MOLECULAR ANALYSIS OF CHANGES IN ABO BLOOD GROUP ANTIGEN EXPRESSION IN HAEMATOLOGICAL MALIGNANCY

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Nothing in this world can take the place of persistence.

Talent will not; nothing is more common than unsuccessful men with talent.

Genius will not; unrewarded genius is almost a proverb.

Education will not; the world is full of educated derelicts.

Persistence and determination alone are omnipotent.

Calvin Coolidge

To Dean

For everything

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Summary

Loss of expression of the A and B antigens of the ABO blood group system from the red cells of patients with haematological malignancy was first observed in the 1950s. Loss of antigen expression *per se* is probably not of direct importance in disease progression, but reflects a genetic alteration that has occurred in the malignant stem cell, as both the leukaemic cells and erythrocytes can be derived from the same precursor.

The recent cloning of the coding region of the ABO gene has made it possible to investigate loss of ABO antigens at the molecular level. This thesis describes the development of techniques to genotype and simultaneously assess allele dosage at the ABO locus using PCR and allele-specific restriction enzyme digestion. Fifteen patients with a variety of haematological malignancies and loss of A and/or B antigen expression were analysed. Loss of antigen expression in two cases was not due to changes occurring at the ABO locus. Four of the remaining thirteen patients had abnormal allele dosage of the ABO gene; one with physical loss of the A allele, one patient with two copies of the O allele and one A allele, and two patients that had lost the O allele. As the O allele of the ABO gene is non-functional, and loss of a non-functional allele can not provide a growth or other advantage to the cell, it was concluded that the changes seen at the ABO locus are an indication of other genetic events occurring in the same chromosomal region.

Analysis of the relative expression of the ABO alleles using RT-PCR and restriction enzyme digestion revealed that the A allele was transcriptionally silenced in four patients. However, these four patients, all of whom genotyped as AO, expressed the O allele. As monoallelic expression of autosomal genes usually indicates imprinting, studies of the patients and their parents were carried out to determine if there was any parental bias in the contribution of the affected allele. Including data from the literature, 11 of 12 patients with loss of A or B antigen expression had lost

expression of the maternally inherited allele of the ABO gene. Therefore, it seems highly likely that imprinting is involved in the etiology of this phenomenon.

Other reports show that some patients with loss of A or B antigens have markedly reduced levels of the enzyme adenylate kinase 1. As both the ABO and AK1 genes are located in chromosome band 9q34, it was possible that the AK1 gene was also affected by the silencing or loss of heterozygosity (LOH) event occurring at the ABO locus. A *Taq*1 restriction enzyme polymorphism in exon 7 of the AK1 gene was identified. Using this polymorphism, it was determined that the loss of heterozygosity event occurring at the ABO gene in one patient, does not extend to the AK1 gene. However, analysis of the allelic expression of the AK1 gene in a patient with severely reduced AK1 enzyme activity and allelic silencing of the ABO gene, revealed decreased expression of one of the alleles, although this patient had normal allele dosage at the AK1 locus. It was therefore concluded that this patient has allelic silencing of both the linked ABO and AK1 genes.

As recurrent LOH in a given region usually indicates the presence of a tumour suppressor gene, the ABL gene, which is located in between the ABO and AK1 genes, was examined. The ABL gene is involved in regulation of the the cell cycle, and therefore it can be considered a potential tumour suppressor gene. Two patients with allelic silencing of the ABO gene and no LOH at the ABO locus were examined. Although both patients had normal allele dosage at the ABL locus, expression analysis of the ABL gene was inconclusive. Although normal controls always produced repeatable results using three different PCR-based methods for the analysis, the relative allelic expression of the ABL gene in the patients was highly variable. The reasons for this are unclear, but are probably due to low mRNA copy numbers of the ABL gene in these patients.

This is the first report of LOH events occurring at the ABO locus and of allelic silencing in haematological malignancy, and represents as yet undescribed genetic and epi-genetic mechanisms for leukemogenesis.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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PUBLICATIONS

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O'Keefe DS, Dobrovic A: A rapid and reliable method for genotyping the ABO blood group (1993). Human Mutation 2:67-70

Dobrovic A, **O'Keefe DS**, Sage RE, Batchelder E: Imprinting and loss of ABO antigens in leukaemia (1993). Blood 1684-1685.

O'Keefe DS, Dobrovic A: A Rapid method for genotyping the A^2 and O^2 alleles of the ABO gene (in preparation).

O'Keefe DS, Dobrovic A: Allelic silencing of the ABO gene is responsible for loss of blood group antigen expression in leukaemia (in preparation).

Publications arising indirectly from the work in this thesis:

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CONFERENCE PRESENTATIONS

International meetings:

Cold Spring Harbour Laboratory Symposium on Quantitative biology: DNA and Chromosomes, June 1993, Cold Spring Harbour, New York. **O'Keefe DS**, Dobrovic A: Allelic silencing is responsible for loss of ABO antigens in haemopoietic malignancy.

National meetings:

The Genome conference, February 1992, Lorne, Victoria. **O'Keefe DS**, Dobrovic A: Loss of the ABO blood group and its association with malignancy.

The Genome conference, February 1993, Lorne, Victoria. **O'Keefe DS**, Dobrovic A: Loss of the ABO blood group in malignancy.

Genetics Society of Australia, July 1993, Adelaide, South Australia. **O'Keefe DS**, Dobrovic A: Loss of the ABO blood group in myeloid malignancy.

Haematology Society of Australia annual meeting, September 1993, Hobart, Tasmania. **O'Keefe DS**, Dobrovic A: Loss of the ABO blood group in malignancy. Australian and New Zealand Journal of Medicine 23: 140.

The Boden conference on the role of DNA methylation in biological processes, February 1994, Thredbo, NSW. **O'Keefe DS**, Dobrovic A: Allelic silencing is responsible for the loss of ABO blood group antigens in haemopoietic malignancy.

(conference presentations continued)

The Genome conference, February 1994, Lorne, Victoria. **O'Keefe DS**, Dobrovic A: Allelic silencing is responsible for the loss of ABO blood group antigens in haemopoietic malignancy.

Haematology Society of Australia annual meeting, September 1994, Perth, Western Australia. **O'Keefe DS**, Dobrovic A: Allelic silencing is responsible for the loss of ABO blood group antigens in malignancy. Australian and New Zealand Journal of Medicine 24: 237.

The Cancer conference, February 1995, Lorne, Victoria. **O'Keefe DS**, Dobrovic A: Simultaneous allelic silencing of linked genes in haematological malignancy.

The Genome conference, February 1995, Lorne, Victoria. **O'Keefe DS**, Dobrovic A: Simultaneous allelic silencing of linked genes in haematological malignancy (winner of the Promega Award).

Adelaide Blood Club annual scientific meeting, April 1995, Adelaide, South Australia. **O'Keefe DS**, Dobrovic A: Simultaneous allelic silencing of the linked ABO and AK1 genes in haematological malignancy.

LIST OF ABBREVIATIONS USED IN THIS THESIS

aa	-	amino acid
AK1	-	adenylate kinase 1
AML	-	acute myeloid leukaemia
bp	-	base pair(s)
BM	-	bone marrow
cDNA	-	complementary DNA
CML	-	chronic myeloid leukaemia
cps	-	counts per second
dH_2O	-	distilled water
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleoside triphosphate
ddNTP	-	di-deoxynucleoside triphosphate
Hb	-	haemoglobin
kb	-	kilobase
LOH	-	loss of heterozygosity
mb	-	megabase
MDS	-	myelodysplastic syndrome
µg	-	microgram
µl	-	microlitre
MNC	-	mononuclear cells
MPD	-	myeloproliferative disorder
mRNA	-	messenger RNA
NATA	-	National Association of Testing Authorities
nt	-	nucleotide
OD_x	-	optical density at x nanometers
PCR	-	polymerase chain reaction
PB	-	peripheral blood
QEH	-	Queen Elizabeth Hospital
RAEB	-	refractory anaemia with excess blasts
RNA	-	ribonucleic acid
RT-PCR	-	reverse transcription-polymerase chain reaction
UV	-	ultra-violet
v/v	-	volume per volume
WCC	-	white cell count
w/v	-	weight per volume

CHAPTER ONE

REVIEW OF THE LITERATURE

1.0 INTRODUCTION

The relationship between the development of cancer and alterations in blood group antigens is poorly understood. Although red cell antigens are of vital practical importance in blood transfusion, their primary functional roles in the physiology of the normal individual are still unknown. What is known however, is that oncogenic transformation in almost all human cancers, is accompanied by numerous changes in cell membranes and cell surface carbohydrate structures. These changes may have a direct effect upon the metastatic potential of the cell in carcinoma, or even upon the uncontrolled proliferation of cells, as seen in haematological malignancies.

Loss of ABO blood group antigens from the surface of carcinoma cells and from the red cells of some patients with haematological malignancy has been observed since the 1950s. It is the aim of this thesis to investigate the molecular mechanisms underlying changes in ABO blood group antigens in carcinogenesis, particularly in haematological malignancies of the myeloid lineage. In order to understand the functional significance of changes in ABO blood group carbohydrate structures in malignancy, it is first necessary to summarise the basic genetic and biochemical principles of the ABO system.

1.1 Genetics and biochemistry of the ABO blood group system

The ABO blood group system was discovered by Karl Landsteiner in 1900, who noted that serum from some individuals agglutinated red cells from other individuals. Later studies revealed that these antigens could be detected on the surface of most endothelial and some epithelial cells, and they were therefore termed the ABH "histo-blood group" antigens. The world wide frequency of the most common blood groups in the Caucasian population is: 45% O group, 32% A^1 group, 10% B group, 9% A^2 group, 3% A^1B group and 1% A^2B (reviewed in Harmening-Pittiglio, 1986).

The gene responsible for ABO blood groups does not code for the specific antigens themselves. Instead, it codes for glycosyltransferases capable of catalyzing the transfer of specific sugars to carbohydrate chains of a precursor molecule (the H antigen; see Figure 1-1).

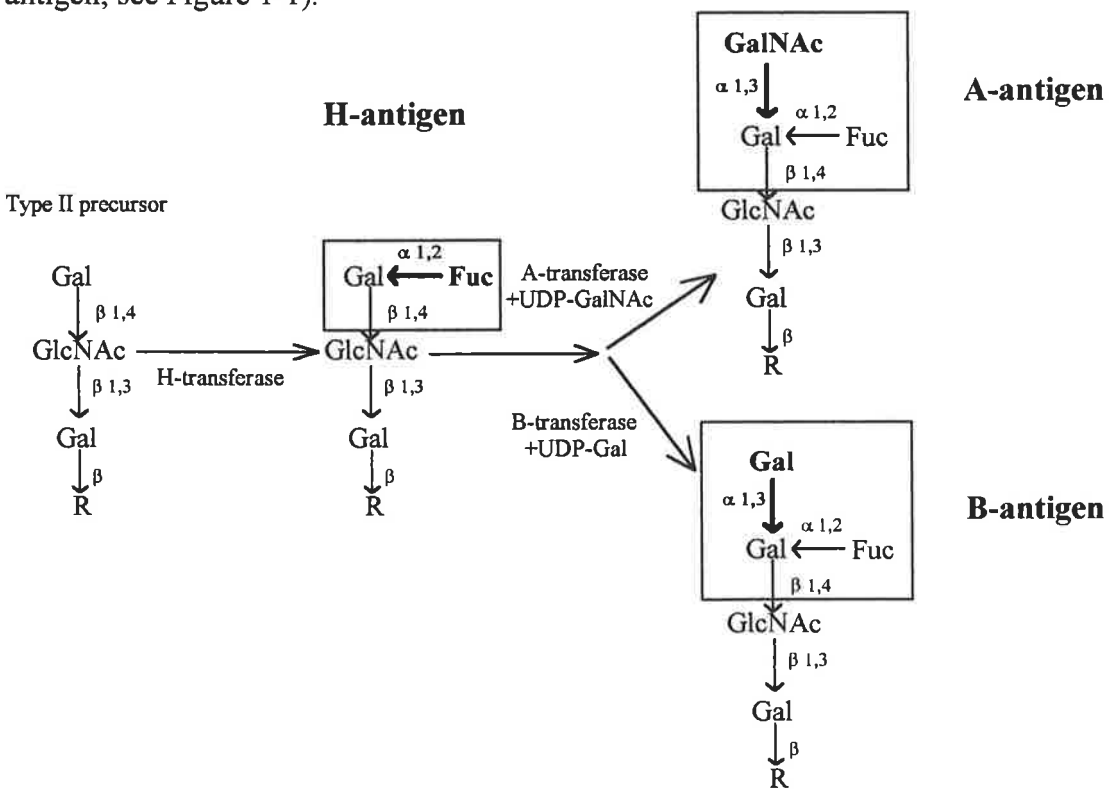

Figure 1-1 Simplified diagram of ABH biosynthesis. Bold type represents the sugar added. R corresponds to the asparagine-linked, serine/threonine-linked or lipid-linked glycoconjugate backbone. The immunodominant regions of the antigens are framed. Sugar abbreviations and complex names of the transferases are as in Table 1-1; GlcNAc is N-acetyl-glucosamine (adapted from Lowe, 1993).

The protein coding region of the ABO gene is 1065 base pairs long and encodes a 354 amino acid glycosyltransferase with a molecular weight of 41 kilo Daltons. The transferase consists of three domains; a short N-terminal, a hydrophobic transmembrane domain, and a long C-terminal domain. The soluble form of the transferase lacks the N-terminal and hydrophobic domains, thus the catalytic domain probably resides in the C-terminus (Yamamoto *et al.*, 1990a).

There are four common alleles of the gene, A^1, A^2, B and O, which are inherited in a simple co-dominant Mendelian fashion. The A^1 and B alleles differ by several single base substitutions, resulting in amino acid changes in the glycosyltransferase, some of which incur differential specificity for nucleotide-sugar donors (see Table 1-1), (Yamamoto *et al.*, 1990b; Yamamoto and Hakomori, 1990). The A^2 allele differs from the A^1 allele at two nucleotide positions, resulting in a transferase that is a weaker catalyst than the A^1 transferase (see discussion on ABO subgroups in section 1.7). The O allele has a single base deletion at position 261, but is otherwise identical to the A^1 allele. This deletion leads to a frameshift mutation, resulting in premature termination of translation due to the formation of a stop codon at position 352 (Yamamoto *et al.*, 1990b). This mutant protein is enzymatically inactive; incapable of converting the precursor H antigen. Therefore the term ABH antigens (as opposed to ABO blood group and the ABO gene), is a more accurate description of this system, and I will refer to it accordingly throughout the rest of this thesis.

Antigen	Glycosyltransferase	Nucleotide (Sugar Donor)	Immunodominant Sugar
H	α-2-L-fucosyltransferase	GDP-Fuc*	L-fucose
A	α-3-N-acetylgalactosaminyltransferase	UDP-GalNAc†	N-acetyl-D-galactosamine
B	α-3-D-galactosyltransferase	UDP-Gal‡	D-galactose

Table 1-1 Donor nucleotides and immunodominant sugars responsible for H, A and B antigen specificities (from Harmening-Pittiglio, 1986).

The ABO glycosyltransferases catalyze the addition of sugars to the H antigen in the Golgi apparatus, however the transferase is also found in a soluble extracellular form that is probably produced by proteolytic cleavage of the membrane-bound region just beyond the hydrophobic region (Weinstein *et al.*, 1987). The soluble form of the transferase might also be determined by the use of an alternative start codon (ATG found at nt 157-159), as this would encode a protein with enzymatic capabilities but lacking the hydrophobic anchor needed to localized the enzyme to the Golgi membrane (Shaper *et al.*, 1988).

1.2 Precursor structures

The blood group oligosaccharides use one of four types of precursor structures synthesised by human cells. Type I and II precursors are at the termini of linear and branched chain oligosaccharides (which are attached to asparagine, serine, or threonine residues, or to membrane-associated lipid molecules), and can be distinguished by the linkage of Galactose to N-acetyl-glucosamine; β1→3 and 1→4 respectively. Type III chains are attached via O-linked glycosylation to serine/threonine-linked oligosaccharides, and type IV chains are only found associated with lipids (reviewed in Lowe, 1993).

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*GDP-Fuc guanosine-diphosphate L-fucose
†UDP-GalNAc uridine diphosphate-N-acetyl-D-galactose
‡UDP-Gal uridine diphosphate galactose

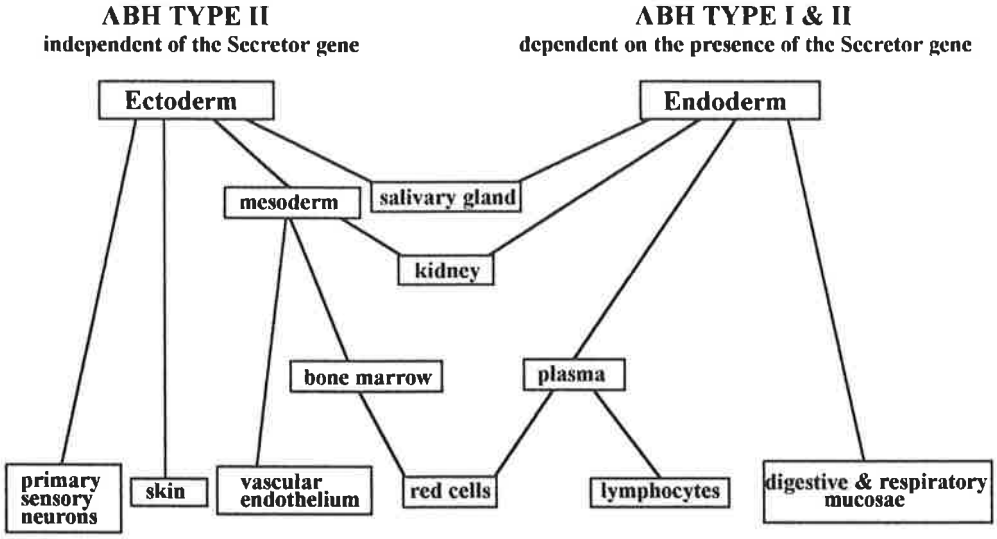

Figure 1-2. Type I and II chain ABH antigen expression in various tissues (adapted from Oriol, 1990).

Type I precursors are found in the epithelia that line the pulmonary, urinary and gastrointestinal tracts, and salivary glands (reviewed in Oriol, 1990), and are converted to ABH antigens in the presence of the secretor gene; subsequently forming secreted ABH molecules. These secreted molecules are then passively adsorbed from plasma onto lymphocytes and erythrocytes. Type II chains are synthesised by the red cells themselves. Therefore red cells, along with the epidermis, mostly express ABH type II determinants (see Figure 1-2),(Oriol *et al.*, 1986; Clausen and Hakomori, 1989).

Type III ABH antigens are carried on mucins synthesised by the gastric mucosa and by cells that line ovarian cysts (reviewed in Sadler, 1984). There are also A-associated type III and IV antigens on glycolipids from A erythrocytes (Clausen *et al.*, 1985), these structures have multiple repeats of the A epitope. Type IV ABH antigens have also been found on red cells (Clausen *et al.*, 1986). The four basic chain types are depicted in Figure 1-3.

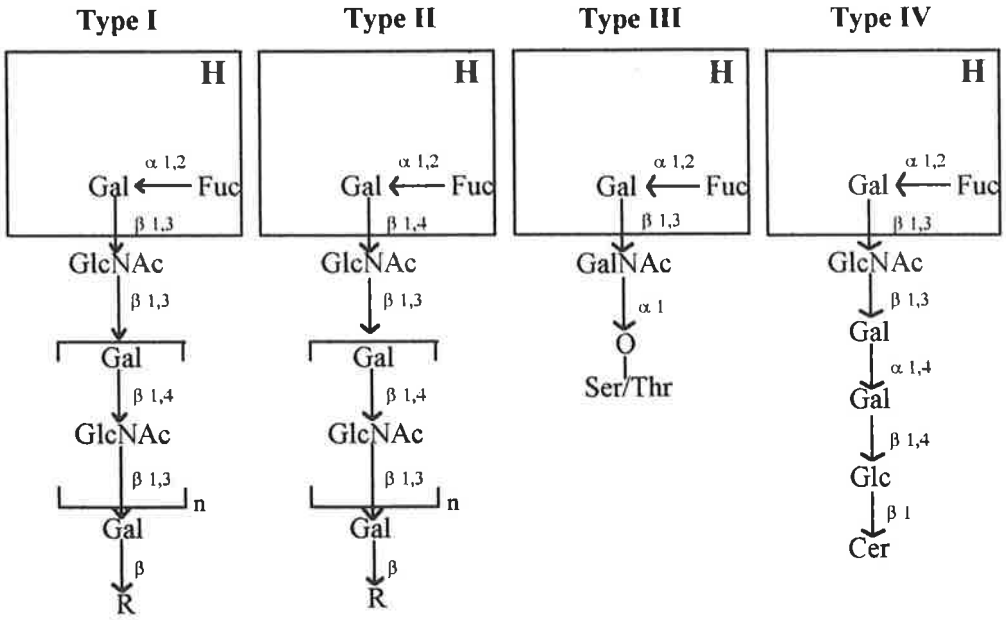

Figure 1-3 Simplified Type I, II, III and IV linear H determinants. The immunodominant region is framed. R corresponds to an asparagine-linked, serine/threonine-linked or lipid-linked glycoconjugate backbone. Single lactosamine units are enclosed in brackets, and may be repeated multiple times within a polymer. Type III determinants are linked via O-glycosylation to the serine/threonine backbone. Type IV chains are linked to lipids, indicated by Cer; ceramides.(reviewed in Clausen and Hakomori, 1989).

1.3 The H antigen

Production of A and B antigens on red cells not only relies on synthesis of the appropriate glycosyltransferases, but also on the presence of the precursor H antigen (refer to Figure 1-1). Expression of the H antigen is in turn controlled by separate genetic systems. The Hh, secretor (Sese) and Lewis (Lele) systems are relevant to this discussion as they are intimately related to the expression of A and B antigens.

The Hh system comprises two alleles, H and an extremely rare null allele, h. The H allele produces the enzyme α-2-L-fucosyltransferase, which modifies a precursor carbohydrate structure by the addition of L-fucose. The resulting structure is then able to be directly converted to the A or B antigen by the appropriate glycosyltransferase. Individuals who inherit two copies of the h allele cannot synthesize the H antigen, and correspondingly cannot produce either A or B antigens on their red cells regardless of whether or not they produce the A or B

glycosyltransferases. This is known as the Bombay Phenotype (reviewed in Watkins, 1980).

The enzyme α-2-L-fucosyltransferase is also coded for at a second locus, by the secretor gene (Oriol *et al.*, 1981). The secretor system is generally considered to control the expression of this enzyme in cells of salivary glands, while the Hh system directs expression of the H antigen on the surface of red blood cells. Although both H and Secretor loci are found on chromosome 19 (Larsen *et al.*, 1990; Ball *et al.*, 1991), the two α-2-L-fucosyltransferases they encode differ significantly in their catalytic properties (Sarnesto *et al.*, 1992).

Biochemical studies (LePendu *et al.*, 1985; Kumazaki and Yoshida, 1984; Betteridge and Watkins, 1985) have revealed that of these two different α-2-L-fucosyltransferases, the enzyme determined by the H allele preferentially converts type II precursor chains, while the secretor determined transferase prefers type I chains. Although both these enzymes are present in gastric mucosa and salivary glands, only the enzyme that prefers type II chains and is determined by the H gene, is found in red cells (Betteridge and Watkins, 1986).

The type II H determinant is present on the surface of all red cells of an individual carrying the H allele. As H is converted to the A and B antigens, people of O blood group have considerably more exposed H antigen on the red cell surface than do people of A and B blood groups. Formation of the A and B antigens by the addition of sugars to the H precursor diminishes red cell reactivity with H-antisera. Therefore, when the reactivity of red cells of various blood groups with anti-H are compared, the strongest reaction is with O cells, and decreasing in the order $A^2>A^2B>B>A^1>A^1B$ (Mollison, 1979). The different glycosyltransferases convert the H antigen with variable efficiency, accounting for the maximum reactivity from

O cells (no conversion of H), and the minimal reactivity with A^1B cells, in which nearly all available H is converted.

1.4 The Lewis system

The Lewis system is also integral to this discussion as the amount of Lewis antigens on red cells is dependent on *ABH* gene expression. Lewis antigens are soluble and found in saliva and plasma (Grubb, 1951) and are normally passively adsorbed from the plasma onto the erythrocyte membrane. The five most common Lewis antigens are Le^a, Le^b, Le^c, Le^d, and Le^x. However, none of these antigens are produced by alleles of the same gene. The Lewis gene (Le), codes for $\alpha 1 \rightarrow 3/1 \rightarrow 4$-L-fucosyltransferase (Johnson *et al.*, 1992). The recessive allele of the gene (le) is a null allele, and individuals homozygous for this allele are known as Lewis negative as opposed to Lewis positive individuals (LeLe or Lele), who express either Le^a or Le^b antigens. This enzyme converts the precursor type I chain by the addition of fucose to N-acetyl glucosamine, and competes with α-2-L-fucosyltransferase (secretor determined) for precursor substrate. Conversion of the precursor substrate by the Lewis encoded enzyme results in the Le^a antigen (Figure 1-4). The Lewis fucosyltransferase and secretor determined α-2-L-fucosyltransferase work sequentially (and only in this order), to produce a di-fucosylated type I structure, the Le^b antigen (Figure 1-4). Therefore Le^b is only found in persons who are secretors and Lewis positive. Subsequently, Le^a and Le^b antigens are expressed in the same tissues that express type I chain H determinants.

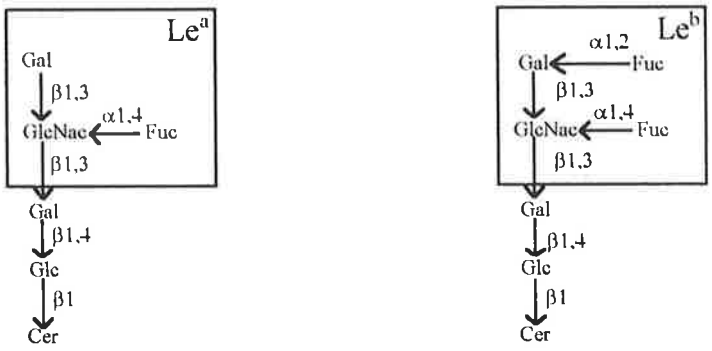

Figure 1-4 Structures of the Lewis A and B glycosphingolipids. The rectangle encloses the immunodominant region of each structure. Cer (Ceramide) links the molecule to the red cell membrane (Lowe, 1993).

The Lewis C (Le^c) antigen was first detected using an antibody isolated from a group O, Le(a-b+) woman (Gunson and Latham, 1972). This antibody reacts with red cells from Lewis negative, non-secretors and with Lewis negative, Bombay phenotypes. Although it is called a Lewis antigen, Le^c is the basic type I precursor chain before the addition of fucose (Watkins, 1980).

Lewis D is also expressed in the absence of the Lewis gene. The Le^d antigen is detected in Lewis negative, H and Se positive individuals (Potapov, 1970). In fact, the structure of the Le^d antigen is type I H, as shown previously in Figure 1-3.

The naming of the Lewis X antigen is somewhat ambiguous. Anti-Le^x was first described by Andresen and Jordal in 1949; it reacts with all blood groups except Le (a-b-). It is thought that Le^x may be Le^a, and that anti-Le^x is able to react with red cells that carry very few Le^a antigens (Schenkel-Brunner and Hanfland, 1981). In 1984 however, Hakomori named another antigen Le^x, which is also known as Stage Specific Embryonic Antigen (SSEA-1; Hakomori, 1984). This is a Lewis fucosylated type II chain, but is not the same antigen that was first detected by Andresen and Jordal in 1949 (see Figure 1-5).

Le^x(SSEA-1)

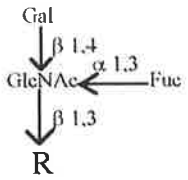

Figure 1-5 Structure of the Le^x antigen, which is otherwise known as the Stage Specific Embryonic Antigen (SSEA1). R represents the underlying glycoconjugate, which is either a protein- or lipid-linked oligosaccharide, or a free oligosaccharide (Lowe, 1993).

1.5 The I system

The ABH genes interact with another blood group system in the formation of their antigenic structures; the Ii system. Type II chain H antigens can be divided into four classes* ; H_1, H_2, H_3 and H_4. The H_1 and H_2 (unbranched) structures demonstrate strong i, but weak I antigenic activity (Hakomori, 1981). The H_3 and H_4 structures are branched glycolipids with strong I antigen activity, as demonstrated by precipitation and inhibition of agglutination reactions (Hakomori, 1981). Koscielak proposed that the I gene codes for the enzyme responsible for the branching of the type II precursor structure, and that addition of the L-fucose by the enzyme encoded by the H gene results in H_3 and H_4 (Koscielak,1977). Thus the i antigen would represent the unbranched or linear type II precursor before fucosylation by the H gene encoded α-2-L-fucosyltransferase (Figure 1-6).

* It is important to realise that Roman numerals are used to describe H antigens based on any of the four precursor structures, ie. HI, HII etc. The four classes of H described as H_1, H_2, H_3 and H_4 are differentiated by minor variations of the type II precursor chain they are carried on; the most important and significant difference being that H_1 and H_2 are formed from linear, unbranched structures while H_3 and H_4 are based on branched type II chains.

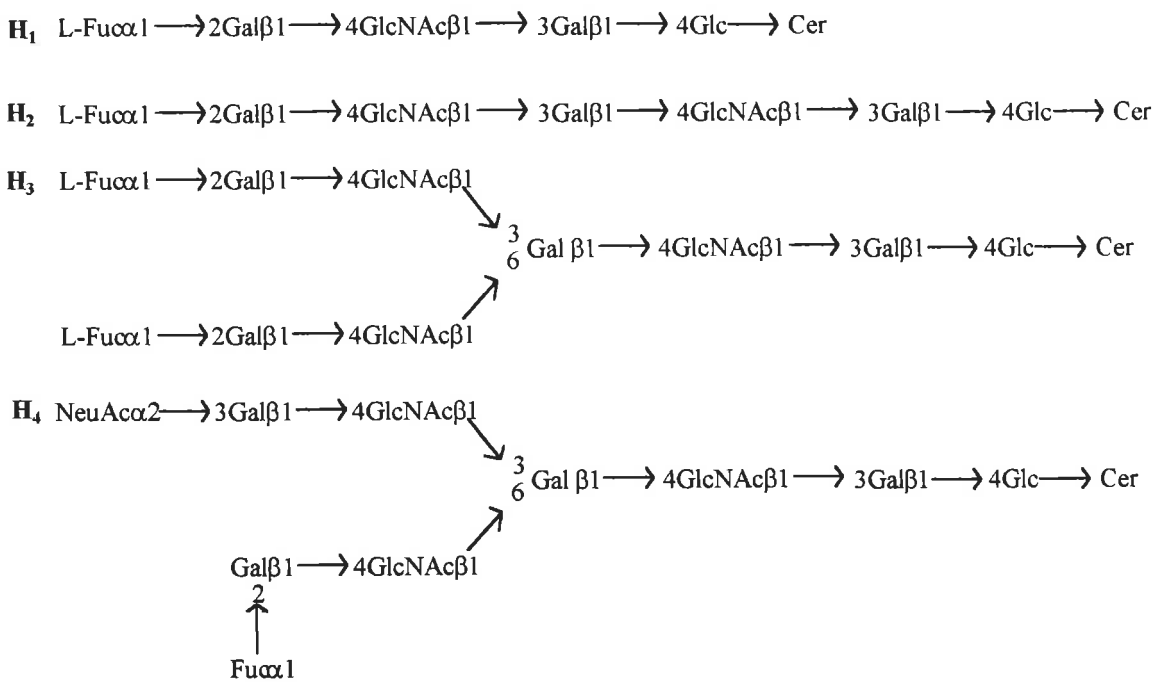

Figure 1-6 Structures of the four different H antigen glycolipids formed from the type II precursor. H_1 and H_2 are linear structures while H_3 and H_4 are branched at the middle galactose; up to five branches may be carried per chain. NeuAc represents a sialosyl group (Hakomori, 1984).

The red cells of newborn infants carry high levels of i, and very little I (Jenkins *et al.*, 1960). However, Marsh (Marsh, 1961) showed that between birth and 18 months of age, the levels of the i antigen gradually decrease, while a reciprocal increase in the I antigen occurs. Marsh also noted that this change corresponds with the gradual disappearance of fetal haemoglobin, and the switch to adult haemoglobin production, however there is no other evidence to link these two events functionally. Although the amount of I and i on the red cells of adults varies, (Jenkins *et al.*, 1960; Marsh *et al.*, 1971), generally there is very little i. However, in some disease states the expression of both I and i is altered. The importance of these changes will be discussed in due course (see section 1.17).

1.6 The molecular basis of the ABO blood group

The cloning of the gene responsible for ABO blood groups has substantially increased our understanding of the mechanisms behind not only the most common groups, but has also made it possible to determine the molecular basis of some of the less common subgroups.

The complementary DNA (cDNA) encoding the A glycosyltransferase (Yamamoto *et al.*, 1990a) was isolated from a cDNA library constructed using RNA from a stomach cancer cell line (MKN45). The library was probed with PCR products which were generated using degenerate primers based on information gained from partial amino acid sequencing of the purified transferase enzyme by Clausen *et al.*(1990). Northern analysis using the A transferase cDNA as a probe revealed multiple transcripts (the number and sizes of the transcripts is not indicated), in one stomach cancer, and three colon cancer cell lines. The blood group phenotypes of the cell lines were A, O, B and AB, indicating that the O gene is expressed. Southern analysis implied that the A transferase is most likely coded for by a single copy gene (Yamamoto *et al.*, 1990a).

The B and O alleles of the ABO gene were subsequently cloned and sequenced, using the A^1 glycosyltransferase cDNA as a probe (Yamamoto *et al.*, 1990b). The B allele differs from the A allele by 7 single base substitutions within the protein-coding region. Four of these differences lead to amino acid (aa) substitutions and correlate with the A or B phenotype of a cell. The remaining 3 substitutions do not alter the aa sequence, and do not consistently associate with a particular phenotype; therefore they probably represent functionally silent polymorphisms. The four amino acid substitutions are found at aa 176 (A, arginine; B, glycine), 235 (A, glycine; B, serine), 266 (A, leucine; B, methionine), and 268 (A, glycine; B, alanine).

Yamamoto and Hakomori (1990), constructed chimeric cDNAs containing various combinations of these single nucleotide substitutions and transfected them into an O genotype cell line. They found that while the residue at amino acid position 176 has little, if any, effect on sugar substrate utilization, the residues at 266 and 268 are vitally important. Constructs containing only the base changes for these two amino acids conferred the appropriate specificity of the A or B transferase, regardless of the other two polymorphism's presence or absence. The authors fail to mention however, the effect (if any) of the amino acid substitution at position 235.

Other alleles at the ABO locus

1.7 The A^2 allele

A common subgroup of A is the A^2 group. Red cells expressing the A^2 antigen are characterised by their ability to react with anti-A, but unlike A^1 cells, they do not react with a plant lectin derived from *Dolichos Biflorus*. The incidence of the A^2 phenotype varies between races, but is approximately 20% of the A group Caucasian population (Yamamoto *et al.*, 1992). The A^2 transferase is a weaker catalyst than the A^1 transferase, and has different pH optima, cation requirements and Km values (Schachter *et al.*, 1973). Yamamoto and his colleagues (1992) used a PCR/sequencing approach on genomic DNA from A^2 blood group individuals.

Only the last two protein-coding exons of the gene were examined, however two differences in comparison to the A^1 sequence were identified. The first of these differences is a single base substitution that results in an amino acid change at position 156; a leucine is present in the A^2 transferase, whereas the A^1 transferase contains a proline. The second change is a single base deletion located at the C-terminus (position 1059-1061 in the A^1 allele), which alters the reading frame and subsequently produces a protein with an extra 21 amino acids. Using cDNA constructs varying only by the single base deletion (all constructs contained the

single base substitution), Yamamoto and his associates demonstrated a 30-50 fold decrease in transferase activity produced from transfected HeLa cells containing the C-terminus deletion. This group did not carry out any transfection experiments to determine the effect (if any) of the single base substitution, although every A^2 allele sequenced contained both the substitution and the deletion, and therefore both alterations may be important.

1.8 The A^3 and B^3 alleles

Yamamoto and his group (Yamamoto *et al.*, 1993a; Yamamoto *et al.*, 1993b; Yamamoto *et al.*, 1993c; Yamamoto *et al.*, 1993d) have extended their molecular analysis of ABO alleles to include some of the weaker and rarer subgroups (see Figure 1-7 for a summary diagram comparing most of the ABO alleles). These subgroups can be distuinguished by their serological reactivity. The A^3 group characteristically shows a mixed-field agglutination with anti-A reagents; that is, not all the red cells agglutinate. Similarly, in red cells of B^3 blood group, approximately two-thirds of the cells are agglutinated. Cells from individuals of both these types of blood groups have virtually no detectable transferase activity, and no transferase is found in the individual's serum, even in secretors (Lopez *et al.*, 1979).

Yamamoto sequenced the last two coding exons of the ABO gene for each of these alleles. This region contains about 91% of the soluble form of the transferases, therefore one would expect most differences between alleles to be contained within this region. It appears that there is significant heterogeneity amongst the A^3 and B^3 alleles. Yamamoto (1993a) reports that in ABO alleles of 2 out of 4 individuals of A^3 blood group, there was a single base substitution resulting in an amino acid change (Asp→Asn), which is predicted to alter the activity of the enzyme. Similarly, an ABO allele from one of the 3 B^3 individuals also had a (different)

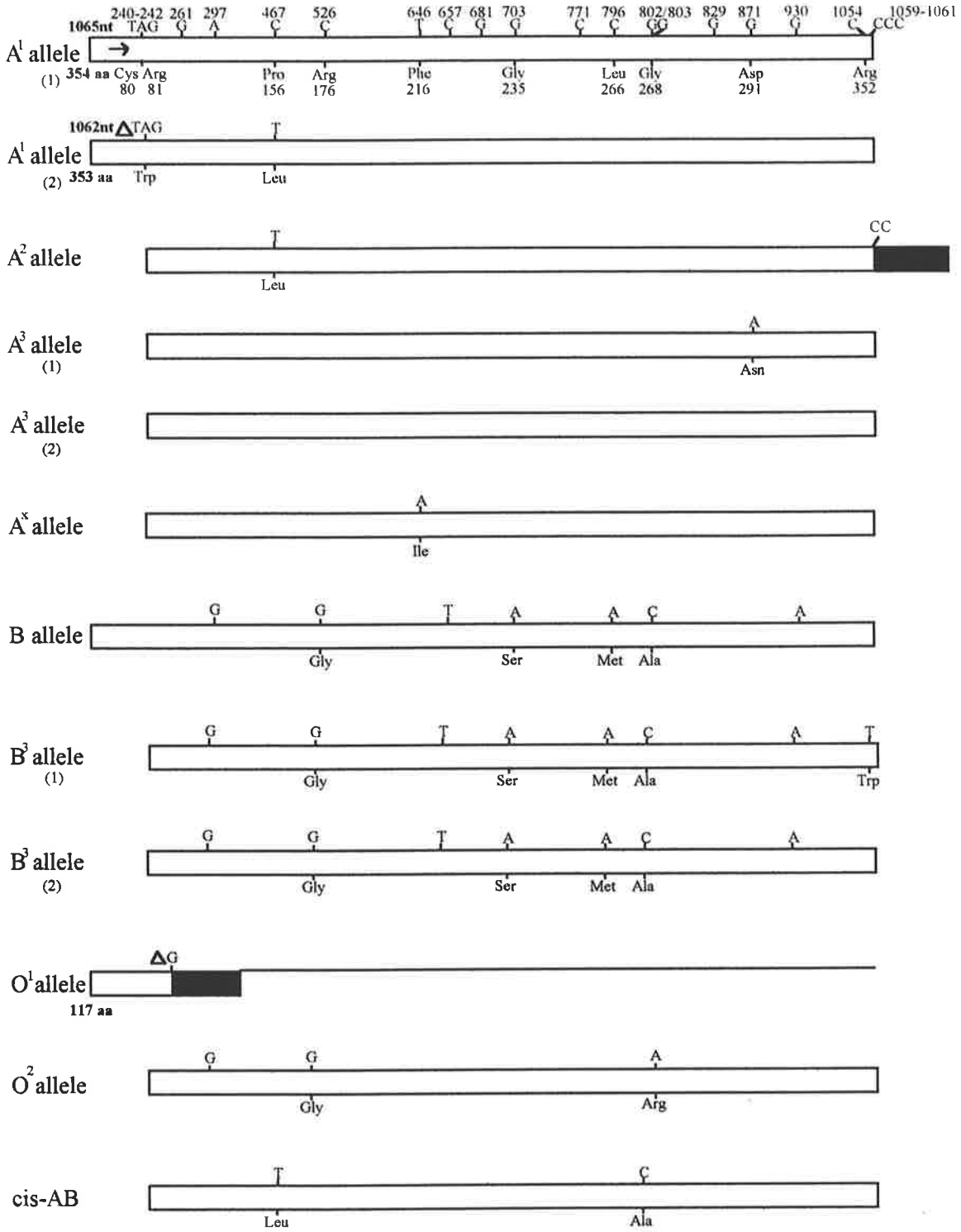

Figure 1-7 Schematic comparison of cDNA nucleotide and deduced amino acid sequences of alleles of the ABO blood group system. A^1 (1) represents most A^1 alleles. The A^1 (2) allele is the originally published sequence found in GenBank and Yamamoto *et al.*, 1990a, and has a trinucleotide deletion (TAG, due to alternative splicing or a splicing error in the cell line from which it was derived; Yamamoto, 1994), as indicated by the delta symbol. The shaded rectangle in the O and A^2 alleles portrays the different amino acid sequence due to frame shifting caused by the single base deletion. The arrow symbol indicates the N-terminal of the soluble form of A^1 transferase. Only the regions sequenced and compared are shown (modified from Yamamoto, 1994).

single base substitution (Arg→Trp). However, in four of the cases, there was no sequence difference between the presumed A^3 and B^3 alleles, and the common A^1 or B alleles respectively. It is possible that there are differences in these alleles located outside the region sequenced. Yamamoto (1993a) suggests that alternative splicing of the normal A^1 and B alleles may affect the activity of the enzymes responsible for A^3 and B^3 blood group antigens, or that lesions may exist in (as yet unknown) ABO modifying genes.

1.9 The A^x and *cis*-AB alleles

A similar approach was employed to determine the molecular basis of the rare A^x and *cis*-AB blood groups (Yamamoto *et al.*, 1993c;Yamamoto *et al.*, 1993d). The A^x blood group is characterised by lack of agglutination of red cells with anti-A, and weak agglutination with anti-A,B (Lopez *et al.*, 1979). Individuals of this subgroup have very weak A-transferase activity in the serum, and anti-A^1 antibodies are usually present. Additionally, A^x secretors carry minute amounts of A substance (Salmon *et al.*, 1984b).

The *cis*-AB blood group was first recognized in 1964 (Seyfried *et al.*, 1964) in a family where the blood groups of the father, mother, their two children and the mother's mother were O, A^2B, A^2B, A^2B and O respectively; suggesting that the A and B alleles were inherited on a single chromosome. Yamaguchi *et al.*(1965) reported an A^2B^3 blood group, showing weak activity of both the A and B antigens. Family studies once again suggested that the A and B genes were inherited together on one chromosome, hence the term *cis*-AB. As both these blood groups are extremely rare, Yamamoto's group only studied one case of A^x, and two unrelated cases of *cis*-AB (Yamamoto *et al.*, 1993c; 1993d). The A^x allele was found to have a single base change relative to the A^1 allele, leading to an amino acid substitution (Phe→Ile). The *cis*-AB alleles from both individuals studied proved to be identical;

both contained the single base substitution found in the A^2 allele, and a substitution at aa position 268, one of the substitutions that differentiates the A and B alleles (Figure 1-7), and is vitally important for B-transferase activity (ie. galactose sugar specificity), (Yamamoto and Hakomori, 1990).

1.10 Another kind of O allele

During these studies, the Yamamoto group encountered another type of O allele, the O^2 allele. Comparison of this allele with the A^1 allele revealed that the single base deletion typical of most O alleles, was not present. There was however, three single base substitutions resulting in two amino acid substitutions. The first two substitutions, at nt297 and 526, are both found in the B allele. The third substitution is found at nt802, and results in the introduction of a charged amino acid, arginine, as opposed to the neutral amino acids, glycine and alanine that are found in the A and B alleles respectively. An expression construct containing these differences exhibited no A^1 enzymatic activity (Yamamoto *et al.*, 1993b), suggesting that the arginine substitution may block catalytic activity. The frequency of this new allele has been determined in the Danish population, and is approximately 3.7% (Grunnet *et al.*, 1994).

1.11 Intron and exon structure of the ABO gene

The molecular basis for regulation of expression of the ABO gene has not yet been determined. There is however, information on the intron/exon structure of the gene (Lee and Chang, 1992; Grunnet *et al.*, 1994; Yamamoto *et al.*, 1995). The ABO gene consists of at least 7 coding exons which span over 18kb of genomic DNA. The six introns begin at nucleotides 28, 98, 155, 203, 239 and 374. Most of the coding region lies in exon 7, which is 688bp and encodes 229 of the 354 amino acids of the transferase. At least 7 alternatively spliced and prematurely spliced cDNAs were also isolated during these analyses; however all of the cDNAs

examined were from tumour cell lines, and the authors concluded that the splice variants seen are probably not representative of the normal situation (Yamamoto *et al.*, 1995).

1.12 Interactions between the different blood group systems

It is now obvious that the different blood group systems are intimately related, with many of the enzymes described utilizing the same substrate. It therefore follows that if the expression of one or more of the enzymes is down-regulated in any way (ie. either by mutation at the DNA level, as in the O allele, or by other mechanisms), there will be more substrate available for the other enzymes to convert, or more antigen available to react with a given antibody. This explains why the blood of O group individuals reacts more strongly with anti-H than that of say, AB individuals. Similarly, if A or B transferase were inactive or down-regulated in a Lewis positive individual, then there would be more precursor H antigen (type II chain) available to the fucosyltransferase encoded by Lewis, and thus more conversion to the Le^x antigen. In fact, an increase in Le^x antigen has been noted in a variety of malignancies, and will be discussed in the ensuing sections.

1.13 Loss of blood group antigens in haemopoietic malignancy

Changes in blood group antigen expression in relation to disease were first observed in acute leukaemia (Wiener and Gordon, 1956; Van Loghem *et al.*, 1957; Stratton *et al.*, 1958; Salmon *et al.*, 1958; Salmon *et al.*, 1959; Gold *et al.*, 1959; Tovey, 1960, and Bhatia and Sanghvi, 1960). Although most of these reports were based on patients with "weakened" expression of the A antigen in acute myeloid leukaemia (AML), many other investigators have also reported loss or weak expression of the A, B and H antigens in AML (Salmon and Salmon,1965;

Kurokowa *et al.*, 1969; Lay *et al.*, 1961; Undevia *et al.*, 1966; Richards, 1962; Bird *et al.*, 1976; Yoshida *et al.*, 1985), in acute lymphatic leukaemia (ALL), the chronic leukaemias (CML, CLL) and pre-leukaemic disorders including the myeloproliferative disorders (MPD), and myelodysplastic syndromes (MDS) (Dreyfus *et al.*, 1969; Ogata and Hasegawa, 1977; Kuhns *et al.*, 1978; Kolins *et al.*, 1978; Salmon *et al.*, 1984). There are also some reports of Ii antigen changes (Salmon *et al.*, 1966; McGinniss *et al.*, 1964; Schmidt *et al.*, 1965; Salmon, 1976) as well as changes in the expression of the Le^a and Le^b antigens (Kolins *et al.*, 1978). In most cases where there is "weakening" or complete loss of the A, B or H antigens from the red cells, the antigens are present in the saliva of the patient (dependent on secretor status)(Renton *et al.*, 1962; Bernard *et al.*, 1965; Kolins *et al.*, 1978; Matsuki *et al.*, 1992), suggesting that the defect leading to loss of the antigens is confined to the haemopoietic compartment, and the patients do in fact have the genetic capability of producing the appropriate antigen.

It is interesting that in at least 50% of the A^3 and B^3 blood group samples they examined, Yamamoto and his group (1993a) found no changes at the DNA level that should cause a reduction in the expression of the appropriate antigen. Both these groups are extremely rare and are characterised by mixed-field reactions, a phenomenon most often seen in leukaemia patients (reviewed in Gunz and Baikie, 1974). It is possible that there is some other mechanism besides mutational inactivation that is down-regulating the expression of the appropriate transferases in these subjects, and that this mechanism may be of importance in the etiology or progression of haemopoietic malignancy.

The loss of ABH antigens *per se* is probably not of importance in the disease progression. Bombay individuals which are deficient in A, B and H antigens (O_h-), show no red cell or other abnormalities (Wallace and Gibbs, 1986); and it is obvious that there is no selection against O blood group individuals considering they make up nearly 50% of the population. Therefore changes in erythrocyte antigens may reflect a genetic disturbance that has occurred in the malignant stem cell, as both leukaemic cells and red cells can be derived from the same myeloid precursor (Fialkow *et al.*, 1981).

Alternatively, loss of the ABH antigens may indicate that a profound change has occurred in the cell membrane, leading to altered spatial distribution of the antigens and subsequently changes in their reactivity with anti-sera. Gottfried (1971), and Hildebrand *et al.*(1971), studied lipid patterns in leukaemic cells and suggested that the expression of lipids reflects the cells' immaturity. Furthermore, they pointed out that even small changes in lipid content can have major affects on the membrane properties, for example physical and electrical attributes. These studies however, were not carried out on erythrocytes, which by definition are terminally differentiated and therefore mature.

1.14 Masking of antigens by sialyl-transferases

In the studies referred to above (eg. Wiener and Gordon, 1956), there are generally dual populations of red cells evident, these can be divided into two categories; normal A and weak A, and normal A and "not A". A third category exists, consisting of a single population of cells that do not agglutinate at all. The first two of these categories are characterised by the appearance of a mixed-field reaction when the red cells are incubated with the appropriate anti-body. The

proportion of normal A cells to weak A or A negative cells is assumed to represent the ratio of normal erythrocytes to those derived from the leukaemic stem cell.

Kassulke *et al.*(1969), compared normal leukocytes to leukaemic cells and red cells, and found that changes in the red cell groups were reflected in the leukaemic cells. In addition, the leukaemic cells expressed the sialic acid-containing M antigen, which is not normally present on leukocytes. This lead the authors to propose that "masking" of the appropriate antigens by a sialyl-transferase had occurred during the malignant transformation, and facilitated the expression of the M antigen. Kassulke's group later tested this hypothesis (Kassulke *et al.*, 1971) by incubating leukaemic cells with neuraminidase, an enzyme which removes sialic acid. They found that in cell populations where A or H antigen expression was decreased, the treatment increased their expression, and decreased the expression of the sialic acid containing M and N antigens. Treatment with neuraminidase produced no changes in antigen expression on normal leukocytes. Evidence contrary to this hypothesis was presented by Renton and his associates (Renton *et al.*, 1962), who described a patient with eosinophilic leukaemia. This patient exhibited four independent populations of red cells, A, B, O and AB. The patient's serum had no antibodies to A or B, implying that before the onset of disease, the blood grouped as normal AB. Family studies indicated that the patient was in fact genetically A^1B. If sialic-acid was masking the antigens, one would expect to see an AB population and an O group population, not four different populations. The argument against the hypothesis that masking of antigens occurs by sialic acid is likewise supported by many other studies of patients in which decreased red cell A or B antigen is accompanied by an increase in the H antigen (eg. Salmon *et al.*, 1984). Further studies are required to understand the role of sialyl-transferases in leukaemia.

1.15 The association between chemotherapy and loss of ABH antigens

Starling and Fernbach (1970) conducted a study of 30 children with acute leukaemia. All the children were blood group A, and changes in A antigen expression were reported in 11 of these patients over the period of one year. The investigators concluded that changes in blood group A antigen expression bore no relation to disease status, but a correlation was found with treatment of the disease. While this may be true for the pediatric leukaemias, in nearly every patient study described in section 1.13, mixed-field reactions representing changes in blood group antigen expression were observed at presentation of the patient; before diagnosis or treatment. Furthermore, changes in antigen expression seem to reflect the disease course, in that blood groups progress towards normal with remission (ie. there was strong agglutination with the appropriate anti-body), whereas relapse is associated with decreased blood group antigen expression (Richards, 1962; McGinniss *et al.*, 1964; Salmon *et al.*, 1969; Kolins *et al.*, 1978; Salmon *et al.*, 1984).

Considering that chemotherapy usually induces remission, it is unlikely that treatment causes loss of the antigens. There is at least one case however, where the patient's blood group returned to normal A^1 at terminal relapse of acute myeloid leukaemia (Hoogstraten *et al.*, 1961). This might be explained by failure of the malignant red cell precursors to differentiate into mature erythrocytes as the disease progressed, with the A^1 red cells being derived from residual normal stem cells that had retained the capacity for differentiation.

1.16 Loss of the H antigen precursor

Section 1.1 described the formation of the A and B antigens by the sequential addition of sugars to a carbohydrate chain. The precursor to A and B is H, and it follows that loss of the H antigen would lead to loss of A and/or B expression on the red cell surface. Some of the cases presented report decreased levels of H antigen on the red cells (ie. Majsky *et al.*, 1967). Other investigators (Kuhns *et al.*, 1978) have reported a general deficiency of α-2-L-fucosyltransferase (H-determined) in the serum of the majority of leukaemic patients (AML and ALL) and a negligible change in the A/B transferases. Furthermore, the levels of serum α-2-L-fucosyltransferase increased to normal values during remission (achieved by chemotherapy), and a decrease in the enzyme was consistent with drug resistance and subsequent relapse. As α-2-L-fucosyltransferase converts the I antigen (type II) precursor into the H antigen, it is likely that there would also be a deficiency of the precursor, H, in these patients.

The widespread occurrence of a decrease in α-2-L-fucosyltransferase suggests that it is unlikely to play a primary role in loss of the ABH antigens in leukaemia, as loss of A or B antigens is a relatively rare event. Salmon *et al.*(1984), considered the Kuhns' group results concerning the negligble changes in the serum A and B transferases to be of little significance, as these transferases are released from many epithelial tissues, while the α-2-L-fucosyltransferase (H-transferase) is predominantly produced by the haemopoietic lineage. Salmon's group therefore decided to study the activity of the A, B and H transferases in the red cell membrane. These studies were carried out on the red cells of eight patients (2 AML M1, AML M2, AML M6, 2 CML, and 2 patients that were in remission).

The results showed that seven of the patients had normal serum H-transferase levels, while one had a significantly higher level. In all these cases, the patients' red cells exhibited mixed-field reactions when tested with anti-A or anti-B anti-sera. The red cells were divided into A (or B) positive and A (or B) negative populations, and the membrane transferase activities determined. Comparisons between the cell populations in each case showed a significant decrease in the activity of the A or B-transferase, while the H-transferase was equally active in both populations. All of the patients did not have blood transfusions for at least three months prior to these experiments, therefore it is likely that the synthesis of the A and B determinants was blocked at the production of the A or B-transferase in the cells derived from the malignant stem cell.

In the same study, Salmon *et al.*(1984) showed that in A antigen negative cell populations, incubation with B-transferase, its metallic co-factor manganese, and galactose, could produce normal B-antigenicity. One of these red cell populations came from a patient with a severe lack of serum H-transferase, yet the red cells were able to be converted to the B antigen. These results confirm the irrelevance of serum transferase levels with respect to loss of ABO antigens from the red cell surface.

1.17 Loss of the I antigen

Loss of the I antigen might also be thought to be related to loss of ABH antigens, as I is the precursor to the H (type II) antigen. Loss of the I (adult) antigen has been observed in many cases of leukaemia (Salmon *et al.*, 1966; McGinniss *et al.*, 1964; Schmidt *et al.*, 1965; Salmon, 1976), but not one of these studies describes loss of the i (fetal) antigen; to the contrary, most of the results show an increase in the

amount of i in leukaemia. As i is the precursor to I (as described in section 1.5), loss of the I-transferase would naturally lead to a deficiency of I and an excess of i. However, the i antigen can also be converted to H and subsequently to the A or B antigen. This is further supported by the fact that red cells from neonates, which almost exclusively express the i antigen, usually react strongly with anti-A or anti-B anti-sera. Therefore loss of I should not lead to loss of ABO.

Schmidt *et al.*(1965), found that the red cells of 38 out of 124 leukaemic patients were I negative. They also found that several mycoplasma organisms that are known to cause pneumonia can block or destroy the I antigen on normal red cells *in vitro*. They suggested that mycoplasmae may be opportunistic secondary invaders in leukaemic patients. Giblett and Crookston, (1964), also found loss of I in the red cells of patients with thalassemia major, and paroxysmal nocturnal hemoglobinuria. The authors concluded that the increased expression of i, and decreased expression of I was the result of decreased marrow transition time, a phenomena which also occurs in the leukaemic marrow (Hillman and Giblett, 1965).

Two erythroleukaemic cell lines, K562 and HEL exhibit strong i activity (Kannagi *et al.*, 1983). A small population of the K562 cells express the I antigen, but the HEL cell line shows no detectable I. It is clear from these studies, which included assaying for Le^x and H activity, that unbranched type II chain accumulation occurs in human leukaemia, possibly due to a blocked synthesis of blood group determinants (Hakomori, 1984). It also is of interest that the i antigen of K562 is converted to I when cells are induced to differentiate by retinoic acid (Testa *et al.*, 1982).

1.18 Intolerance to "self" antigens

In a report by Gold *et al.* (1959), a patient suffering from AML was described. At presentation, approximately 2% of the patient's red cells were agglutinated by a strong anti-A anti-body. Clinical remission followed treatment, as did a return to 35% "normal" A_2 red blood cells. When the patient subsequently relapsed, 8% of red cells in peripheral blood and marrow agglutinated with anti-A, and there were no anti-A anti-bodies in the patient's serum. However, an *in vivo* experiment showed that A_1 red cells radioactively labelled with chromium-51 and transfused into the patient survived for a much shorter time in the patient's circulation than similarly labelled group O cells. Unfortunately the patient died at this point, and the possibility that he was intolerant to small amounts of A_1 blood could not be confirmed. Interestingly, family studies indicate that the patient's true blood group was indeed A_1.

1.19 Intrinsic factors that may modify ABH antigens

It is possible that glycosidases (enzymes which destroy A or B antigens), or other intrinsic factors may exist in at least some patients with altered antigen expression, and modify the A or B determinants. To test this idea, Salmon *et al.* (1961) transfused normal A_1 cells into a leukaemic patient with weakened A expression. Separation of the normal transfused cells from the leukaemic cells *in vitro* indicated that the transfused cells retained their original antigenic strength. These results exclude the possibility of some intrinsic factor (such as glycosidases) modifying the A antigen, at least in this patient.

1.20 A genetic basis for loss of ABH antigens

From the information presented, there is strong support for a genetic origin of this phenomenon. Several investigators have suggested that there are chromosomal alterations in some of these patients, and that these changes may be associated with loss of the ABH antigens. Ducos *et al.* (1964), discussed an AML patient that lost expression of the A antigen, and also exhibited a pair of constricted chromosomes. In 1964, cytogenetic techniques to distuinguish chromosomes were not available, thus Ducos and his associates were limited to inferring that the gene corresponding to the loss of the A antigen resided on one of the medium group of chromosomes (group 6-12). Ragen *et al.* (1968), also described a patient with acute myelomonocytic leukaemia, with decreased expression of A antigen and a chromosomal abnormality. However, the cytogenetic changes in these reports vary, and it would therefore be of interest to study these patients now, given the advances in cytogenetics since the 1960s.

Salmon *et al.* (1968) produced perhaps the strongest evidence for a genetic basis of loss of ABH blood groups. The patient described by Salmon exhibited two distinct populations of red cells. Over 13 years, the patient's grouping changed very little (ie. approximately 45% of cells agglutinated with anti-A^1, and about 70% with anti-H). Although the patient had been permanently exposed to benzene in her youth, she had no signs of disease. Benzene is an agent which is known to cause leukaemia, in particular acute leukaemia (Gunz and Baikie, 1974). The fact that this woman was exposed to a leukaemogenic agent and exhibited blood group changes characteristic of leukaemic patients, yet did not develop leukaemia, is extremely interesting. Whether or not this patient was pre-leukaemic will be considered elsewhere in this discussion (section 1.21).

The red cell populations from this patient were separated (by agglutination with anti-A) and assayed for activity of a number of enzymes unrelated to the ABH system. Changes in the activity of the red cell enzyme, adenylate kinase I (AK1)*, were noted. The A-positive cells had normal activity of both AK_1 and AK_2 (enzyme polymorphisms of AK1), while the A-negative cells were AK_1 positive and AK_2 deficient (see Figure 1-8). The ABH system is not functionally related to AK1. However, the ABO gene is located in chromosome band 9q34.1-34.2, while the AK1 gene is in 9q34.1 (Attwood *et al.*, 1994).

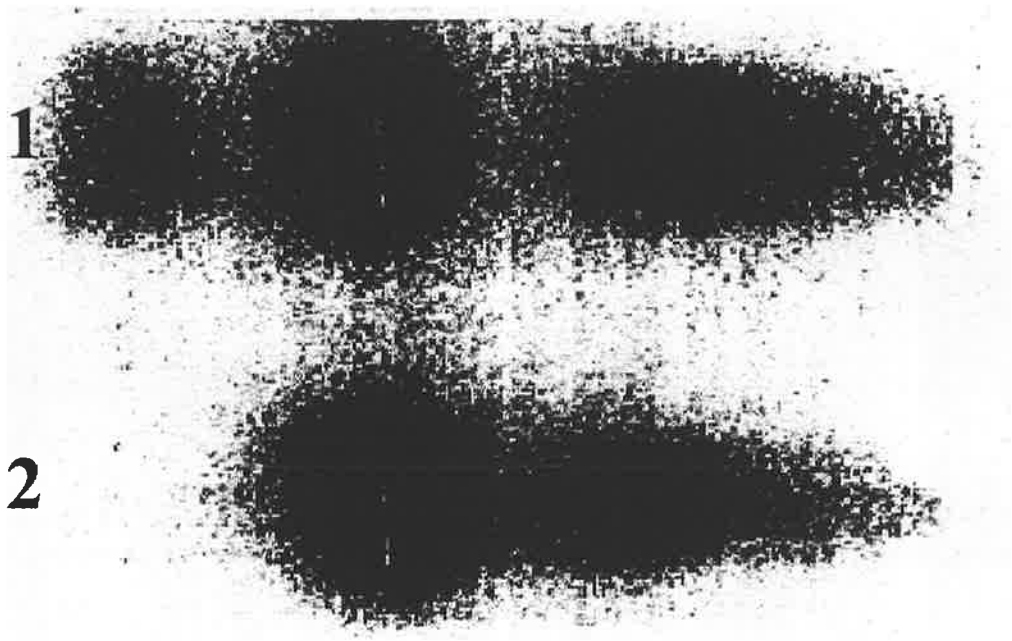

Figure 1-8 Red cell adenylate kinase I enzyme electrophoresis results from a patient with loss of A antigens. Lane 1: AK_2AK_1 phenotype from A^1 red cells. Lane 2: AK_1AK_1 phenotype from weak A red cells from the same patient (Salmon *et al.*, 1968).

Only two other groups have investigated the possibility of concurrent changes in ABH and AK1. Kahn *et al.* (1971), described a patient with erytholeukaemia that exhibited a mixed-field-reaction with anti-A. Although only 50% of the patient's red cells agglutinated with anti-A, the unagglutinated "O" cells were strongly H-

* Adenylate kinase I is a phosphotransferase, and catalyses the reversible reaction: 2ADP ⇔ ATP + AMP (Fildes and Harris, 1966).

positive. Again, AK1 activity was significantly decreased in the A-negative population (by approximately 80%), compared to the relatively normal value of the A-positive population.

A more recent report (Marsden *et al.*, 1992) describes a patient with acute non-lymphocytic leukaemia (ANLL), loss of A antigen and a significant decrease in AK1 activity (66IU/gHb; the normal range is 220-340IU/gHb). Assuming there is a genetic basis for changes in ABH blood groups, it appears more than coincidental that the expression of these two linked genes has simultaneously decreased.

1.21 Loss of A or B antigens - an indicator of pre-leukaemic status?

The patient described by Salmon and his colleagues in 1968 exhibited loss of A antigen with a simultaneous depression in AK1 activity. This patient however, was supposedly healthy, although she had been exposed to the leukaemogenic agent benzene all her working life. It is possible that loss of A or B antigens is indicative of some genetic event predisposing the individual to frank leukaemia.

Support for this hypothesis lies in the report of another patient, studied by Lopez and associates (Lopez *et al.*, 1986). The patient was a four year old girl who underwent a routine blood group examination. Her red cells exhibited a mixed-field reaction with anti-A, and subsequent studies revealed a decrease in membrane bound A-transferase activity. Blood and bone marrow smears showed a moderate dysmyelopoietic state. Eighteen months later, the child developed AML. At this point in time, 70% of her cells grouped as O, whilst the other 30% appeared to be A^2. Family studies revealed that she should have inherited an A^1 gene. Although

changes were also observed with the I antigen (loss of I, increase in i), agglutination with anti-H was slightly less than in normal controls.

These results suggest that loss of ABO antigens may be one of the first indicators of disease. Assuming loss of antigens reflects a genetic change that has occurred in the malignant stem cell, these changes may be one of the first of many in a possibly multi-step process leading to the development of frank leukaemia.

1.22 Family studies of patients with loss of A or B antigens

Family studies of patients with loss of A or B antigens often indicate that the patient should have inherited an A^1 gene, yet the cells that agglutinate with anti-A behave as A^2 cells, as they do not agglutinate with the plant lectin from *Dolichos biflorus*. Salmon (1976), compared the activity of the A-transferase in two of his patients, and their offspring that must have inherited the same gene. The patient's partners were both blood group O, and the offspring both A, therefore the offspring must have inherited the A genes from their respective leukaemic parents. In both these cases, the patients' enzymes were significantly less active than the enzyme produced by their offspring.

The A^2 enzyme is very similar to the A^1 enzyme (see section 1.7). However, the A^2 transferase is less efficient at converting substrate, and this results in fewer A antigen sites. These studies imply that although a normal A^1 allele is present in these patients (or at least was present before onset of disease), the glycosyltransferase it produces is somehow less efficient at converting the H-

substrate and subsequently acts in a similar manner to the glycosyltransferase produced by the A^2 allele.

There are a number of possible explanations that could account for the altered behaviour of the ABO gene. Somatic mutation is an obvious one, as is altered transcription of the gene (either reduced transcription or disruption of the normal mRNA by alternative splicing), or the presence of modifying genes. Until the recent cloning of the ABO cDNA in 1990 (Yamamoto *et al.*, 1990a), it was not possible to determine if the gene was mutated in these patients, or to analyse its transcriptional status; these studies are yet to be carried out.

Rubinstein *et al.* (1973) accounted for the altered behaviour of the ABO gene by proposing the existence of a dominant suppressor of the A and B genes. In a family study, they found a healthy blood donor of blood group O, who was had no anti-A in his serum. The subject was also a secretor, and had a reduced amount of A substance in his saliva. The red cells carried the normal amount of H antigen. Adsorption/elution tests on the red cells with anti-A^1 (a sensitive method for determining if minute amounts of antigen are present), revealed that the cells in fact eluted anti-A^1. These results indicate that the patient is actually A^1, although his red cells do not agglutinate with anti-A at all. Similar studies were carried out on the donor's maternal half-brother, who was also blood group O but who lacked anti-B in his serum. Red cells from this individual eluted anti-B, and therefore he appears to genetically be blood group B. It can be seen from the pedigree in Figure 1-9 that the subjects (7 and 10) must have inherited the A and B alleles from their respective fathers, but the expression of these genes is modified in some way by a gene inherited from their mother.

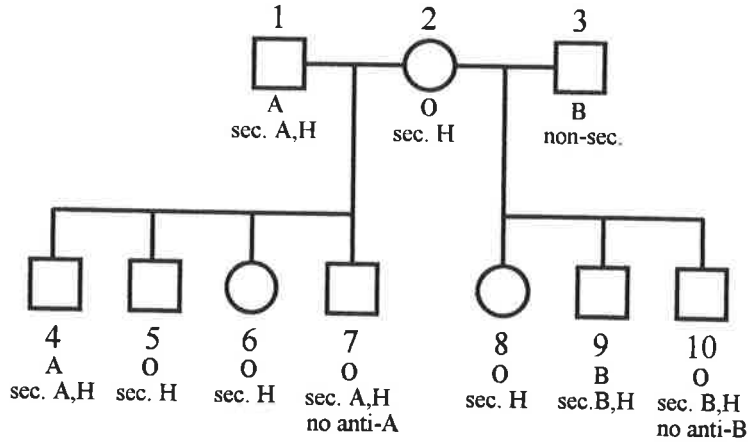

Figure 1-9 Pedigree described in Rubinstein *et al* (1973), to support the hypothesis that a dominant suppressor of the A and B genes exists. sec.=secretor ; non-sec.=non-secretor of ABH substance in saliva. Subject 7 and 10 are group O, yet secretor, serum antibodies and family data indicate that they should be A and B blood group respectively. All the subjects are healthy donors.

It must be remembered that in all of the cases discussed, there is loss of antigens and sometimes a corresponding increase in the antigen's precursor, but (with one exception), the appearance of neo-antigens has not been described. This suggests that there is a block in the synthesis of the appropriate carbohydrates, most likely occurring at the genetic level.

There is one case where a patient exhibited neo-antigens. Hocking (1971), presented a female AML patient who prior to disease had consistently typed as group O. During her disease, the patient developed septicaemia and 70% of her red cells agglutinated with anti-A. Treatment with Daunorubicin resulted in recovery from the septicaemia, and a subsequent return to normal O blood group. It is likely in this case that bacteria produced some kind of A-like transferase, thus converting the patients O cells temporarily to A.

1.23 Loss of the ABH antigens in carcinoma

Changes in A, B and H determinants in a large variety of human cancers was originally observed in Masamune's laboratory (Masamune *et al.*, 1958; Masamune and Hakomori, 1960). Other reports involved changes in blood group antigen expression in ovarian carcinoma (Metoki *et al.*, 1989), prostrate cancer (Idikio and Manickavel, 1991), gastric cancer (David *et al.*, 1993), lung carcinoma (Miyake *et al.*, 1992), cervical carcinoma (Davidsohn *et al.*, 1969;Griffin and Wells, 1993), the urothelium of patients with chronic cystitis (Orntoft *et al.*, 1989), pancreatic cancer (Davidsohn *et al.*, 1971), squamous cell carcinoma of the head and neck (Wolf *et al.*, 1990), oral malignant and premalignant lesions (Dabelsteen *et al.*, 1983;Dabelsteen *et al.*, 1991), uterine/endometrial carcinoma (Tsukazaki *et al.*, 1991); testicular germ cell tumours (Dabelsteen and Jacobsen, 1991), transitional cell carcinoma of the bladder (Mandel *et al.*, 1992) and colon carcinomas (Dahiya *et al.*, 1989).

There are a number of differences between the results on blood group changes in the haemopoietic disorders and the epithelial cell carcinomas described above. Firstly, it appears that the phenomenon occurs much more readily in carcinoma, with some authors proposing a direct relationship between metastasis and loss of ABH antigens (Davidsohn, 1972). Secondly, the tumours often express incompatible antigens, that is patients of O or B blood group have tumours that express "A" antigens (Metoki *et al.*, 1989). Although changes in ABH antigens in carcinoma and haemopoietic disease may arise from two completely different mechanisms, it is important to analyse the data in both areas, as there may be significant similarities in the etiology of this phenomenon.

The study of ABH antigens in carcinoma is in some ways less complicated than studying antigen expression in the leukaemias. A major problem with leukaemia is the diseased cells may be (and usually are) mixed with normal cells. It is therefore difficult to compare normal and malignant cells from the one person. On the other hand, solid tumours are more easily separated from the surrounding normal tissue; providing a control for comparison using immunohistochemical and other techniques (for example mixed agglutination on cell suspensions, and specific red cell adherence assays). Perhaps this is why changes in ABH antigens have been far more extensively studied in the carcinomas than in the haematological malignancies.

1.24 Relationship between loss of ABH antigens and tumour aggressiveness

Davidsohn's group reported that A and B antigens are lost in all anaplastic tumours, but retained in differentiated tumours and benign adenomas. This was first seen in gastric tumours and later in cancers of the ovary, skin, tongue, larynx, bladder and cervix (Davidsohn *et al.*, 1966; Kovarik *et al.*, 1968). Idikio and Manickavel (1991) repeated this study on prostate carcinomas, and they also examined the tumours for expression of the H antigen. They found that loss of ABH antigens correlated with increasing grade of carcinoma, with 1 out of 3 grade 2 tumours positive for the A antigen, 1 out of 6 grade 3 tissues positive for the B antigen, and no grade 4 or 5 carcinomas positive for the A, B or H antigens (out of 4). The largest and most convincing study comes from Davidsohn (1972). Davidsohn developed the specific red cell adherence test, which allowed him to study tissue sections that were up to 35 years old. He studied 355 primary carcinomas of the uterus, cervix, lung, pancreas and stomach, and 578 metastatic carcinomas from these tumours (Table 1-2). These results show that with few exceptions, loss of ABH antigens precede the formation of distant metastases.

		Staining for A, B or H antigens		
Tumour type	**total**	**negative (%)**	**partial (%)**	**positive (%)**
lung-primary	104	99 (95)	1 (1)	4 (4)
lung-metastases	206	204 (99)	1 (0.5)	1 (0.5)
uterine/cervix-primary	102	89 (87)	11 (11)	2 (2)
uterine/cervix-metastases	123	114 (93)	9 (2)	0
pancreatic-primary	54	45 (83)	4 (8)	5 (9)
pancreatic-metastases	79	66 (84)	1 (1)	12 (15)
stomach-primary	95	70 (74)	20 (21)	5 (5)
stomach-metastases	170	149 (88)	13 (7)	8 (5)
total primary tumours	**355**	**303 (85)**	**36 (10)**	**16 (15)**
total metastases	**578**	**533 (92)**	**24 (4)**	**21 (4)**

Table 1-2 Expression of A, B and H antigens on primary tumour tissues and metastases derived from these tumours (modified from Davidsohn, 1972).

1.25 Biochemical studies of blood group antigens in tumours

Originally, blocked biosynthesis of A antigen from the H_1 glycolipid was observed in gastric cancer tissue, as compared with normal mucosa, by analysis of glycolipid patterns and *in vitro* conversion of H (in the A negative tumour tissue) to A by the

A transferase (Stellner and Hakomori, 1973). Furthermore, in tissues where synthesis of A and B antigens was blocked due to lack of a functional transferase, there was accumulation of the precursor type I and type II H chains, and the Le^x antigen in some of the tumours. Some other cases of adenocarcinoma accumulated Le^a structures, as well as carbohydrates with Le^b activity due to an enhanced fucosyltransferase activity (Hakomori and Andrews, 1970). Studies using monoclonal antibodies directed against Le^x have shown specific reactivity with human colonic, gastric and lung cancers as well as myelogenous leukaemia (Brockhaus *et al.*, 1982; Gooi *et al.*, 1983; Huang *et al.*, 1983a and Huang *et al.*, 1983b). Le^x is widely distributed in various normal tissues and cells, however, the reason behind its accummulation in a large variety of human cancers is unknown.

1.26 Studies using monoclonal antibodies that detect the A and B glycosyltransferases

White *et al.* (1990), produced an antibody (WKH1), specific to the A^1 transferase. Characterisation of this antibody revealed that it cross-reacted with the B transferase; a not unexpected result considering the genetic similarity of the two transferases. This antibody does not detect any protein in O blood group individuals, supporting the hypothesis that the single base deletion in the O allele leads to a truncated and immunologically distinct protein (Yamamoto *et al.*, 1990b). Mandel *et al.* (1990), used this antibody to study the biosynthetic regulation of A and B antigens in epithelial tissue, using oral epithelia as a model. They concluded that the presence of the transferase in the cytoplasm (probably the golgi apparatus) of the cells directly correlated with the appearance of the antigens on the cell surface, and suggested that expression of the A and B blood group antigens is directly regulated at the transcriptional level of the ABO glycosyltransferase.

Further analyses using the WKH1 antibody were carried out to compare the expression of the transferases in normal and malignant epithelia (Mandel *et al.*, 1992). Of 9 bladder tumours examined, the corresponding normal bladder tissue was found to co-express both A or B antigens, and the A/B transferase. No staining with either antibodies to the transferases or the antigens was observed in the tumour tissue from 3 patients with grade III tumours, and in 2 patients with grade II tumours. However, tumour tissues from 2 other grade II patients, and 2 patients with grade I tumours, were postitive for the antigens and the transferases.

Mandel *et al.* (1992) also examined normal and malignant oral epithelium, although the results are dependent on secretor status and more difficult to interpret than those from the bladder carcinomas. In secretors, normal oral tissue was found to co-express both the A and/or B antigens and the transferase. However, in non-secretor tissue, there was expression of the transferase but no corresponding expression of the antigens; probably due to the lack of type I H precursor that is normal in these individuals (see Section 1.3). From the staining patterns, it appears that all of the squamous cell carcinomas derived from oral epithelia examined in this study were from patients of positive secretor status. In 8 out of the 18 carcinomas, neither the antigens or the transferase were present. In the other 10, staining was sporadic with antibodies to both the antigens and the transferase. Interestingly, 2 carcinomas had more intense staining with both antibodies when compared to the normal tissue. Dysplastic tissue adjacent to the carcinoma was examined in 3 patients; one of which showed loss of both the antigens and the transferase, the other two had sporadic staining.

The third group of tumours examined in this study were from colonic epithelium. These tumours were all from patients of either A or B blood group, and four of the tumours were from the distal colon, while 2 were from the proximal region of the colon. It should be noted here that tumours from the distal colon do not exhibit loss of the A and B determinants, conversely they show onco-developmental expression of these antigens, as normal distal tissue does not express A and B antigens (Yuan *et al.*, 1985). Mandel *et al.* (1992), showed that in the carcinomas derived from the proximal region, both the A and B antigens were expressed in the same cells as the transferase; as they were also in the normal adjacent tissue. In the samples from the distal colon, however, both the normal and malignant tissue expressed the transferases, although only the tumour tissue expressed the antigens. Mandel *et al.* (1992), concluded that the onco-developmental expression of the A and B antigens in the distal colon is not regulated by the expression of the transferase, and is probably dependent on the presence of the precursor H antigen.

These studies indicate that, at least in malignant epithelia, loss of A and B antigens is due to loss of the appropriate glycosyltransferase, and that this loss is probably directly regulated by the transcriptional regulation of the gene encoding the transferase. This might also explain the accumulation of the Le^x antigen in epithelial carcinomas and AML described in the preceding section, as loss of the A/B transferase would diminish competition by the Lewis encoded fucosyltransferase for the H precursor substrate.

1.27 Genetic analysis of urothelial cell lines with loss of blood group A antigen expression

Meldgaard *et al.* (1994) examined the ABO gene in two cell lines that had loss of A antigen expression. The cell lines were derived from normal ureter (Hu 609) and bladder mucosa (HCV 29), adjacent to tumours. In both cases, the patients from whom these cell lines originated, had been treated with irradiation. The authors reported that the cell line Hu 609 genotyped as A plus a mutated allele (the mutation was not defined), and the cell line HCV 29 genotyped as AA. Southern analysis of genomic DNA from both cell lines did not reveal any gross changes at the ABO locus, and Northern analysis indicated the ABO gene was expressed, although biochemical analyses indicated no detectable A-transferase activity.

There seems to be a number of problems with these experiments. Firstly, the authors do not explain why they chose to use cell lines derived from normal mucosa, and not from the tumours themselves. There is no mention of whether or not the tumours themselves had lost expression of the A antigen, although the authors admit that loss of A antigen in cell lines derived from normal tissue is peculiar, and hence may not reflect the phenomenon seen *in vivo*. The fact that the ABO gene is expressed, but active transferase is not, suggests some kind of mutational inactivation of the gene, which could have been introduced by the irradiation treatment received by the patients from whom these cell lines originated.

1.28 Expression of incompatible A antigen in carcinoma

In tumours of O or B patients, neo-expression of A-like antigens have been observed (Hakomori *et al.*, 1967), although the molecular basis of this expression is unclear. Yokota *et al.* (1981), suggested that the A-like antigen could be a fucose-less A determinant, while Breimer, 1980, suggested it could be a di-fucosylated A structure, based on mass spectometry results. It has long been

known that the Forsmann antigen cross-reacts with blood group A, and hence the possibility that the A-like antigen is in fact the Forsmann antigen has been studied by several groups. The enzyme responsible for synthesis of the Forsmann antigen is greatly increased in most squamous cell carcinomas and in some adenocarcinomas (Taniguchi *et al.*, 1981). Mori *et al.* (1983) used immunofluorescence studies to show that Forsmann antigen is expressed in various human lung, breast and gastric cancer cell lines. However, the A-like antigen expressed in gastrointestinal tumours from O or B group individuals is structurally different from the Forsmann antigen (Hakomori *et al.*, 1977).

Recently, David *et al.* (1993) used the monoclonal antibody developed to the A/B transferase to confirm the presence of the true A antigen in 3/31 cases of gastric tumours from blood group O individuals. The histologically unaffected tissue adjacent to the tumours did not stain with the antibodies, and genotyping of the ABO gene in the tumour tissue revealed an OO genotype. Five other cases had an A-like antigen, but did not stain with the antibodies. Based on these results, it was postulated that alternative splicing of the ABO gene may have occurred. If the so-called second exon (the intron/exon structure of the 5' end of the gene is not entirely known), was spliced out, the reading frame of the O allele would remain intact and could theoretically produce a glycosyltransferase capable of converting the H antigen to A. Interestingly, one of the patients examined in this study had two tumours, one positive for the true A antigen, the other tumour negative.

The evidence so far presented in this discussion indicates that changes in the ABO gene could explain loss of the A and B antigens in carcinoma and the haemopoietic malignancies, at least in some patients. Some leukaemias are reported to have

disturbance of, or translocations in 9q34, the same chromosomal band that the ABO gene is located in. It is therefore pertinent to include a discussion of the haemopoietic malignancies.

1.29 The haemopoietic malignancies

Leukaemia is almost always fatal, however little is known about the origin of the disease itself. In his introduction to a chapter on classification of the leukaemias, Edward Henderson provides a quote from an eminent haematolologist of the 19th century, Virchow, made in 1849; "*This is what we know about leukaemia: during normal blood cell production the cells differentiate into specific types. In a pathologic situation the differentiation into specific cells is blocked. This disturbance of normal differentiation - so called leukaemia - is a disease sui generis. We know the sequelae of this disease, we don't know its origin.*". Henderson goes on to state that this is essentially still the case today (Henderson, 1990).

Leukaemia may best be defined as the abnormal proliferation of haematopoietic precursor cells that do not retain the capacity to differentiate normally to mature blood cells (Figure 1-10). As a consequence, the leukaemias have been divided into two main categories according to the predominant cell type; lymphoid and myeloid. This distinction is made on morphological grounds, although certain cases of acute leukaemia defy this kind of classification, in which case the disease is classified as acute undifferentiated leukaemia (AUL).

There are also other haematological malignancies that do not strictly classify as leukaemias, as they still retain the capacity for differentiation, but exhibit a clonal expansion of cells (ie. myeloproliferative disorders), or, as in the myelodysplastic syndromes, there is no uncontrolled proliferation, yet the differentiation of the cells

to their mature forms is blocked at some point. Both the myeloproliferative disorders (MPD) and the myelodysplastic syndromes (MDS) can lead to acute leukaemia (reviewed in Sawyers *et al.*, 1991).

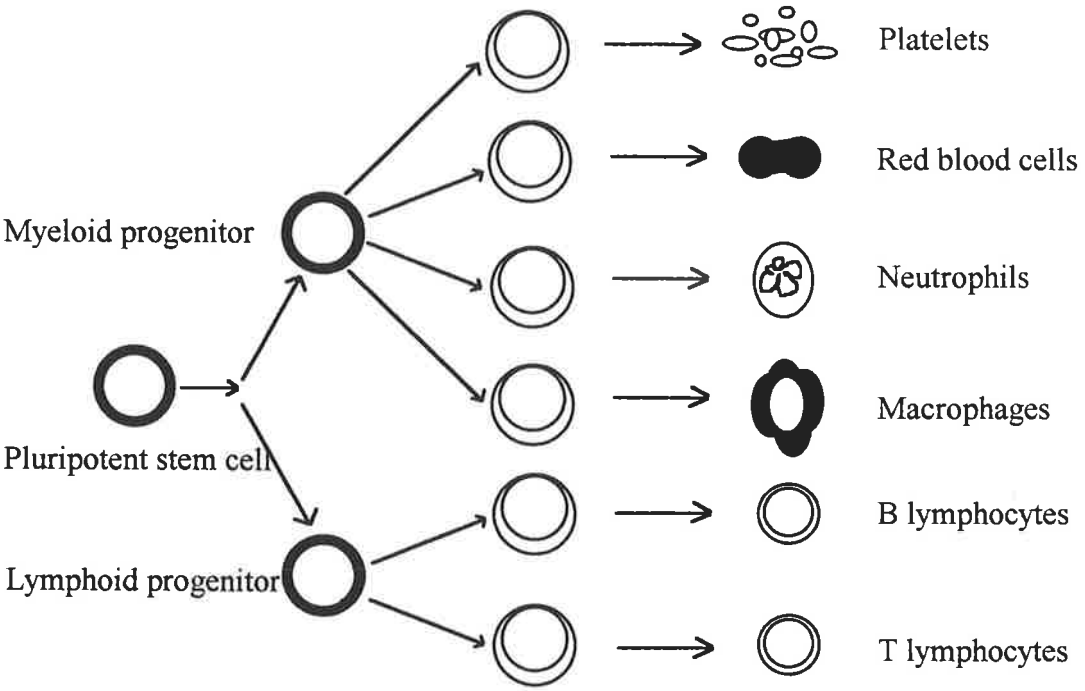

Figure 1-10 Simplified version of the cell lineages of the haemopoietic system. In leukaemia, differentiation of the progenitor cells to mature blood cells is blocked at some point (from Sawyers *et al.*, 1991).

Loss of A and B antigens occurs mainly in the myeloid malignancies, which include chronic and acute myeloid leukaemia, the myelodysplastic syndromes, and myeloproliferative disorders. At onset, chronic myeloid leukaemia is essentially a myeloproliferative disorder in which the cells retain their full capacity for differentiation. This chronic phase is eventually followed by a blast crisis, in which differentiation of the cells is arrested at a progenitor stage.

There are eight subtypes of acute myeloid leukemia, classified according to the French-American-British (FAB) scheme; AML M0 to M7. A summary of the

classifications and simplified features of the various subtypes of AML and the myelodysplastic syndromes referred to in this thesis is shown in Table 1-3 below.

FAB classifications of AML	Simplified Description
AML M0	acute undifferentiated leukaemia; stem cell leukaemia
AML M1	acute myeloid leukaemia or acute non-lymphocytic leukaemia without maturation
AML M2	myeloblastic leukaemia with maturation
AML M3	hypergranular promyelocytic leukaemia
AML M4	myelomonocytic leukaemia
AML M5	monocytic leukaemia
AML M6	erythroleukaemia
AML M7	megakaryoblastic leukaemia
FAB subsets of myelodysplastic syndrome	**Characteristics**
refractory anaemia (RA)	<1% blasts in PB; < 5% blasts in BM
refractory anaemia with ringed sideroblasts (RARS)	<1% blasts in PB; <5% blasts with ringed sideroblasts comprising >15% of nucleated cells in BM
chronic myelomonocytic leukaemia (CMML)	<5% blasts with peripheral monocytosis (>1 x 10^9/l) in PB; 5-20% blasts in BM
refractory anaemia with excess blasts (RAEB)	<5% blasts in PB; 5-20% blasts in BM
refractory anaemia with excess blasts in transformation (RAEB-t)	>5% blasts in PB; 20-30% blasts in BM

Table 1-3 French-American-British classification of acute myeloid leukaemia and myelodysplastic syndrome (from Henderson, 1990). PB indicates peripheral blood; BM is bone marrow.

1.30 Disruption of the ABL proto-oncogene at 9q34.1 in CML

CML cells have a characteristic translocation t(9;22), which is found in the malignant pluripotent haemopoietic stem cells and all their progeny. The translocation, disrupts the Abelson oncogene (ABL) on chromosome 9 (9q34.1), and the BCR (break-point cluster region) gene on chromosome 22 (22q11.2)(reviewed in Dobrovic *et al.*, 1991). The derivative chromosome 22 is known as the Philadelphia chromosome (Baikie *et al.*, 1960). The Philadelphia chromosome is also found in 1% of AML and 20% of adult ALL cases (reviewed in Dobrovic *et al.*, 1991), and some myeloproliferative disorders (Heim and Mitelman, 1987).

The ABL gene codes for a tyrosine kinase which is predominantly localised in the nucleus (Van Etten *et al.*, 1989). The BCR-ABL fusion protein exhibits enhanced (cytoplasmic) tyrosine kinase activity, possibly due to the loss of a negative regulatory domain from the 5' end of the gene (Konopka *et al.*, 1984; Konopka and Witte, 1985; Kloetzer *et al.*, 1985; Clark *et al.*, 1987; Jackson and Baltimore, 1989). Daley *et al.* (1990), introduced the protein produced from the fusion of the 5' region of the BCR gene, and the 3' end of the ABL gene into cultured murine bone marrow cells, and transplanted them into syngeneic mice; the mice subsequently developed a disease similar to CML, confirming that the BCR/ABL protein is a potent leukaemogenic agent.

Although ABL is located between the AK1 and ABO genes in 9q34 (Attwood *et al.*, 1994), it is unlikely that disruption of this region by the translocation leads to loss of ABO antigens, as approximately 90% of CML patients have the Philadelphia chromosome (Nowell and Hungerford, 1960; Rowley, 1973), but loss of A and B antigens seems to be a relatively rare event.

1.31 Disruption of other genes at 9q34 in leukaemia

A subset of patients with AML carry a t(6;9)(p23;q34) translocation. Von Lindern *et al.* (1992), cloned an aberrant transcript produced in these patients and concluded that the 5' part of the DEK gene from 6p23 forms a fusion protein with the 3' region of the CAN gene at 9q34. The CAN gene lies approximately 360kb distal to ABL (Von Lindern *et al.*, 1990). Kraemer *et al.* (1994), inferred from the cDNA sequence of the CAN gene that there was partial homology (at the amino acid level) to the putative rat nucleoporin (nuclear pore complex) protein. Monoclonal antibodies were subsequently developed to the rat protein, and used to probe proteins produced by *E. coli* expressing the CAN cDNA construct. The results lead the investigators to conclude that the CAN gene probably encodes a nucleoporin,

and is an oncogene involved in leukaemogenesis. However, confirmation of this awaits further molecular analyses.

Disruption of the CAN gene could be related to loss of ABO antigens. However, no translocation or other cytogenetic changes have been associated with patients in which blood group antigens have been observed to date. Therefore if the CAN gene is disrupted in loss of ABO patients, it is either due to microdeletions not detected during cytogenetic analysis, or by some other means, for example gene silencing.

Another gene found in 9q34.3 that is also disrupted in leukaemia is the Translocation-Associated "Notch" homolog, known as the TAN1 gene. The gene was found by cloning the translocation breakpoint [t(7;9)(q34;q34.3)], from a patient with acute T-cell lymphoblastic leukaemia (T-cell ALL),(Ellisen *et al.*, 1991). TAN1 is highly homologous to the Drosophila Notch gene, hence its name. Ellisen *et al.* (1991) found that the (7;9)(q34;q34.3) translocation resulted in truncation of the TAN1 transcript in 3 cases of T-cell ALL, and interpreted the observations to mean that TAN1 is integral to normal lymphocyte function. At least one other "Notch" homolog is expressed in human bone marrow, suggesting that TAN1 and other members of the "Notch" family could be involved in haematopoiesis (Milner *et al.*, 1994).

1.32 Tumour suppressor genes and loss of ABO antigens

Tumour suppressor genes (TS genes), might best be defined as recessive oncogenes; that is functional loss of both copies of the gene is required for transformation (reviewed in Stanbridge, 1990). The presence of a TS gene is generally deduced by recurrent deletions and loss of heterozygosity (LOH) in a region of the genome.

Loss of ABO antigens might in fact be due to deletion or inactivation of an entire region of 9q34; this is supported by the AK1 data. As loss of ABO or AK1 is not likely to be important in the aetiology of these leukaemias, these results may indicate the presence of a TS gene in the 9q34 region. The AK data presented by Salmon *et al.*, 1968 (see Section 1.20), suggested that there was allelic loss of the AK1 product. If there were allelic loss of AK1 and ABO, one would expect allelic loss of the presumed TS gene (ie. loss of heterozygosity). The other "normal" copy of the TS gene would have to also have undergone some alteration in expression to satisfy TS criteria. This could be due to mutational inactivation, as is seen in the P53 tumour suppressor gene, or epigenetic inactivation of the normal copy of the gene.

An example of epigenetic inactivation of a tumour suppressor gene is the human H19 gene. The H19 gene is normally imprinted, that is only the maternally inherited copy of the gene is expressed (Zhang *et al.*, 1993). However, in Wilm's tumours, both copies can be inactivated (Moulton *et al.*, 1994) or the maternal (active) allele lost (Zhang *et al.*, 1993). It appears that genomic imprinting and transcriptional silencing of genes might also be involved in the pathogenesis of a number of other human cancers (reviewed in Tycko, 1994).

There is some evidence that a TS gene for leukaemia may exist on the long arm of chromosome 9. Deletions of 9q11-9q32 repeatedly occur in AML (Trent *et al.*, 1989). The region 9q22 appears to be the minimal recurrent deletion in a variety of AML, MPD, and MDS disorders, and a putative TS gene (Growth Arrest Specific 1; GAS-1) has been mapped to this region (9q21.3-q22) (Evdokiou *et al.*, 1993). Perhaps a more likely candidate for a TS gene on 9q is in fact the ABL gene. Recent evidence suggests that the nuclear tyrosine kinase encoded by ABL

negatively regulates cell growth; and therefore, loss of both copies of ABL would lead to growth deregulation (Sawyers *et al.*, 1994; Mattioni *et al.*, 1995).

1.33 AIM OF THIS PROJECT

The aim of the project described within this thesis is to determine the molecular basis of loss of ABO antigens in haematological malignancy. Expression of the A and B antigens is directly dependent on expression of the ABO glycosyltransferase, and this transferase appears to be reduced or absent in the red cells of some patients with haematological malignancy. As loss of A and B antigens *per se* is unlikely to be important in cancer progression, and as these changes reflect a genetic event that is occurring in the malignant stem cell, they may indicate the presence of the tumour suppressor gene in the region of 9q34. It is therefore important and relevant to examine the molecular changes at the ABO locus which result in this phenomenon.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.0 REAGENTS AND SOLUTIONS

All reagents were of analytical grade and, unless otherwise stated, were obtained from distributors of Ajax chemicals (Sydney, Australia), Amersham Australia (North Ryde, Australia), Amrad-Pharmacia Biotech (Melbourne, Australia), BDH Laboratory Supplies (Poole, England), Biorad Laboratories (North Ryde, Australia), Boehringer-Mannheim (North Ryde, Australia), Bresatec (Thebarton, Australia), Commonwealth Serum Laboratories (CSL, Parkville, Australia), Difco (Detroit, USA), Gibco-BRL Life Technologies (Australia), Sigma Chemical Co. (St Louis, USA), New England Biolabs (Genesearch, Brisbane, Australia), Progen Industries (Darra, Australia), Promega (Melbourne, Australia), Selby (Australia), and Qiagen (Chatsworth, USA).

2.1 SOLUTIONS USED FOR THE ANALYSIS OF RED CELLS

Antibodies

Anti-A, anti-B, anti-AB (Epiclone ® murine monoclonal antibodies for blood grouping), anti-H (*Ulex europaeus* lectin), and anti-A^1 (*Dolichos biflorus* lectin) were all obtained from CSL, and used neat.

30% solution of polyvinyl pyrrolidone (PVP)

30g of PVP (MW 40,000) was dissolved in 100mls of a 0.6% saline solution.

Phosphate-buffered saline (PBS)

137mM NaCl
2.7mM KCl
4.3mM $Na_2HPO_4.7H_2O$
1.4mM KH_2PO_4

2.2 SOLUTIONS USED FOR CELL ISOLATION AND CULTURE

Trypsin-versene
0.1% Trypsin (Difco)
0.1% EDTA
99.8% RPMI1640 (Gibco-BRL)

Growth Media

Media (DMEM or RPMI 1640; Gibco-BRL), was diluted in dH_2O according to the manufacturer's instructions, and supplemented with 3.7g/l $NaHCO_3$ (Sigma), 60μg/ml benzylpenicillin (Sigma), 50μg/ml dihydrostreptomycin (Glaxo), and 100μg/ml L-glutamine (Sigma), 3.5g/l D-glucose (added to DMEM only; Sigma). HAT medium was essentially DMEM containing 1 x HAT solution (Gibco-BRL). All media were filter sterilized using a 0.22μM disposable filter (Cornwell), before the addition of fetal calf serum (CSL) to a final concentration of 10%.

2.3 SOLUTIONS FOR MOLECULAR BIOLOGY

EDTA (ethylenediamine tetra-acetic acid), 0.5M
186.1g of $Na_2EDTA.2H_2O$ was dissolved in 700ml H_2O and the pH adjusted to 8.0 with 10M NaOH, before adjusting the total volume to 1litre.

TEN1 buffer
50mM Tris-HCl, pH8
2mM EDTA
400mM NaCl

Phenol

2-3 grains (0.1%) of 8-hydroxyquinoline (Sigma), were added to 100mls of phenol (Sigma). The phenol was equilibrated by washing it 100mls of 0.5M Tris-HCl (pH 8.0). The phenol was drawn from the Tris-HCl, and washed with 100mls of 0.1M Tris-HCl (made with DEPC treated H_2O; pH 8.0). This step was repeated until the phenol was pH 7.8. Finally, 0.2% v/v β-mercaptoethanol (Sigma) was added and the phenol stored at 4°C and used within 4 weeks, or stored at -20°C until required.

TE buffer
10mM Tris-Cl, pH 8.0
1mM EDTA, pH 8.0

TES1 buffer
10mM Tris-Cl pH 8.0
1mM EDTA
100mM NaCl

Solution D
4M guanidinium isothiocyanate (Sigma)
25mM sodium citrate, adjusted to pH 7 with citric acid
0.5% sarcosyl
0.1M 2-mercaptoethanol (Sigma)

DEPC (diethylpyrocarbonate) treated H_2O

0.2mls of DEPC (Sigma) was added to 100mls of ultrapure H_2O. The solution was shaken vigorously every 10 minutes for 1hr, before autoclaving.

Loading buffer (6x)
50% glycerol
0.2M EDTA, pH 8.3
0.05% bromophenol blue

5x Tris-Borate (TBE) buffer

To 1600mls of dH_20, 108g of Trizma base, 55g of boric acid, and 40mls of 0.5M EDTA (pH 8.0) was added. The volume was then adjusted to 2 litres with dH_20.

20x SSPE
3.6M NaCl
0.2M Sodium phosphate
0.02M EDTA pH 7.7

10mg/ml Salmon Sperm DNA

100mgs of salmon sperm DNA (Sigma), was dissolved in 10mls of dH_20 to which 200µl of 10N NaOH was added. The mixture was boiled for 10 minutes and sheared using an 18-gauge needle.

Sephadex column

0.25g of Sephadex G50 (Pharmacia) was suspended in 6mls of dH_20 and allowed to swell for at least 2hrs at 65°C. A Poly-Prep chromatography column (Biorad), was filled with 2mls of Sephadex solution and placed inside a 50ml tube. Excess liquid was removed from the column by gravity flow. The column was then flushed with 190µl of 200mM β-mercaptoethanol and 10µl of 10mg/ml salmon sperm, and centrifuged at 70 x *g* for 10 minutes, at which time the column was ready for use.

Prehybridization mix
5xSSPE
0.5% SDS
5x Denhardt's solution

100x Denhardt's solution
2% Ficoll (Flow laboratories)
2% bovine serum albumin
2% PVP (MW 40,000)

Stripping solution
5mM Tris-Cl, pH8
200µM EDTA
0.02% SDS

40mM dNTP solution

A 40mM stock solution of dNTPs containing approximately 10mM of each nucleotide was prepared. 50mgs of each dNTP (2'-deoxy-adenosine-5'-triphosphate disodium salt; 2'-deoxy-cytidine-5'-triphosphate disodium salt; 2'-deoxy-guanosine-5'-triphosphate disodium salt and 2'-deoxy-thymidine-5'-triphosphate tetrasodium salt; Boehringer-Mannheim), were pooled and dissolved in 9mls of DEPC treated water. The pH of the solution was adjusted to pH 7.0 by drop-wise addition of 1M Tris-base, after which the final volume of the solution was adjusted to 10mls.

Ligase 10x buffer
300mM Tris-HCl, pH 7.5
100mM $MgCl_2$
100mM DTT
10mM ATP

IPTG stock solution (0.1M)
1.2g IPTG (Sigma)
H_20 to 50mls, filter sterilized

X-Gal (50mg/ml)
50mg of x-gal powder (Progen), was dissolved in 1ml of Dimethyl Formamide (Sigma).

Luria Broth (LB) Medium
10g Bacto-tryptone (Difco)
5g Bacto-yeast extract (Difco)
5g NaCl
dH_20 to 1 litre
brought to pH 7.0 (using 10N NaOH), and autoclaved.

Preparation of LB plates with ampicillin/IPTG/X-Gal

15g of agar (Difco) was added to 1 liter of LB medium and boiled. When the medium had cooled to 50°C, ampicillin (Sigma), was added to a final concentration of 100μg/ml, IPTG to a final concentration of 0.5mM, and X-gal (Progen) to a final concentration of 80μg/ml. Approximately 35mls of medium was poured into each 85mm petri dish and allowed to set. The plates could be used immediately or stored at 4°C for up to one month.

2.4 TECHNIQUES USED FOR SEROLOGICAL ANALYSIS OF RED CELLS

Red cells from patients were generally not available, although blood groups and in some cases other test results were available from the various laboratories that supplied the samples. In some cases, frozen red cells were able to be obtained from Dr Alex Dobrovic. Where fresh or frozen samples were available, preparation of red cells and blood grouping was carried out as described in the methods below.

2.4.1 Preparation of red cells for long-term storage

Red cells were separated from the other components of the blood or bone marrow by density gradient centrifugation (see 2.5.1). The cells were then resuspended in a two-fold excess of PBS, and washed by centrifugation at 300 x *g* for 10 minutes. The supernatant was removed and the cells washed twice more. All traces of PBS were removed, and a 1:1 mix of packed red cells in a 30% solution of PVP. The mixture was allowed to stand for 10 minutes, before being frozen dropwise in liquid nitrogen.

2.4.2 Thawing frozen red cells

When red cells were required, two to five frozen pellets were dropped in 10mls of 37°C PBS, inverted and sedimented by centrifuging at 300 x *g* for 10 minutes. Depending on the amount of haemolysis, this step was repeated until the supernatant was clear. The supernatant was then removed and the cells resuspended in PBS to produce an approximate concentration of 5% v/v red cells.

2.4.3 Blood grouping

One drop of anti-serum was added to one drop of red cell suspension (5% red cells in PBS). If the tile method of typing was being used, the cell/anti-sera mixture was dropped onto a tile, gently agitated for 1-2 minutes and then given a score of 1-12 based on the NATA approved* method of quantifying agglutination, as shown in Figure 2-1. If the tube method was used, the cell/anti-sera mixture was centrifuged for 20 seconds at 600 x *g*. The cell button was then gently dislodged, and the sample scored as above for agglutination. Positive and negative controls were included in all tests. If thawed red cells were being analysed, blood from healthy individuals that had been frozen for a minimum of three weeks was used for the controls.

If a mixed-field reaction was observed, the non-agglutinating cells were aspirated and washed in 300µl of PBS three times (700 x *g* for 20 seconds). The cells could then be resuspended in PBS (usually about 50µl), and tested for agglutination with other anti-bodies (eg. anti-H lectin).

* NATA (National Association of Testing Authorities, Australia). All tests performed in Australian hospital laboratories must be NATA certified.

	Score	Grade	Macroscopic Appearance
	12	++++	Complete agglutination, a single agglutinate of strongly reactive red cells.
	10	+++	Strong reaction, 1 or 2 large agglutinates.
	8	++	Strong reaction, a number of large agglutinates.
	5	+	Many small agglutinates.
	3	(+)	Weak granularity in cell suspension.
	0	0	An even cell suspension.

Figure 2-1. Scoring agglutination reactions. The examples shown use the "tube" method for blood grouping, however the scoring system is the same for both the tube and the tile methods.

2.5 CELL ISOLATION, CULTURE AND REGULAR MAINTENANCE

2.5.1 Isolation of the mononuclear cell fraction from patient samples

Peripheral blood or bone marrow samples were obtained from patients and centrifuged for 5 minutes at 500 x *g*. The plasma was removed and the remaining cells diluted in a two-fold excess of PBS. A density gradient was prepared by carefully underlaying the suspension with an appropriate volume of sterile Ficoll-Hypaque (Flow laboratories), generally 10mls of Ficoll-Hypaque to a 40ml suspension of cells, plasma and PBS. Centrifugation was carried out for 25 minutes at 300 x *g*. The mononuclear cells were then isolated by aspiration from approximately the middle of the gradient, and washed at least two times in a two-fold excess of PBS (500 x *g*, 10 minutes).

2.5.2 Cell lines

Cell lines were maintained in either HAT medium, DMEM or RPMI 1640, at 37°C in a humidified atmosphere supplemented with 5% CO_2. When cells reached confluency, they were either detached from their support by treating them with trypsin solution (adherent cell lines), or placed directly into 10ml tubes (suspension cell lines). Details of the specific cell lines used in this study are shown in Table 2-1; all cell lines were from laboratory stocks, except for the LIM series of cell lines, which were kindly provided by Dr. R.H. Whitehead from the Ludwig Institute for Cancer Research at the Royal Melbourne Hospital, Melbourne, Victoria, Australia. Karyotypes are not included as they were either not available, or inconclusive because there were so many marker chromosomes and/or unidentified double-minute chromosomes. It is however, worthy to note that the cell line K562 does not contain a normal chromosome 9, although it does contain a Philadelphia chromosome.

Cell line	Type	culture type	Reference
K562	CML - erythroleukaemia	suspension	ATCC* database
HiMeg	CML - megakaryocytic leukaemia	suspension	unpublished; personal communication with Dr. Alex Dobrovic
KCL22	CML	suspension	Kubonishi and Miyoshi, (1983)
HEL	erythroleukaemia	suspension	ATCC database
HL60	promyelocytic leukemia	suspension	ATCC database
HT29	colon carcinoma	adherent monolayer	ATCC database
SW480	colon carcinoma	adherent monolayer	ATCC database
LIM 1863	colon carcinoma	organoid/suspension	Whitehead *et al.*, 1992.
LIM 2412	colon carcinoma	suspension	Whitehead *et al.*, 1992.
LIM 1215	colon carcinoma	adeherent monoalyer	Whitehead *et al.*, 1992.
LIM 2405	colon carcinoma	adeherent monoalyer	Whitehead *et al.*, 1992.
LIM 2463	colon carcinoma	adeherent monoalyer	Whitehead *et al.*, 1992.

Table 2-1. Details of cell lines used in this study.

2.5.3 Freezing and thawing of cells

Isolated cells were prepared for freezing by resuspending them at a concentration of 10^7 cells per ml in RPMI 1640 (Gibco), containing 20% fetal calf serum (CSL), or autologous plasma. DMSO (dimethyl-sulfoxide, Sigma) was then added dropwise to a final concentration of 10%. The cells were then placed in 1.5ml aliquots in cryopreservation tubes (Nunc), and stored in liquid nitrogen.

Thawing of the cells was accomplished by agitating the ampoules in a water bath at 37°C. As soon as they were thawed, the cells were pelleted (2 minutes at 12,000 x *g*), and either DNA or RNA prepared as described below (see Sections 2.6.1 and 2.6.2). If it was required that the cells maintain viability after thawing, they were instead placed in a tube to which 10mls of the appropriate growth media was added drop-wise. The cells were then washed three times in growth media (10 minutes at 300 x *g* per wash), to remove any last traces of DMSO.

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* ATCC is an abbreviation for the American Tissue Culture Collection

2.6.1 Preparation of genomic DNA

Two methods were used to isolate genomic DNA from leukocytes, tumour samples and cultured cells. The phenol-chloroform method was used to isolate DNA for use in Southern hybridization experiments, while the "salting out" procedure was used to prepare DNA for use in PCR experiments.

Phenol-Chloroform extraction

Standard techniques (Sambrook et.al, 1989) were combined and modified as follows: Up to 20 million cells (or the equivalent of macerated tumor tissue) were resuspended in 500µl of TEN1 buffer to which 100µl of 10mg/ml Proteinase K (Sigma) and 100µl of 20% SDS (sodium dodecyl sulfate) was added. Following an overnight digestion at 37°C, the DNA was extracted with 500µl of phenol, and the resulting aqueous phase was collected and extracted once with phenol:chloroform:isoamylalcohol (50:49:1) and finally with 500µl of chloroform:isoamylalcohol (49:1). The DNA was precipitated with either an equal volume of isopropanol or two volumes of absolute ethanol, by centrifuging in a 1.5 ml microfuge tube at 12,000 x *g* for 30 minutes. The resulting DNA pellet was then washed in 70% ethanol, vacuum dried and dissolved in 400µl of TE buffer or distilled water, and stored at 4°C.

Salting out procedure for extracting DNA

This procedure was modified from Miller et. al., 1988. Cell lysis was carried out as described for the phenol-chloroform extraction method, with the exception that TES1 buffer was substituted for the TEN1 buffer. After digestion, an equal volume of 3M NaCl was added to the solution, and the tube shaken vigorously.

The tube was then placed on ice for 10 minutes, before centrifuging for 10 minutes at 7,000 x *g*. The supernatant containing the DNA was removed, whilst carefully avoiding the protein pellet at the bottom of the tube. The DNA was then precipitated and redissolved in the same manner as described for the phenol-chloroform extraction.

2.6.2 Preparation of total RNA

Total RNA was prepared from lymphocytes, bone marrow, cultured cells or tumor tissue for use in RT-PCR and Northern hybridization experiments using a modified version of the method published by Chomczynski and Sacchi (1987). Cells were counted and five to ten million were lysed by vortexing briefly in 500µl Solution D. Where RNA was to be prepared from tumours, the fresh tissue was snap-frozen in liquid nitrogen and then fixed in Tissue-TEK O.C.T compound (Miles Diagnostics, USA). A cryostat machine was then used to slice 15-30 sections of 17µm thickness, which were then placed directly in Solution D. The presence of fixative did not appear to adversely affect the RNA isolation. Specimens were stored in Solution D at -20°C indefinitely.

When RNA was required, the specimens in Solution D were thawed on ice. Extraction was carried out by sequentially adding 50µl 2M NaAc (pH 4.5), 500µl Phenol (saturated with DEPC treated water) and 100µl chloroform:isoamylalcohol (49:1). The cell suspension was then vortexed thoroughly and allowed to rest on ice for 15 minutes. The organic and aqueous phases were separated by centrifuging for 8 minutes at 7,000 x *g*. After centrifugation, the top 400µl of the aqueous phase was transferred to another tube and mixed with 400µl of isopropanol, then placed at -80°C for 20 minutes to overnight to precipitate the RNA. The RNA was sedimented at 12,000 x *g* for 20 minutes before the pellet was washed in 70%

ethanol and vacuum dried. The dried pellet was dissolved in 50µl of DEPC treated water.

2.6.2.1 Modification of the RNA isolation method for isolating RNA and DNA from limited patient samples

If $5x10^6$ cells or less were available from patients, the RNA extraction method described in 2.6.2 was scaled down appropriately, and carried out in a 0.5ml tube. After the top ¾ of the aqueous phase was removed for RNA precipitation, an equivalent amount of TEN1 buffer was added to the remaining aqueous and organic phases. The mixture was then vortexed and centrifuged for 5 minutes at 7,000 x *g* to separate the phases. Almost all of the aqueous phase was then removed, and DNA precipitated using two volumes of absolute ethanol, or one volume of isopropanol. The pellet was washed and dried as described in 2.6.2.

2.6.3 Quantitation of DNA and RNA

To quantitate DNA and RNA, 5µl of sample was diluted in 1000µl of water and the optical density at 260nm was determined using the Genequant DNA calculator (Pharmacia). Concentration of DNA was based on one OD unit being equivalent to 50µg of DNA per ml, while for RNA the concentration was calculated on the basis that one OD unit was equivalent to 40 µg of RNA per ml of solution.

2.6.4 Polymerase Chain Reaction (PCR)

Primer Design

Primers were designed with the assistance of the Primer (version 0.5), computer program, (copyright 1991, Whitehead Institute for Biomedical Research). Criteria requested by the program were set to:

Optimal primer length:	24
Minimum primer length:	22
Maximum primer length:	26
Optimal primer melting temperature:	70.0°C
Minimum acceptable primer melting temperature:	65.0°C
Maximum acceptable primer melting temperature:	75.0°C
Minimum acceptable primer GC%	20
Maximum acceptable primer GC%	80
Salt concentration (mM)	65
DNA concentration (nM)	50
Maximum number of unknown bases (Ns) allowed allowed in primer	0
Maximum acceptable primer self-complementarity (number of bases)	12
Maximum acceptable 3' end primer self-complementarity (number of bases)	8
GC clamp- how many 3' bases	0
Product range	variable

Synthesis and isolation of oligonucleotides

Oligonucleotides were synthesised on 0.2μM columns (Pharmacia-LKB) in a Pharmacia-LKB Gene Assembler Plus DNA synthesizer according to the manufacturer's instructions by Mr Terence Gooley at the The Queen Elizabeth Hospital. Initially, excess synthesis reagents were removed from the column by centrifuging in 1.5ml Sarsdedt tubes for 1 minute at 1,000 x *g*. The oligonucleotides were cleaved from their support using 28% NH_4OH; the column was placed in 1.5mls of -80°C NH_4OH, and centrifuged again for 1 min at 1,000 x *g* to soak the column. Cleavage was achieved by incubation at 37°C overnight. The ammonia solution was then removed and vacuum dried for approximately 2 hours, after which time the oligonucleotide pellet was resuspended in 1ml of TE.

The concentration of the oligonucleotides was determined as described for DNA (see 2.6.3), with the exception that one OD unit was assumed to represent an oligonucleotide concentration of 33μg/ml.

Standard amplification conditions

Unless stated otherwise, all PCR reactions were carried out as follows. Genomic DNA was isolated as described in section 2.6.1 of this chapter. Target DNA (100ng), was placed in a 0.5ml reaction tube in a total reaction volume of 50μl containing 100ng of each of the oligonucleotide primers to be used, 0.8mM dNTPs, 0.5 units of *Taq* DNA polymerase (Boehringer-Mannheim), in the presence of the buffer supplied by the manufacturer. The reaction was overlaid with one drop of mineral oil (Sigma), to prevent evaporation. The DNA was then denatured by heating the reaction to 94°C for 5 minutes, before amplification was achieved by 35 cycles of 96°C for 24 seconds, 60°C for 30 seconds, and 72°C for 1 minute. If the amplified product was to be used for cloning, a final extension of 72°C for 5

minutes was added to the standard protocol. The reaction was carried out in a water-cooled thermal cycler (ARN Electronics, Belair, South Australia).

2.6.5 Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated as described in section 2.6.2 of this chapter. Reverse-transcription was then accomplished as follows: 1μg of RNA was mixed with 0.5μg of random-hexamers (Pharmacia) in a total volume of 20μl of DEPC treated water. The RNA/random primer mix was then denatured by heating to 60°C for 5 minutes, followed by cooling on ice for 3 minutes. Twenty micro-litres of a reaction master-mix was then added, bringing the total reaction volume to 40μl, containing 200 Units of MMLV reverse-transcriptase (Gibco-BRL), 1 x First strand buffer (Gibco-BRL), 10mM DTT (Gibco-BRL) and 2mM dNTPs. The reaction was placed at 37° C for one hour, after which time 60μl of sterile (but not DEPC treated), water was added. The resulting cDNA was then stored at -20°C. RT-PCR was carried out using 5μl of the cDNA mix as target, under the same amplification conditions described for genomic DNA in section 2.6.4 of this chapter.

Removal of contaminating DNA from RNA prior to reverse-transcription

In some cases, it was desirable to remove contaminating DNA from RNA preparations. RNA (1μg), random hexamers (0.5μg), 1μl of 10 x DNAse I buffer (Gibco), and 1 unit of Amplification grade DNAse (Gibco), were incubated for 12 minutes at room temperature in a total volume of 10μl. To stop the reaction, 10μl of 2mM EDTA was added, and the mixture heated to 70°C for 10 minutes. The reaction was then cooled on ice for three minutes, and the reverse transcription master mix added as described above.

2.6.6 Gel electrophoresis

Agarose gel electrophoresis

PCR products were routinely resolved on 1.5% horizontal agarose slab gels (Progen), containing 100ng/ml of ethidium bromide (Biorad), in 0.5 x TBE buffer. Bands were visualised by subjecting the gel to UV (254nm) light, and photographed using Polaroid 665 film. The resulting negatives were scanned on a G670 densitometer (Biorad), for densitometric analysis and transfer to this thesis. The scans in this thesis are shown as the negative image to enhance them.

Polyacrylamide gel electrophoresis

Digested PCR products were generally resolved on 8% vertical polyacrylamide gels (29:1, acrylamide:bis ratio). A 40% stock solution of acrylamide (29:1; acrylamide: *bis*-acrylamide ratio), obtained from Biorad, was diluted in 1x TBE buffer, to which 420µl of a 10% solution of ammonium persulfate (Biorad) and 10µl of TEMED (BRL), was added. Bands were visualised by staining the gel in ethidium bromide (5µg/ml) and subjecting the gel to UV (254nm) light.

2.6.7 Southern blot analysis

Preparation of samples

Genomic DNA was prepared and quantitated as described (2.6.1 and 2.6.3). Restriction digests were then performed on the DNA according to the manufacturers' instructions. Generally, 10µg of DNA was digested with 20 units of enzyme in the appropriate buffer for 12 hours (total volume 70µl). The restricted DNA was then concentrated by the addition of 1/10th volume 3M NaAc, 2 volumes of ethanol, and precipitation at -20°C overnight. After centrifugation and washing

in 70% ethanol, the dried DNA pellet was resuspended in 15µl of H_2O and 3µl of 6x loading buffer.

Electrophoresis

The digested DNA samples were electrophoresed in a 0.9%, 0.5xTBE agarose gel, at 40V overnight. The gel was stained with ethidium bromide (0.5µg/ml) and photographed next to a ruler to allow graphical determination of band sizes later. The gel was then placed in 0.25M HCl for 20 minutes to depurinate the DNA, after which time it was rinsed in distilled water in preparation for the alkali blotting procedure.

Alkali blotting procedure

A tray was filled with alkali transfer buffer (0.4M NaOH) and covered with a sheet of glass on which three sheets of Whatman 3MM filter paper were placed to form a wick. The gel was placed top side down onto the wick and surrounded with cling film to prevent buffer leakage. A sheet of Hybond N+ membrane (Amersham), was placed on top of the gel, and three more pieces of Whatman paper (wetted with transfer buffer) and a stack of absorbent paper towels placed above. A glass plate laid on the paper towels and weighted with a 100ml bottle of liquid ensured an even transfer from the whole gel to the membrane. The transfer was allowed to proceed for between three to twelve hours. After blotting, the membrane was rinsed briefly in 2 x SSPE, wrapped in cling film and stored at 4°C.

Radio-labelling of probes

The Gigaprime DNA labelling kit (Bresatec), was used to label DNA with α-^{32}P-dCTP (Bresatec) by the random-priming method. Up to 200ng of DNA was labelled according to the manufacturer's instructions. The labelled probe was then isolated from unincorporated nucleotides and primers by placing it on a prepared Sephadex column, and centrifuging for 6 minutes at 90 x *g*. The eluate containing the purified probe was retrieved and used immediately.

Procedure for membrane hybridization

Hybridizations were carried out in bottles in a rotary incubator (Hybaid). The membrane was prehybridized at 65°C for one to five hours in 25mls of prehybridization mix. Sheared and denatured salmon sperm (100µg),was added as a blocking agent. After prehybridization, 200ng of the labelled probe was denatured by heating to 100^{0}C for five minutes, snap-cooled on ice, and added to the bottle. Hybridization was then carried out at 60-65°C for at least 12 hours.

Washing the membrane

Following hybridization, the membrane was washed twice in 2xSSPE, 0.1%SDS at room temperature for ten minutes. The wash solution was then replaced with 1xSSPE, 0.1%SDS and incubated at 65°C for 15 minutes, after which time the counts emitted from the filter were measured. If the counts were between two and twenty counts per second (cps), the filter was wrapped in cling film and autoradiographed. Membranes with higher cps were sometimes washed in a high stringency wash (0.1xSSPE, 0.1%SDS), again at 65°C for fifteen minutes before autoradiography.

Autoradiography

The membrane was wrapped in cling film and placed in a cassette next to X-ray film (Hyperfilm, Amersham), and exposed at -80°C for a minimum of twelve hours. The film was then developed using an automatic developer (Kodak Australia).

Re-use of blots

Membranes could be stripped and re-probed by immersing them in a solution of boiling 0.5% SDS, and allowing to cool to room temperature. If this method failed to remove the probe, the membrane was boiled in stripping solution (described in 2.3) for 1hr.

2.6.8 Purification of PCR products for use in cloning and sequencing experiments

PCR products for use in cloning or sequencing were either directly purified or isolated from a low-melting point agarose gel (Progen Australia), before purification. Primers, nucleotides and non-specific products were removed using the Wizard (Magic) PCR Preps kit (Promega). To isolate PCR product from agarose, the desired band was excised and melted at 70°C. When the agarose was thoroughly melted, one ml of Wizard resin was added, and the slurry vortexed for 20 seconds. To purify PCR products directly, 30-300µl of PCR product was added to 100µl of direct purification buffer, vortexed, one ml of Wizard preps resin added, and vortexed briefly 3 times over a one minute period. The DNA/resin slurry was then passed though a Wizard minicolumn attached to a 2ml syringe (Terumo), after which the column was washed by flushing it with 2mls of 80% isopropanol (Sigma). The column was dried by placing it in a microcentrifuge tube

and centrifuging it for 20 seconds at 12,000 x *g*. The DNA was eluted from the column by adding 50µl of TE and centrifuging it for 20 seconds at 12,000 x *g*.

2.6.9 Cloning of PCR products

The pGEM-T vector (Promega) is prepared by digesting pGEM-5Zf(+) (Promega) with EcoRV and the addition of a 3' terminal thymidine to both ends. As Taq DNA polymerase contains template-independent terminal A transferase activity, this makes the pGEM-T vector an ideal plasmid for cloning of PCR products, therefore we chose to use this system for cloning of PCR products.

Preparation of competent cells, transformation and analysis of recombinant plasmid DNA was carried out essentially as described in Promega's "Protocols and Applications Handbook", with slight alterations as described below.

Ligation of purified PCR product into the pGEM -T Vector

Vector (50ng), and a variable amount of purified PCR product (in a 1:1 - 1:3 molar ratio respectively) were incubated for 3hrs at 15°C, in ligase buffer with one Weiss unit of T4 DNA-ligase enzyme (Promega), in a total reaction volume of 10µl. The reaction was terminated by heating to 72°C for 10 minutes.

Preparation of competent bacterial cells

XL1-Blue (XL1-B) bacterial cells (Bullock *et al.*, 1987) were used for cloning of PCR products. A single colony was used to inoculate 5mls of LB (containing 10µg/ml Tetracycline) and incubated overnight at 37°C in a shaking waterbath. Of the overnight culture, 1ml was introduced into 25mls of LB (containing

tetracycline). The cells were harvested while in the exponential growth phase, as determined by the optical density at 600nm (0.4-0.6, approximately 2hrs after inoculation). The cells were then cooled on ice for 10 minutes, before they were sedimented by centrifugation at 3,000 x *g* (4°C) for 5 minutes. The resultant pellet was resuspended in 2mls of 0.1M $CaCl_2$/20mM $MgCl_2$, and incubated on ice for 1-2 hours before transformation.

Transformation of XL1-Blue cells

The competent cells were transformed with 2µl of ligation mix, or the equivalent of 10ng of vector DNA. An aliquot of cells were also transformed with supercoiled plasmid DNA (eg. pBluescript) as a positive control. Briefly, 200µl of competent cells were placed on ice with ligated DNA and vector. The mix was then incubated at 42°C for 2 minutes in a water bath. The transformed cells were then placed on ice for ten minutes, then rested at room temperature for a further ten minutes. LB containing 20mM glucose (900µl) was then added, and the cells allowed to recover at 37°C for one hour. The transformed cells were plated onto agar plates containing tetracycline (10µg/ml) and ampicillin (50µg/ml), 0.5mM IPTG and 80µg/ml X-Gal, and incubated overnight at 37°C.

Analysis of Transformants

Standard colour selection allowed white (recombinant) colonies to be differentiated from colonies not containing parent vector (blue). The colonies were picked from the plate using sterile toothpicks, which were then used to inoculate firstly 5mls of LB broth containing tetracycline and ampicillin (20 and 50µg/ml respectively), and secondly PCR reaction mix containing universal sequencing primers (see below). The inoculated cultures were incubated overnight.

Screening bacterial colonies for recombinant plasmids using PCR

The standard PCR conditions described in 2.6.4 were used to detect the presence of recombinant plasmids from freshly picked bacterial colonies, with the following exception. The amplification reaction was carried out in a Peltier-cooled thermal cycler (PTC-100, MJ Instruments, USA). An initial denaturation of 5 minutes was followed by 35 cycles of 94°C for 42 seconds, 55°C for 1 minute, and 72°C for 1 minute. The primers used for this reaction were the universal forward and reverse pUC/M13 sequencing primers (Promega), which span nt 2,944-177 of the pGEM-T vector sequence. The size of the products were estimated from 1.5% agarose gels. Parent vector produced a 236bp band, while vector plus insert produced a band of 236bp plus the size of the inserted fragment.

Small scale isolation of recombinant plasmid

Of each of the overnight cultures, 1.5mls was placed in a microcentrifuge tube and centrifuged at 12,000 x *g* for 1 minute. The supernatant was aspirated and the pellet resuspended by vortexing in 100µl of ice-cold lysis buffer (25mM Tris-HCl, pH 8.0, 10mM EDTA, 50mM glucose). After incubation for 5 minutes at room temperature, 200µl of 0.2N NaOH/ 1% SDS was added and mixed with the cells by inversion. After incubation on ice for 5 minutes, 150µl of ice-cold potassium acetate solution (pH 4.8) was added, mixed by inversion, and again incubated on ice for 5 minutes. Bacterial cell debris and DNA was then sedimented by centrifugation at 12,000 x *g* for 5 minutes and the supernatant containing the plasmid DNA aspirated and placed in a fresh tube. Bacterial RNA was then degraded by the addition of DNase-free RNase A (Boehringer-Mannheim), at a final concentration of 20µg/ml and incubation at 37°C for half an hour. Degraded RNA and the RNase A were then removed from the preparation by phenol/chloroform extraction. The solution was extracted once with an equal

volume of phenol/chloroform:isoamylalcohol (49:1) by vortexing for 1 minute before centrifugation 12,000 x *g* for 5 minutes. The aqueous phase was then extracted once with an equal volume of chloroform: isoamylalcohol (49:1) and again vortexed and centrifuged. The plasmid DNA was precipitated by the addition of 2.5 volumes of ethanol and incubation at -80°C for 10 minutes. This was followed by centrifugation for 5 minutes at 12,000 x *g*, and the DNA pellet was then rinsed with 70% ethanol before drying under vacuum. The dried plasmid DNA pellet was then dissolved in 30µl of sterile water.

Larger scale isolation of recombinant plasmids

If plasmids were to be used for sequencing, purification was carried out from a 25ml bacterial culture using Qiagen tip-100 Plasmid Midi-prep kit, according to the manufacturer's instructions. Briefly, the bacteria were pelleted by centrifugation at 15,000 x *g*, at 4°C for 10 minutes. The bacterial pellet was resuspended in 4ml of the supplied buffer P1. Following the addition of 4mls of the supplied P2 buffer, the mixture was incubated at room temperature for 5 minutes. A 4ml aliquot of the supplied buffer P3 (chilled) was added, and the mixture incubated on ice for 15 minutes. Bacterial debris was pelleted by centrifuging the mixture at 30,000 x *g*, at 4°C for 30 minutes. The supernatant was immediately removed and centrifuged again (30,000 x *g* at 4°C for 30 minutes) to pellet any remaining bacterial debris. A Qiagen tip-100 was equilibrated with 4mls of the supplied buffer QBT. The supernatant containing the plasmid was then applied to the tip, and the tip washed twice with 10mls of the supplied buffer QC. The plasmid DNA was then eluted from the tip with 5mls of the supplied buffer QF, and precipitated with 0.7 volumes of isopropanol (Sigma), by centrifugation at 15,000 x *g* at 4°C for 30 minutes. The resultant DNA pellet was washed with 5mls of cold 70% ethanol and air dried before being dissolved in 50µl of TE.

2.6.10 Sequencing

Automatic sequencing

Sequencing of PCR products was performed at Flinders Medical Centre, Bedford Park, South Australia on an ABI model 373A automatic sequencer (Applied Biosystems). The sequence was read by the software supplied with the sequencer (version 2.0.1S). The sequence was also read manually as the high background fluoresence of the red label (thymidine), was not taken into account by the program. Subsequently, bases that were called as "N" by the program could often be resolved manually.

Denaturation of plasmid template for manual sequencing

Plasmid DNA was diluted in 20µl of H_2O containing 20µg of RNase A (Boehringer-Mannheim), and incubated at 37°C for 15 minutes. The mixture was then incubated a further 15 minutes with the addition of 5µl 1M NaOH/ 1mM EDTA. A mini-spin column was then prepared by piercing a 1.5ml tube with a needle, into which a small plug of fibre-glass wool was placed, overlayed with 500µl of Sepharose CL-6B (Pharmacia). The column was centrifuged for 3 minutes at 500 x *g*, and the DNA mixture then added and the spin repeated; the eluate being collected in a clean 1.5ml tube placed underneath the tube containing the column. An aliquot (7µl) of the denatured plasmid was used in the sequencing reaction.

Manual sequencing

Denatured plasmids (4µg) were manually sequenced using the Sequenase version 2.0 DNA sequencing kit (Amersham-USB), according to the manufacturer's instructions.

Briefly, the denatured plasmid, 2µl of the supplied reaction buffer, and 0.5pmol of the sequencing primer (universal pUC/M13 forward or reverse sequencing primers as described in 2.6.9), were brought to a total volume of 10µl by the addition of dH_2O. The primer was annealed by heating the mixture for 2 minutes at 65°C, and then slowly cooling it to room temperature (20-30 minutes). The reaction was chilled on ice, and a mixture of 1µl 0.1M DTT, 2µl labeling mix (dGTP, diluted 1:5 just before use), 1µl ^{35}S dATP (10µCi/µl and 10µM dATP at 1000Ci/mmol; Bresatec), and 2µl of diluted Sequenase Polymerase (diluted 1:8 in the supplied glycerol enzyme dilution buffer just before use). Primer extension was accomplished by incubating the reaction at room temperature for 5 minutes. The reactions were then terminated by the addition of 3.5µl of the labeling reaction to each of 4 tubes (labelled A,C,G and T), containing 2.5µl of the appropriate supplied termination mixes (ddATP, ddCTP, ddGTP, ddTTP, at room temperature), and incubated at 37°C for 5 minutes. The reactions were then stopped by the addition of 4µl of the supplied Stop solution. The reactions were concentrated and denatured by heating them to 95°C with the lids open for a few minutes before loading 3µl per lane onto a polyacrylamide sequencing gel.

Sequencing gels

Sequencing reactions were resolved on 6% polyacrylamide gels containing 7M urea. A 40% stock solution of acrylamide (19:1; acrylamide: *bis*-acrylamide), obtained from Biorad, was diluted in 1 x TBE buffer, to which 420µl of a 10% solution of ammonium persulfate (Biorad) and 70µl of TEMED (BRL), were added. Electrophoresis was carried out at 55 watts for 2-3 hours, after which time the gel was soaked in 5% acetic acid, 15% methanol to remove the urea. The gel was then gently attached to Whatman gel drying paper and dried under vacuum at 60°C. The dried gel was then exposed to Hyperfilm-MP (Amersham) overnight.

CHAPTER THREE

RED CELL SEROLOGY

3.0 INTRODUCTION

The A and B antigens of the ABO blood group system are formed by sequential addition of specific sugars to precursor carbohydrate structures. It follows that changes in A or B antigen expression might be due to any break in this chain of events. For example, if the precursor H antigen was absent, there would be loss of A and/or B antigens, regardless of whether or not the ABO gene was expressed. As the aim of this project was to specifically examine the ABO gene for molecular changes in patients with full or partial loss of A or B antigens, it was necessary to determine the presence/absence of the H antigen on red cells from these patients.

The aim of this chapter is to provide essential background to the rest of this thesis, and to describe difficulties encountered during this project, that in some ways explain why the observation of loss of A and B antigens is so rare.

3.1 CONSIDERATIONS AND DIFFICULTIES ENCOUNTERED WHEN COLLECTING SAMPLES FROM PATIENTS WITH CHANGES IN ABO BLOOD GROUP STATUS

Patients with changes in ABO blood group are relatively rare, and samples are difficult to obtain for a number of reasons. One major difficulty is that blood group changes in patients are often not recognized. Changes in blood group antigen expression are usually characterized by what is termed a "mixed-field reaction" (mfr). This refers to a partial agglutination of red cells with the appropriate antibody, implying the presence of cells that do not express the corresponding A or B antigen. Alternatively, if some of the cells are expressing lower amounts of A or B antigen, a weak reaction may be seen. In a weak reaction, all of the cells agglutinate, however the agglutinates are very small and easily disrupted. In most cases with loss of A or B antigen expression, there is a combination of cells that appear to express small amounts of antigen, cells that do not express any antigen, and cells expressing antigen. Weak and mixed-field reactions are rarely reported and can be difficult to determine, particularly considering the methods routinely used for ABO blood group typing.

There are two common methods used to determine ABO blood group. In the "tile" method, one drop of diluted blood is mixed with one drop of any of the commonly available antisera and placed on a transparent plastic tile over an illuminated box. Agglutination is easily observed, as is non-agglutination of cells. In Figure 3-1, the tile method has been used to show various amounts of O blood group cells combined with decreasing numbers of A group red cells; simulating mixed-field reactions of varying strengths. Using this method, it is possible for the practiced eye to determine the presence of as low as 5% O cells in 95% A red cells. However, the majority of modern blood transfusion laboratories use the "tube" test. This is considered to be more sensitive, although my results show that this is only true in the case of agglutination; a positive result. Briefly, the tube test consists of

adding one drop of diluted blood to one drop of antibody. The mixture is centrifuged, and the red cell pellet is then gently dislodged, and agglutination scored on a scale of 1 to 12 (see Section 2.4.3), where a score of 12 represents complete and strong agglutination.

3.1.1 Evidence that mixed-field reactions are not perceived

Towards the end of the practical work of this thesis, I was demonstrating ABO blood grouping to another student. As it happened, the sample I chose (patient SR) exhibited a definite mixed-field reaction with anti-A, but not with anti-B, or anti-A,B (see Figure 3-2). I immediately contacted the Blood Transfusion Unit at the QEH, who had also grouped the same specimen. I was surprised to learn that the unit had scored the sample as 12 for all three antibodies. As always, two staff members had independently grouped the specimen and both had obtained the same results. At my request, they again grouped the sample. This time the scores were 12 for anti-B and anti-A,B, and 10 for anti-A. I was assured that I was mistaken in believing this to be a mixed-field reaction. Later the same day, the sample was grouped yet again, this time by the supervisor of the laboratory, who scored 12 for anti-B and anti-A,B, but 6 for anti-A.

These events led me to carry out a small experiment. Blood samples consisting of 30% and 50% O blood group cells respectively in A group cells, were scored blindly by the Blood Transfusion Unit, using the standard methods accepted by the National Association of Testing Authorities (NATA). The samples scored 12 and 10 respectively. The sample with the score of 12 (ie. the sample with a 70:30 mix of A:O cells) was unequivocally grouped as A blood group in this case. In practice, the sample with the score of 10 (ie. the sample that was a 1:1 mix of O and A cells), should be further investigated by the laboratory supervisor, however from my experience this is not always the case.

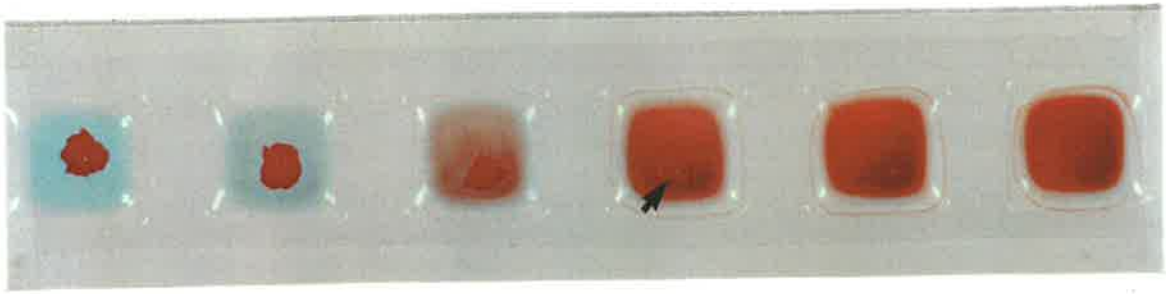

Figure 3-1. Simulation of mixed-field reactions of varying strengths. A^1 red cells were mixed with O group red cells and agglutinated with anti-A using the tile method. Note that as few as 5% O cells can be detected, by comparing the background color of the mixture with that of the control 100% A cells. In normal A (including A^1, A^2 and AB) individuals, the background color is always crystal clear blue. However in the panel with 5% O cells, it can be seen that the background is a slightly "muddy" off-blue color. The intensity of the blue background decreases as the percentage of O cells increases, until no agglutination can be seen in the mixture of 95% O cells and 5% A cells. The arrow indicates the agglutinate in the mixture of 30% A and 70% O red cells.

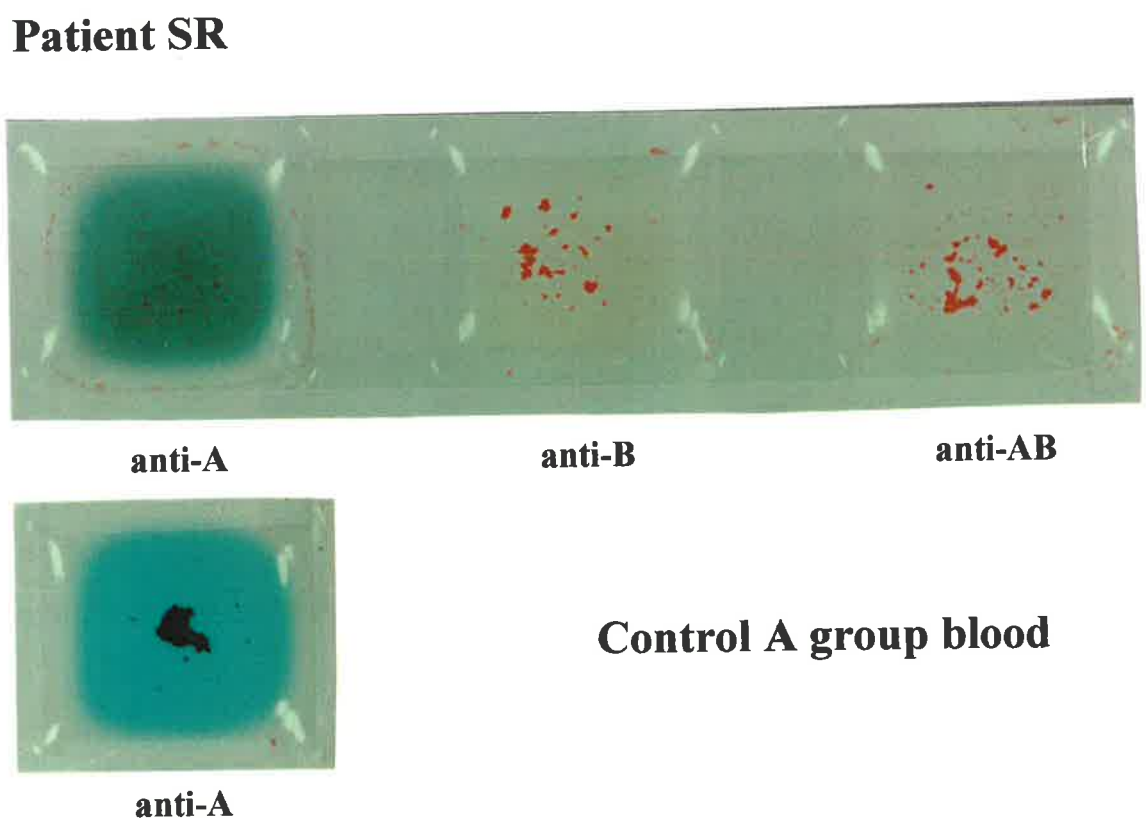

Figure 3-2. Agglutination of red cells from the patient SR with anti-A, anti-B and anti-AB, compared with a normal A^1 control. Note that all cells are agglutinated with anti-B and anti-AB. However, there is a weak and mixed-field reaction with anti-A. Incubation of this reaction for a further five minutes revealed several larger agglutinates, but a significant proportion of the cells did not agglutinate at all. These results indicate loss of the A antigen, but not the B antigen from the red cells of this patient.

The main function of a blood transfusion laboratory is to provide appropriate blood packs for use in a transfusion, and the most important information regarding transfusions is obtained from the cross-match between patient serum and donor red blood cells. Therefore it is the presence of ABO antigens and the lack of antibodies in the patient's serum that is of consequence to the blood transfusion staff, as opposed to our interest in loss of ABO antigens. As these methods are NATA certified, there is no reason to believe that this is not the case throughout Australia.

A mixed-field reaction does not always indicate loss of ABO antigens from the patient's own red cells. Although patients are generally transfused with their own blood group, they may be transfused with several units of O blood in an emergency. It is therefore possible that blood taken from an A or B group patient transfused within the last 120 days (the red cell life time) with O blood, may exhibit a mixed-field reaction. In this case the non-agglutinated red cells would be the donor O red cells. Patients who have undergone an allogeneic bone marrow transplant from a different blood group donor could also exhibit a mixed field reaction once the donor stem cells engraft, the resultant haemopoietic cells mixing with the patients own cells. It is therefore important to determine the transfusion and transplantation status of patients' samples.

It is of some interest to note that every time I presented this work at conferences where personnel from blood transfusion laboratories were present, I was informed that this phenomenon was not as uncommon as previously supposed. It appears that weak and/or mixed-field reactions are actually seen at least once a year in most laboratories but are usually considered of no consequence.

Given that approximately 50% of individuals are O blood group - and therefore changes occurring at the ABO locus would not be observed by blood grouping

methods, and the evidence that mixed-field reactions are rarely reported, the molecular events that lead to loss of A and B antigens are probably much more common than has been previously realised.

3.2 PATIENTS USED IN THIS STUDY

Patient samples reported to exhibit mixed-field reactions or weak antigen expression, were obtained from several Australian hospitals. Table 3-1 details these patients, including cytogenetic results, samples obtained, and blast counts in bone marrow or peripheral blood. Blast, and sometimes white cell counts (WCC), were used as a crude estimate of the proportion of cells in a particular sample derived from the malignant clone for the molecular studies. There are however, significant problems with this method, and these will be discussed in further detail in Chapter 4. To the best of our knowledge, none of these patients had received a blood transfusion from a different blood group donor in the three months prior to a peripheral blood sample being taken, or had had an allogeneic bone marrow transplant.

3.2.1 Patients with haematological malignancy and loss of A or B antigens

Fifteen patients with loss of A or B antigen expression and some form of haematological malignancy were obtained for this study. Fourteen had either AML or MDS, although in the AML patients there was no noticeable clustering to any particular FAB subtype. One patient had CML. Examination of the age at presentation of the patients with haematological malignancy revealed two distinct age groups; 8 of the patients were 35 years old or under, while 7 were over 55 (see Figure 3-3). According to the South Australian cancer registry's report on the epidemiology of cancer over the last eighteen years (published July, 1995), the median age at presentation for patients with AML is 65-69 (range 0 to 85+), while

for MDS, the median is 75-79 (range 0 to 85+). Excluding the patients with ALL, CML and two patients with AML and loss of the precursor H antigen (which will be addressed elsewhere in this discussion), 3 of the 7 patients with loss of A and/or B antigen expression and AML were aged under 35 at the date of presentation, while 2 of the 4 patients with loss of A and/or B antigen expression and MDS were aged under 32. Obviously, patient numbers are small, and therefore may not be truly representative of a large population of patients with loss of A/B antigen expression and haematological malignancy.

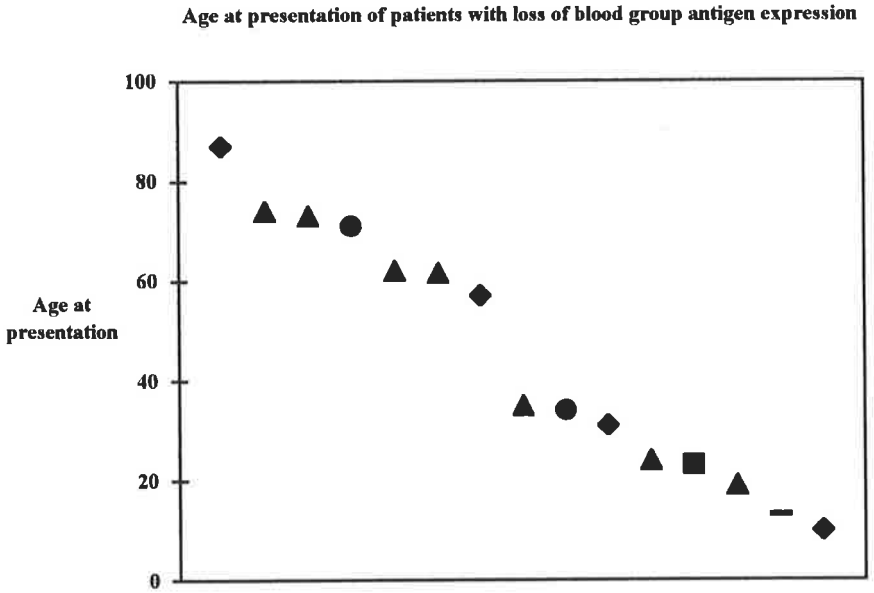

Figure 3-3. Scattergram showing the age distribution of patients with loss of A, B or H antigens and haematological malignancy. ◆ indicates patients with MDS; ▲ indicates patients with AML; ● indicates patients with AML and loss of the precursor H antigen; ■ is a patient with ALL and – indicates a patient with CML.

There are no reports in the literature of a correlation between loss of blood group antigen expression and age at presentation, however most of the studies have consisted of only one or two patients. By combining data from the literature where age at presentation was included in the report (Salmon *et al.*, 1958; Salmon *et al.*, 1959; Gold *et al.*, 1959; Hoogstraten *et al.*, 1961; Salmon *et al.*, 1961; Renton *et al.*, 1962; Undevia *et al.*, 1966; Ragen *et al.*, 1968; Dreyfus *et al.*, 1969; Bird *et al.*,

1976; Kolins *et al.*, 1978; Yoshida *et al.*, 1985; Lopez *et al.*, 1986; Matsuki *et al.*, 1992) it is possible to determine that of twenty patients with AML or MDS and loss of A or B antigen, 8 were 38 years old or under at presentation (ages 4, 19, 20, 24, 26, 32, 35 and 38), and 12 were 45 or older (45, 51, 52, 52, 58, 58, 59, 60, 66, 70, 76 and 82). Two cases also had a reduction in H antigen expression (ages 4 and 51). Including our patients (but excluding patients with loss of H), the median age at presentation of patients with loss of A/B antigens and AML or MDS is 52. It seems, therefore, that the age distribution seen in our group of patients with loss of A/B antigen expression and haematological malignancy is reflected in the literature.

As AML and MDS usually affects an older age group, but patients with loss of ABO antigens seem to be affected indiscriminately of their age, is it possible that antigen loss is in fact an indication of a different type of disease? It is not possible to determine from the literature if there are other characteristics of the diseases suffered by patients with loss of antigen expression that distinguish them from patients with haematological malignancy and no loss of antigen expression.

3.2.2 Ante-natal patients with loss of A antigen expression

Samples from three patients with loss of A antigen expression that had no haematological malignancy, and who were supposedly healthy, were obtained for this study. Interestingly, all three of these patients were pregnant. Furthermore, one of these patients was grouped at several stages throughout two pregnancies. At two and five months of the respective pregnancies, the patient grouped as A^1, however at the birth of both children, she grouped as A^3. This suggests that loss of antigen expression in this patient is directly related to pregnancy, as after the birth of her first child, the patient's blood group returned to normal A^1. Subsequently, the mechanism by which loss of antigen expression occurs during pregnancy is probably not related to changes seen in haematological malignancy.

3.2.3 Red cell analysis

From the information provided from the respective blood transfusion laboratories, it was clear that loss of B antigen expression was due to loss of H antigen expression in two cases (patients TF and SC). These patients were subsequently excluded from the rest of the study. However, it is interesting to note that for patient TF, the red cells that had lost H antigen expression had also lost I (adult) antigen expression. Furthermore, these cells expressed the fetal i antigen, and also carried fetal haemoglobin. The cell population in this patient that expressed the B, H and I antigens, also expressed adult haemoglobin. This suggests that some of the red cells in this patient had undergone an onco-fetal change; reflecting the expression pattern of antigens on the red cells from some new borns* .

Two other patients, DH and KN, exhibited mixed-field reactions with anti-A, anti-B and anti-A,B. The cells that did not agglutinate with anti-A were aspirated, washed, and mixed with anti-B (as described in Section 2.4.3). Most of the cells agglutinated, however there was still a population that did not agglutinate. These cells were then aspirated, and tested against anti-AB. Again, none of the cells agglutinated; the cells were then tested against anti-H. All the cells agglutinated strongly with anti-H. These experiments were repeated by taking the cells that did not react with anti-B in the original grouping reaction. It appeared that in both of these patients, there were four distinct populations of red cells; A, B, O and (presumably) AB. This appears to be similar to the case reported by Renton *et al.*, 1962 (see Section 1.14).

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* Expression patterns of the A, B and H antigens in newborns is extremely variable. While red cells from some neonates may exhibit a very weak reaction with A and B antibodies, red cells from other neonates agglutinate just as strongly as red cells from adults (personal experience).

Serum transferase activities were measured in the patient WD (Geoff Magrin; personal communication); activity of the A-transferase was between that of A^1 and A^2 controls, while B-transferase activity was normal. However, the activity of the H-transferase in the serum was 20% of normal controls. Although this might indicate that loss of A antigen expression in this patient is due to absence of the precursor H antigen, normal agglutination of red cells was seen with anti-B, implying that there was sufficient precursor available. As discussed in section 1.16, serum transferase activities are not a reliable indicator of membrane transferase activities, and decreases in serum H-transferase occur in the majority of leukaemic patients while loss of A and B antigens are relatively rare.

Auto-antibodies directed against the A antigen were detected in the serum of patients MR and GN. One might propose that loss of the A antigen occurred first, and due to constant exposure to intestinal flora that expresses A-like antigens, the patients developed A-antibodies, because the A antigen was no longer recognised as "self". Alternatively, it might be proposed that the patients developed the auto-antibodies first, and therefore selected against red cells expressing the A antigen.

Patients with concurrent loss of A or B antigens and reduced red cell adenylate kinase 1 activity have been described in the literature (see Section 1.20). We were able to obtain samples from one such patient, who was described by Marsden *et al.*, 1992. AK1 activity was not measured in any of the other patients in this study.

3.2.4 Cytogenetics

Finally, one last item of important information can be gained from Table 3-1. None of the patients have any changes affecting chromosome 9, much less 9q34, as determined by cytogenetics. The only exception to this is the patient CG, who had

the Philadelphia chromosome characteristic of his disease (CML). In this case, it is interesting to note that the patient at one stage had two distinct leukaemic clones. At presentation, cytogenetics revealed only one Philadelphia chromosome. However, by 21/12/91, approximately 50% of metaphases had one Philadelphia chromosome, while the remainder had two Philadelphia chromosomes, (and a normal chromosome 9). The sample we obtained from this patient was dated 9/12/93, nearly two years after this finding. As no cytogenetic data was available for this date, the assumption was made that the new clone had completely replaced the original leukaemic clone by this time. As the ABO gene is located distally to the ABL gene, it is translocated with the distal end of the ABL gene to chromosome 22 to form the Philadelphia chromosome. Therefore, it was concluded that the patient CG had three copies of the ABO gene per leukaemic cell.

3.2.5 Quality of samples

Progress on this project was seriously hampered by two major factors. Firstly, it was difficult to obtain more than a couple of million cells from each of the patients from other hospitals. The second major problem was the quality of the samples when we received them. Most of the samples were sent to us by courier on dry ice, however couriers "lost" the packages for anything from a few hours to several days. In one case (CG), whole peripheral blood samples arrived in our laboratory 14 days after the blood was taken from the patient! It was also difficult to obtain remission or other samples from these patients, as in all the cases the patient either declined further treatment, left the country, underwent an allogeneic transplant, or died.

Table 3-1. Details of patients with loss of A or B antigens obtained for this study. When red cells were available from the patients, blood grouping was performed to confirm and sometimes add to the information supplied with the sample (indicated by *).

Patient	Sex	Date of Birth	Age at Presentation	Diagnosis	Cytogenetics	Details	Samples obtained	Source
MR	M	2/11/28	57	RAEB	BM 6/3/89, 14/15 metaphases 46,XY, del(20)(q11)	The patient was previously blood grouped as A, but on 6/3/89 there was a mfr with anti-A (approx. 12% cells were A negative). The direct Coombs test revealed the presence of an auto-antibody.	BM 6/3/89 67% erythroblasts	Elaine Batchelder, St. Vincent's Hospital, Fitzroy, Victoria.
AN	M	16/5/60	31	RAEB	BM 46 XY, no. of metaphases scored unknown	Weak A^2 antigen expression. Agglutination of the red cells with *Ulex europaeus* lectin scored 9, as opposed to control cells which scored 0 (A^1), 8 (A^2) and 12 (O). The patient's disease progressed to AML M2 31/3/93.	BM 27/9/92 (presentation) 19% blasts 38% erythroblasts 54% CD34+	Rick Tocchetti, Institute for Medical and Veterinary Science, Adelaide, South Australia.
GN	F	13/12/77	10	MDS	PB, 11/4/88, 15/15 metaphases 46,XX BM 28/1/92, 7/7 metaphases 45,XX,-7 BM 23/6/92, 34/34 metaphases 45,XX,-7	At presentation the patient's blood group was A^1. By July, 1992, the patient' s group was O by forward typing, A by reverse grouping. Auto-antibodies appeared to be present as the patient exhibited a reaction to A group blood. The patient received a bone marrow transplant from an O blood group donor on 9/9/92.	BM 21/8/92 All cell lines showed abnormal morphology; blast cells 5.8%; promyelocytes 15.6% PB 4/9/92 neutrophils and lymphocytes low, monocytes normal BM 15/1/93 (post-transplant), remission.	Dr. George Kannourakis, Royal Children's Hospital, Melbourne, Victoria.

Table 3-1 (continued)

Patient	Sex	DOB	Age at Presentation	Diagnosis	Cytogenetics	Details	Samples obtained	Source
PM	M	10/1/67	24	AML M6	BM 19/12/91, 2 metaphases 46,XY/ 21 metaphases 45,XY,-7	The patient's red cells exhibited a mfr with anti-A^1. Both cell populations agglutinated strongly with anti-H. The patient succumbed to the disease approximately one month later.	Whole PB (frozen). 10/12/91 case notes unavailable.	Elaine Batchelder, St. Vincent's Hospital, Fitzroy, Victoria.
TF	F	19/1/56	34	AML M6	BM 3/5/90, 29/29 metaphases 46,XX,del (20)(q13.1).	Red cells separated into two distinct populations, B and O. The B cells were H and I positive, while the O cells were H and I negative, but i positive. The B cells had adult haemoglobin, however the O cells carried fetal haemoglobin.	BM 2/5/90 73% erythroblasts, 18% blasts.	Elaine Batchelder, St. Vincent's Hospital, Fitzroy, Victoria.
NR	M	3/3/61	19	AML M2	46 XY throughout the disease except BM 22/6/90, 8/15 46 XY; 4/15 45 XY -7; 3/15 45 XY, -7 t(12;X)(p13;p11)	The patient had thrombocytopenia since age 3. It is not clear when the patient's red cells first exhibited a mfr with anti-A. The cells were separated into A+ and A- populations; both populations expressed the H antigen*. By late 1990, the cells grouped as O.	BM 7/3/89 > 85% blasts PB 11/1/91 >85% blasts Pleural effussion 15/1/91 - mostly leukaemic cells.	Dr. RE Sage, The Queen Elizabeth Hospital, Woodville, South Australia.
MA	F	1954	35	AML M1	BM 14/2/89 15/19 metaphases 46,XX. 2 metaphases had 45 chromosomes, while 2 metaphases had 44. The hypodiploidy was non-clonal.	Weak A-antigen expression. H antigen status unknown.	PB 18/2/89 >35% blasts	Geoff Magrin, Alfred Hospital, Prahran, Victoria.

Table 3-1 (continued)

Patient	Sex	DOB	Age at Presentation	Diagnosis	Cytogenetics	Details	Samples obtained	Source
WM	F	1913	73	AML M0	BM 18/3/88, 13/19 metaphases 46,XX,t(1;3)(p36 ;q21) 6/19 metaphases 46,XX,t(1;3)(p36 ;q21),t(14;17)(q3 2;q21). BM April, 1989 15/15 metaphases 46,XX,t(1;3)(p36 ;q21),t(14;17)(q3 2;q21).	The patient presented with primary acquired sideroblastic anaemia which evolved into AML with erythroid features. In July 88, 50% of her red cells grouped as A^2, 50% as O. Red cell H antigen expression was normal, however serum A blood group transferase activity was low. In August 1989, red cell adenylate kinase I activity was 66IU/gHb (normal range 220-340IU/gHb). One mono-clonal antibody of several tested revealed that the patient's red cells expressed the Lewis B antigen; on the 14/7/88 the patient was Le (a-b-), however on the 5/12/88, the type was Le (a-b+) (Marsden *et al.*, 1992).	PB 9/6/89 97% blasts.	Dr. Kathryn Marsden, University of Tasmania, Hobart, Tasmania.
SR	F	26/5/23	71	AML M3	20/20 metaphases 46,XX	On the 18/2/93 the patient presented for a routine surgical procedure, at which time she grouped as normal AB. However, when the patient presented in 1994 with AML, her red cells showed a mfr with anti-A, but not anti-B or anti-A,B. The cells expressed the H antigen, but did not agglutinate with *Dolichos biflorus* lectin*.	PB 17/8/94 94% blasts.	The Queen Elizabeth Hospital, Woodville, South Australia.

Table 3-1 (continued)

Patient	Sex	DOB	Age at Presentation	Diagnosis	Cytogenetics	Details	Samples obtained	Source
CG	M	28/11/72	13	CML	BM 17/1/85 12/12 metaphases 46,XY,t(9;22)(q34;q11). BM 21/12/91 11/24 metaphases 46,XY,t(9;22)(q34;q11)/13/24 48,XY +8, t(9;22)(q34;q11) + der (22)t(9;22)(q34;q11).	25-30% of the patient's red cells agglutinated with anti-A. The patient's red cells expressed the H antigen, however the agglutinates were easily disrupted. It is possible that this was related to the fact that the cells were received by our laboratory 14 days after phlebotomy*. Blast crisis occurred in April, 1993.	PB 9/12/93 WCC 125 x 10^9/l, 50% blast cells.	Helen Haysom, Royal Melbourne Hospital, Melbourne, Victoria.
WD	F	unknown	23	ALL LI	BM 5/11/87 2/2 metaphases 46,XX.	The patient's red cells exhibited a weak reaction with anti-A, but a normal reaction with anti-B. Serum transferases were measured, and revealed normal levels of the B transferase, but the level of the A transferase was between normal A^1 and A^2. Serum H transferase activity was 20% of the normal controls.	PB 26/11/87 case notes unavailable.	Geoff Magrin, Alfred Hospital, Commercial rd., Prahran, Victoria.
SC	M	23/9/29	62	AML M?	BM 8/3/93 9/9 metaphases 46,XY.	The patient's red cells exhibited a mfr with anti-B. The unagglutinated red cells did not agglutinate with anti-H.	BM 21/6/93 case notes unavailable.	Peggy Nelson, Royal Prince Alfred Hospital, Camperdown, New South Wales.

Table 3-1 (continued)

Patient	Sex	DOB	Age at Presentation	Diagnosis	Cytogenetics	Details	Samples obtained	Source
KN	F	24/8/32	62	AML M2	BM 7/94, 46XX	The patient's red cells exhibited a mfr with anti-A, anti-B, and anti-A,B. There was no reaction with *Dolichos biflorus* lectin. Agglutination with anti-H was strong (score 5, compared to 1 for a normal AB control). The cells were able to be separated into 3 distinct populations; A, B and O*.	BM 5/7/94 30% myeloblasts 18% erythroblasts	Rick Tocchetti, Instiitute for Medical and Vetinary Science, Adelaide, South Australia.
DH	M	22/1/18	74	AML M1	BM 28/5/92, 35/35 metaphases 46,XY	The patient's red cells exhibited a mfr with anti-A, anti-B, and anti-AB. Three distinct red cell populations could be distinguished; A, B, and O. The red cells expressed the H antigen*.	PB 15/6/92 WCC 13.7, 77% blast cells.	Elaine Batchelder, St. Vincent's Hospital, Fitzroy, Victoria.
BA	M	30/9/07	87	Suspected MDS	not done	No diagnostic marrow was taken due to the patient's age. No detectable A-antigen was on red cells; however there was no anti-A in serum, and the patient secretes normal amounts of soluble A-antigen. The patient's red cells express H-antigen and were able to absorb and elute anti-A.	PB 16/12/94 WCC 12.1 72% neutrophils, 10% lymphocytes and 18% monocytes.	Pam Pussell, Institute of Clinical Pathology and Medical Research, Westmead Hospital, Westmead, NSW 2145.

Table 3-1 (continued)

Patient	Sex	DOB	Age at Presentation	Diagnosis	Cytogenetics	Details	Samples obtained	Source
GB	F	1953	34	Pregnant	Not done.	Blood grouped as normal A^1 2/9/87, with no anti-bodies detected. On the 6/4/88 the patient underwent a caesarian section, and required a blood transfusion. At this time she grouped as A^2, with some anti-Le^a detected in her serum and was transfused with A^2, Le^a negative blood. On the 22/4/88 the patient was grouped as A^3 and once again anti-Le^a positive. The patient was again blood grouped on the 1/2/89, at which time she was once again a normal A^1 and had no anti-bodies in her serum. The cell blood picture was normal. On the 30/6/89, the patient again presented for another caesarian section, and again typed as A^x/A^3. This time she was transfused with A, Le^a- blood. Blood collected on the 30/6/89 was also grouped again, 4 days later by another technician, who this time grouped it as O. In our laboratory, incubation with anti-A or anti-A,B for 10 minutes at room temperature revealed weak agglutination, however all the aggregates were very small, and easily disrupted. The cells strongly agglutinated with anti-H*. The patient's saliva contained H-substance, but no A substance.	PB 30/6/89 No abnormalities in peripheral blood; alterations consistent with pregnancy.	David Roxby and Alec Morley, Flinders Medical Centre, Bedford Park, South Australia.

Patient	Sex	DOB	Age at Presentation	Diagnosis	Cytogenetics	Details	Samples obtained	Source
SK	F	1963	31	Pregnant	Not done	Previously grouped as B, now groups as A^2B*. The Kleihauer test showed no fetal blood contamination. We were asked to determine if this patient had acquired the A-antigen.	PB 19/8/94 Blood film did not indicate haematological disorder.	David Petersen, The Queen Elizabeth Hospital, Woodville, South Australia.
HT	F	1975	19	Pregnant	Not done.	This patient was detected during routine blood grouping of ante-natal patients. Previously she had grouped as normal AB (June, 1992). On the 16/3/94 the patient's red cells agglutinated normally with anti-B and anti-A,B, however there was a mixed-field reaction with anti-A. Agglutination with *Dolichos biflorus* lectin was absent, suggesting no A^1 antigen on the patient's cells*. The Kleihauer test confirmed the absence of fetal cells in the maternal blood.	PB 16/3/94 Blood film did not indicate haematological disorder.	David Petersen, The Queen Elizabeth Hospital, Woodville, South Australia.

SUMMARY

Bone marrow or peripheral blood samples from eighteen patients with loss of A and/or B antigen expression were collected from hospitals around Australia. Of these, fourteen had AML or MDS and one had CML, reflecting the bias of patients with loss of ABH antigens to the myeloid malignancies, and in particular to AML that is seen in the literature. Two patients that had loss of B antigen expression, also had loss of the precursor H antigen expression, and were subsequently excluded from the rest of this study. Of the patients with haematological malignancy, there seemed to be an over-representation of individuals under the age of 35, compared with the median age at presentation for AML and MDS, which is 65-69 and 75-79 respectively. The abnormal age distribution seen in our group of patients is reflected in the literature, suggesting an as yet unknown relationship between loss of antigen expression and disease.

Interestingly, although the ABO gene is located on chromosome 9, none of the patients with loss of ABO antigens and AML or MDS had any cytogenetic changes involving chromosome 9. However, the patient with CML had two copies of the Philadelphia chromosome, and one normal chromosome 9, indicating that there is probably three copies of the ABO gene per leukaemic cell.

Two of the patients with AML had been previously grouped as normal AB, but during their disease exhibited three distinct populations of red cells simultaneously; A, B and O. Presumably a fourth population of AB cells was also present, but our blood grouping techniques could not conclusively prove this. This suggests that multiple clones of red cells are present in these patients, and that there is some form of selection occurring against the ABO gene, or possibly a closely linked gene.

Three patients with loss of A antigen expression supposedly had no haematological malignancy, however all three were pregnant. One of these patients was grouped throughout two pregnancies. The patient reverted to normal blood group A after her first pregnancy, but at the birth of her second child, she again exhibited loss of A antigen expression. It therefore appears that pregnancy somehow influences A antigen expression, and that loss of antigen expression during pregnancy is probably not related to that seen in haematological malignancy.

The ensuing chapters of this thesis detail a molecular analysis of the ABO gene in patients with abnormal A/B antigen expression.

CHAPTER FOUR

GENOTYPING THE ABO LOCUS

4.0 INTRODUCTION

Classical methods for ABO genotyping rely on a combination of red cell serology and family studies. These methods were not appropriate for this study as they are limited by the availability of family members, and are restricted to typing of red cells. Even if family members are available, the genotype of a particular individual can not always be determined. The major aim of the experiments contained within this chapter was to develop a sensitive method for ABO genotyping directly from DNA, and to subsequently examine patients with haematological malignancy, both with and without blood group changes, for loss of heterozygosity at the ABO locus.

4.1 DEVELOPMENT OF A SENSITIVE METHOD FOR MOLECULAR ABO GENOTYPING

At the beginning of this study two methods existed for molecular genotyping of the ABO locus. Both were described by Yamamoto *et al.* (1990b). The first method involved Southern blot analysis of genomic DNA, while the second was a multistep method entailing PCR, restriction endonuclease digestion, blotting and hybridisation with radio-labelled probes. These methods were considered unacceptable for the present study for several reasons. Southern blotting requires large amounts of intact genomic DNA (typically 10μg or more per lane). As only limited amounts of DNA was available from patients exhibiting blood group changes, Southern analysis was not a viable option for genotyping. The PCR method described by Yamamoto *et al.* (1990b), is time consuming, requires 1μg of target DNA, and involves the use of radio-active nucleotides. Therefore the aim of this set of experiments was to develop a rapid and reliable method for ABO genotyping that was sensitive enough to avoid the use of radio-active probes.

4.1.1 Rationale

Analysis of the primers used by Yamamoto *et al.* (1990b), revealed markedly different melting temperatures (Tms) within the primer sets, which results in inefficient amplification of the target DNA. It was not possible to alter these primers, as they were based on unpublished intron sequences. Subsequently, completely new sets of primers with matched Tms were designed, based on the published cDNA sequence (Yamamoto *et al.*, 1990a;b).

As the intention was to amplify genomic DNA and only the cDNA sequence was available for the gene, an attempt was made to determine intron/exon boundaries using consensus splice sites (Shapiro and Senapathy, 1987). The consensus splice junction sequence for cDNA is 5' AGG 3', which theoretically occurs approximately once in every 64 base pairs of random sequence. Nevertheless, two sets of primers

were designed flanking (1) the site at 261 which distinguishes the O, and A/B alleles, and (2) position 703, which differentiates the B and O/A alleles[1]. Theoretically, the three major alleles could be distinguished by restriction enzyme digestion of the two amplified products (see Figure 4-1). To avoid amplifying intron and therefore unknown sequence (and possibly too large a region to amplify), the primer sets were designed to amplify small regions of DNA, containing as few AGG sites as possible.

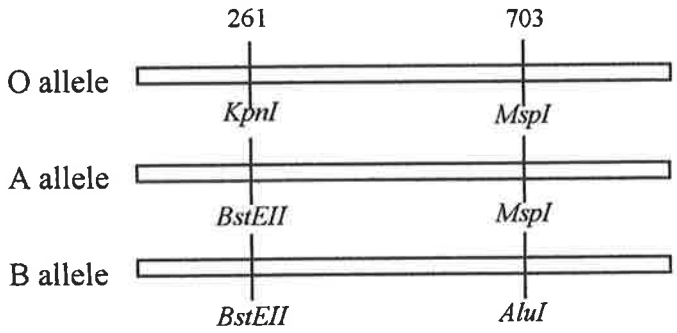

Figure 4-1 Diagrammatic representation of the three major ABO alleles. Two fragments spanning positions 261 and 703 of the cDNA sequence are amplified by PCR. Each allele can be differentiated by its unique pattern of restriction sites.

[1] This method for ABO genotyping has since been published (O'Keefe and Dobrovic, 1993). At this stage however, all base pair numbering was based on the published cDNA sequence (Yamamoto *et al.*, 1990a;b); the single base pair deletion in the O-allele was at nt 258. Since that time Yamamoto has published a review on the molecular genetics of the ABO blood group (Yamamoto, 1994). He states that the sequence published in the 1990 paper was based on a cDNA clone, FY-59-5, which unlike most A^1 alleles, lacks the trinucleotide TAG at position 240-242. This sequence is however, present in genomic DNA, and Yamamoto suggests that the absence of these three residues in the cDNA may be due to alternative splicing or a splicing error. Consequently, the new numbering is used throughout this thesis, however numbering in our paper is based on the originally published sequence.

4.1.2 Materials and Methods

A multiplex PCR reaction in a total volume of 75µl was performed on 100ng of target DNA, using 100ng of each of the following primers under the conditions described in section 2.6.4.

AO1	5' TGACACCGTGGAAGGATGTCCTCGT 3'	(sense)
AO2	5' TGAACTGCTCGTTGAGGATGTCGA 3'	(anti-sense)
AB1	5' CGCATGGAGATGATCAGTGACTTC 3'	(sense)
AB2	5' GCTCGTAGGTGAAGGCCTCCC 3'	(anti-sense)

Diagnostic restriction enzyme digestions were performed for 10-16 hours on four 10µl aliquots of each PCR product using 7 units of *Kpn*I, *Bst*EII, *Msp* I (all from New England Biolabs), and *Alu* I (Boehringer-Mannheim), in a total volume of 14µl, according to the manufacturer's instructions. The restricted products were then resolved on 8% polyacrylamide gels (see Section 2.6.6) and stained with ethidium bromide in order to visualise the bands.

4.1.3 Results and Discussion

Two regions of the ABO glycosyltransferase gene are amplified, each containing a diagnostic restriction enzyme site (Fig. 4-1). Primers AO1 and AO2 amplify the region from nt 230-328 of the published cDNA sequence. They utilise the polymorphic *Kpn*I and *Bst*EII sites to differentiate the O allele from A and B alleles, and generate a product of either 98 or 99bp, depending on whether or not an O allele is involved (although this size difference was not resolved on either acrylamide or agarose gels). The second pair of primers are termed AB1 and AB2, and amplify a 181bp product (nt 562-742). Digestion of this product with *Msp*I and *Hpa*II distinguishes the A and O alleles from the B allele. All four primers were designed with melting temperatures matched to within 3.5°C to allow multiplex

amplification, and therefore facilitate rapid screening of large numbers of genomic DNA samples. PCR conditions were varied to allow optimal amplification of both products within the multiplex reaction.

Each ABO genotype has a unique pattern following digestion of the multiplex product (Table 4-1, Fig. 4-2, Fig. 4-3). The success of this method was tested by applying it to 40 (non-randomly selected) DNA samples from healthy individuals, of known blood group; 14 O, 10A, 10B, and 6AB blood group individuals.

ABO genotype	*Kpn*I	*Bst*EII	*Msp*I	*Alu*I
OO	181,67	181,98	140,98	181,98
AA	181,99	181,68	140,99	181,99
AO	181,99,67	181,98,68	140,98/99	181,98/99
BB	181,99	181,68	159,99	140,99
BO	181,99,67	181,98,68	159,140,98/99	181,140,98/99
AB	181,99	181,70	159,140,99	181,140,99

Table 4-1 Pattern of restriction fragments (sizes in base pairs) visualised after digestion of the multiplex PCR product.

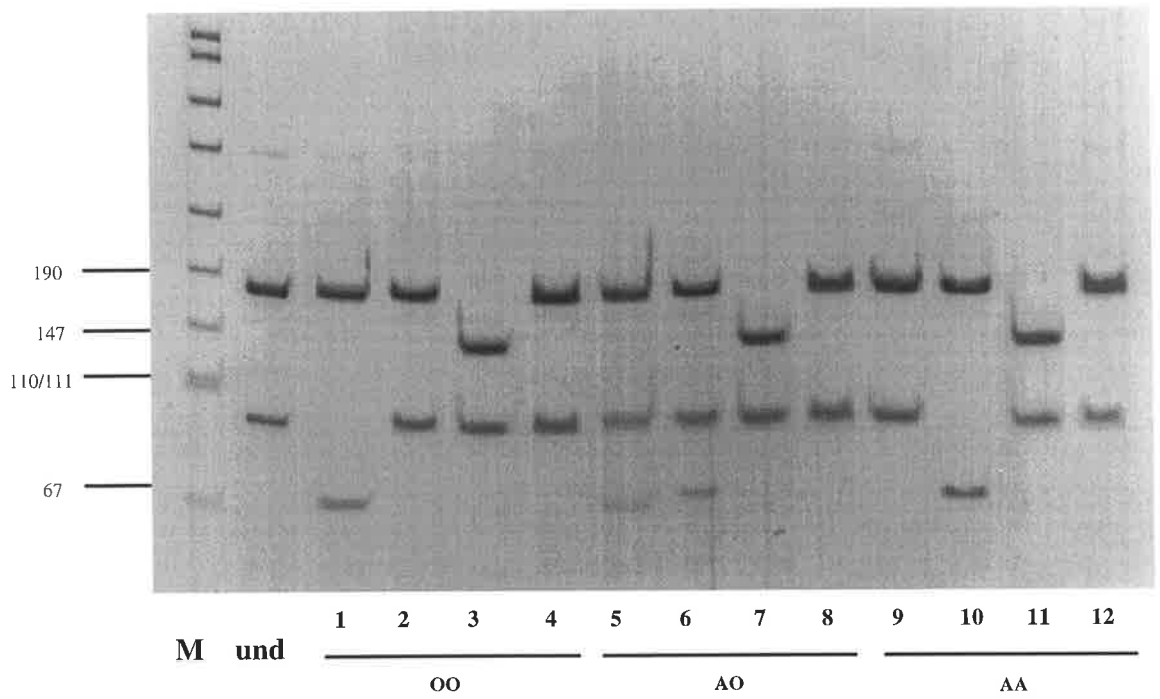

Figure 4-2. ABO genotyping. Diagnostic restriction enzyme digestion of multiplex PCR amplified product yields a unique pattern of bands for each of the genotypes; OO, AO and AA. Lanes 1,5 and 9 are PCR product that has been digested with *Kpn*I; 2,6 and 10 are *Bst*EII digests; 3,7 and 11 are *Msp*I digests; 4,8 and 12 are *Alu*I digests. Multiplex PCR product before digestion is indicated by "und". The marker (M) is pUC19 plasmid digested with *Hpa*II; Sizes of the relevant marker bands shown are in base pairs.

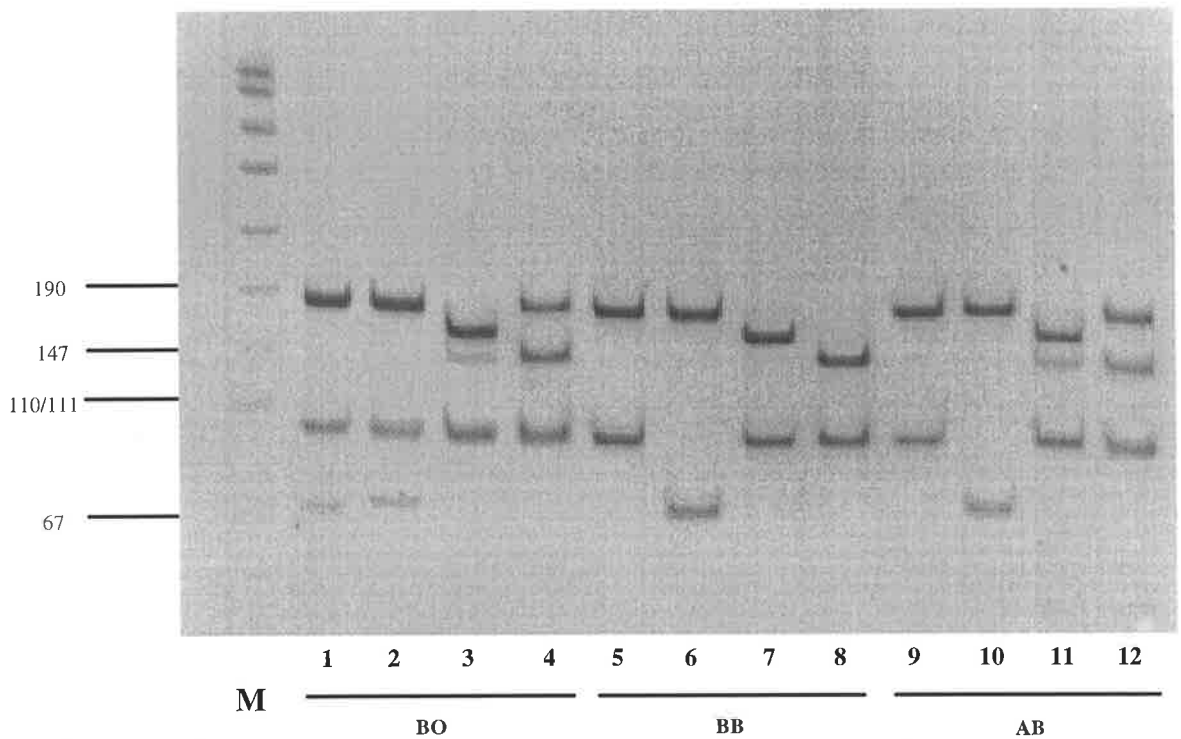

Figure 4-3. ABO genotyping. Diagnostic restriction enzyme digestion of multiplex PCR amplified product yields a unique pattern of bands for each of the genotypes; BO, BB and AB. Lanes 1,5 and 9 are PCR product that has been digested with *Kpn*I; 2,6 and 10 are *Bst*EII digests; 3,7 and 11 are *Msp*I digests; 4,8 and 12 are *Alu*I digests. The marker (M) is pUC19 plasmid digested with *Hpa*II. Sizes of the relevant marker bands shown are in base pairs.

To ascertain if this method was applicable to the Australian aboriginal population, 96 DNA samples from a population of Central Australian aborigines kindly provided by Professor Boettcher of Newcastle University, NSW, were analysed. The results are shown in Table 4-2.

	ABO genotype		
Blood group	OO	AO	AA
O (n=43)	43	0	0
A (n=53)	1	42	10
B (n=0)	-	-	-

Table 4-2 Blood group versus ABO Genotype of an Australian aboriginal population. Aboriginals of B blood group are extremely rare, and none were found in this particular population (pers. comm. Professor Boettcher).

The use of complementary pairs of restriction enzymes to control for incomplete digestion is necessary for unequivocal genotyping. For example, to type O, the PCR product is digested with both *Kpn*I and *Bst*EII. Normally, cutting with *Kpn*I alone is sufficient to type the O allele. However if digestion were to be absent or incomplete; OO, AO or BO individuals could be falsely typed. The use of *Bst*EII controls for absent or incomplete *Kpn*I digestion. Where there is no *Kpn*I digestion, there should be full *Bst*EII digestion.

An analogous situation holds for *Msp*I and *Alu*I digestion at position 703. Here, the use of both enzymes is important as the *Msp*I digested product in B/non-B heterozygotes consistently has a markedly more intense upper band, resembling incomplete digestion (Fig. 4-3, lanes 3 and 11). Alternatively, digestion with *Alu*I approximates the expected 1:1 ratio.

As a control for *Msp*I cutting, the AB2 primer (anti-sense primer) was positioned so that it contained another *Msp*I site, 19bp downstream from the diagnostic site. The

primer site cuts completely even though the diagnostic *Msp*I site is cut less than expected 50% in B/non-B heterozygotes. In samples that are homozygous for the diagnostic *Msp*I site (OO or AA or AO), complete digestion is typical (Fig 4-2, lanes 3, 7 and 11) at both sites. Identical results are obtained with increased concentrations of *Msp*I or with the use of the *Msp*I isoschizomer, *Hpa*II (data not shown). Therefore the apparent incomplete digestion of the non-B allele in heterozygotes is not due to insufficient enzyme.

Incomplete digestion in heterozygotes can be explained by the presence of a proportion of heteroduplexes with one strand containing the *Msp*I recognition site and the other lacking the recognition site. Different restriction enzymes appear to be adversely affected by heteroduplex formation to different extents. This point is accentuated by the fact that *Alu*I digested products from heterozygous BO and AB individuals yields an approximate 1:1 ratio (Figure 4-3, lanes 4 and 12). However, after these characteristics are taken into account, the correct genotype can be readily deduced.

After this method was established, two other laboratories published ABO genotyping methods. Uggozoli and Wallace (1991), presented an elegant method using 8 primers in a multiplex PCR approach. However, this method required the use of radio-labelled probes to detect specific signal. Chang *et al.* (1992), independently developed a PCR based method similar to ours, yet it varies from our method in two important respects. We use a multiplex PCR approach which is the method of choice when samples and time are limited. Our method also uses complementary pairs of enzymes to eliminate the assignment of incorrect genotype as a consequence of incomplete or absent digestion, whereas Chang *et al.* (1992) carry out two separate amplification reactions, and digest only with *Kpn*I or *Msp*I.

One would assume that each of our primer pairs are contained within the same exons, as the PCR products are of the correct size as predicted from the cDNA sequence. A recent paper (Yamamoto *et al.*, 1995), describes the intron/exon structure of the gene. Surprisingly, the primer AO1 encompasses an intron/exon boundary.

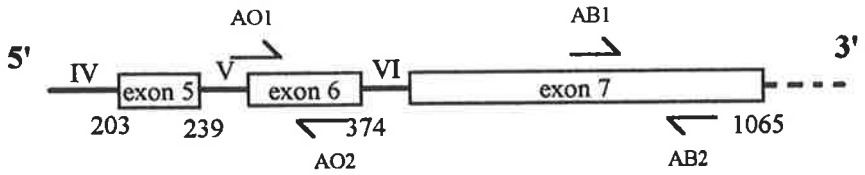

Figure 4-4 Schematic representation of the genomic structure of the ABO gene beginning from intron 4, modified from Yamamoto *et al.*, 1995 to include the positions of the ABO genotyping primers. The boxes represent exons, the solid bars introns, and the dashed line unknown sequence. The (coding) exons are numbered; Roman numerals identify introns.

As the primer AO1 is designed from nt 230-257 of the cDNA sequence, 9 base pairs could not bind to the genomic DNA (Figure 4-4). Furthermore, when this primer was designed, the TAG sequence in the O, B and common A^1 alleles was not known. Subsequently, this primer probably anneals poorly to the target DNA at this site, forming a structure as shown in Figure 4-5. This may explain the poor amplification of the 98/99bp product in the multiplex PCR relative to the 181bp fragment amplified by primers AB1 and AB2.

Figure 4-5. Probable structure formed by primer AO1 and the known genomic sequence of the ABO gene. The bold type in the primer represents sequence now known to be contained in exon 5, whilst the rest of the primer is derived from exon 6. The extra trinucleotide TAG now known to be part of the real A^1/O/B cDNA sequence is represented by bold type, while the lower case letters represent the published intron 5 sequence.

As this information was published subsequent to the completion of the experimental work of this thesis, a new primer was not designed to replace primer AO1.

However, although this sequence data does explain the less efficient amplification of the 98/99bp product, our conclusions are not affected.

Although this technique reliably predicted the blood groups of 40 healthy individuals, a single discrepancy in the Australian aboriginal population was detected. As shown in Table 4-2, one individual genotyped as OO, yet the blood group of this person was supposedly A. This result was repeatable, and may be explained by a simple sample mix-up at the point of collection of the blood. Alternatively, a number of complex hypotheses could be proposed to explain this result (ie. alternative splicing of the O allele to produce a functional A transferase). However, no other samples (either DNA or RNA), were available from this individual, and therefore this matter could not be investigated any further.

4.2 ABO GENOTYPING OF PATIENTS WITH MALIGNANCY

The aim of this set of experiments was to use the ABO genotyping method described above, to compare the ABO genotype with blood group data in three major groups of patients. The first group of samples were from patients who had presented at the QEH over the last 8 years, and included patients with a wide range of haematological malignancies. This group also included patients with colon carcinoma. It was hoped that this comparison might lead to some discrepancies between ABO genotype and the patient's reported blood group, which could increase the number of patients with loss of blood group antigens for this study. The second group of samples the ABO genotyping method was applied to were those supplied to us from other hospitals from patients with haematological malignancies and observed loss of blood group antigen expression. The third group consisted of "normal" individuals, which during routine blood grouping procedures exhibited

mixed-field reactions or were described as having "acquired antigens". These particular patients were all pregnant, but were presumably healthy.

4.2.1 Materials and Methods

Specimens taken at presentation of the patients with haematological malignancy were used where possible. If mononuclear cells from bone marrow aspirates were not available, peripheral blood mononuclear cells were analysed. DNA samples from histologically normal mucosa adjacent to colon tumours, and whole tumour specimens were kindly provided by Wendy Hart from the Dept. of Gastroenterology at the Queen Elizabeth Hospital.

Genotyping of patients AN, GN, MR, MA, CG and PM that had loss of A antigen expression was carried out on 2-5µl of DNA isolated as described in Section 2.6.2.1. As DNA isolated by this method is contaminated by RNA, no attempt was made to quantitate the sample. Furthermore, amplification of DNA from all the patients with abnormal antigen expression and the corresponding normal controls was carried out for 45 cycles, as opposed to 35 cycles that was used for the original technique. Each sample was amplified on at least 3 separate occasions; a multiplex reaction was carried out the first time but on subsequent occasions only the 181 or the 98/99bp band was amplified to allow more efficient amplification of the particular target (this was really only necessary for amplification of the 98/99bp band; amplification of which was inhibited due to competition with the 181bp fragment as described previously).

4.2.2 Results and Discussion

4.2.2.1 Patients with malignancy

Eighty-five patients with haematological malignancy were randomly chosen for this study. The blood groups of the patients appeared to represent the normal distribution, in that 50.5% were O, 36.4% were A, 9.4% were B and 3.5% were AB.

Comparison of blood group and ABO genotype revealed discrepant results in three patients (all patient details and results are tabulated in Appendix A, Table 1). Of the 31 patients whose blood group was A, 23 genotyped as AO, while 8 were AA. Therefore the genotype of all A group people agreed with their blood group, as did the genotype of the 8 patients whose blood group was B (7 BO and 1 BB), and the three patients whose blood group was AB. However, of the 43 patients whose blood group was O, 3 did not genotype as OO.

One patient (RR), was examined at 6 different stages of disease (ALL). The presentation specimen genotyped as normal OO, in accordance with the patient's blood group. However, in remission, the patient genotyped as AO, although compared to normal AO samples, there was virtually no O allele. Again in relapse, the patient genotyped as normal OO. This problem was resolved by examination of the patient's case notes, which revealed remission was induced by an allogeneic transplant from the patient's sister. Cytogenetic analysis revealed that in remission, the (male) patient's bone marrow had 29/30 XX metaphases, with one XY metaphase. This suggests that the transplanted marrow had engrafted and replaced the patient's own bone marrow. Presumably the sister, who was A blood group, genotypes as AA. These results revealed another use for the ABO genotyping method; that is detection of engraftment of an allogeneic marrow.

The two other patients with discordant serology/genotyping results were MCG and DN. ABO genotyping clearly showed a normal AO genotype, but both patients were unequivocably blood group O. Fortunately, blood group results on patient MCG from 16 years previously were available; at which time the patient's blood group was also O. At this time, (the first report of an O^2 allele had not been published), these patients were retained as candidates for loss of ABO antigens to be analysed at the RNA level.

It should be considered that these experiments may not have detected some patients with loss of ABO antigens. If loss of antigen expression was due to physical loss of one or both alleles of the ABO gene, **and** the proportion of leukaemic cells in the sample analysed was sufficient to mask the contribution of normal cells, **and** the red cells were also affected by this change (that is, mature red cells were derived from a malignant stem cell), **then** perhaps some patients with loss of antigen expression would have been overlooked.

An effort to determine the proportion of leukaemic cells in each of the 85 samples examined was made, however it was discovered that this was virtually impossible for the majority of patients. A significant number of the blood and bone marrow samples that had been sent to our laboratory had not been analysed using the differential cell count that might have given an indication of the number of leukaemic blast cells in the sample (for peripheral blood samples), or in the case of bone marrow specimens, the specimen was considered to poor to examine. In the cases where differential cell counts or bone marrow reports were available, it was still impossible to determine the number of leukaemic derived cells, simply due to the nature of leukaemia and haematological malignancies. In some cases, it was safe to assume that virtually all the cells in the sample were from the leukaemic clone, for example where bone marrow contained 95% blast cells (compared to a normal maximum of 5%). However, as this only occurred in a limited number of cases, this data was left out of this thesis.

The ABO genotyping method was also applied to 17 colon carcinomas, and the results compared those obtained from histologically normal mucosa adjacent to each of the tumours, and with the patient's blood group (see Appendix A, Table 2). The choice of patients was biased towards "not O" blood group, with the aim that tumours that had loss of heterozygosity at the ABO locus might be detected. Of the

10 patients of blood group A, 7 of the corresponding tumours genotyped as AO, while three genotyped as AA. Of the other tumours, 4 from O, 2 B and 1 AB blood group patients, the genotype corresponded with the patient's blood group. In each case the results from the tumour and normal tissue were identical.

In all of the patients that were heterozygous at the ABO locus, visual comparison of the relative intensities of the alleles compared to normal controls did not reveal any imbalance, suggesting that none of the patients had loss of heterozygosity.

4.2.2.2 Patients with haematological malignancy and loss of ABO antigens

ABO genotyping of the patients with haematological malignancy and loss of A and/or B antigens described in Chapter 3, revealed that all were heterozygous at this locus. This made it possible to examine the patients for loss of heterozygosity, which produced some interesting results (Table 4-3, Figures 4-6 and 4-7). As only a single base pair differentiates the ABO alleles, amplification of the polymorphic regions of the alleles should represent allele dosage in the original DNA sample. There should not be preferential amplification of one allele, because at the binding sites of the primers, the alleles are not distinguished. Furthermore, the sizes of the amplified products are virtually identical, therefore size of the amplified product should not influence the representation of any particular allele in the resulting PCR product. Nevertheless, as a control for artifactual amplification, all patient samples were amplified and digested on at least 3 separate occasions, and normal heterozygous controls were always amplified at the same time.

Loss of heterozygosity in AO genotype patients was defined as over-digestion of the 98/99bp fragment with either *Kpn*I or *BstE*II, and under-digestion with the alternative enzyme. For example, in Figure 4-6, genotyping of patient MR reveals slight digestion of the 98/99bp fragment with *Kpn*I, and almost complete digestion

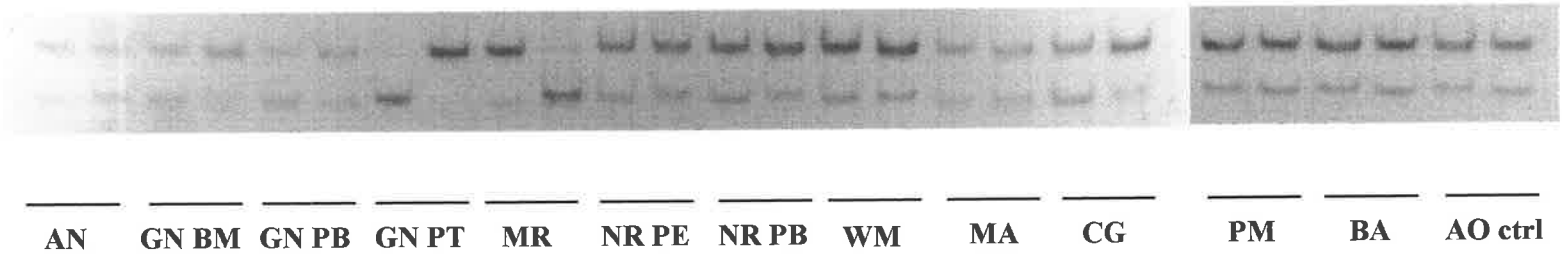

Figure 4-6. Allele dosage of the ABO gene in patients who genotype AO. Samples are paired digests (*Kpn*I, *Bst*EII) of the 98/99bp product. When more than one sample of a patient was analysed, the sample is identified (BM- bone marrow; PB- peripheral blood; PT - post-transplant; PE- pleural effusion).

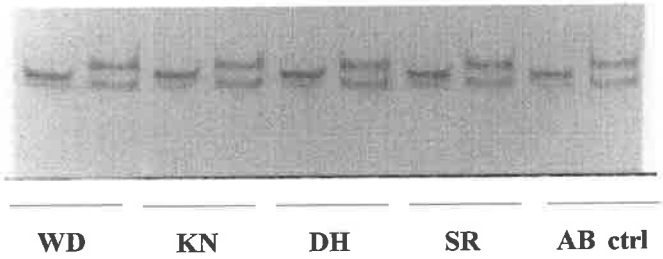

Figure 4-7. Allele dosage of the ABO gene in patients who genotype AB. Samples are paired digests (*Msp*I, *Alu*I) of the 181bp product.

with *BstE*II. In normal heterozygous controls, the relative intensity of the two bands is always the same, regardless of whether the product was digested with *Kpn*I or *BstE*II. Consequently, it was concluded that the partial digestion of PCR product generated from patient MR with *Kpn*I, and over-digestion with *BstE*II, represented loss of heterozygosity of the O allele in this patient.

Patient	Sample Type	Date	ABO change	ABO geno	Abnormal allele dosage
AN	BM	27/2/92	A→O	AO	reduction in O
GN	BM	4/9/92	A→O	AO	reduction in A
GN	BM	21/8/92	A→O	AO	reduction in A
GN	BM	15/1/93	A→O	AO	reduction in A
MR	BM	6/3/89	A→O	AO	reduction in O
NR	PE	21/2/91	A→O	AO	-
NR	PB	11/1/91	A→O	AO	-
NR	BM	7/3/89	A→O	AO	-
WM	PB	9/6/89	A→O	AO	-
MA	PB	18/2/89	A→O	AO	-
CG	PB	10/12/93	A→O	AO	reduction in A
PM	whole blood	10/12/91	A→O	AO	-
BA	PB	16/12/94	A→O	AO	-
WD	PB	26/11/87	AB→B	AB	-
KN	BM	5/7/94	ABmfr	AB	-
DH	PB	15/6/92	ABmfr	AB	-
SR	PB	17/8/94	AB→B	AB	-

Table 4-3. ABO genotyping of patients with loss of antigen expression and haematological malignancy. Normal allele dosage is indicated by a dash in the appropriate column.

Of the nine AO genotype patients examined, two had loss of heterozygosity of the A allele (GN[#] and CG[*]), and two had loss of the O allele (AN and MR). Of course, PCR genotyping can only reveal an imbalance in the representation of the alleles. Although in most cases this would be due to loss of heterozygosity, duplication of an allele would give the same results. All of these experiments were carried out

[#] The differences in the relative intensities of the alleles (in patients GN and AN) are subtle but clearly seen by examination of the negative from which Figure 4-6 was produced. Photographs prepared from the negative did not retain the resolution of the original picture, and were the same as the scanned negative.

[*]The sample from patient CG shown in Figure 4-6 is probably incompletely digested with *Kpn*I, as three repeats of this experiment in each case revealed a significantly more intense lower band.

without knowledge of the patient's karyotype, or the percentage of blast cells in the particular sample (except for patient WM). It was therefore obvious when the cytogenetic results on patient CG became available, that unequal allele dosage was in fact due to duplication of the O allele of the ABO gene. As shown in Chapter 3, Table 3-1, this patient had CML, and 2 Philadelphia chromosomes as well as one cytogenetically normal chromosome 9. As the ABO gene is translocated with the ABL gene to join chromosome 22 and form the Philadelphia chromosome, it follows that there would be 3 copies of the ABO gene. As ABO genotyping indicates there are more copies of the O allele than there are of the A allele in this patient, presumably the chromosome 9 carrying the O allele has taken part in the translocation event. If this is so, and therefore the leukaemic cells in this patient still carry a normal A allele, why did this patient lose expression of the A antigen?

To date, nothing is known about the regulation of the ABO gene. One could postulate a feed-back mechanism; that is when levels of the ABO transferase are high enough, the gene "switches" off. In this case, two expressed copies of the O allele might lead to down-regulation of the A allele on the normal chromosome 9. The problem with this theory however, is that the O allele does not produce a full length product. It appears then that either the protein product that is produced by the O allele before truncation is in some way sufficient to lead to down-regulation of the A allele, or that regulation of the ABO gene takes place at the RNA level. Alternatively, the A allele in the leukaemic cells of this patient may carry a mutation, making it non-functional.

Loss of the A allele in patient GN is probably due to loss of heterozygosity of the ABO gene, and explains loss of the A antigen from her red cells. Although samples taken from three different time points in this patient's disease were genotyped, and all showed loss of the A allele, it should be remembered that the sample from

15/1/93 (sample GN PT), was taken four months after the patient underwent a bone-marrow transplant and was in clinical remission. The patient was transplanted with bone-marrow from her sister, who was blood group O. Subsequently, it appears that the allogeneic marrow has almost completely replaced the patient's own marrow, as evidenced by a faint non-digested band in the *Kpn*I digested sample, and the corresponding faintly visible digested band in the *BstE*II digested lane, which is indicative of the presence of a small amount of A allele.

In the remaining five AO genotype patients, the relative intensity of the allelic bands was identical to that of normal controls, suggesting that there was no loss of heterozygosity at the ABO locus. However, it might be argued that the proportion of leukaemic cells in the samples from these patients was too small for loss of heterozygosity to be detected. The number of blast cells in each of the particular samples was used to estimate the minimum number of leukaemic-derived cells, as shown in Table 4-3. As loss of heterozygosity of the ABO gene was able to be determined in patient GN, who had the least number of blast cells of all the patients, it was concluded that the five patients with normal relative intensity of the alleles did in fact have equal allele dosage. In the cases of patient PM and BA, blast cell counts were unavailable. However, a bone marrow report from patient PM indicated >80% blast cells at approximately the same time that the PB sample was taken. Furthermore, this patient succumbed to the disease shortly after, indicating that there was probably a significant proportion of leukaemic derived cells in the patient's PB sample. The patient BA, had no detectable A antigen on his red cells, and succumbed to his disease several months after the PB sample was taken. The assumption was therefore made that if LOH was occurring, it would have been detected.

To test the sensitivity of the ABO genotyping method, varying proportions of AO and OO genotype cells were mixed, and DNA prepared from them. Differences in the relative intensity of the bands following PCR/digestion, could be determined (visually) from samples with as few as 18% OO cells and 82% AO cells. Theoretically, this mixture would represent a sample in which 18% of the cells had physical loss of the A allele and duplication of the O allele by mitotic recombination. If loss of heterozygosity was due to a simple deletion, the mixture of 18% OO and 82% AO cells would represent a sample with 30% O/- cells, and 70% AO cells. Densitometry was not used to increase the sensitivity of these experiments, as placement of the base-line is too subjective when differences between the band intensities are so subtle they can not be determined visually.

Comparison of the genotyping results from patients GN and AN implies that both samples have approximately equivalent amounts of cells with LOH. However, the corresponding BM reports on these patients would suggest that there were more cells derived from the leukaemic clone in patient AN (19% blast cells compared with 5.8% blasts in the bone marrow sample from GN). These results are probably an indication of the inadequacy of using blast cell counts to quantitate the number of leukaemic cells. Furthermore, if LOH was only occurring in 5.8% of the cells from the patient GN, it would have been below the threshold for detection.

The same principles were applied to the examination of AB genotype patients for LOH. In these cases, digestion of the 181bp fragment with *Msp*I and *Alu*I was compared to that of normal AB controls. In all four cases, the relative intensity of the allelic bands was identical to the normal controls, indicating no difference in allele dosage. Although the samples from 3 of these patients had more than 30% blast cells, a differential cell count on the sample from patient WD was not available, and therefore no conclusions could be made from the results on this

patient. It was concluded that the patients KN, DH and SR did not have loss of heterozygosity at the ABO locus.

4.2.2.3 Ante-natal patients with abnormal A antigen expression detected during routine screening

Three ante-natal patients (BG, HT, and SK) exhibited abnormal blood group serology, as described in Chapter 3. Briefly, BG had grouped on different occasions as A^1, A^2, A^x/A^3 and **O**. HT had grouped on two different occasions as normal AB, and subsequently as **weak A, normal B**. SK has previously grouped as B, and subsequently as **A^2B** (described by the Blood Transfusion Unit at the QEH as acquired A antigen). The samples used for genotyping these patients were all from the most recent time point (indicated by bold type). Genotyping of the patients revealed that all three possessed an A allele; BG genotyped as normal AO, while HT and SK genotyped as AB (data not shown).

These results show that the patient SK did not have acquired A antigen, but was in fact was a true AB blood group. However, these results did not explain the weak expression of the A antigen in these patients. It could be hypothesised that the patients carried the A^2 allele, which encodes a transferase that is less efficient at converting the precursor H antigen than the A^1 transferase. These patients were therefore retained for further analysis, to see if they did in fact possess the A^2 allele.

4.3 Development of a method to genotype the A^2 allele

Approximately 10% of all individuals fall into the A subgroup, A^2. A^2 can be defined as a weak reaction with anti-A, and no reaction with *Dolichos biflorus* lectin. As a weak reaction with anti-A might sometimes be the result of loss of the A^1 glycosyltransferase, a molecular method to define the A^2 allele was subsequently developed to determine if some of the patients who had been blood grouped as A^2

did in fact carried the A^2 allele or could possibly have low expression of the A^1 glycosyltransferase.

4.3.1 Rationale

The A^2 allele is characterised by two differences from the A^1 allele. There is a single base substitution at nt 467, resulting in a leucine at amino acid 156 in the A^2 glycosyltransferase, where normally a proline residue exists in the A^1 allele. Secondly, there is a single base deletion in one of the three C residues at nucleotides 1059-1061, close to the carboxyl terminal of the gene (Yamamoto *et al.*, 1992). Analysis of the single base change at nt 467 (C in A^1, T in A^2) reveals that the base change destroys an *Msp*I restriction enzyme site present in the A^1 allele, and creates a *Pvu*II site in the A^2 allele (see Figure 4-8). Primers spanning this region of DNA were subsequently designed.

467

A^1 allele GACCAGC**C**GGCC

A^2 allele GACCAGC**T**GGCC

Figure 4-8 Comparison of the A^1 and A^2 allele sequences. The C→T transition at nt 467 is represented by bold type. The *Msp*I restriction enzyme recognition site in the A^1 allele is underlined, as is the *Pvu*II site in the A^2 allele.

4.3.2 Materials and methods

Primers spanning the region of interest, nt 467 of the ABO cDNA sequence, were designed. Genomic DNA was isolated from 4 healthy individuals of A^2 blood group, and amplified using the following primers:

A10	5' TTCCTGAAGCTGTTCCTGGAGACG 3'	(sense)
AB2	5' GCTCGTAGGTGAAGGCCTCCC 3'	(anti-sense)

PCR was carried out as described in Section 2.6.4. Ten microliter aliquots of the resulting PCR products (357bp), were digested for 8 hours in a total volume of 20µl, with 10 units of *Msp*I or *Pvu*II restriction endonuclease (New England Biolabs), according to the manufacturer's instructions. The digested samples were then resolved on a 2% agarose or 8% polyacrylamide gel (Section 2.6.6).

4.3.3 Results and Discussion

The primers chosen amplify the region from nt 385-742, and the resultant 357bp PCR product was digested with *Msp*I and *Pvu*II. This method was applied to DNA samples from 4 healthy individuals of blood group A^2, and also samples from O, A^1 and B individuals. Using the ABO genotyping method for the three major alleles, it was determined that all the A^2 blood group samples were heterozygous for the A and O alleles. Digestion with *Msp*I or *Pvu*II resulted in a number of different sized bands, as the region amplified contains at least one other site for each enzyme. The predicted band sizes as determined from the cDNA sequence (Yamamoto *et al.*, 1992) are shown in Table 4-3.

Genotype	*Msp*I	*Pvu*II
OO/A^1A^1	204, 82	234,123
A^1O	204, 82	234,123
A^2O	204,113,82	234,123,82
A^2A^2	204,113	234,82

Table 4-4. Fragment sizes (bp) of visualised restricted PCR products amplified by primers A10 and AB2. The O, A^1 and B alleles contain identical restriction sites (with the exception that the B allele has one less *Msp*I site), hence all possible genotypes are not listed. The fragment amplified is 357bp, however this region contains other restriction sites for both *Msp*I and *Pvu*II, which act as a control for digestion; hence if there is complete digestion the 357bp fragment will not be present.

Analysis of DNA samples from individuals of various blood groups (including A^2 blood group) confirmed the reliability of this technique (see Figure 4-9).

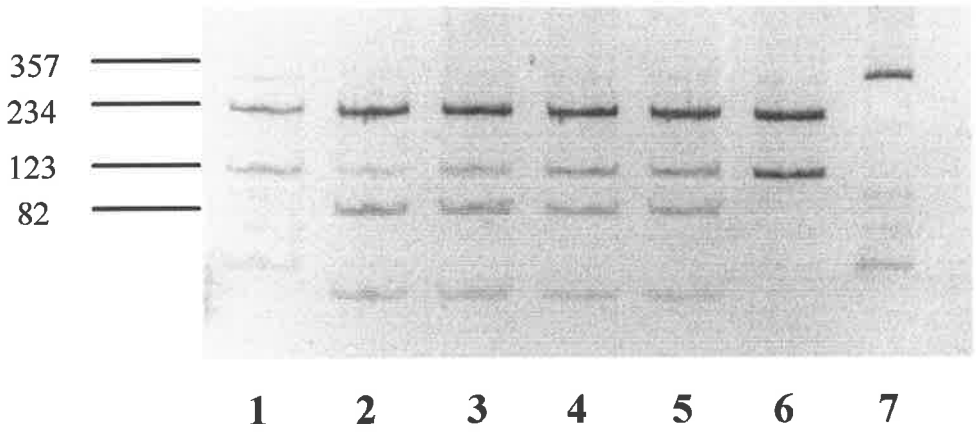

Figure 4-9. Detection of the A^2 allele of the ABO gene. Genomic DNA was amplified using primers A10 and AB2, and digested with *Pvu*II. Lanes 1 and 6 are from OO and A^1A^1 genotype individuals respectively. Lanes 2-5 are products generated from A^2 blood group individuals, all four genotype as A^2O. Lane 7 is undigested PCR product. Note that the 82bp band only appears in the A^2 individuals. The sizes of the bands are indicated in base pairs.

Digestion with *Msp*I produced the predicted band sizes for all genotypes (results not shown). However, this enzyme seemed particularly refractory to digesting heteroduplexes, as digested bands were poorly visible, reminiscent of the results obtained with *Msp*I digestion for the ABO genotyping method. As digestion with *Pvu*II detects the A^2 allele, and incomplete digestion is controlled for by the existence of another *Pvu*II site within the PCR product (at nt 508), it is not necessary to digest with both enzymes. Subsequently, all further experiments using this PCR method only included digestion with *Pvu*II.

Close examination of Figure 4-9 reveals that although this genotyping method reliably detects the A^2 allele, digestion with *Pvu*II does not approximate the expected 1:1 ratio. In fact, *Pvu*II does not seem to be sensitive to heteroduplex formation at all, with over half of the product digested in two of the lanes (lanes 2 and 3). It seems that, unlike most of the other enzymes used in this study, *Pvu*II can

digest heteroduplexes. Digestion conditions were varied, however there was either very little digestion, or over-digestion. Conditions ranged from 7 units of enzyme over 5 hours (lack of digestion as evidenced by the presence of a strong band at 357bp, and a weak band at 82bp), to 10 units for 5 hours to overnight (digestion of heteroduplexes). Regardless of the source of this variation in digestion, the technique was used to analyse patient samples for the A^2 allele, however it was not considered a reliable indicator of allele dosage.

4.4 A^2 GENOTYPING OF PATIENTS WITH LOSS OF A ANTIGEN EXPRESSION

This method was applied to patients from our collection of specimens for whom blood group data had been recorded as either A^2 or A^2B. Patients that had been provided to us as "loss of ABO patients" were also tested, as were the ante-natal patients, which had all been grouped as either A^2 or A^2B at least at one stage in their case history.

4.4.1 Results and Discussion

4.4.1.1 Patients of A^2 blood group

Normally the blood transfusion unit at the QEH does not divide patients of A blood group into the subgroups A^1 and A^2. However, if there is a notably weak reaction with anti-A, or anti-A^1 appears in the patient's serum, further analysis on that particular sample will be carried out. Further analysis usually consists of testing the sample for agglutination with the lectin, *Dolichos biflorus*. Lack of agglutination with this lectin results in the blood group A^2 being recorded. Of the 34 A and AB blood group patients with haematological malignancy described in 4.2.2.1, one patient was recorded as A^2B (ID 068152); no other patients were reported as either A^2 or A^2B. The A^2 genotyping method was applied to this patient to determine if the patient was truly A^2B, or possibly had weak A^1 expression. To determine if the source of weak A antigen in the three patients from the ante-natal clinic might be

explained by the presence of the A^2 glycosyltransferase, these patients were also examined using this method.

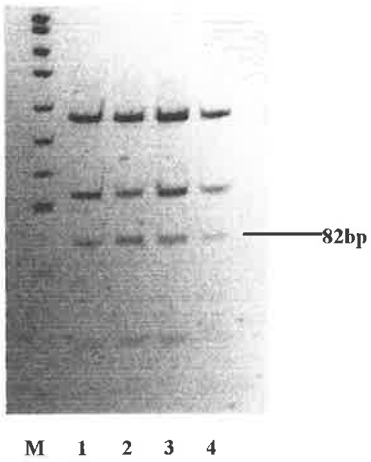

Figure 4-10. PCR A^2 genotyping of patients with weak A antigen expression or blood group A^2. Lane 1, patient 068152; lane 2, BG; lane 3 HT; lane 4, SK. The marker (M), is pUC19/*Hpa*II. All four patients exhibit the 82bp band indicative of the ABO A^2 allele.

PCR genotyping revealed the A^2 allele in all four patients (see Figure 4-10). It was therefore concluded that the patient 068152 was truly A^2B blood group, SK and HT were A^2B, and BG was A^2O. It is interesting to note that the patient BG, had previously been blood grouped as A^1. Regardless of the original blood group of this patient, does the presence of the A^2 allele explain the weak A antigen expression seen in the red cells of these patients?

Economidou *et al.*(1967), made quantitative measurements of the number of A antigen sites on red cells in ABO blood group heterozygotes (see Table 4-5). Although these measurements reveal a large range in A antigen expression for each of the groups, there is approximately a ten-fold difference in the number of A antigen sites in A^1B and A^2B heterozygotes. This probably explains the weak A antigen expression in the AB patients examined here. The fact that one of these patients (SK) had previously grouped as B however, suggests there may be an intrinsic variability in the normal expression of the A antigen, perhaps controlled by

as yet unknown factors. This hypothesis might also explain the findings with the patient BG, who had been previously grouped as A^1 but subsequently grouped as A^2, A^x/A^3 and O. One common factor between these 3 patients is that they were all pregnant, suggesting that perhaps pregnancy may induce changes in ABO antigen expression.

Blood group	A antigen sites
A^1B	4-8 x 10^5
A^1O	8-12 x 10^5
A^2B	4-12 x 10^4
A^2O	15-40 x 10^4

Table 4-5. Number of A antigen sites on red cells from individuals heterozygous for the ABO alleles (Economidou *et al.*, 1967).

An association between low expression of A antigen and pregnancy has been documented. Schachter *et al.*(1971), assayed the level of N-acetylgalactosyltransferase (the A transferase), from the serum of two pregnant women who were blood group A^1, and found that the levels were significantly reduced compared to normal controls. Tilley *et al.* (1978), later confirmed these results by assaying serum levels of the A transferase in six A^1 and three A^2 group women who had given birth within the last 48 hours. In the A^1 women, enzyme levels were 47% of that of the normal A^1 controls, while in the A^2 women, levels were approximately 67% of normal A^2 controls. Serum H-transferase activity was also assayed, and although there was a slight decrease in the new mothers compared to normal controls, it was not significant. Interestingly, Tilley *et al.*(1978), also found that the levels of A transferase in the group A infants of these mothers was at least as high (if not higher), than that of the normal adult controls.

Weak expression, or complete loss of the Lewis B antigen is commonly seen in pregnant women (reviewed in Wallace and Gibbs *et al.*, 1986; personal

communication with personnel in the Blood Transfusion Unit at the QEH). However, in the case of Lewis antigens, the situation is quite different to that of the loss of A antigens. Hammer *et al.* (1981), found that the level of Le^b glycolipid in the blood of pregnant women was the same as normal controls. Furthermore, two thirds of the Le^b antigen was attached to plasma lipoprotein, with the remaining third bound to red cells. The authors concluded that loss of Le^b antigen from the red cells was due to the increased ratio of lipoprotein to red cell mass (more than four-fold), that occurs during pregnancy. Therefore, more of the Le^b antigen could bind to lipoproteins.

Obviously loss of A and Le^b antigen expression during pregnancy occurs by completely different mechanisms. It seems that the only explanation for the decrease in A transferase activity and the corresponding loss of A antigen expression during pregnancy is that there is inhibition of the A transferase, perhaps due to hormonal changes.

4.4.1.2 A^2 genotyping of patients with loss of A antigen expression and haematological malignancy.

A^2 genotyping of patients with loss of, or weak A antigen expression and haematological malignancy revealed that four of the nine A blood group patients (44.4%), and two of the four (50%) AB group patients, carried the A^2 allele (see Table 4-6). As only 24% of A and 34% of AB blood group individuals are A^2 and A^2B respectively (Adelaide Blood Bank statistics), it seems that these results show a correlation between the A^2 allele and patients with loss of A antigen expression.

Patient	ABO genotype
AN	A^2O
GN	A^2O
MR	A^2O
NR	A^1O
WM	A^1O
MA	A^1O
CG	A^2O
PM	A^1O
BA	A^1O
WD	A^2B
KN*	A^1B
DH*	A^1B
SR	A^2B

Table 4-6. Results following ABO genotyping of the A^2 allele in patients with loss of A antigen expression and haematological malignancy. * patients that exhibited loss of A and B antigen expression.

Both the ante-natal patient BG, and the patient GN had previously been blood grouped as A^1. As both the patients genotype as A^2O, it seemed there is a discrepancy between the genotype and the patients' original blood groups. These concerns were discussed with Dr. Hay and other personnel from the Adelaide Blood Bank, who confirmed that if the *Dolichos* lectin used for grouping was either old or incorrectly diluted, it could agglutinate A^2 red cells; subsequently A^2 group cells could be incorrectly grouped as A^1. When asked if they had ever seen changes of this type in regular blood donors, they admitted that in the past eight years two donors had exhibited dramatic changes in expression of the A antigen. In both of these cases the donors had been referred to haematology clinics for further testing. Both donors were subsequently diagnosed with leukaemia.

4.5 Confirmation of Allele Dosage using Southern Blotting

As further confirmation that the group of patients with loss of ABO antigens had no change in allele dosage, Southern blotting was carried out on specimens of which there was sufficient amounts of DNA available. As no probe was available for the

ABO gene, the first aim in this section was to clone a segment of the coding region for subsequent use as a probe.

4.5.1 Rationale

Yamamoto *et al.* (1990) used Southern blotting of genomic DNA to genotype individuals at the ABO locus. As the probe used (a full length cDNA clone) was not available to us, it was necessary to generate a probe. The Southern results from Yamamoto's group revealed two major bands of 10 and 6kb, in AO or BO genotype samples when digested with *BstE*II. Theoretically, a 4kb band should have been detected with the 6kb band, however the authors do not explain the lack of this band in their Southerns (it appears the 4kb band has been cut out of the picture). Nevertheless, these results indicated that no *BstE*II sites other than the diagnostic site at nt 261 were detected by the full length cDNA probe. Therefore, it was feasible to generate a smaller probe using primers already in the laboratory. The primers chosen to generate ABO PCR product for cloning span nt 562-742 of exon 7 of the ABO cDNA sequence.

4.5.2 Materials and Methods

Genomic DNA from an individual of genotype BO was amplified using primers AB1 and AB2 as described in 2.6.4. The resultant product was purified and cloned into the modified pGEM5zf(+) (pGem-T), cloning vector as described in section 2.6.9. Manual sequencing was then performed in both directions to confirm the presence of the appropriate insert (2.6.10). Southern blot analysis of *BstE*II digested genomic DNA, was carried out as described in section 2.6.7, the blot was washed in 0.1xSSC, 0.1% SDS at 65°C, for 15 minutes. The blot was then exposed at -80°C for 3 days.

4.5.3 Results and Discussion

Two clones were analysed (6J and 7J). Sequencing revealed that 6J contained the B-allele, in a sense orientation for the T7 RNA polymerase transcription initiation site, and 7J contained the O allele, in a sense orientation for the SP6 RNA polymerase transcription initiation site. The clone 7J was used as a probe for the Southern blot, and revealed two bands (10kb and 4kb), in all individuals tested (see Figure 4-11).

There was enough DNA to examine patients NR, WM and CG. However, analysis of genomic DNA from patient CG on a 0.8% agarose gel revealed laddering of the DNA, characteristic of apoptotic specimens. This is probably explained by the fact that this blood specimen was received at the QEH 14 days after it was taken from the patient. Regardless, the specimen was analysed on the Southern, however it yielded faint bands of 10 and 4kb amongst a smear of non-specific background; therefore no conclusions could be drawn from Southern blotting of this specimen. The remaining two patients however, showed the 10 and 4kb bands seen in the normal AO genotype control.

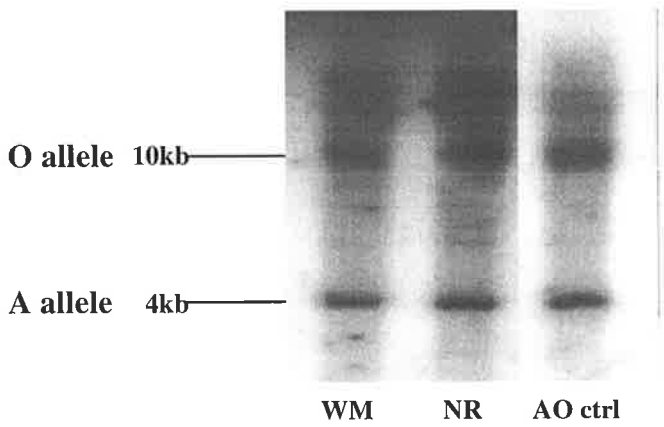

Figure 4-11. Southern blot analysis to determine allele dosage of the ABO gene. Lane 1 is patient WM, lane 2 is patient NR (BM 7/3/89), and lane 3 is DNA from a normal AO genotype individual.

Given that the samples analysed from WM and NR contained 97% and 85% blast cells respectively, it is clear that neither of the patients has loss of heterozygosity of the ABO gene, compared to the normal control. Unfortunately, when this gel was being prepared for Southern blotting, the acid-nicking step was accidently omitted. Other Southerns where the gel had been acid-treated yielded much clearer 10kb bands, however this Southern was not considered important enough to waste another 10μg of precious patient DNA repeating it.

SUMMARY

Methods for distinguishing the four major alleles of the ABO blood group gene were developed. Subsequent genotyping of samples from patients with haematological malignancy and colon carcinomas from patients of known blood group revealed discrepancies in two cases. Two patients, both with CML, genotyped as normal AO, but had always blood grouped O with no indication of any A antigen expression. These patients were retained for further analysis. Sixteen patients with abnormal antigen expression were analysed; thirteen had a haematological malignancy, and three were pregnant, but otherwise healthy. Four of the patients with haematological malignancy had unequal allele dosage of the ABO gene; two had loss of the A allele, and two had loss of the O allele. In one of the patients with "loss" of the A allele, cytogenetics revealed the presence of two Philadelphia chromosomes, and hence it was assumed that the Philadelphia chromosome carried the O allele. Loss of the A allele in the other patient explains the weak A antigen expression seen in this patient. In the patients who were pregnant, two genotyped as A^2B. It was postulated that weak antigen expression in these patients could be explained by the fact that the transferase encoded by the A^2 allele is significantly less efficient at converting the precursor H antigen when competing with the B transferase. This argument does not explain the weak A antigen expression seen in the third ante-natal patient, who genotyped as A^2O, as expression was so weak in this patient that she at one stage was considered blood group O. It was concluded that A transferase activity can be inhibited during pregnancy, probably by an as yet undefined mechanism induced by hormonal changes.

Finally, ABO genotyping of eleven of the patients with weak antigen expression and haematological malignancy was not able to yield an explanation, as nine of the patients had no detectable loss of heterozygosity at the ABO locus, and two had loss of the non-functional O allele.

Although loss of antigen expression in these eleven patients might be due to a mutation in the ABO gene, the fact that two patients had loss of the O allele indicates some kind of larger event is occurring. Furthermore, these results suggest that the ABO gene is not the primary locus involved in this event, as there would be no selective advantage for a cell that had lost expression of a non-functional allele.

As loss of antigen expression in most of the patients was not able to be explained by this PCR analysis of the DNA encoding the ABO gene, the next step in this project was to examine the expression of the gene.

CHAPTER FIVE

EXPRESSION OF THE ABO GENE

5.0 INTRODUCTION

In the previous chapter, patients with loss of blood group antigens were examined for loss of heterozygosity at the ABO locus. ABO genotyping revealed that nine of the patients had no loss of heterozygosity, and two of the patients had loss of the O allele. As these results could not provide an explanation for loss of antigen expression in these patients, the next logical step in this study was to analyse expression of the ABO gene at the molecular level.

The relative expression of the various alleles of the ABO gene at the mRNA level has not been described in the literature. Therefore, the first aim of the experiments contained within this chapter was to develop a sensitive molecular method for ABO phenotyping, and subsequently determine the expression of the various ABO alleles in healthy individuals. The second major aim of this set of experiments was to examine expression of the ABO alleles in leukaemic cells from patients with loss of ABO blood group antigens.

5.1 DEVELOPMENT OF A SENSITIVE METHOD FOR ABO PHENOTYPING

Yamamoto *et al.* (1990a), used Northern analysis to examine expression of the ABO gene. Northern analysis and RNase-protection assays were considered for this study, however disregarded due to the large amount of intact mRNA required for these types of analyses. Consequently, we decided on an RT-PCR approach, followed by allele specific restriction enzyme digestion.

5.1.1 Rationale

At this stage of this project, the intron-exon structure of the ABO gene had not been elucidated. As a result, primers spanning a region of approximately 500bp of the cDNA sequence were designed, to increase the chance of the amplified region spanning one or more introns and therefore specifically amplifying cDNA. The first consideration before attempting RT-PCR of the ABO gene however, was to develop an appropriate positive control for the reverse-transcription step.

5.2 POSITIVE CONTROLS FOR THE REVERSE-TRANSCRIPTION REACTION

5.2.1 Assessment of β-actin as a positive control for the reverse-transcription reaction

Initially, it was considered that β-actin expression would be an appropriate control for these experiments. β-actin is assumed to be constitutively expressed in all cells, and therefore would make an ideal positive control for reverse-transcriptions. Primers were designed to the β-actin gene so that they spanned an intron-exon boundary, and the expression of the β-actin gene in peripheral blood examined.

5.2.2 Materials and methods

RNA was isolated from peripheral blood from healthy volunteers, reverse-transcribed and subjected to PCR as described (see Sections 2.6.2 and 2.6.5), using the following primers:

β-actin A 5' TGTACGCCAACACAGTGCTGTCT 3' (sense)
β-actin B 5' CGTCATACTCCTGCTTGCTGATCC 3' (anti-sense)

PCR conditions were: 94°C for 5 minutes, followed by 35 cycles of 96°C for 30 seconds, and 60°C for 30 seconds (a "bounce" PCR). Aliquots (7μl) of the resulting PCR products were resolved on a 1.5% agarose gel.

5.2.3 Results and Discussion

The primers designed span nt 2638-2962 of the β-actin genomic DNA sequence (Genbank accession number M10277). This region contains part of exon 5, intron E and exon 6 of the gene. The predicted size of PCR product generated from genomic DNA is 324bp, while PCR product generated from cDNA would be expected to be 213bp (intron E is 111bp). It is clear from Figure 5-1 that the 213bp product is obtained from amplification of cDNA (lanes 1 and 2), however amplification of genomic DNA (lanes 3, 4 and 5) also produces a band of 213bp. This finding could not be explained by contamination with PCR product, as the β-actin gene had never been amplified in this laboratory before, and samples without DNA were always negative. It is interesting to note that amplification of DNA also produced a faint smear at approximately 324bp (Figure 5-1, lanes 3, 4 and 5 as indicated by the arrow).

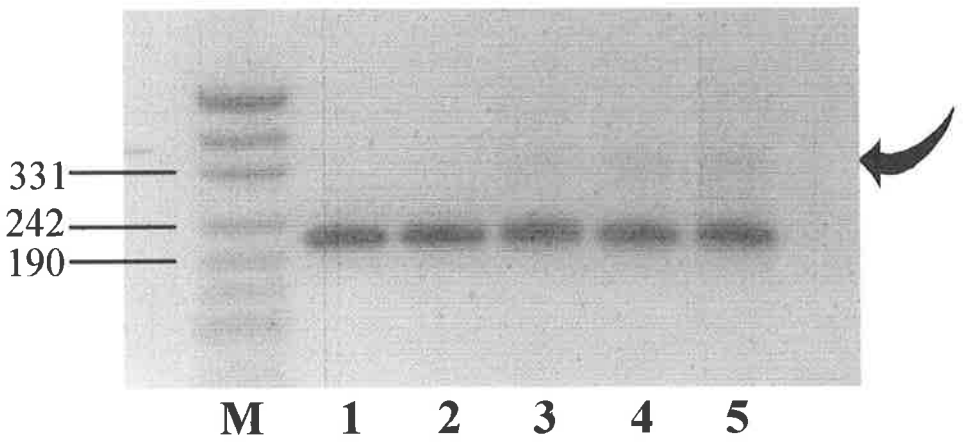

Figure 5-1. PCR amplification of β-actin. The marker is pUC19/*Hpa*II (M). PCR products were generated from normal peripheral blood cDNA (lanes 1 and 2), and genomic DNA (lanes 3, 4 and 5). The expected band of approximately 312bp is generated from cDNA, however a similar size band is generated from genomic DNA. A very faint smear of approximately 324bp, the expected product size from genomic DNA can be seen in lanes 3, 4 and 5, and is indicated by the arrow.

Consultation of the literature and Genbank revealed that there are at least 19 processed β-actin pseudogenes in the human genome (Leavitt *et al.*, 1984), which have approximately 95% homology with the β-actin cDNA sequence. Therefore, it was concluded that β-actin was not a suitable positive control for RT-PCR, as products generated from cDNA and contaminating genomic DNA in RNA preparations could not be distinguished. Since these results were obtained, other laboratories have published similar findings (Taylor and Heasman, 1994).

5.2.4 Assessment of β-globin as a positive control for the reverse-transcription reaction

It was expected that cells expressing the ABO gene would also express the β-globin gene; therefore primers were designed to the β-globin gene and analysed using RT-PCR.

5.2.5 Materials and Methods

RNA was isolated from peripheral blood from healthy volunteers, reverse-transcribed and subjected to PCR as described (see Sections 2.6.2 and 2.6.5), using the following primers:

β-globin A 5' GCAAGGTGAACGTGGATGAAGTTG 3' (sense)

β-globin B 5' GCTTGTCACAGTGCAGCTCACTCA 3' (anti-sense)

PCR conditions were: 94°C for 5 minutes, followed by 35 cycles of 96°C for 30 seconds, and 60°C for 30 seconds. Aliquots (7μl) of the resulting PCR products were then electrophoresed on a 1.5% agarose gel.

5.2.6 Results and Discussion

The primers designed amplify nt 62,236 - 62,605 of the genomic DNA sequence of the β-globin gene cluster region on chromosome 11 (Genbank accession number U01317). The sense primer is located in exon 1 of the β-haemoglobin gene, while the anti-sense primer is located in exon 2. The primers span intron 1, which is 131bp long. Therefore the expected size of amplified product from genomic DNA is 369bp, while a product of 238bp should be generated from cDNA.

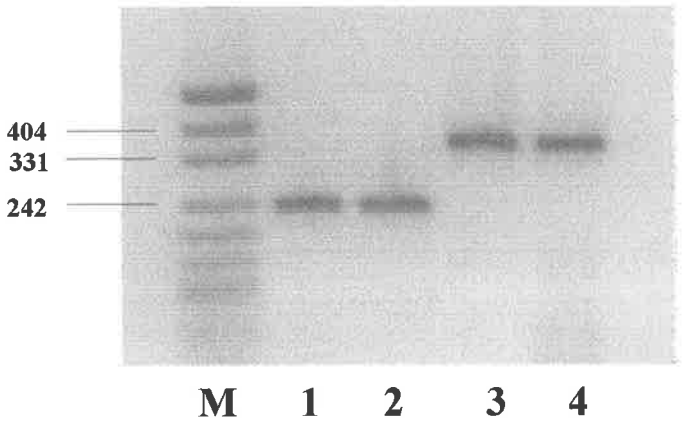

Figure 5-2. PCR amplification of β-globin. The marker is pUC19/*Hpa*II (M). PCR products were generated from normal peripheral blood cDNA (lanes 1 and 2), and genomic DNA (lanes 3 and 4). The expected band of approximately 238bp was generated from cDNA, while a band of 369bp was generated from genomic DNA.

It can be seen from Figure 5-2 that amplification using the chosen β-globin primers produces the expected bands of 238bp from peripheral blood cDNA (lanes 1 and 2), and 369bp from genomic DNA (lanes 3 and 4). However, expression of β-globin is supposedly restricted to the red cell lineage. As we were repeatedly able to amplify β-globin from normal peripheral blood mononuclear cells, either the cell preparations were contaminated with reticulocytes, or this method is detecting illegitimate transcription of the β-globin gene. Transcription of the β-globin gene has been shown before in a number of inappropriate cell types, including lymphocytes (reviewed in Kaplan *et al.*, 1992). Regardless of the nature of the transcript (either legitimate or illegitimate), amplification of β-globin was consistent, and therefore considered an appropriate positive control for the reverse-transcription reaction, and was used as such throughout the rest of this study.

5.3 Expression of the ABO gene

Initially, the primers used to examine the relative expression of the ABO alleles were two of the primers used in the ABO PCR genotyping method described in the previous chapter. The primers, AO1 and AB2, span nt 231-742 (exons 5-7) of the cDNA sequence of the ABO gene (Yamamoto *et al.*, 1990a; Yamamoto *et al.*, 1995). The predicted size of the amplified product generated from cDNA using these primers is 512bp. Table 5-1 shows the predicted fragment sizes after digestion with the restriction enzymes *Kpn*I and *Bst*EII for various phenotypes.

	*Kpn*I	*Bst*EII
OO	481, 30	512
AO/BO	512, 481, 30	512, 481, 30
AA/BB/AB	512	481, 30

Table 5-1. Predicted fragment sizes after digestion of the 512bp ABO RT-PCR product with the diagnostic restriction enzymes *Kpn*I and *Bst*EII, for various phenotypes.

5.3.1 Materials and methods

RNA was isolated from peripheral blood leukocytes of healthy volunteers, cell lines, and peripheral blood mononuclear cells from patient MCG (taken when the patient was in blast crisis). RT-PCR was carried out as described (see Sections 2.6.2 and 2.6.5), using the following primers:

AO1	5' TGACACCGTGGAAGGATGTCCTCGT 3'	(sense)
AB2	5' GCTCGTAGGTGAAGGCCTCCC 3'	(anti-sense)

The optimal conditions for this reaction were difficult to determine, however the final conditions chosen were: 94°C for 5 minutes, followed by 45 cycles of 94°C for 1 minute, 65°C for 1 minute and 72°C for 1.5 minutes. The PCR reaction was carried out in a Corbett Research FTS-1 thermal sequencer. Aliquots (10μl), of each of the reactions were then digested overnight in a total volume of 14μl, with 7 Units of *Kpn*I or *Bst*EII, and resolved on an 8% polyacrylamide gel as described (see Section 2.6.6).

5.3.2 Results and Discussion

No product was obtained from amplification of cDNA derived from the peripheral blood leukocytes of normal individuals. This is not surprising as leukocytes only express small amounts of ABH antigens, and it is thought this occurs by adsorption of the antigens from plasma (see Chapter 1). As expected, no product was obtained from genomic DNA. Products of the expected size were obtained from peripheral blood from the patient MCG, and all of the cell lines tested, excluding HL60 and HiMeg. Digestion of the products with *Kpn*I and *Bst*EII revealed two bands of the expected sizes, from which the phenotype of the cells could be determined (as shown in Figure 5-3 and Table 5-2).

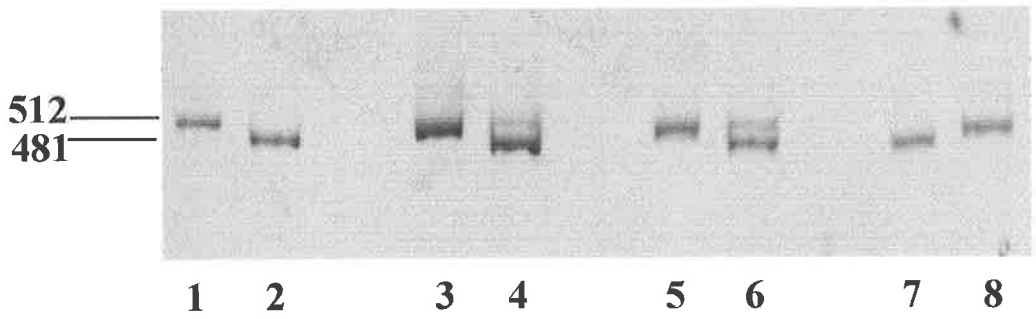

Figure 5-3. ABO phenotyping. RT-PCR product generated from cDNA was digested with *Kpn*I (lanes 1, 3, 5, and 7) or *Bst*EII (lanes 2, 4, 6, and 8), and resolved on an 8% polyacrylamide gel. The samples were from the cell lines SW480 (genotype AA, lanes 1 and 2), and HT29 (genotype AO, lanes 3 and 4), the patient MCG (genotype AO, lanes 5 and 6), and the cell line K562 (genotype OO, lanes 7 and 8).

RT-PCR revealed that both K562 and HEL (both genotype OO) expressed the O allele (Figure 5-3, lanes 7-8; HEL is not shown), and SW480 (genotype AA) expressed the A allele (lanes 1 and 2). The phenotyping results for HT29 and the patient MCG were unclear (lanes 3-6); both of these samples genotyped as AO, however there was no digestion of the RT-PCR product with *Kpn*I, suggesting no expression of the O allele in these samples. Identical results were obtained for all the heterozygous cell lines tested, regardless of whether the genotype was AO or BO (see Table 5-2). However, both K562 and HEL exhibited complete digestion with *Kpn*I, and therefore it could be concluded that the O allele is expressed.

Cell Line	Type	ABO genotype	ABO phenotype
K562	CML - erythroleukaemia	OO	O
KCL22	CML	AO	A
HiMeg	CML-Megakaryocytic leukaemia	AO	no expression
HEL	erythroleukaemia	OO	O
HL60	promyelocytic leukaemia	AO	no expression
HT29	colon carcinoma	AO	A
SW480	colon carcinoma	AA	A
LIM 1863	colon carcinoma	OO	O
LIM 2412	colon carcinoma	AO	A
LIM 2463	colon carcinoma	OO	O
LIM 1215	colon carcinoma	BO	B
LIM 2405	colon carcinoma	OO	O

Table 5-2. ABO phenotyping results from cell lines as deduced by RT-PCR and diagnostic restriction enzyme digestion of ABO mRNA.

Digestion of the RT-PCR products from MCG and HT29 with *Bst*EII, which digests the A and B alleles, revealed a small undigested fraction of the product - although SW480 digested completely with this enzyme. This result was repeatable, and can be explained by the presence of a small amount of O allele. If this were so, heteroduplex formation could be sufficient to completely inhibit digestion with *Kpn*I, however digestion with *Bst*EII would be expected to be incomplete as it too is refractory to heteroduplex digestion. The most likely explanation for the relatively low amount of O allele present in heterozygous samples is that the O allele mRNA is less stable than that of the A or B alleles; this point will be discussed in further detail elsewhere in this chapter.

Although DNA from the patient MCG genotyped as AO, blood group results from this patient 16 years before the onset of disease reported the patient as unequivocally blood group O. However, the results shown here suggest that the patient exclusively expressed the "A" allele, and are identical to that of the cell lines HT29, LIM 1215, LIM 2412 and KCL22, all of which genotyped as AO or BO. Two conclusions were made from this set of experiments. Firstly, the primers chosen for this analysis were unsuitable for these experiments as the difference

between the 512 and 481bp fragments was too subtle. Secondly, the patient MCG could possess an new kind of O allele, not characterised by the single base deletion at nt 261.

5.4 SEQUENCING OF THE NEW "O" ABO ALLELE

As the phenotyping results on patient MCG were inconclusive, it became clear that there could be a mutation in the "A-like" allele, that made it non-functional. As the fragment amplified spans approximately half of the coding region of the gene, it seemed appropriate to begin with sequencing this region. It appeared that there was no significant amount of contaminating transcript from the "normal" O allele, as evidenced by lack of digestion of the PCR product with *Kpn*I, therefore sequencing was carried out directly on the ABO RT-PCR product generated from this patient.

5.4.1 Materials and Methods

The ABO RT-PCR product generated from the patient MCG in section 5.3.1 was purified and sequenced on an automatic sequencer using the primers AO1 (sense sequence), and AB2 (anti-sense sequence), as described in Sections 2.6.8 and 2.6.9.

5.4.2 Results and Discussion

The product was sequenced in both directions, and a consensus sequence derived from both the sequences (for the original sequence data, see Figures 5-4 and 5-5 over the page). Comparison of the consensus and nt 262-707 of the A^1-allele sequence revealed three single base substitutions, two of which would confer amino-acid changes on the protein. Figure 5-6 summarizes the differences found:

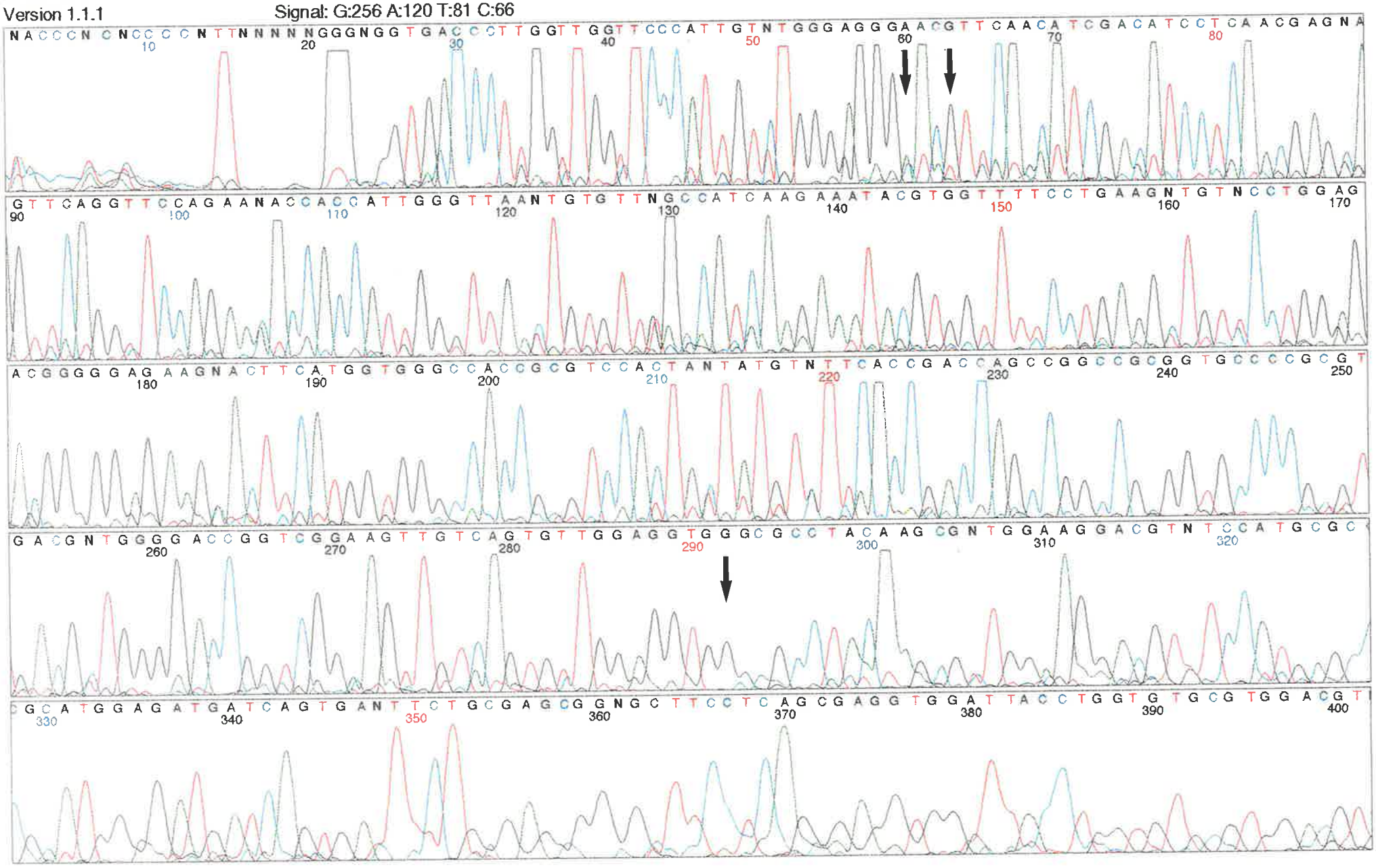

Figure 5-4. Raw sequencing data (sense direction) of the new O allele from patient MCG. Three bases deviate from the published A[1] sequence, as indicated by the arrows.

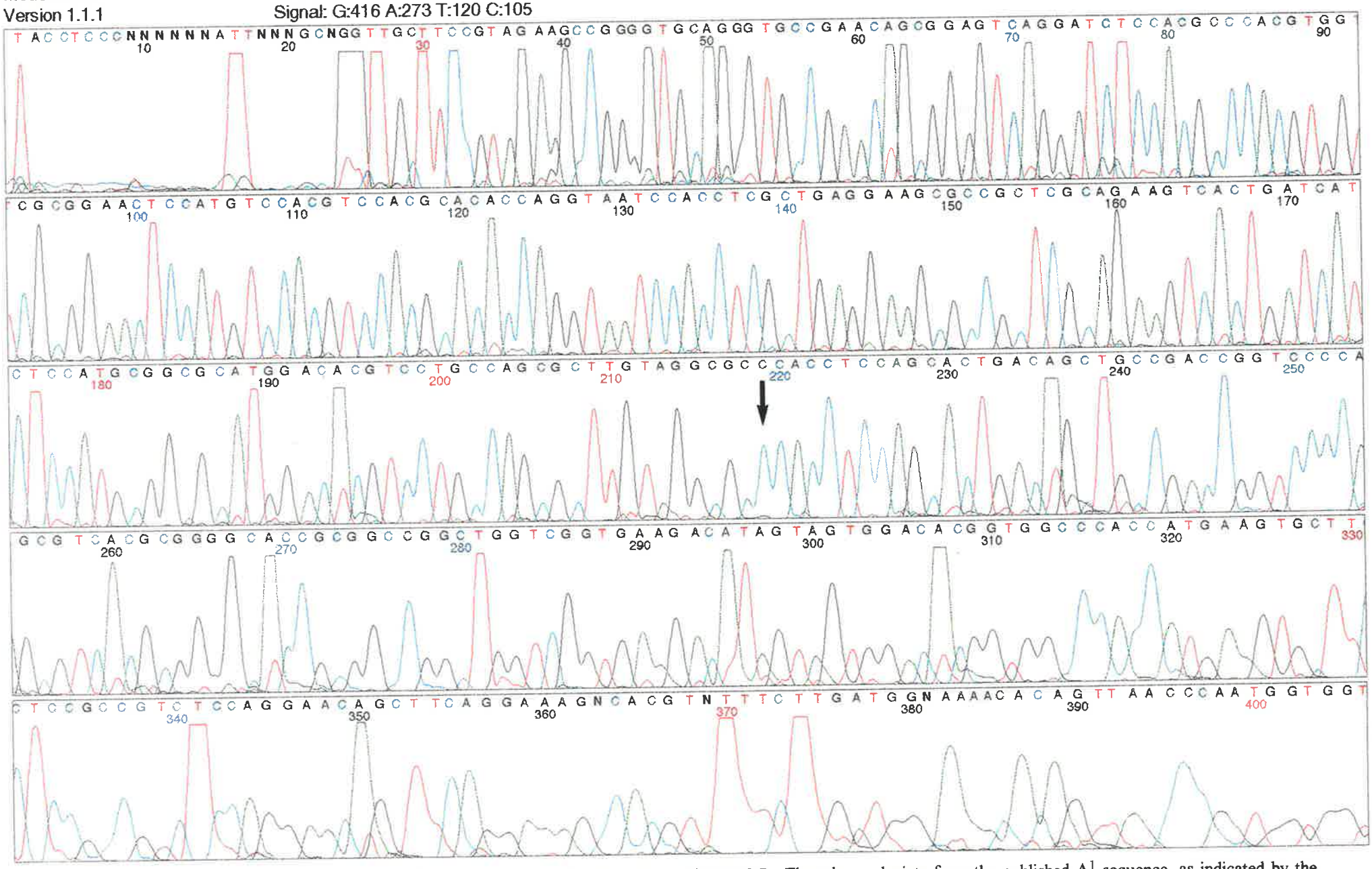

Figure 5-5. Raw sequencing data (anti-sense direction) of the new O allele from patient MCG. Three bases deviate from the published A^1 sequence, as indicated by the arrows (continued over page).

Applied Biosystems
Model 373A
Version 1.1.1

24 MC GDNA 3'
DyeTerminator{AnyPrimer}
Lane 24
Signal: G:416 A:273 T:120 C:105

Points 647 to 6900 Base 1: 647
432 MATRIX FILE
D.O'Keefe QEH

Tue, Dec 15, 1992 2:13 PM
X: 0 to 6517 Y: 0 to 1200

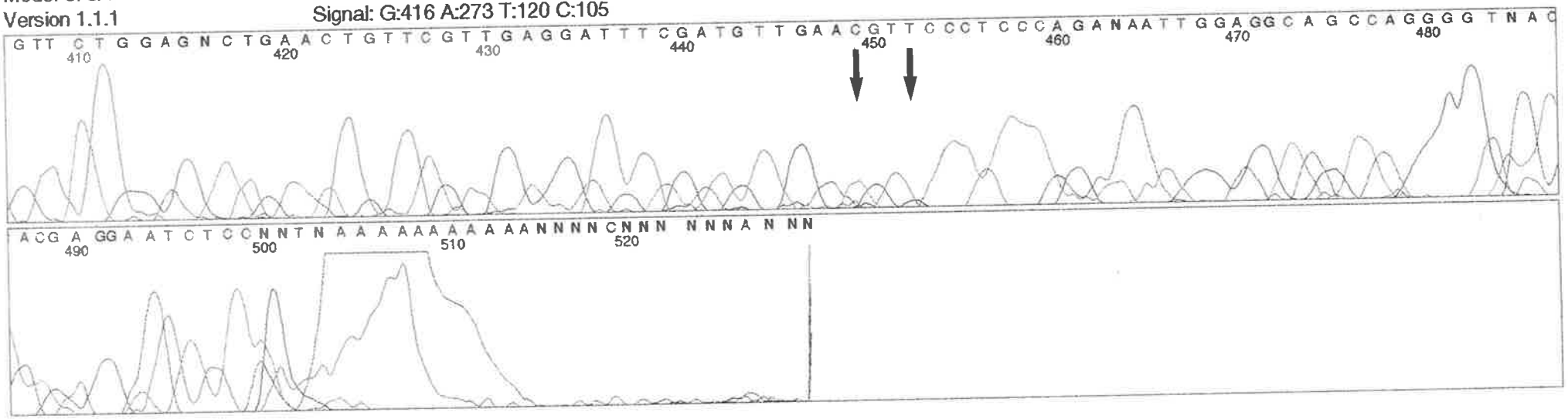

Figure 5-5 (continued)

		Gly Thr		
292	5'	GGC ACA	3'	A^1 allele
		GG**A** AC**G**		patient MCG
		Gly Thr		
		Arg		
526	5'	CGC	3'	A^1 allele
		GGC		patient MCG
		Gly		

Figure 5-6. Comparison of the patient MCG sequence and the common A^1 allele. Nucleotide and inferred amino-acid differences are indicated by bold type.

It is interesting that the second substitution in this new allele, A→G at position 297, is also found in the B allele, as is the third substitution, C→G at position 526 (a.a. 176, arginine→glycine; Yamamoto *et al.*, 1990b).

Some time after this work was completed, another type of O allele was published; the O^2 allele (Yamamoto *et al.*, 1993b). This sequence was compared with our new O allele. The substitutions at positions 297 and 526 were the same in both alleles, however the substitution in our new O allele at position 294 was not reported in the O^2 allele. Later, another group sequenced 3 more O^2 alleles from the Danish population (Grunnet *et al.*, 1994). Once again, the change we found at position 294 was not reported in these alleles. As only 4 O^2 alleles have been sequenced in this region, it is possible that this base change is another (silent) polymorphism of the ABO gene.

Alternatively, close examination of the raw sequencing data (see Figures 5-4 and 5-5 position 60 and 452 respectively), reveals that in both the forward and reverse sequence, there is a smaller peak corresponding to nt 294 of the ABO sequence. In the forward sequence, the smaller peak indicates a cytosine, while in the reverse sequence, the smaller peak indicates the presence of guanine. As the common A allele has a cytosine at nt 294, it is possible that at some stage early in the

amplification reaction, *Taq* DNA polymerase incorrectly incorporated adenine instead of a cytosine. This scenario is unlikely for two reasons. Firstly, it is obvious that most of the transcripts sequenced have an A at nt 294; given that presumably there were a reasonable number of ABO cDNA transcripts at the start of the reaction, there should not be such a bias to one particular mutated transcript. Secondly, smaller peaks are produced by background fluorescence throughout the sequence, and as such it is possible that the smaller peaks seen beneath the adenine peak at 294 in the forward sequence, and the thymine in the reverse sequence, are due to non-specific background.

It now remained to be determined whether or not the other patient who genotyped as AO by our standard method, yet was blood group O (patient DN), also possessed the O^2 allele. As no frozen samples were available from this patient, it was not possible to carry out RT-PCR and sequencing as for patient MCG. Therefore a method to determine the presence of the O^2 allele directly from genomic DNA was developed.

5.5 Development of a PCR method to detect the O^2 allele

5.5.1 Rationale

Grunnet *et al.* (1994) used PCR genotyping to determine the presence of the O^2 allele in individuals of O blood group. The method described however, required 1μg of DNA target for amplification, and is followed by restriction digestion of the product, blotting and hybridisation with a radio-labelled probe. We wished to develop a quick, sensitive method to distinguish the O^2 allele from the A allele, preferably avoiding the use of radio-active probes.

Given that samples we wished to examine for the O^2 allele were from blood group O individuals, who had previously genotyped as AO (as described in Chapter 4), it

was only necessary to examine the gene at one site to distinguish the A and O^2 alleles. We chose to use the substitution at nt 526, which is a C in the A^1 allele, but a G in the B and O^2 alleles. As the samples were already genotyped as AO, it does not matter that this method does not distinguish between the B and O^2 alleles. The base substitution in the O^2 allele creates a *Nar*I restriction enzyme site, where there is a *Bss*HII restriction enzyme site in the A^1 allele, as shown in Figure 5-7 below:

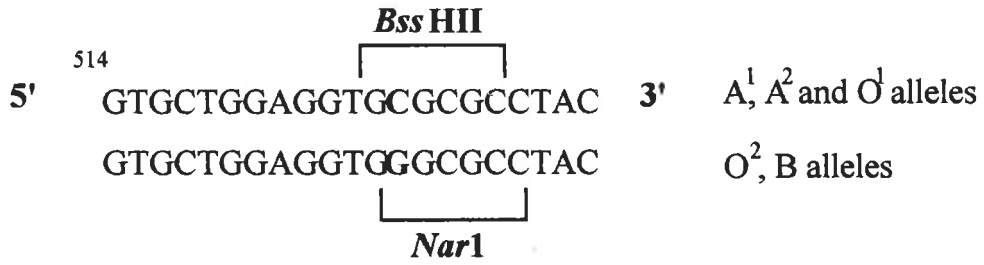

Figure 5-7. Comparison of the nucleotide sequence of the 5 major ABO alleles at position 526. The A^1, A^2 and O alleles can be distinguished by digestion with the restriction enzyme *Bss*HII, from the O^2 and B alleles, which digest with the enzyme *Nar*I.

5.5.2 Materials and methods

Genomic DNA from normal individuals that had previously been ABO genotyped using the PCR genotyping method described in Section 4.1.2, and the patients MCG and DN, was amplified using the primers A10 and AB2 as described for the A^2 genotyping method (Section 4.3.2). Aliquots of the resultant PCR product (10µl), were digested with 10 Units of the restriction enzyme *Bss*HII (New England Biolabs), according to the manufacturer's instructions. The digested products were resolved on 1.8% agarose gels (Section 2.6.6).

5.5.3 Results and Discussion

DNA from individuals of genotype OO, AO, AA, BO and BB was amplified and digested with the enzyme *Bss*HII. Figure 5-8 shows the expected pattern of bands from the various genotypes, that is, digestion of O and A alleles, and no digestion of B alleles. Digestion of the site at nt 526 produces two bands, of 216 and 141 base pairs.

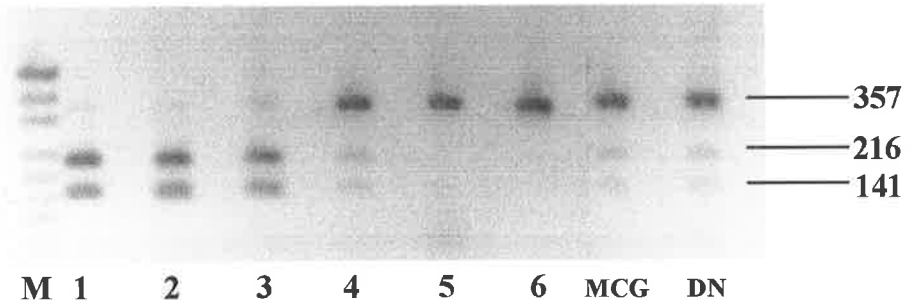

Figure 5-8. Detection of the O^2 allele. PCR product generated from genomic DNA was digested with *Bss*HII. Complete digestion is seen in individuals of OO (lane 1), AO (lane 2), and AA (lane 3) genotype, while partial digestion is seen in an individual of BO genotype (lane 4), and no digestion in a BB individual (lane 5). Undigested product is shown in lane 6, while samples from the patients MCG and DN, who genotype AO, exhibit the same pattern as the BO individual, indicating the presence of the O^2 allele. The marker (M) is pUC19/*Hpa*II.

As the O^2 allele, like the B allele, does not have a *Bss*HII restriction site within this PCR product, it would be expected that O^1O^2 heterozygous individuals would produce the same pattern of bands as a BO^1 heterozygous individual following application of this method. Figure 5-8 shows the application of this method to samples from the patients MCG and DN. Although the enzyme *Bss*HII is extremely sensitive to heteroduplex formation (as was the enzyme *Nar*I; data not shown), it can be seen that the patient MCG genotypes as O^1O^2, which was expected from the sequencing data described in Section 5.4. Patient DN, who blood grouped unequivocally as O, yet was genotyped as AO using the genotyping method described in Section 4.1.2, also genotypes as O^1O^2 . Therefore, as the apparent anomaly between the blood groups and ABO genotypes of these patients had been explained, they were excluded from the rest of this study.

5.6 DEVELOPMENT OF A MODIFIED METHOD TO ASSESS THE RELATIVE EXPRESSION OF THE A/B AND O ALLELES.

The RT-PCR method described in Section 5.3 was considered inadequate for the purpose of this study, as the difference between digested and undigested fragments was too subtle, even when the fragments were resolved on 8% polyacrylamide gels. Therefore the aim of this set of experiments was to develop a new RT-PCR method to assess expression of the ABO gene. Primers to a smaller region of the ABO cDNA sequence were designed; shorter regions of cDNA amplify more efficiently than larger regions, especially when the target RNA for the reverse-transcription reaction is partially degraded.

When designing primers to a smaller region of the gene, it was necessary to make the concession that only one of the diagnostic restriction sites could be included in the region to be amplified. Consequently, the primers used for this set of experiments span nt 12-329 (exons 1-6) of the ABO cDNA sequence (Yamamoto *et al.*, 1990a; Yamamoto *et al.*, 1995). Following restriction enzyme digestion of the resulting RT-PCR product with the enzymes *Kpn*I and *Bst*EII, the relative expression of the A or B and the O alleles should be able to be determined (see Table 5-3 for predicted fragment sizes).

	*Kpn*I	*Bst*EII
OO	249	316
AO/BO	249,316	249,316
AA/BB/AB	316	249

Table 5-3. Predicted fragment sizes after *Kpn*I or *Bst*EII digestion of the 316bp ABO RT-PCR product for individuals of various phenotypes. The numbers shown in grey indicate band sizes expected from analysis of the cDNA sequence, that were not detected in AO and BO heterozygotes using the ABO RT-PCR method described in Section 5.3.

5.6.1 Materials and methods

RNA was isolated from histologically normal bone marrow taken from patients with breast cancer, umbilical cord-bloods, and peripheral blood stem cell collections from patients with breast cancer. The RNA was reverse-transcribed and subjected to PCR as described (see Sections 2.6.2 and 2.6.5), using the following primers:

PA5'	5' TTGCGGACGCTGtCCGGAAAAC 3'	(sense)
AO2*	5' TGAACTGCTCGTTGAGGATGTCGA 3'	(anti-sense)

The primer PA5' was designed by a student in the laboratory, Peter Laslo, for cloning purposes, and subsequently contains an artificially created *Stu*I restriction site (a T replaces a G in the original sequence; indicated in lower case).

Aliquots of the PCR product (10µl), were then digested for 8-16 hours with 10 units of each of the enzymes *Kpn*I and *Bst*EII. The digested products were then resolved on 8% polyacrylamide gels or 2% agarose gels (see Section 2.6.6).

5.6.2 Results and Discussion

No PCR product was generated from DNA, or from peripheral blood mononuclear cells derived from normal individuals. Therefore it was concluded that the primers spanned at least one intron (the intron/exon structure had not been published when these experiments were carried out), and the mononuclear cell fraction from peripheral blood of normal individuals does not express ABO mRNA. Digestion of the RT-PCR product generated from normal bone marrow mononuclear cells from individuals of AO, BO and OO genotype is shown in Figure 5-9.

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* AO2 is one of the primers also used for the ABO PCR genotyping method described in Chapter 4.

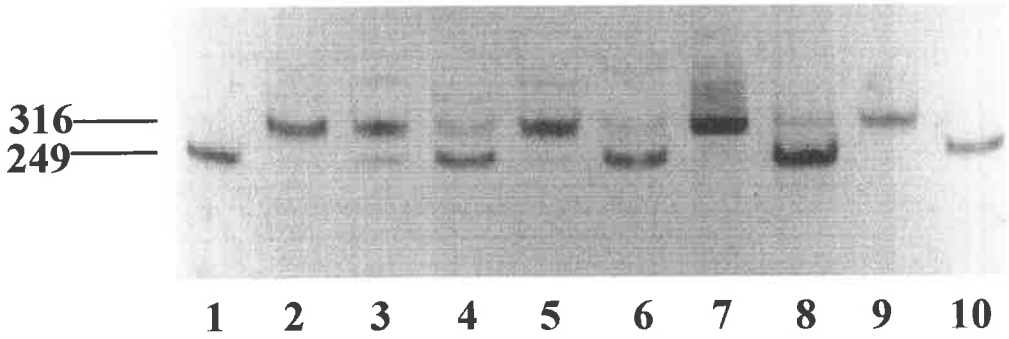


Figure 5-9. RT-PCR of the ABO gene after diagnostic restriction enzyme digestion. RT-PCR products were generated from individuals whose genotype was OO (lanes 1 and 2), AO (lanes 3-6), BO (lanes 7 and 8) and AB (lanes 9 and 10). The product was digested with *Kpn*I (lanes 1,3,5,7 and 9), or *Bst*EII (lanes 2,4,6,8 and 10).

The results obtained for the cell lines in Section 5.3, were confirmed using the modified RT-PCR. Normal bone marrow from 7 adults, and 16 fetal umbilical cord bloods were also examined for ABO expression. All results were repeated at least three times from the reverse-transcription step, and were always the same for a given individual. Of the 5 OO genotype individuals, all expressed the O allele. Two AA genotype individuals both expressed the A allele; while the one AB individual examined showed the expected pattern (complete digestion with *Bst*EII). Ten AO, and five BO genotype individuals were examined, and in all cases the results were identical to that shown in Figure 5-9. It seems that although the O allele is expressed in OO genotype individuals, in individuals heterozygous for the O allele, the majority of the steady-state mRNA encoding the ABO glycosyltransferase consists of the A or B allele.

This finding is most easily explained by the proposed relative stability of the alleles. The O allele has a single base deletion which leads to a premature stop-codon (see Chapter 1). The instability of transcripts with premature stop-codons has been well documented for a number of genes (Dunn *et al.*, 1989; Lim *et al.*, 1992; Cheng and Maquat, 1993, and references therein). Lim *et al.*, 1992 quantitated a decrease in steady state levels of mutant mRNAs of the human β-globin gene during a block in

RNA polymerase II catalysed transcription. This lead to the proposal that premature stop-codons leading to early termination of translation reduces the stability of cytoplasmic mRNA, as has been observed for a number of genes including the Rous sarcoma virus *gag* gene, the rabbit β-globin gene, and the yeast URA3 and His4 genes.

The mechanism by which such transcripts are more susceptible to nucleolytic cleavage is not understood, as the transcripts are polyadenylated, although a single nucleotide change may alter the secondary structure of the mRNA (Lim *et al.*, 1992). It is possible that the actual translation process influences degradation of mRNAs with premature stop-codons. For example, when a ribosome is stalled at a stop-codon, perhaps some kind of structure is formed that enhances the mRNA's susceptibility to RNase attack. Alternatively, structural changes might take place following translation of a complete reading frame, such that the recently translated mRNA has increased stability (Lim *et al.*, 1992).

Alternatively, analysis of human triosephosphate isomerase (TPI) gene transcripts with premature stop-codons revealed that the cytoplasmic half-life of these mRNAs was the same as non-mutant alleles, however the mutant transcript only constituted approximately 20% of the total TPI mRNA in both the nuclear and cytoplasmic cell fractions (Cheng and Maquat, 1993). These authors also found that both the mutant and normal alleles were transcribed at the same rate, hence they proposed that some kind of metabolic process occurs in the nucleus that affects the stability of mRNAs with premature stop-codons, as has been suggested for the DHFR gene and the minute virus of mice.

Studies on inherited mutant alleles of the retinoblastoma tumour suppressor gene (RB1), correlate most closely to the results seen here for the ABO gene. Dunn *et al.*

(1989), found that in the normal lymphocytes of patients with retinoblastoma tumours, mutant alleles with premature stop-codons were not detected, but the normal allele was. However, in tumours from these patients where the normal allele was physically lost, only the mutant allele was expressed. The authors propose that the functional RB1 gene product either directly or indirectly regulates its own transcription. When the functional product is lost, transcription of the mutant allele is increased to compensate for loss of the functional product. Subsequently, the mutant transcript would be detected, despite its instability, because it is being over-expressed.

In all of these cases, a reduction in mRNA stability was observed, regardless of whether the premature stop-codon was generated by a frame-shift or a nonsense mutation. The premature stop-codon in the O allele of the ABO gene, is due to a single base deletion at nt 261, leading to formation of a stop-codon at nt 352 and subsequently early termination of translation at a.a.118. Presumably the stability of the O allele mRNA is reduced, and like the RB1 gene, it could be proposed that the functional A or B allele product regulates ABO transcription. In OO genotype individuals, transcription of the gene would be enhanced to compensate for the lack of gene product, allowing detection of the transcript using RT-PCR. This hypothesis is further supported by the RT-PCR results obtained for patient MCG, whose genotype was O^1O^2. The O^2 allele, which although it is non-functional does not contain a premature stop-codon, was expressed, while no O^1 allele RT-PCR product could not be detected in this individual. Alternatively, perhaps the levels of O allele mRNA are so low that it can only be detected by RT-PCR when there is no competition with the A or B alleles. This seems a less likely explanation however, as other investigators have demonstrated approximately equivalent amounts of ABO mRNA in cell lines derived from A, B and O blood group individuals, using Northern analysis (Yamamoto *et al.*, 1990a).

Interestingly, two samples (both AO genotype), repeatedly seemed to express slightly more O allele relative to the A allele than seen in other heterozygous samples. One sample (Figure 5-9, lanes 3 and 4), was from an umbilical cord blood sample, while the other (not shown) was from a peripheral blood stem cell collection (PBSC). It was possible that these samples were from A^2O heterozygotes, and that the stability of the A^2 allele mRNA is altered by the single base deletion and subsequent frame-shift at the three prime end of the coding region. However, both samples genotyped as A^1O. Furthermore, four of the AO samples that showed virtually no expression of the O allele were genotyped; two genotyped as A^2O.* It is possible that in the individuals from which the cord blood and PBSC samples were derived, the promoter of the A allele is less active than that of the O allele, perhaps due to a polymorphism.

Further analysis of the transcriptional regulation of the ABO gene is required to support these hypotheses, however for the purposes of this project, the normal relative expression of the ABO alleles was sufficient to continue with a comparative analysis of normal individuals and patients with loss of A or B antigen expression.

5.7 Detection of a minor splice variant of the ABO gene

During the RT-PCR analyses described above, it was noted that some samples *sometimes* exhibited a different size band to the expected 316bp (see Figure 5-10; lane 2). The result was not consistently repeatable from the same reverse-transcription, although it seemed to occur approximately once in every ten times a

* It was not possible to genotype all the AO control samples for the A^2 allele, as the A^2 genotyping method was the final new technique developed for this project, and by this stage most of the samples had been exhausted.

particular sample was amplified. The band (approximately 500bp) was usually seen in addition to the 316bp fragment, and was detected in control bone marrows, umbilical cord bloods, cell lines and the PBSC samples.

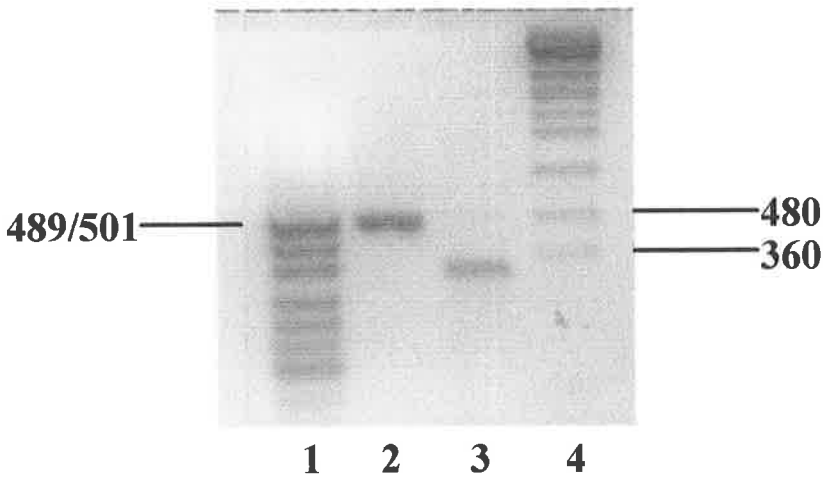


Figure 5-10. RT-PCR of ABO mRNA. Lane 1: pUC19/*Hpa*II marker; lane 2: a splice variant of the ABO gene; lane 3: RT-PCR product of the expected size; lane 4: SPP1/*Eco*RI marker. The sizes of relevant marker bands are given in base pairs.

Products of this size from individuals of various genotypes were digested with *Kpn*I and *Bst*EII (data not shown); digestion resulted in the same pattern and relative intensity of bands as digestion of the 316bp product from the same individuals (ie. in AO/BO individuals there was complete digestion with *Bst*EII and no digestion with *Kpn*I), with the exception that the fragment sizes were different.

To determine if this band was truly a splice variant of the ABO gene, PCR product generated from a cord blood was cloned and (partially) manually sequenced by Peter Laslo. Analysis of the sequence obtained revealed complete identity between nt 12 and nt 98 for the ABO cDNA sequence and the variant sequence (see Figure

5-11). However, after nt 98, there was approximately 200bps* of sequence that did not correspond to the ABO sequence; followed by sequence corresponding to nt 99-329 of the ABO cDNA sequence. When the genomic organization of the gene was published (Yamamoto *et al.*, 1995; Bennett *et al.*, 1995), it became clear that the point at which the sequences diverge (nt 99), is an intron/exon boundary. Therefore the variant contains exon 1, exon 2, followed by approximately 200bps of sequence probably from intron 2, then exons 3-6.

```
ABO       1 ATGGCCGAGG TGTTGCGGAC GCTGGCCGGA AAACCAAAAT GCCACGCACT TCGACCTATG
                        -------- ---------- ---------- ---------- ----------
variant   1               TTGCGGAC GCTGGCCGGA AAACCAAAAT GCCACGCACT TCGACCTATG

ABO      61 ATCCTTTTCC TAATAATGCT TGTCTTGGTC TTGTTTGGTT ACGGGGTCCT AAGCCCCAGA
            ---------- ---------- ---------- --------
variant  61 ATCCTTTTCC TAATAATGCT TGTCTTGGTC TTGTTTGGGT GTGGGACGTG GCGAGGCGCT

ABO     121 AGTCTAATGC CAGGAAGCCT GGAACGGGGG TTCTGCATGG CTGTTAGGGA ACCTGACCAT

variant 121 GAGCTTTGCT GAGAACTTGC CCTACCTGCC TCGAGGCCTT GCAGCTTCAC CGGGAACTCT

ABO     181 CTGCAGCGCG TCTCGTTGCC AAGGATGG

variant 181 GTGCTCACGC TGCTGGCCGC ACATGCAC
```

Figure 5-11. Comparison of the ABO cDNA sequence and the first 208bps of sequence obtained for the ABO splice variant. The sequences are identical for the first two exons of the gene, but diverge after nt 98.

Intron 2 is about 900bps long, and although the first bases of the 200bp sequence do not correspond to the first bases of the intron sequence, the last nine base pairs seem to correspond to the last nine base pairs of intron 2 (only the last nine bases

---

* It was calculated that there was approximately 200bps of additional sequence in the variant from the relative fragment sizes obtained from RT-PCR (around 500 compared with the expected 316bp). However, only 120bps of the 200 were sequenced.

have been published; Bennett *et al.*, 1995). Although our sequence lacks a T in this region (see Figure 5-12), this is probably because the resolution of the bands was limiting; hence two Ts versus three Ts in a row was difficult to determine. However, two separate sequencing reactions did not reveal a G, where the last base of intron 2 is a G. Further sequencing of this region is required to confirm this, but at this stage we concluded that the extra 200bps in our variant sequence was the final 200bps of intron 2. A similar splice variant isolated from an adenocarcinoma cell line has been reported (Yamamoto *et al.*, 1995). This variant (known as clone FY-69-7), also contains part of the second intron sequence, although the actual sequence has not been reported. However, this clone also lacked exon 5, while our variant contains exon 5 (see Figure 5-13).

Figure 5-12. Comparison of the ABO genomic sequence and the 3' end of the extra 200bp sequence seen in the ABO variant.

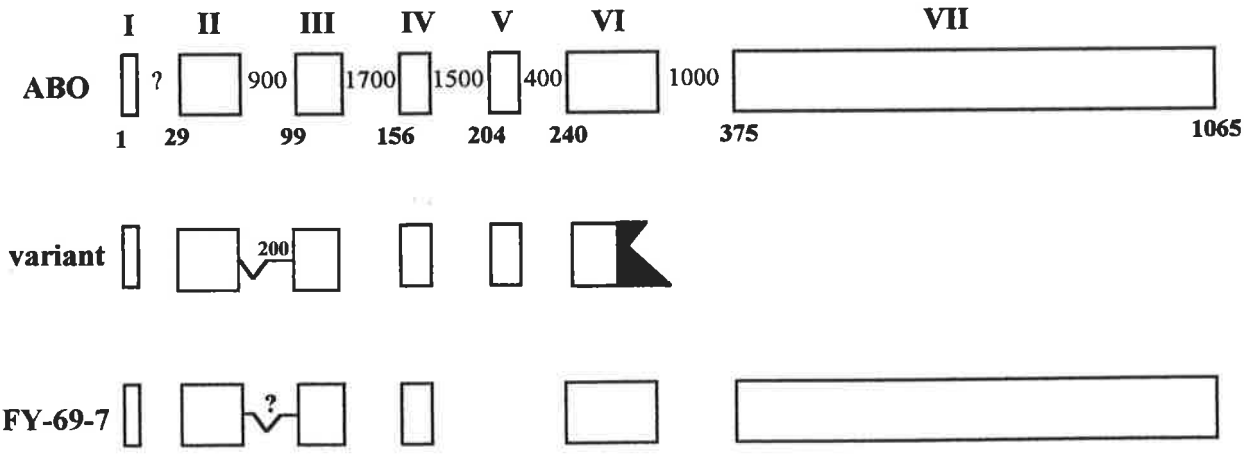


Figure 5-13. Comparison of the genomic organization of the ABO gene and sequences of the variant clone, and clone FY-69-7. Open boxes indicate coding exons. Numbers in bold indicate the nucleotide in the cDNA sequence at which a particular exon begins. Numbers between exons are approximate sizes of introns according to Bennett *et al.*, 1995. Only the sequence obtained for our variant clone is shown, while the v-shaped line in the variant and FY-69-7 represent intron sequence spliced into the ABO transcript.

It appears that our variant, if translated, is non-functional, as a premature stop-codon exists in frame at nt 130 (see Figure 5-14). Furthermore, the amino-acid sequence deviates from that of the maturely spliced ABO transcript after 32 residues. Similar findings have been reported by Yamamoto *et al.* (1995).

```
             9          18          27          36          45          54
5' ATG GCC GAG GTG TTG CGG ACG CTG GCC GGA AAA CCA AAA TGC CAC GCA CTT CGA
   Met Ala Glu Val Leu Arg Thr Leu Ala Gly Lys Pro Lys Cys His Ala Leu Arg

            63          72          81          90          99         108
   CCT ATG ATC CTT TTC CTA ATA ATG CTT GTC TTG GTC TTG TTT GGG TGT GGG ACG
   Pro Met Ile Leu Phe Leu Ile Met Leu Val Leu Val Leu Phe Gly Cys Gly Thr

           117         126
   TGG CGA GGC GCT GAG CTT TGC TGA 3'
   Trp Arg Gly Ala Glu Leu Cys ***
```

Figure 5-14. Predicted amino acid sequence encoded by the ABO splice variant. Translation would be prematurely terminated as a result of the stop codon at nt 130, producing a non-functional protein product.

5.8 Analysis of ABO gene expression in patients with colon cancer

ABO gene expression was examined in the tumours genotyped in chapter 4. Although the genotype of these tumours in each case agreed with the blood group of the patient, it was possible that the ABO gene was not being expressed, leading to loss of A/B antigen expression that is commonly seen in carcinoma (see Section 1.23). The aim of this set of experiments was to determine whether or not these tumours expressed the ABO gene, using RT-PCR and immunohistochemistry.

5.8.1 Expression of the ABO gene in colon tumours

RT-PCR was carried out as described in Sections 5.3 and 5.6. All 17 of the tumours expressed the ABO gene, and digestion of the RT-PCR product revealed the expected pattern and/or relative intensity of the bands in 15 cases. However, tumours from 2 patients (ID Q232171 and 508864), repeatedly exhibited more O

allele derived product than seen in normal controls (approximately 40-50% of the total RT-PCR product; data unable to be shown). The tumours were graded as Duke's stage B and C and genotyped as AO and BO respectively. It appeared therefore, that the A and B alleles of these tumours were being down-regulated, as genotyping had previously shown no difference in allele dosage relative to that of the normal mucosa adjacent to the tumour and to normal controls. Although DNA isolated from normal tissue adjacent to the tumour was available, neither RNA nor actual tissue was available to compare the expression patterns found in the tumours with normal expression in the patient.

If the A or B alleles were being down-regulated, it would be expected that antigen expression in the tumours was also suppressed. To determine if this was the case, immunohistochemistry was attempted by Ms Vivian Pascoe, Dept. of Histopathology at the QEH. However, staining of the tumours and normal stomach mucosa was not repeatable; therefore no conclusions could be made from these experiments. Furthermore, it was not clear how much normal tissue contaminated the tumour samples.

Although these results indicated disruption of ABO gene expression *may* play a role in colon carcinoma, we did not have the appropriate skills to carry out a conclusive study. Furthermore, we had knowledge that several major groups were (and still are) specifically examining expression of the ABO gene in carcinoma (ie. Henrik Clausen, Fumi-ichiro Yamamoto and Sen-itiroh Hakomori), subsequently, we decided not to pursue this avenue of investigation any further.

5.9 Analysis of ABO gene expression in patients with haematological malignancy and loss of antigen expression

To determine if the ABO gene was being expressed in patients with haematological malignancy and loss of antigen expression, samples were examined using the method described in Section 5.6., with the exception that cDNA was amplified for 45 cycles, using 1U of *Taq* DNA polymerase (as opposed to 35 cycles and 0.5U of *Taq* normally used). Normal controls were always amplified simultaneously, and produced the expected results. ABO RT-PCR product could be generated from all but three samples (GN PB 4/9/92; PM and BA), although strong bands were obtained following amplification of cDNA from patients PM and BA with the β-globin primers (not shown). The fact that ABO RT-PCR product was able to be generated from PB samples from some of the other patients (eg. WM, NR and CG), but not from normal PB samples indicates the different cell populations present in PB of patients with haematological malignancy. For example, blast cells are not normally found in PB. Furthermore, there could be increased numbers of other immature cell forms in the PB of these patients that express the ABO gene.

RT-PCR product generated from samples from AO genotype patients was digested with *Kpn*I and *Bst*EII (see Figure 5-15 and Table 5-4). All but two of the samples solely expressed the O allele; there was no expression of the A allele of the ABO gene. For the sake of simplicity, the results have been combined with loss of heterozygosity studies in Chapter 4, and are described on a case by case basis.

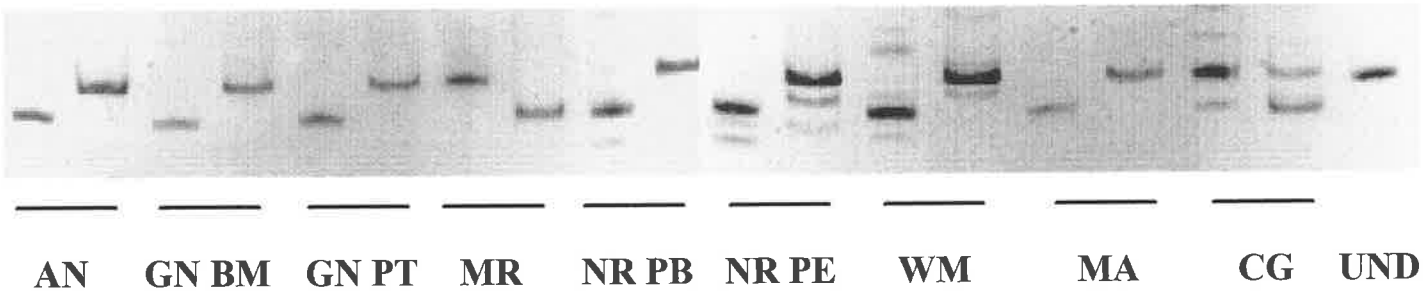


Figure 5-15. RT-PCR and differentiation of the alleles of the ABO gene in patients with loss of A antigen expression and haematological malignancy. Samples are paired digests (*Kpn*I, *Bst*EII) of the 316bp ABO RT-PCR product. When more than one sample of a patient was analysed, the sample is identified (BM- bone marrow; PB- peripheral blood; PT - post-transplant; PE- pleural effusion). "UND" is undigested PCR product. Complete digestion of the product with *Kpn*I, as seen in patients AN, GN, NR, WM and MA indicates expression of the O allele, and is identical to the pattern of bands obtained from a normal O blood group individual. Complete digestion of the product with *Bst*EII, as seen in patient MR indicates sole expression of the A allele, as is seen in normal A blood group individuals. The pattern of bands in patient CG reveals the presence of O allele transcripts, but the majority of the ABO mRNA in this patient is derived from the A allele. The weak bands seen in the samples from patients NR and WM have resulted from digestion of an ABO splice variant, and probably indicate transcription of the variant has also occurred from the O allele of the ABO gene, as the digestion pattern is the same as that seen of the variant in normal blood group O individuals.

Patient	Antigen "lost"	ABO genotype	LOH	ABO RT-PCR product	ABO expression
AN	A	A^2O	O	√	O
GN	A	A^2O	A	√	O
MR	A	A^2O	O	√	A
NR	A	A^1O	-	√	O
WM	A	A^1O	-	√	O
MA	A	A^1O	-	√	O
CG	A	$A^2O + O$	-	√	AO
PM	A	A^1O	-	-	n/a
BA	A	A^1O	-	-	n/a
WD	A	A^2B	-	√	nd
SR	A	A^2B	-	√	nd
KN	A/B	A^1B	-	√	nd
DH	A/B	A^1B	-	√	nd

Table 5-4. Summary of the results obtained from analysis of the ABO gene in patients with loss of A/B antigens and haematological malignancy. LOH indicates the allele lost if LOH had occurred, a dash indicates normal allele dosage. The assumption that patient CG had 3 copies of the ABO gene is indicated by his genotype. A tick below the heading ABO RT-PCR product indicates a positive result; a dash indicates no ABO RT-PCR product was able to be amplified, however the samples from these patients expressed the β-globin gene. n/a=not applicable; nd=alleles were not distinguished.

Patient AN

Genotyping of this patient revealed that most cells were probably AO, but some cells had physically lost the O allele. The red cells of the patient however, had lost expression of the A antigen. RT-PCR and restriction enzyme digestion (herein termed ABO phenotyping), revealed that of the cells in this patient's bone marrow, those expressing the ABO gene only expressed the O allele. These results are best explained by the presence of multiple leukaemic clones, at least one of which had LOH at the ABO locus, and one had transcriptional silencing of the A allele of the ABO gene. As the cells expressing the ABO gene only expressed the O allele, loss of A antigen expression from the patient's red cells could be explained.

Patient GN

Three different samples from this patient were examined. ABO genotyping revealed that in pre-transplant BM and PB samples, the A allele of the ABO gene was physically lost. The post-transplant (remission) BM sample genotyped OO, reflecting replacement of the patient's marrow with that of her OO genotype donor. In accordance with the genotyping results, ABO phenotyping only showed expression of the O allele in both BM samples; once again indicating that in the cell population expressing the ABO gene, only the O allele was expressed. This was probably the same cell population that had physically lost the A allele. RT-PCR of the pre-transplant PB sample of this patient was unsuccessful as determined by lack of β-globin RT-PCR product. This was almost certainly due to RNA degradation, which was confirmed by gel analysis. Loss of A antigen expression from the red cells of this patient was therefore due to loss of the A allele of the ABO gene.

Patient MR

Genotyping of this patient revealed physical loss of the O allele, probably in most cells. ABO phenotyping showed the normal pattern of expression for an AO heterozygote; that is expression of the A allele. These results do not seem to explain loss of A antigen expression from the patient's red cells. However, analysis of the patient's serological details described in Chapter 3 (Table 3-1), indicates that only 12% of the red cells did not express the A antigen. Furthermore, H antigen expression was not determined in this patient. There are two possible explanations for the results obtained for this patient: the cells that had lost A antigen expression in fact had lost expression of the precursor H antigen, or the major leukaemic clone in this patient had physical loss of the O allele, while a minor clone existed that either had physical loss or transcriptional silencing of the A, or both the A and the O alleles.

Patients NR and WM

The results from both of these patients are most striking. Both genotyped as AO, and both had loss of A antigen expression from their red cells. Equal allele dosage was confirmed by Southern blotting of samples from the patients that contained >85% blast cells, and therefore were almost purely derived from the leukaemic clone. ABO phenotyping revealed that the O allele was expressed, while there was no indication of A allele expression. Furthermore, digestion of the ABO splice variant described in Section 5.7, from both patients revealed that it too was transcribed from the O allele, as the product completely digested with *Kpn*I. Subsequently, loss of A antigen expression in these patients was explained by transcriptional silencing of the A allele of the ABO gene, an allele specific event, as the O allele was expressed.

Patient MA

ABO genotyping again revealed equal allele dosage of the A and O alleles, however ABO phenotyping revealed that in the cell population expressing the ABO gene, there was transcriptional silencing of the A allele. Hence, loss of A antigen expression was explained.

Patient CG

This patient was diagnosed with CML. By combining ABO genotyping and cytogenetic results, we concluded that the leukaemic cells of the patient each carried two copies of the O allele, and one copy of the A allele. ABO phenotyping revealed expression of both the A and the O alleles, although the O allele represented significantly more of the total ABO RT-PCR product than usually seen in normal individuals. This could be explained by the presence of two copies of the O allele being simultaneously transcribed with the A allele. This hypothesis does not, however, explain loss of A antigen from 70% of patient's red cells. As 30% of

the patient's cells were expressing A antigen, it is possible that the results are complicated by the presence of cells expressing normal relative amounts of the alleles. Analysis of the ABO gene in this patient would best be determined by erythrocyte culture; in which case individual colonies expressing the ABO gene could be examined.

Patient PM

No ABO RT-PCR product could be generated from the PB sample obtained from this AML patient. However, a strong band was obtained following amplification of the cDNA with the β-globin primers; indicating the success of the reverse-transcription reaction. As the sample used was PB, it is possible that lack of ABO amplification was merely a representation of the normal situation, as PB from healthy individuals does not contain significant numbers of cells that express the ABO gene. Alternatively, it is more likely that the PB sample from patient PM contained cells that would normally express the ABO gene, and the gene has been inactivated as part of the pathogenic process. For the duration of this project, amplification of cDNA from PB samples from patients with AML and CML (without loss of antigen expression) has always resulted in ABO RT-PCR product, which probably indicates the expression pattern of blast cells. Therefore it was concluded that the most likely explanation for loss of A antigen expression in this patient was inactivation of both alleles of the ABO gene.

Patient BA

Genotyping of this patient did not reveal abnormal allele dosage, although the percentage of malignant cells in the PB sample examined was unclear. Like the patient PM, there was no amplification of the ABO mRNA, although β-globin RT-PCR produced a strong band. Unlike the patient PM, patient BA had (suspected) MDS. The differential cell count of the PB sample examined did not report any

blast cells or erythrocyte precursors. Subsequently, no conclusion could be reached concerning loss of A antigen expression in this patient without examination of a bone marrow sample, which was not available.

Patients WD and SR

Both of these patients genotyped as AB and had lost expression of the A antigen from their red cells. ABO RT-PCR was not attempted on patient WD, as no RNA could be isolated from the patient's sample. While patient SR expressed an ABO RT-PCR product, our method does not distinguish the A and B alleles, and hence the relative expression of the alleles was not determined. Attempts to develop such a method were terminated, as only one control AB sample was available. It is possible that the weak A antigen expression seen in these patients was due to the fact that both carried the A^2 allele. As described in Chapter 4, the A^2 transferase is significantly less efficient at converting the precursor H antigen than the B transferase. Therefore, whether or not there was actual antigen loss, or A^2 antigen expression in these patients was at the lower end of the normal range, remains unresolved.

Patients KN and DH

These patients both exhibited distinct populations of red cells; A, B, O and (presumably) AB. No abnormal allele dosage could be shown, and samples from both patients expressed the ABO RT-PCR product. Even if a method had been developed to determine the relative expression of the A and B alleles, it would not have been useful due to the multiple red cell populations. The best way of examining these patients would be erythrocyte culture, in which case single BFU-E (Blast-Forming Units, Erythroid) colonies could be analysed both for allele dosage and ABO gene expression. This was attempted as part of Ms Michelle Pedler's

honours project in the laboratory, but no colonies were obtained, probably due to technical problems with the procedure.

Although some of the patients were reported to have weak A antigen expression, no expression of the A allele was detected (eg. patient MA). One explanation for this is that the defect in ABO transcription is limited to the haemopoetic lineage. Subsequently, secreted antigens could be adsorbed by the red cells, leading to weak A antigen expression. However, secretor status was unknown in most of the patients. Another possible explanation is conversion of the precursor H antigen on the cell's surface by serum (A/B) transferases. However, this would not explain the complete loss of antigen expression seen patients GN, BA, and NR.

Perhaps the most likely explanation for the weak antigen expression seen in some of these patients is the time point in their disease at which they were examined. As described in Chapter 3, mixed-field and weak reactions are rarely observed. By the time such a reaction is noted, a significant number of the cells are usually affected. As antigen expression in such patients tends to decrease with progression of the disease, it is possible that the red cells in the patient's circulation consist of a mixture of "old" cells normally expressing the A antigen that are derived from a normal progenitor cell, while those with loss of antigen expression are derived from the leukaemic progenitor. In this situation, large agglutinates of the "old" cells would take longer to form because they would be interspersed by red cells not expressing the A antigen. Therefore, if the agglutination reaction was allowed to continue (with mixing) for longer than usual, the cells would form larger agglutinates more characteristic of a mixed-field reaction. In fact, this is exactly what occurred with the blood-grouping reaction on patient SR shown in Figure 3-2.

If this hypothesis was correct, it would follow that the patient MA was examined during a transition period, at the point where although cells expressing the A antigen remained in the circulation, the only cells expressing the ABO gene were derived from the leukaemic clone.

Finally, it should be considered that when PB samples from patients with loss of antigen expression were examined, cells were present that expressed the ABO gene, whereas in normal individuals no expression is seen. Therefore one would expect that the cells expressing the ABO gene in the PB of these patients are in fact solely derived from the leukaemic clone. The exception to this is patient CG, whose PB sample expressed the A allele. However, it could be argued that excess reticulocytes or other ABO expressing cells derived from normal progenitors were in the patient's circulation as a specific feature of his disease (CML); the numbers of erythroid progenitors are known to be increased in the blood of CML patients (Henderson and Lister, 1990).

What appears to be transcriptional silencing of the A allele may in fact be due to an abnormal alternative splicing event. The A allele could be spliced in such a way that one (or both) of the regions containing the primers is spliced out; therefore amplification would only be able to occur from the normally spliced O allele. This would imply an allele-specific splicing event, which is possible if there is a mutation altering a splice site. However, two of the patients were also examined using the RT-PCR method described in Section 5.3. Both patients (NR and WM; data not shown), only expressed the O allele. As this method uses two different primers and all four primers used for these two methods are in different exons (1, 5, 6 and 7), it is unlikely that such an event has occurred.

One obvious explanation for the results seen is that the A allele has a mutation in the promoter region, or that the promoter region is deleted. Alternatively, there could be mutation leading to a premature stop-codon and subsequently disrupted mRNA stability, although this would require the transcript to be less stable than the O allele. While these events can not be ruled out, the findings in Chapter 4 that LOH is occurring at the ABO locus strongly suggest that loss of A/B antigens is an indication of a larger event that is occurring. It was therefore concluded that the most likely explanation for loss of expression of the A allele, was transcriptional silencing of a single allele of the ABO gene probably due to some kind of epigenetic change.

5.10 Imprinting and loss of ABO antigens?

The results obtained in the previous section show transcriptional silencing of a single allele of the ABO gene has occurred in several cases. Mono-allelic expression of an autosomal gene is almost always indicative of imprinting. Although the ABO gene is obviously not normally imprinted or subject to random allelic exclusion (otherwise AB, AO and BO individuals would exhibit distinct cell populations), it is possible that at some stage during embryonic development, or during development of some cell lineages, imprinting of the ABO gene occurs. Furthermore, during a period of this project, it was thought that the ABL gene, which is closely linked to the ABO gene, was imprinted. Haas *et al.* (1992) used cytogenetic polymorphisms of the chromosome 9 centromere to show that in CML patients, the chromosome 9 portion of the Philadelphia chromosome was almost always paternally derived. However, imprinting or random allelic exclusion of the ABL gene was not supported by molecular studies (Melo *et al.*, 1994; Melo *et al.*, 1995). Whether or not ABL is imprinted is still being argued in the literature (Haas, 1995; Litz, 1995). As our results indicate possible imprinting of the ABO gene, we

attempted to determine if there was any parental bias in the contribution of the affected allele.

5.10.1 Results and Discussion

We were able to obtain the parental blood groups of four patients with loss of A antigen expression (Table 5-5). Three cases were informative, and in all three cases, the silenced allele was maternally derived.

Patient	Blood group	Mother's blood group	Father's blood group
AN	A	A	O
NR	A	A	O
PM	A	A	B
GN	A	A	A

Table 5-5. ABO blood groups of patients with haematological malignancy and loss of A antigen expression, and their parents.

Some of the reports of patients with loss of A or B antigens in the literature include family studies. We were able to find nine such cases, all of which were informative (see Table 5-6). In eight of the nine cases, expression of the maternally inherited ABO allele was affected.

Patient's blood group	Age	Diagnosis	Mother's blood group	Father's blood group	Reference
$A^1O \rightarrow O$	?	AML	A^1	OO	Van Loghem *et al.*, 1957
$A^1O \rightarrow O$	32	AML	OO	A^1O	Salmon *et al.*, 1958
$A^1O \rightarrow O$	?	AML	A^1O	A^2O	Salmon *et al.*, 1959
$A^1 \rightarrow O$	26	AML	A^1B	*	Salmon *et al.*, 1959
$A^1O \rightarrow O$	19	AML	A^1	OO	Gold *et al.*, 1959
$A^2O \rightarrow O$	10	EL	A^2	OO	Salmon *et al.*, 1961
$AB \rightarrow A$	40	CLL	BO	AO	Richards, 1962
$B \rightarrow O$	24	AML	A^1B	?	Undevia *et al.*, 1966
$A^2O \rightarrow O$	?	leukaemia	A^2O	OO	Salmon, 1976

Table 5-6. ABO blood group changes/inferred genotypes of patients with haematological malignancy and their parents, as deduced from the literature. EL refers to erythroleukaemia; CLL is chronic lymphatic leukaemia. * indicates the father's blood group was not reported, however the authors state that the A^1 allele of the patient was maternally inherited. For the sake of simplicity, the blood group changes have been summarised to indicate the antigen lost; eg. "$A^1O \rightarrow O$" as opposed to "a mixture of A^1 and weakly A positive cells".

Including our data, the maternally derived ABO allele was affected in 11/12 patients. If the parental contribution of the affected allele was randomly determined, the probability of observing loss of expression of the same sex parent's allele in 11 or more of the patients is 0.006. These results indicate that silencing of A or B alleles may occur as a result of imprinting. This would imply that the imprint was not erased following the neo-natal period, at least in some cells. Subsequently, it is possible that loss of antigen expression is merely a reflection of the clonal expansion that occurs in malignancy, and in fact has no role in the pathogenesis of the disease. Alternatively, other linked genes might be silenced in addition to the ABO gene. If one of these genes was a tumour suppressor gene, physical loss of, or a mutation in the expressed copy, would lead to leukaemogenesis.

The latter hypothesis is further supported by the data on patient AN. In Section 5.9, it was proposed that there were two leukaemic clones in this patient, one of which had physically lost the O allele, and one of which had silencing of the A allele. It is possible that silencing of the A allele occurred first, and physical loss of the O allele occurred in one such cell as a "second hit", producing the second clone. Subsequently, there would be a clone of cells in which the ABO gene, and possibly closely linked genes were completely knocked out, and a clone of cells in which a putative tumour suppressor gene was still active.

This hypothesis however, does not explain the results seen for patient MR, who genotyped AO, but had physically lost the O allele from the majority of his cells. Although this patient expressed the A allele, only 12% of his red cells had actually lost A antigen expression. If loss of A antigen expression from these cells was due to silencing of the A allele, then the implication would be that the first event was LOH, and the second event silencing. Could it be possible that the mechanism by

which maintenance of appropriately imprinted genes occurs, can be "switched on" under abnormal conditions?

If such a phenomenon could occur, it might explain the results from patients KN and DH. These patients both exhibited four distinct populations of red cells, which could be determined by serological analysis because both patients originally grouped as AB. While three populations of red cells could easily be explained by proposing silencing of one allele, and evolution of the clone by deletion of the other ABO allele, it is difficult to explain the presence of four red cell populations. Furthermore, whatever events are leading to this phenomenon, could be occurring in the patients who genotype AO and have loss of A antigen expression, as serological analysis would not expose this kind of event in these patients. However, it could be argued that the event occurring in the patients with four red cell populations does not happen very often, as there is only one similar case reported in the literature (Renton *et al.*, 1962).

SUMMARY

An RT-PCR method utilising restriction enzyme digestion to differentiate the A/B and O alleles of the ABO gene was established. Examination of normal AO and BO genotype individuals revealed that the majority of steady-state ABO mRNA is derived from the A or B allele. The stability of O allele mRNA is probably reduced due to the presence of a premature stop-codon, a phenomenon which is commonly seen in other genes. In individuals of OO genotype however, the O allele was expressed, implying that transcription of the ABO gene is directly regulated by its own gene product.

In the previous chapter, two individuals who genotyped as AO but were unequivocally blood group O were identified. Expression of the ABO gene was examined in one of these patients, and the expressed allele sequenced. Sequence analysis revealed several single base substitutions, one of which leads to an amino-acid change. This allele was later cloned and sequenced by another group, and identified as the O^2 allele. We subsequently developed a PCR method to distinguish the O^2 allele from the A^1 allele, and analysis of the second patient who had previously genotyped AO revealed that her true genotype was O^1O^2.

During analysis of ABO gene expression in normal individuals, it became clear that a splice variant of the gene existed, probably constituting of a minor proportion of the total ABO mRNA, as it could not be consistently amplified from the same reverse-transcription reactions. This variant was cloned and sequenced, and comparison of the sequence obtained with that of the ABO cDNA sequence revealed that approximately 200bps of intron 2 sequence was spliced into the transcript at the intron/exon boundary of the 3' end of exon 2. A similar variant has been cloned by another group, although there are two important differences between the variants. Firstly, ours was cloned from normal tissue (umbilical cord

blood), while the other variant was cloned from an adenocarcinoma cell line. Secondly, the other variant does not contain exon 5, while our variant does. The variant is predicted to be non-functional, as a stop-codon exists in frame, and would lead to termination of translation after just 43 residues.

Analysis of ABO gene expression in samples from patients with loss of A and/or B antigens and haematological malignancy showed that 10/12 expressed the ABO gene. Of the nine AO genotype patients examined, seven solely expressed the O allele, while one expressed significantly more O allele than controls, and the final patient had normal A allele expression. It was concluded that although the A allele was physically present in four of the samples, it was transcriptionally silenced. One patient had lost the A allele, presumably from the cells that were expressing the ABO gene. The results in the two patients in which expression of the A allele was detected are best explained by the presence of multiple leukaemic clones and/or the presence of significant amounts of normal cells.

As mono-allelic expression of autosomal genes is usually associated with imprinting, data from the patients presented here and compiled from the literature was investigated to determine if there was any bias in the parent contributing the affected allele. The results were highly significant; the affected allele was maternally derived in 11/12 cases.

As it is unlikely that the ABO gene is the primary locus at which this event is occurring, the next stage in this project was to define the affected region, beginning with a study of the linked AK1 gene.

CHAPTER SIX

ANALYSIS OF THE ADENYLATE KINASE 1 GENE

6.0 INTRODUCTION

The previous chapters have shown that in at least in some patients with loss of blood group antigens, loss of antigen expression is due to transcriptional silencing or physical loss of a single allele of the ABO gene. Salmon *et al.* (1968), and Kahn *et al.* (1971) reported a simultaneous decrease in red cell antigens and the red cell enzyme, adenylate kinase 1 (AK1; see Section 1.20). Patient WM, who was also described by Marsden *et al.* (1992), also had simultaneous loss of A antigen expression and a decrease in AK1 activity. The AK1 gene is located approximately 15 megabases closer to the centromere than the ABO gene, at 9q34 (Attwood *et al.*, 1994). Considering the proximity of these two genes, and the biochemical evidence suggesting concurrent loss of ABO antigens and AK1 activity, we wished to examine the AK1 gene at the molecular level.

Prior to this study, none of the reported polymorphisms in the AK1 gene had been localised to the transcribed region. Consequently, it was not possible to examine the relative expression of the alleles, using RT-PCR. Although there are two electrophoretically distinguishable isozymes of AK1, AK^1 and AK^2 (Fildes and Harris, 1966), and the genetic sequence of the AK^1 variant has been determined (Matsuura *et al.*, 1989), the sequence of the AK^2 isozyme has not been reported.

This chapter describes the localization of a known restriction enzyme polymorphism to the transcribed region of the AK1 gene, and subsequent studies using this and other polymorphisms to examine the AK1 gene for LOH and mono-allelic expression in patients with loss of A or B antigen expression.

6.1 RATIONALE

A literature search revealed three papers describing restriction enzyme polymorphisms in the AK1 gene. Puffenberger and Francomano (1991), described *Dde*I and *Kpn*I polymorphisms within intron 5 of the gene. As a cDNA polymorphism was sought, this information was temporarily disregarded. Bech-Hansen *et al.* (1989), described a *Taq*I polymorphism detected by Southern analysis, using a probe containing a region of the AK1 gene. Schuback *et al.* (1991), used the same probe to detect a *Ban*I restriction enzyme polymorphism. Comparison of the probe sequence used by Bech-Hansen *et al.* (1989), and Schuback *et al.* (1991), and the known genomic sequence of AK1 (Matsuura *et al.*, 1989), allowed an approximate localization of the polymorphic *Ban*I and *Taq*I sites.

6.2 THEORETICAL LOCALIZATION OF THE *BAN*I RESTRICTION ENZYME POLYMORPHISM IN THE AK1 GENE

The probe described by Bech-Hansen *et al.* (1989), was a 3.25kb *Bam*HI genomic DNA fragment, known as phAK1B3.25, which spans exons 2-5 (nt 3,463-6,794) of the AK1 gene. Schuback *et al.* (1991), reported that Southern blotting of *Ban*I digested DNA with the phAK1B3.25 probe detected a constant band of 1.7kb, and two polymorphic bands of 2.2 and 1.6kb (frequency 0.76 and 0.24 respectively), in a study carried out on individuals from the North American population. Analysis of *Ban*I restriction sites in the genomic sequence of the AK1 gene revealed two possible positions for the polymorphic *Ban*I site (see Figure 6-1).

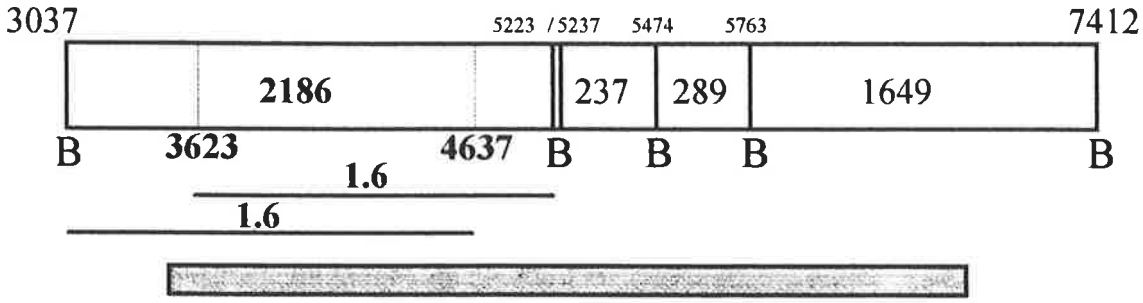


Figure 6-1. Diagrammatic representation of the AK1 gene, from nt 3,037-7,412. *Ban*I restriction endonuclease sites are indicated by "B". The numbers within the boxes are the predicted fragment sizes in base pairs. The shaded grey box represents the phAK1B3.25 probe used by Schuback *et al.*, 1991, to probe *Ban*I digested DNA. The dotted lines represent possible sites for the *Ban*I polymorphisms, to produce the 1.6kb band (based on the sequence in Matsuura *et al.*, 1989).

The origin of the constant 1.7kb band can be determined from Figure 6-1 (ie. the 1649bp fragment). The 14, 237 and 289bp fragments were probably not detected due to their small size. The identity of the 2.2kb polymorphic band can also be determined from Figure 6-1. In order to produce the 1.6kb fragment, the polymorphic site must be in the vicinity of either nt 3,623 or nt 4,637 (indicated by the dotted lines in Figure 6-1). These nucleotides are located within introns 1 and 2 of the gene respectively, with several hundred base pairs of intron sequence on either side. Therefore it was considered unlikely that the *Ban*I polymorphic site existed within the transcribed region of the gene. However, to test this, cDNA specific primers spanning exons 1-5 of the AK1 gene were designed, and the amplified product examined for a *Ban*I polymorphism.

Bech-Hansen *et al.* (1989), reported that the phAK1B3.25 probe did not detect any polymorphisms in genomic DNA digested with the following enzymes: *Bam*HI, *Bgl*II, *Bst*EII, *Eco*RI, *Hind*III, *Mbo*I, *Msp*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I and *Xba*I. As the region of the AK1 gene that was to be amplified to test for a *Ban*I polymorphism comprises about 46% of the entire transcribed region of the gene, the AK1 RT-PCR product was also examined for polymorphisms using the following restriction enzymes: *Taq*I, *Hha*I, *Bst*NI, *Dde*I, *Alu*I, *Kpn*I and *Msp*I.

6.2.1 Materials and Methods

RNA was isolated from 12 umbilical cord bloods and reverse-transcribed as described previously (see Sections 2.6.2 and 2.6.5). Amplification of 5µl of cDNA under the standard conditions was carried out using the following primers:

AKCD A 5' CACCCCTCCCCAGAGAGCACTGAC 3' (sense)

AKCD B 5' ATTGACTTTGGCCACCATGGCATC 3' (anti-sense)

Digestion of 10µl aliquots of the resulting RT-PCR product with 10 units of the restriction enzymes: *Ban*I, *Taq*I, *Hha*I, *Bst*NI, *Dde*I, *Kpn*I, *Msp*I (New England Biolabs), and *Alu*I (Boehringer-Manneheim), was carried out according to the manufacturer's instructions. The restricted products were resolved on 8% polyacrylamide gels as described in Section 2.6.6.

6.2.2 Results and Discussion

The primers designed span nt 971-6703 of the AK1 genomic DNA sequence (Matsuura *et al.*, 1989). The sense primer is located in exon 1 and 2 (nt 28-51 of the cDNA sequence), while the anti-sense primer is in exon 4 (nt 329-306 of the cDNA sequence). Therefore it was predicted that these primers would generate a 302bp band from cord blood cDNA, and no product from genomic DNA. The results obtained confirmed this, as is shown in Figure 6-2. No AK1 product was generated from peripheral blood mononuclear cells.

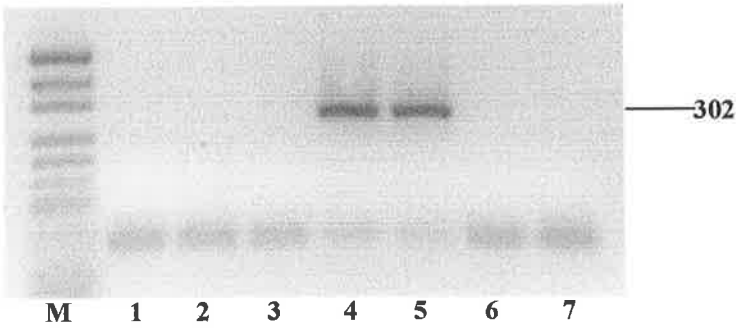


Figure 6-2. Amplification of AK1 using the primers AKCDA and AKCDB. No product is generated from genomic DNA (lanes 1, 2 and 3), or from peripheral blood mononuclear cell cDNA (lanes 6 and 7). A 302bp product was generated from umbilical cord blood cell cDNA (lanes 5 and 6). The marker (M) is pUC19/*Hpa*II.

The 302bp product generated by the primers AKCDA and AKCDB has a single *Ban*I restriction site (position 5,763 of the genomic AK1 sequence). This was used as an internal control for *Ban*I digestion. Of the 12 samples tested, none contained an additional *Ban*I site (data not shown). This is not surprising, as exons 2 and 3 comprise only 76bp; compared with introns 1 and 2, which consist of approximately 4,500bp. From the reported allele frequencies (Schuback *et al.*, 1991), one would expect four of the 12 individuals examined to be heterozygous for this polymorphism, if it were contained within the region amplified. It was therefore concluded that the *Ban*I polymorphic site was probably in either the first or second intron of the AK1 gene.

Digestion of the AK1 product with the other enzymes yielded the predicted sizes and number of bands; *Alu*I (5), *Bst*NI (3), *Dde*I (3), *Hha*I (1), *Kpn*I (1), *Msp*I (4) and *Taq*I (1) (data not shown). It was concluded that the region amplified by the AKCDA and AKCDB primers was not highly polymorphic for any of these enzymes.

6.3 THEORETICAL LOCALIZATION OF THE *TAQ*I RESTRICTION ENZYME POLYMORPHISM IN THE AK1 GENE

Bech-Hansen *et al.* (1989), reported that the phAK1B3.25 probe detected two polymorphic bands of 6.4 and 5.5kb in *Taq*I digested genomic DNA, with allele frequencies of 0.79 and 0.21 respectively. Other (constant) bands detected were a strong 1.3kb and two weaker 0.3kb and 1.4kb bands. Combined with the information gained from the genomic sequence, the restriction map shown in Figure 6-3 was constructed.

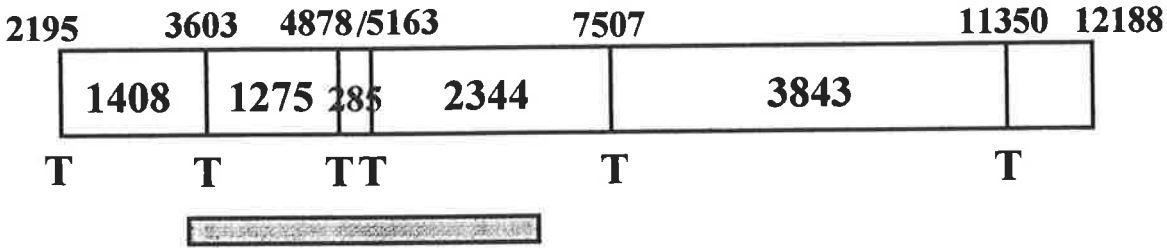


Figure 6-3. Diagrammatic representation of the AK1 gene, from nt 2,195-12,188 (the end of the known sequence). *Taq*I restriction endonuclease sites are indicated by "T". The numbers within the boxes are the predicted fragment sizes in base pairs. The shaded grey box represents the 3.25kb *Bam*HI fragment used by Bech-Hansen *et al.* (1989), to probe *Taq*I digested DNA. The map is drawn to scale.

The origin of the constant 1.3, 0.3 and 1.4kb bands described by Bech-Hansen *et al.* (1989), can be determined from Figure 6-3, implying that the *Taq*I polymorphism is in the three prime end of the gene. However, considering this map, it would be expected that one of the polymorphic bands was approximately 2.3kb. The polymorphic bands detected by Bech-Hansen *et al.* (1989), however, were estimated at 6.4 and 5.5kb. However, if the *Taq*I restriction site at nt 7,507 did not exist, a fragment of about 6.2kb would be detected. This was considered a likely scenario, therefore the first step in these experiments was to test if the *Taq*I site at nt 7,507 did in fact exist in the general population.

6.3.1 Materials and Methods

Primers spanning the region of interest, nt 7,507 in the AK1 gene, were designed. The sequence of these primers was based on (but different to) those described by Puffenberger and Francomano, (1991), and amplify nt 7,405-8,424. These primers also span the *Dde*I and *Kpn*I polymorphisms within intron 5 of the AK1 gene. Genomic DNA from 5 individuals was amplified using the following primers:

AKGEN2A	5' CAGGATGGCACCCCACACACG 3'	(sense)
AKGEN2B	5' CGGCACCTACAACAGCCCTGGAAG 3'	(anti-sense)

PCR was carried out as described in Section 2.6.4 with the exception that the following amplification cycles were employed; 94°C for 5 minutes, followed by 35 cycles of 96°C for 30 seconds, 63°C for 30 seconds, and 72°C for 1 minute. Ten microliter aliquots of the resulting PCR products (1019bp), were digested overnight in a total volume of 20µl, with 10 units of *Taq*I restriction endonuclease (New England Biolabs), according to the manufacturer's instructions. As a control for *Taq*I digestion, DNA isolated from lambda phage was also digested. The restricted samples were then resolved on a 2% agarose gel (Section 2.6.6).

6.3.2 Results and Discussion

None of the five samples digested with *Taq*I, although the control, Lambda phage DNA completely digested (data not shown). These results suggested that the *Taq*I restriction enzyme site at nt 7,507 in the AK1 sequence did either (i) not exist; was simply a sequencing error, or (ii) was in fact the polymorphic site, or (iii) was either unique to the individual or the Japanese population the original sequence was determined in.

If the site at nt 7,507 was the polymorphic site, then the 6.4kb band detected by Bech-Hansen *et al.* (1989), could be explained. However, the presence of the 5.5kb band would require both loss of the *Taq*I site at nt 7,507, and gain of another *Taq*I site at about nt 10,500, *simultaneously*. This was therefore considered unlikely.

Based on the assumption that the *Taq*I site at nt 7,507 was rare or non-existent, at least in the South Australian population, the following updated restriction map was produced:

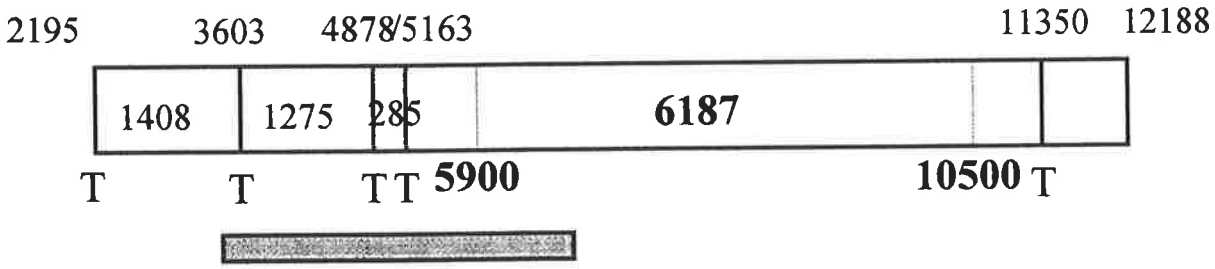


Figure 6-4. Updated *Taq* I restriction map of the region nt 2,195-12,188 of the AK1 gene. The AK1 probe is indicated by the grey rectangle. Constant *Taq*I sites are marked by "T". The proposed 6.4 (6.2)kb band detected by Bech-Hansen *et al.* (1989), is shown in bold, whilst the two possible polymorphic sites are indicated by dotted lines at nt 5,900 and 10,500.

As indicated in Figure 6-4, there were two possible sites for the *Taq*I polymorphism. The 5.5kb band detected by Bech-Hansen *et. al.* (1989), could be accounted for if a polymorphic site existed at either nt 10,500, or at nt 5,900. However, if the site was indeed at nt 5,900, one would also expect to see a smaller band, of around 700bp. As bands as small as 300bp were able to be detected by the Bech-Hansen group, there is no reason a band at 700bp would not consistently appear with the 5.5kb band. Based on this reasoning, it was postulated that the polymorphic *Taq*I site existed within several hundred base pairs of nt 10,500.

Primers were subsequently designed to span nt 10,194-11,073 of the AK1 gene. The sense primer (AKTA), was located in exon 6, while the anti-sense primer (AKTB), was positioned in exon 7 (see Figure 6-5). Subsequently, the predicted size of the amplification products generated from genomic DNA and cDNA were 879bp and 637bp respectively.

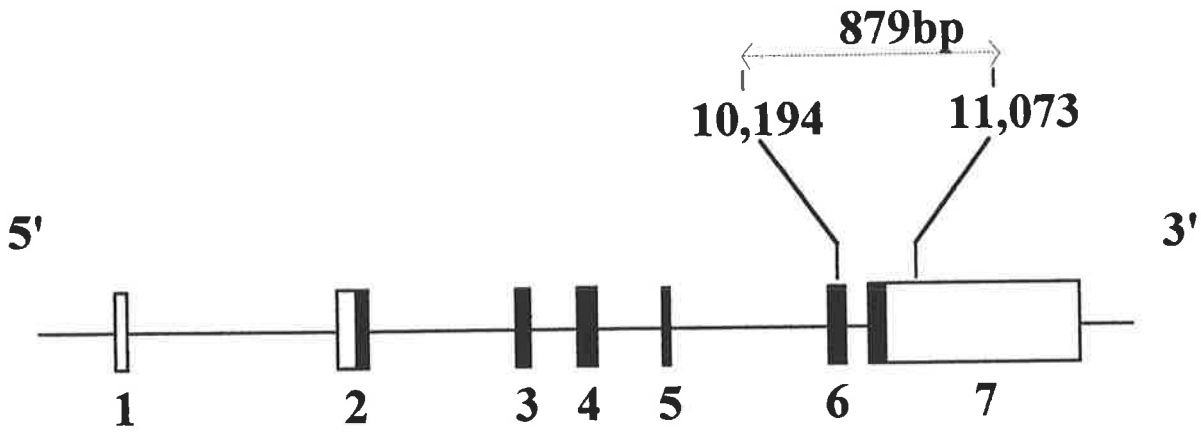


Figure 6-5. Genomic organization of the AK1 gene. Boxes represent exons, and the exon numbers are shown below the boxes. Black boxes indicate the coding region while white boxes represent untranslated regions. Exon 1: nt 944-983; exon 2: 3,948-3,988; exon 3: 5,534-5,569; exon 4: 5,742-5,905; exon 5: 6,656-6,772; exon 6: 10,075-10,266; exon 7: 10,508-12,188. The protein coding region of the gene begins at nt 3,982 and ends at 10,577 (Matsuura *et al.*, 1989). The position of the AKTA and AKTB primers and the 879bp fragment they amplify from genomic DNA is shown.

6.4.1 Materials and Methods

Genomic DNA from 30 unrelated normal individuals was isolated and amplified as described (see Sections 2.6.1 and 2.6.4), using the following primers:

AKTA	5' GCGGCTGGAGACCTATTACAAGGC 3'	(sense)
AKTB	5' CAAGGCAGGCATGGAACATATGCT 3'	(anti-sense)

Following amplification, 10µl aliquots of PCR product were digested overnight in a total volume of 20µl with 7 units of the restriction enzyme, *Taq*I (New England Biolabs), in the buffer supplied by the manufacturer, according to the manufacturer's instructions. The restricted products were then resolved on 1.5% agarose gels (Section 2.6.6).

6.4.2 Results and Discussion

Of the 30 individuals analysed, digestion of the AK1 product with *Taq*I produced two fragments in 12 of the samples, of approximately 879 and 430 base pairs (see Figure 6-6 for an example). None of the individuals tested were homozygous for the 430bp fragment, however the cell line K562 was. As K562 supposedly does not contain a normal chromosome 9 (ATCC database), and AK1 is located proximally to the ABL gene and therefore not translocated to form the Philadelphia chromosome, it appears that one of the many marker chromosomes in this cell line carries the AK1 gene. This is supported by the fact that according to the ATCC, K562 expresses AK1 enzyme. As there is no *Taq*I restriction site between nt 10,194-11,073 of the published AK1 sequence, and the fact that only some of the individuals tested were found to possess this restriction site, it was concluded that the region amplified did in fact encompass a polymorphic *Taq*I site. Bech-Hansen *et al.* (1989), examined 100 unrelated Caucasians for this polymorphic site. Using the allele frequencies determined by Bech-Hansen *et al.* (1989), it can be calculated that approximately 33% of individuals should be heterozygous (2pq; 2 x 0.79 x 0.21). In this study of the South Australian population, 12/30 (40%) individuals were heterozygous for the *Taq*I polymorphism. It was therefore concluded that the polymorphisms detected by the Bech-Hansen group, and the polymorphism detected by this PCR method were the same. Subsequently, the alleles are referred to as D (no *Taq*I site) and d (contains the *Taq*I site), according to the precedent set by Bech-Hansen *et al.* (1989).

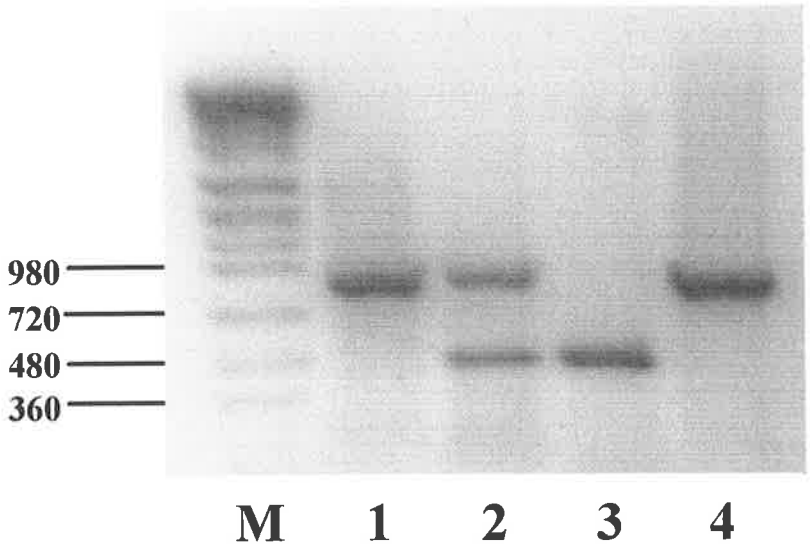


Figure 6-6. Determination of the *Taq*I polymorphism in the AK1 gene. Digestion of the 879bp PCR product generated from genomic DNA by the primers AKTA and AKTB with *Taq*I reveals a polymorphism; homozygous (DD) individual (lane 1), heterozygous (Dd) individual (lane 2), the cell line K562 is homozygous for the allele containing the *TaqI* site (dd; lane 3), undigested product is in lane 4, (M) is SPP-1/*Eco*RI. Sizes of relevant marker bands are shown.

Digestion of the product should, theoretically, yield two bands. As the digested band seen was around 430bp, and the PCR product was originally 879bp, it was concluded that the polymorphic *Taq*I site was in the middle of the amplified region. If the site was in the middle of the product, then the two bands expected could run together, and appear as one 430/440bp fragment.

6.5 Expression analysis of the polymorphic *TaqI* site

To determine if the polymorphism existed in exon 6, intron 6 or exon 7 of the AK1 gene, cDNA from Dd individuals, and the cell line K562, was analysed.

6.5.1 Results and Discussion

As expected, K562 expressed AK1. RT-PCR and digestion with *Taq*I revealed that in normal bone marrow, both alleles are expressed (see Figure 6-7). These results also mean that the polymorphism must be contained within the transcribed region

of the AK1 gene. To determine the precise localization of this polymorphism, RT-PCR product generated from K562 was sequenced.

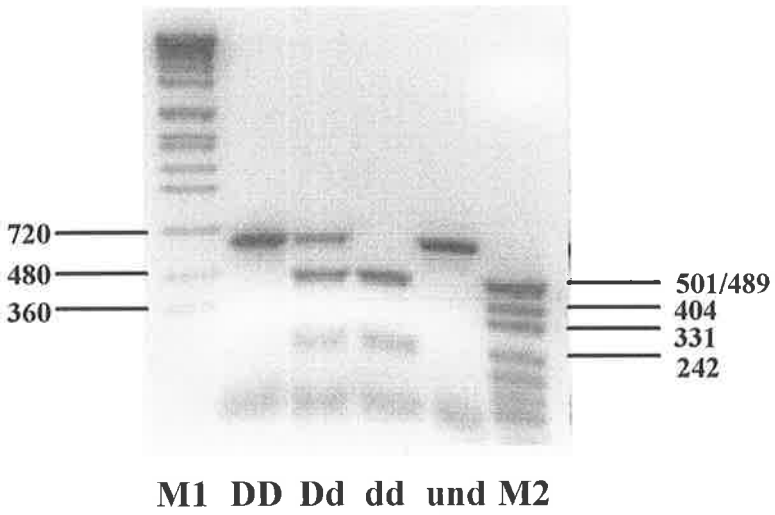


Figure 6-7. AK RT-PCR product from DD and Dd genotype individuals and K562 (dd), digested with *Taq*I. Sizes of the relevant marker bands (M1; SPP-1/*Eco*RI and M2; pUC19/*Hpa*II) shown are in base pairs. "und" is undigested RT-PCR product.

6.6 SEQUENCING OF THE NEW AK1 ALLELE

To determine the precise location of the polymorphic *Taq*I site, sequence analysis was carried out using the cell line K562, which only contained the allele with the *Taq*I site (the d allele).

6.6.1 Materials and Methods

AK1 PCR products were generated from genomic DNA and cDNA from the cell line K562 as described in Sections 6.4.1 and 6.5.1. The products were purified and sequenced on an automatic sequencer using the primers AKTA (forward sequence), and AKTB (reverse sequence), as described in Sections 2.6.8 and 2.6.9.

6.6.2 Results and Discussion

Analysis of the four sequences derived from the AK PCR products allowed construction of a consensus sequence spanning nt 10,285-11,019 of the AK1 genomic DNA sequence. Only one difference between the consensus sequence and the published sequence (Matsuura *et al.*, 1989), was detected. This difference, a single base substitution (A→C at nt 10,630; Figures 6-8 and 6-9), was confirmed in three of the four sequences obtained. The fourth sequence (AK RT-PCR product with the reverse primer, AKTB), was unreadable in this region.

```
10,629    TAGA    AK1
          | ||
          TCGA    K562
```

Figure 6-8. Comparison of the nucleotide sequence of the published AK1 gene, and the sequence derived from the cell line K562. A single base substitution at nucleotide 10,630 (A→C), creates a *Taq*I restriction endonuclease recognition site.

The A→C substitution at nt 10,630 of the AK1 sequence is located in the 3' untranslated region of the gene, and as expected creates a *Taq*I restriction site (TCGA). The substitution does not create a new polyadenylation site, and does not seem to alter the stability of the mRNA, as evidenced by the fact that in the normal heterozygote, both alleles are expressed (Figure 6-7). Furthermore, as the polymorphism is within the 3' untranslated region, it is not the change responsible for production of the AK^2 isozyme.

Applied Biosystems Model 373A Version 2.0.1S

Sample 15 K562cDNA AKTA

Lane 15

Signal G:133 A:53 T:41 C:32
DyeTerminator{AnyPrimer}
432 MATRIX FILE
Points 141 to 7750 Base 1: 141

Thu, Apr 6, 1995 12:30 PM
Wed, Apr 5, 1995 5:13 PM
Spacing: 11.00

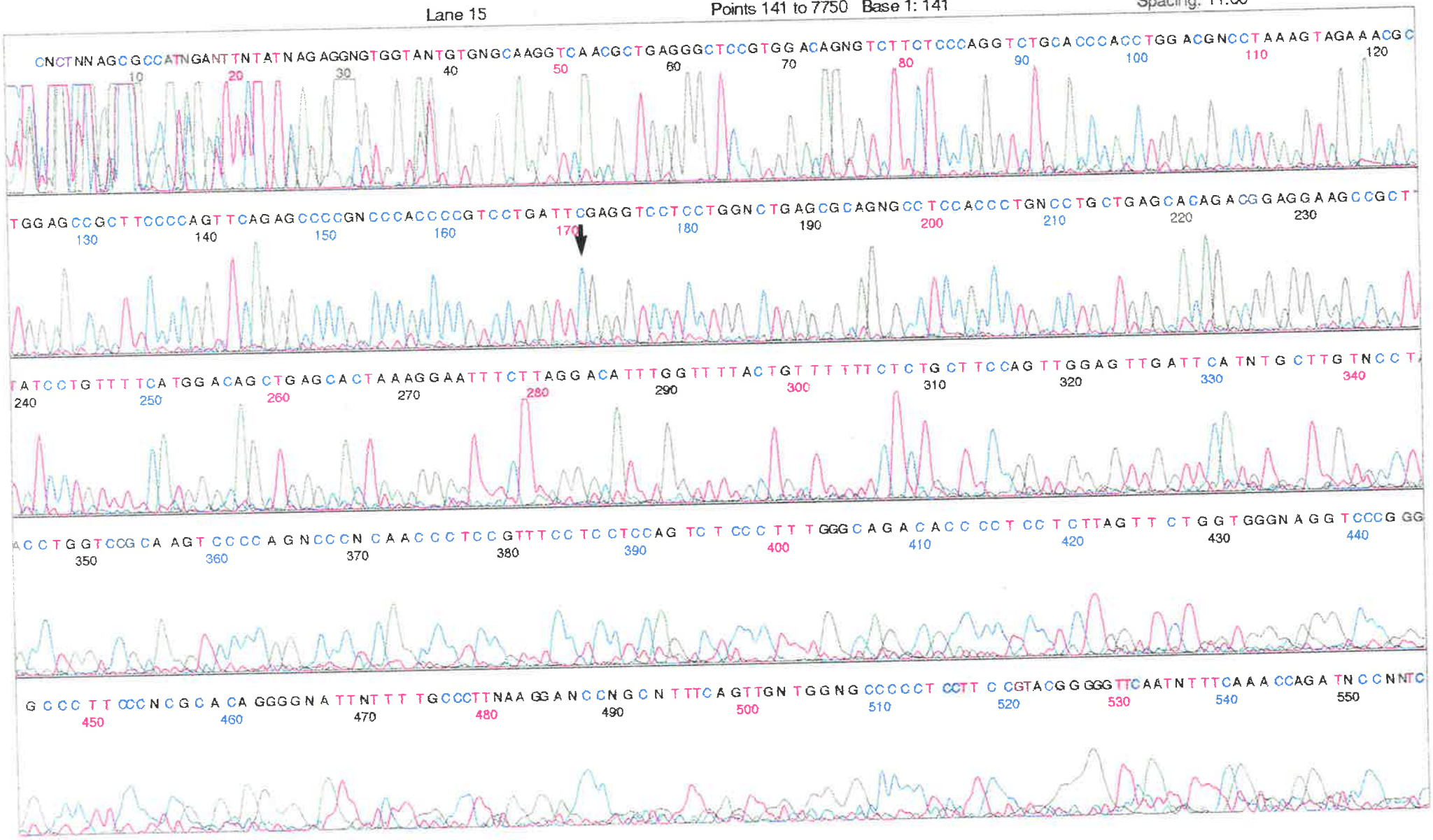


Figure 6-9. Raw sequencing data (sense) derived from PCR product generated from cDNA of the cell line K562, using the primers AKTA and AKTB. The single nucleotide difference to the published AK1 sequence is indicated by the arrow.

Localisation of the polymorphic site allowed calculation of the band sizes following digestion of the PCR product. Product generated from genomic DNA of a *dd* homozygous individual would yield two bands of 436 and 443bps. The size difference of 7bps would not be resolved on an agarose gel, and explains the results seen in Figure 6-6. Product generated from cDNA from a *dd* individual would yield bands of 443 and 194 following digestion with *Taq*I.

6.7 Analysis of patients with loss of A/B antigens for loss of heterozygosity of the AK1 gene

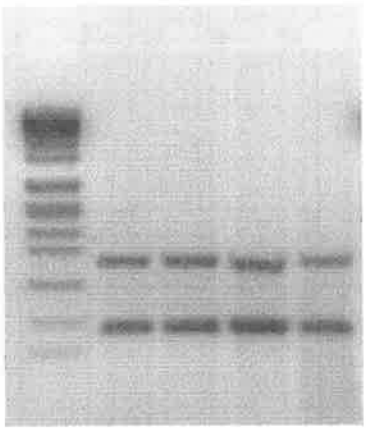
Analysis of genomic DNA from the thirteen patients with loss of A/B antigen expression and haematological malignancy was carried out to determine if LOH was occurring at the AK1 locus. To increase the number of informative patients, the *Taq*I and the published *Kpn*I and *Dde*I polymorphisms (Puffenberger and Francomano, 1991) were investigated.

6.7.1 Materials and Methods

To analyse the *Kpn*I and *Dde*I polymorphisms, PCR was carried out as described in Section 6.3.1. An aliquot of the resulting PCR product (10µl), was then digested overnight with 10 units of *Kpn*I or *Dde*I in a total volume of 20µl, according to the manufacturer's instructions (New England Biolabs). The bands were resolved on 1.5% agarose gels. The *Taq*I polymorphism was examined as described in Section 6.4.

6.7.2 Results and Discussion

The genotyping results for the AK1 gene are shown in Figures 6-10, 6-11 and 6-12, and are summarised in Table 6-1. Complete digestion with *Taq*I and *Kpn*I was controlled for by spiking the reactions with the 98bp ABO product generated from an OO genotype individual (using the primers AO1 and AO2 as described in Chapter 4). This product contains a single *Taq*I site, and in OO individuals, a *Kpn*I site. Due to the small fragment sizes produced by digestion of the control PCR product, it was necessary to resolve the bands on acrylamide or higher percentage agarose gels (the digest was divided in two and run on different gels; not shown). Complete digestion with *Dde*I was controlled for by the presence of a non-polymorphic site within the PCR product.



M WM MR MA ctrl

Figure 6-10. Digestion of PCR product generated from genomic DNA using the primers AKTA and AKTB with *Taq*I. Patient samples are annotated; ctrl is a normal heterozygous individual. The marker (M) is SPP-1/*Eco*RI.

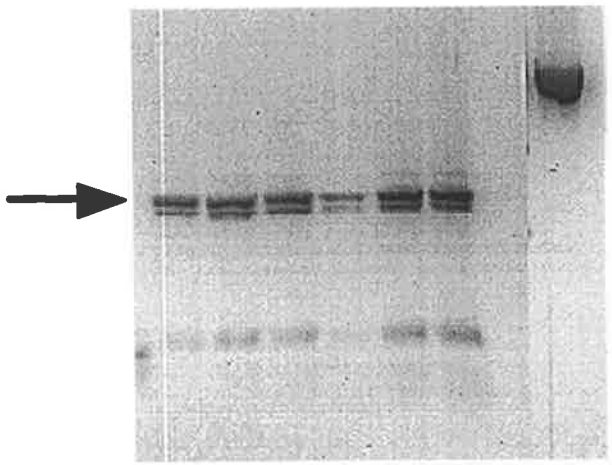


Figure 6-11. Digestion of PCR product generated from genomic DNA using the primers AKGEN2A and AKGEN2B with *Dde*I. Patient samples are indicated; C1 and C2 are normal heterozygous individuals; und is undigested PCR product. The allelic bands are marked by the arrow.

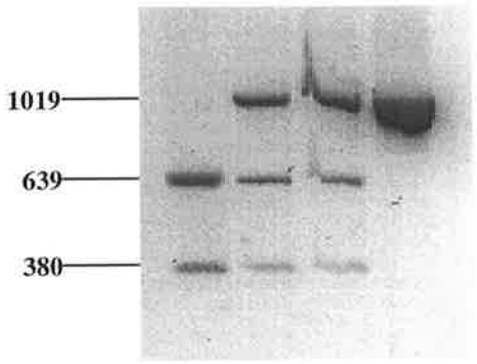


Figure 6-12. Digestion of PCR product generated from genomic DNA using the primers AKGEN2A and AKGEN2B with *Kpn*I. C1 is an individual homozygous for the *Kpn*I containing allele, and C2 is a normal heterozygous individual; und is undigested PCR product. The pattern and relative intensity of the bands is identical in the patient MA and the normal heterozygous control. Band sizes are shown in base pairs.

Patient	AK genotype (*Taq*I)	AK genotype (*Kpn*I)	AK genotype (*Dde*I)
AN*	*DD*	-	-
GN*	*DD*	-	-
MR*	*Dd*	-	-
NR	*DD*	-	-
WM	*Dd*	-	het
MA	*Dd*	het	het
CG	*DD*	-	-
WD	*DD*	-	het
KN	*DD*	-	-
DH	*DD*	-	het
PM	*DD*	-	-
BA	*DD*	-	-
SR	*DD*	-	het

Table 6-1. Results from analysis of the AK1 gene. "Het" indicates the patient was heterozygous for the polymorphism, "-" indicates the patient was homozygous. The standard *Dd* notation was used for the *Taq*I polymorphism.

Digestion of the AKGEN2 product with *Dde*I can detect four different alleles between 500 and 700bps in size. Only two of the alleles were seen in our patients, which is not surprising as the frequencies of the two most common alleles is 53 and 40%. The frequency of the allele containing the *Kpn*I site is 90%, while the frequency of the allele lacking the site is 10% (Puffenberger and Francomano, 1991). Digestion of the allele containing the *Kpn*I site produces two fragments, of 639 and 380bps.

Allele dosage was determined by comparing the relative intensity of the allelic bands with that of normal controls. Seven of the thirteen patients examined were informative for at least one of the three polymorphisms, and all had equivalent allele dosage. Only one of the three patients that had LOH at the ABO locus (AN, GN and MR) was informative; patient MR. This patient was heterozygous for the *Taq*I polymorphism, and although LOH at the ABO locus was obvious in this patient, there is clearly equivalent allele dosage at the AK1 locus. As the region of

chromosome nine that was physically lost in patient MR did not include the AK1 gene, it was concluded that the proposed tumour suppressor gene is located distally to the AK1 locus.

6.8 Analysis of AK1 gene expression in patients with haematological malignancy and loss of antigen expression

Although the LOH study described in the previous section excluded involvement of the AK1 locus, it was possible that the silencing event occurring at the ABO locus could also affect the AK1 gene. For this reason, the aim of the next set of experiments was to determine the normal relative expression of the *D* and *d* alleles of the AK1 gene, and to subsequently examine the patients who were heterozygous for the *Taq*I polymorphism for mono-allelic expression of the gene.

The three patients who were heterozygous for the *Taq*I polymorphism were MA, MR and WM. WM is the patient who had significantly decreased red cell AK1 activity (Marsden *et al.*, 1992).

6.8.1 Materials and Methods

Amplification of RT-PCR product was carried out essentially as described in Section 6.5, with the exception that all samples were DNase treated before reverse-transcription (see Section 2.6.5). This was necessary, as contaminating DNA in the RNA preparation amplified in addition to the cDNA target. Following digestion of the PCR product, the relative intensities of the cDNA-derived bands could not be calculated as digestion of the 879bp PCR fragment generated from DNA could also contribute to the intensity of the 443bp band. As DNase treatment seems to adversely affect the efficiency of the reverse-transcription reaction, samples were subjected to 45 cycles of PCR. The rest of the experiments in this chapter were

carried out using ultra-pure water from Biotech International (Western Australia), as opposed to water from the hospital Millipore purification systems.

6.8.2 Results and Discussion

At this point in the project, only one "normal" BM control that was heterozygous for the *Taq*I polymorphism was available (this was a remission marrow from a non-Hodgkin's lymphoma patient). However, it was noted that the different water being used in the laboratory (see Section 6.8.1), significantly increased the efficiencies of some PCR reactions. Furthermore, the efficiency of the AK1 RT-PCR reaction was increased, as it was now possible to generate product from normal peripheral blood mononuclear cell cDNA. Subsequently, peripheral blood samples from normal individuals were used as controls for this part of the study. Eight heterozygous individuals were examined, in order to determine the normal relative expression of the alleles. However, the results were not repeatable; aliquots of the same reverse-transcription reaction amplified simultaneously often revealed markedly different relative intensities of the allelic bands. This was not due to incomplete digestion, as often the allele containing the *Taq*I site was more intense than the allele without the site, and the control fragments always digested completely. Furthermore, the results could not be explained by star-activity of *Taq*I, as the digestion pattern was the same after 1 hour as it was after 22 hours.

To see if the non-repeatability of these results was in fact due to the DNase treatment of the RNA prior to the reverse-transcription reaction, identical RNA samples, both treated and non-DNase treated, were reverse-transcribed simultaneously. Digestion of PCR product obtained from these experiments with *Taq*I revealed significant differences between the relative intensities of the allelic bands following DNase treatment (Figure 6-13). This is mostly aptly demonstrated in the sample from control individual 3, (C3; lanes 3 and 6 after the marker). In the

DNase treated sample from this individual, there is complete digestion of the RT-PCR product, which would indicate a *dd* phenotype. However, in the sample that was not DNase treated, there is quite obviously equal expression of both alleles.

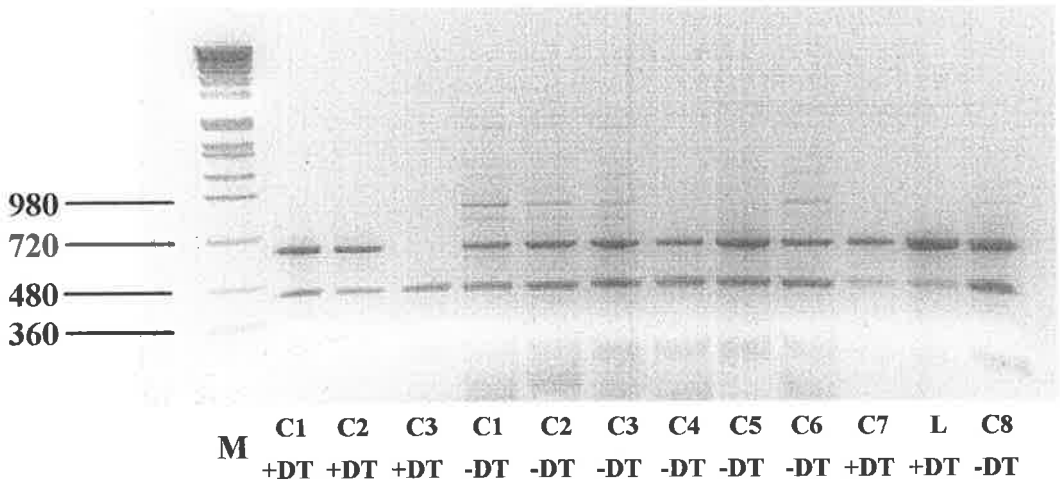


Figure 6-13. *Taq*I digestion of the AKTAQ RT-PCR product generated from DNase treated (+DT), and non-DNase treated (-DT) RNA from eight control individuals (C1-C8), and the cell line LIM 1215 (L), all of which genotyped *Dd* for the *Taq*I polymorphism. The marker (M) is SPP-1/*Eco*RI. Sizes of the relevant marker bands are in base pairs.

The most likely explanation for the inconsistencies seen in DNase treated specimens is that the number of mRNA copies of the AK1 gene in normal PB mononuclear cells is extremely low. As DNase digestion severely decreases the efficiency of the RT-PCR reaction (at least in our system; multiple methods of DNase treatment and inactivation were tested to try and increase the efficiency, to no avail), the obvious way to avoid this problem was to omit the DNase treatment of the RNA. While excluding the DNase treatment revealed repeatable results in the control samples, and minimal contamination of DNA, the patient RNA samples we wished to analyse had significant DNA contamination.

To avoid this problem, the patient and control samples were amplified following RT-PCR without DNase treatment. The products were resolved on 0.9% low

melting point agarose gels, and the product generated from cDNA isolated and purified using the Wizard PCR preps kit (see Section 2.6.8). Unfortunately, digestion of the purified product was severely inhibited, presumably by one of the ingredients of the kit. Increasing digestion times to 24 hours, and enzyme concentrations (up to 20 units per reaction), did not result in complete digestion of the control PCR products.

The most obvious way to solve this problem would be to avoid contaminating DNA altogether, by designing another primer to replace AKTA. If the sense primer was located in exon 5 instead of exon 6, amplification of DNA contaminating RNA samples would be unlikely to occur, as this region spans just over four kilobases. Although this approach would be simple and effective, there was insufficient time to carry out the experiments.

Although DNase-treatment of RNA from the control individuals did not yield consistent results, two of the three patients with loss of A antigen always produced the same result. While no product at all could be generated from DNase-treated RNA from the patient MA, strong bands were obtained from the patients WM and MR. Presumably the cell populations in the samples from these patients exhibit higher expression of the AK1 gene, as product was able to be obtained before the implementation of the "new" water and subsequent increase in the efficiency of the RT-PCR reaction. Furthermore, the sample from MR was from bone marrow consisting of 67% erythroblasts. If one was to accept that both alleles of the AK1 gene are expressed in equal amounts, it would appear that the patient MR has normal expression (see Figure 6-14). However, digestion of the RT-PCR product from patient WM indicated that expression of the *d* allele was down-regulated relative to the *D* allele (see Figure 6-14). Identical results were obtained from four different (DNase treated) reverse-transcription reactions of this patient.

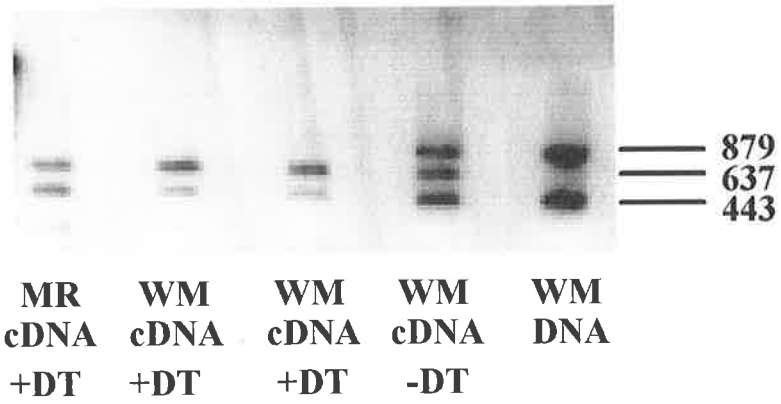


Figure 6-14. *Taq*I digestion of the AKTAQ PCR product generated from reverse-transcribed, DNase-treated RNA in the patients MR and WM (indicated by cDNA +DT), and from non-DNase-treated RNA from patient WM (cDNA -DT). An estimate of the relative intensities of the allelic bands in patient WM cDNA (non-DNase treated) can be made by correcting for the presence of contaminating DNA by comparing the digested RT-PCR product with the digested PCR product generated from genomic DNA of this patient. After correcting for the presence of genomic DNA, it appears that the *d* allele of the AK1 gene is reduced in expression relative to the *D* allele in this patient. The two DNase-treated samples from patient WM were generated from different RTs.

Non-DNase treated RNA from patient WM was also subjected to RT-PCR, and although a band generated from DNA is visible, digestion of the product reveals an imbalance in the relative intensities of the alleles (see Figures 6-14 and 6-15).

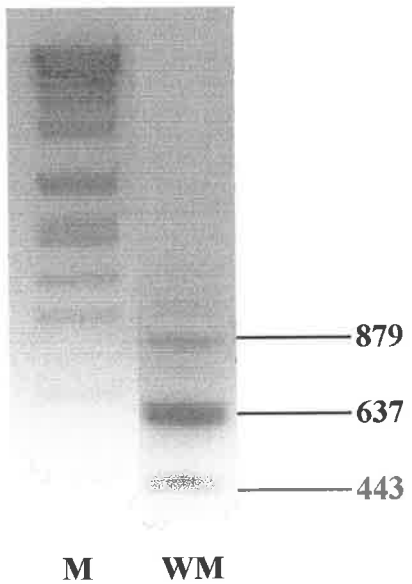


Figure 6-15. *Taq*I digestion of the AKTAQ RT-PCR product generated from non-DNase treated RNA from the patient WM. The marker (M) is SPP-1/*Eco*RI. The 879bp band generated from DNA is visible; although digestion of this band would contribute to the intensity of the 443bp band, the 637 band generated from cDNA is much stronger in intensity than the 443 band.

Although *Taq*I digestion of the product amplified from DNA would contribute to the intensity of the lower (443bp) band, the intensity of this band is still significantly less than that of the 637bp band. Given that non-DNase treated controls show approximately equal intensity of the two bands, it appears that there is transcriptional silencing of the *d* allele of the AK1 gene in the patient WM. If these results were in fact a true indication of the relative amounts of the AK1 alleles, it would appear that although the A allele of the ABO gene is completely silenced in patient WM, there is only partial silencing of the AK1 gene. This could be explained by an overall higher expression of AK1 than of the ABO gene. The expression of both genes is supposedly restricted to red cell precursors, however although AK1 expression could be detected in normal PB samples using the "new" water, expression of the ABO gene still remained undetected. Therefore, while expression of the ABO gene from normal cells in the PB of patient WM is not able to be detected, perhaps expression of AK1 from normal cells is - leading to detection of transcripts (albeit, imbalanced in their relative intensities), from both alleles.

Non-DNase treated RNA from the patient MA was reverse-transcribed, and the band corresponding to cDNA product isolated and purified using Wizard Preps, as described above. The purified product was then amplified in three separate PCR reactions. Digestion of the product was complete; implying transcriptional silencing of the *D* allele (data not shown). The results on patient MA must be taken with extreme caution; expression of the AK1 gene was limiting as no product could be generated from DNase-treated RNA, and only a very weak band could be generated from non-DNase treated RNA. Therefore the product that was purified and re-amplified might not be a true indication of the relative expression of the AK1 alleles.

SUMMARY

A previously known *Taq*I RFLP in the AK1 gene was localised and sequenced. A single base change (A→C at nt 10,630) was responsible for the polymorphism, and is located in the 3' untranslated region of the gene. Subsequent studies using this and two other polymorphisms of the gene to examine patients with loss of A/B antigens and haematological malignancies showed equal allele dosage at the AK1 locus in all seven informative patients. One of these patients had LOH at the ABO locus. As this patient had equivalent allele dosage of the AK1 gene, it was concluded that if a tumour suppressor gene is in this region, it is located distally to the AK1 gene.

As the *Taq*I polymorphism is in exon 7 of the AK1 gene, attempts were made to determine the normal relative expression of the AK1 allele. Although a number of technical problems were experienced when using DNase-treated RNA, it appears that in normal individuals, both alleles are equally expressed. The patient MR also seems to have normal expression of the AK1 gene. Unfortunately, the results in patient MA are not convincing as expression of the AK1 gene appeared to be extremely low in this patient, which could lead to preferential amplification of one allele and subsequently not represent the true relative amounts of the AK1 alleles. The patient WM, who also had significantly reduced red cell AK1 activity, consistently gave the same result from DNase-treated, and non-DNase treated reverse-transcription reactions; markedly reduced expression of the *d* allele of the AK1 gene. Until the results can be confirmed using a different set of primers (and the problem of DNA contaminating RNA samples can be avoided), it was concluded that the patient WM has transcriptional silencing of single alleles of the linked AK1 and ABO genes.

As the region of chromosome 9q that is physically lost in patient MR does not include the AK1 gene, the next step in this project was to further define the affected region. Subsequently, the next chapter describes the analysis of the ABL gene, which is located between the ABO and AK1 genes.

CHAPTER SEVEN

ANALYSIS OF THE ABL GENE

7.0 INTRODUCTION

The ABL gene is located 5mb closer to the centromere than the ABO gene, and is approximately 10mb distal to the AK1 gene (Kwiatkowski *et al.*, 1993). Examination of the ABL locus was undertaken primarily because of the close proximity of the ABL and ABO genes, and secondly due to the evidence suggesting that ABL is involved in regulation of the cell cycle and can act as a tumour suppressor gene (Sawyers *et al.*, 1994). The ABL gene is ubiquitously expressed, at low levels. There are two major splice variants of the gene, which contain one of two alternative first exons, known as 1a and 1b (Shtivelman *et al.*, 1986), although differential expression of the variants in haematopoietic cells has not been reported. Until December of 1994, it was not possible to study allelic expression of the ABL gene, as there were no known exonic polymorphisms. Melo *et al.* (1994), described a polymorphic marker in exon 11 of the ABL gene. The polymorphism is a single base pair silent mutation, from cytosine (allele "C") to guanine (allele "G"), in the third base of codon 784 of the ABL type 1a transcript. This chapter describes the analyses of patients, both with and without altered blood group antigens, for LOH and altered allelic expression using this polymorphism.

7.1 ANALYSIS OF THE ABL POLYMORPHISM IN NORMAL INDIVIDUALS

The first aim of these experiments was to confirm that the alleles distinguished by the *Bst*NI polymorphism are equally expressed in peripheral blood mononuclear cells from normal individuals.

7.1.1 Rationale

Approximately 16% of normal individuals are heterozygous for the ABL polymorphism described by Melo *et al.* (1994), the most common allele of which is the "C" allele. The polymorphism is able to be detected by restriction enzyme digestion of PCR product generated by ABL exon 11 specific primers with *Bst*NI, as shown in Figure 7-1 below.

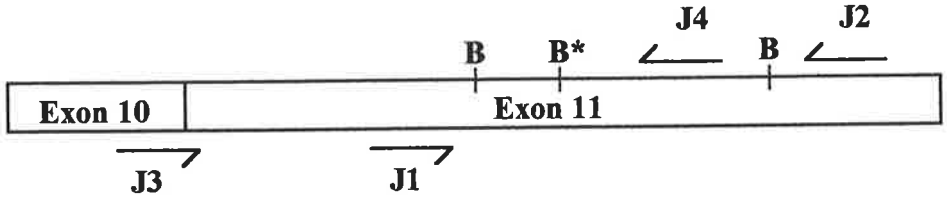


Figure 7-1. Diagrammatic representation of the primers used by Melo *et al.* (1994), to determine the relative allelic expression of the ABL gene relative to intron-exon boundaries. Restriction enzyme digestion of PCR product generated using the primers J1 and J2 with *Bst*NI was used to genotype individuals. The relative expression of the ABL alleles in individuals heterozygous for the *Bst*NI polymorphism (indicated by B*; B indicates non-polymorphic *Bst*NI sites) was determined using a nested PCR approach. The first round of cDNA amplification was carried out using the primers J3 and J2; the PCR product was then diluted 1:1000 and 1µl used as template for amplification using the primers J1 and J4.

The oligonucleotide primers J1 and J2 used by Melo *et al.* (1994) to genotype individuals for the *Bst*NI polymorphism, are both contained within exon 11 of the ABL gene. Consequently, both DNA and cDNA can be amplified with the same set of primers. To avoid amplification of genomic DNA when examining allelic expression of the gene in peripheral blood mononuclear cells, Melo *et al.* (1994), used a nested PCR approach. The first round of amplification was carried out using a primer spanning the intron/exon junction of exons 10 and 11, (J3; nt 1657-1681 of the ABL 1a transcript; Genbank accession number UO7653), and a primer from

within exon 11 (J2; nt 2615-2640). Theoretically, these primers should specifically amplify reverse-transcribed mRNA, and not contaminating genomic DNA in RNA preparations (see Figure 7-2).

```
                                    10<<>>11
      TCAC TCCAAGGGCCAGGGAGAGAGCGAT CCTC
1653                                      1685
```

Figure 7-2. Positioning of primer J3 so that it spans the intron/exon boundary of exons 10 and 11 of the ABL transcript (the dinucleotide "AT" at the 3' end of the primer is in exon 11). The primer is distinguished from surrounding sequence by bold type.

The PCR product generated by the primers J3 and J2 was then diluted (1:1,000), and 1μl of the dilution used as template for a second round of amplification using primers specific to exon 11 (J1; nt 2180-2205 and J4; nt 2563-2588). To control for carry-over genomic DNA contamination, ABL intron specific primers were used on the diluted RT-PCR product. Using this approach, Melo *et al.* (1994) reported that although the PCR product generated in both genotyping and phenotyping reactions was refractory to digestion due to heteroduplex formation, the ratio of the alleles was approximately the same in both cases. The authors subsequently concluded that both the C and G alleles were equally expressed in normal individuals (see Figure 7-3).

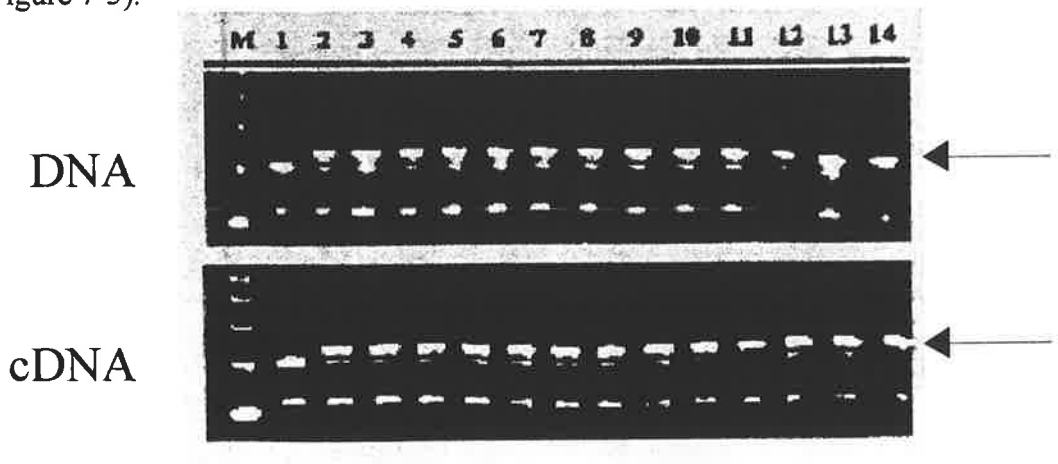


Figure 7-3. Relative expression of ABL alleles distinguished by the BstNI polymorphism in normal individuals as determined by Melo *et al.*, 1994. The arrows indicate the allelic bands; the lower band in constant. Lane 1 is a CC individual; lanes 2-13 are CG individuals while lane 14 is a GG individual. As the relative intensities of the bands generated from cDNA are identical to that from DNA, it was concluded that the C and G alleles are equally expressed in normal individuals.

To analyse the ABL locus, the approach described by Melo *et al.* (1994), was followed, with two exceptions. To genotype individuals for the *Bst*NI polymorphism, amplification was carried out using the primers J1 and J4 (compared with J1 and J2 used by Melo *et al.*, 1994). The second alteration to the procedure described by Melo *et al.* (1994), was relatively minor. Primers J1 and J4 were altered slightly, by the addition of an A and a T respectively to the 5' ends of the oligonucleotides. This was done primarily to enhance the binding of the primers to the appropriate region in exon 11. A diagrammatic representation of the fragments sizes obtained after digestion of the PCR product is shown in Figure 7-4.

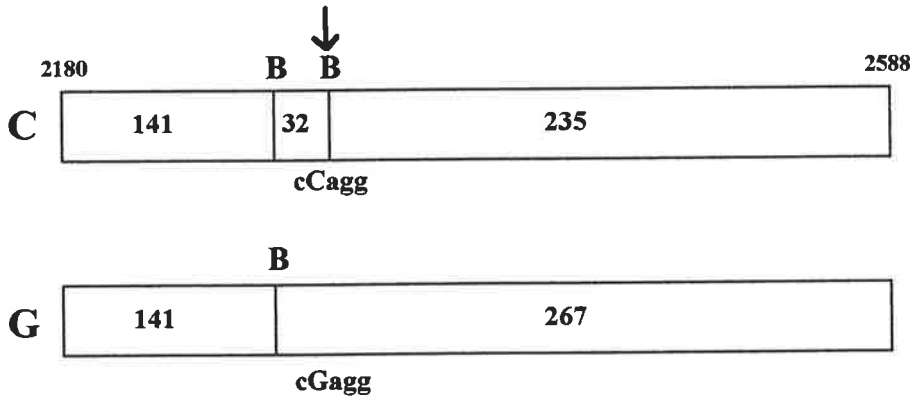


Figure 7-4. Diagrammatic representation of the ABL exon 11 region amplified by the primers J1 and J4. *Bst*NI restriction sites are indicated by "B"; the polymorphic site that is present in the "C" allele is indicated by an arrow. Fragment sizes after digestion of the 408bp PCR product generated from the "C" and "G" alleles are shown in base pairs.

7.1.2 Materials and Methods

Genomic DNA was prepared from 30 unrelated individuals as described (see Section 2.6.1). PCR was carried out as described in Section 2.6.4, with the following primers:

J1 5' AAGGACACGGAGTGGAGGTCAGTCA 3' (sense)

J4 5' TTGGTGCACCTGAGCCTGCTTTGCT 3' (anti-sense)

Diagnostic restriction enzyme digestions were performed on 10µl aliquots of each sample, in a total reaction volume of 20µl containing 7 units of *Bst*NI (New England Biolabs), in the buffer supplied by the manufacturer, according to the manufacturer's instructions. The restricted products were resolved on 2% agarose gels (see Section 2.6.6).

To analyse the relative expression of the ABL alleles in individuals heterozygous for the *Bst*NI polymorphism, RNA was prepared from peripheral blood mononuclear cells, DNase-treated and reverse-transcribed as described in Sections 2.6.2 and 2.6.5. Five µl of cDNA was then amplified as described above, with the exception that 45 cycles of PCR were employed (as opposed to 35 cycles used for genotyping). To ensure the absence of genomic DNA in the reverse-transcription, parallel reactions without reverse-transcriptase were also amplified.

7.1.3 Results and Discussion

Of the 30 normal individuals examined, 26 (86.67%) were CC homozygotes, and 4 (13.33%) were CG heterozygotes. No GG homozygotes were detected. Comparison of the relative intensities of the allelic bands obtained from DNA and cDNA in the 4 heterozygous individuals revealed approximately the same ratios in all samples. As the relative intensities of the bands obtained from amplification of

DNA from normal individuals presumably represents a 1:1 ratio of the alleles, it was concluded that there was equal expression of the alleles in normal heterozygous individuals. The relative intensities of the alleles differ slightly to that reported by Melo *et al.*, 1994 (compare Figures 7-5 and 7-3). This is probably due to increased amounts of heteroduplex formation obtained by the different cycling conditions used by Melo *et al.*, 1994.

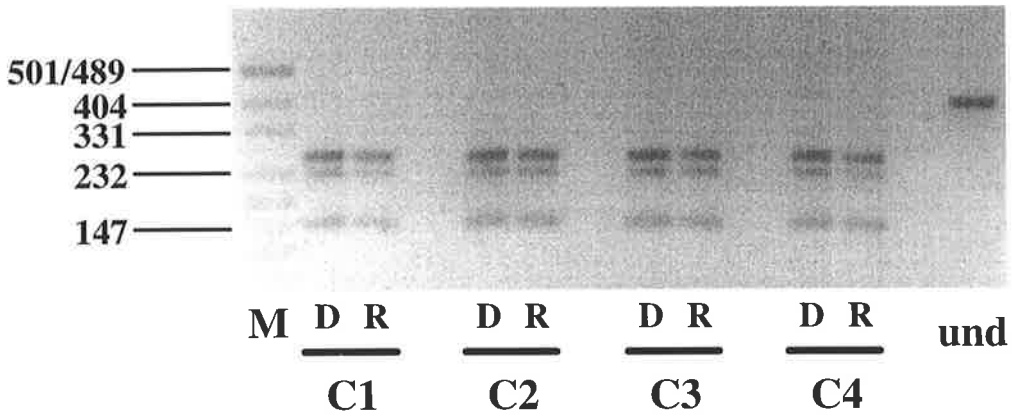


Figure 7-5. Relative expression of the ABL alleles distinguished by *Bst*NI restriction enzyme digestion in peripheral blood of four normal individuals (C1-4). Genomic DNA (D) and RNA (DNase-treated, reverse-transcribed; R) samples from each of the individuals are paired. Sizes of the relevant pUC19/*Hpa*II marker (M) bands are indicated in base pairs. "und" is undigested PCR product.

7.2 Analysis of the ABL gene in patients with haematological malignancy and loss of ABO antigen expression

Two patients with haematological malignancy and loss of ABO blood group antigen expression were heterozygous for the ABL polymorphism. Both patients (WM and NR), had equal allele dosage at the ABO locus, but transcriptional silencing of the A allele. Similarly, both patients had equal allele dosage at the ABL locus. Furthermore, initial RT-PCR experiments showed loss of expression of the G allele of the ABL gene (see Figure 7-6). Only samples where the negative control for DNA contamination (ie. the parallel reverse-transcription reaction without reverse-transcriptase added) was completely negative following amplification with the

primers J1 and J4 were considered. However, it was noted during these experiments that digestion of DNA contaminating the RNA samples was not always complete; some of the DNase-treated negative controls still produced PCR product. Furthermore, the results from multiple reverse-transcriptions in the patients WM and NR gave varying results. It appeared that although the intensity of the band corresponding to the G allele was reduced relative to that seen in the normal controls, the amount of reduction seen from multiple RT-PCR reactions was not always the same.

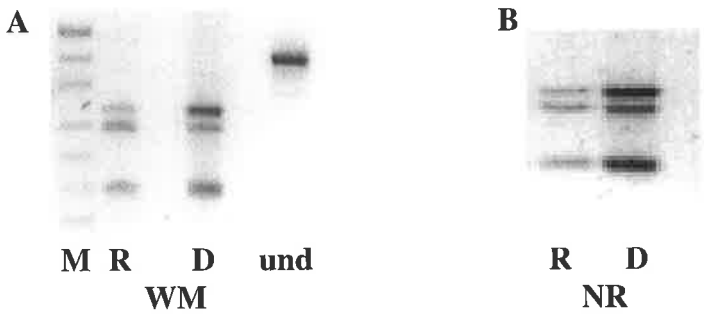


Figure 7-6. Analysis of the ABL gene in patients with transcriptional silencing of the ABO gene. Panel A shows digestion of the PCR product with *Bst*NI from DNA (D) and DNase-treated, reverse-transcribed RNA (R) from patient WM. Panel B shows digestion of the product obtained from DNA and DNase-treated reverse-transcribed RNA from patient NR. The marker (M) is pUC19/HpaII, "und" is undigested PCR product. While there appears to be abnormal allelic expression of the ABL gene in both patients, independent RT-PCR reactions did not give repeatable results.

As the varying results in the patient samples could be due to contaminating DNA, the DNase incubation was altered from 12 minutes at room temperature, to 10 minutes at 37°C, to increase activity of the enzyme. Lack of contaminating DNA was confirmed by amplifying the samples with intron-specific primers to the AK1 gene (AKGEN2A and B; as described in Section 6.3.1). No bands corresponding to the intron-specific product could be detected following the enhanced DNase

treatment and 50 cycles of PCR in any samples. Although ABL RT-PCR product could be generated from cDNA prepared from RNA treated in such a way from normal individuals, no product could be generated from the patient WM and NR samples. The amount of RNA in the reverse-transcription was increased from 1μg to 2-2.5μg but only very weak bands could be generated. It seemed therefore, that expression of the ABL gene is reduced in these patient samples. As experiments in this thesis have already shown that a combination of low gene expression and DNase-treatment can lead to non-repeatable results (as described in Chapter Six of this thesis), a more sensitive method was required to analyse the ABL gene in these patients; avoiding DNase treatment altogether.

7.2.1 Optimization of the RT-PCR method to assess allelic expression of the ABL gene

The nested PCR method described by Melo *et al.* (1994) was used on non-DNase treated reverse-transcribed RNA. The J2 primer was modified by the addition of two nucleotides, GT, to the 5' end. The addition of these two nucleotides theoretically increases the melting temperature of the J2 primer, such that it is closer to the melting temperature of its pair, the J3 primer.

7.2.2 Materials and Methods

Amplification of cDNA specific product was achieved by a nested PCR reaction, as described (Melo *et al.*, 1994). The first round of amplification was carried out for 35 cycles in a total reaction volume of 50μl, containing 5μl of cDNA, as described in Section 2.6.4, with the following primers:

J3 5' TCCAAGGGCCAGGGAGAGAGCGAT 3' (sense)

J2 5' GTGCTTGTGCCTCCTCACTCTGGAC 3' (anti-sense)

The resulting PCR product was diluted 1:1000 in water, and 1µl used for the second round amplification for 45 cycles with the primers J1 and J4, and digested with *Bst*NI as described above (7.1.2). To control for carry-over genomic DNA contamination, the diluted RT-PCR product was subjected to PCR using the AKGEN2A and B primers for 45 cycles.

7.2.3 Results and Discussion

Normal controls were again examined, and independent RT-PCR/restriction enzyme digestion gave the same results as shown in Figure 7-5. Again, analysis of the patients WM and NR showed reduced expression of the G allele relative to the C allele (not shown). Given these results, and the fact that ABL could be a tumour suppressor gene, patients with haematological malignancy and no loss of ABO antigen expression were examined for relative allelic expression of the ABL gene.

7.3 Analysis of the ABL gene in patients with malignancy and no loss of ABO antigen expression

7.3.1 Analysis of allele dosage

Samples from eighty-four patients with a broad range of haematological malignancies were genotyped for the ABL polymorphism. Most, but not all of the patients were the same as those genotyped at the ABO locus and described in Appendix A; patients with CML were excluded from this analysis because one would expect that there was differential expression of the ABL alleles, given that one allele is driven by the promoter of the BCR gene after the formation of the Philadelphia translocation. Furthermore, increased expression of the ABL allele involved in the BCR/ABL translocation relative to that of the "normal" ABL allele in CML patients has recently been shown (Melo *et al.*, 1995). Fifteen of the patients (17.8%) were CG heterozygotes; 68 (80.9%) were CC homozygotes, and one patient genotyped GG (1.19%). It is unlikely that any patients who were

recorded as homozygous were in fact heterozygous but had LOH, as the number of CG heterozygotes obtained was slightly higher than that reported in the normal individuals (17.8% compared with 13% of the normal individuals examined in this study, and 16% reported by Melo *et al.*, 1994).

Normal tissue from thirty-five patients with colon cancer was also examined for the ABL polymorphism. Interestingly, only one of the patients was heterozygous (2.9%). Thirty-three (94%) of the patients genotyped as CC, while one patient genotyped as GG. Analysis of tumour tissue from the heterozygous patient showed normal allele dosage at the ABL locus. The low number of heterozygous patients with colon cancer is presumably due to sampling variation; although it would be of interest to see if this trend continues by examining more patients.

The cell lines LIM 2405, LIM 1863, LIM 1215, LIM 2412, HT29, HEL, KCL22, HiMeg, HL60, EM2, JK, and K562 were also examined for the ABL polymorphism; all were CC homozygotes or C hemizygotes. The colon-carcinoma cell line SW480 however, was a CG heterozygote and had normal allele dosage.

None of the 15 heterozygous patients with haematological malignancy showed abnormal allele dosage at the ABL locus, although no regard was given to the amount of leukaemic cells in each of the particular samples. To ensure that there was equal allele dosage at the ABL locus in these patients, different samples from 13 of the patients were examined. Every effort was made to analyse samples that had a minimum of 50% blast cells, although in one case the number of blast (or leukaemic) cells in the samples could not be estimated as no patient results were available. Descriptions of these patients and the samples analysed have been tabulated in Appendix B. Analysis of these samples again showed normal allele dosage at the ABL locus (see Figure 7-7). As at least 12 of these patients had a

minimum of 50% leukaemic-derived cells, and subsequently abnormal allele dosage should be obvious, it was concluded that there was no LOH occurring at the ABL locus.

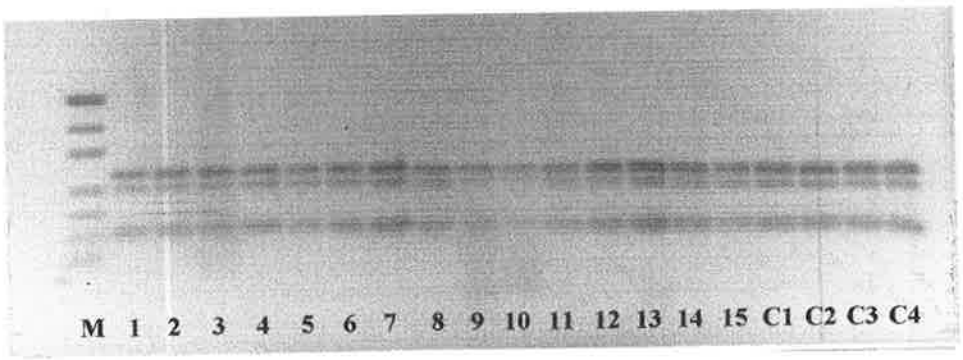


Figure 7-7. Allele dosage of the ABL gene in 15 patients with haematological malignancy as revealed by the *Bst*NI polymorphism. Patients 4 and 5 are the patients WM and NR (the PB sample is shown; the results from the PE sample of this patient were identical), that had loss of blood group antigen expression; the rest of the patients are described in Appendix B of this thesis. The marker (M) is pUC19/*Hpa*II. C1-4 are normal controls.

7.3.2 Analysis of ABL expression in patients with haematological malignancy and no loss of blood group antigen expression.

Eleven of the thirteen patients examined using the nested RT-PCR method described in Section 7.3.1 clearly had abnormal relative expression of the alleles, with complete loss of both the C and the G alleles in different patients (data not shown). However, multiple PCR reactions carried out on the same (and different) RT reactions did not reveal consistent results. In fact, for all but two of the thirteen patients and one cell line (SW480) examined using this method, the relative intensities of the allelic bands showed extreme variation in the same individual. In the two patients that produced consistent results (patients 6 and 8), both patients were examined 9 times, from 3 different reverse-transcription reactions, and the relative intensities of the allelic bands were always the same as seen in the normal controls (data not shown).

The inconsistencies seen in most of the patients could not be explained by variations in the efficiency of the reverse-transcription reactions, or the PCR reactions, as normal controls were always reverse-transcribed and subjected to PCR simultaneously, and always gave the expected results. Although RNA from the normal controls was not isolated at the same time as that from the patients, RNA from the two patients which consistently showed normal expression of the ABL gene was isolated at the same time as that from the patients that gave inconsistent results. Furthermore, analysis of the RNA preparations for integrity by gel electrophoresis showed that the RNA from patient 6 was extremely degraded. Therefore, the abnormal results in the other patients could not be explained by the presence of partially degraded RNA.

To determine if it was possible to generate the ABL PCR product from genomic DNA following the nested protocol, 100ng of DNA was subjected to amplification. In fact, the ABL PCR product could be amplified from the diluted product obtained using the cDNA "specific" primers J3 and J2, although nothing could be amplified from the same diluted product using primers to an intron in the AK1 gene. The first round PCR product could not be visualised on a gel (even after 45 cycles of amplification), so it was possible that non-specific amplification of the ABL gene was occurring. In fact, closer analysis of the J3 primer revealed another possible binding site within exon 11 of the ABL gene (Figure 7-8).

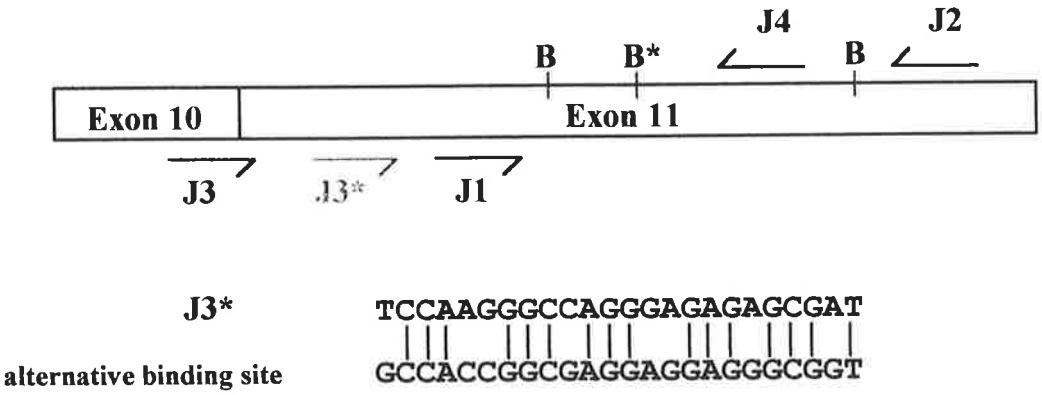


Figure 7-8. Alternative binding site of the primer J3 (indicated by *). Sixteen of the twenty-four bases in the primer can bind to nt 2095-2119 of the ABL type 1a transcript. Therefore, amplification of J1/J4 product can occur from genomic DNA following the nested protocol described Melo *et al.*, 1994.

Therefore, the first round of PCR with the primers J3 and J2 *could* give rise to a product from genomic DNA, although this region would probably not amplify very efficiently as 8 of the 24 base pairs of the J3 primer are mismatches. The nested RT-PCR method was subsequently considered unsuitable for analysis of expression, as DNA contaminating the reverse-transcriptase reaction could also give rise to a transcript. This result has interesting implications for the conclusions made by Melo *et al.* (1994), who used this method to show bi-allelic expression of the ABL gene in colony-forming unit granulocyte/macrophage colonies (CFU-GMs). Although these authors do not describe how they carried out reverse-transcriptions on the colonies, presumably they lysed single colonies to isolate RNA. Subsequently, genomic DNA would have been present in the reverse-transcription and could have given rise to ABL PCR product.

In an attempt to avoid using the nested PCR reaction, the primers J3 and J4 were used to amplify cDNA. After 45 cycles of amplification, the expected 930bp product could be detected in the normal controls and the two patients that consistently showed bi-allelic expression of the ABL gene (patients 6 and 8). However, no PCR product at all could be detected in any of the other patient

samples. The results indicate that the most likely explanation for the non-repeatable results in most of the patient samples is a combination of low ABL gene expression, and amplification of DNA contaminating RNA preparations. Incidentally, amplification with the primers J3 and J4 sometimes also gave rise to a shorter product of around 500bp, which is the size expected if the J3 primer was binding non-specifically to genomic DNA.

To ensure that the ABL gene was being expressed in these patients, and as shorter regions of cDNA amplify more efficiently, a new set of primers spanning exon 7-11 of the ABL gene were designed. The primers amplify a 590bp product, from nt 1141-1730 of the ABL type 1a transcript, and do not span the *Bst*NI polymorphism. Although these primers are termed J5 and J6, it should be noted that they are not related to the primers of the same name described by Melo *et al.*, 1995.

J5	5' TGATTTTGGCCTGAGCAGGTTGAT 3'	(sense)
J6	5' GCTCTTTTCGAGGGAGCAATGGAG 3'	(anti-sense)

Following 45 cycles of amplification under the standard conditions described in Section 2.6.4 of this thesis, the 590bp band was amplified in all of the patient samples. Although this method is not quantitative, the intensities of the bands were the same as that of the normal controls (except for patient 15 in which the intensity of the band was significantly weaker). No product could be amplified from genomic DNA using this set of primers. It was subsequently concluded that the ABL gene was expressed in the patient samples.

It was not possible to design cDNA specific primers spanning a shorter region of the ABL gene that also spanned the polymorphic site. In a final attempt to analyse the relative expression of the ABL alleles, another primer, J10 was designed. This

primer lies in exon 10, 5' of the J3 primer (nt 1615-1641 of the ABL type 1a transcript).

J10 5' TGCAGAGCACAGAGACACCACTGACG 3' (sense)

Amplification of cDNA was carried out using the primers J10 and J2, for 45 cycles (94°C, 1'; 60°C, 1'; 72°C 1'). The product was then diluted (1:100) and 1µl used as template for 45 cycles of amplification with the primers J1 and J4. The resultant PCR product was then digested with *Bst*NI as described above. Up to 200ng of genomic DNA subjected to this nested PCR reaction did not give rise to a PCR product.

7.3.3 Results and Discussion

For this final round of experiments, only the patients that had loss of blood group antigen were examined (WM and NR). Again, repeatable results were obtained from amplification of cDNA from normal individuals. However, the results from multiple reverse-transcriptions of patients WM and NR were not consistent (Figure 7-9), although amplification of cDNA with the primers J5 and J6 revealed the presence of ABL mRNA (see Figure 7-10; the relative positioning of the primers is shown in Figure 7-11). Ultimately, no conclusions concerning the relative allelic expression of the ABL gene could be drawn from these analyses. The most likely reason for this is a founder effect, as a result of low cDNA copies of exon 11 mRNA in the patients; hence amplification of the region using the PCR technique gives unreliable results because of the initial target copy number.

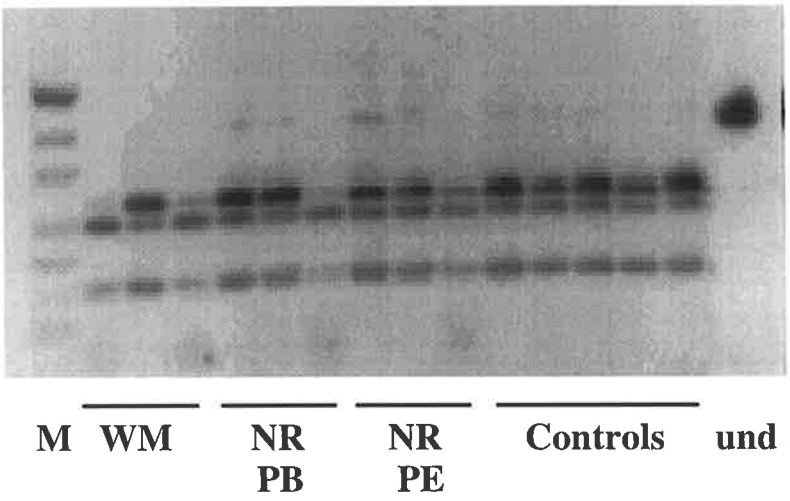


Figure 7-9. Non-repeatability of the ABL RT-PCR method for examining relative allelic expression in the patients WM and NR. Three reverse-transcriptions from WM, NR peripheral blood (PB) and NR pleural effusion (PE) samples were selected at random and subjected to PCR, as were five reverse-transcriptions from normal individuals. Digestion of the PCR product with *Bst*NI reveals markedly different relative intensities of the allelic bands in the different reverse-transcriptions from the patient samples, while the normal controls show relatively constant band intensities. "und" is undigested PCR product; the marker (M) is pUC19/*Hpa*II.

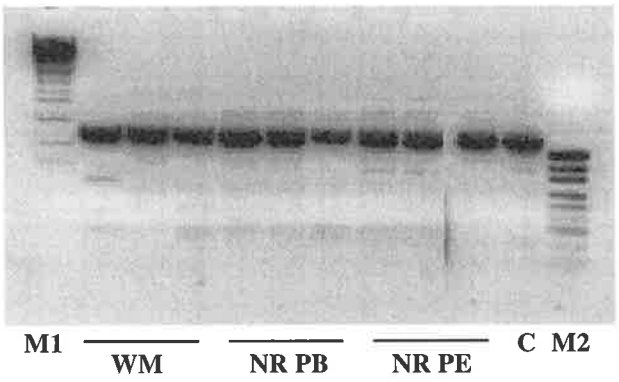


Figure 7-10. Expression of the ABL gene in the patients WM, and NR as determined by RT-PCR of the same reverse-transcriptions analysed in Figure 7-9 above. The markers are SPP1/*Eco*RI (M1) and pUC19/*Hpa*II (M2). "C" indicates a control individual.

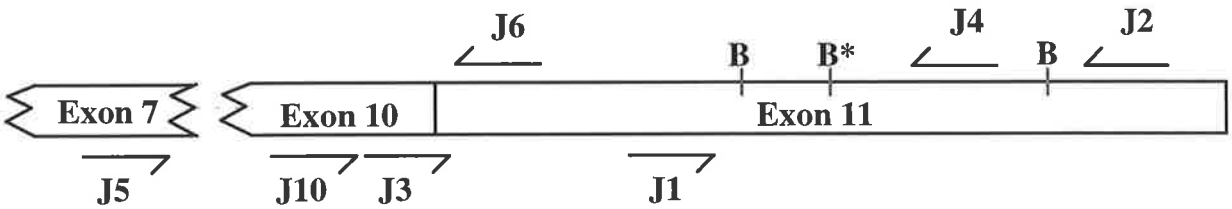


Figure 7-11. Relative positioning of all the primers used for analysis of the ABL gene.

SUMMARY

Analysis of the ABL gene in two patients with haematological malignancy and transcriptional silencing of the A allele of the ABO revealed equal allele dosage in both patients. Furthermore, normal allele dosage was determined in 13 patients with haematological malignancy and no loss of blood group antigen expression.

A number of different methods were attempted to examine the relative allelic expression of the ABL gene, but although normal controls and two patients with haematological malignancy consistently showed equal expression of both alleles, none of the methods produced repeatable results in the rest of the patients. The most likely explanation for these results is that the ABL gene is expressed at low levels in these patients, or at least cDNA copies of mRNA corresponding to exon 11 of the gene is present at very low levels. The reason for this remains elusive, but should be investigated further by using improved RT-PCR techniques or other (as yet unknown) polymorphisms in the gene. To improve upon the RT-PCR technique the primer J2 could be used to prime the reverse-transcription instead of random-hexamers, and thus enrich the cDNA for the specific region of ABL being examined. Therefore, the founder effect that appears to be occurring could be avoided. Another improvement would be to use a thermostable reverse-transcriptase such as "Superscript" (Gibco), which is active at higher temperatures. Carrying out the reverse-transcription at a higher temperature would help to eliminate any abnormal secondary structure of the ABL exon 11 mRNA which might be affecting the efficiency of the reactions in these patients.

CHAPTER EIGHT

CONCLUDING DISCUSSION

The aim of this thesis was to determine the molecular basis for loss of ABO blood group antigens from the red cells of patients with haematological malignancy. While loss of antigen expression is rarely reported, the actual molecular events leading to this phenomenon might be an indication of a more common genetic event occurring in haematological malignancies.

At least three different mechanisms are responsible for loss of blood group antigen expression. The first, loss of the precursor H antigen was responsible for loss of B antigen expression in two patients. Physical loss (loss of heterozygosity), of the A allele of the ABO gene which has been localised to chromosome band 9q34, was responsible for loss of A antigen expression in one case, while transcriptional silencing of the A allele of the gene was responsible for loss of A antigen expression in four other cases. One of the most interesting findings from the analysis of the ABO gene in patients with loss of antigen expression was physical loss of the non-functional O allele. As loss of a non-functional allele cannot provide a growth or other advantage to the cell, it is an indication of a larger event occurring that some times includes the ABO locus. As recurrent loss of heterozygosity events indicate the presence of a tumour suppressor gene, other genes were analysed in these patients to define the recurrently lost region.

Analysis of the linked AK1 gene revealed that loss of heterozgosity occurring at the ABO locus (at least in one patient) does not include the AK1 gene. As the AK1 gene is closer to the centromere than the ABO gene, this also excludes involvement of the candidate tumour suppressor gene, the Growth Arrest Specific 1 (GAS1) gene. The GAS1 gene, which we mapped to 9q21.3-q22 (Evdokiou *et al.*, 1993), is in a region recurrently deleted in AML, MDS and MPD (Trent *et al.*, 1989).

Analysis of the relative allelic expression of the AK1 gene in a patient with allelic silencing of the ABO A allele, revealed transcriptional silencing of a single allele of the AK1 gene. Presumably other genes in this 15mb region are also silenced, including a tumour suppressor gene. Given that some of the genes in this region are expressed in haematopoietic cells (eg. TAN1), it could also be hypothesised that silencing and subsequent reduced expression of these genes also contributes to the overall features of the disease.

The finding of both physical loss of the O allele of the ABO gene and transcriptional silencing of the A allele in one patient, indicates that the presumed tumour suppressor gene in this region is closely linked to the ABO gene. Following this hypothesis, one copy of the gene would be transcriptionally silenced, and the other physically lost. Subsequently, the gene would be completely functionally lost from the cell. In the patients with transcriptional silencing of a single allele of the ABO gene, it is proposed that there is also transcriptional silencing of a linked tumour suppressor gene on the same chromosome. Functional loss of the other copy of the tumour suppressor would either be by deletion, mutational inactivation, or another silencing event.

A likely candidate for the tumour suppressor gene in the 9q34 region is the ABL gene. However, if ABL is indeed a tumour suppressor, it might have been expected that at least one of the patients with haematological malignancy and no loss of blood group antigen expression had loss of heterozygosity at the ABL locus. Alternatively, it could be possible that the ABL gene is more susceptible to transcriptional silencing or other events than physical loss, for reasons as yet unknown. The finding that ABL, or at least the cDNA copies of mRNA transcripts containing the region examined in exon 11 of the ABL gene, appear to be under-expressed in most patients with haematological malignancy relative to that of

normal controls, could be important. It is possible that the majority of precursor stem or "blast" cells normally express very low amounts of ABL, although in two patients with haematological malignancy and large numbers of blast cells, ABL appeared to be normally expressed (patients 6 and 8 in Appendix B). Furthermore, in three other patients (patients 3, 14 and 15 in Appendix B), the majority of cells in the samples examined were lymphocytes. As the cells examined from normal control individuals were also mainly lymphocytes, it seems that reduced expression of the ABL gene is not merely a consequence of the cell type examined.

Future Directions

Comparatively little is known about the pathogenic process involved in the haematological malignancies relative to that of solid tumours. Most of what we know about leukaemia and other haematological malignancies has come from studies of the genes involved in translocation breakpoints (eg. CML). The work in this thesis demonstrates the difficulties involved when working with samples from patients with haematological malignancy, in which the "tumour" is completely disseminated and interspersed with normal cells. The best way of avoiding this problem and allowing comparisons between normal and malignant cells in the same individual is to analyse single haematopoietic colonies (eg. CFU-GM, BFU-E) using PCR and RT-PCR. An additional advantage of this technique would be that different/ evolved leukaemic clones could also be distinguished at the molecular level.

To further localise the tumour suppressor gene, analysis of the patients with loss of heterozygosity at the ABO locus using micro-satellite markers which are highly polymorphic and found in almost all genes should prove to be very useful. Similarly, the patients with allelic silencing of the ABO gene could be examined for

monoallelic expression of other genes in the 9q34 region using exonic polymorphisms.

Transcriptional silencing of the ABO gene is most likely to be due to allele-specific methylation of the promoter region. The recent cloning of the 5' untranslated region of the gene (Yamamoto *et al.*, 1995), will now permit analysis of methylation patterns in the patients with transcriptional silencing of the A allele of the ABO gene and no loss of heterozygosity. Similarly, the promoter region of the AK1 gene could also be examined for methylation in the patient that appears to have transcriptional silencing of both the ABO and AK1 genes. It would also be interesting to determine if the silenced alleles in this patient are on the same chromosome, which could be achieved using somatic cell hybrid analysis. Given that 11 of 12 patients with loss of A or B antigen expression and haematological malignancy had lost expression of the maternally inherited allele, it seems highly likely that imprinting of the 9q34 region is involved in this phenomenon.

In conclusion, this thesis provides the first insight into the molecular basis for loss of ABO blood group antigens in haematological malignancy, and the first report of loss of heterozygosity at the ABO locus in haematological malignancy. The finding that transcriptional silencing of a single allele of the ABO gene is more often responsible for loss of antigen expression than physical loss of the gene is intriguing and may represent an as yet uncharacterised epi-genetic event occurring in malignancy.

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APPENDIX A

Table 1. Comparison of ABO genotype with ABO blood group of patients with a wide range of haematological malignancies.

Key:

ALL -	acute lymphoblastic leukaemia
AML -	acute myeloid leukaemia
AUL -	acute undifferentiated leukaemia
ABL -	acute bi-phenotypic leukaemia
MDS -	myelodysplastic syndrome
MDS-t-	myelodysplastic syndrome in transformation to acute leukaemia
RAEB -	refractory anaemia with excess blasts
RAEB-t-	refractory anaemia with excess blasts- in transformation to acute leukaemia
MPD -	myeloproliferative disorder
CML -	chronic myeloid leukaemia
CLL -	chronic lymphoblastic leukaemia
CMML-	chronic myelomonocytic leukaemia
HCL -	hairy cell leukaemia
TP -	thromobcytopenia
WM -	Waldenstrom's macroglobulinaemia
BM -	bone marrow aspirate
PB -	peripheral blood

spleen/thyroid refers to biopsies of these organs

Where it was specified in bone marrow reports, FAB subtypes (ie. M1-M7), were also included.

Patient ID	Diagnosis	ABO genotype	ABO group	Sample ID	Sample type	Date
016232	AML	OO	O	-	BM	2/6/94
160825	AML	OO	O	C04088	BM	7/5/88
207290	AML	BO	B	-	PB	10/7/92
221257	AML	OO	O	-	BM	25/3/92
318122	AML	OO	O	120763	BM	21/12/90
323213	AML	OO	O	-	BM	23/3/92
510832	AML	AA	A	-	BM	18/8/92
214890	AML M1	OO	O	-	PB	10/4/92
482499	AML M1	OO	O	120714	PB	9/11/90
438680	AML M2	OO	O	120476	BM	2/7/90
529175	AML M2/M4	OO	O	-	BM	presentation
327298	AML M3	AO	A	120084	BM	24/10/90
451028	AML M3	AO	A	-	BM	25/5/92
072084	AML M4	AA	A	120461	BM	18/6/90
146612	AML M4	OO	O	120849	BM	11/2/91
151907	AML M4	OO	O	121575	BM	21/5/92
337200	AML M4	AO	A	120860	BM	14/2/91
426168	AML M4	AO	A	-	BM	16/9/92
434471	AML M4	OO	O	C04083	PB	4/5/88
493577	AML M4	OO	O	121534	BM	8/1/92
509612	AML M4	AA	A	-	PB	10/8/92
510023	AML M4	OO	O	-	PB	5/8/92
521522	AML M6	OO	O	112504	BM	21/5/93
052295	AML M7	AO	A	-	BM	8/8/92
495102	ABL	AO	A	121277	BM	16/8/91
406326	AUL	OO	O	C04248	BM	6/10/91
413314	AUL	AO	A	-	PB	4/11/92
463260	AUL.	OO	O	121322	BM	5/9/91
031686	MDS	AO	A	-	PB	30/4/92
043638	MDS	OO	O	121410	BM	29/10/91
081282	MDS	BO	B	-	PB	29/3/93
110030	MDS	OO	O	-	BM	13/11/92
167069	MDS	AO	A	120052	BM	17/1/90
185998	MDS	AA	A	-	BM	1/1/93
188346	MDS	AA	A	-	PB	8/7/92
224836	MDS	BO	B	-	BM	8/12/93
279492	MDS	AO	A	-	PB	5/11/92
400151	MDS	OO	O	C00428	BM	23/11/88
433924	MDS	OO	O	-	BM	13/5/92
437720	MDS	AB	AB	121206	BM	10/7/91
448281	MDS	OO	O	-	BM	18/8/92
482245	MDS	OO	O	120717	BM	9/11/90
525193	MDS	AA	A	-	BM	12/9/93
FMCDT	MDS	BO	B	-	BM	presentation
459580	MDS-t	OO	O	121356	BM	4/10/91
068152	RAEB-t	AB	AB	C04532	BM	21/8/89
310971	RAEB-t	OO	O	120596	BM	28/8/90
066179	MPD	OO	O	CO4636	BM	23/11/89
263600	MPD	BO	B	120668	spleen	15/10/90
458245	MPD	AO	A	-	spleen	9/11/92
168727	CMML	AA	A	120644	PB	2/10/90
187003	CMML	AO	A	120640	BM	1/10/90

Patient ID	Diagnosis	ABO genotype	ABO group	Sample ID	Sample type	Date
043200	ALL	OO	O	C04047	BM	14/3/88
264630	ALL	OO	O	120974	BM	9/4/91
334321	ALL	OO	O	121795	BM	5/6/92
480781	ALL	OO	O	120854	BM	12/2/91
RR	ALL L2	OO	O	C04110	PB	7/6/88
024568	CLL	OO	O	121744	BM	13/5/92
18316	CLL	AO	A	121782	BM	2/6/92
195788	CLL	AO	A	121437	Thyroid	13/11/91
215842	CLL	AO	A	C04084	BM	5/5/88
318179	CLL	BO	B	C04115	BM	16/6/88
415132	CLL	AO	A	CO4052	BM	25/8/93
447214	CLL	BO	B	104325	BM	21/12/88
474631	CLL	AB	AB	121474	BM	29/11/91
154706	CML	AO	A	120598	PB	30/8/90
169032	CML	OO	O	121758	PB	22/5/92
210507	CML	BB	B	C04268	PB	14/11/88
278035	CML	AO	A	120474	PB	29/6/90
299578	CML	OO	O	120974	PB	11/1/91
420701	CML	OO	O	CO4596	PB	19/10/89
428501	CML	OO	O	121705	BM	24/4/92
455201	CML	AO	A	C04455	BM	26/5/89
456001	CML	OO	O	C04472	BM	15/6/89
457096	CML	AO	A	CO4550	BM	11/9/89
457442	CML	AO	A	C04497	BM	13/7/89
465208	CML	OO	O	121469	BM	27/11/91
507817	CML	AA	A	121802	PB	10/6/92
527787	CML	AO	A	-	PB	presentation
DN	CML	AO	O	121413	BM	25/9/91
QMCG	CML	AO	O	-	BM	7/5/90
469521	HCL	AO	A	120663	BM	15/10/90
125332	TP	OO	O	C04523	BM	15/8/89
500395	TP	OO	O	121668	PB	30/3/92
226193	WM	OO	O	120656	BM	10/10/90

Table 2. Comparison of ABO genotype of tumours and blood group of patients.

Patient ID	Sample	Tumour Duke's stage	ABO Genotype	ABO Group
508254	recto-sigmoidal tumour	B	AO	A
Q232171	transverse colon tumour	B	AO	A
223966	ascending colon tumour	B	AA	A
181262	ascending colon tumour	B	AO	A
513154	transverse colon tumour	C	AA	A
196384	ascending colon tumour	C	OO	O
501579	recto-sigmoidal tumour	A	OO	O
084895	ascending colon tumour	C	OO	O
500162	ascending colon tumour	B	AB	AB
316369	transverse colon tumour	B	AA	A
508864	ascending colon tumour	C	BO	B
Q23183	ascending colon tumour	B	AO	A
130623	ascending colon tumour	B	AO	A
505233	ascending colon tumour	D	AO	A
300521	ascending colon tumour	B	BO	B
152896	ascending colon tumour	B	AO	A
084625	recto-sigmoidal tumour	B	OO	O

*Duke's stages are classified according to Carter *et al.*, 1982. A simplified guide to the classification is:

Duke's A - lesion confined to the mucous membrane (non-invasive)
Duke's B - penetration of the muscularis propria
Duke's C - metastases to the lymph nodes
Duke's D - advanced; metastases to liver, lung, bone etc.

APPENDIX B

Table 1: Patients analysed at the ABL locus.

Patient	ID	Diagnosis	Sample Type	Sample Date	Blast Cells (%)
1	068152	RAEB-t → AMLM4 or M5	BM	7/5/90	66
2	490580	MDS-t	BM	4/10/91	>95
3	188346	MDS	PB	22/7/92	95.7#
6	526084	AML M1	PB	6/9/93	72
7	426168	AML M4	BM	16/9/92	50
8	101998	AML M4	BM	23/11/94	95
9	434471	AML M1	BM	30/9/88	95
10	016232	ALL L2	BM	2/6/94	>95
11	410885	ALL L2	BM	25/6/90	100
12	Stv	AML M3	PB	6/94	N/A
13	480781	ALL L2	BM	3/10/90	64
14	447214	CLL	PB	16/8/91	97*
15	226193	WM	BM	30/4/90	98*

The abbreviations used in this table are defined in Appendix A.

The figure given refers to the white cell count of the patient (normal range is 4-10.5).

*The figure given refers to the percentage of lymphocytes in the patient sample.

ERRATA

p.36 Table 1-2: The percentage of uterine/cervix metastases with partial staining should be 7% and the percentage of positively staining primary tumours (total) should read 5%.

p. 172, line 4. The statistical test used is a binomial expansion.

p. 194, line 9. The sentence should read: "Six of the thirteen patients..." (rather than seven).