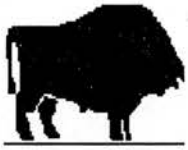

Variations in immune response as determined by MHC class I polymorphism in cattle

by

Lisa Murphy

A thesis submitted for the degree of Doctor of Philosophy

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"The cow is of the bovine ilk, one side is moo, the other is milk" Ogden Nash

Abstract

Major histocompatibility complex (MHC) class I molecules play an essential role in the defence against intracellular pathogens. CD8+ T cells recognise antigenic peptides in association with self-MHC, a process known as MHC restriction. In this study we examined bovine MHC class I genes using both molecular and cellular approaches. There is evidence for the existence of five or six classical class I loci in cattle, with the number of genes expressed varying between haplotypes. Most alleles are putatively assigned to a locus according to their grouping following phylogenetic analysis. Here we applied reference strand mediated conformational analysis (RSCA), a high resolution typing method, to four of these groups: 1, 2, 3 and 6. Using group-specific primers and two reference strands 22 potential new alleles were identified with one probable pseudoallele in group 1. Based on the results obtained and previous phylogenetic analysis, groups 1 and 3 appear to be the most polymorphic.

The need for a reliable typing method for MHC class I genes in cattle can be seen through studies of CD8+ T cell responses to *Theileria parva*. *T. parva* is an intracellular parasite against which CD8+ CTL have been shown to be the principal effector cells in infected cattle. Previous studies identified a clear bias in class I restriction of CTL to either the maternal or paternal haplotype, and showed that haplotypes differ in their ability to restrict this response. We have extended these studies using animals with well-characterised haplotypes. We showed that the A14 and A18 haplotypes appear to be dominant over the A10 and A31 haplotypes respectively. A14 expresses three class I genes, D18.1, D18.4 and D18.5. Assays testing an A14-restricted CTL clone against D18.1 and D18.4 transfected target cells proved inconclusive indicating a need for further investigation and the inclusion of D18.5 in any studies.

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To everyone in Lab F1B, past and present, their friendship and encouragement was a vital part in me finishing this work and finally getting to leave Compton – thanks! To my other friends at the institute (you know who you are!) who were always there for me and supplied much needed chats (and pints!), I owe you a beer!

To Liam thanks for putting up with me over the years despite everything – look I've finally finished! Biology PhDs are definitely harder than physics.....
Hopefully life can start getting a bit more normal now whatever that is!

.

Finally, the biggest thanks go to my parents to whom this thesis is dedicated. Their constant support is fantastic and I couldn't have done it without them.

Declaration

This thesis is submitted for examination for the degree of Doctor of Philosophy to the University of Edinburgh. It has not been submitted to any other university or for any other degree or professional qualification. The work presented here is entirely my own unless where specifically stated in the text and acknowledgements.

Signed .

Date.....6/6/05.....

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Abbreviations

aa	Amino acid
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
β_2m	β_2-microglobulin
BLAST	Basic local alignment search tool
BoLA	Bovine leucocyte antigen
bp	Base pair(s)
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDR	Complementary determining regions
cM	Centi Morgan
CTL	Cytotoxic T lymphocyte
°C	Degrees Celsius
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dNTP	Deoxynucleotide
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulphoxide
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediaminetetra-acetic acid
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
FACS	Fluorescence activated cell sorter
FBS	Foetal bovine serum
FITC	Fluorescein isothyiocyanate
FLR	Fluorescent-labelled reference strand
g	Force of gravity
HA	Heteroduplex analysis
H-2	Murine histocompatibility complex
HLA	Human leucocyte antigen
IEF	Isoelectric focusing
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
ILA	ILRI antibody
IL	Interleukin
IPTG	Isopropyl β-D-thiogalactosidase

kb	Kilobase
KCl	Potassium chloride
kDa	Kilodalton
KH_2PO_4	Potassium phosphate
LB	Luria-Bertani broth
LMP	Low molecular weight protein
L	litre
Mb	Megabases
mAb	Monoclonal antibody
mRNA	Messenger ribose nucleic acid
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
μg	Microgram
μl	Microlitre
mg	Milligram
ml	Millilitre
mM	Millimolar
M	Molar
MW	Molecular weight
NaCl	Sodium Chloride
Na_2PO_4	Sodium phosphate
NaOH	Sodium hydroxide
NK	Natural killer cell
nt	Nucleotide
PAGE	Polyacrylamide gel electrophoresis
PBM	Peripheral blood mononuclear cells
PBR	Peptide binding region
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-SSP	PCR-sequence specific primers
PCR-SSOP	PCR-sequence specific oligonucleotide probes
p.i.	Post infection
RBC	Red blood cell
RNA	Ribose nucleic acid
RSCA	Reference strand mediated conformational analysis
SSCP	Single-stranded conformational analysis
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate EDTA
TAP	Transporter associated with antigen processing
TBE	Tris-borate EDTA
TCR	T cell receptor
TE	Tris-EDTA
T. parva	*Theileria parva*
TM	Transmembrane
Tris	2-amino-2-(hyromethyl)propane-1,3 diol

µl	Microlitre
UTR	Untranslated region
UV	Ultraviolet
V	Voltage
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactosidase

Bases

A	Adenine
G	Guanine
C	Cytosine
U	Uracil
T	Thymine

Amino acids

A	alanine	**G**	glycine	**M**	methionine	**S**	serine
C	cysteine	**H**	histidine	**N**	asparagine	**P**	proline
T	threonine	**D**	aspartic acid	**I**	isoleucine	**V**	valine
E	glutamic acid	**K**	lysine	**Q**	glutamine	**W**	tryptophan
F	phenylalanine	**L**	leucine	**R**	arginine	**Y**	tyrosine

Chapter 1

General Introduction

1.1. The Major Histocompatibility Complex

1.1.1 Identification of the MHC

The MHC can be defined as a genetic region containing tightly linked genes involved functionally with both the adaptive and innate immune systems. It was first discovered by Gorer (1936) then further characterised by Snell (1958) as a set of polymorphic genes encoding molecules involved in the rejection of foreign transplanted tissue in mice. Snell proposed the name based on the evidence that this system was the most important genetic determinant for the outcome of transplants between individuals. Evidence for MHC in humans was provided by Dausset *et al.* (1958) who found that blood from multiparous women (immunised by paternal antigens on foetal cells) produced antibodies that agglutinated lymphocytes.

A more precise function of the MHC came through the work of Zinkernagel and Doherty (1974). They showed that T cells are restricted by the MHC, with CD8+ cytotoxic T cells (CTL) only recognising antigens associated with self-histocompatibility molecules. This led to the now well-established role of MHC molecules in presenting both self and non-self antigens to T cells.

MHC genes have been found in all jawed vertebrates including the oldest group, cartilaginous fish, but not beyond this (Flajnik and Kasahara, 2001). Instead, lower groups such as the cyclostomes, including the hagfish and lamprey, are found to have allorecognition loci that are not orthologous to classical MHC genes.

1.1.2 MHC – genetic location and organisation

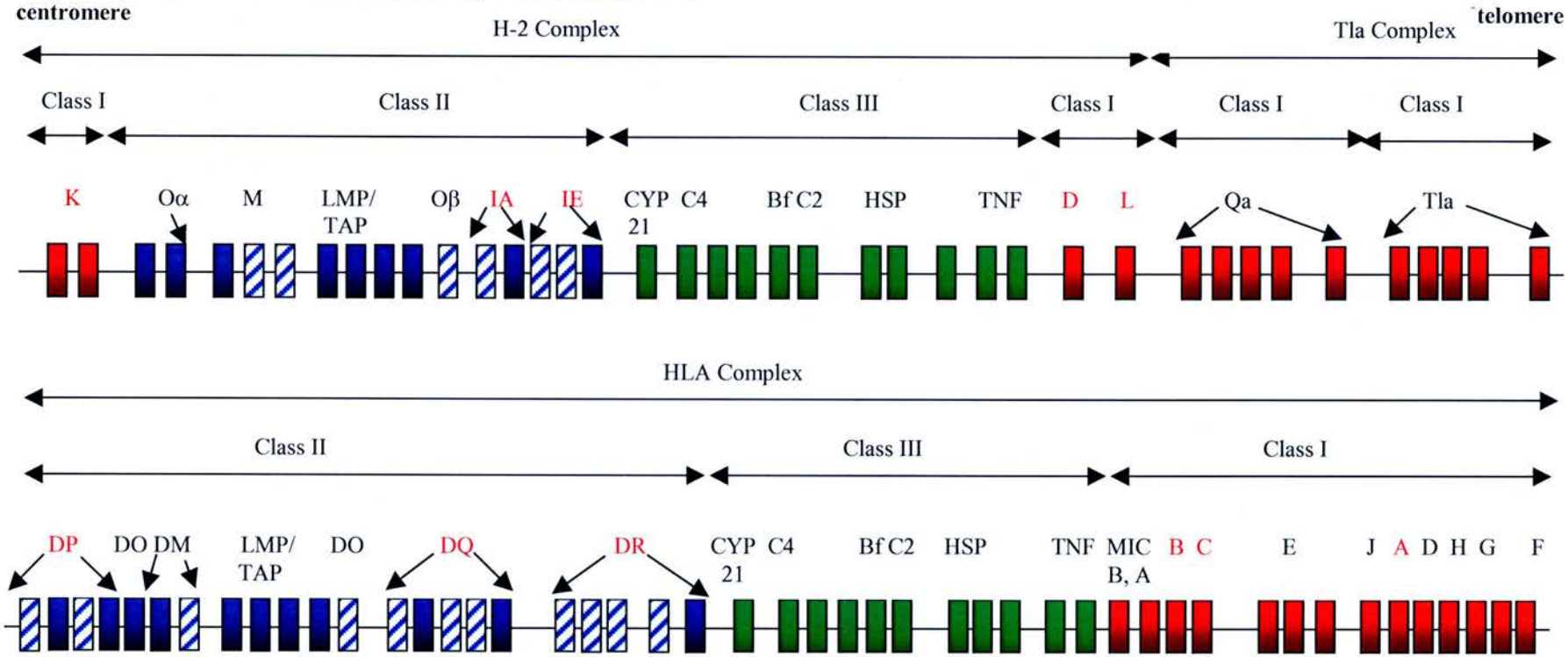
The MHC region in humans and mice has been extensively studied with mapping data establishing the basic organisation of both. The human MHC, or Human Leukocyte Antigen (HLA), is found on the short arm of chromosome 6 (band 6p21.3) while the mouse MHC, also known as the H-2 complex has been mapped to chromosome 17. Both regions have a centromere to telomere orientation, and can be divided into three gene clusters, classes I, II and III. Their organisation is shown in fig. 1.1. The major difference observed between the two species is the presence of MHC class I genes (H-2K) centromeric the class II region in mice. The genetic linkage between the class I and II regions observed in humans and mice has also been seen in all other species studied to date including chickens and sharks. Bony fish appear to be the exception to this (reviewed by Kumanovics *et al.*, 2003).

Sequencing of the HLA was completed in 1999 and provided a large amount of information regarding the gene content of this region, including pseudogenes and gene fragments. Within the 3.6Mb stretch are at least 224 genes, including 128 that are believed to be expressed (MHC sequencing consortium, 1999). Approximately 40% of these expressed genes are thought to have an immunological function (Trowsdale, 2001).

Both the class I and II regions contain genes which code for functional MHC molecules while the class III region contains genes which code for various components of the immune system. Six classical genes are found in humans, three in

Figure 1.1 Comparative map of the murine (chromosome 17) and human (chromosome 6) MHC regions

This diagram shows the MHC genes present in the MHC of humans and mice, both classical (red) and non-classical (black). Class I loci are shown in red, class II in blue and class III in green. Genes encoding α strands are shown as filled while those encoding β strands are striped. (Adapted from MHC sequencing consortium, 1999)



the class I region, A. B and C and three in the class II region DP, DQ and DR. Mice also have three in their class I regions H-2K, D and L, but two class II genes, IE and IA. Their DP equivalents are pseudogenes. Class I genes are single genes encoding the α chain of class I molecules. Class II genes generally exist in pairs, encoding either the α or β chain of the class II heterodimeric molecule although more than one gene can encode for each chain, for example there are two DPβ genes in humans. The number of class I and II genes is variable amongst all species studied to date as a result of species or order-specific amplification of genes.

These MHC genes are marked by their extensive polymorphism, the most observed in higher vertebrates (Parham *et al.*, 1988). The type of MHC expressed is dependent on the haplotype i.e. the combination of alleles on each strand of DNA (two haplotypes making up the genotype).

1.2 Function and structure of class I molecules

1.2.1 Function of class I molecules

MHC class I molecules act to present endogenously derived peptides from, for example, viruses or intracellular parasites to CD8+ T cells thereby indicating that something is wrong within the cell. They are constitutively expressed on the majority of cells in the body, since all cells are susceptible to viral invasion, which allows constant surveillance for infection. Upon recognition of the foreign peptide by the binding of the T cell receptor (TCR) to the peptide-MHC complex, CD8+ T

cells are stimulated to expand clonally and differentiate to CTL which then trigger apoptosis of the target cell. Apoptosis is the preferred method of killing since this prevents pathogen replication and release.

Two main mechanisms of apoptosis are used, the granule secretion pathway and Fas-mediated apoptosis. During the differentiation from CD8+ T cells to CTL a large number of modified lysosomes known as lytic granules are formed inside the cell. Upon interaction between the CTL and target cell these granules are secreted and delivered onto the target cell surface. Perforin is released from the granules which produces transmembrane pores through which granzymes can enter (Young *et al.*, 1986). Granzymes are serine proteases which act to cleave cell proteins which in turn activate nucleases and other enzymes thereby inducing apoptosis within the target. In addition binding of the Fas ligand on CTL to the target causes transmission of signals which activate death caspases (Metkar *et al.*, 2002).

1.2.2 Basic structure of class I molecules

Investigations by Bjorkman *et al.* (1987a) led to the first elucidation of the crystal structure of a class I molecule, HLA-A2. It was found to consist of a 45kDa heavy (α) chain encoded by the MHC genes, non-covalently complexed to a small 12kDa protein β-2-microglobulin (β2m), encoded by a gene on chromosome 15 (chromosome 2 in mice, Goodfellow *et al.*, 1975, Smith *et al.*, 1975). The heavy chain has three extracellular domains α1, α2 and α3 (each approximately 90 amino acids, aa, long), a short transmembrane region of 35 to 40 aa and a cytoplasmic tail

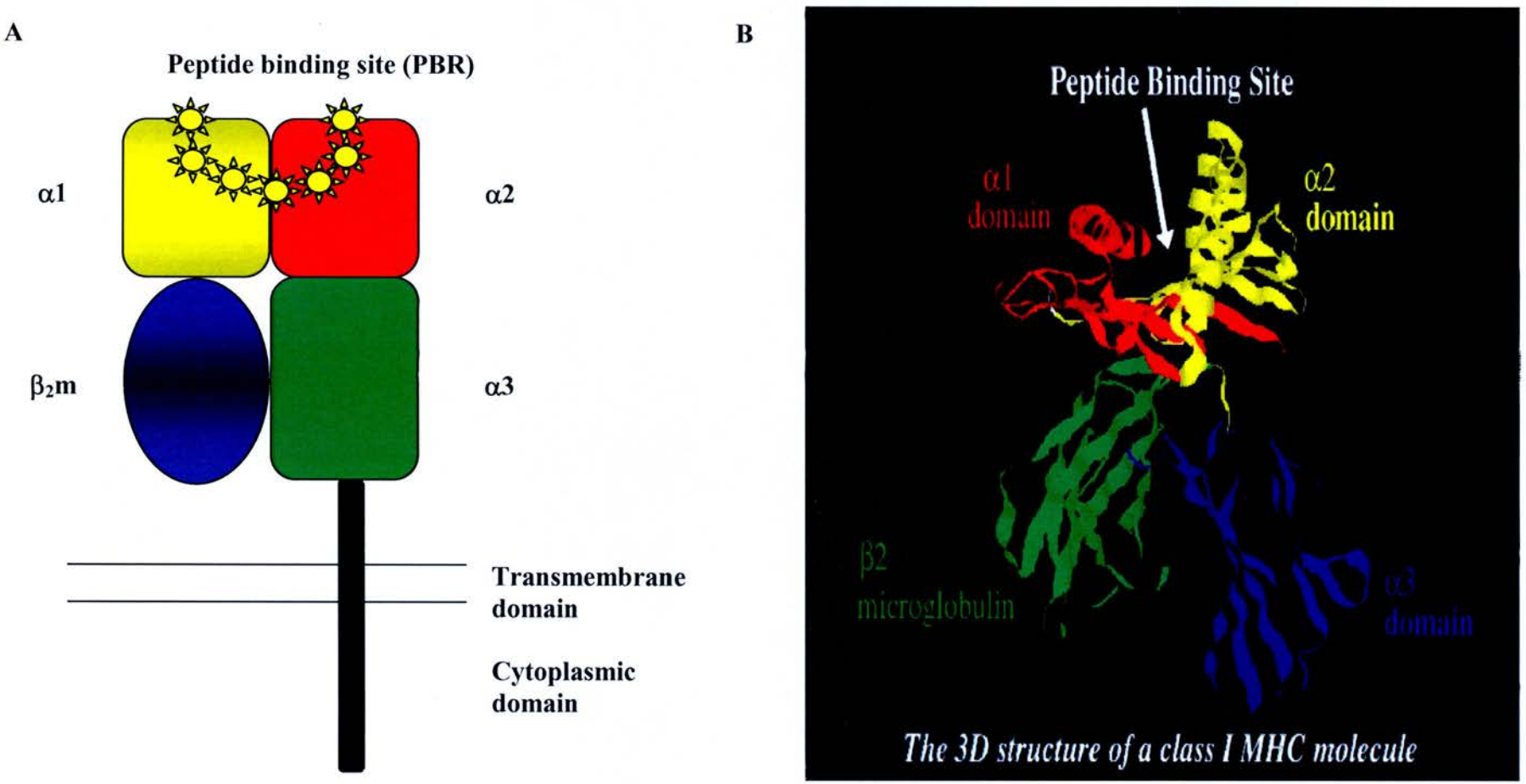
of approximately 30 aa. The α1 and α2 domains, supported by the α3 domain and β2m, are furthest from the cell surface and are folded very similarly, both having a 4-strand β sheet with an α helix at the top. The two domains interact to form the peptide binding region (PBR), a cleft with 8 anti-parallel β strands supporting two anti-parallel α helices (see section 1.2.3). The α3 domain and β2m both consist of two antiparallel β sheets connected by a disulphide bond, a structure previously seen in the constant regions of immunoglobulins (Bjorkman *et al.*, 1987a). Although not involved in peptide binding, α3 is important for attachment of CD8, a co-receptor on CTL, helping to strengthen and prolong contact between the cells (Salter *et al.*, 1989, see fig. 1.2).

1.2.3 Structure of the peptide binding region

The cleft formed between the α1 and α2 domains was found to contain 'extra' electron density in the crystal structure, which could not be accounted for from the class I sequence. Bjorkman *et al.* (1987b) suggested this to be the site of peptide binding because of its position at the top of the molecule and also because the polymorphisms of the MHC molecules that determine TCR recognition are focused here. Later work by Madden *et al.* (1991) allowed further speculation on this theory by using X-ray crystallography to examine the MHC molecule HLA-B27 which exhibited clear, interpretable electron density. They showed that the bound nonamer peptide would be restricted at various positions by interactions between side chains of the peptide and pockets in the cleft, and also that some kinking of the backbone

Figure 1.2: Structure of MHC class I molecules

Figure 1.2a shows a schematic diagram of the structure of a class I molecule. A ribbon diagram showing the three dimensional structure is shown in figure 1.2b. The peptide-binding site is also indicated. (Figure 1.2b was taken from **www-immuno.path.cam.ac.uk)**



could be accommodated. In addition, conserved atoms at either end of the cleft would bind the terminal ends of the peptide.

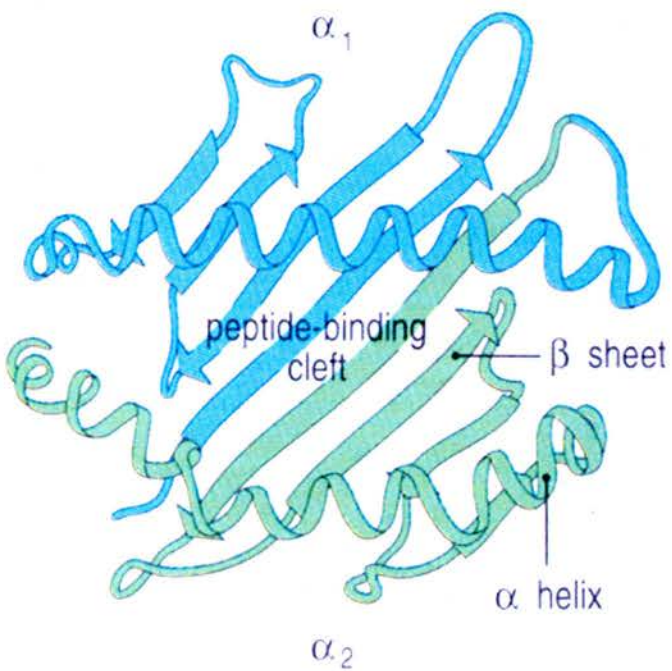
Specificity for sequence motifs in peptides bound to class I molecules is determined by pockets in the cleft formed by the polymorphic residues of the MHC protein (fig. 1.3). The binding region has 6 pockets, A to F, with A, B, C and F being deep while D and E are shallow (Saper *et al.*, 1991, Matsumura *et al.*, 1992). Differences in the sequence of the $\alpha 1$ and $\alpha 2$ domains cause a change in the shape and charge of these pockets which determines the peptides that can bind. Pocket A, at the left boundary of the cleft, binds the amino terminal of the peptide, with its specificity determined by four conserved tyrosine residues (positions 7, 59, 159 and 171). Pocket F binds the carboxyl terminal with hydrogen bonds formed between the COO- group and tyrosine, threonine, lysine and tryptophan residues (positions 84, 143, 146 and 147 respectively). The strong linkage in these pockets affects binding by determining the position and orientation of the peptide and also restricts the length of the peptide bound. Pocket B has been found to bind the second residue of peptides in all human MHC structures so far known (Smith *et al.*, 1996b). The remaining pockets interact with peptide side chains, their importance varying depending on the allele (Jardetzky, 1996).

Generally octamers or nonamers bind to class I molecules but some variation can occur with the peptide bulging to accommodate the extra residues. A combination of flexibility of the polymorphic residues at the bottom of the cleft and water molecules underneath the peptide allows for sequence diversity in the middle of the peptide,

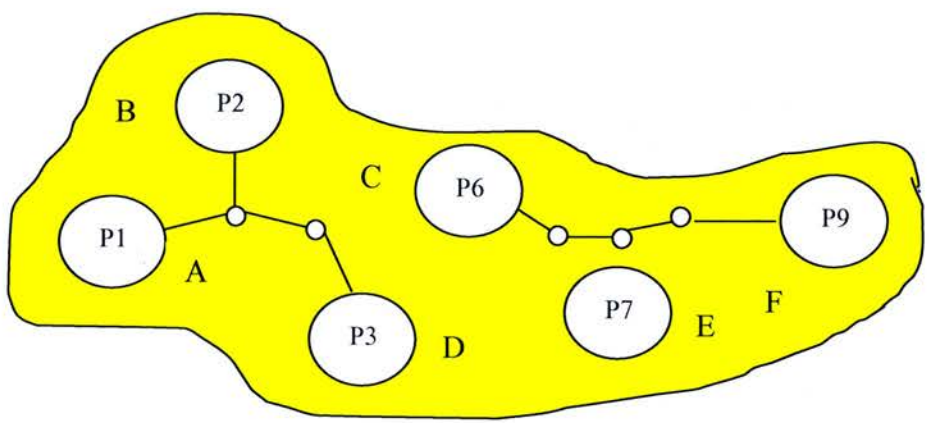
Figure 1.3: Structure of the peptide binding region of MHC class I molecules

Figure 1.3a shows the PBR viewed from above. Six pockets are formed in the bottom of this into which the side chains of bound peptides fit. A schematic diagram showing how a nonamer can fit into the groove is shown in figure 1.3b. P1, 2, 3, 6, 7, 9 represent the side chains of the amino acids at the numbered positions of the peptide. Longer peptides can be accommodated by the backbone of the peptide bulging out of the groove (adapted from Matsumura *et al.*, 1992).

A



B



with the water molecules helping peptides to fit into the binding groove better. They can also compensate for octamers allowing them to bind more efficiently (Smith *et al.*, 1996a,b).

Comparison of peptides eluted from MHC molecules identified conserved residues amongst those from the same molecule (Falk *et al.*, 1991). These anchor residues which represent the allele-specific peptide motif are generally invariant or change to closely related residues (Matsumura *et al.*, 1992). Most alleles appear to have at least two, usually at the second or fifth residue and the last residue of the peptide (Rammensee *et al.*, 1993). For example, in most human peptide binding motifs known position two is an anchor residue with three main specificities, arginine, proline and aliphatic residues (Rammensee *et al.*, 1995, Smith *et al.*, 1996a). It has been speculated that those aa which point up from the PBR may be important for TCR recognition and binding (Falk *et al.*, 1991).

1.2.4 β-2-microglobulin

β2m is extremely important since the heavy chain needs association with it to enable the protein to assemble correctly and be transported to the cell surface (Degen *et al.*, 1992, Sugita and Brenner, 1994). Alteration of this protein can lead to a decrease in expression as shown by Tatake *et al.* (1992) who combined human MHC molecules with mouse β2m in a β2m-null cell line and found only 20-30% expression despite human and mouse β2m having approximately 70% homology. Altering the amino terminal of the protein showed that this region contained the major class I regulatory

elements. Interestingly this region also includes the major points of contact between the β2m and the heavy chain suggesting that the problems with expression may be due to how the whole MHC molecule is assembling (Trymbulak and Zeff, 1997).

Alteration of β2m can also lead to a reduced repertoire of peptides that can be presented. Changing a residue at the interface between β2m and α2 from alanine to aspartic acid was shown to affect binding of some peptides, resulting in decreased recognition by specific CTL (Perarnau *et al.*, 1990).

While β2m is not polymorphic in humans alleles have been identified in mice and cattle, however these generally contain substitutions with similar amino acids or are non-coding (Hermel *et al.*, 1993, Ellis *et al.*, 1995).

1.2.5 Glycosylation and phosphorylation

Both human and murine MHC molecules have a glycosylation site at residue 86 (asparagine, ASN), which lies in a loop between α1 and α2. This residue has a Man9GlcNAcGlc sugar attached which is important for assembly of class I molecules, peptide loading and cell surface expression through its association with chaperone proteins (discussed in section 1.4, reviewed in Rudd *et al.*, 2001). Some studies however have shown it not to be essential. (Ploegh *et al.*, 1981, Santos-Aguado *et al.*, 1987). Mice have a further glycosylation site at Asn176 (and Asn 256 in certain alleles, Kimball and Coligan, 1983). The Asn 176 in α2 is required for binding to the NK receptors Ly49A and C. Since this site is only found in mice it

indicates that Ly49 has evolved simultaneously with murine class I genes (Lian *et al.*, 1998).

Comparison of human and murine cytoplasmic domains identified a conserved Ser-Asp/Glu-X-Ser(P)-Leu motif which contains conserved serine residues at position 332 and 335. Serine 335 has been shown to be the site of phosphorylation *in vivo* in both species (Guild and Strominger, 1984). Phosphorylation of this serine residue is thought to be important for the modulation of class I molecule movement from the endoplasmic reticulum (ER) to the cell surface via the Golgi and recycling at the cell surface via endosomes (Pitcher *et al.*, 1999). Phosphorylated class I molecules are found on the cell surface, late intracellular compartments and recycling endosomes, with phospohrylation thought to occur outside the golgi (Capps and Zuniga, 2000, Lippe *et al.*, 1991). Some viruses have adapted mechanisms to interfere with the expression of class I molecules. For example, adenoviruses produce a protein E3/19K which binds to class I molecules in the rough ER and prevents transportation to the cell surface by stopping phosphorylation (Lippe *et al.*, 1991).

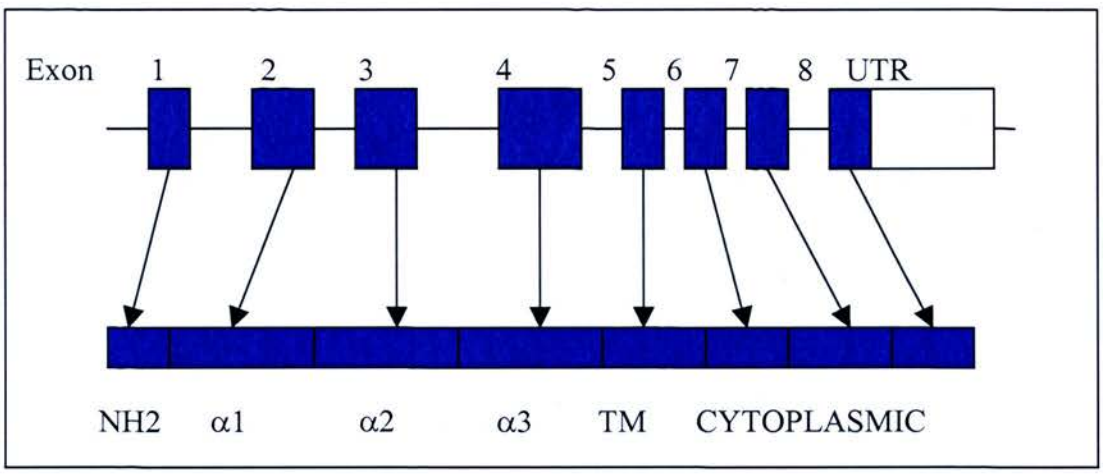
1.2.6 Exon arrangement of MHC class I genes

Exon arrangement of the class I genes is well conserved amongst the jawed vertebrates studied. Class I genes contain eight exons and a 3' untranslated region (fig. 1.4). Exon one encodes the leader peptide of approximately 30 nucleotides. This is involved in directing the construct to the ER and is also important for providing peptides for the non-classical MHC molecule HLA-E to present and

helping it to reach the cell surface. Exons two, three and four code for the α1, α2 and α3 domains respectively. Exon five corresponds to the transmembrane domain that anchors the MHC molecule to the cell surface. The cytoplasmic region is generally encoded by exons 6, 7 and or 8 although complete excision of exon 7 has been observed in H-2D (Fahrner *et al.*, 1987).

Figure 1.4: Intron/Exon arrangement of MHC class I genes

Figure 1.4 shows a schematic diagram of the arrangement of introns and exons in a MHC class I gene and the regions of the molecule that the exons code for.



1.3 Structure and function of class II molecules

MHC class II molecules act to present exogenously derived peptides to T cells expressing the CD4+ co-receptor (T helper cells). These peptides originate from pathogens ingested by phagocytic and B cells or residing in macrophage vesicles. Two types of CD4+ cells exist, Th1 and Th2. Th1 cells act to produce cytokines including IL-2 which activates macrophages allowing them to take up pathogens by phagocytosis and destroy them. In contrast Th2 cells secrete IL-4 which encourages the maturation and differentiation of B cells which then produce specific antibodies.

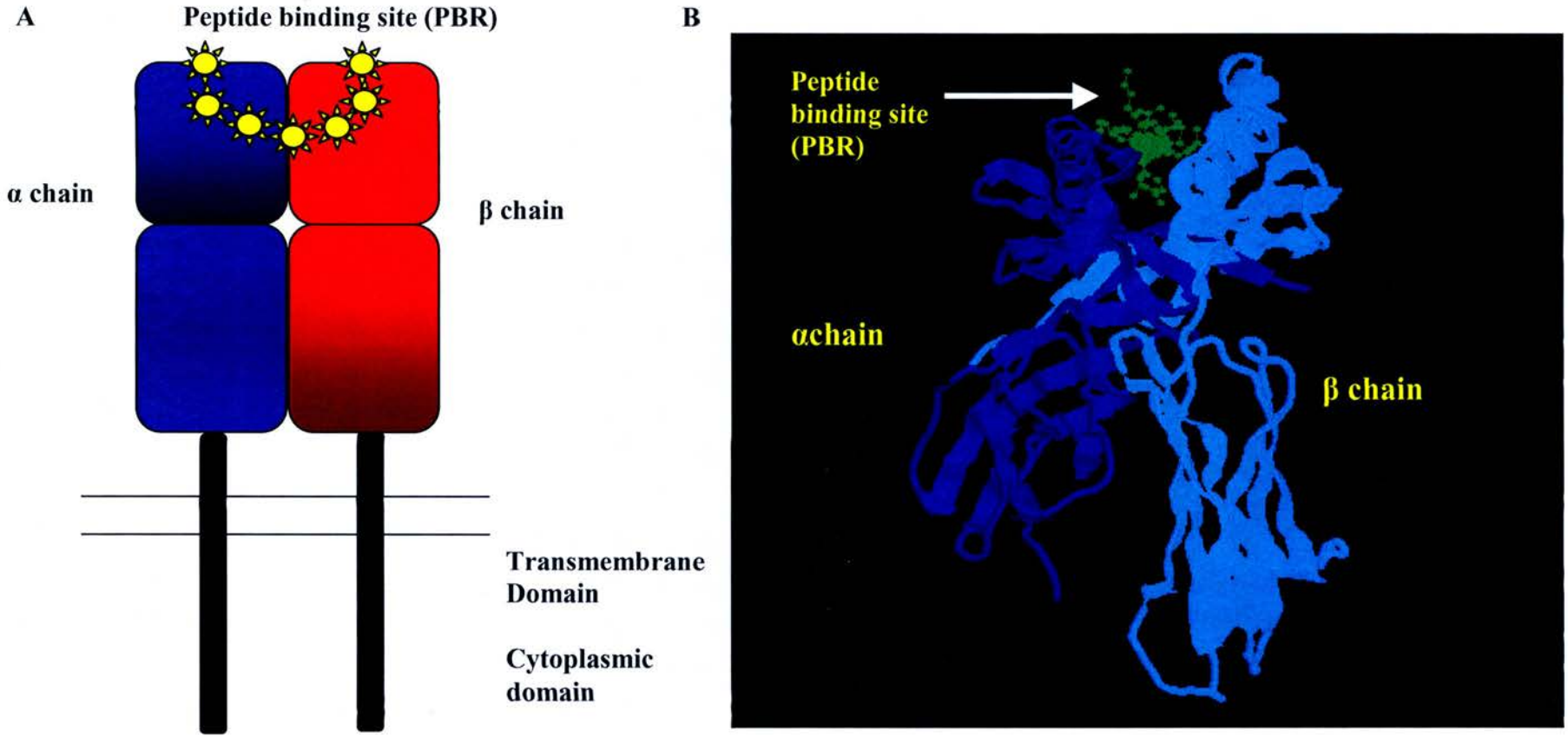
Class II expression is consistent but limited to professional antigen presenting cells including macrophages, dendritic cells and B cells and the thymic epithelium but can be induced on other cell types by cytokines, including interferon-γ.

MHC class II molecules have a similar overall structure to class I molecules despite relatively low sequence homology (fig. 1.5, Brown *et al.*, 1988). The α and β genes in the class II region code for α and β chains which join by disulphide bonds to form heterodimers. The α chain has a molecular weight of 30-34kDa while the β chain ranges from 26-29kDa. Each chain contains two extracellular domains, $\alpha 1$ and $\alpha 2$, $\beta 1$ and $\beta 2$ anchored by a transmembrane and cytoplasmic region. The two membrane proximal domains, $\alpha 2$ and $\beta 2$, have immunoglobulin like structures similar to $\alpha 3$ and $\beta 2m$, while $\alpha 1$ and $\beta 1$ are similar to the $\alpha 1$ and $\alpha 2$ domains of a class I molecule. $\alpha 1$ and $\beta 1$ interact to form a super domain which is extremely similar to the PBR of a class I molecule having a floor of eight antiparallel β sheets with an alpha helix contributed by each chain as walls (Brown *et al.*, 1993, Stern and Wiley, 1994).

Class II molecules can bind longer peptides than class I since the residues involved in binding the ends of peptides in class I molecules are absent here, so no binding of the terminal ends takes place. Instead peptides are bound along their length in the middle of the cleft by conserved residues and allowed to extend out at either end. Like class I molecules these structures also have pockets in the binding region which side chains of the peptide extend into. These pockets are lined with polymorphic residues which determine the sequence of which peptides can bind (Jardetzky, 1996).

Figure 1.5: Structure of MHC class II molecules

The structure of MHC class II molecule is shown in schematic form in figure 1.5a. A ribbon diagram showing the three dimensional structure is shown in fig. 1.5b. The peptide-binding site is also indicated.
(Figure 1.5 b was taken from **http://webmed.unipv.it/immunology/mhcstr.html**)



1.4 MHC class I – antigen processing and presentation pathway

The production of mature MHC class I molecules presenting peptides can be divided into three steps; degradation of protein to produces antigenic peptides, transportation of peptides into the ER lumen, and assembly of heavy chain with β2m and peptide to form stable molecules (fig. 1.6).

1.4.1 Peptide production and the proteasome

The proteasome is a large endo-peptidase found in the cytosol, which has broad proteolytic activity and is the key enzyme for intracellular protein degradation (Goldberg and Rock, 1992). It has a cylindrical structure consisting of four stacked rings each containing seven subunits, with the proteolytic sites buried on the inside of the structure preventing indiscriminate degradation of cytosolic proteins (fig. 1.7). Three of the subunits δ, Z and X found in the inner rings have protease activity. Upon IFNγ stimulation of cells these subunits are replaced by low molecular mass polypeptide 2 (LMP2), LMP7 and multicatalytic endopeptidase complex like-1 (MECL-1) forming an immunoproteasome (Griffin *et al.*, 1998).

The role of the proteasome in antigen processing has been suggested by the fact that the genes encoding LMP2 and 7 are found in the MHC class II region adjacent to the transporter associated with antigen processing genes, TAP1 and TAP2 (Glynne *et al.*, 1991, Martinez and Monaco, 1991, Kelly *et al.*, 1994). In addition, both human and mouse knock-out cells deficient in either or both LMP 2 and 7 were found to have

Figure 1.6: MHC class I antigen processing and presentation pathway

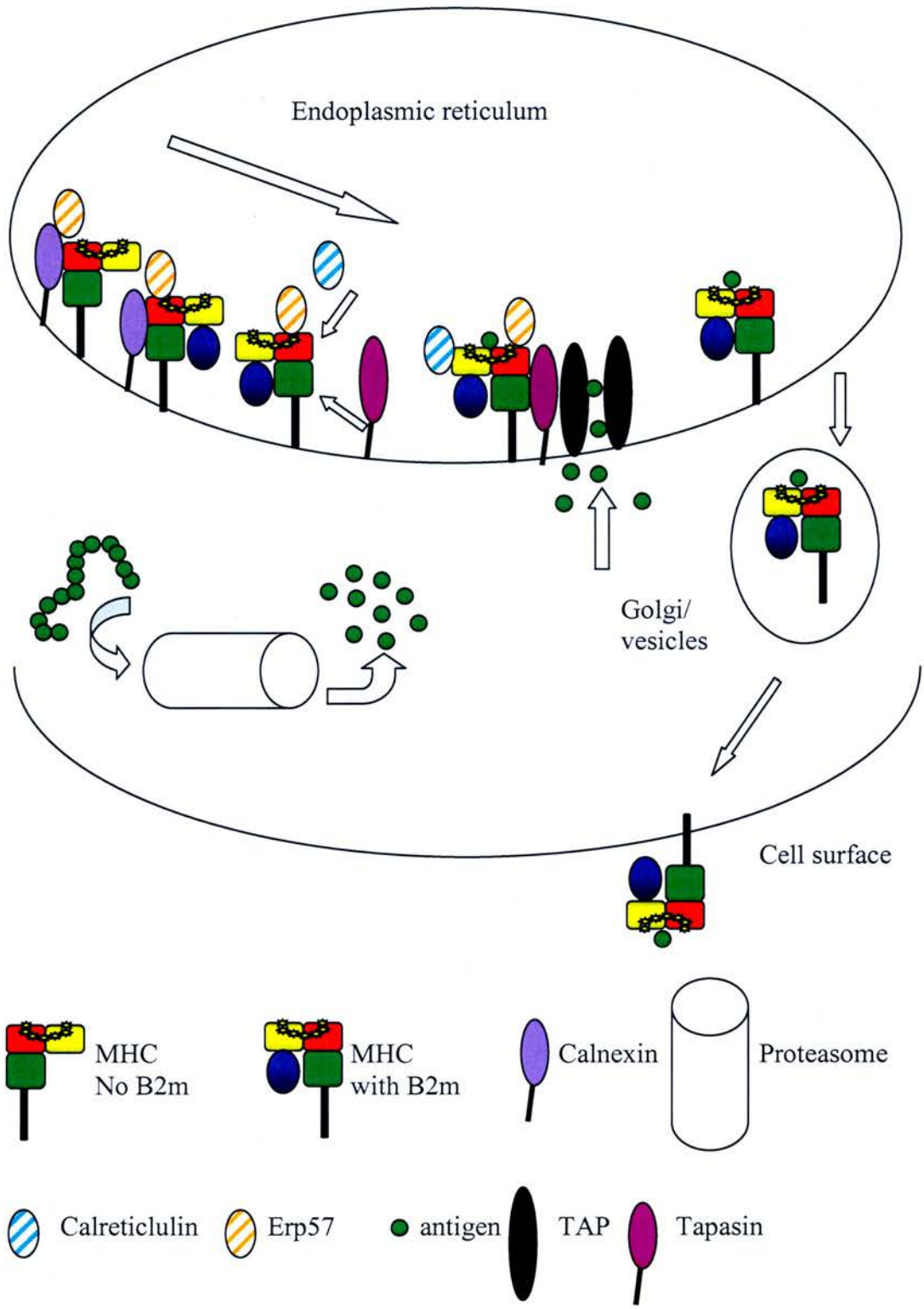
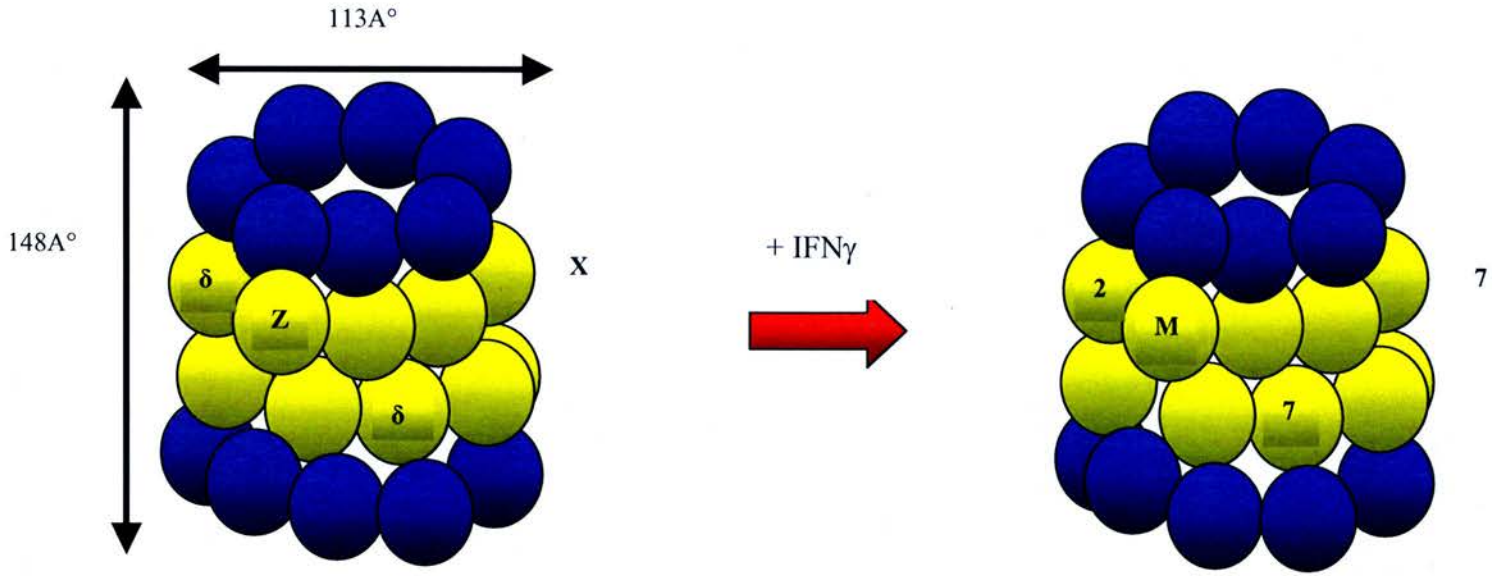


Figure 1.7: Structure of the proteasome and immunoproteasome

The house-keeping proteasome consists of four rings, each with seven subunits. Upon IFNγ stimulation immunoproteasomes are expressed. These differ from house-keeping proteasome in that they have the subunits LMP2, LMP7 and MECL-7 (M) instead of X, δ and Z in their inner rings.



impaired antigen presentation (Fehling *et al.*, 1994, van Kaer *et al.*, 1994, Cerundolo *et al.*, 1995). Despite these observations it is possible for human cells to compensate for loss of proteasome function as seen in EL-4 cells which can develop resistance to proteasome inhibitors (such as lactacystin) by overexpression of an alternative proteolytic molecule (Glas *et al.*, 1998).

Differences in the cleavage patterns are observed between normal and immunoproteasomes, with immunoproteasomes having higher activity in cleavage of peptides with hydrophobic and positively charged carboxyl terminals which are ideal for presentation by class I molecules (Driscoll *et al.*, 1993). As a result LMP2 and 7 may act to increase the availability of specific groups of peptides for class I binding.

1.4.2 Peptide transport into the ER

The peptides produced by proteasome are released into the cytosol and pass into the lumen of the ER via the TAP complex. This complex consists of TAP1 and TAP2 subunits, which interact to form a heterodimer. They are very similar in structure both lying in a head-head/tail-tail orientation with three domains, heterodimeric peptide binding domain facing onto the cytosol, heterodimeric pore domain through which the peptide travels to enter the ER and a nucleotide binding domain on each (Vos *et al.*, 1999, 2000). These molecules were found to be part of a superfamily of transporter proteins known as the ATP binding cassette or ABC family, which rely on ATP binding to the nucleotide binding domains to function. Binding of ATP to TAP2 induces a (probably considerable) conformational change in the complex

allowing ATP to bind to TAP1 and the pore to open (Knittler, 1999). Hydrolysis of ATP at TAP2 also powers peptide transfer (Williams, Au Peh and Elliott, 2002). The TAP complex is vital for MHC class I function since TAP deficient cells do not express class I molecules on their surface. This phenotype can be rescued by addition of functional genes (Spies and DeMars, 1991).

Through a combination of peptide libraries, competition binding assays and assays using peptides with an N-glycosylation site at one end and a tyrosine residue for radio-iodination at the other it was possible to show that TAP is selective in the peptides it transports preferring to transport 8-16 amino acid peptides, with 9 to 12mers transported with highest efficiency (van Endert *et al.*, 1994, Koopman *et al.*, 1996). When considering that proteasomes also preferentially produce peptides of approximately this length it shows that selection is occurring at a number of stages in the antigen processing and presentation pathway to ensure provision of peptides of optimal length for binding to MHC class I molecules.

1.4.3 Assembly and peptide loading of MHC class I molecules

In the ER newly produced heavy chain is initially found associated with two chaperone proteins, calnexin (a 64.5kDa phosphorylated transmembrane protein) and Erp57. These molecules interact to aid in folding of the heavy chain, with Erp57, a thiol-dependent oxido-reductase encouraging disulphide bond formation between $\alpha 2$ and $\alpha 3$ (Farmery *et al.*, 2000). This then allows the heavy chain to associate with $\beta 2m$ followed by substitution of calnexin with another chaperone protein, calreticulin

(Sadasivan *et al.*, 1996). As with calnexin, calreticulin also helps Erp57 in completing the folding of the MHC molecule. Calreticulin is also though to have a role in retention of empty MHC molecules within the ER as shown by a mutant HLA-A2 molecule which had lost its ability to associate with calreticulin but is capable of leaving the ER without a bound peptide (Lewis and Elliott, 1998). This calreticulin/Erp57/class I complex then associates with tapasin.

Tapasin is a 48kDa type 1 transmembrane protein with an ER retention signal, coded for by a gene resident in the MHC class II region in humans (Grandea and Van Kaer, 2001). It acts to bridge class I/β2m complexes to TAP by having a heavy chain binding site in its N-terminal region and a TAP binding site in its C-terminal region (Bangia *et al.*, 1999). Tapasin is also thought to increase the amount of, and encourage peptide binding to TAP (Sadasivan *et al.*, 1996, Ortmann *et al.*, 1997). Tapasin has been shown to be essential for class I processing through investigation of a defective cell line 721.220 which has a functional TAP complex yet expresses only a small amount of MHC class I. When soluble tapasin was added this allowed reconstitution of normal class I assembly and normalised antigen presentation (Grandea *et al.*, 1995, Lehner *et al.*, 1998).

Binding to TAP ensures that the MHC complex is in a region high in concentration of specific peptides to ensure peptide loading. However there is some evidence to show that binding to TAP is not an essential event. Analysis of interactions of various MHC molecules with TAP showed that while the majority of HLA-A and C molecules studied bound to TAP effectively HLA-B molecules were more variable,

with a large proportion binding very ineffectively or not at all (Neisig *et al.*, 1996). The authors suggested that by having two sets of MHC molecules, some associated to TAP and others free in the ER, this mechanism would ensure that the majority of peptides were able to bind to a class I molecule as quickly as possible.

Once the MHC class I molecule has bound a specific peptide it dissociates from TAP and enters the Golgi secretory pathway whereupon it is transported to the cell surface for expression. A lack of specific peptides within the ER can result in retention of the molecule and continued association with the TAP complex (Neisig *et al.*, 1998, Knittler *et al.*, 1998).

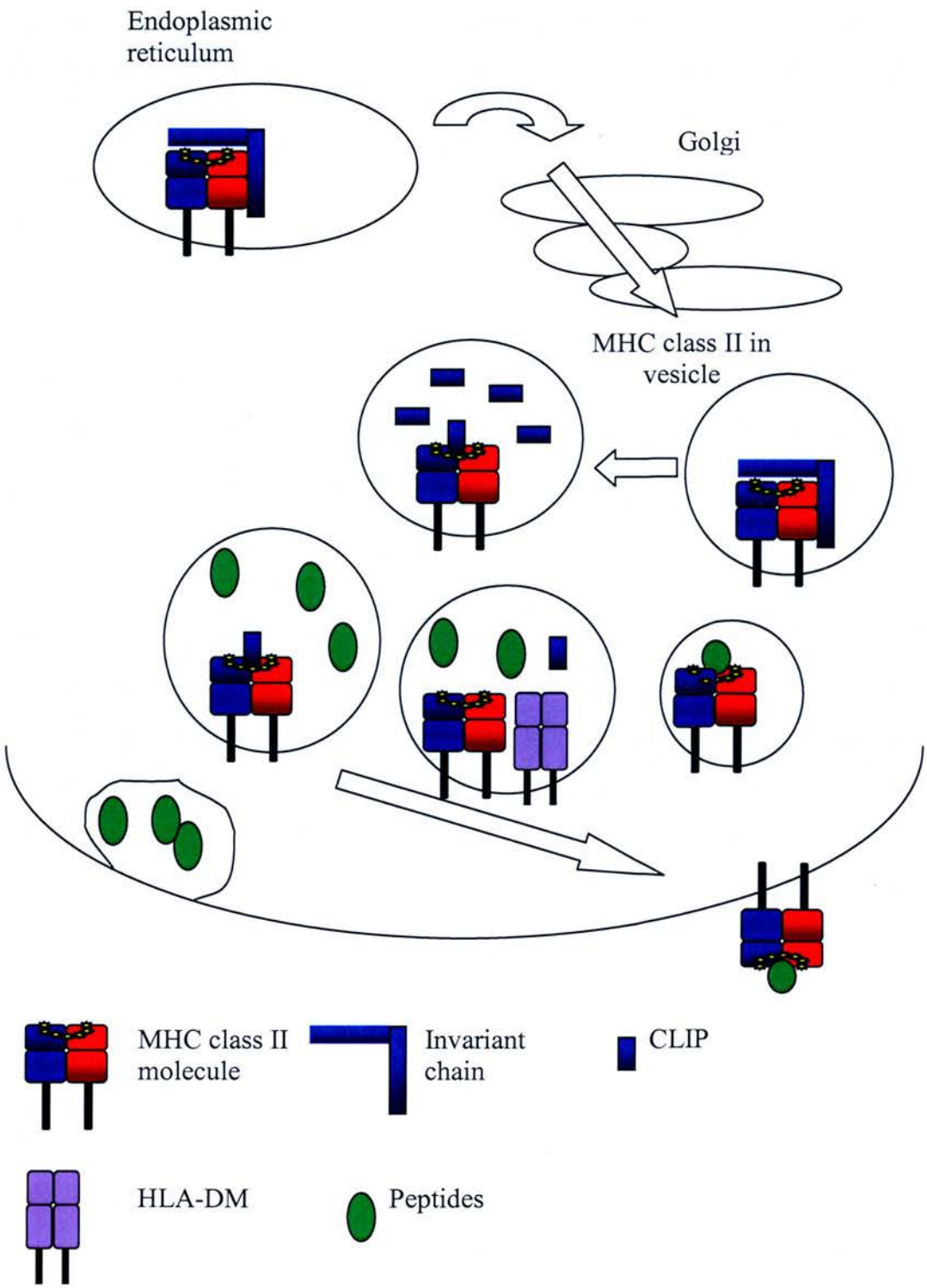
1.5 MHC class II – antigen processing and presentation pathway

The majority of peptides presented by MHC class II molecules are derived from external antigens that have been taken up by the cell by receptor mediated endocytosis or phagocytosis and broken down along the endocytic pathway by endocytic proteases. MHC class II molecules are loaded with these peptides within endosomal compartments (fig 1.8, Unanue, 1992).

Following translation the class II heavy chains are translocated into the ER where they bind to an invariant chain molecule (Ii). This is a transmembrane glycoprotein whose expression is generally limited to antigen presenting cells (Reber *et al.*, 2002). A portion of this is inserted into the peptide-binding region, which allows completion of class II folding. The Ii also acts to prevent peptides from binding to class II

Figure 1.8: MHC class II antigen processing and presentation pathway

MHC class II molecules are assembled in the ER along with an invariant chain then moved into the endocytic pathway where the invariant chain is broken down to CLIP which blocks the PBR. In a vesicle HLA-DM acts to remove CLIP and facilitates loading of the class II molecule with exogenously derived peptides.



molecules in the ER and provides signals targeting the class II-Ii complex into the secretory pathway and then into an endosome (Teyton *et al.*, 1990, Lamb *et al.*, 1991).

Endosomes are membrane-bound vesicles formed by invaginations of the cell surface, which contain extracellular and surface-bound molecules such as bacteria. Following internalisation these endosomes become part of the vesicle system which transports materials to and from the cell surface. The interior of endosomes increases with acidity as they move through the cytosol and accumulate proteases that act to break down the endosome contents (Blum and Cresswell, 1988). In the endosome the invariant chain is degraded by proteases to a CLIP peptide (class II-associated invariant chain peptide). DM, a nonclassical class II molecule, is believed to catalyse the dissociation of CLIP from class II molecules leaving the PBR free to bind peptides within the endosome. DM also acts to stabilise the MHC molecule following CLIP release and is believed to help in the removal of peptides that do not fit well into the pocket, ensuring that only peptides with high affinity are bound (van Ham *et al.*, 1996). Another MHC class II non-classical molecule, DO, modulates the peptide loading by DM. Cell surface expression of the MHC-peptide complex occurs when the endosome fuses with the cell membrane.

1.6 TCR recognition of MHC molecules

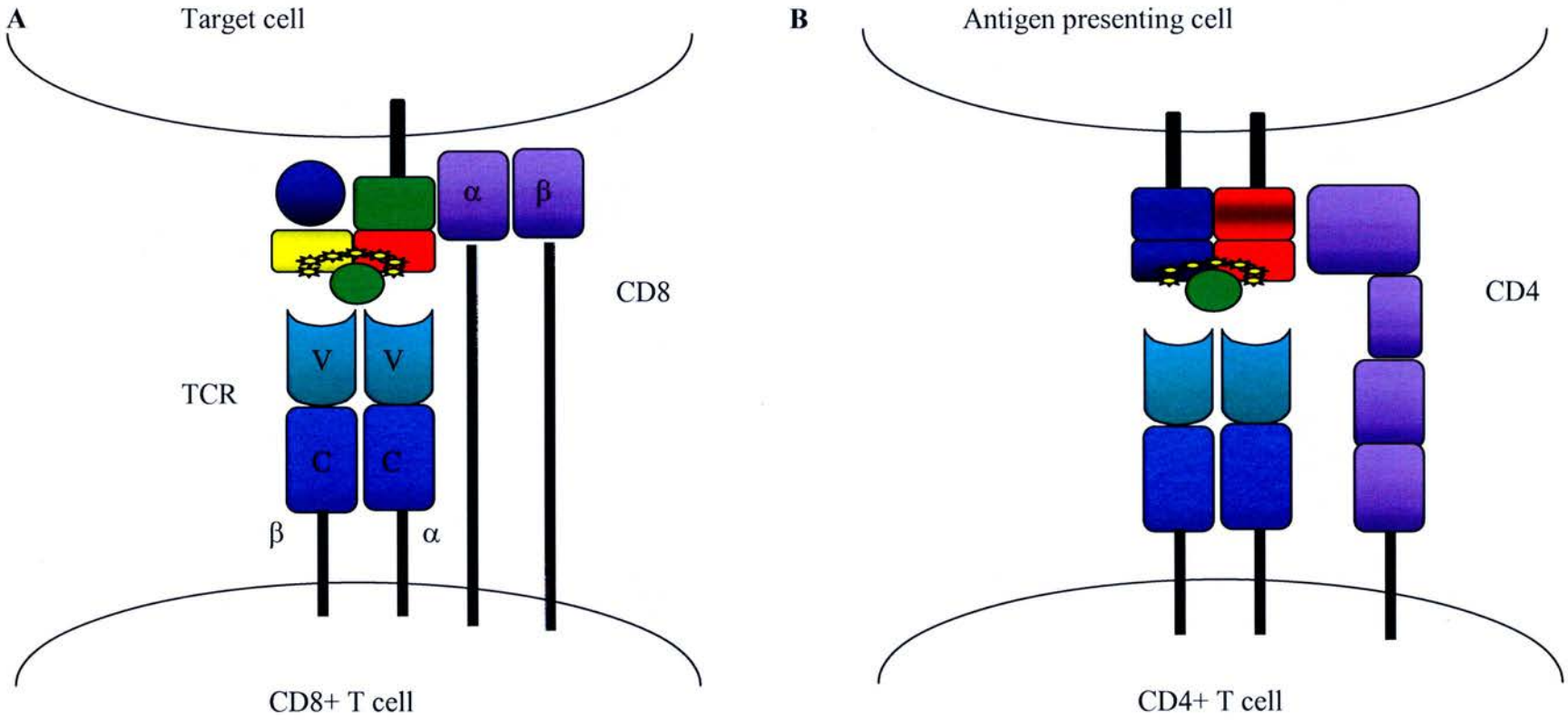
The specificity of the T cell response to infection requires recognition of antigenic peptides bound to MHC class I or II molecules by the TCR (fig. 1.9). Two types of TCR exist consisting of disulphide-linked heterodimers of either αβ or γδ chains.

1.6.1 αβ T cells

The majority of T cells in the peripheral blood are αβ T cells which play a direct role in antigen recognition and determine the outcome of an immune response. αβ TCRs are present on the cell surface in conjunction with the CD3 complex, one CD3γ, one CD3δ and two CD3ε proteins. Two copies of another protein, the ζ chain, are also involved. Together these proteins form the functional TCR complex, with CD3 and the ζ chain being important for internal signalling. αβ T cells can be divided into two groups depending on the co-receptor they express. CD4+ cells recognise MHC class II with the CD4 co-receptor binding to the β2 domain of the MHC molecule. CTL recognise MHC class I, with the CD8 co-receptor binding to the α3 domain of the MHC molecule. CD4 and CD8 help to increase the avidity with which the TCR binds the MHC/peptide complex and reduce the flexibility of interaction between the two surfaces helping to activate the T cell more efficiently. They both also recruit tyrosine kinases to their cytoplasmic domains which are important components of the signalling pathway, enhancing signalling transduction following stimulation of the TCR (reviewed in Gao and Jakobsen, 2000).

Figure 1.9: Interactions between αβT cell receptors and MHC class I and II molecules

The standard T cell receptor consists of two chains, α and β, each of which have a constant (C) and a variable (V) region. The variable region is distal to the cell membrane and is the site of interaction with MHC molecules. MHC class I molecules are recognised by T cells that carry the co-receptor CD8 (A) while MHC class II molecules are recognised by T cells with the CD4 co-receptor (B).



1.6.2 γδ T cells

γδ T cells were originally discovered in 1986 by Brenner *et al.* but to date, despite study in a number of species, their function still remains unclear. In rodents and mammals γδ T cells represent less than 10% of the T cells in the body, with the majority of these residing within mucosal sites such as the skin and gastrointestinal tract (Haas *et al.*, 1993). In direct contrast ruminants have been shown to have large numbers of γδ T cells especially in new born calves where they make up to 50% of the circulating Peripheral Blood Mononuclear cells (PBM, Hein and Mackay, 1991). The reason for this variance is not known but possible suggestions include selective advantage or as a result of a biochemical event occurring around gestation (Howard *et al.*, 1999).

γδ T cells are a heterogeneous population, with their functions differing depending on their location and local environment, the structure of their TCR and how and at what stage they become activated during an immune response (Carding and Egan, 2002).

Recognition of antigen by γδ T cells occurs in a different manner from that of αβ T cells with no antigen processing or MHC restriction required (Schild *et al.*, 1994). As a result γδ T cells are not constrained to bind to MHC and so are able to recognise a greater variety of antigen. γδ T cells in the blood tend to respond to antigens constitutively expressed on the surface of microbial pathogens and host cells such as non-protein phosphorylated nucleotides and alkylamines and homologues of a heat

shock protein (Tanaka *et al.*, 1995, O'Brien *et al.*, 1992). In comparison γδ T cells in tissues respond to induced antigen such as MHC class I related chain MICA and MICB which are expressed by cells during periods of stress (Bauer *et al.*, 1999).

Studies on γδ T cells identified two principal functions, production of cytokines and chemokines and cytotoxicity. They are believed to have both effector and regulatory roles in the immune response which is supported by the kinetics of the γδ T cell response. Following infection a first wave of γδ T cells are seen along with the innate immune response. These produce pro-inflammatory cytokines and chemokines helping to control the natural killer cells and macrophages and also development of the adaptive response. Later on in the infection the cells then act to downregulate inflammation through the inhibition of αβ CD8+ T cell migration and responses and killing of activated macrophages (Hayday, 2000, Born *et al.*, 1999, Guan *et al.*, 2002).

1.6.3 TCR structure

The TCRs are polypeptides very similar to immunoglobulins. Their genes are assembled in a similar manner consisting of interchangeable V (variable), J (joining) and C (constant) gene segments. In addition the β and δ chains also have D (diversity) segments. Specific V, D and J segments fuse to form a complete V region, which then combines with the C region creating a functional chain. This combination of different gene segments along with the addition of nucleotides where they join acts to increase the diversity of the T cell repertoire. Unlike

immunoglobulins however, TCRs do not undergo somatic hypermutation and affinity maturation to increase their affinity for antigen, instead they have more J segments and junctional diversity (Davis and Bjorkman, 1988). When these chains fold the variable region is found distal to the cell surface. This region is equivalent to the hypervariable region in immunoglobulins, with three folds known as complementarity determining regions (CDR) providing the points of contact to the MHC peptide complex.

The crystal structure of a TCR bound to a MHC molecule has been determined for a number of human and mouse models (reviewed in Garcia *et al.*, 1999). These showed that all six CDR loops interact, with the CDR1s interacting with the peptide and the α helices of the MHC molecule, CDR2s just the helices and CDR3s primarily the peptide but with some contact on the helices as well. On a wider perspective the variable domains of the polypeptide chains lie in a diagonal orientation above the PBR, with the variable portion of the α chain (Vα) lying over the N-terminus while Vβ is over the C-terminal. Interactions with the peptide vary between the mouse and human models with the human TCRs having more intimate contact with the peptide (although this is thought to reflect the antigenicity of the peptide used since a self-peptide involved in thymic selection was used in the murine model).

1.6.4 T cell development

Following production in the bone marrow T cell precursors must migrate, via the blood, to the thymus to complete their development before entering circulation as functional cells. These precursors have no CD4 or CD8 receptors and the TCR is not fully formed. Once in the thymus the TCR genes become rearranged with the expression of a preTCR receptor. The cells then proliferate followed by another round of rearrangement which results in cells expressing αβ or γδ receptors. The αβ T cells then go on to express both CD4 and CD8. These double positive cells are found in the inner cortex of the thymus where they are surrounded by a network of cortical epithelial cells which express MHC/self peptides on their surface. Initially only a small proportion of these T cells have TCRs specific for self-MHC. If interaction between these cells and the epithelial cells occurs within three to four days of cells gaining their receptors then a positive signal is delivered to the T cell allowing it to mature. If no such signal occurs then the cell dies by apoptosis and is removed by macrophages. This positive selection also determines the fate of the T cells, with those interacting with class I MHC molecules becoming CD8+ cells, while those that interact with class II molecules become CD4+ cells.

Following on from this the cells that have survived go through a negative selection process. In the cortico-medullary junction they are mixed with professional antigen processing cells (APC) such as dendritic cells. T cells which bind to these APC with high affinity are removed, since these could become alloreactive if allowed into circulation. This process ensures the release into circulation of a repertoire of T cells

that recognise self MHC molecules but which do not bind with a high affinity such as to cause autoimmunity (reviewed in Anderson, Hare and Jenkinson, 1999). In addition the combination of many types of self peptide and a large TCR repertoire ensures a diverse array of T cells leave the thymus.

1.6.5 T regulatory cells

The process of T cell activation is under tight control to prevent or reduce reactivity to self antigens and limit the response to pathogen-derived antigen as uncontrolled responses to these can lead to the development of autoimmune disease and infection. While thymic selection described in section 1.6.4 ensures that the T cells released into the system are self tolerant another system exists in the periphery to help control the T cells – T regulatory (Treg) cells. Two populations of regulatory cells exist, natural and inducible. Natural Treg cells have a CD4+ CD25+ phenotype and develop in the thymus before migrating to the periphery (Sakaguchi *et al.*, 2001). Within the thymus their development appears to depend on the strong TCR interaction and co-stimulation through CD28. Once in the periphery repeated co-stimulation through CD28 is required to ensure survival of this cell population (reviewed in Bluestone and Abbas, 2003). These cells are anergic but are able to suppress the activation and proliferation of CD4+CD25- and CD8+ T cells, possibly by cell to cell contact through the TCR following recognition of self-antigens on APC (Cozzo *et al.*, 2003). There is also evidence for the involvement of CTLA4 (CTL antigen 4, a marker on the Treg cell) which binds to CD80/86 on the APC and delivers a negative signal for T cell activation (Read *et al.*, 2000)

Inducible Treg cells can be produced from naïve CD4+CD25- and CD8+CD25- T cells in the periphery when they interact with antigen presented by a population of dendritic cells. Unlike natural Treg cells inducible Treg cells act by producing immunosuppressive cytokines including IL-10 and TGFβ. These cytokines cause reduced expression of MHC and co-stimulatory molecules and suppress inflammatory cytokine release by the APC. These changes in APC function then result in decreased proliferation and cytokine production by Th1, Th2 and CD8+ T cells which are reacting to the peptides presented on the surface of the APC (reviewed by Wills, 2004).

1.6.6 Natural Killer Cells

Another type of cell within the body which also interacts with MHC class I molecules is the Natural Killer (NK) cell. These cells are classed as lymphocytes and are derived from the same common progenitor cell as T cells (Rodewald *et al.*, 1992) but are considered to be a fundamental part of innate immunity, becoming activated through constitutively expressed receptors prior to clonal expansion and differentiation of T cells.

Two families of NK receptors have been identified, Ig-like receptors and C-type lectins. These are polymorphic, polygenic families encoded in two regions, the LRC (leukocyte receptor complex) and the NKC (natural killer complex) respectively. These regions are orthologous in both humans and mice (reviewed in Barten *et al.*, 2001). Ig-like receptors include human killer cell Ig-like (KIR) receptors and Ig-like

transcripts (ILTs). C-type lectins include the murine Ly49 receptors and NKG2 receptors, the majority of which are found as heterodimers in association with CD94 on both murine and human cells. Within both families are activatory and inhibitory receptors which tend to occur in pairs on the cells surface, comprising one of each set. Inhibitory receptors have ITIM (immunoreceptor tyrosine-based inhibition motifs) in their cytoplasmic domains which transduce inhibitory signals into the cell (Long, 1999). Activatory receptors have truncated cytoplasmic tails and so lack this motif instead coupling with an ITAM (immunoreceptor tyrosine-based activation motifs)-bearing adapter molecule DAP12 in the transmembrane allowing positive signals to be passed into the cell for activation (Isakov, 1998). The balance between the two types of receptors determines the outcome of the NK cell response.

NK cells act to monitor the health of cells in the body by determining the level of class I molecules on the cell surface as loss or a decrease in class I expression is frequent in tumour and infected cells (Ljunggren and Karre, 1990). Direct interaction between Ly49 and KIRs with classical MHC molecules causes an inhibitory signal to be sent to the NK cell stopping the cell becoming activated. CD94/NKG2 heterodimers interact with the non-classical MHC molecules HLA-E in humans and Qa1b in mice (Braud *et al.*, 1998, Vance, 1998). Analysis of these molecules has shown that they present the leader peptides of classical MHC molecules thereby allowing the NK cells to detect class I expression indirectly.

NKG2D is an NK cell receptor only distantly related to the other NKG2 receptors. Present also on NK, γδ and activated CD8+ T cells it is expressed as a homodimer

and is found associated with the activating adapter protein DAP10 (Wu *et al.*, 1999). Like γδ T cells NKG2D ligands are MICA and MICB (and retinoic acid early inducible receptors in mice). Since these factors are found on stressed or tumorigenic cells this allows the NK cell to be activated even when there is no decrease in class I expression (Bauer *et al.*, 1999, Cerwenka, 2000).

1.7 Non-classical MHC

Following discovery of the classical class I genes in the class I region other genes were identified which showed significant homology to the classical genes but differed in both polymorphism and expression. These genes were called non-classicals or class Ib. In the mouse the non-classicals were termed Qa and TL and mapped telomeric to the classical genes (see fig. 1.1). These genes encoded unusual molecules, for example, Qa2, which is attached to the cell surface by a glycophosphoinositol anchor, and Q10, which is a soluble protein. In humans three non-classicals were initially identified, HLA-E, F and G. The molecules were found to be structurally similar to classical class I molecules including association with β2m suggesting key roles in immunological recognition although they have limited polymorphism and tissue distribution. Further investigation of both genomes identified other class I like molecules both within and outside the MHC region, including other chromosomes.

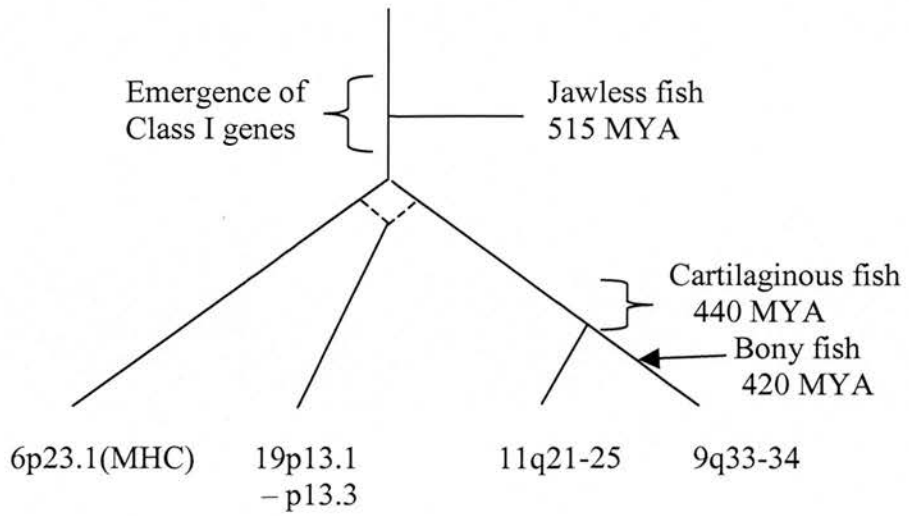
1.8. Evolution of the MHC region

As discussed above, the MHC region is highly complex, containing approximately 224 genes, only a proportion of which are directly involved in the immune system (the MHC sequencing consortium, 1999). How such a set of genes came to be associated together, in such a distinct configuration, has been the subject of much interest. Analysis of the subunits of the proteasome in humans led to the discovery that a section of chromosome 9 (q33-q34) to which one of the subunits Z mapped, contained at least ten genes which had high homology to those found in the MHC region. Thus it appeared that these two regions were paralogous i.e. they contained closely linked pairs of duplicated genes (Kasahara *et al.*, 1996a, Kasahara, 1999a, Katsanis *et al.*, 1996). Further paralogous regions were also identified on chromosome 19p13.1-13.3 and chromosome 1q21-q25/p11-p32 (Katsanis *et al.*, 1996, Kasahara *et al.*, 1997).

To date more than 20 gene families have been identified across the paralogous regions (see fig. 1.10 for examples). It should be noted that the regions are not exact replicas of each other, with deletions following gene inactivation, inversions and intrachromosomal translocations all taking place following establishment of the region (see below, Kasahara *et al.*, 1999b). Regions paralogous to the MHC were also identified in the mouse genome (Kasahara *et al.*, 1996b, Katsanis *et al.*, 1996) however these are more disjointed than the humans, possibly as a result of more rearrangements.

Figure 1.10 : Emergence of the four paralogous regions identified in the human genome

This figure shows the believed relationship between the paralogous regions with the estimated emergence time for each class of vertebrates indicated in million years ago (MYA). Paralogous genes present in each of the regions are shown on the same line with blank spaces indicating the no genes for that group have been identified. Adapted from Kasahara *et al.*, 1997).



RXRB		RXRG	RXRA
COL11A2		COL11A1	COL5A1
RING3			RING3-like
LMP			PSMB7
TAP			ABC2
NOTCH4	NOTCH3	NOTCH2	NOTCH1
PBX2		PBX1	PBX3
TNX		TNR	HXB
CYP21	CYP2		
C4	C3		C5
HSPA1		HSPA6/7	GRP78
MHC class I		CD1	
	VAV1		VAV2
	LMNB2	LMNA	
		SPTA	SPTAN1
		ABL2	ABL1

There are two arguments for how these paralogous regions have evolved. One dictates that block duplication of sections of or whole chromosomes has occurred resulting in the inheritance of all members (Kasahara *et al.*, 1996a, Kasahara, 1999a, Abi-Rached *et al.*, 1999). The other argues against this, instead suggesting that selective pressure has acted to group these genes, which have arisen by individual duplication, for some functional reason (Hughes, 1998). The former is the more widely accepted opinion since it is difficult to imagine a mechanism that could cause such a large amount of gene manipulation to occur.

Since all jawed vertebrates have a MHC region it is likely that the duplications giving rise to these paralogous regions occurred in a common ancestor prior to the divergence of this group. Furthermore it appears that jawless fish appear to have two paralogous regions which would indicate that the first round of duplication took place in an ancestor to all vertebrates, with a further round occurring after the division of jawed and jawless groups (reviewed in Flajnik and Kasahara, 2001).

It has been suggested that the duplications forming the MHC paralogous regions could be part of a much larger genome-wide duplication. A hypothesis exists that genome duplication occurred in vertebrates early in evolution to produce the complex genomes now seen (Ohno, 1973). The timings of these duplications and the number of duplications match those of the paralogous regions indicating this as a distinct possibility for their evolutions.

Recent work by Amadou (1999) identified the presence of non-MHC genes in the class I region of humans and mice which appeared to define a framework, with the areas in between determining class I permissive regions. Examples of these genes include ZNF173 which encodes a nucleic acid binding protein (Chu *et al.*, 1995) and MOG, myelin oligodendrocyte glycoprotein. Some of these anchor genes have also been identified in the MHC region of cattle (Archibald, 2002). Although the class I genes evolved independently in both species the anchor genes are conserved and their order maintained indicating that they may have existed in this order in a predecessor of humans and mice. This may be useful when considering how the class I region has evolved. It is important that the framework contains genes with essential functions to warrant conservation.

1.8.2 Maintenance of polymorphism

The MHC genes are the most polymorphic loci identified in the animal kingdom. Indeed it was this characteristic rather than their function which was first discovered (reviewed in Hughes and Hughes, 1995). The elucidation of their role in presenting peptides, with different alleles presenting a different panel of antigens, led the authors to suggest that heterozygote advantage would occur in a population under threat by various pathogens, thereby acting to increase diversity in the MHC (Zinkernagel and Doherty, 1974). The evolution of methods allowing in-depth sequence analysis has supported this theory, with patterns of nucleotide substitutions indicating that both the MHC class I and II genes are under overdominant selection which acts to maintain polymorphism within a species. Two main pieces of evidence

point to this. Firstly in neutrally evolving loci it would be expected that fixation of nucleotides would occur over time as a result of genetic drift with an allele fixed at each locus with approximately 100% frequency and the others all at low frequencies. However with MHC loci there are no dominant alleles found, instead there is a large number of alleles all of intermediate frequency, although it should be noted that in some cases, for example isolated populations, higher frequencies of particular alleles can occur (Maruyama and Nei, 1981). Secondly, comparing patterns of nucleotide substitutions it would be expected that a higher proportion of synonymous (non-aa altering) mutations than non-synonymous would occur since these are likely to be deleterious and thus removed. This is the case for the majority of the MHC gene sequences (both class I and II) with the exception of the peptide-binding region where the opposite occurs indicating that this region is under selection, acting to increase polymorphism in both mice and humans (Hughes and Nei, 1988).

The fact that selection is acting at the PBR, encouraging an increase in the diversity of peptides that can bind means it is reasonable to assume that the evolution of the system has been influenced by pathogens which may be driving an increase in polymorphism. Unfortunately there is little actual evidence to support this theory. Evidence for long lasting polymorphisms, including those prior to speciation, have been identified, for example, between humans and chimps (Lawlor *et al.*, 1988). These can only be supported by selection since neutral polymorphisms are not likely to survive that long (Takahata and Nei, 1990).

1.8.3 Generation of polymorphism

Comparison of MHC class I sequences in humans and mice indicates that diversity was generated by point mutations which were then propagated by intra- and/or inter-locus-specific recombination. Recombination alone can play a role but a basic level of polymorphism needs to be in place for this method to have any effect.

Intra-locus recombination has been identified in HLA-A and B and to a lesser extent in HLA-C (Parham *et al.*, 1988, Yeager and Hughes, 1999) as shown by the presence of locus-specific characteristics. Both large (involving whole exons) and small scale recombinational events have been identified in HLA-A and HLA-B while HLA-B has been found to have undergone recombinational events twice as often as HLA-A, with these more likely to be in the PBR. As a result HLA-B has more alleles (Hughes *et al.*, 1993).

In contrast little or no interlocus recombination has been identified in humans. However, in mice and rats this is relatively frequent (Weiss *et al.*, 1983) leading to a reduction in the number of locus-specific characteristics found in the alleles of each locus.

1.9 Bovine MHC

1.9.1 Origin of cattle

The ancestor of modern day cattle is the aurochs, or *Bos primigenius*. These existed as distinct populations in central and northern Europe, Asia (*Bos nomadicus*) and possibly North Africa (*Bos opisthoriomalus*), each of which were evolving in distinct conditions with different endemic pathogens. These animals are now extinct, thought to have died out approximately 2000 years ago (Bradley *et al.*, 1996). Modern cattle are believed to have their roots in two main areas of domestication, in the Near East and Northern India and Pakistan. The first domestication events are believed to have taken place approximately 10,000 years ago. Analysis of mitochondrial DNA shows there to be two main lineages of cattle which diverged between 200,000 and 1 million years ago, one containing all European (*Bos taurus*) and African (*Bos indicus*) breeds and the other the Asian *B. indicus* breeds (Loftus *et al.*, 1994). The African and European breeds are believed to have diverged between 180 and 250,000 years ago. It is not clear whether *B. indicus* and *B. taurus* are distinct species or related subspecies although they appear to be subspecies based on their ability to interbreed and from studies into their evolution (Lewin *et al.*, 1999). These animals can be differentiated by the presence of a hump on *B. indicus* cattle.

1.9.2 Bovine MHC region

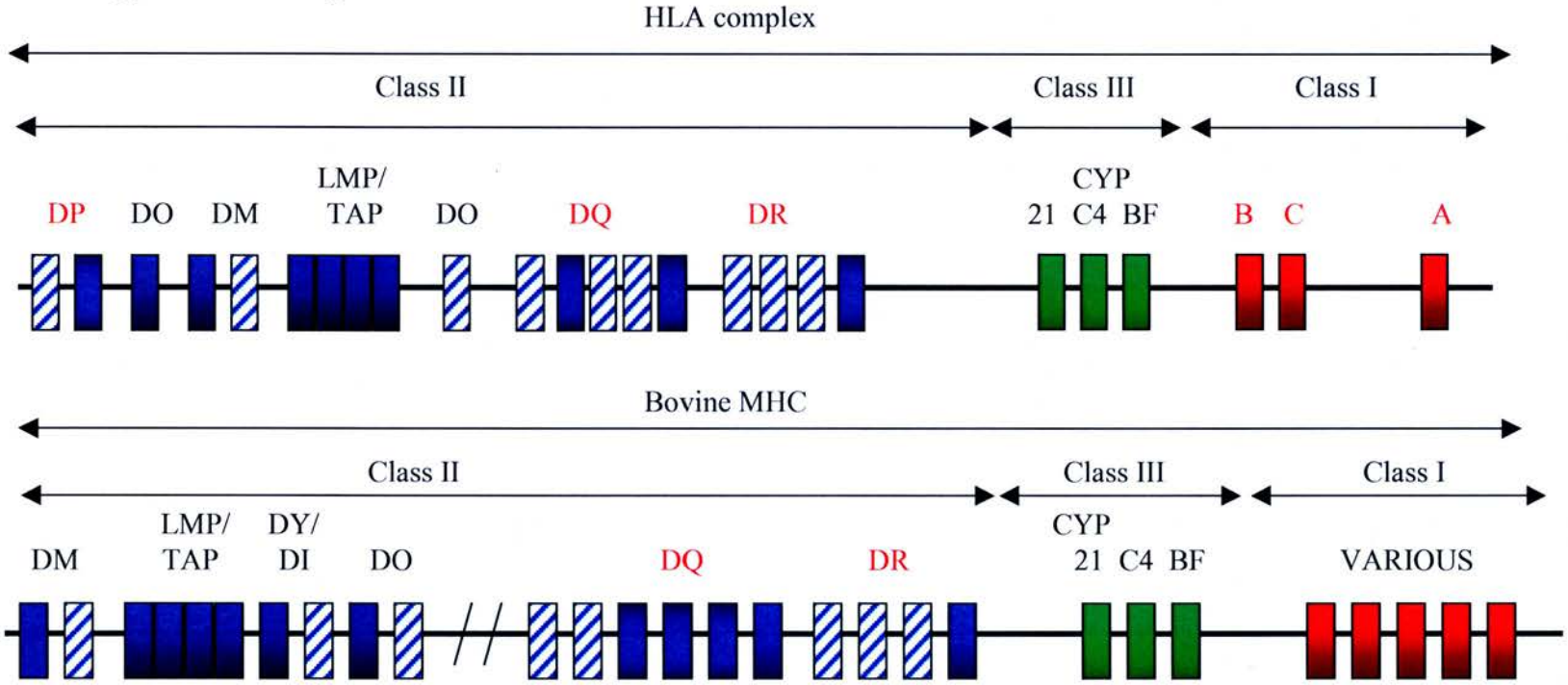
The bovine MHC or BoLA system was located to chromosome 23 (BTA23) by *in situ* hybridization using a cloned sequence derived from a pig MHC class I gene (Fries *et al.*, 1986). It has the same basic organisation as human and mouse with class II, class III and class I in a centromere to telomere orientation (fig. 1.11). One major difference exists however in that the class II region is divided into two sections, termed class IIa and class IIb, by a genetic distance of 17cM (Andersson *et al.*, 1988, Hess *et al.*, 1999). Comparison of the gene order between humans, cattle and mice indicates that this division is likely caused by a single large chromosomal inversion (Band *et al.*, 1998). Gallagher *et al.* (1994) found that families related to the bovines including *Giraffidae* and *Antilocaprodae* had similar banding patterns on their BTA 23 homologues indicating that these animals may also have the class II division.

1.9.3 Class II region

Investigation of the classical class II genes in cattle has been relatively straightforward due to the high levels of homology that exist between class II genes in mammals. The class IIa region contains the genes DR and DQ which code for functional class II molecules. These genes were originally identified using human class II probes on Southern blots (Andersson *et al.*, 1988, Andersson and Rask, 1988). No cattle homologues of the DP genes have been found suggesting that these have been deleted from the genome. DP genes are also absent from ovine MHC

Figure 1.11 Comparative map of the bovine and human MHC regions

This diagram shows the MHC genes present in the MHC of humans and cattle, both classical (red) and non-classical (black). Class I loci are shown in red, class II in blue and class III in green. Genes encoding α strands are shown as filled while those encoding β strands are striped.



indicating that this may be a common feature of the ruminant MHC (Deverson *et al.*, 1991). Possibly as a compensatory mechanism it has been shown that approximately half of the common haplotypes in cattle have duplicated DQ genes which are expressed (Glass *et al.*, 2000).

The DR region consists of a monomorphic DRA gene and at least 3 DRB genes. DRB1 is known to be a pseudogene as shown by the presence of a number of stop codons within its sequence (Groenen *et al.*, 1990) while DRB2 is transcribed, but at low levels. In contrast DRB3 is actively transcribed, expressed at high levels and is polymorphic, particularly in the PBR region with at least 60 alleles identified (Van Eijk *et al.*, 1992). Each haplotype contains one DR gene pair, DRA and DRB3.

Analysis of the DQ region containing polymorphic DQA and DQB genes shows it to be highly variable with the number of DQ genes varying between haplotypes. Three haplotypes have been described with different DQA genes, DQA1, DQA1 and 2, DQA2 and 3 with at least two DQB loci found (Andersson and Rask, 1988). Variation in the number of DQB genes expressed has also been described (Marello *et al.*, 1995).

The class IIb region contains the LMP2, LMP7 and TAP2 genes along with the non-classicals DOB, DOA, DIB and DYA, which show limited homology to human class II genes (Stone and Muggli-Cockett, 1990, van der Poel *et al.*, 1990). The functions of these are currently unknown but it has been shown that DYA and DIB are transcribed at low levels in dendritic cells (Ballingall *et al.*, 2001). DYA and DIB

have also been identified in sheep and goats but not pigs, humans or mice suggesting it to have been produced by gene duplication following divergence of these groups and be ruminant specific. Recently it has been shown that these genes form a pair similarly to other class II genes since they are closely linked, separated by approximately 18kb (Ballingall *et al.*, 2004). As a result the authors suggested that DIB be renamed DYB to reflect this close association. Analysis of the products of these genes shows them to code for class II α and β chains which, although similar to classical chains, differ by 16 unique aa in the peptide binding region.

1.9.4 Class III region

Little information is available regarding the class III region in cattle probably due to a lack of interest in the genes residing here. Its position between class I and II was determined based on the finding that the linkage disequilibrium between a class III gene C4 and either class I or II genes was approximately equal. Five genes have so far been identified, C4, Bf, CYP21, HSP70 and TNFα. C4 and Bf are complement proteins while CYP21 is a 21 hydroxylase. These are also components of the human MHC and were mapped to the bovine MHC by linkage analysis (Andersson *et al.*, 1988, Teutsch *et al.*, 1989) and analysis of somatic cell hybrids (Skow *et al.*, 1988). The presence of HSP70 and TNFα was shown by the fact that they co-localise with CYP21 using fluorescent *in-situ* hybridisation (FISH) indicating tight linkage (McShane *et al.*, 2001).

1.9.5 Class I region

The MHC class I region is more difficult to study than class II due to the mix of expressed genes, pseudogenes and gene fragments found here. Initial studies of the class I region used alloantisera produced by skin grafting or lymphocyte inoculation in microlymphocytotoxicity assays for identifying class I genes (Spooner *et al*, 1979, Ennis *et al.*, 1988). By 1987 25 distinct specificities had been identified by this method and approved by the International BoLA workshop. This number was subsequently extended to 50 (Bernoco *et al.*, 1991). Further studies led to the limitations of serology for typing the bovine class I genes being uncovered. Testing antisera on single gene transfectants it was shown that the sera were detecting only one gene on a haplotype with any others being serologically blank. As a result it was originally assumed that all specificities mapped to the same locus. It was also found that some sera were cross-reactive between alleles for example A10 and JSP1 which differ by 15 aa (Pichowski *et al.*, 1996). Some of the antisera used recognise more than one haplotype, indicating that they are specific for a conserved region between alleles, for example the w6 sera recognises the haplotypes A17, A18 and A19. Thus while serology it useful for determining the frequency of specificities it is clearly not specific enough to allow complete characterisation of haplotypes.

The development of biochemical and molecular techniques allowed more in-depth analysis of the class I region and provided evidence for the existence of more than one class I locus. Ennis *et al.* (1988) were the first to adapt such methods. They used a HLA-B probe to screen a cDNA library produced from a bovine B cell line. From this they identified two long clones, BL3-6 and BL3-7, which appeared to code

for all the characteristics of MHC class I proteins including phosphorylation and glycosylation sites. Examination of the 3'untranslated region (3'UTR) of these clones showed them to have 86% homology, approximately 10% lower than that observed for alleles from the same locus in humans and mice, suggesting that these sequences were derived from different loci.

Serological evidence for two classical class I loci was provided by Toye *et al.* (1990). They transfected genomic DNA from an animal homozygous for w10/KN104 into L cells then screened the transfectants with monoclonal antibodies specific for each specificity. From this they identified two populations, one expressing KN104 and one expressing w10 indicating that these specificities are products of different genes. Further work on this haplotype was carried out by Bensaid *et al.* (1991). They produced a cDNA library from a MHC homozygous animal from which two distinct cDNA strands were isolated and cloned. When these clones were used as probes in Northern blots on mRNA from the transfectants mentioned above, each of the clones were found to be specific for one or other transfectant, conclusively showing that w10 and KN104 are products of two different genes. Following on from this they were able to map the genes to within 210kb of each other using field inversion gel electrophoresis (FIGE).

Evidence for a third locus was provided by Garber *et al.* (1993) through phylogenetic analysis of the class I sequences available at this time. He found that the sequences appeared to fall into two groups, with the exception of the allele KN104 which was equally similar to both groups. Given that KN104 is encoded by a separate gene to

the other specificities (Bensaid *et al.*, 1991) and had been shown to be important in MHC restriction of T cells against the parasite *Theileria parva* (Goddeeris *et al.*, 1990) it can be taken that this allele codes for a functional MHC molecule and thus there are at least 3 classical class I loci. More conclusive evidence for a third locus was provided by Garber *et al.* (1994) who used reverse-transcriptase Polymerase Chain Reaction (PCR) and RACE (rapid amplification of cDNA ends) PCR to identify six cDNA clones from a heterozygous animal.

1.9.6 Class I haplotype analysis

Despite attempts to investigate the class I region in cattle the exact number of class I genes has not yet been determined. In 1996 Ellis *et al.* initiated a series of studies using a herd of cattle that had been specifically bred to produce MHC-homozygous animals expressing common serologically defined class I specificities. Using a combination of cDNA cloning, sequence analysis and transfection/expression studies they investigated individual common haplotypes in great detail. Four class I haplotypes were characterised, A18, A31, A14 and A11. Two of the haplotypes had two classical genes, A31 and A11, with the A18 haplotype found to express only one gene. The A14 haplotype was found to have three transcribed genes conclusively showing that there are at least three classical class I loci in cattle. The genes identified on each of the haplotypes are summarised in table 1.1.

Table 1.1: Genes identified on MHC class I haplotypes

Haplotype	Genes
A10/KN104	A10, KN104
A18	HD6
A31	HD1, HD7
A11	D18.2, D18.3
A14	D18.1, D18.4, D18.5

The presence of locus-specific characteristics within class I coding sequences from humans and primates allows relatively easy locus assignment with all the sequences falling into distinct groups representative of loci when compared phylogenetically (Knapp *et al.*, 1998, Parham *et al.*, 1989). Studies of cattle sequences have not identified such characteristics within the coding region (Ellis *et al.*, 1999, Holmes *et al.*, 2003) with analysis of 22 class I sequences failing to fall into such distinctive groups (see fig. 3.1). Instead there appeared to be five or six potential groups of alleles. The pattern of distribution of these alleles does match the haplotype configurations identified, with alleles on the same haplotype not grouping together suggesting a different origin i.e. products of different loci. A study of the promoter region of a proportion of these alleles found that they also appeared to fall into the previously observed groups (Smith, 2000).

Together these data provide evidence for the existence of five or six classical class I genes in cattle, of which two or three are polymorphic. Interestingly it can be seen through haplotype comparison that not all of the genes are expressed in all haplotypes, with the number of expressed genes varying between one and three and

no gene consistently expressed. Whether all genes are present and a proportion turned off, or deletions have occurred to remove them from the genome has not yet been determined.

The variation in class I haplotype composition observed in cattle does not in itself appear to be particularly unusual. Variation has also been observed in the bovine class II DQ region and the murine and rat class I genes (Joly *et al.*, 1996, Wroblewski *et al.*, 1994). Rather, the standard three gene expression seen in humans appears to be unusual and in no way represents the situation in all mammals. However cattle may be unique in the level of variation seen, in particular the fact that none of the genes identified are found on all haplotypes.

1.9.7 Polymorphism in bovine MHC class I genes

Since the total number of class I genes and assignment of alleles to loci has not been confirmed it is difficult to comment on the level of polymorphism in cattle. To date approximately 40 alleles have been identified by various groups, 30 of which have been submitted to the BoLA database (www.projects.roslin.ac.uk/bola). Given that the majority of studies have been done on inbred herds or very small numbers of animals it is possible that the polymorphism may be quite extensive. Analysis of available BoLA class I sequences showed that, like humans, d_N is greater than d_S in the $\alpha 1$ and $\alpha 2$ regions while the opposite is seen in the remainder of the sequence which indicates that the PBR is under selection to maintain diversity (Garber *et al.*, 1993).

Through comparison of the coding sequence from all bovine MHC class I alleles it is possible to see a patchwork of substitutions with shared motifs between alleles. This shows that segmental exchange was involved in creating diversity of the class I loci. In depth analysis using statistical packages showed that interlocus recombination has also taken place, with two alleles, BL3-7 and D18.4 changing groups in both neighbourhood joining and maximum likelihood phylogenetic trees constructed with different gene sections (Holmes *et al.*, 2003). Furthermore the authors were able to locate the breakpoints within the sequences. Other alleles were also found to move position on neighbourhood joining trees but maximum likelihood trees did not support this. It is a possibility that the interlocus recombination observed with the bovine MHC class I genes may account for the failure of distinct gene-specific groups to form when alleles are compared.

1.9.8 Bovine MHC class I genes and molecules

Analysis of MHC class I transcripts isolated from bovine PBM showed them to contain the characteristic features of functional MHC molecules when compared to those from other species (Ennis *et al.*, 1988, Garber *et al.*, 1993). These include cysteine residues in α2 and α3 for intramolecular disulphide bonds (positions 101 and 164 and 203 and 259 respectively) and an N-linked glycosylation site in α1 at position 86 which is also found in human and murine class I molecules (Ploegh *et al.*, 1981). A conserved potential site for phosphorylation was identified in all the transcripts at serine 333 in the cytoplasmic domain.

Through the use of immunoprecipitation bovine MHC class I molecules have been shown to consist of a 44kDa heavy chain and a 12kDa β_2m molecule (Hoang-Xuan *et al.*, 1982). These sizes are comparable with human and murine class I molecules (Creswell *et al.*, 1973).

1.9.8.2 Promoter

Initial attempts to sequence the promoter region of an MHC class I gene identified essential components including the enhancer A/ Interferon Response Sequence (IRS) region, enhancer B, TATA and CAAT boxes for RNA Polymerase II binding and a transcription initiation site (Harms and Splitter, 1995), all of which are found in the human promoter region. Surprisingly, comparison of HLA-A2 and a bovine promoter (BoLAenh-9) showed that while both were capable of driving transcription they shared only 59% homology (Harms and Splitter, 1994).

1.9.8.3 B_2-microglobulin

The sequence of bovine β_2m was originally elucidated by Groves and Greenberg (1982). Comparison to the published human, murine, rabbit and guinea-pig sequences showed the bovine sequence to be one aa shorter due to missing a conserved valine at position 49. Overall comparison of sequences shows that bovine β_2m has between 24 and 32 aa differences from these species with guinea-pig and mouse being most different. A total of 48 residues are completely unchanged however, showing that β_2m is a highly conserved molecule between species.

Further work on bovine β_2m was carried out by Ellis *et al.* (1995). They also sequenced β_2m but found that the sequence differed by 3 aa to that previously obtained suggesting that allelic variation was occurring as with the murine β_2m (Hermel *et al.*, 1993). Following on from this they sequenced the majority of the coding region of β_2m from a range of European, African and Indian animals. The European and Asian animals all had identical sequences to that previously found by this group. Of the six African animals studied two also had this sequence. Two allelic variants were identified in the remaining animals, each carrying at least one of the variants. One animal was found to have all three variants, which the authors suggested could indicate the presence of two genes for β_2m. Alternatively it could be chimaeric since this animal is a twin. One of the variants identified had a non-coding change while the other had this plus a coding change. It is possible that these changes may affect the binding of β2m to an MHC molecule.

1.9.8.4 Peptide binding region

It is generally accepted that CTL play an important role in the immune response against intracellular pathogens. In cattle there is evidence for their action against *Theileria parva*, respiratory syncytial virus and bovine herpes virus (Eugui and Emery, 1981, Gaddum *et al.*, 1996, Splitter *et al.*, 1988). CTL specifically recognize the combination of antigenic peptides attached to self-MHC molecules and respond accordingly. A major impetus in the drive towards vaccine design is the identification of epitopes recognized by CTL which could then be produced synthetically and used to induce a CTL response.

As discussed previously peptides bound to MHC class I molecules are typically nonamers. Despite the low level of different MHC molecules expressed by an individual, each type of molecule is able to present an array of peptides. These peptides are limited only in the requirement for specific anchor residues located along the peptide which determine the peptide binding motif of each class I molecule. Thus by determining the anchor residues it is possible to predict the sequences of peptides that should bind. This is usually done by eluting and sequencing self peptides from the class I molecules which bind in the absence of antigenic peptides. An increase in the frequency of a residue at any position suggests preferential binding and indicates this to be an anchor residue. This data should also provide some information about the affinity of the peptide for the MHC molecule and also the affinity of CTL for the peptide and MHC combination.

To date the peptide binding motif has been determined for HD1, HD6, HD7, D18.3 and an unknown gene from an animal typed serologically as A20 (Gaddum *et al.*, 1995, Hegde *et al.*, 1995, Bamford *et al.*, 1995). Further motifs are also available for A10 and KN104 (unpublished data, S. Ellis personal communication). These motifs are summarised in fig. 1.12. Surprisingly neither HD1 nor HD6 have an anchor residue near the carboxy terminal which is unusual when compared to most motifs. HD7 has a tyrosine anchor residue at position 10 which suggests it may be able to present larger peptides than normal.

Figure 1.12: Peptide binding motifs of bovine MHC class I molecules

The peptide binding motifs of HD1, HD6, HD7, D18.3 and A20 has been determined by elution and sequencing of self peptides. Anchor residues are shaded in red, with auxillary anchor residues which were also identified but in reduced frequency shaded in blue. Other preferred residues are shown in green.

	1	2	3	4	5	6	7	8	9	10
HD6		Q I L	K Y H	P	H K		H		R H	
HD1		E Q T	I L M F	P H	N					
HD7		V I A Q S T	P L M	P M T Q	F	Q	I	Q	H V	Y
A20	A I	K	K	D P	D P	K I P V D	I V L	R Q	R	
D18.3		P							I V	

1.10 Typing MHC genes

Polymorphisms of the human MHC system have been traditionally detected by complement mediated microcytotoxicity. While this method is still widely used (Kurz *et al.*, 1999) it is not able to accurately define all MHC class I antigens due to lack of specificity and reagents. Typing now relies on either sequence-based or conformational methods or a combination of both. Examples of both are discussed below.

1.10.1 Sequence-based methods

Three main approaches can be included in this grouping; PCR using sequence-specific primers (PCR-SSP), PCR followed by probing of gels with an oligonucleotide probe (PCR-SSOP) and direct sequencing. These methods generally rely on the identification of a particular motif within the target sequence. Evolution of MHC class I genes through both inter- and intra-locus recombination has resulted in each allele consisting of a unique combination of sequence motifs, but with motifs shared between alleles (Parham *et al.*, 1995). As a result, targeting a particular section of sequence can prove difficult. The presence of pseudogenes and gene fragments within the MHC region can also hinder this. In terms of practicality with such a large number of alleles (309 HLA-A, 563 HLA-B and 167 HLA-C, IMGT/HLA database, www.ebi.ac.uk/imgt/hla) in the MHC class I region the requirement for reagents such as allele-specific primers means the process can become unwieldy, with many reagents required for typing an unknown sample. In

addition the system needs to be constantly reviewed to incorporate new allele-specific polymorphisms that can be used for a specific primer or probe. It is possible to directly sequence every sample using for example locus-specific primers but this approach is not only time consuming and expensive but also has the added problem of discerning to which allele the sequence belongs to in a heterozygous individual (cis/trans distinction). Sequence-based typing and the other PCR-based systems are also subject to interference from PCR errors and intra-PCR and intracloning recombination which further adds to their unreliability (Ennis *et al.*, 1990, Longeri *et al.*, 2002).

PCR-SSP has been successfully used to type for a panel of MHC class I alleles in cattle using allele-specific primers (Ellis *et al.*, 1998). These primers were tested on animals of known haplotype which carried either the target allele, alleles with high levels of homology to the target, or unrelated alleles. In all cases these primers were able to amplify a product in an allele-specific manner. However the authors did speculate on the intrinsic usefulness of such a system since it is likely that the alleles included in this represent only a small proportion of the total polymorphisms in the bovine MHC class I. However with research concentrating on inbred herds and small numbers of animals this method is applicable for small scale typing. The use of PCR-based typing systems is also limited due to potential problems with class I allele sequence variation between breeds, including breed-specific differences in the class I types represented (Bull *et al.*, 1989).

1.10.2 Conformational methods

The problems associated with sequence-based typing have been largely overcome through the use of conformational methods which are based on the behaviour of DNA as it moves through a polyacrylamide gel. Two widely used examples of this are single strand conformation polymorphism techniques (SSCP) and heteroduplex analysis (HA). With SSCP DNA is normally amplified in a group-specific manner (i.e. using primers that amplify a range of alleles so as to reduce the number of primers required), then denatured and run on a non-denaturing polyacrylamide gel. Alternatively, single strands are produced by having one primer in excess in the PCR. After the low concentration of the other primer is used the reaction continues producing only the target strand (asymmetric PCR). Differences in sequence between samples cause the single strands to fold differently with intrastrand base pairing leading to loops and compaction which affects their mobility through the gel (Orita *et al.*, 1989). The bands can be visualised in a variety of ways including the use of radiolabels or fluorescent tags on the primers in the PCR, or by silver or ethidium bromide staining. It is important that the conditions are optimised to ensure optimal migration since the differences between samples may be subtle. This method is particularly useful in, for example, comparing the class I alleles between donors and recipients for bone marrow transplants by comparing the banding patterns. The advantages of SSCP include the fact that this is a simple method with no difficult reactions and is relatively inexpensive. However this method is limited in its use since only small fragments (up to 300bp) can be analysed (Sheffield *et al.*, 1993). In addition it has been observed that single strands with identical sequences

can adopt multiple conformations meaning banding interpretation can be difficult. The reproducibility is also poor with inter and intra gel variation occurring.

HA has been successfully used for analysing class I genes (Martinelli *et al.*, 1996), although its main application is for screening potential bone marrow donors. Duplexes are formed between, for example, a test sample and a known control sample during the final stages of PCR along the length of which are regions of complementarity and also differences depending on how similar the sequences are. At positions where the sequences do not match the DNA bulges or loops out of the duplex which affects the electrophoretic mobility of the duplex. These heteroduplexes can be detected as extra slow moving bands in polyacrylamide gels compared to homoduplexes. Under defined conditions each duplex has a unique conformation with no multiple conformations occurring due to the increased stability of duplexes compared to single strand DNA. This technique has the same advantages as SSCP but can analyse longer fragments. Problems include the inability to distinguish samples that differ by only one or two aa and difficulty in distinguishing heteroduplexes from homoduplexes due to the strong intensity of the homoduplex signal.

1.10.3 Reference strand-mediated conformational analysis – RSCA

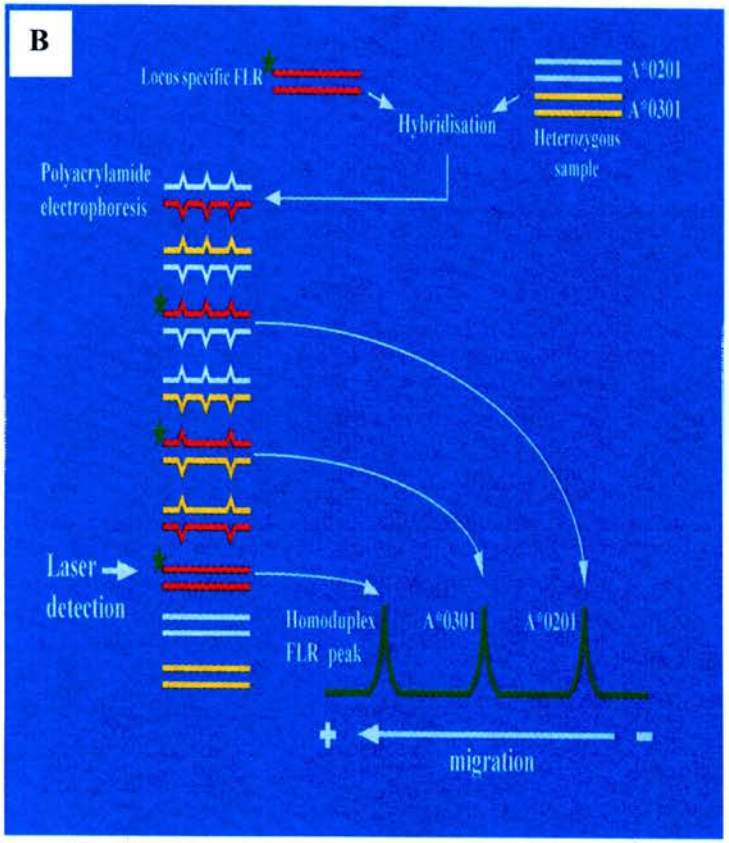
This method was designed at the Anthony Nolan Research Institute in 1998 (Arguello *et al.*, 1998a) to combine the advantages of HA with a laser-based

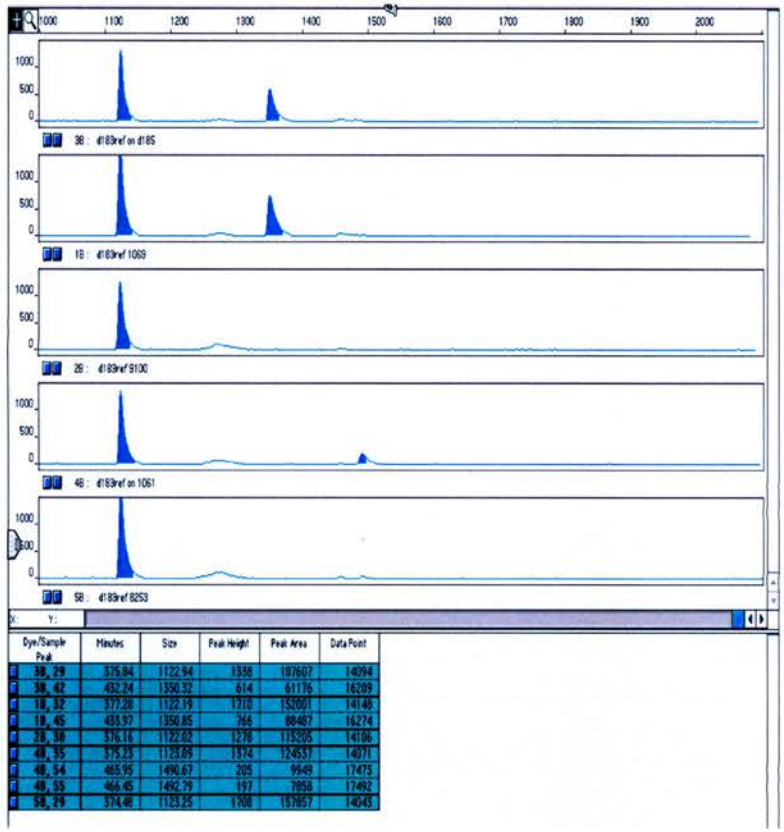
detection system and computer analysis packages which allow the detection of alleles which differ by only one nucleotide (Arguello *et al.*, 1998b).

RSCA uses a fluorescently labelled locus-specific reference strand (FLR) produced by PCR with locus-specific primers. The fluorescent tag is introduced by labelling the 5' end of the forward primer. The primers amplify the entire exons 2 and 3 and intron 2. This should allow detection of all alleles since the majority of polymorphisms are located here. The FLR is then hybridised to the test sample amplified with the same, but unlabelled, primers. The duplexes formed are then loaded and run on a non-denaturing polyacrylamide gel in an automated DNA sequencer, with the laser only detecting the duplexes with the label attached. This overcomes the problems of interpreting the complex banding patterns observed in HA and SSCP. Markers or size standards are included in each of the lanes and the duplexes are assigned a mobility value relative to the marker as determined by an analysis package such as Genescan (Applied Biosystems, Cheshire). For each sample tested the number of duplexes identified corresponds to the number of alleles present in the sample since each duplex generated will have a unique mobility i.e. heterozygous samples give two heteroduplexes plus the FLR homoduplex while homozygous samples give only 1 heteroduplex. There is a possibility that the inclusion of the intron sequence may affect the mobility of the duplex with any intra-allelic polymorphisms being misinterpreted as a new coding polymorphism. This does not appear to be the case however since analysis of 3800 alleles from different samples identified only one case of this (Arguello *et al.*, 1998b). A diagram of this method is shown in fig. 1.13.

Figure 1.13 Reference strand mediated conformational analysis (RSCA)

Samples and reference strands are hybridised to each other forming a heteroduplexes and homoduplexes. These are then loaded and run on a polyacrylamide gel. The size of the duplex determines how fast it moves, with mismatches between the strands affecting the size. Only duplexes with the fluorescent label attached can be detected by the laser (a, adapted from Arguello *et al.*, 1998). These samples are then analysed using genescan software (b).





1.10.3.2 Advantages of RSCA

RSCA has been designed in such a way as to overcome the disadvantages associated with other conformational methods discussed previously. The use of known reference strands allows the conformation of duplexes to be manipulated, with the use of different references meaning it should be possible to identify all allelic variants. The inclusion of a fluorescent label and the laser detection system has a number of advantages. In other methods variation in signal intensity can mean that some samples are not detected and this also prevents direct comparison between two samples. RSCA is much more sensitive allowing the detection of weak signals. It is also possible to compare between samples differing in signal intensity with the analysis package compensating for lane to lane variation. In addition much less DNA is required than with HA and SSCP since the resolution of duplexes is inversely proportional to the amount of DNA loaded.

Problems with inter- and intra-gel variability are eliminated by the inclusion of a marker or size standard in each lane since the ratio of mobility of each duplex is constant in relation to the standard regardless of lane variation. This means that it is possible to normalise the samples across and between gels.

Resolution of samples is also a problem associated with both HA and SSCP. DNA samples all migrate at different rates depending on their size with bigger fragments moving more slowly through gels. Since resolution depends on how far the DNA has migrated during electrophoresis large samples will be poorly resolved. With

RSCA all samples are detected after they have migrated the same distance (by having a fixed detection system) so removing this problem. Similarly, it is very difficult to resolve heteroduplexes and homoduplexes in HA where the test sample is very similar to the reference strand since the homoduplex signal will be much more intense. By varying the ratio of the reference strand to the test allele in RSCA it is possible to reduce the homoduplex signal and increase the heteroduplex signal thereby allowing detection.

RSCA has been shown to be highly reproducible, with a minimum amount of variation of mobility values observed between gels (Arguello *et al.*, 1998b).

1.11 *Theileria parva*

Theileria parva is a tick-borne apicomplexan parasite which infects domestic cattle (*Bos taurus* and *Bos indicus*) and buffalo (*Syncerus caffer*, Morrison *et al.*, 1989). Prevalent in sub-Saharan Africa, it causes a severe lymphoproliferative disease known as East Coast Fever which results in the death of up to 1 million cattle a year and severe economic losses, estimated to be around US $168 million per year (Mukhebi *et al.*, 1995). Some animals can recover from infection and are found to have solid immunity to challenge with the same strain of parasite (Burridge *et al.*, 1972). Infection in buffalo is less devastating, forming a persistent infection with no clinical signs of disease. As a result the buffalo acts as a reservoir of infection (Norval *et al.*, 1992). Control of infection has largely relied on treatment of animals with acaricides to prevent ticks from attaching. The reliability of this method has

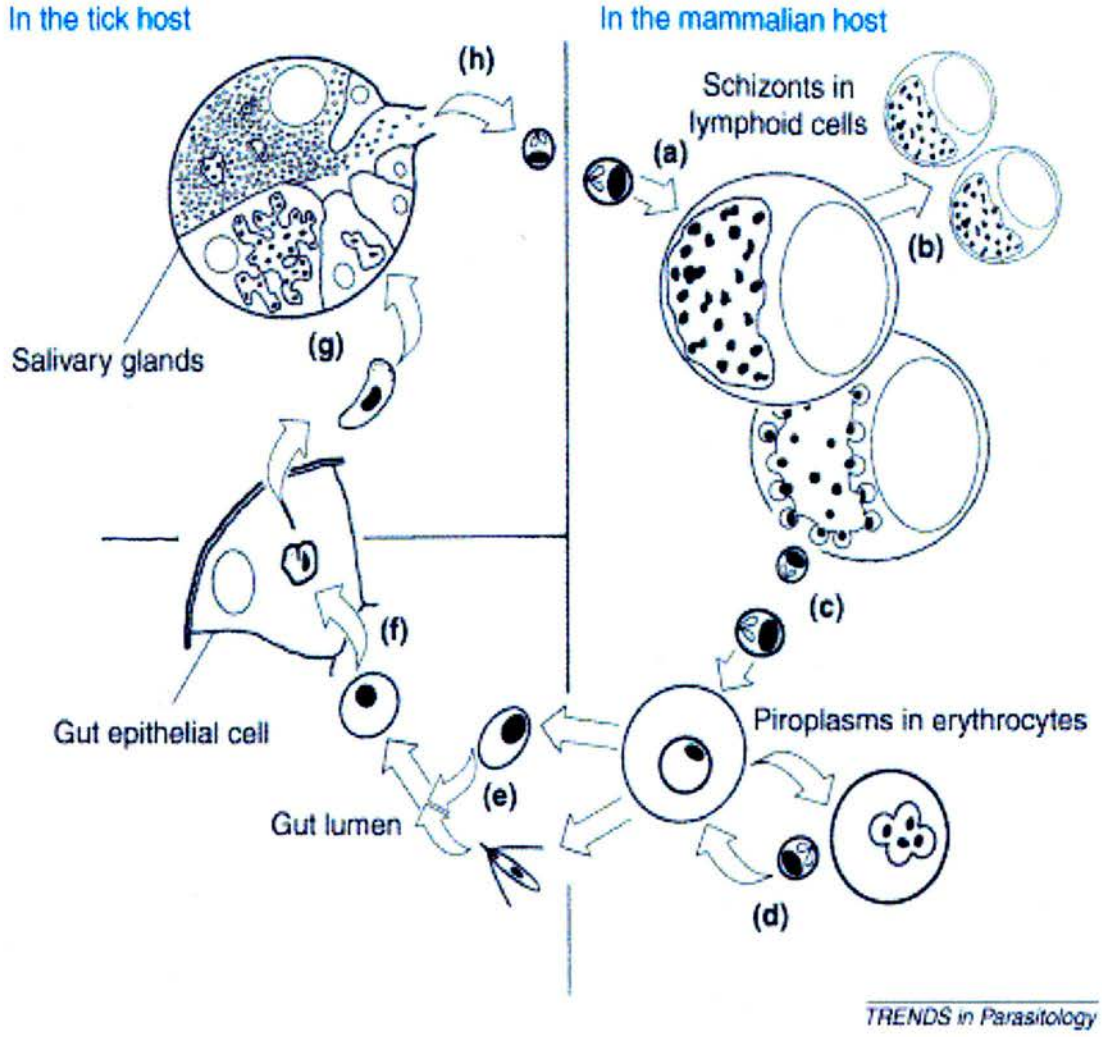
dropped however due to reduced public spending by governments, increased costs of acaricides and poor control over management of treatments (Dolan, 1999). As a result the development of alternative methods, such as vaccination, would prove invaluable.

1.11.2 Biology of the parasite

Theileria parasites are obligate intracellular parasites belonging to the phylum Apicomplexa which includes the Plasmodium and Eimeria parasites (reviewed in Irvin and Morrison, 1987). The life cycle of this parasite is shown in fig. 1.14. *T. parva* is transmitted by the three host tick *Rhipicephalus appendiculatus* which delivers the parasite to its main host when taking a blood meal. On entering the blood stream, the infective stage, sporozoites, quickly infect lymphocytes. This initial interaction is a chance event since the parasites are non-motile. Parasites are thought to bind and enter the cell by receptor-mediated endocytosis (Fawcett *et al.*, 1984) with a zippering process occurring between the two membranes allowing complete internalisation of the parasite (Shaw, 1999). Unlike a number of other intracellular parasites, *T. parva* does not remain within a parasitophorous vacuole but instead lies free within the cytoplasm. The contents of the rhoptries and microspheres, organelles within the parasite, are released which separate the parasite and host membranes, followed by the establishment of a host-derived microtubule array around the parasite (reviewed by Shaw, 2003).

Figure 1.14: Life cycle of *Theileria parva*

Taken from Shaw, 2003



On taking a blood meal sporozoites are released from the tick and enter the blood stream where they infect lymphocytes (a). Within the cell the parasite multiplies to form a schizont which stimulates the cell to divide with the parasite passing into both daughter cells (b). Some of the schizonts undergo merogony releasing merozoites which infect RBC (c). Some *Theileria* parasites but not *T. parva* can undergo replication in the RBC (d). Infected RBC are taken up by a tick when it next feeds. Within the tick the merozoites differentiate to gametes which then fuse to form zygotes (e). These pass into the epithelial cells of the gut (f) and further differentiate into kinetes which migrate to the salivary glands (g). Sporozoite production is induced when the tick next feeds (h).

Over the next 24 to 48 hours the parasite multiplies forming a multinucleated schizont. This stimulates the host cells to undergo blastogenesis during which the schizont associates with the mitotic spindle and divides, with parasites passing into both daughter cells (Hulliger *et al.*, 1964). *T. parva* is able to immortalise the cells in which it resides meaning that infected lymphoblasts (produced *in vivo* or *in vitro*) can be grown continuously *in vitro* as parasitised cells (Brown *et al.*, 1973). Multiplication is largely by clonal expansion with infected cells disseminating throughout the lymphoid tissues rapidly followed by extensive lysis of both infected and uninfected cells.

The life cycle within the animal is completed when some of the macroschizonts differentiate to microschizonts. Merogony then takes place, with merozoites released which infect erythrocytes forming piroplasms. When a tick next feeds it ingests infected red blood cells with the bloodmeal. Within the tick gut these cells are lysed, releasing the piroplasms, which differentiate to male and female gametes, then fuse to form zygotes. The zygotes then invade the gut epithelial cells and develop to motile kinetes. These are released into the haemocoel and migrate through the haemolymph to the salivary gland where sporogony takes place, resulting in the production of large numbers of sporozoites (Mehlhorn and Schein, 1984, Fawcett *et al.*, 1982).

1.11.3 Course of the disease

Once animals are infected, several days lapse prior to the appearance of the parasites as schizont-infected lymphocytes in the lymph nodes draining the site of infection but by day ten to fourteen parasites are evident in most lymphoid tissues (Morrison *et al.*, 1989). Extensive damage of lymphoid cells ensues with a large drop in the number of circulating lymphocytes. Death can occur three to four weeks after the initial infection (Morrison *et al.*, 1987). Animals which die from infection are found to have large numbers of parasitised cells in their lymphoid system with an overall decrease in lymphocytes in both the circulating pool and lymphoid tissue (Irvin and Morrison, 1987, Jarrett *et al.*, 1969, Emery, 1981).

1.11.4 Target cell types and pathogenicity

Early studies on the cell type infected by *T. parva* were restricted to the use of parasitised cells taken from infected cattle and cell lines. Using reagents specific for bovine antibodies, *T. parva*-infected cells were tested and found not to express any surface immunoglobulins (sIg), suggesting that the parasite infects T cells (Duffus *et al.*, 1978). Pinder *et al.* (1981) tested a panel of lectins and monoclonal antibodies on *T. parva*-infected cell lines in an attempt to further classify the surface phenotype. They found that the parasitised cell lines were positive for markers found only on a subpopulation of T cells.

The development of lymphocyte-sorting techniques utilising lineage-specific monoclonal antibodies was able to show that *in vitro T. parva* is capable of infecting and transforming αβ T cells, γδ T cells and B cells (Baldwin *et al.*, 1988). Following infection of PBM with high concentrations of sporozoites, parasitised B cells were evident in large numbers for the first week but were quickly overgrown by T cells (Morrison *et al.*, 1996). Also *in vivo* studies showed that parasitised cells taken from animals given a lethal dose of *T. parva* predominantly expressed T cell markers (Emery *et al.*, 1988) with three populations observed, CD4+ CD8-, CD4+ CD8+ and CD4- CD8+. Together these data show that T cells dominate the infection.

1.11.5 Immunity to *T. parva*

Investigation into the immune response against *T. parva* has been greatly aided by the development of the infection and treatment regime. Animals are inoculated with a lethal dose of viable sporozoites, generally obtained from a cryopreserved stabilate, and simultaneously treated with oxytetracycline (Radley *et al.*, 1975). As a result animals are able to mount an immune response and recover, displaying only mild, asymptomatic infections. These animals are immune to challenge with the same isolate but small numbers of infected cells are evident in the draining lymph node at approximately the same time as in susceptible animals. This suggests that immunity does not prevent infection of cells but rather acts against the infected cells.

A sporozoite-specific antibody response can be detected in the serum of animals undergoing heavy challenge in endemic areas (Musoke *et al.*, 1982). *In vitro* studies

have shown that, amongst others, the p67 surface protein induces antibodies that prevent sporozoites from infecting cells (Musoke *et al.*, 1984). This humoral response is not thought to play a role in the response of naive cattle to *T. parva* however (Morrison, 1996).

Considering the intracellular location of the parasite and the fact that attempts to identify parasite-derived antigens on the surface of lymphocytes have failed (Morrison and McKeever, 1998), it seems likely that the major immune response produced against *T. parva* is cell-based.

Evidence for such a response was provided by Muhammed *et al.* (1975) who found that transferring serum from an immune animal to a naive animal did not transfer protection to *T. parva*. However when leucocytes were transferred, the naive animal was able to make a response and recover from infection (Emery, 1981). Infection and treatment has been shown to result in the production of a strong cytotoxic response in cattle around the time of remission of infection. PBM taken from calves immunised with *T. parva* then challenged four weeks later with either the same isolate or with sporozoites adsorbed on autologous PBM were tested for cytotoxicity on a range of target cells in a standard ^{51}Chromium release assay. Results showed that both challenge protocols produced CTL against autologous cells but not against allogeneic or xenogeneic infected cells. Thus there appeared to be a specific set of cytotoxic cells that develop following challenge that appear to be genetically restricted (Eugui and Emery, 1981).

These cytotoxic cells are now known to be part of the CD8+ subset of lymphocytes as shown by transfer studies between identical calves produced by embryo splitting. One calf from each set was immunised and samples of efferent lymph lymphocytes taken over a length of time following challenge with a lethal dose of parasites (McKeever *et al.*, 1994). These efferent lymph cells were then depleted of CD4+ T cells, γδ T cells and B cells and transferred daily to the infected calf that had been infected. This allowed the animal to control the emerging parasitosis and recover. In contrast the control animals developed severe symptoms. To rule out the possibility of any remaining factors in the transfer samples other than CD8+ cells that may be affecting the results, CD8+ cells were also removed. Transfer of the remainder did not provide protection. These results clearly show that protection against *T. parva* is mediated by CD8+ cytotoxic T cells.

1.11.6 Role of cytokines

Whether CD8+ T cells mediate protection alone is not known, however to date none of the cytokines tested including interleukin (IL)-1, IL-2 and IL-4 were able to inhibit the proliferation of infected cell lines (Preston *et al.*, 1992). Some inhibition of early development of the parasite within the cell may occur (Morrison and McKeever, 1998).

The cytokines produces by infected cells have been found to vary, with only IL-10 constitutively expressed (McKeever *et al.*, 1997). IL-2-like components produced by transformed cells have also been identified (Brown and Logan, 1986). Both of these

cytokines may have a role in encouraging proliferation (Morrison and McKeever, 1998).

1.11.7 Role of CD4+ and γδ T cells

Following immunisation, parasite-specific CD4+ T cells have been detected which are thought to be capable of lysing infected cells (Baldwin *et al.*, 1987, McKeever *et al.*, 1999). In addition, CD4+ T cells are known to produce IL-2, which can then act directly on CD8+ cells allowing them to proliferate and lyse infected cells. This rapid CD8+ response can then allow early control of the infection.

γδ T cells have also been shown to be involved in the immune response to a primary *T. parva* infection. They are able to proliferate in response to lymphoblasts and lyse infected cells but in an MHC-unrestricted manner. This response can be detected before the CD8+ T cell response (Morrison *et al.*, 1995) and is thought to be as a result of the γδ T cells recognising stress proteins on the infected cell surface (Daubenberger *et al.*, 1999).

1.11.8 Parasite strain heterogeneity

Heterogeneity was originally detected in cross-protection studies where animals were immunised with one isolate of parasite, then challenged with another. Generally it was found that animals were protected from the isolate with which they had been

immunised but varied in their susceptibility to others. In addition protection against one strain was not always reciprocal (Radley *et al.*, 1975).

Using rare cutting enzymes and pulse-field gel electrophoresis differences between isolates were identified showing that there is heterogeneity between strains (Morzaria *et al.*, 1990). A major problem with cross-protection studies is the presence of more than one population within a stock for example the Marikebuni stock is thought to contain at least five strains (Taracha *et al.*, 1995). In contrast, the Muguga stock is known to be highly homogenous (Morrison, 1996).

1.11.9 MHC restriction of *T. parva*-specific CTL

As with the human system, the MHC in cattle has been shown to restrict the ability of T cells to recognise antigens. Three CD8+ clones isolated from PBM from an animal infected with *T. parva* (Muguga stock) were tested for cytotoxicity against 32 infected cell lines. Only cells with the KN104 specificity were killed, with both autologous and allogeneic cells affected. Administration of specific antibodies and alloantisera to the expressed specificities blocked killing, confirming the involvement of MHC class I molecules in restricting cytotoxicity (Goddeeris *et al.*, 1986a).

In order to investigate this restriction further, the CTL derived from ten immunised animals were tested for cytotoxicity on a panel of parasitised cells with matched or mismatched specificities. In all cases killing of mismatched cells was minimal, with

cells from nine out of ten animals preferentially killing cells sharing BoLA specificities. Within this, there was a clear bias towards killing of one specificity over another with a hierarchy of preferentially killed cells evident overall (Morrison *et al.*, 1987).

Further work involved the combination of a limiting dilution assay with statistical analysis to allow the quantitation of memory CTL precursors (CTLp) in the blood of immunised MHC heterozygous animals expressing known specificities. T cells from these animals were restimulated *in vitro* with parasitized cell lines then tested for cytotoxicity against a panel of MHC homozygous targets in an Indium oxine release assay. Based on the isotope release values and the number of T cells per well it was possible to determine the CTLp frequency specific for each target. In most cases the CTLp were almost entirely restricted to one haplotype, with the A6, KN8 and A10/KN104 haplotypes appearing dominant overall. Within these, A10/KN104 was dominant over KN8 while A6 was dominant over both (Taracha *et al.*, 1995). Furthermore it was found that the CTLp of Muguga-immunised animals were restricted entirely by the KN104 specificity, with all but one animal producing a strain-specific response. This one animal was found to also produce CTL capable of reacting to Marikenbuni 3219, a strain of the Marikebuni stock. The presence of cross-reactive KN104-restricted CTLp in this animal suggests that its class I molecules are capable of presenting conserved antigens between strains of parasites.

T. parva is a large complex parasite and so it would easily be assumed that a wide range of antigenic peptides would be available for presentation. However these

results seem to point to a limited repertoire, with restriction being controlled by only one type of MHC molecule in most animals (Taracha *et al.*, 1995). In addition, animals which were shown to have KN104-restricted, strain-specific CTL responses following immunisation with the Muguga strain developed cross-reactive CTL after challenge with Marikebuni 3219, a proportion of which were also restricted by KN104. Two identical calves were also infected, one with the Muguga strain and one with Marikebuni 3219. The Muguga infected animal was found to produce a strain-specific response and the other a cross-reactive response, despite both being restricted by KN104 (Taracha *et al.*, 1995). Since these animals are genetically identical this shows that the Muguga infected animal is capable of responding to determinants in both strains of parasite, but for some reason produces a strain specific response.

1.11.10 Immunodominance

In an attempt to explain the above results it has been suggested that immunodominance is occurring whereby different MHC-peptide combinations differ in their ability to elicit an immune response, with some being dominant over others. The variation in specificity following challenge with Marikebuni 3219 suggests there are either a limited number of dominant determinants or the immune response is focused on only a few of the most dominant (Morrison, 1996). Possible reasons for immunodominance include levels of expressed MHC class I molecules, affinity for peptide binding and variations in the concentrations of peptides, possibly as a result of competition in the antigen processing pathway.

1.11.11 Consequences of immunodominance and hierarchy of MHC Class I restriction

These results have important implications for both vaccine design and breeding programmes. Theoretically it appears that vaccines should be directed to those conserved antigens (rather than strain-restricted epitopes) resulting in cross-reactive responses. In addition, by identifying the dominant MHC types that provide protection it may be possible in the long term to introduce breeding strategies to ensure that these dominant haplotypes are distributed among herds at risk. However it must be noted that these experiments were carried out on a limited number of animals and may not be representative of the general cattle population.

1.12 Aims of this research

Available evidence suggests that the bovine MHC class I system may be the most complex of all mammals studied to date with variable numbers of haplotypes and a lack of defined loci. Based on phylogenetic analysis and comparison of haplotypes, there appears to be six classical class I genes in cattle, none of which are consistently expressed. In addition, the number of classical genes on a haplotype can vary, with three genes the most found so far. The principle aim of this project was the establishment of an effective method for typing bovine class I alleles based on the RSCA method described in section 1.10.3. This would allow not only typing of known alleles but also detection of new ones. In addition it would provide us with another tool for future investigation of, for example, the variability of haplotype

composition within herds and determination of levels of polymorphism for each gene.

Within this study we aimed to establish and optimise RSCA for a number of the bovine class I genes followed by a small study on untyped animals to prove that the method was working. We proposed that the introns adjacent to exons 1 and 2 of class I genes would contain locus-specific nucleotides around which primers could be designed for locus-specific amplification and then used in conjunction with published protocols to attempt to type class I genes.

A second component of this study focussed on the function of MHC class I molecules, investigating the genetic restriction of CTL in response to the intracellular parasite of cattle *T. parva*. Previous studies found the CTL response to be almost entirely restricted by either the maternal or paternal haplotype and by analysis of a panel number of heterozygous animals a hierarchy of dominance was determined. At the time of these studies little information was available regarding nature of the class I genes present on the different haplotypes. The second aim of this project concentrated on continuing this work, testing the hypothesis that CTL from our animals would also show a bias in MHC restriction. In addition since the haplotypes being investigated have been fully characterised and their classical class I genes identified we could further investigate dominant haplotypes using newly available techniques and resources such as monoclonal antibodies and transfection methods in an attempt to define the genes responsible for restricting the immune response.

Chapter 2

General materials and methods

This section lists those methods that were used routinely throughout this project. Specific methods are described in more detail in the individual results chapters. All materials were obtained from Invitrogen (Paisley, Scotland) unless otherwise stated. All kits were used according to the manufacturers' guidelines.

2.1. Materials

2.1.1 Buffers/solutions

All buffers and media were prepared using MilliQ ion-exchange purified water (Media department, IAH, Compton). Chemicals were obtained from BDH Laboratory Supplies, Poole, England.

Buffer	Reagents
Tris-acetate/EDTA (50xTAE)	2M Tris base, 1M glacial acetic acid, 0.05M EDTA (pH 8.0)
Tris-Cl/EDTA (TE)	10mM Tris-Cl, 1mM EDTA (pH 8.0)
Tris-borate/EDTA (10xTBE)	0.9M Tris base, 0.9M boric acid, 0.02M EDTA (pH 8.0)

Buffer	Reagents
Gel loading buffer (6x)	0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water
Phosphate buffered saline (PBS)	0.15M NaCl, 2.5mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4 (pH 7.4)

2.1.2 Bacterial Growth Media

Luria-Bertani (LB) medium	1% bactotryptone, 0.5% bacto yeast extract, 0.5% NaCl
SOB medium	2% bactotryptone, 0.5% bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$
SOC medium	As SOB plus 20mM glucose

LB agar plates were prepared by adding 15g of Bacto-agar to 1 litre of LB medium. This was then supplemented with 0.5mM IPTG and 80μg/ml of X-gal prior to plating.

2.1.3 Kits

QiaAmp DNA blood minikit	Qiagen UK Ltd., Crawley, UK
Qiagen gel extraction kit	
Qiagen miniprep kit	
Dynal mRNA extraction kit	Dynal A.S., Oslo, Norway
Invitrogen first strand cDNA synthesis kit	

2.2 General methods

2.2.1 Blood sampling and extraction of PBM

Venous blood was collected from animals into syringes containing 100IU of Heparin sodium (Leo Laboratories Ltd., Bucks) for every 10ml of blood collected. Using glass pipettes blood was slowly layered on top of histopaque 1083 (Sigma, St Louis, USA) at a ratio of 5:3 in a sterile 50ml Falcon tube. Samples were carefully loaded into a refrigerated centrifuge (Heraeus multifuge 3 S-R) and spun at 2000xg, 4°C for 40 minutes (min) without the brake applied. This allows the samples to separate into the following layers; plasma and platelets at the top, then lymphocytes (visible as a cloudy white interface), then histopaque and red blood cells (RBC). Lymphocytes were removed using a pastette, ensuring no RBC were taken up, and transferred to a clean 25ml Universal tube.

PBM were then washed by centrifugation with phosphate buffered saline (PBS, media department, IAH, Compton) at 400xg for 5 min, then 2 to 3 times more at 300xg for 5 min. On the final wash the cells were resuspended in 10ml of PBS and counted using a haemocytometer (see section 2.2.2). For cDNA and genomic DNA extraction purposes cells were pelleted in 15ml Falcon tubes (~5 x 10^6 cells) and all liquid removed prior to snap freezing on dry ice. Samples were then stored at -80°C until required. Alternatively cells were pelleted and all PBS removed before resuspension in culture media for use in cell culture.

2.2.2 Quantitative determination of cell viability

The number of live cells present in a sample was determined by dye exclusion with Trypan Blue (Sigma). Cells were mixed with Trypan Blue at a 1:1 ratio and counted using a Neubauer haemocytometer. Taking into account the dilution factor, 100 cells per 16 square grid is the equivalent of $1x10^6$ cells per ml. At least two grids were always counted to enable a good estimation.

2.2.3 Genomic DNA isolation

Genomic DNA was prepared from lymphocytes using the QIAamp DNA blood mini kit (Qiagen). Briefly, $5x10^6$ cells (either frozen pellet or fresh) were lysed with 20µl Qiagen proteinase (20mg/ml) followed by addition of a high salt containing binding

buffer. This solution was then passed through a QIAamp spin column containing a silica gel membrane, allowing the DNA to bind to the membrane. The columns were washed twice to remove residual contaminants and the DNA eluted from the column in 200μl elution buffer (10mM TrisCl, 0.5 mM EDTA) and stored at -20°C. $5x10^6$ PBM produced approximately 6µg DNA.

2.2.4 Isolation of mRNA

Messenger RNA was extracted from lymphocytes using the Dynal mRNA direct kit. This is based on the system of Edmonds (1971) which follows the principle that since most eukaryotic mRNA's carry poly A (adenine) regions at their 3' ends they can be separated from the RNA by affinity chromatography on oligo (dThymine, dT) cellulose i.e. oligo dT's linked to a supporting matrix. This kit utilises magnetic beads coated with oligo dT's. $5x10^6$ cells were lysed in Dynal lysis binding buffer (100mM Tris-HCl pH 7.5, 500mM LiCl, 10mM EDTA pH8 1% LiDS, 5mM dithiothreitol, DTT). The lysate was then passed repeatedly through a 21-gauge needle using a 1ml syringe to shear the DNA and mixed with the oligo dT-coated beads to isolate the mRNA. The beads were then fixed by a magnet and washed with various buffers to remove any non-poly A RNA's and other residual components. mRNA was then eluted in 20µl of 10mM Tris-HCl. 5 x 10^6 lymphocytes produced approximately 2µg RNA for use in cDNA synthesis. Sterile conditions were adhered to throughout this procedure to prevent the addition of RNases.

2.2.5 First strand cDNA synthesis

First strand cDNA was produced using the Invitrogen Superscript™ first strand synthesis kit immediately following mRNA isolation. An RNA-dependent DNA polymerase reverse transcriptase (Superscript™ II RNase H^-) and poly dT primer act on the mRNA producing a hybrid mRNA-cDNA molecule, followed by phenol-chloroform extraction to remove proteins and purify the DNA. The mRNA is removed later during the first stages of PCR. First strand cDNA was produced by adding approximately 2μg of mRNA to 5x first strand buffer (100mM Tris-Hcl pH 8.4, 250mM KCl, 15mM $MgCl_2$), 10mM dNTP mix-10mM each dATP, dCTP, dGTP, dTTP (Amersham Biosciences, N.J., USA), 0.1M DTT and 50U reverse transcriptase to a final volume of 25μl followed by incubation at 37°C for 1 hour.

2.2.6 Phenol chloroform extraction

The mRNA-cDNA solution was made up to 100μl with DEPC water (media department, IAH, Compton) and an equal volume of phenol chloroform added (one part phenol to one part chloroform). This was vortexed until a white emulsion formed, centrifuged at 15000xg for 5 min in an IEC micromax benchtop microfuge (IEC, MA, USA) to separate the aqueous and organic phases and the top aqueous layer removed to a clean eppendorf. An equal volume of chloroform was added to this, vortexed and the top layer removed to a fresh tube for DNA precipitation.

2.2.7 Ethanol precipitation

10% 3M Sodium acetate (Sigma) and 2.5x volume 100% cold ethanol was added to the DNA solution (approximately 100µl) and incubated at -20°C overnight to precipitate the DNA. This was then pelleted by centrifugation (15000xg for 15 min) and washed once with 300µl of 70% cold ethanol to remove any residual salts. Excess ethanol was carefully removed and the pellet allowed to dry at room temperature. Following this, the DNA was resuspended in 20µl of TE buffer and stored until use at -20°C.

2.2.8 Polymerase chain reaction (PCR)

2.2.8.1 PCR program

PCR was employed throughout this project for amplification of specific regions within MHC class I sequences. The standard program used was as follows with the annealing temperature (X) adjusted for optimal annealing of primers: 95°C for 5 min, 30 cycles of 95°C for 30 seconds (s), X°C for 50s, 72°C for 1 min with a final extension step of 72°C for 5 min. All samples were run in a Peltier thermal cycler 200 (MJ Research, MA., USA).

2.2.8.2 PCR reaction

Standard reactions contained 1.5 mM $MgCl_2$, 1x PCR buffer, 0.25mM of each dNTP's (Amersham), 1μM of each primer, 0.5 units of Taq polymerase and 6ng DNA with DEPC-treated water (media department, IAH, Compton) to a final volume of 25 or 50μl.

2.2.8.3 Primer design

Primers were designed according to the following criteria:

(1) Length between 18 and 30 nucleotides, with a GC content between 40% and 60%.

(2) The annealing temperature (T_m) of the primers should be between 55°C and 75°C, with both primers of a pair having similar temperatures. T_m is estimated by the following calculations:

4(G+C) + 2(A+T) for primers of less than 20 base pairs (bp)

62.3°C + 0.41°C (%GC) – 500/length - 5°C for more than 20 bp

(3) The 3' base of the oligo should be a G or C if possible, preferentially with an A or T before it. This increases the specificity of binding and reduces the formation of hairpin structures or primer dimers.

(4) There should be low complementarity between members of a primer pair.

(5) There should be no inverted repeat sequences or self-complementarity sequences greater than 3 bp to prevent the formation of hairpins structures which can stop oligos from annealing to the target DNA.

All oligos were ordered from either MWG Biotech (UK) Ltd. (Milton Keynes, UK) or Invitrogen.

2.2.9 Electrophoresis of DNA

PCR products were premixed with 6x gel loading buffer and loaded onto 1% agarose gels containing 0.5μg/ml Ethidium Bromide. Gels were run in 1x TAE buffer at 5V/cm with the running time determined by the agarose concentration and the predicted PCR product size (Sambrook *et al*, 1989). Samples were run alongside a φX174 Hae III digest marker or a 1Kb plus DNA ladder (Sigma) to allow the products to be sized. Gels were visualised under UV light using a UVP transluminator and the images recorded on a UVP GDS5000 camera/printer system.

2.2.10 DNA extraction from agarose

Bands of the correct size that required sequencing were cut with the gel with a scalpel blade, and DNA recovered using the Qiagen gel extraction kit. This works on a similar principle to those kits already described, with the agarose digested by the enzyme agarase followed by binding of the DNA to a spin column. The bound DNA was then washed with a range of buffers of varying pH and salt concentrations prior to elution in 30μl of elution buffer (10mM Tris-HCl pH 8.5). Eluted samples were then stored at -20°C.

2.2.11 Ligation into the pGEM-T vector

Purified DNA was ligated into the pGEM-T vector system according to manufacturers instructions with recommended controls also included (Promega UK Ltd., Southampton, UK). This vector has a multiple cloning site within the β-galactosidase gene with "sticky" thymidine overhangs ideal for the insertion of PCR products generated by the Taq enzyme used. Insertion of DNA interrupts the coding sequence β-galactosidase allowing recombinant clones to be detected by white/blue selection. pGEM-T has two bacteriophage promoters in opposite directions which flank the cloning region (Short *et al*, 1988). These primers, T7 and SP6, can be used as primers for specific sequencing of the inserted product. 3μl of DNA (approximately 20ng) was added to 2x T4 DNA ligase buffer, 50ng of PGEM-T vector and 3U of T4 DNA ligase, mixed by pipetting and incubated overnight at 4°C.

2.2.12 Transformation of JM109 competent cells with recombinant pGEM-T vectors

2μl of each ligation reaction was added to 50μl of JM109 competent cells (Promega) in 15ml Falcon polypropylene tubes and the tube gently flicked to allow mixing. The cells were incubated on ice for 20 min, heat shocked at 42°C for 50 s and then placed directly back onto ice for 2 min to allow entry of the plasmid. 900μl of SOC medium was added to each tube and the cells incubated for 1 hour at 37°C in an orbital-shaking incubator

(150rpm). Samples were plated out in duplicate (100µl) on LB plates containing ampicillin, IPTG and X-gal, the plates inverted and incubated overnight at 37°C. White colonies (indicating positive ligations) were then picked using a sterile pipette and placed in 50ml Falcon tubes containing 5ml LB and 100µg/ml ampicillin. Samples were then grown overnight at 37°C with shaking.

2.2.13 Minipreparations of purfied DNA from JM109 transformed cultures

Purified DNA was extracted from the transformed cultured using the QIAprep spin miniprep kit (Qiagen). This works by using alkaline lysis and SDS detergent (Birnboim and Doly, 1979) to lyse the bacteria and denature chromosomal DNA and proteins with plasmid DNA being released into the solution. The denatured material was then removed by centrifugation and the supernatant applied to a Qiagen spin column. The column was washed several times and the DNA eluted in 10mM Tris-Cl (pH 8.5). This was stored at -20°C until further use.

2.2.14 Sequencing

DNA was sequenced using an ABIPrism 377 automated sequencer (Applied Biosystems, Warrington, UK) with a 14-hour program based on the dideoxy method. Reactions were set up with the BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied

Biosystems) as follows: 2µl of Big Dye, 1µl of milliQ water, 1pmol of primer (either forward or reverse) and 125ng DNA to a final volume of 5µl.

These reactions were run under the following conditions: ramp of 1°C/s to 96°C, 96°C for 10 s, 1°C/s to 50°C, 50°C for 5 s, 1°C/s to 60°C, 60°C for 4 min, repeat for 25 cycles. DNA was precipitated by adding 1µl of 3M ammonium acetate (pH 4.6) and 25µl of 95% ethanol, incubating at room temperature for 15 min then centrifuging at 2900xg for 30 min. Supernatants were removed and the pellets washed with 70% ethanol, then allowed to dry. Sequencing reactions were resolved on denaturing polyacrylamide gels by the zoology department, University of Oxford, Oxford. Results were analysed using the GCG 10/Wisconsin Package Version 10.3 (Accelrys Inc., San Diego, CA) and Genedoc (http://www.psc.edu/biomed/genedoc) programs.

Chapter 3

Identification and testing of group-specific primers for use in RSCA

3.1 Introduction

To date typing of MHC class I genes in cattle has been greatly hampered by the lack of information available, with only a relatively small number of alleles identified and sequenced. In addition, since the total number of classical class I loci has not been confirmed, assignment of alleles to loci is difficult. A PCR typing system was previously devised at Compton which utilised allele-specific primers based on coding sequences obtained from a herd containing a few well characterised MHC haplotypes (Ellis *et al.*, 1998). While this method is useful for typing individual animals in such a herd, the requirement for cDNA and large number of primer pairs makes it inappropriate for wide-scale typing, both in terms of cost and time. Identification of new alleles if other animals were introduced to the herd would also prove difficult.

A typing system termed reference strand-mediated conformational analysis (RSCA) has been designed at the Anthony Nolan Research Institute. This allows rapid and easy typing of MHC class I and II genes for assessing histocompatibility between patients and donors for bone marrow and other organ and tissue transplants (Arguello *et al.*, 1998, Corell *et al.*, 2000). This method has also been successfully used in sheep, dogs and cats for typing the functional DQB and DRB genes respectively, allowing identification of both known and previously undiscovered alleles (Feichtlbauer-Huber *et al.*, 2000, Kennedy 2000, Kennedy *et al.*, 2003). RSCA and its advantages are discussed in greater detail in section 1.10.3.2. Samples to be typed are amplified by PCR from genomic DNA using group or locus-specific primers,

which negates the need for a large number of primer pairs required by systems such as PCR-SSP. It is important that these primers amplify polymorphic regions of the genes to ensure detection of all possible alleles. In humans, sequencing of introns 1 and 3 surrounding the polymorphic exons 2 and 3 of the classical loci identified so-called 'locus-specific characteristics' i.e. conserved nucleotides amongst alleles from a locus. Primers designed around these bases were shown to have excellent locus specificity which was confirmed by hybridisation of the PCR products with 10 oligonucleotide probes specific for both classical and non-classical MHC class I genes (Cereb *et al.*, 1995). These primers have been successfully used in RSCA for locus-specific amplification and are now routinely used for typing (Arguello *et al.*, 1998, Turner *et al.*, 1999, Turner *et al.*, 2001).

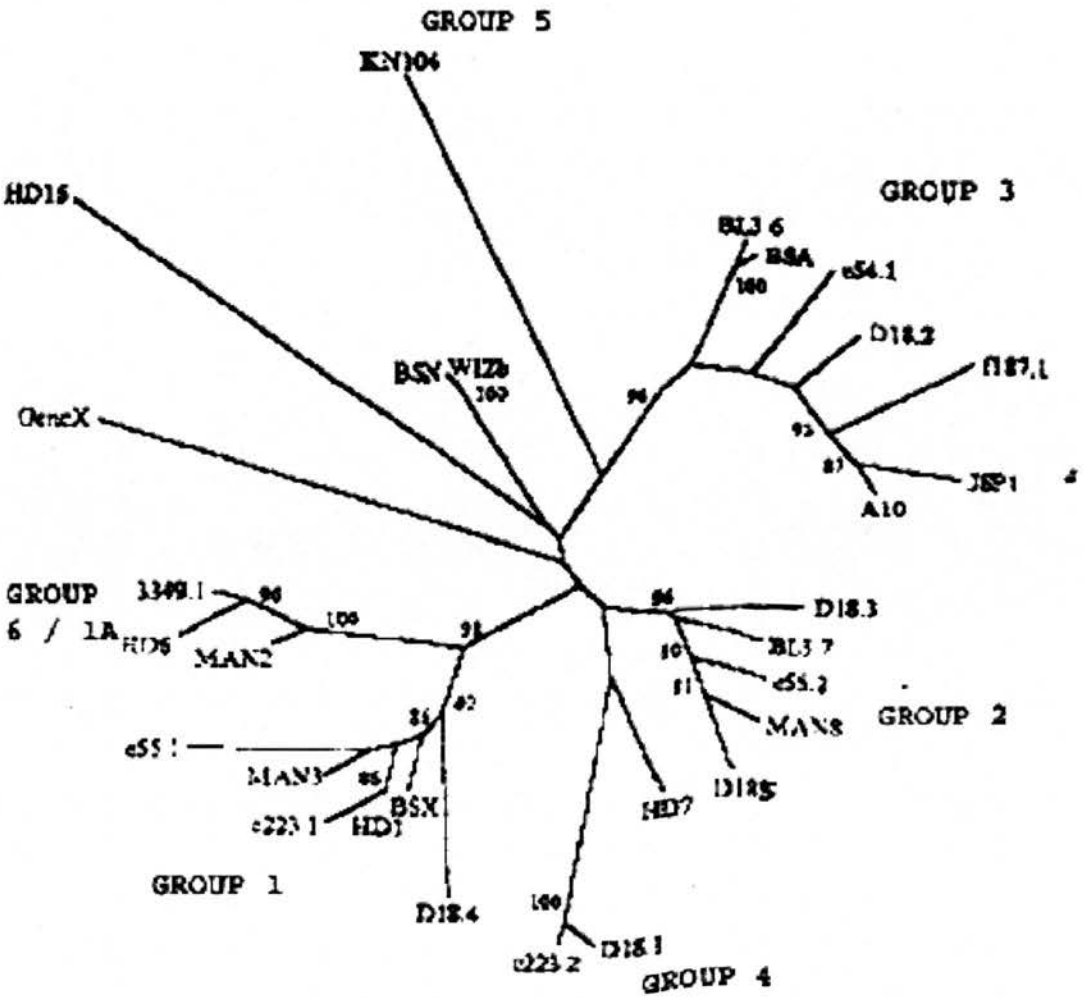
In order to use RSCA as a typing method in cattle we had to initially determine if the introns flanking exons 2 and 3 in bovine MHC class I genes also contain locus-specific characteristics around which primers could be designed. Only five full length class I genomic sequences are currently available from the Genbank database; BSA, BSX, BSC, BSF and BSN which correspond to published cDNA sequences (Garber *et al.*, 1993, 1994). As a result, no studies on bovine introns have taken place.

Since we cannot assign alleles to loci accurately we have instead inferred which alleles are derived from the same locus based on a combination of phylogenetic comparisons of coding sequence (Ellis *et al.*, 1999), mapping data and detailed analysis of haplotypes using MHC-homozygous animals. These will be referred to

throughout as groups and are indicated in fig. 3.1. Five main groups are evident including one group with a single allele, KN104. It is not clear if the alleles HD6, MAN2 and 3349.1 are a subgroup of group 1 or instead form their own group but analysis of intron sequences may make this situation clearer. For this reason we have chosen to refer to these alleles as group 6. Choosing two representatives from each group (and one from group 4) we aimed to sequence introns 1 and 3 and attempted to identify any residues which may be group-specific. The alleles chosen are indicated in table 3.2 with their corresponding haplotypes shown. Following on from this, potential group-specific primers would be designed and tested against a range of genomic DNA samples prior to use in RSCA. It was hoped that not only would this information be useful for typing but would also later provide data to help in the process of locus identification. In addition, it is important to study introns since they are likely to provide information on how polymorphism has been generated, and will allow investigation into how alleles are related to each other (Kotsch and Blasczyk, 2000).

Figure 3.1: Maximum likelihood tree of full length cDNA cattle MHC class I sequences indicating putative 'groups' or loci

Comparison of available full length cDNA sequences for bovine MHC class I alleles shows that they appear to fall into 5 to 6 groups which may be indicative of class I loci. Group 6 may be a subgroup of group 1. Figure adapted from Ellis *et al.*, 1999.



3.2 Materials

3.2.1 Animals used in this study and details of their haplotypes

All work described in this chapter used genomic DNA which had been prepared from PBM (section 2.2.3). Blood samples were obtained from animals at the IAH farm, Compton which are part of an inbred herd. These animals were chosen based on their MHC haplotypes, which had been inferred by a combination of PCR-SSP typing, serology, flow cytometry and parentage. The animals and their corresponding haplotypes are listed in table 3.1, with a summary of the haplotypes and characterised alleles given in table 3.2.

3.2.2 Primers designed for intron amplification

Primers for allele-specific intron amplification were designed based on the coding sequence of the alleles listed in table 3.2. The location of these primers is shown in fig. 3.2. All primers were obtained from either Invitrogen (Paisley, Scotland) or MWG (Germany). To ensure specificity primers for introns 1 and 3 were designed with one or more allele-specific nucleotides at the 3' end for forward primers and the 5' end for reverse primers. The primers used are summarised in table 3.3 and 3.4. The PCR conditions employed are described in section 2.2.8.

Table 3.1: Summary of genomic DNA used in this study for sequencing of introns and optimisation of group-specific primers

An asterix beside the haplotype indicates this sample is homozygous e.g. A11*.

Animal	Haplotype
200630	A31*
A11	A11*
A17	A17*
6152	A10/A20
704	A31*
7022	A14/A31
6009	A10*
1184	A18/A17
7189	A17/A31
4229	A18*
5403	A19/A10
61/96	A18*
5072	A11*
6045	A14/A11
999	A18/A10
9159	A10*
4277	A18/A14
5383	A10/A11
Manus	A19/A12
10069	A14*

Table 3.2: Summary of haplotypes and alleles used in this study

This table lists the common haplotypes found within the herd at IAH, Compton and their corresponding expressed classical MHC class I genes. Haplotypes not fully characterised are marked with a question mark. The accession number relates to the full length coding sequence from the Genbank/EMBL databases. The alleles included in the intron study are highlighted and colour coded to indicate those from the same group/gene. Group 1 are shown in red, group 2 in green, group 3 in blue, group 4 in yellow and group 6 in purple.

Haplotype	Genes identified	Accession Number
A14	D18.1 D18.4 D18.5	Y09205 Y09208 AJ010867
A31	HD1 HD7	X80933 X80935
A11	D18.2 D18.3	Y09206 Y09207
A18	HD6	X80934
A10	A10 (JSP.1)	X92870
A19?	MAN2 MAN8	AJ010861 AJ010866
A17?	3349.1	AJ010862
A20?	unknown	

Table 3.3: Primers designed for amplification of intron 1
F= forward primer, R= reverse primer

Allele	Primer name	Sequence (5' – 3')	Tm (°C)
D18.1	F Prom 2	GGA CTC TGC TTC TTC CCA	51
	R D18.1 (L)	TCG AGG CCG GGC CGG GA	49
D18.2	F D18.2int1forsp3	TCC TCC TGC TGC TCT CGG	60.5
	R D18.2int1revsp2	TAG CCG ACT TCC AGG TAC	56
D18.3	F D18.3int1forsp1	CCA CTG GGT GTT CAG TTC	56
	R D18.3int1revsp2	GTC GCT GTC GAA CCG TGT	58.2
D18.4	F Prom 3	CCC GGA CTC TGC TTC TCG	55
	R D18.4(L)	TTC CTT CTC TAT CCA CGG	49
D18.5	F Prom 2		
	R D18.5(L)	TGT GAA CTG CGT GTC GTC CA	47
HD1	F Prom 3		
	R HD1 (L)	CCC GGA CTC TGC CTT CTC G	59
HD6	F Bov21(a/g)	ATG G(A)GG CCG C(G)GA A(G)CC	

	R HD6(L)	CCT CGG ATC CCG GGC GT	55
HD7	F Prom 2		
	R HD7(L)	TCG TCC ACG TAG CCG ACA	53
A10	F A10int1prom4	AGG TCT CCA CCG ACC CGT	60.5
	R A10int1revsp	CAG GCT CAC TCG GAA AAA T	54.5
MAN2	F Promoter*	TCA AGG GCG GTG TCT GGG	60
	R MAN2int1revsp	GCG TTT TCC TTG GAG ATT TGA	55.9

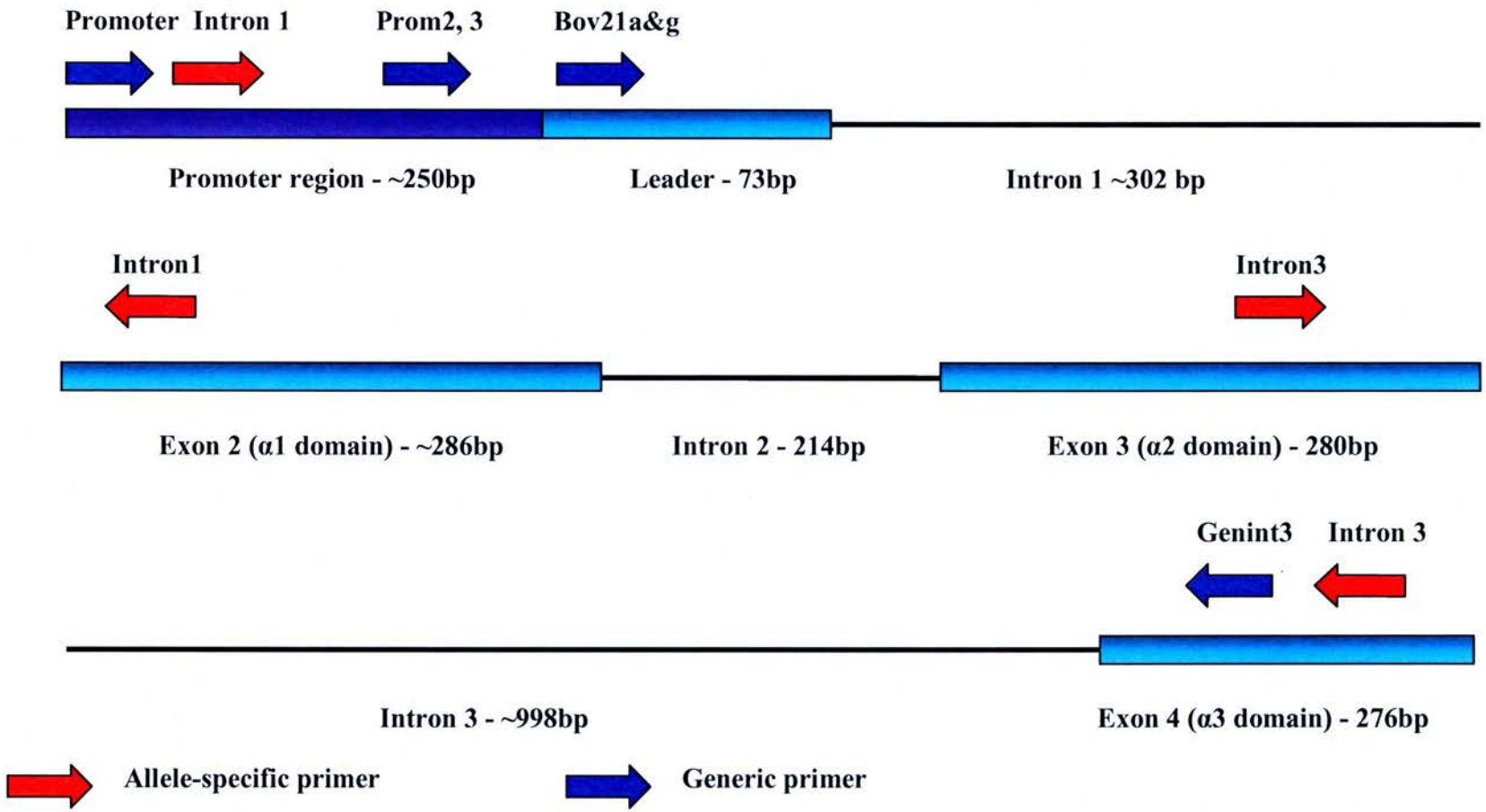
*Designed by N Barker

Table 3.4: Primers designed for amplification of intron 3
F =forward primer R= reverse primer

Allele	**Primer name**	**Sequence (5'-3')**	**Tm (°C)**
D18.1	F D18.4int3(5')	AAG GTT ATG CTG AGT CTT TGA	54
	R genint3(3')	TGC CAG GTC AGT GAG ATC TCC	61.8
D18.2	F D18.2int3forsp2	AAG TGG GAG GCG GGA GGT GCT	60.5
	R D18.2int3revsp1	GGT CTG GTC CTC CCC ATT	58.2
D18.3	F D18.3int3forsp2	AAG TGG GAG GCG GCA GGT GAG	65.7
	R D18.3int3revsp1	TCT GGT CCT CCC CTT CGT	58.2
D18.4	F D18.4int3(5')	AAG GTT ATG CTG AGT CTT TGA	54
	R D18.4int3revsp1	CAG CTA CTA CGA GGA GAA CCA	59.8
D18.5	F D18.5int3(5')	CAG GTG CTG CGG AGG GCG AGA	67.6
	R genint3		
HD1	F HD1int3forsp1	CAG GTG ATG CGG AGA GAT TCA	59.8
	R HD1int3revsp1	GGT CTG GTC CTC TCC ATT	56
HD6	F HD6int3forsp3	GAA CTA CCT GGA GGG CAC	58.2
	R HD6int3revsp2	ACC TCA GGG TGA CCT CAT	56
HD7	F HD7int3(5')	CAG GTG CTG CGG AGA GAT TAA	59.8
	R genint3		
A10	F A10int3forsp1	GTG AAG CTG AGG TAC AGA	53.7
	R A10int3revsp5	CGG TCA GAG ATG GGG TGA TGC	63.7
MAN2	F MAN2int3forsp2	CGC CTC CTC AGC GGG TTC ACG	67.6
	R genint3		

Figure 3.2: A schematic diagram of a bovine class I gene from the promoter region to exon 4 (alpha 3 domain).

Positions of generic and allele-specific primers used to amplify introns 1 and 3 of the bovine alleles are shown (sequences are listed in tables 3.3 and 3.4).



3.2.3 Design of group-specific primers

Group-specific primers were designed based on conserved residues in introns 1 and 3 between representative alleles from the same group. In the case of group 1 a generic primer in exon 3 had been designed prior to availability of intron 3 sequences: this is group-specific when used in conjunction with group 1 primer 4. These primers are listed in table 3.5. Primers 2a and 6e consist of a mixture of two primers with one nucleotide difference reflecting the difference in sequence between the two alleles analysed. These bases are highlighted in red and explained below the table. Reactions were run under the conditions described in section 2.2.8.1 with the exception of group 1 which used the following PCR program: 98°C for 20s, 35 cycles of 96°C for 20s and 68°C for 1min 30s then a final step of 72°C for 5 min.

3.3 Results

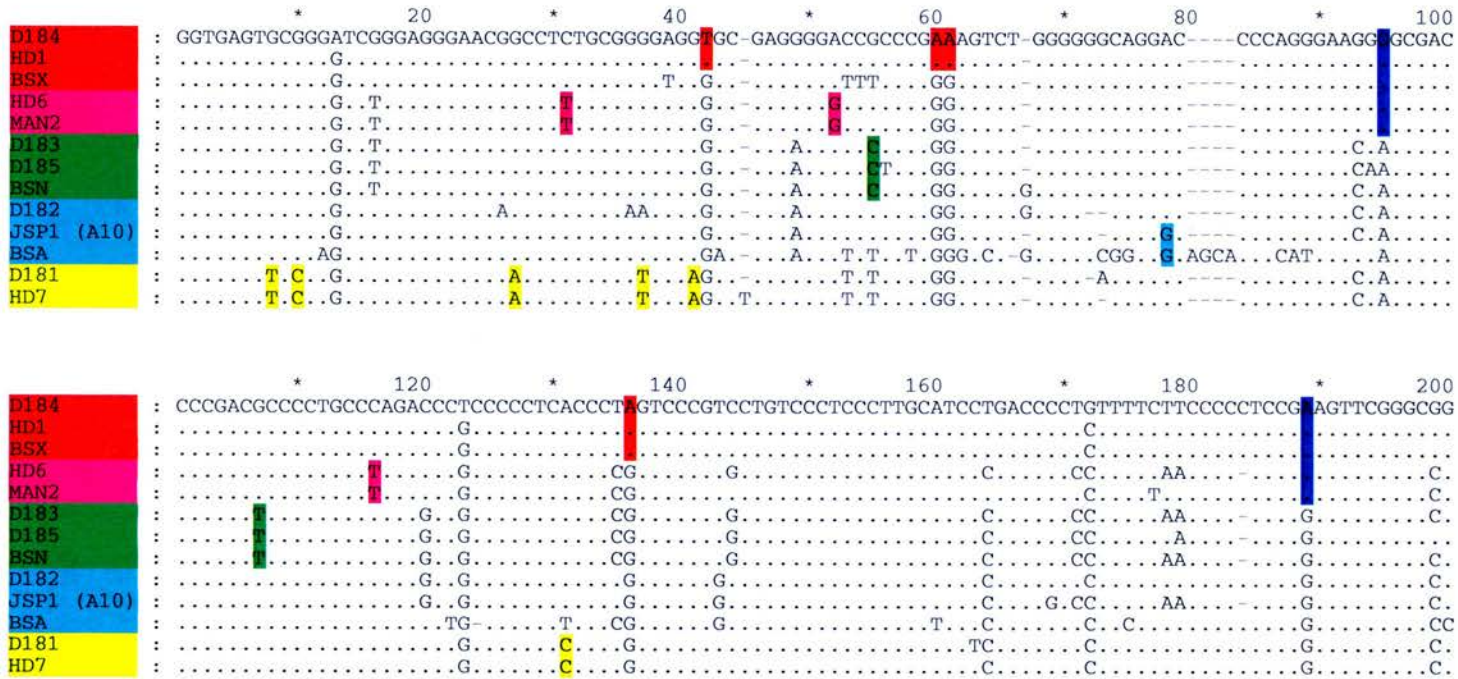
3.3.1 Amplification and sequencing of introns

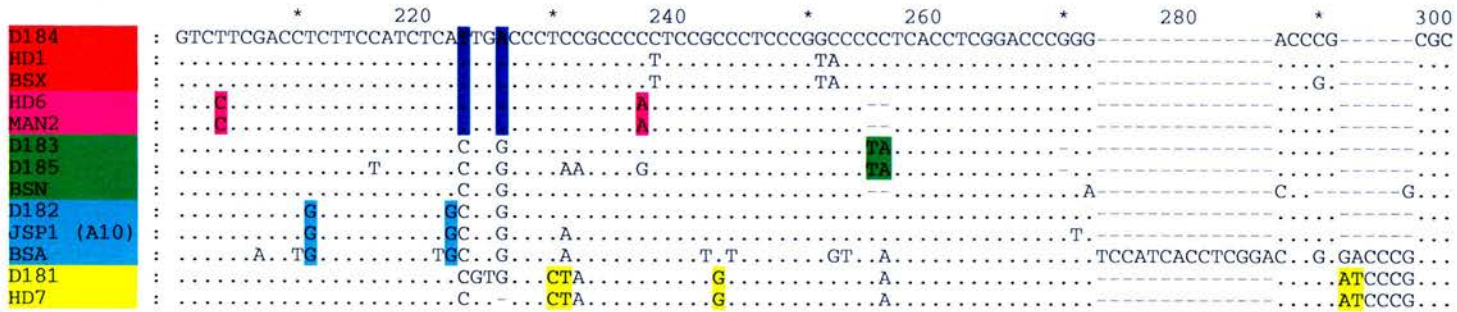
Using primers located in the surrounding exons or promoter regions we were able to successfully amplify introns 1 and 3 of ten bovine MHC class I alleles. The positions of these primers are shown in fig. 3.2. At least 3 clones of each sample were sequenced in both directions and a consensus made. Fig. 3.3 shows the whole of intron 1 while fig. 3.4 shows the first 420 bases of intron 3 in which any locus specificity should reside. Three other bovine MHC class I alleles, BSX, BSN and BSA which appear to fall into groups 1, 2 and 3 respectively (see fig. 3.1) have also been included (Garber *et al.*, 1993, 1994).

The presence of a large number of non-classical genes, pseudogenes and gene fragments within the MHC region made specific amplification from genomic DNA particularly difficult, with extensive optimisation and re-designing of primers required before the correct product was obtained. Where possible genomic DNA from cattle homozygous for class I was used. Initially it was hoped that the combination of an allele-specific and a generic primer would work so as to reduce the number of primer pairs required. This approach had some success with two forward primers, Prom 2 and 3, amplifying five of the alleles in conjunction with their specific reverse primers for intron 1 and genint3 amplifying intron 3 for four alleles with specific forward primers. Primers were designed to include at least one allele-specific base and also to amplify enough exon sequence to allow comparison

Figure 3.3: Alignment of entire intron 1 sequence of 13 alleles representative of 4 putative bovine MHC class I genes

The 10 alleles included in the intron study are shown aligned together with 3 alleles that have previously been sequenced, BSX, BSN & BSA (Garber *et al.*, 1993, 1994). Potential locus-specific nucleotides shared amongst alleles from the same group have been highlighted. Dots (.) indicate that the sequence is the same to the top allele while dashes (-) denotes no base present at that position. Group 1 alleles are shown in red, group 2 in green, group 3 in blue, group 4 in yellow and group 6 in purple. Bases shared between groups 1 and 6 are shown in dark blue since it is unclear if group 6 forms a distinct locus.




220
240
260
280
300
D184 : GTCTTCGACCTCTTCCATCTCATTGACCCTCCGCCCCCTCCGCCCTCCCGGCCCCCTCACCTCGGACCCGGG----------ACCCG------CGC
HD1 : .....T.....TA.....----------.....------...
BSX : .....T.....TA.....----------...G.------...
HD6 : ...C.....A.....--.....----------.....------...
MAN2 : ...C.....A.....----------.....------...
D183 : .....C..G.....TA.....----------.....------...
D185 : .....T.....C..G....AA....G.....TA.....----------.....------...
BSN : .....C..G.....--.....A----------C..------G...
D182 : .....G.....GC..G.....----------.....------...
JSP1 (A10) : .....G.....GC..G....A.....T.----------.....------...
BSA : .....A..TG.....TGC..G....A.....T.T.....GT..A.....TCCATCACCTCGGAC..G.GACCCG...
D181 : .....CGTG...CTA.....G.....A.....----------.....ATCCCG...
HD7 : .....C..-...CTA.....G.....A.....----------.....ATCCCG...

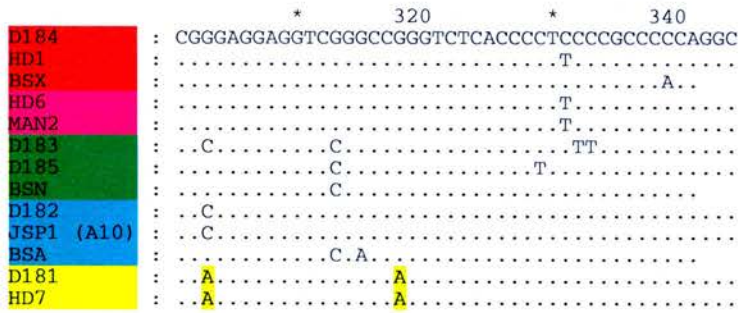
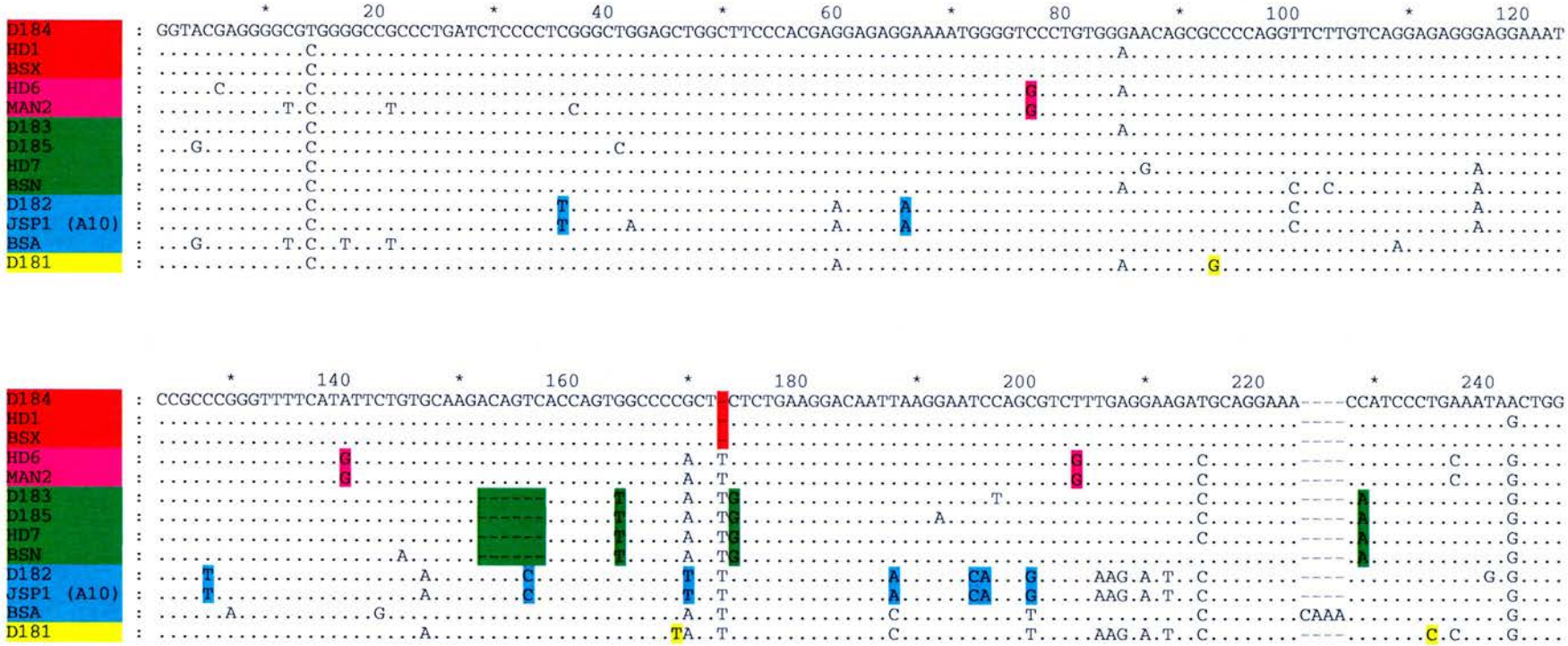
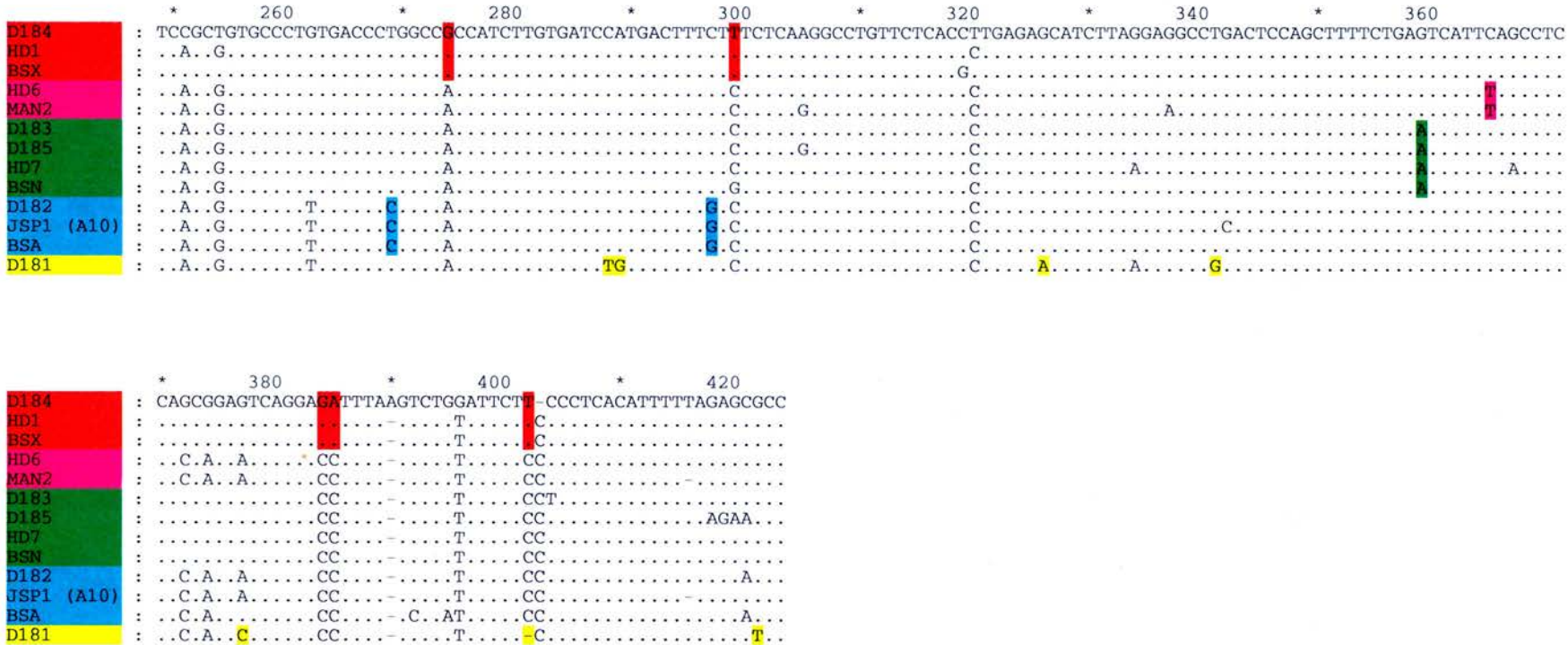

320
340
D184 : CGGGAGGAGGTCGGGCCGGGTCTCACCCCTCCCCGCCCCCAGGC
HD1 : .....T.....
BSX : .....A..
HD6 : .....T.....
MAN2 : .....T.....
D183 : ..C.....C.....TT.....
D185 : .....C.....T.....
BSN : .....C.....
D182 : ..C.....
JSP1 (A10) : ..C.....
BSA : .....C.A.....
D181 : ..A.....A.....
HD7 : ..A.....A.....

Figure 3.4: Alignment of partial intron 3 sequence of 13 alleles representative of 4 putative bovine MHC class I genes

The alignment shows the sequence for the first 420bp of intron 3 (~1000bp). These sequences were generated using allele-specific and generic primers described in table 3.3.2, or obtained from the Genbank database. Potential group-specific nucleotides have been highlighted. Group 1 alleles are shown in red, group 2 in green, group 3 in blue, group 4 in yellow and group 6 in purple.




260
280
300
320
340
360
D184 : TCCGCTGTGCCCTGTGACCCTGGCCGCCATCTTGTGATCCATGACTTTCTTTCTCAAGGCCTGTTCTCACCTTGAGAGCATCTTAGGAGGCCTGACTCCAGCTTTTCTGAGTCATTCAGCCTC
HD1 : ..A..G.....C.....
BSX : .....G.....
HD6 : ..A..G.....A.....C.....C.....T.....
MAN2 : ..A..G.....A.....C.....G.....C.....A.....T.....
D183 : ..A..G.....A.....C.....C.....A.....
D185 : ..A..G.....A.....C.....G.....C.....A.....
HD7 : ..A..G.....A.....C.....C.....A.....A....
BSN : ..A..G.....A.....G.....C.....A.....
D182 : ..A..G.....T.....C....A.....G.C.....C.....
JSP1 (A10) : ..A..G.....T.....C....A.....G.C.....C.....C.....
BSA : ..A..G.....T.....C....A.....G.C.....C.....
D181 : ..A..G.....T.....A.....TG.....C.....C.....A.....A.....G.....
380
400
420
D184 : CAGCGGAGTCAGGAGATTTAAGTCTGGATTCTT-CCCTCACATTTTTAGAGCGCC
HD1 : .....-.....T.....C.....
BSX : .....-.....T.....C.....
HD6 : ..C.A..A.....CC....-.....T.....CC.....
MAN2 : ..C.A..A.....CC....-.....T.....CC.....-.....
D183 : .....CC....-.....T.....CCT.....
D185 : .....CC....-.....T.....CC.....AGAA...
HD7 : .....CC....-.....T.....CC.....
BSN : .....CC....-.....T.....CC.....
D182 : ..C.A..A.....CC....-.....T.....CC.....A...
JSP1 (A10) : ..C.A..A.....CC....-.....T.....CC.....-.....
BSA : ..C.A.....CC.....-.C..AT.....CC.....A...
D181 : ..C.A..C.....CC....-.....T.....-C.....T..

with coding sequences from databases to ensure we had the correct product. Where incorrect product was obtained this was usually due to mispriming. These products were not studied any further other than to compare them to the other classical class I alleles present on the haplotype to determine if the primers were amplifying the wrong allele.

A comparison of the introns amongst the ten alleles shows the presence of a number of indels (insertions/deletions), which is surprising considering the non-dynamic nature of introns, although this could be due to the alleles representing different loci. These tend to occur mainly around strings of base repeats for example intron 1 position 73 (fig. 3.3). This suggests a sequencing error however reanalysis of at least three clones shows these indels to be real. In intron 1 one main insertion occurs, between HD7 and D18.1 at position 291. Phylogenetic analysis of full length coding sequence without the PBR suggests D18.1 to represent a locus with little polymorphism since it has relatively few similarities to other alleles identified so far. However when just the PBR sequences are analysed D18.1 is found near HD7 from group 2 (Ellis *et al.*, 1999). In intron 1 these alleles share a number of bases that are not found in any of the others studied but in contrast few similarities are observed in introns 3. This suggests that HD7 may represent an intermediate allele that has arisen by interlocus recombination between groups 2 and 4.

Analysis of intron 3 (fig. 3.4) shows a large group-specific deletion for group 2 at position 152, the significance of which is unknown. The largest insertion amongst the sequences is seen in intron 3 for D18.4 and HD1 where approximately 590 bases

have been added (data not shown). This insertion is also seen in the full-length sequence of BSX, another member of group 1 but not in any other sequences (Archibald, 2002) suggesting it to be locus-specific. Further investigations are required in order to confirm this. Attempts are currently being made by our laboratory to design a locus-specific probe around this insertion for screening of genomic DNA samples in southern blots, concentrating initially on haplotypes where we believe the gene to be absent e.g. A18. This should hopefully provide an indication as to whether genes are silenced or absent between different haplotypes.

3.3.2 Presence of group-specific nucleotides

Analysis of introns 1 and 3 shows the presence of a number of conserved nucleotides between members of the same group. The inclusion of BSA, BSN and BSX in the alignments allows speculation on the locus-specificity of the nucleotides highlighted in figs 3.3 and 3.4. In most cases these are shared with the BS sequences however there a few cases where the base is only shared by two of the alleles for example in intron 1 at bases 60 and 61 where BSX differs from HD1 and D18.4. There are also a number of bases shared between BSX and either HD1 or D18.4 but not both for example position 238 in intron 1. This indicates that caution should be taken when assuming specificity of bases if only two alleles from each group are sequenced. Where possible the group-specific primers designed here included all members of the group but emphasis was placed on including the alleles found within the herd at Compton since no functional information is available about the BS sequences and they may not represent true alleles. This was particularly the case with group 3

where BSA was quite diverse from D18.2 and A10 (and all the other alleles included here) and had a number of unique insertions and deletions.

As discussed in the introduction, there is no clear indication that group 6 is a true group. In intron 1 a number of bases were found to be conserved amongst both the group 1 and group 6 members (shown in dark blue). Primers were designed around these and tested on a panel of DNA with a conserved reverse primer but were found to be non-specific (data not shown). The lack of conserved bases in intron 3 and the remaining coding sequence prevents a specific reverse primer for group 1 and 6 being designed. As a result of this it is necessary to consider group 6 separately.

3.3.3 Group-specific primer design

The primers chosen for group-specific amplification are summarised in table 3.5.

Table 3.5: Group-specific primers

This table lists those primers that have been tested and shown to have group-specificity.

Group	Primer name	Sequence (5'-3')	Tm (°C)
Group 1	F Group 1 primer 4	TGC GAG GGG ACC GCC CGA	65.1
	R Generic primer C	AGC GCA GGT CCT CGT TCA	58.2
Group 2	F 2a	GAA CRA GCG ACC CCG ACT*	59.4
	R 2c	CAA GTG GGG CAA CTG GTC	58.2
Group 3	F 3c	TCG ACC GCT TCC ATC TCG	58.2
	R 3e	GAA CAG GCC TTG AGA GAC	56
Group 6	F 6d	TCA TTG ACC CTC CGC CCA	58.2
	R 6e	GGC GCT GTT YCC ACA GGC**	61.7

* R = A or G ** Y = C or T

3.3.3.1 Group 1

Prior to sequencing intron 3 we attempted to design sets of group-specific primers using forward primers based around the specific bases identified in intron 1 with generic primers located at the 3' end of exon 3. Five forward primers were designed for group 1 and initially tested with three reverse primers at 55°C (using the PCR program described in section 2.2.8) on four samples, two positive for D18.4 and HD1 and two negative for both. Of the fifteen possible combinations only two sets appeared to be specific, primer 4 with generic primer a or c (see fig. 3.5). Attempts were then made to optimise this primer pair to the conditions for RSCA previously described (Arguello *et al.*, 1998). A combination of the PCR reaction and program described in sections 2.2.8.1 and 3.2.3 were found to provide optimal results with distinct, clean products only observed from samples positive for group 1 alleles (see fig. 3.6). We then tested primer 4 and generic c against a panel of genomic DNA samples with a range of haplotypes to confirm the specificity (see fig. 3.7). From this PCR only two amplicons were observed in samples positive for D18.4 and HD1, confirming this primer pair as being group-specific.

3.3.3.2 Groups 2, 3 and 6 – primer design and testing

Similar approaches were initially taken to design primers in exon 3 for groups 2, 3 and 6 but no primer combinations allowing group-specific amplification were found. Sequencing of intron 3 (fig. 3.4) showed a number of group-specific nucleotides around which further primers could be designed.

Figure 3.5: Testing putative group 1 primers

Group 1 primer 4 was tested with 3 reverse generic primers a, b and c on four genomic DNA samples, two positive for group 1 alleles (A14 and A31) shown in lanes marked 1 and 2 and two negative in lanes 3 and 4 (A10 and A11). PCR products were run on a 1% agarose gel at 5V/min for 40 min.

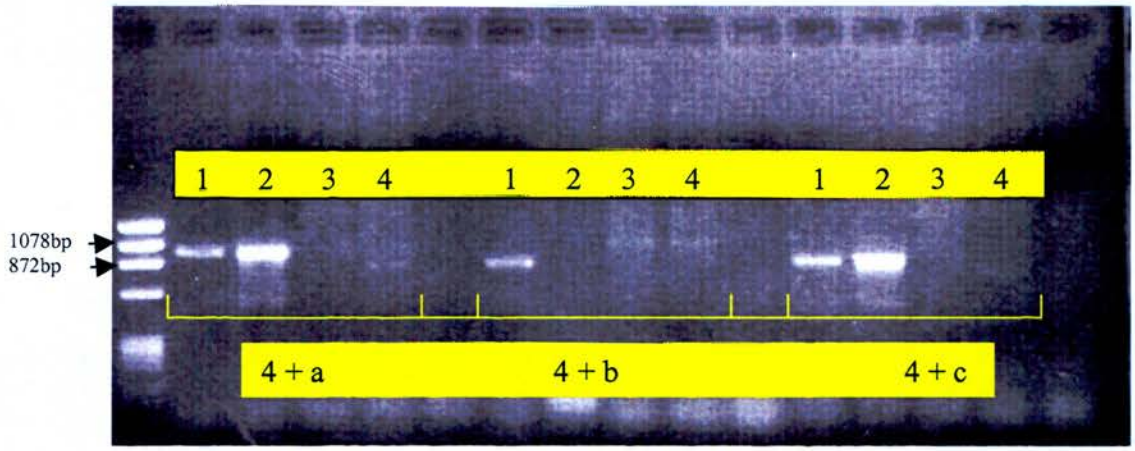


Figure 3.6: Optimisation of group 1 primers 4 and c

The primer pair 4 and c were tested using combinations of the PCR programs (P) and reactions (R) described in sections 2.2.8.1 (PL) and 2.2.8.2 (RL) and Arguello *et al.*, 1998 (PA and RA) on two positive (A14 and A31, lanes 1 and 2) and two negative (A10/A20 and A10, lanes 3 and 4) samples.

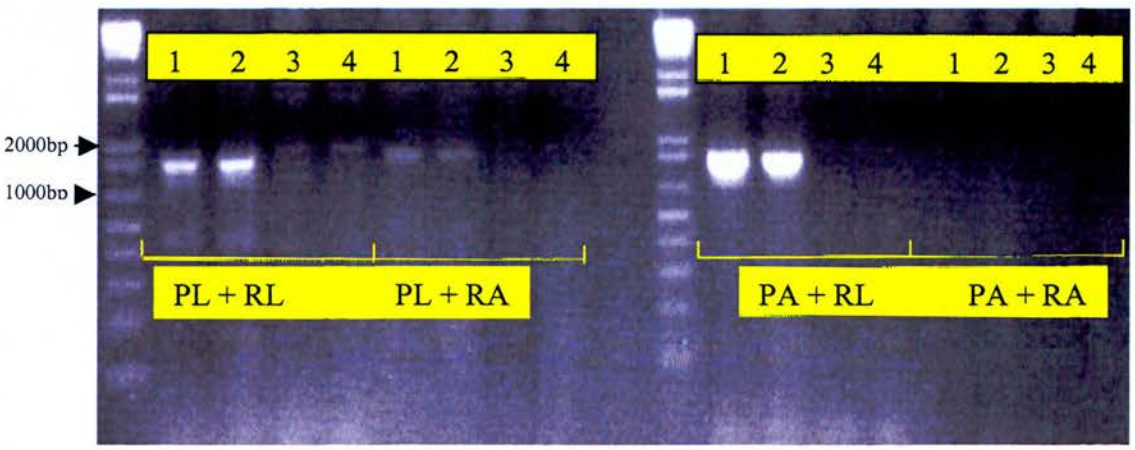
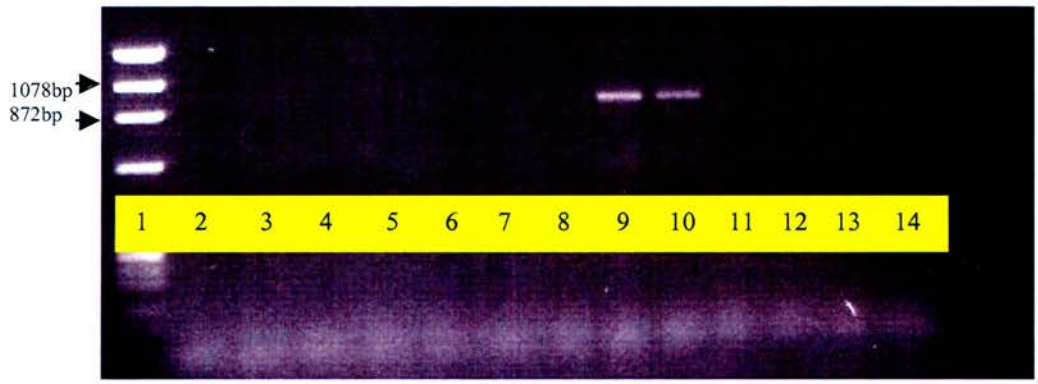


Figure 3.7: Amplification of D18.4 and HD1 using group 1-specific primers – group1 primer 4 and generic primer C

Group 1 primer pair 4 and c were tested on a panel of genomic DNA samples carrying different haplotypes to ensure they amplified only group 1 alleles (A14 and A31 haplotypes). 1: phi x 174 ladder, 2: A18/A10, 3: A10/A10, 4: Manus DNA, 5: chicken DNA (negative control), 6: A17/A17, 7: A18/A18, 8: A18/A17, Lane 9: A31/A31, 10: A11/A14, 11: A10/A20, 12: A11/A11, 13: A10/A10, 14: no DNA control



Four forward and reverse primers were designed for group 2, three forward and two reverse for group 3 and four forward and reverse for group 6 giving sixteen possible combinations for groups 2 and 6 (A-P) and six for group 3 (A-F). These primer combinations were initially tested on three samples, two positives for alleles from that group and one negative. The annealing temperature in each reaction was chosen as an intermediate between the values for each of the primers given by MWG.

As can be seen from fig. 3.8 the results of the different primer combinations were quite variable, with some working extremely well e.g. group 6 pair I while others gave multiple banding patterns, smearing, or amplified a product from the 'negative' sample e.g. 6H, 2I. Two primer pairs for groups 6 and 3 and one for group 2 were identified as being potentially group-specific since they only amplified single bands of the correct size in the 'positive' samples. These were pairs 6I and 6M, 3B and 3F and 2A. A further eight pairs for group 6 and two for group 2 were kept in reserve since it was possible they could be optimised to increase their specificity.

3.3.3.3 Group 2, 3 and 6 – determining group specificity

Primer pairs 6M, 2A and 3F were chosen for further study. As with the group 1 primers we attempted to confirm the group specificity by testing the pairs against a panel of genomic DNA samples positive for a range of haplotypes. The expected results from these tests are shown in table 3.6, with the actual results shown in figs 3.9 to 3.11 and summarised in table 3.7.

Figure 3.8: Choosing putative group-specific primers for groups 2, 3 and 6

Primer pairs for groups 2, 3 and 6 were tested on three genomic DNA samples, two positives and one negative as described in section 3.3.2.2. PCR products were then run on a 1% agarose gel at 5V/cm for 40 min. The results of each primer pair are labelled with those chosen for further investigation marked by an asterix (*). Those kept in reserve are marked by a double asterix (**). Samples in the first two lanes of each set should be positive and the third lane negative.

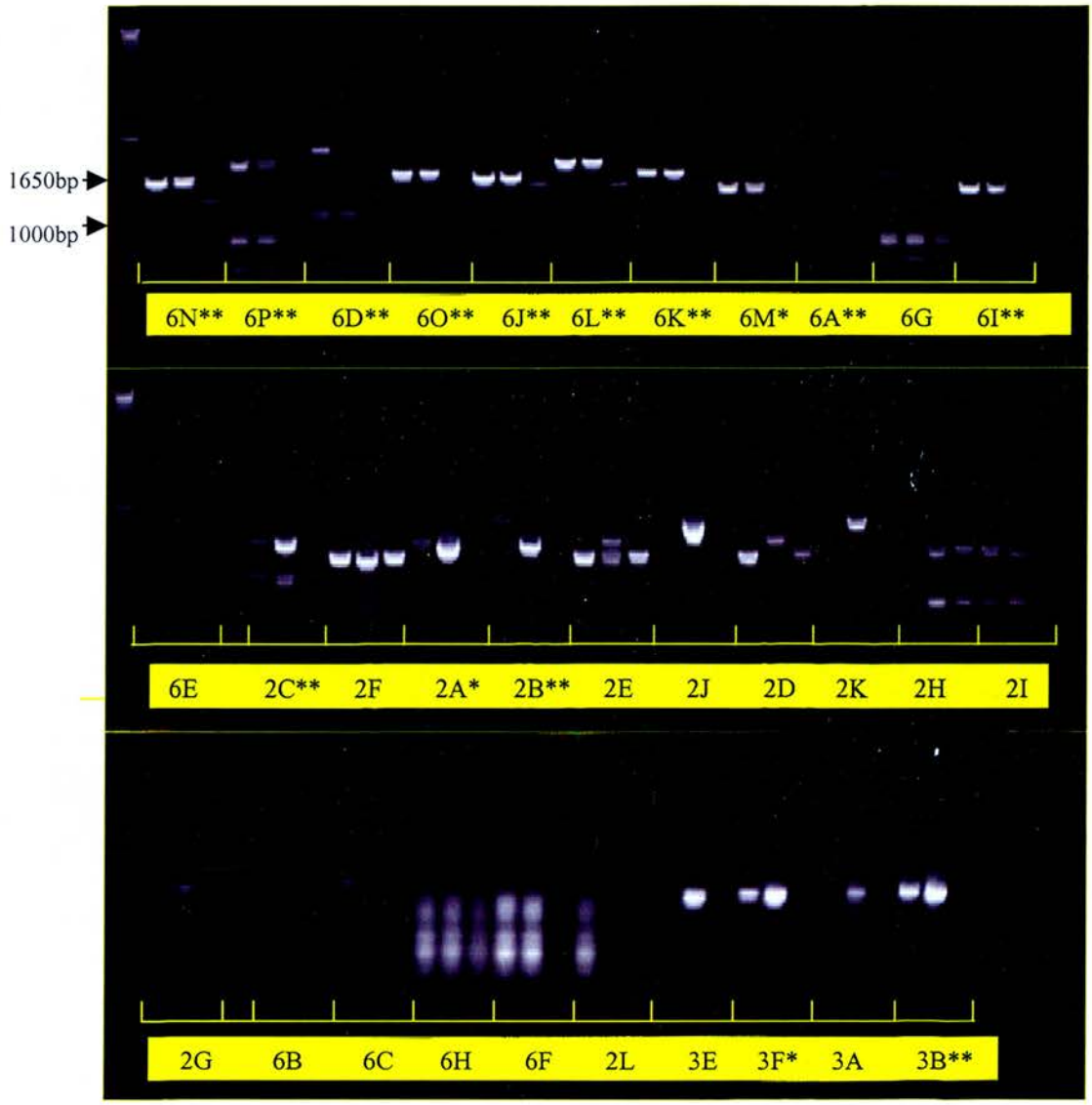


Table 3.6: Predicted results from group-specific primer testing

Primers were tested on a panel of genomic DNA samples with known haplotypes to confirm group-specificity. This table shows the predicted results with X marking those haplotypes that we believe to be positive for each group. Haplotypes marked with a question mark indicates them to be partially characterised, with only some alleles identified.

PREDICTED RESULTS	HAPLOTYPE							
GROUP PRIMERS	A14	A31	A18	A10	A20	A17	A11	A19
1	X	X			?	?		?
2	X				?	?	X	X
3				X	?	?	X	?
6			X		?	X		X

Figures 3.9-3.11: PCR results for testing putative group-specific primers

Primer pairs 2A, 3F and 6M were tested on a panel of genomic DNA samples carrying a range of haplotypes to determine their specificity. PCR products were then run on a 1% agarose gel at 5V/cm for 40 min. Samples marked in red were sequenced for further analysis.

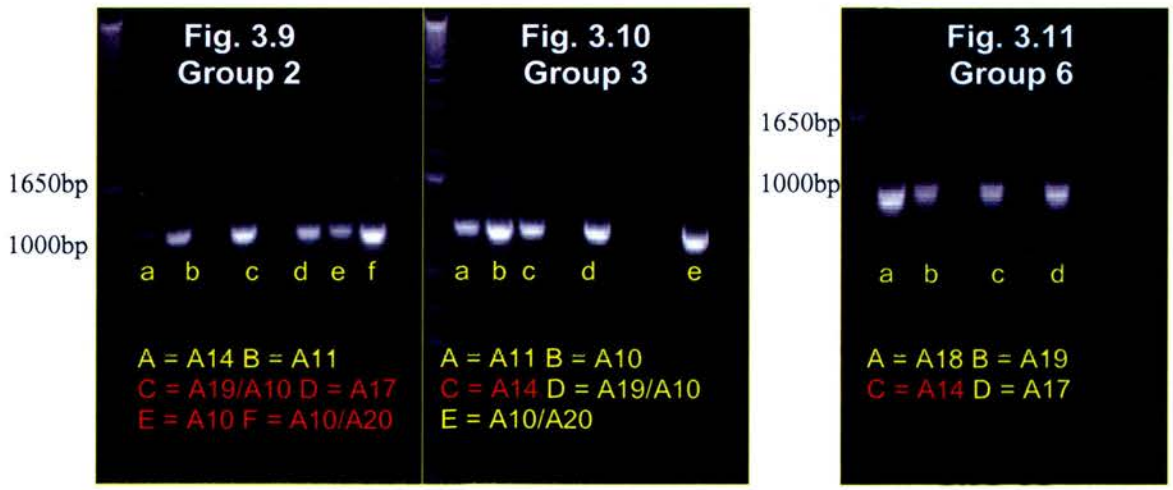


Table 3.7: Summary of group-specific primer testing results

This table summarises the results of the PCRs shown in fig. 3.9 to 3.11. Samples which gave a positive result when tested with the different group-specific primers are indicated by X.

TEST RESULTS	SAMPLES – MHC CLASS I HAPLOTYPES							
GROUP PRIMERS	A14*	A31*	A18*	A10*	A10/A20	A17*	A11*	A19/A10
1	X	X						
2	X			X	X	X	X	X
3				X	X		X	X
6			X			X		X

Primer pair 2A correctly amplified products from the A14 and A11 samples, which have the group 2 alleles D18.5 and D18.3 respectively. In addition they amplified a product from an A19/A10 sample. The A19 haplotype is known to carry the allele MAN8 which appears to be a group 2 allele based on phylogenetic analysis (see fig. 3.1). When α1 and α2 of the A19/A10 product was sequenced it was found to be identical to MAN8 except for 1 aa difference suggesting either an error in this or the database sequence (fig. 3.12). Alternatively this may be an allelic variant of MAN8.

Amplicons were also obtained with samples A17*, A10* and A10/A20. Sequencing of these products was carried out in order to determine which MHC class I alleles were being amplified. A17* was found to have the same MAN8 allele as the A19/A10 animal, including the variant aa, which does indicate that there is an error on the database (fig. 3.12). We believe this to be the first finding of a shared allele between class I haplotypes in cattle. Blast screening of the two new sequences from A10/A20 and A10 against the Genbank database showed them both to be bovine MHC class I sequence. The sequence found in A10/A20 was found to be most similar to BL3-7, another group 2 allele which suggests that we have identified a new class I allele from group 2. The absence of this sequence from other samples with the A10 haplotypes shows that it is almost certainly present on the A20 haplotype. A pile-up of this predicted coding sequence (α1 and α2) against other group 2 sequences indicates this to be a true class I allele since the majority of amino acid substitutions are also found in other alleles (see fig.3.13). The new sequence from A10 was found to be similar to MAN2 (group 6) and an African allele E55.2 (group 2). Again it appears to be a real allele when compared to other coding sequences

(fig. 3.14). This sequence was surprising in two ways. Previously it was believed that the main A10 haplotype within the herd (derived from a known bull) carried only one gene – JSP.1. Further investigations of other A10 animals using the group 2 primers have also detected this allele showing that A10 is not a single gene haplotype as believed. It may be that low levels of transcription have resulted in the failure to detect this previously since the majority of investigations have been carried out on cDNA. It was also interesting to note that the new allele was not also found on the A10/A20 sample however this may be due to the fact that only 3 clones were sequenced (at least 6 clones were sequenced when other A10 positive animals were tested). It should be taken into consideration however that although this resembles a classical class I allele only a portion of sequence was analysed. It may be that it is not a full length sequence and so is not expressed.

Primer pairs 3F and 6M correctly amplified samples positive for alleles from each group as expected. Both pairs also amplified from an A14 homozygous sample. Sequencing of these products showed the sample to contain JSP.1, 3349.1 and MAN2, which suggests that either the sample had either been incorrectly typed or contaminated. This sample was removed from the stock of genomic DNA and not included in any further assays.

Figure 3.12: Alignment of sequences from A19/A10 and A17 samples amplified with group 2 primers

The α1 and α2 domains of PCR products amplified from A19/A10 and A17 using the group 2 specific primers were sequenced and the predicted amino acid sequence compared to published sequences. They both coded for the allele MAN8 with a single amino acid change (highlighted) and were found to be very similar to BSF, another group 2 allele. Since the haplotype A19 is known to carry the MAN8 allele this indicates an error in the database sequence. The scale shown indicates the amino acid number in the transcript, with a line included to show the division between the exons.

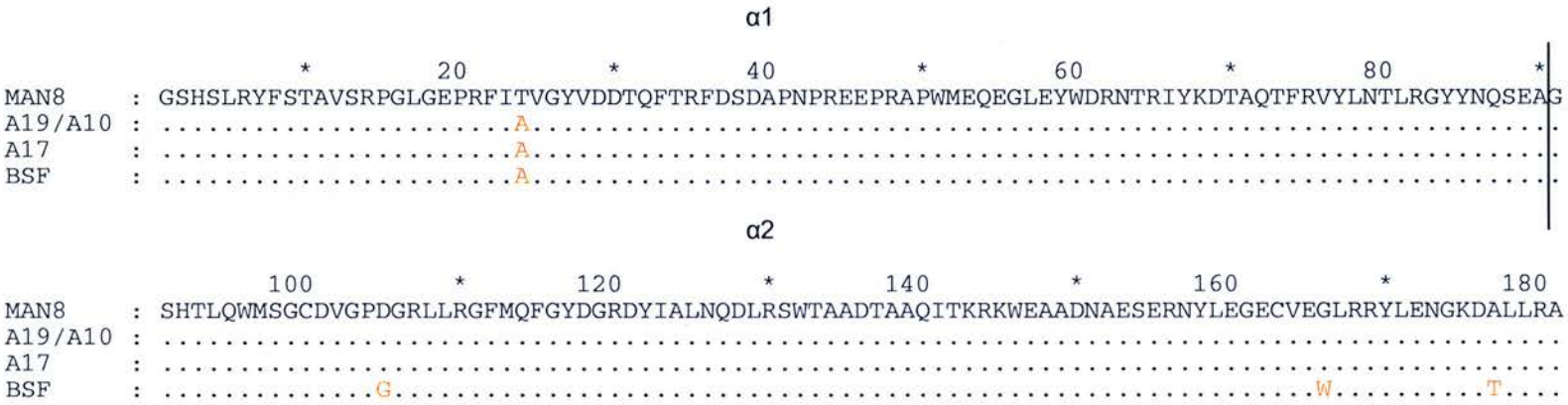


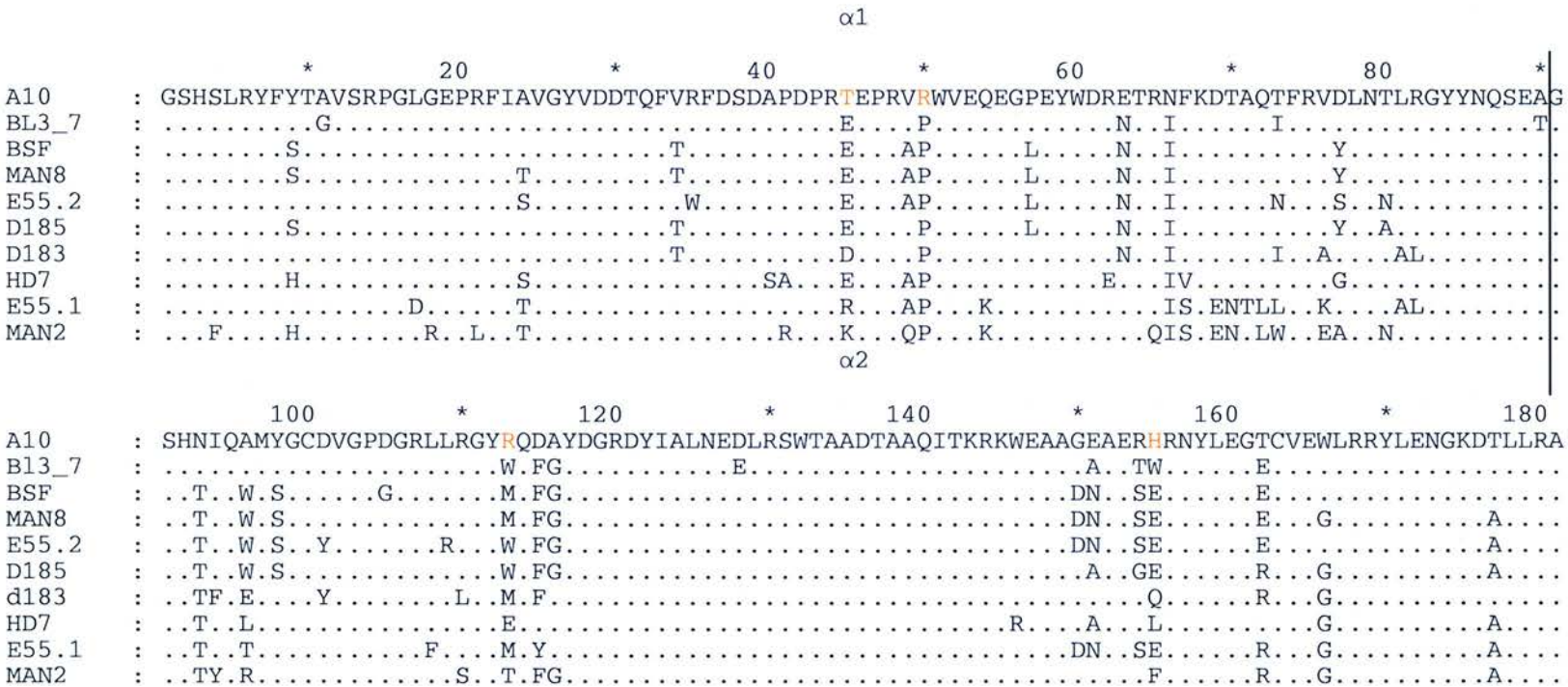
Figure 3.13: Alignment of predicted α1 and α2 sequence of the product amplified from an A10/A20 sample using group 2-specific primers with published allele sequences

The genomic sequence amplified from an A10/A20 sample using group 2 primers was blasted against the Genbank database. The alignment shows the predicted amino acid sequence of this product aligned with the alleles to which it had closest similarity. The exon division between α1 and α2 is shown. Any unique amino acids in the A10/A20 sequence are highlighted.

```
                                                    α1
                 *         20         *         40         *         60         *         80         *
A10/A20  : GSHSLRYFYTGVSRPGLGEPRFIAVGYVDDTQFTRFDSDAPNPREEPRVPWMEQEGPEYWDRETRISKETAQTFRVDLNTLRGYYNQSEAG
BL3_7    : ..............................V.............................N...Y.D...I................T.
MAN8     : ........S.A............T.........................A.......L.....N...Y.D.......Y.............
E55.2    : ..........A............S.........VW.............A.......L.....N...Y.D...N...S..N...........
E55.1    : ..........A.....D......T.........V..........R...A....K..............NTLL..K....AL.........
BSF      : ........S.A.....................................A.......L.....N...Y.D.......Y.............
D185     : ........S.A.....................................L.....N...Y.D.......Y..A.....
D183     : ..........A.................................D.................N...Y.D...I..A....AL.........
HD7      : ........H.A............S.........V.....SA.......A.............E....V.D.......G.............
MAN2     : ...F....H.A......R..L..T.........V......R...K...Q....K..........Q....N.LW..EA..N...........
                                                    α2
                  100         *         120         *         140         *         160         *         180
A10/A20  : SHTIQEMYGCDVGPDGRFLRGYEQYGYEGRDYIALNEDLRSWTAADTAAQITKRKWEAADYAESLRNYLEGRCVEGLRRYLENGKDALLRA
BL3_7    : ..N..A...........L....W....D.........E.....................GA...W......E...W..........T....
MAN8     : .....W.S.........L....M....D.....................................N...E......E...................
E55.2    : .....W.S..Y......LR...W....D.....................................N...E......E...W..............
E55.1    : .....T.................M..A.D.....................................N...E.........................
BSF      : .....W.S......G..L....M....D.....................................N...E......E...W..........T....
D185     : .....W.S.........L....W....D.....................................GA..GE.......................
D183     : ...F......Y......L.L..M..A.D.....................................GE..RQ.....................T....
HD7      : .....L...........L......DA.D.....................................R...GA..R.......T..................
MAN2     : ...Y.R...........L.S..T....D.....................................GE..RF.......................
```

Figure 3.14: Alignment of predicted α1 and α2 sequence of the product amplified from an A10 sample using group 2-specific primers with published allele sequences

The genomic sequence amplified from an A10 sample using group 2 primers was blasted against the Genbank database. The alignment shows the predicted amino acid sequence of this product aligned with the alleles to which it had closest similarity. The exon division between α1 and α2 is shown. Any unique amino acids in the A10 sequence are highlighted.



3.4 Discussion

Sequencing of introns 1 and 3 from ten alleles, representative of five of the six phylogenetic groups evident from bovine MHC class I sequences revealed a number of potentially group-specific nucleotides for four of the groups. Primers designed around these bases were tested and found to have good group specificity since not only are they correctly amplifying known alleles they are also capable of identifying new unknown alleles as shown by the group 2 pair. This allows us to develop RSCA as a method for typing MHC class I genes in cattle.

It is unclear at present whether these groups are good representatives of the loci that exist in the bovine MHC – this should become more evident when RSCA has been used a number of times to type a larger number of animals. It may be that the primers designed here are specific only for the alleles that the introns were sequenced from. This could be resolved however by testing them on samples containing other alleles from the groups (see fig. 3.1) or by sequencing introns 1 and 3 from these to determine if the group-specific bases are present.

While the group 1 primers have been optimised it may be that optimisation (particularly annealing temperature) is required for the other pairs. Unfortunately time restrictions prevented this. It would be ideal to normalise the pairs to the same PCR program to enable all samples to be run together to make the process more efficient. It is also important that a specific reverse primer be designed for group 1 in intron 3 since we may be missing alleles by not amplifying the whole of α1 and α2.

The presence of a large number of group-specific bases in the introns, while useful, is slightly surprising considering how the different alleles are created. In humans new MHC class I alleles arise primarily as a result of intra-locus recombination. Since introns are not under selection these recombinational events followed by fixation through genetic drift lead to homogenisation of the introns over time with the maintenance of any locus-specific nucleotides (Parham *et al.*, 1989, Cereb *et al.*, 1997). In contrast, phylogenetic analysis of varying regions of the class I sequences and direct comparison of sequences suggests that some degree of inter-locus recombination between MHC class I genes is occurring in cattle, particularly around exons 2 and 3 (Ellis *et al.*, 1999, Holmes *et al.*, 2003). It would therefore be expected that any locus specificity would be lost over time but this does not appear to be the case. HD7 is the exception however. As mentioned in the results section, HD7 normally groups with D18.3 and D18.5 when full length sequences are used for phylogenetic analysis (group 2) but is more similar to D18.1 across $\alpha 1$ and $\alpha 2$ regions. A comparison of introns 1 and 3 from this allele show it to be most like D18.1 in intron 1 but then reverts back to a group 2 phenotype in intron 3. This suggests that HD7 is the product of inter-locus recombination, and as a result has no group 2-specific nucleotides in intron 1.

More recent studies also found evidence of interlocus recombination occurring in the allele D18.4 (Holmes *et al.*, 2003). D18.4 is considered to be a group 1 allele when full length and 3' sequence is included for phylogenetic analysis. However when only 5' sequence is used D18.4 is found to group more closely to D18.1. This sequence incorporates exons 3 and 4 encoding the $\alpha 2$ and $\alpha 3$ domains. It would be

interesting to include each of these exons separately in analysis to see if there is a difference since group 1-specific bases are found in intron 3 which lies between the two exons. It would be expected that exon 3 is more like other group 1 sequences and exon 4 more like D18.1 showing a recombination occurring somewhere in intron 3. The position of D18.4 and D18.1 have been mapped on a BAC contig derived from an A14 homozygous animal and are believed to be less than 130kb apart (Di Palma *et al.*, 2002). This small genetic distance could allow unequal crossing over to occur between chromosomes resulting in the (recombinational) pattern observed.

Aside from the identification of group-specific characteristics, examination of the intron sequences could provide important information to gain an understanding of the evolutionary mechanisms that have resulted in the generation of polymorphism within the PBR. Unfortunately the lack of alleles identified and sequenced so far, along with problems with locus assignment, currently prevent this. It would prove interesting to investigate this further when more information becomes available to determine, for example, if lineages involving groups of alleles from the same locus are evident as with human alleles, since these are indicative of alleles of similar origin (Gomez-Casado *et al.*, 1999, Elsner *et al.*, 2002). The best approach would include phylogenetic analysis of the introns separately and together and also with different sections of the corresponding allele coding sequences. Comparison of bovine and human introns may also provide some useful data although they are likely to be quite different based on work by Cereb *et al.* (1997) who found that chimpanzee Patr-B and HLA-B intron 2 sequences formed two distinct species-specific groups when compared phylogenetically.

Chapter 4

Use of RSCA for typing MHC class I genes in cattle

4.1 Introduction

One of the major aims of this project was to develop a method for typing the MHC class I genes in cattle. This not only allows us to type a large population of unknown animals quickly but should also generate data indicating how polymorphic the genes are and how much genetic diversity there is within a population. The requirements for such a system are largely for research purposes at present, but there are a number of factors that could result in typing having a significant economical effect.

In-depth analysis of the small inbred herd at Compton identified the presence of a haplotype with a single class I gene, A18 (Ellis *et al.*, 1996). Assuming this is a 'normal' MHC class I gene i.e. restricted in the number of peptides it can present, then it would be expected that animals with only this haplotype would be at a distinct disadvantage compared to others in terms of the range of antigens they can present. Thus it is important to determine how common these single gene haplotypes are. This is worrying when considering that current breeding strategies in the UK rely heavily on artificial insemination using a relatively small number of bulls (approximately 100, Cogent website, www.cogentuk.com) as donors. It may be that selecting for positive traits such as milk and meat production has an adverse effect on the MHC, resulting in a reduction of diversity of the MHC repertoire within herds, particularly if many bulls carry haplotypes like A18. As a result the herds in Britain and elsewhere are potentially becoming more susceptible to pathogens.

The recent move toward epitope-based vaccines based on peptides presented by class I molecules makes it important to be able to type large numbers of animals to ensure that any vaccines developed will be efficacious on a wide scale. It may also be possible to define BoLA supertypes where several alleles share overlapping peptide specificities.

Lastly there is some evidence from a number of species to suggest that MHC compatibility between mother and foetus can lead to foetus rejection or problems during the pregnancy (Joosten *et al.*, 1991, Wegman, 1987, Ober, 1992). This could potentially have a large impact on the number of calves being born, particularly where embryo transfer and artificial insemination are used and no information regarding compatibility between the dam and bull is available.

Chapter 3 described how a range of specific primers were designed and tested for four of the groups in cattle which we believe to be indicative of MHC class I loci. Using these primers we aimed to establish the RSCA method within the laboratory for typing known alleles and also potentially for the detection of new class I alleles. This chapter describes the processes required to do this and also shows the method being used to type a panel of unknown samples obtained from a range of breeds including Charolais, Simmental, Holstein Friesian and some African *Bos indicus* samples from Africa. Four buffalo sequences were also included to determine if this method was applicable for typing MHC class I in related species.

4.2 Materials and methods

4.2.1 DNA samples

Genomic DNA was extracted from PBM obtained from animals in the farm herd at IAH as described in section 2.2.3 (NB these animals are not inbred). Other samples were provided by Dr. P. Chavatte-Palmer (INRA, France), Dr. W. Gerner (Tuebingen, Germany) and Dr. N.D. MacHugh (formerly ILRI, Kenya, now University of Edinburgh).

4.2.2 PCR conditions

Fragments containing exons 2 and 3 were amplified from the unknown samples using the group-specific primers described in chapter 3. Reactions were set up as in section 2.2.8.2 and run under the following conditions:

Group 1: 98°C for 20s, 35 cycles of 96°C for 20s, 68°C for 1min 30s and a final extension step of 72°C for 5 min

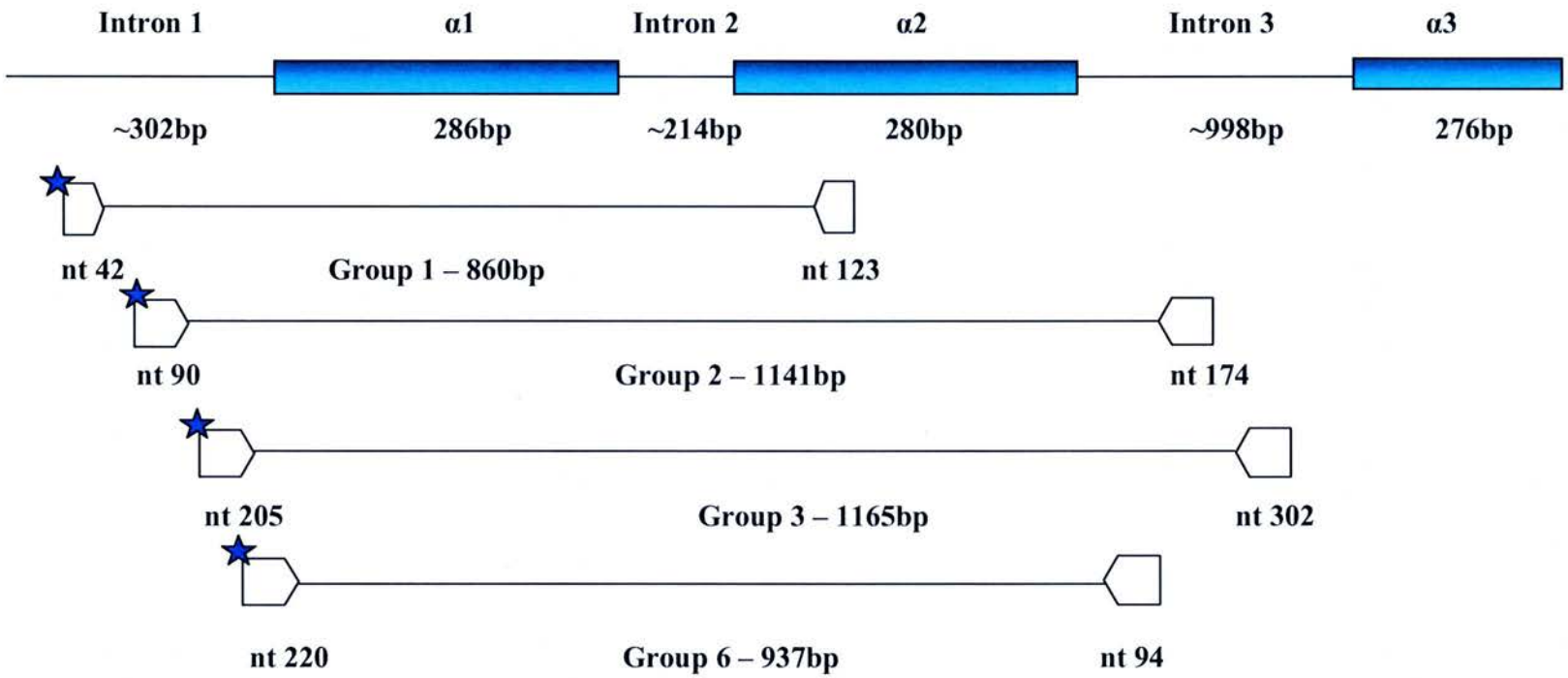
Group 2 and 3: 95°C for 5 min, 30 cycles of 95°C for 30s, 58.3°C for 50s, 72°C for 1 min with a final extension step of 72°C for 5 min.

Group 6: As group 2 and 3 but 59.8°C as annealing temperature.

The sizes of the products and their locations are shown in fig. 4.1.

Figure 4.1: Reference strands for RSCA – position and length

Reference strands were amplified from clones using group-specific primers with a FAM label (★) attached to the 5' primer. All the reference strands include the α1 and α2 sequences except group 1 since the 3' reverse primer is located within α2.



4.2.3 Preparation of reference strands

Two alleles were chosen from each group to act as reference alleles, these are listed in table 4.1. Alleles were amplified from genomic DNA using the group-specific primers as described above, ligated into the pGEM-T vector, transformed into JM109 competent cells and sequenced with T7 and SP6 primers (sections 2.2.12 – 2.2.15). Sequences were then compared to Genbank-derived coding sequence using the GCG 10 program to ensure amplification of the correct product. Minipreparations of bacterial clones containing the correct products were stored at -20°C to form a reference bank. Fluorescent labelled reference (FLR) strands for each group were produced as in the PCR above except that the forward primers had a FAM label attached (MWG Biotech, Germany). A 1/100 dilution of the minipreparation DNA in water was used as the template DNA.

4.2.4 Duplex formation

3µl of PCR product was added to 1µl of FLR, vortexed to mix then run at the following program to allow the formation of duplexes: 95°C for 4 min, 55°C for 5 min, 15°C for 3 min. Samples were then centrifuged at 300xg for 1 min to ensure all reagents were at the bottom of the tube. 0.5µl of 2500-ROX Genescan size standard (Applied Biosystems) and 2µl of Ficoll loading buffer (15% Ficoll and 0.25% bromophenol blue, both Sigma) were then added to each sample which was vortexed then centrifuged.

Table 4.1: Alleles chosen as reference strands

This table lists the alleles chosen from each group as reference strands with their Genbank accession number also given. The alleles were amplified with their corresponding group-specific primers, the products sequenced to ensure accurate amplification and the minipreparations stored at -20°C for use in RSCA.

Group	Allele	Accession No
1	D18.4 HD1	Y09208 X80933
2	D18.3 D18.5	Y09207 AJ010867
3	D18.2 A10	Y09206 X92870
6	HD6 MAN2	X80934 AJ010861

4.2.5 Gel preparation and electrophoresis

0.2mm, 12% non-denaturing polyacrylamide gels were used to separate the duplexes. 48cm plates were cleaned thoroughly with Alconox (Alconox Inc., NY., USA) dissolved in warm tapwater, and then rinsed with hot tapwater, MQ water and finally 100% ethanol to remove all gel residue and dust. Plates were then assembled and raised at a slight angle from the horizontal to aid gel pouring. Gels were prepared by adding 7.2ml Long Ranger™ gel solution (Cambrex Bioscience, ME, USA) to 46.8ml Milli-Q water and mixing for 5 min with 2g of Amberlite (Sigma). The gel solution was then filtered through a 0.2μm Nalgene filter with 6ml of 10xTBE and de-gassed for 20 min. 450μl of ammonium persulphate and 50μl TEMED (both Pharmacia Biotech, Sweden) was added to polymerise the gel, which was swirled to mix then poured between the plates using a 50ml syringe. A 36-well, square-tooth comb was then secured into the top, the gel sealed and damp towels placed at the bottom to stop the gel shrinking.

After two hours the comb was removed and the sample loading area cleaned to remove excess acrylamide. The laser read area on the plates was carefully cleaned with damp kimwipes to remove any dust and the gel cassette placed into an ABIPrism 377 automated sequencer. A plate check was then carried out to ensure no interference of the laser detection was occurring. 3µl of duplex sample was loaded onto the gel using capillary tips and electrophoresed for 10 hours at 20 watts (1800 volts, 60 milliamps). Temperature was maintained during electrophoresis at 40°C.

4.2.6 Sample analysis

Samples were analysed using the Genescan software from Applied Biosystems. Duplexes are observed as blue peaks due to the FAM label while the size standard shows up as red peaks (ROX label). Initially one lane was chosen and the peaks of the standard within this were assigned sizes (in kb) based on information from Applied Biosystems. This was then used to normalise the standard in each of the other lanes to ensure correct and accurate sizing of products in each sample. Not only does this allow comparison of samples run on the same gel but also allows different gels to be compared, thereby removing the need for controls to be run on each gel. Following on from this each of the blue peaks were assigned a size. In each lane the homoduplex of the reference strand should be the first duplex to run past the laser, since it will be smaller than any heteroduplexes formed, and is visualised as the first blue peak in the analysis package. This duplex should have a size approximately equal to the original PCR product. An arbitrary mobility value is

then assigned to each of the heteroduplexes observed by subtracting the homoduplex size from the heteroduplex size.

4.3 Results

4.3.1 Production of reference strands

The choice of alleles for reference strands can greatly affect the outcome of RSCA. Ideally at least two references should be used per locus, with these alleles having quite diverse sequences. This should allow detection of all or most alleles at this locus since they should differ by a number of nucleotides from at least one of the references. Due to lack of availability of sequenced alleles we were restricted in our choice of references. Two alleles were chosen from each group which are known to be true alleles coding for functional MHC class I molecules and for which a full coding sequence is available. These alleles are listed in table 4.1.

In humans reference strands are derived from a panel of homozygous B-lymphoblastoid cell lines that are readily available (Marsh *et al.*, 1997). Since no equivalent is available for bovine samples we chose to form a bank of minipreparation DNA containing each reference allele. This approach has also been successfully used for typing MHC genes in cats (Kennedy *et al.*, 2003). Alleles were amplified from the minipreparations using the group-specific primers so that exon 2 and 3 of the alleles were included and the sequences compared to Genbank to ensure that the products were correct and no PCR errors were incorporated. The sequences of the reference strands are shown in appendix a with the group-specific primers highlighted.

4.3.2 PCR on genomic DNA samples

The products used in the RSCA process are derived by PCR of bovine class I genes from genomic DNA with group-specific primers. Since the expressed MHC class I loci can vary between haplotypes and therefore between samples it is presumed that all four sets of primers will not work on every sample. As a result it was decided to run the PCR products on agarose gels to confirm the presence of an amplicon prior to running the RSCA gel. PCR was carried out on a range of untyped DNA samples from European cattle breeds including Holstein Friesians, Normandy cattle, Simmentals, Charolais and Brown Swiss cattle. DNA from partially characterised *Bos indicus* cattle was also included along with some buffalo DNA. The results of these PCRs are summarised in table 4.2.

The pattern of positives was found to be quite variable, with no sample positive for all groups. This indicates that the primers are behaving in a specific manner and also provides more evidence for the variable nature of the haplotypes in different breeds. Since these PCR reactions were carried out on genomic DNA it suggests that rather than all loci being present on all haplotypes and some silenced, some genes appear to be deleted. Alternatively it may be that the genes are all there but that their introns have changed, which is possible as the primers are relying on only a few specific bases. Another possibility is that the primers designed are specific for the alleles sequenced in chapter 3 but not for all alleles of the group.

Table 4.2: PCR results for group-specific primers tested on untyped samples

Group-specific primers for groups 1, 2, 3 and 6 were tested on a panel on untyped samples from various breeds in PCR reactions carried out as described in section 4.2.2.

Breed	Sample	Group			
		1	2	3	6
Holstein Friesian	O89	X		X	
	9183	X		X	
	1069	X	X		
	9100		X	X	
	161		X	X	
	8253		X	X	
	9159			X	
	9163	X	X	X	
Buffalo *Syncerus caffer*	buff1			X	
	buff2			X	
	buff3			X	
	buff4			X	
Unknown IAH farm	98	X			
	99	X			
	101315	X			
	4188	X	X	X	
Normandy	B755	X	X		
	B818	X	X		
	B668		X	X	X
Charolais	B891		X		X
Rouge des pres	B822		X		
African	D409		X	X	
	8392		X	X	
	T3.5		X		X
	E55	X		X	
	G277				X
	G310		X	X	
	E54		X		
	E182	X	X		
Brown Swiss	813		X		X
Simm/HF cross	252				X
Brown Swiss	129		X	X	

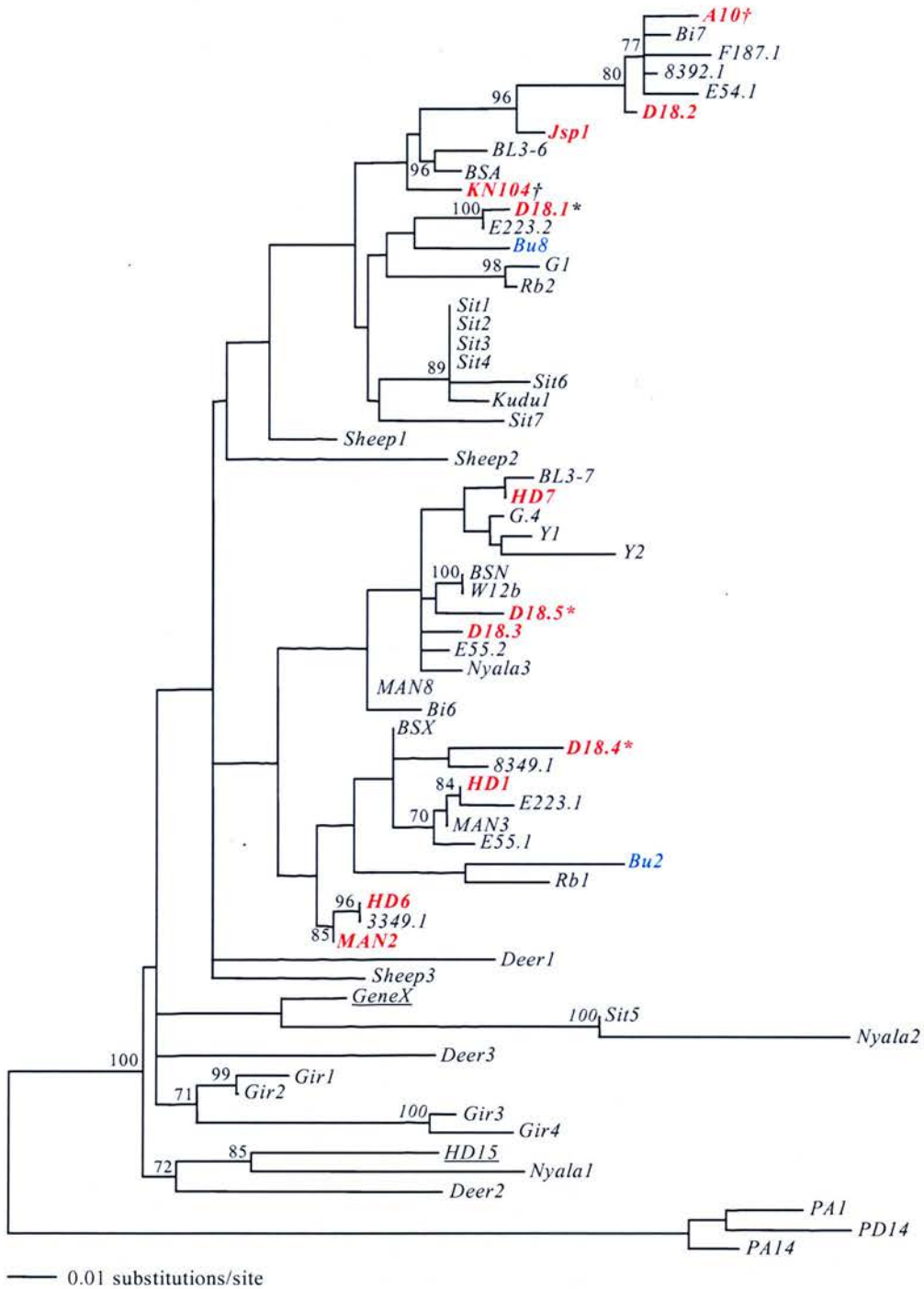
Previously it was thought that gene silencing was occurring to produce the variable haplotypes. Southern blot analysis of DNA derived from animals homozygous for the A14 and A10 haplotypes following digestion with random-cutting enzymes showed them to have approximately the same number of bands. This suggested that no large deletions were occurring in the A10 haplotype that was believed to have only one MHC class I gene (Stephen Archibald, personal communication). This can now be explained with the results from chapter 3 where we found that the A10 haplotype does appear to have another class I gene which may or may not be expressed.

While the majority of the samples included were positive for two or more groups the buffalo samples included were found to be positive only for group 3. This is surprising since previous phylogenetic analysis which included two buffalo sequences found one of them to group with D18.1 and the other with group 1 (see fig. 4.2, Holmes *et al.*, 2003). While it is difficult to conclude anything from this with such a small number of samples and the relation of the animals unknown it indicates that buffalo have at least some similar genes to cattle and that variable haplotypes are likely to occur. However it is likely that we are missing genes in these animals probably as a result of differences in the intron sequences.

It was interesting to note that groups 1 and 6 appeared to be mutually exclusive and not found on the same haplotypes, which might suggest that they actually represent a single locus. As discussed in chapter 3 no distinct groups are evident when phylogenetic analysis of coding sequence from a range of class I alleles is carried

Figure 4.2: Maximum likelihood tree linking 3' sequences from 13 species of Cetartiodactyls

This tree was adapted from Holmes *et al.*, 2003. Pig sequences were chosen to root the tree due to their divergence from the other species included. Cattle class I alleles are shown in red, buffalo sequences are shown in blue.



out. Instead we have had to group alleles based on loose associations. It was unclear however whether the three alleles HD6, MAN2 and 3349.1 were part of group 1 or formed a separate group. Phylogenetic trees constructed with 3' (Holmes *et al.*, 2003) or full length sequence found these alleles formed a sub-group of group 1 whereas inclusion of 5' sequence shows them to be more diverse. Comparisons of the intron 1 sequences identified a number of conserved bases between the HD6 and MAN2 and the group 1 alleles (see fig. 3.3) but none were found in intron 3. As a result we chose to separate these alleles to a new group, group 6. An alternative view is that the primers designed for group 6 could be lineage-specific rather than locus-specific which might explain why such a small number of positive results were obtained. Clearly more samples need to be studied and alleles identified in order to investigate this group further.

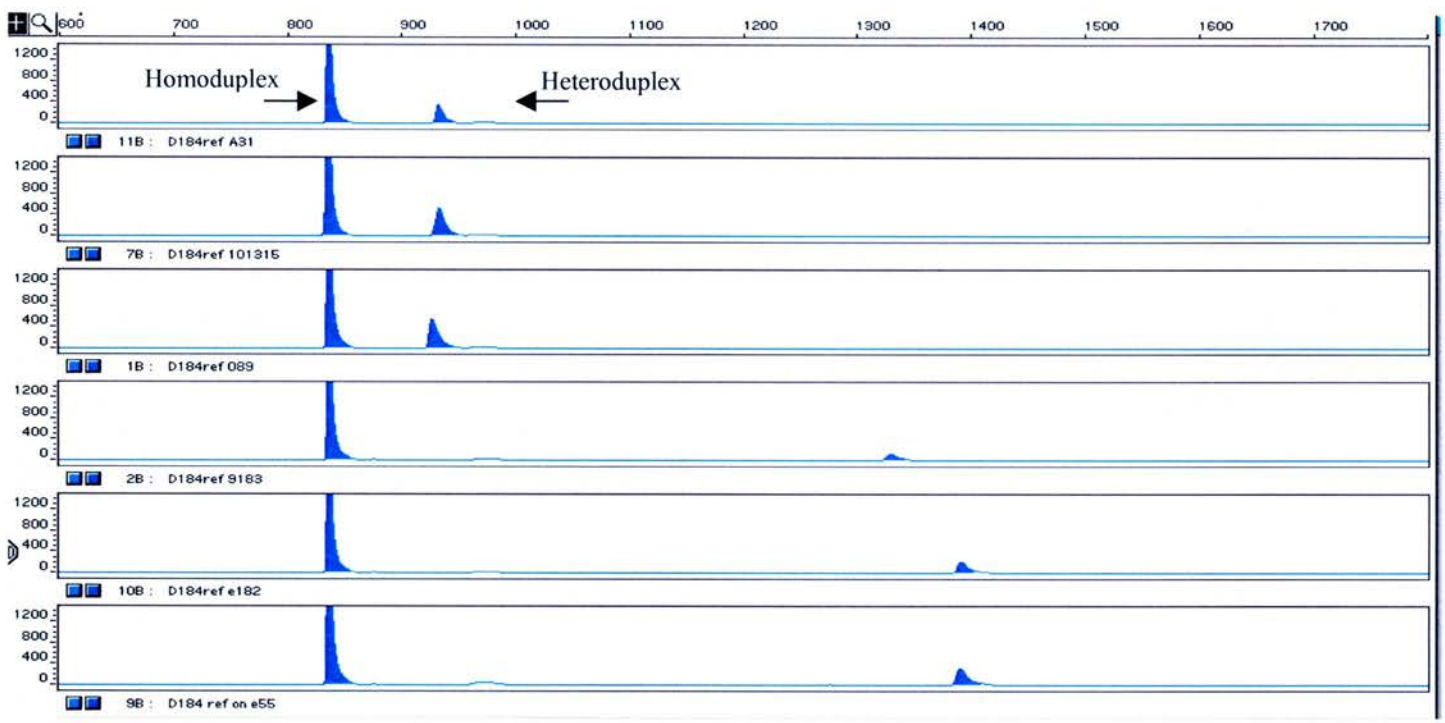
4.3.3. RSCA results – group 1

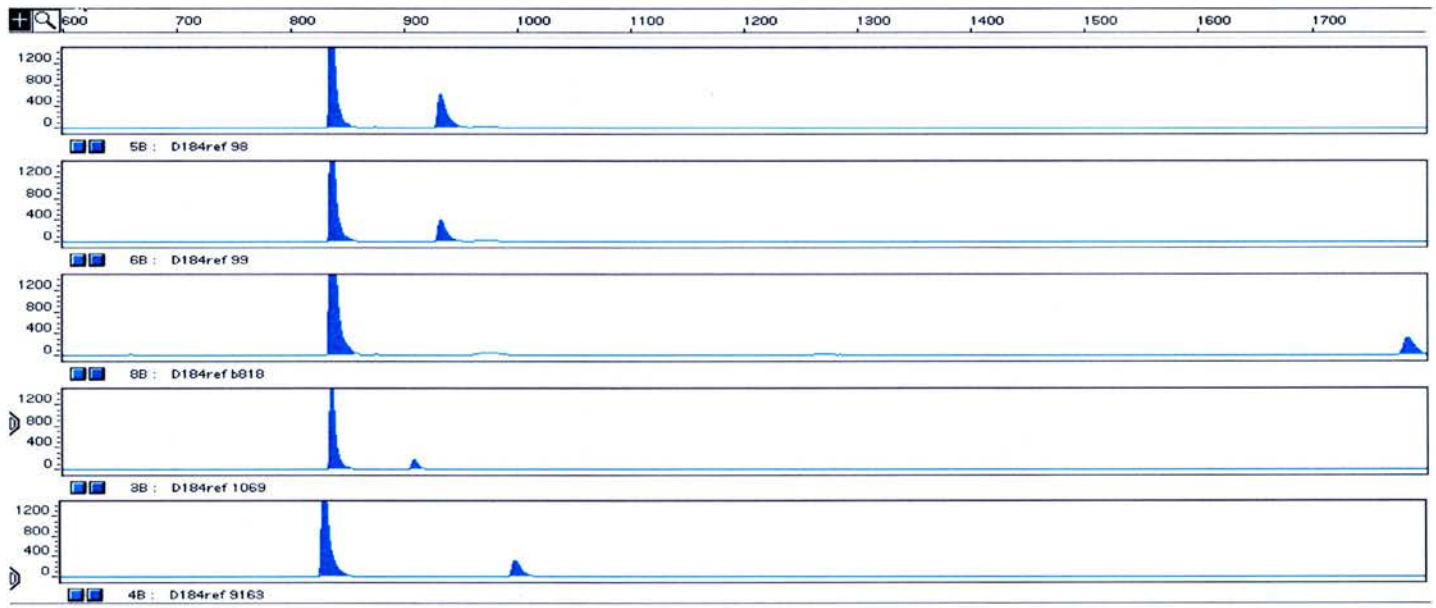
Of the 32 samples tested with the group 1 primers twelve were found to be positive. These samples were analysed by RSCA using both the D18.4 and HD1 FLRs. In addition, samples positive for D18.4 and HD1 were included to assign a mobility value for each using the alternative reference strand. Examples of the traces obtained are shown in fig. 4.3 (D18.4 FLR) and fig. 4.4 (HD1 FLR).

When a sample positive for HD1 was duplexed to D18.4 FLR a mobility value of 96.2 was obtained. By comparing this value to those obtained for the remaining samples it can be seen that 98 and 99 have similar values (96.2 and 95.9 respectively)

Figure 4.3: RSCA profile with group 1 D18.4 FLR

Samples were amplified with group 1 primers and the products then tested on RSCA gels duplexed to D18.4 FLR. Duplexes with a fluorescent tag attached are detected by a laser in a automated sequencer which is then viewed as a peak on the Genescan software. Intensity of the fluorescence is shown on the Y-axis of each sample trace as peak height, while the size of the duplex is shown on the X-axis scale in base pairs. The first peak in each trace is the FLR homoduplex, any other peaks represent heteroduplexes formed between the FLR and the sample. An arbitrary mobility value for the heteroduplexes formed is shown. This is determined by subtracting the homoduplex size from the heteroduplex size. The sample name and FLR are shown under each trace.





Mobility Values:

98 – 96.2

99 – 95.9

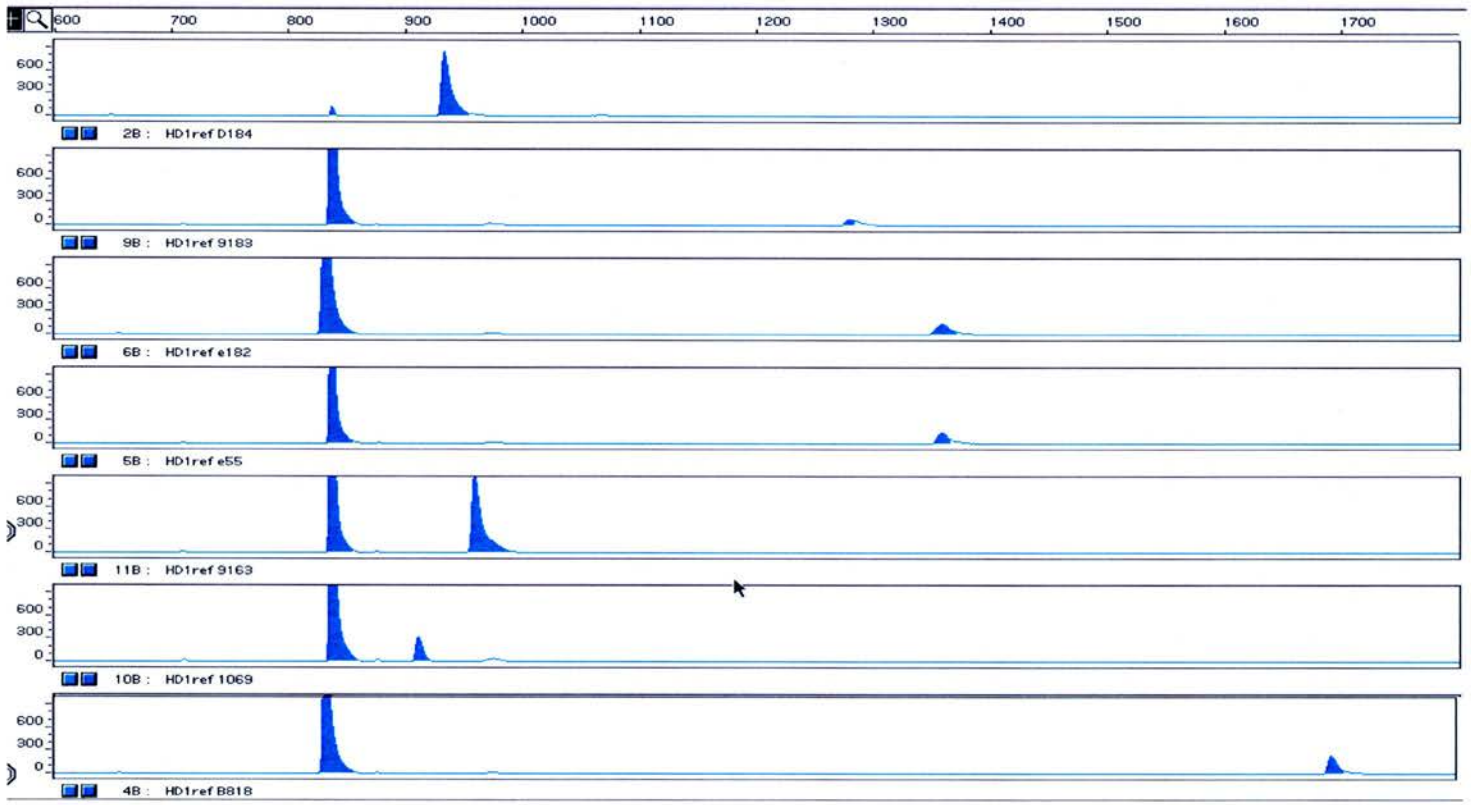
B818 – 858.2

1069 – 72.7

9163 – 168.2

Figure 4.4: RSCA profile with group 1 HD1 FLR

Samples were amplified with group 1 primers and the products then tested on RSCA gels duplexed to HD1 FLR.



showing these to have the HD1 allele or a slight variant. This is supported by the fact that no heteroduplex is observed when the samples are run with the HD1 FLR (not shown). 98 and 99 have previously been typed as either A31* or A31/W12A. This result confirms the presence of the A31 haplotype but does not exclude W12A about which relatively little is known, although it is believed to carry a group 1 allele. Sequencing of a large number of PCR clones would be required to detect the presence of further group 1 alleles other than HD1 in these animals.

When the 101315 and 089 samples were run with the D18.4 FLR they were found to have mobilities of 97.1 and 90.7. When considering the values above and the slight variation seen it is likely that these samples are allelic variants of HD1. When run with HD1 FLR, a heteroduplex was formed with 089 (showing it to be different to HD1) but not with 101315. Sequencing of these samples (see fig. 4.5) showed 089 to differ by four aa to HD1 in the α1 region while 101315 differed by three. This clearly shows the importance of having more than one FLR since with the HD1 FLR alone 101315 would have been typed as positive for HD1.

When the remainder of the samples were run with both of the FLRs 9183, 9163, B818 and 1069 all appeared to have new group 1 alleles while E182 and E55 had similar mobility values indicating they may have the same allele. Sequencing of the α1 region of the latter two supported idea (fig. 4.5), although α2 would need to be sequenced to confirm this. The other sequences were similar to each other, with the majority of the amino acid changes appearing to be real since they are also found in either HD1 or D18.4. The presence of the KN and TAL motifs in a few of

Figure 4.5 : Complete α1 sequence of new group 1 alleles

Group 1 specific primers were tested on a panel of untyped genomic DNA samples. The α1 region of those that were positive was then sequenced. These are shown aligned to HD1, with two other group 1 alleles D18.4 and E55.1 included for comparison. Dots (.) indicate that the sequence is the same to the top allele.

```
                *        20        *        40        *        60        *        80        *
HD1     : GSHSLRYFYTAVSRPGDGEPRFITVGYVDDTQFVWFDSDAPDPRKEPRTPWIEKEGPEYWDRETRISKENTLVYRGSLNNLRGYYNQSEA
4188    : ................................................................................
101315  : ..................................................C.........Y.....................D.......
D184    : ................L.................R.........E...A.............................E.............
9183    : ...................................R.............A............D.............KN..T..........
E182    : ................L.................R.............A............................KN..TAL........
E55.1   : ...................................R.............A............................KN..TAL........
E55     : ................L.................R.............A............................KN..TAL........
9163    : ................L...................................A............................KN..TAL........
1069    : ................L.................R.........M...A.....................................
089     : ..........................................................................K.....EN..T..........
B818    : ..........G.....L.....FA........IAR.....W...M...A....QK.....EEM..DA...QQRSQLC..T..........
```

these sequences would suggest that sub-groups or lineages of alleles are present within group 1. In contrast to the others B818 had an extremely diverse sequence suggesting this to be a pseudoallele. It would be interesting to sequence the remainder of this product to see if the intron and α2 sequences are also highly variable.

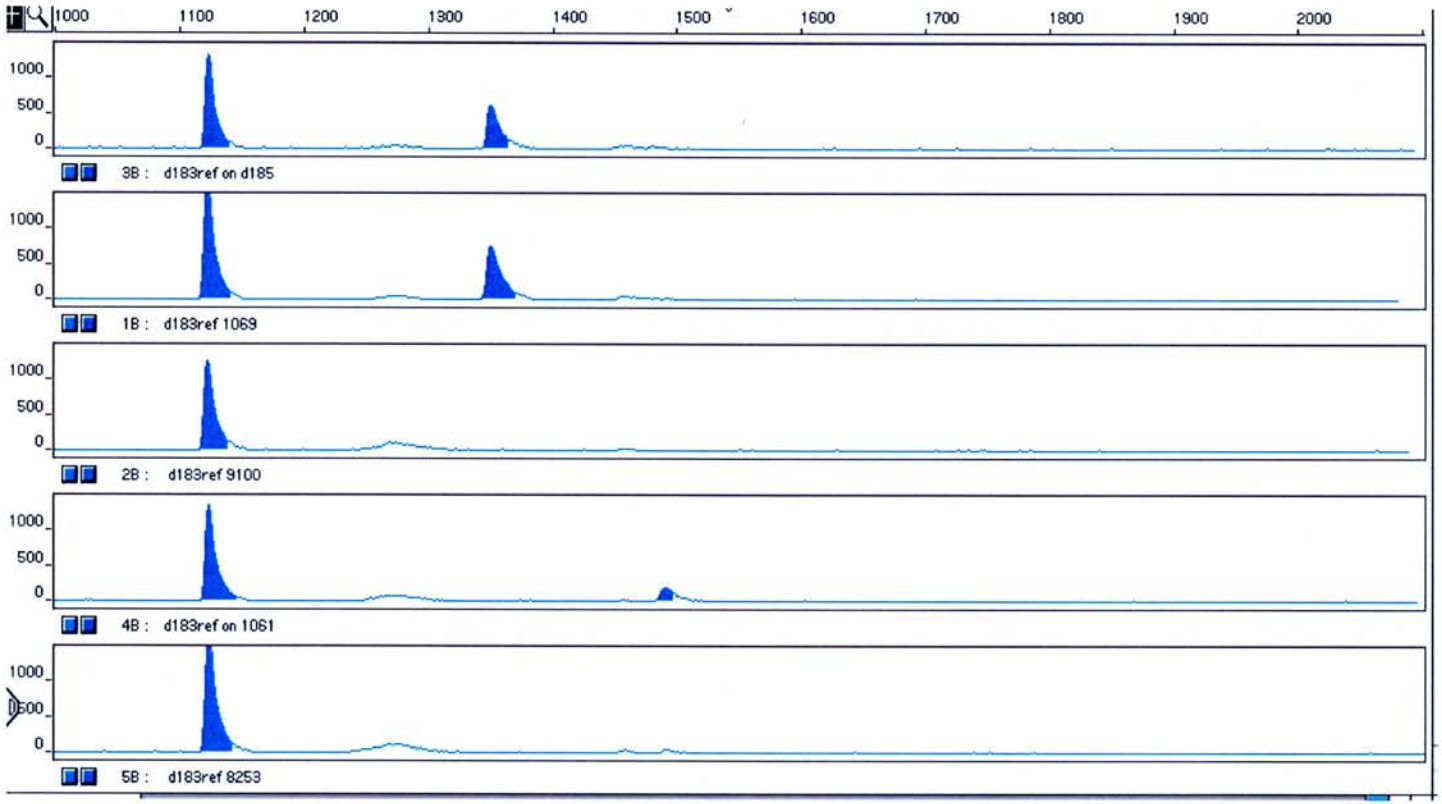
It should be noted that some of the sequences analysed here were sequenced directly from the PCR product without cloning which prevents identification of any PCR errors and any other sequences that may be present (although these should have been visible in the RSCA traces as another heteroduplex peak).

4.3.4. RSCA results – group 2

Nineteen positives were obtained for group 2 of which ten were later sequenced. These samples were run with both D18.3 and D18.5 FLRs (see fig. 4.6 and 4.7 for examples). As with group 1, D18.3 and D18.5 positive samples were included to determine mobility values for these alleles. These were found to be 168.4 and 227.4 respectively. Based on the RSCA profiles alone we were successfully able to type sample 1069 as carrying D18.5, which was confirmed by sequencing. As with the samples typed as HD1 some variability in mobility values was observed, with 1069 having a mobility value of 228.6 compared to 227.4. While these values only differ by 0.8 units larger differences were seen with the African sample T3.5. When this was run against D18.5 FLR a mobility of 158.1 was obtained compared to 168.4 for a D18.3 positive sample. However when T3.5 was sequenced it was found to match

Figure 4.6: RSCA profile with group 2 D18.3 FLR

Samples were amplified with group 2 primers and the products then tested on RSCA gels duplexed to D18.3 FLR.



Mobility Value

D18.5 – 227.4

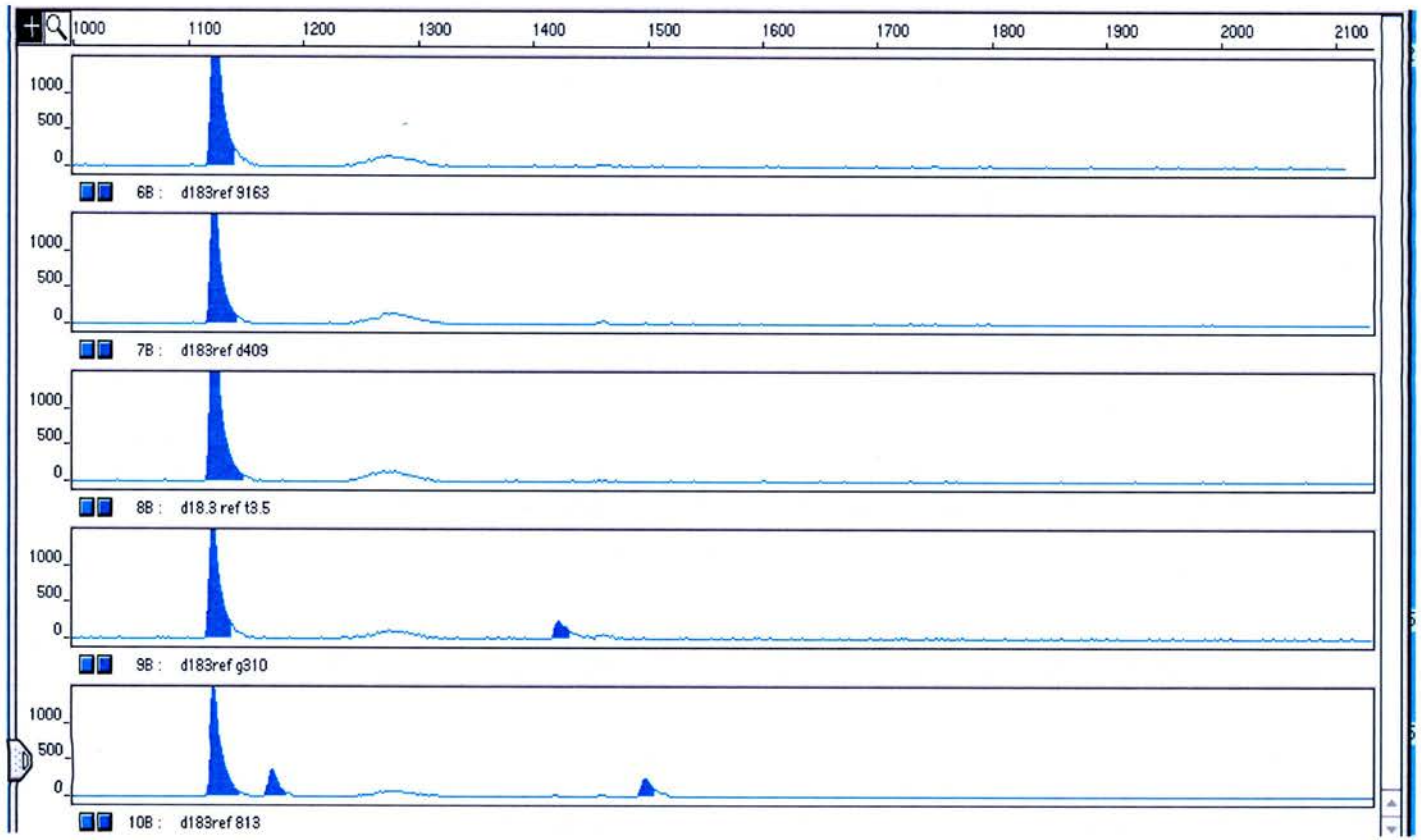
1069 – 228.7

9100 – no peak

0161 – 367.6

8253 – no peak

Figure 4.6 cont'd



Mobility Value

9163 – no peak

D409 – no peak

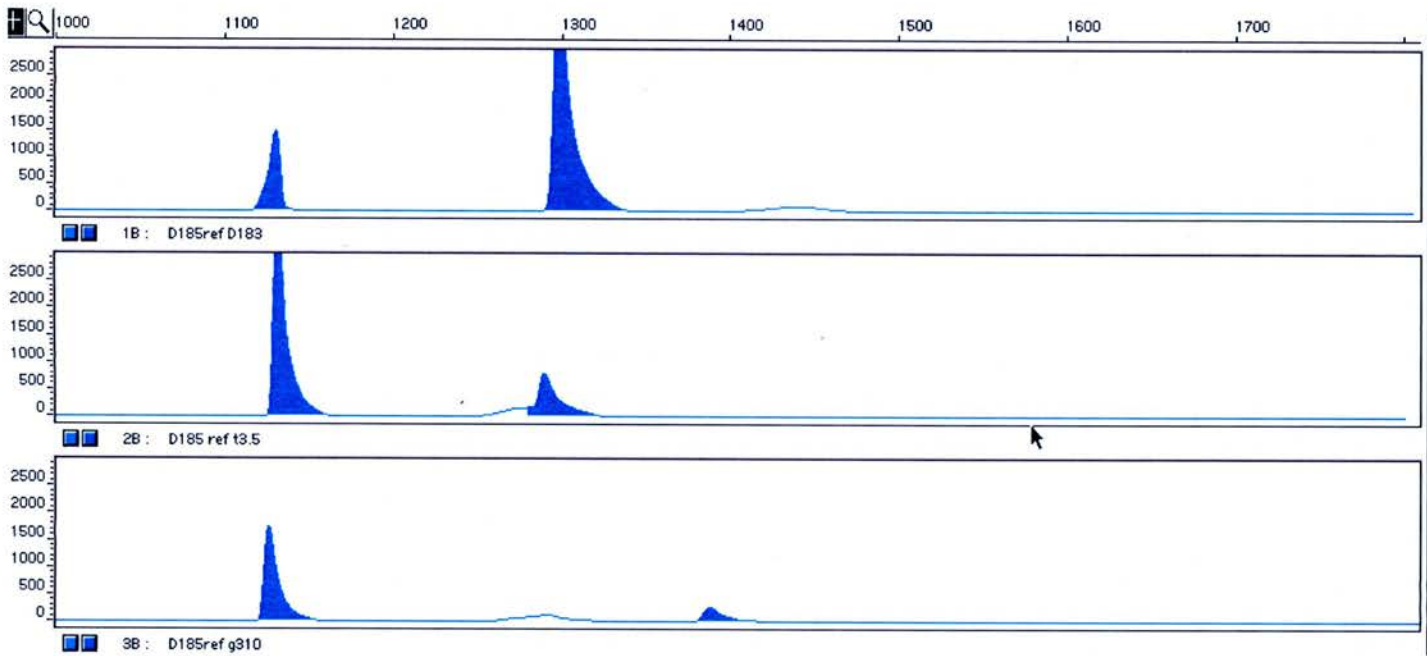
T3.5 – no peak

G310 – 300.3

813 – 49.5 & 375.5

Figure 4.7: RSCA profile with group 2 D18.5 FLR

Samples were amplified with group 2 primers and the products then tested on RSCA gels duplexed to D18.5 FLR.



D18.3 in the whole of α1 and α2. This larger difference is possibly due to the fact that the D18.3 positive sample used to get a mobility value was the D18.3 FLR. Since four times the amount of fluorescence was detected than in normal samples (1μl of FLR with 3μl of labelled sample compared to 3μl of unlabelled sample) this would affect how the signal was detected which probably results in the distorted peak observed in fig. 4.7. This heteroduplex peak can be seen as a large dye blob on the actual gel image (see fig. 4.8).

From the remaining RSCA profiles samples 0161, G310 and 813 appeared to have new group 2 alleles. Sequencing of the α1 and α2 regions of the PCR products showed them to have quite diverse sequences from the other animals (see fig. 4.9). However when these sequences were compared to all available bovine class I sequences the different aa were also found in other alleles showing that we have found real alleles. This indicates that the group 2 primers are specific and capable of amplifying diverse alleles rather than lineages of alleles.

Through sequencing we were able to identify a further five samples positive for D18.3 which had been suggested by the absence of a heteroduplex peak in their RSCA profiles with the D18.3 FLR (fig. 4.9). Interestingly these included another African animal, D409. Both T3.5 and D409 have been typed previously by serology as A10, KN104/A7 and A6/A7 respectively. Since neither the A10, KN104 or A6 haplotypes are believed to have group 2 genes it is likely that D18.3 is derived from A7.

Figure 4.8: RSCA gel image of group 2 samples run with D18.5 FLR

This figure is a standard RSCA gel image which was generated by the ABIPrism 377 collection package. It is representative of the results obtained when samples amplified with group 2 primers are duplexed to the D18.5 FLR. Duplexes are shown as blue bands, with the Genescan 2500-Rox standard shown in red. In lane 10 (highlighted) the D18.5 FLR was duplexed to D18.3 FLR to obtain a mobility value for D18.3. The band is much more intense than the others due to the excess of fluorescence which is shown as a large thick peak when analysed in Genescan (fig. 4.7).

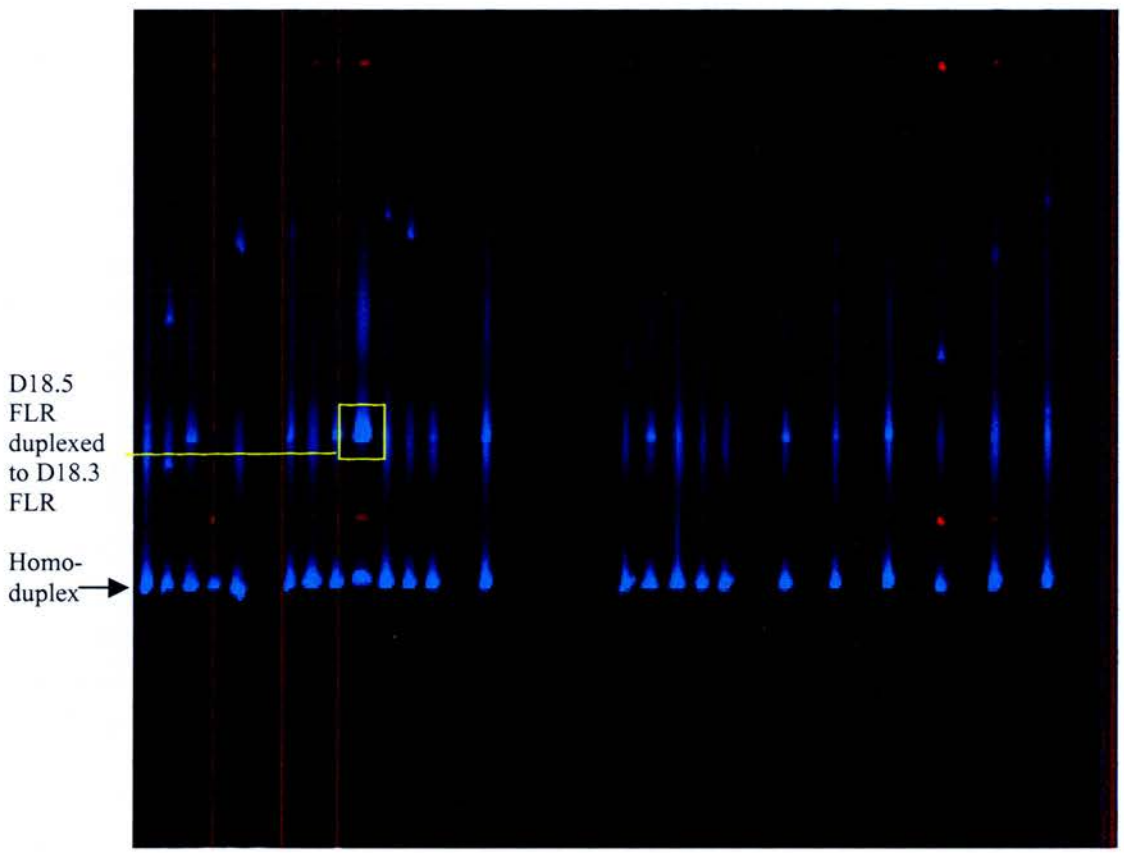


Figure 4.9: Partial α1 and α2 sequence of new group 2 alleles

Group 2 specific primers were tested on a panel of untyped genomic DNA samples. The α1 and α2 region of those that were positive was then sequenced. The sequences obtained are shown aligned to D18.3, D18.5 is also included for comparison. Dots (.) indicate that the sequence is the same to the top allele, dashes (-) indicate that no sequence was available. The division between α1 and α2 is shown as a black line.

```
                *        20        *        40        *        60        *        80        *
D18.3   : GSHSLRYFYTAVSRPGLGEPRFIAVGYVDDTQFTRFDSDAPNPRDEPRVPWMEQEGPEYWDRNTRIYKDTAQIFRANLNTALGYYNQSEAGSHTF
9100    : ..........................................................------------------------------------
8253    : ..................................................------------------------------------------
9163    : ..................................................------------------------------------------
T3.5    : ..........................................................................................
B818a   : ------------------------------------------------------------------------------------------
B818b   : ----------------------------------------------------------------------------..............
D409    : ------------------------------------------------------------------------------..............
D18.5   : ........S.....................................E..........L..............T..VY..ALR.........L
1069    : ........S.....................................E..........L..............T..VY..ALR.........-----
G310    : ..........G.....................V..........E.....................V....LR.........NI
0161    : ------------------------------------------------------.E..NS.E...T.....................V
813     : ..........G.............I.........V.....T....M...AR...........QE...S.GA..T..----------------
813     : ------------------------------------------------------------------------------------------

          100        *       120        *       140        *       160        *       180
D18.3   : QEMYGCYVGPDGRLLLGFMQFAYDGRDYIALNEDLRSWTAADTAAQITKRKWEAAGEAERQRNYLEGRCVEGLRRYLENGKDTLLRA
9100    : ----------------------------------------------------------------------------------------
8253    : ----------------------------------------------------------------------------------------
9163    : ----------------------------------------------------------------------------------------
T3.5    : ........................................................................................
B818a   : ----------------------------------------------------------------------------------------
B818b   : ........................................................................................
D409    : ........................................................................................
D18.5   : .W.S..D........R..W..G.................................A..G.....................A....
1069    : ----------------------------------------------------------------------------------------
G310    : ......D......F.R..R.D.................................E.Y..SL.....................A....
0161    : ......D........R....D.................................D..TW..........W..........A....
813     : ----------------------------------------------------------------------------------------
813     : ..................................................DN..G.....................
```

The high incidence of D18.3 amongst such a small set of samples indicates this may be a common allele in herds however it should be noted that three of the animals were from the Compton herd and are likely to be related.

4.3.5. RSCA results – group 3

18 of the 32 samples tested were positive with the group 3 primers. These were run on RSCA gels with A10 and D18.2 FLRs and from these results we chose to sequence 15 of the products. When the A10 and D18.2 alleles were tested with the FLRs they were found to mobility values of 121.7 and 239.6 respectively. Based on the RSCA profiles shown in fig. 4.11 we were able to type 9159, 9183 and 9100 for A10. When the 9159 and 9100 samples were sequenced (fig. 4.12) 9159 matched A10 exactly confirming the RSCA typing of this sample. In contrast 9100 was found to have two aa differences from A10 despite having the same mobility value as 9159 and no heteroduplex forming with A10. Similarly sample 4188 was typed as positive for D18.2 with the A10 FLR (fig. 4.10) but resulted in a peak when run with the D18.2 FLR. Sequencing of this product showed 4188 to be similar to D18.2 in $\alpha 1$ and $\alpha 2$ but with fourteen aa changes (see fig. 4.12). These data show the need for more diverse reference strands to allow better distinction of alleles in RSCA although it may be that this problem could be resolved by repeating the samples. It also shows that there is a need to combine sequencing with RSCA at this early stage of development to confirm the typing of samples with confusing RSCA profiles.

Figure 4.10: RSCA profile with group 3 D18.2 FLR

Samples were amplified with group 3 primers and the products then tested on RSCA gels duplexed to D18.2 FLR.

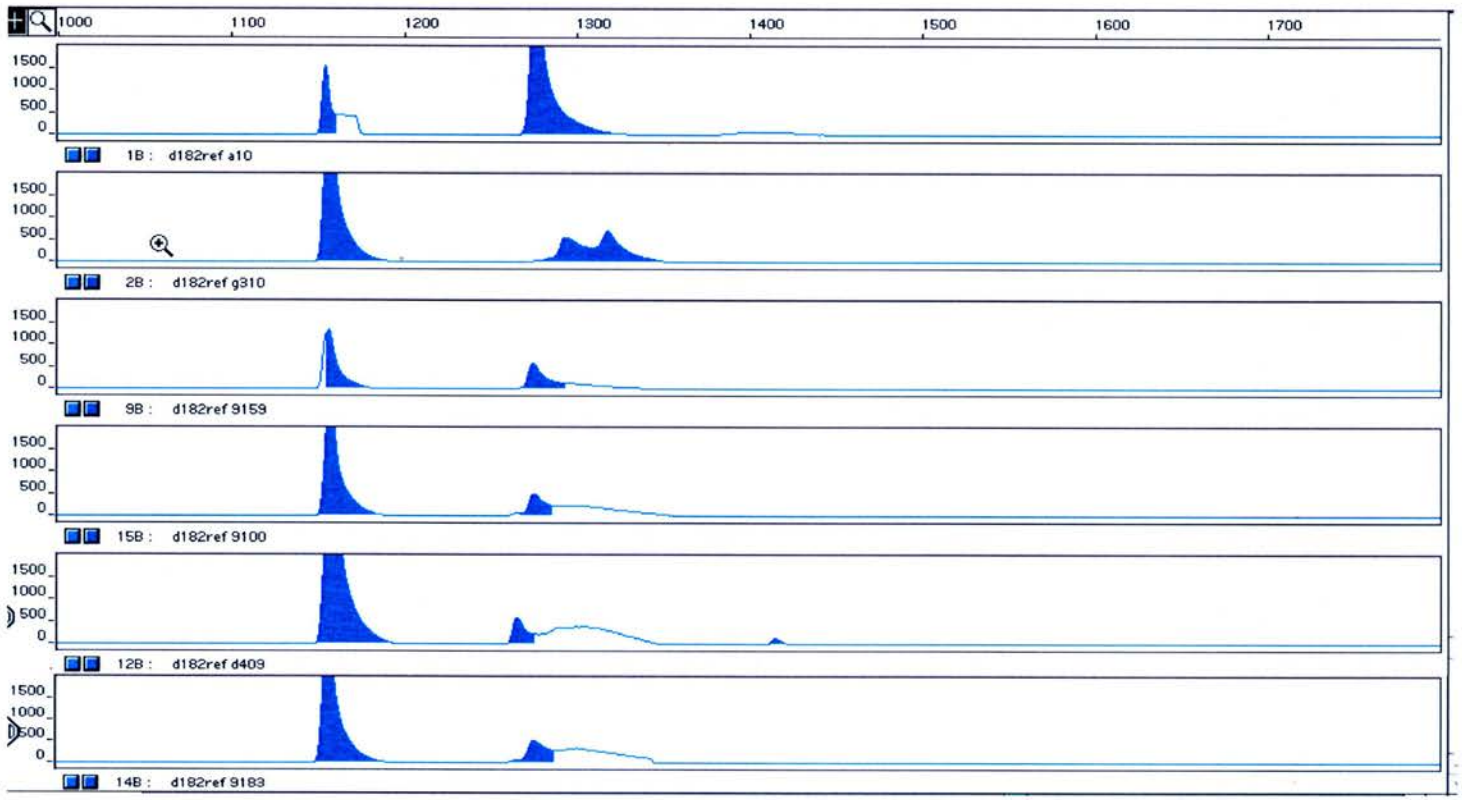


Figure 4.10 cont'd

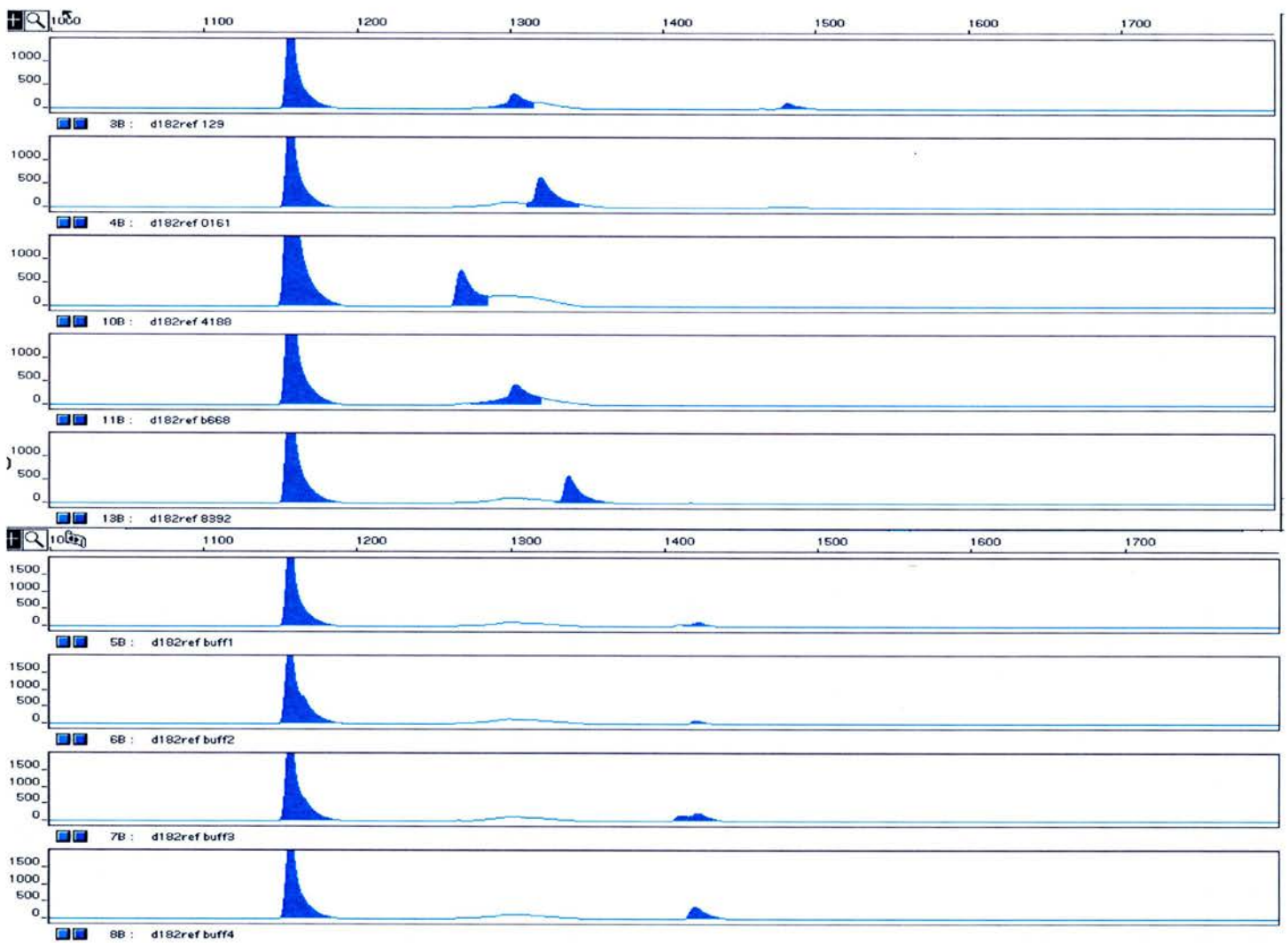
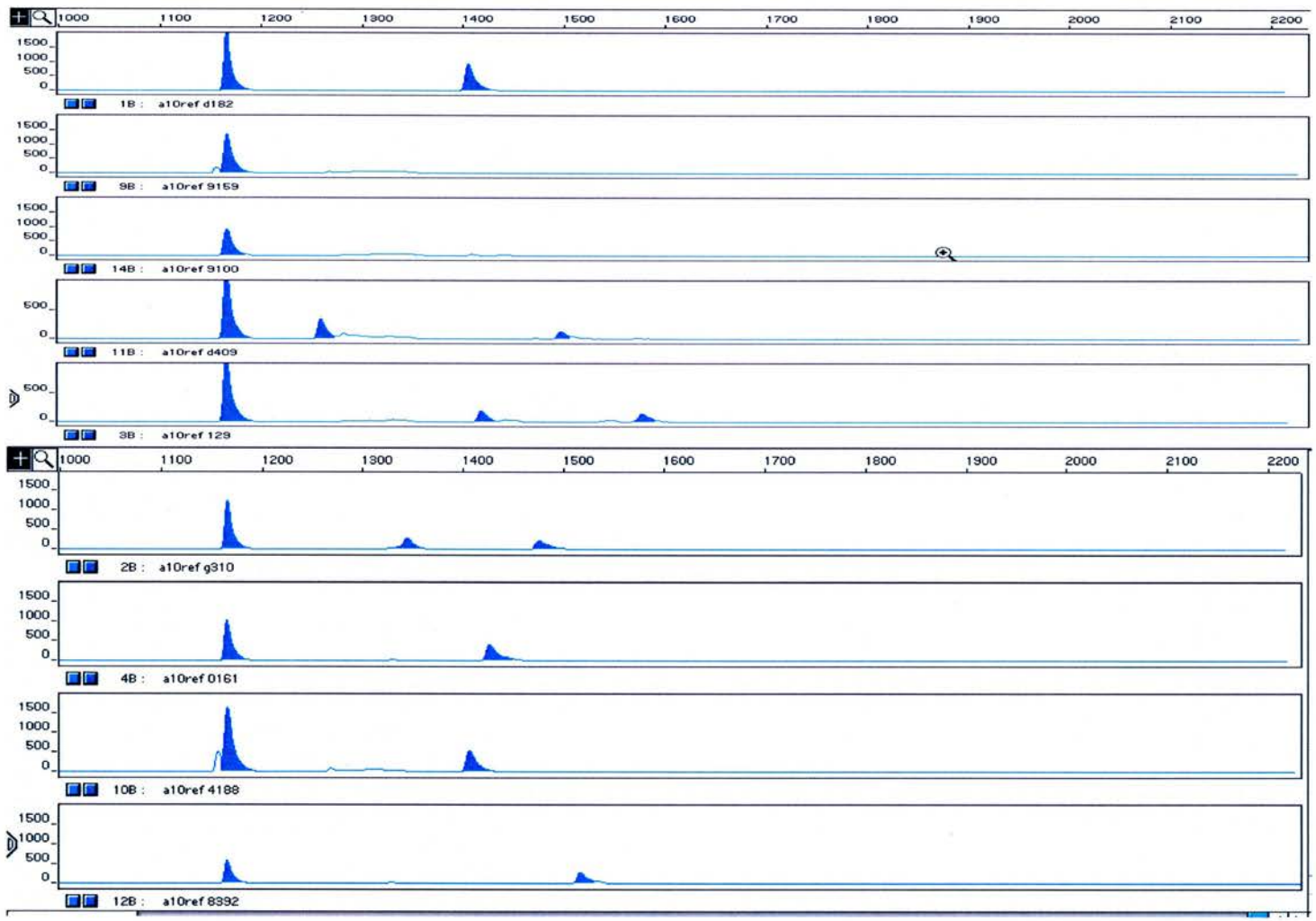


Figure 4.11: RSCA profile with group 3 A10 FLR

Samples were amplified with group 3 primers and the products then tested on RSCA gels duplexed to A10 FLR.



Mobility Value

D18.2 – 239.6

9159 – no peak

9100 – no peak

D409 – 93.2 & 330.8

129 – 252.4 & 411.3

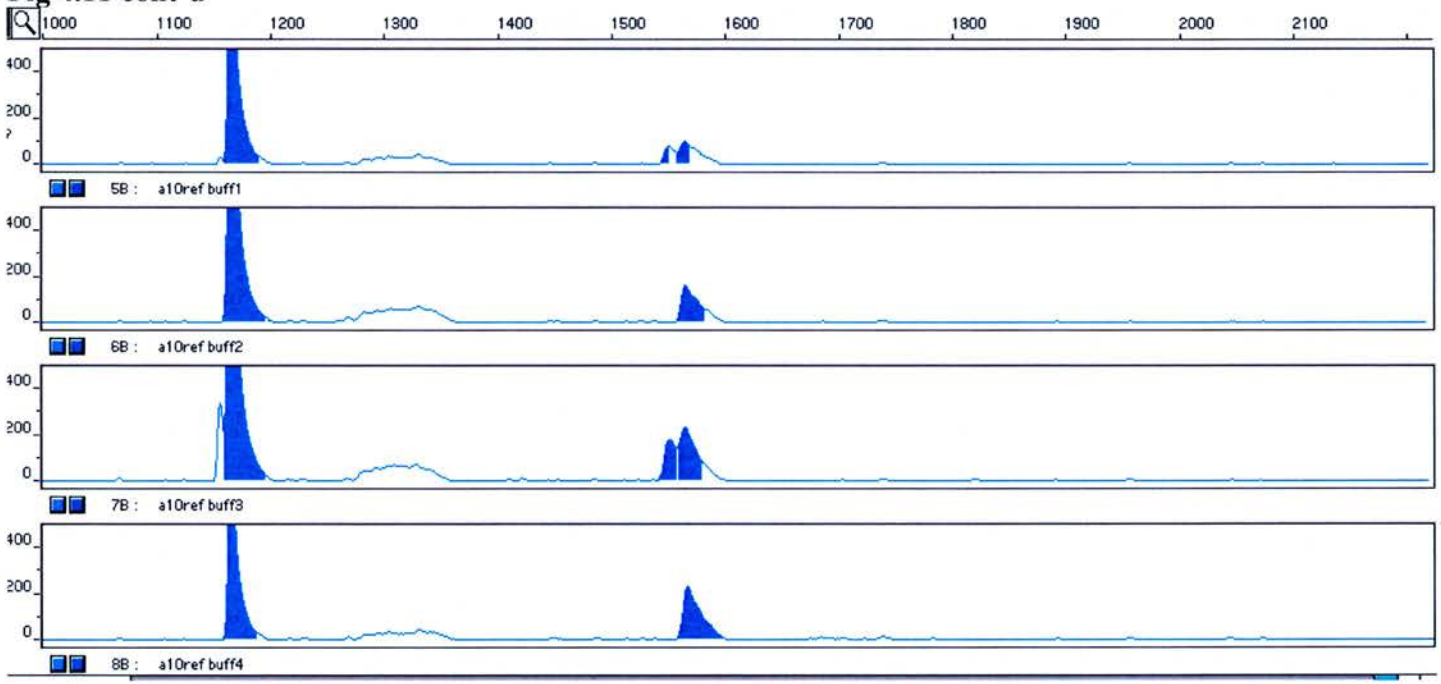
G310 – 179 & 309.8

0161 – 261.4

4188 – 240.5

8392 – 351.1

Fig 4.11 cont'd



Mobility Value

Buff 1 – 398.3 & 399.2

Buff2 – 399.2

Buff3 – 385.6 & 398.91

Buff 4 – 401.33

Figure 4.12: Partial α1 and α2 sequence of new group 3 alleles

Group 3 specific primers were tested on a panel of untyped genomic DNA samples. The α1 and α2 region of those that were positive was then sequenced. The sequences obtained are shown aligned to A10, D18.2 is also included for comparison. Dots (.) indicate that the sequence is the same to the top allele, dashes (-) indicate that no sequence was available. The division between α1 and α2 is shown as a black line.

```
                *         20         *         40         *         60         *         80         *
A10     : GSHSMRYFLTAVSRPGFGEPRYLEVGYVDDTQFVRFDSDAPNPRMEPRARWVEQEGPEYWDQETRKAKGNAQFFRVSLNNLRGYYNQSEAGSHTL
9159    : ..........................................................................................|.....
089     : ..........................................................................................|.....
9100    : ..........................................................Q...............................|.....
D18.2   : ........S.......L.........................R.......E............R..Q.......I..................|....F
8253    : ........S.......L.........................R.......K............R..Q.......I..................|....F
9163    : ........S.......L.........................R.......K............R..Q.......I..................|....F
4188    : ........S.....................................................R..Q.......T...N..T..............|.....
Buff2   : ........S.......L.....................................................RN..N......S...N..T..............|.....
Buff4   : ........S.......L.....................................................RN..N......S...N..T..............|.....
Buff3   : ........S.......L.....................................................RN..N......S...N..T..............|.....
Buff1   : ........S.......L.....................................................RN..N..V...S...N..T..............|.....
0161    : ........Y.G.....L......A.....................................RN..N..DA..T...N..T..............|....F
8392    : ........Y.G.....L......A.....................................RN...T.DA..T...N..T..............|.....
129     : ........Y.......L......I.................G.....................DT......G..T..............|.....
G310    : ........Y.....................................................RN..N......DS..G..T..............|-----
D409    : ........Y.......L......I.....................................R..Q.......................|.....
D409b   : ........Y.......L......I.....................................RN..IS.DT..S...............|.....
```

```
          100         *       120         *       140         *       160         *       180
A10     : QLMSGCYVGPDGRLRRGFMQFGYDGRDYLALNEDLRSWTAVETVAQISKRKMEAAGEAEVQRNYLEGRCVEWLRRYLENGKDTLLRA
9159    : ..................................----------------------------------------------------
089     : ..............................----------------------------------------------------------
9100    : ..............L...............----------------------------------------------------------
D18.2   : .W.Y..D.......LG..E.....................G..E.......W....A..R.........................
8253    : .W.Y..D.......LG..E...........----------------------------------------------------------
9163    : .W.Y..D.......LG..E...........----------------------------------------------------------
4188    : .W.Y.........F....R.DA........----------------------------------------------------------
Buff2   : .W....D..............................G..E.......W..E.Y..........E...................
Buff4   : .W....D.....................----------------------------------------------------------
Buff3   : .W.....................................G..E.......W..E.Y..........E...................
Buff1   : .W.....................................G..E.......W..E.Y..........E...................
0161    : ...Y..D.......L...E...........----------------------------------------------------------
8392    : .W.Y..D.......L...E.............----------------------------------------------------------
129     : .W.C..D--------------------------------------------------------------------------------
G310    : --------------------------------------------------------------------------------------
D409    : .W.............................----------------------------------------------------------
d409b   : .W.............................----------------------------------------------------------
```

When both reference strands were run with four buffalo samples (buff 1-4) double peaks were observed for samples buff 1 and buff 3 and single peaks for buff 2 and 4. The samples all appear to share the same allele based on the mobility values, with buff 1 and buff 3 having a second variant of this. When the products were sequenced (fig. 4.12) buff2 and buff 4 were identical with one aa difference at position 100 from buff 1 and buff 3 which only differ from each other at position 69. Even though the PCR products were cloned and sequenced no second sequence corresponding to the extra peak in the traces for samples 1 and 3 was found. To determine if this extra peak is true it is necessary to repeat the initial PCR on these samples and sequence larger number of clones.

As with the buffalo sequences double peaks were also found for 129 and G310 indicating the presence of two group 3 alleles in these samples. However, despite cloning and sequencing, only one sequence was obtained from each of these. Again it is necessary to repeat these samples to identify further sequences. D409 also has two heteroduplexes when tested with by RSCA and was the only such sample we were able to identify two sequences from, D409 and D409b (fig. 4.12). These sequences differ by seven aa indicating them to be different alleles. D409b differs from the remainder of the sequences shown so this may potentially be a pseudoallele. To confirm this is real allele it is necessary to compare the sequence obtained to that of other class I alleles to determine if any of the aa are found in other sequences.

Sequencing of range of products typed two samples, 8253 and 9163 as having D18.2 while a further five potential new alleles were also identified from 4188, 0161, 8392,

129 and G310 (fig. 4.12). These sequences share many aa with A10 and D18.2 indicating them to be true alleles.

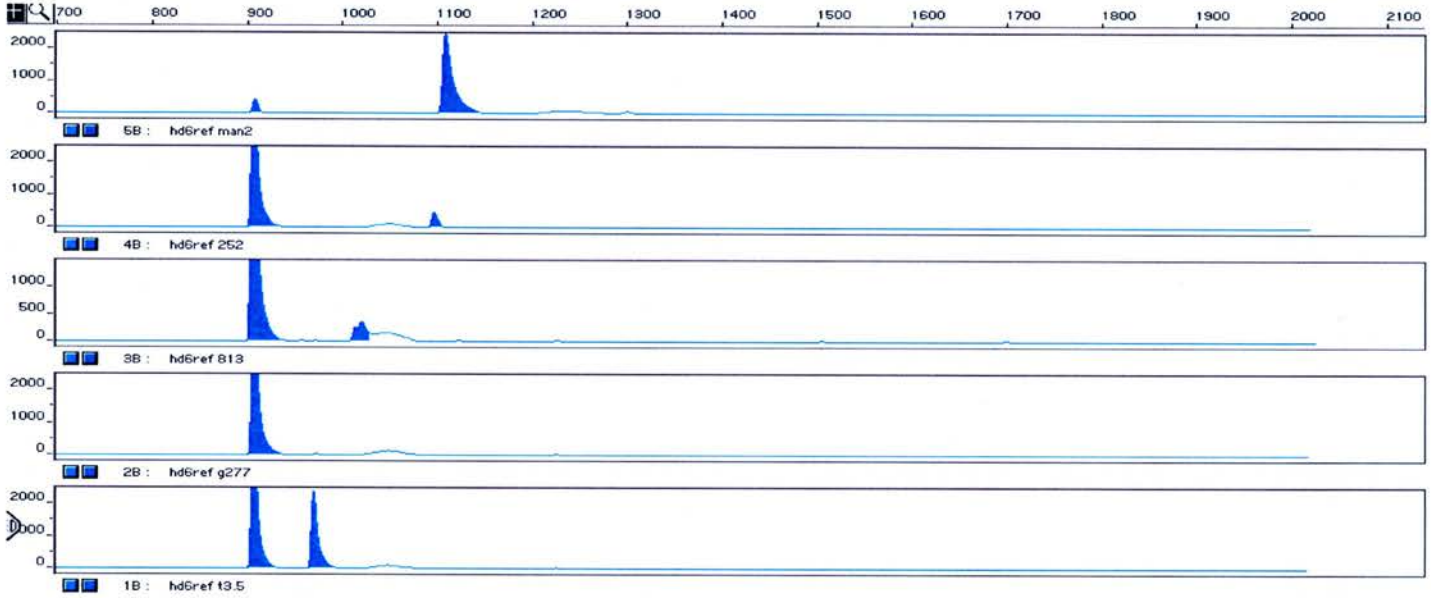
Comparing the sequences obtained it can be seen that the buffalo sequences are very similar, sharing all their aa with the other alleles identified with the exception of a single valine at position 69 in the buffalo 1 sequence. This indicates that the group-specific primers (and RSCA) may be applicable for typing class I genes in buffalo and other related species.

4.3.6 RSCA results - group 6

Only six of the 32 samples amplified with the group six primers of which two were later sequenced. From the RSCA traces using HD6 and MAN2 FLRs (figs 4.13 and 4.14) animal 252 typed as having MAN2 which was then confirmed by sequencing (fig. 4.15). None of the samples appeared to have HD6 although sample G277 did not work when run on the RSCA gel. Sample T3.5 was found to have a variant of another group 6 allele 3349.1 with only one aa difference identified. Sample 813 had two heteroduplexes with MAN2 but only one with HD6 which would suggest the sample is heterozygous with A18 as one of the haplotypes. This does not appear to be the case however as neither of the duplexes has a mobility value similar to HD6 when run with the MAN2 reference strand.

Figure 4.13: RSCA profile with group 6 HD6 FLR

Samples were amplified with group 6 primers and the products then tested on RSCA gels duplexed to HD6 FLR.



Mobility Value

MAN2 – 199.4

252 – 189.3

813 – 113.6

G277 – no peak

T3.5 – 62.8

Figure 4.14: RSCA profile with group 6 MAN2 FLR

Samples were amplified with group 6 primers and the products then tested on RSCA gels duplexed to MAN2 FLR.

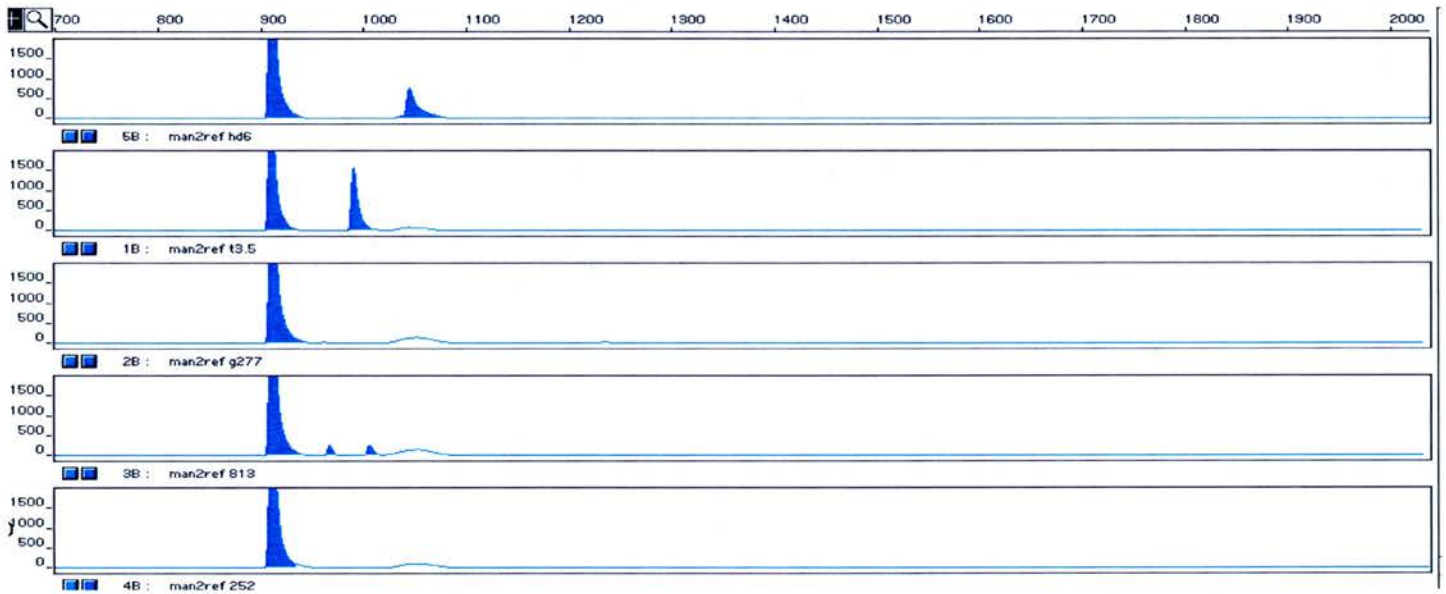


Figure 4.15 : Partial α1 and α2 sequence of new group 6 alleles

Group 6 specific primers were tested on a panel of untyped genomic DNA samples. The α1 and α2 region of those that were positive was then sequenced. The sequences obtained are shown aligned to MAN2, HD6 and 3349.1 are also included for comparison. Dots (.) indicate that the sequence is the same to the top allele, dashes (-) indicate that no sequence was available. The division between α1 and α2 is shown as a black line.

```
                    *        20         *        40         *        60         *        80
MAN2    : GSHFLRYFHTAVSRPGLREPLFITVGYVDDTQFVRFDSDARDPRKEPRQPWMEKEGPEYWDRETQISKENALKYREALNILRG
252     : ................................................................................
3349.1  : ...S....Y.G.....................................................W......N...
T3.5    : ...S....Y.G.....................................................W......N...
HD6     : ...S.....................................T.....................W......N...

                  *       100         *       120         *       140         *       160
MAN2    : YYNQSEA|GSHTYQRMYGCDVGPDGRLLSGFTQFGYDGRDYIALNEDLRSWTAADTAAQITKRKWEAAGEAERFRNYVEGRCVE
252     : .......|.....................................--------------------------------
3349.1  : .......|....L.L.............R.....................................M....D...Q.........
T3.5    : .......|....L.L.S...........R.................--------------------------------------
HD6     : .......|....L.E.......S....RR..E.............................M....A.........T...

                *       180
MAN2    : WLRRYLENGKDALLRA
252     : ----------------
3349.1  : G..........T....
T3.5    : ----------------
HD6     : ..........T....
```

4.4 Discussion

Previous attempts to study the polymorphism of cattle MHC class I genes relied upon serological methods, flow cytometry using specific antibodies and clonal sequencing from cDNA amplicons. In this chapter we successfully used the group-specific primers designed in chapter 3 for RSCA for typing the most complex class I system studied to date. As well as correctly typing a number of samples using this method we were also able to distinguish alleles differing by only a few nucleotides and identified new alleles or allelic variants for groups one, two and three by sequencing PCR products. Assuming these groups are representative of loci then it would appear that the majority of animals tested here have only two of these genes (although they may have others such as D18.1, HD7 and KN104 which were not included in this study). Only two animals were positive for all three groups.

A number of new alleles were detected for each group which all appear to be real i.e. their aa substitutions are also found in other previously identified alleles. Only the allele amplified from B818 with group 1 primers appears false. This could be further investigated by designing allele-specific primers and testing cDNA from this animal. The pattern of aa distribution between the sequences amplified by a set of primers appears to be more diverse than that observed with human alleles of the same gene (http://www.ebi.ac.uk/imgt/hla/). When more sequences have been identified it should be possible to align all the allele sequences available and identify shared motifs between genes which should give us some idea as to how the alleles have been generated. Also, by inclusion of the intron sequences we may be able to

identify break-points in the sequences where recombinations between the genes have taken place.

There were some discrepancies between the RSCA profiles and the sequencing results e.g. group 3 primers with 129 and G310 where two heteroduplexes were formed but only one sequence obtained from the clones. It is likely that we are missing some alleles, particularly since not all of the PCR products were cloned, instead just sequenced directly from product due to time restrictions. These would need to be cloned in future in order to confirm the sequences.

The method used to generate FLRs has also previously been used by Kennedy *et al.*, (2003). They were able to choose a 'rare' cat DRB allele based on previous analysis and included a lion allele to ensure optimal detection of alleles. In addition they used five FLRs in total to reduce possible detection of the FLR allele in animals. Based on this, future work could include the use of buffalo or other related species alleles as FLRs for each of the groups. For group 1 the sequence from B818 could also be used as a FLR since this was very different from the other alleles identified. It will be possible to include more than two FLRs once the new alleles have been confirmed as real by amplification from cDNA. Another alternative is to generate artificial FLRs (using techniques such as those used to produce primers) which would allow us manipulate the compatibility between the FLR and test samples.

One of the major points to arise from the attempts at RSCA is the level of variability of mobility values observed which made it difficult to type some samples as the

limits of this variance was unknown. This variability is not unusual however and has been detected in all the other RSCA systems used. In all cases it was found that intra-gel variance is relatively low but increases between gels, probably as a result of differences in gel composition. Kennedy *et al.* (2002, 2003) used a program called Genotyper (Applied Biosystems) in addition to Genescan which enables them to place 'bins' or confidence limits on known alleles based on the level of variance detected over numerous runs. By doing so it allows easier assignment of alleles and increases confidence in the typing without the need for further sequencing. In order to develop RSCA further for cattle it is important to incorporate a similar system in order to increase the accuracy of typing between gels.

With a number of samples such as sample 9183 with the HD1 FLR (fig. 4.4) there was a problem with resolution of heteroduplexes with the level of fluorescence detected only slightly above background. This could be overcome by increasing the amount of labelled primer used resulting in excess labelled strand. By doing so the level of fluorescence observed should be greater. Less FLR could also be used to increase the resolution of the heteroduplex peaks compared to homoduplex peaks. In these experiments we did not quantify the amount of DNA used at any point which further affects the size of the heteroduplexes formed and the resolution of samples, for example where we have more of one product than another. When the concentration of DNA is too high the bandings on the gels become smeared which affects how the analysis package reads the gels while in some samples no heteroduplex peaks were detected at all even though sequencing identified them as positive for that particular group. Future work should ensure that the amount of

DNA present in each sample and FLR is determined and the quantity used optimised for all assays. This is particularly important considering the small volumes of DNA used in RSCA, where there is an increased need for accuracy.

Investigation of more samples should lead to a greater understanding of the bovine class I system. Based on results from cat and dog studies (Kennedy *et al.*, 2002, 2003) it is likely that variation between breeds in allele frequency and distribution will be identified. Indeed comparison of class I serological specificities between east African and European animals has identified differences in specificity frequencies (Kemp, Spooner and Teale, 1988). It may be the case that cross-breeding of cattle that have been derived from different ancestors and have different class I genes has lead to the introduction of genes into the MHC, resulting in the variable number of genes per haplotype observed or alternatively breeds may have lost some of their class I genes over time. The study of both pure breed and cross-breed animals may allow us to investigate these ideas further.

In conclusion, RSCA is a simple method allowing rapid and easy typing of the class I genes with high resolution. We have shown its application for cattle, allowing comparison of individual animals and providing new information about the bovine class I genes. By incorporating the adjustments suggested and with further optimisation this could quickly be used for wide scale typing of animals, although there is clearly a need to continue sequencing new alleles. It is also important that we extend the method to incorporate the other genes to ensure full typing of animals i.e. group 4 which includes D18.1, found on the A14 haplotype, and E223.2, an

African allele and group 5 includes KN104, also African, and an allele identified from an Indian animal. This requires the sequencing of their introns 1 and 3 to identify any group-specific bases followed by primer design and testing.

Chapter 5

Analysis of MHC class I restriction of *T. parva*-specific CTL in heterozygous cattle

5.1 Introduction

Theileria parva is an apicomplexan parasite capable of infecting and multiplying in bovine lymphocytes. Following development to the schizont stage the parasite induces division of the host cell and passes into daughter cells by association with the mitotic spindle. In susceptible cattle this leads to a disease status marked by extensive parasitosis throughout the animals followed by lymphocytolysis and leukopaenia (Morrison, 1996) with death occurring around three weeks post infection (p.i.). Some animals can recover from infection and have been shown to be immune to challenge with a homologous strain of parasite (Burridge *et al.*, 1972).

Experimental investigation of *T. parva* infection has been greatly aided by the development of infection and treatment regimes whereby animals are given a sublethal dose of parasite (normally in the form of ground-tick stabilate) with simultaneous treatment with oxytetracycline (Radley *et al.*, 1975). Animals are observed to develop mild parasitosis indicated by an increase in temperature and swelling of the draining lymph node but recover between ten and fourteen days p.i.. Challenge of animals infected in this manner indicates that they maintain high levels of strain-specific immunity even at three years p.i. (Burridge *et al.*, 1972).

The major mechanism in the immune response against *T. parva* is widely believed to be CD8+ cytotoxic T cells (CTL) which can be detected at low levels in peripheral blood during recovery from infection. CTL isolated from immune animals have been shown to lyse autologous *T. parva* infected cells *in vitro* but not allogeneic cells

indicating genetic restriction of the immune response (Eugui and Emery, 1981). While little was known about the MHC class I in cattle at this time the authors were able to suggest this as a possible mechanism of restriction by comparison to mouse experiments. Immunisation of various strains of mice with Lymphocytic Choriomeningitis (LCM) virus resulted in the production of specific CTL which were shown to only kill infected targets which shared at least one H-2k haplotype in a ^{51}Chromium release assay (Zinkernagel and Doherty, 1974).

The role of MHC class I molecules in restricting CTL in cattle was later confirmed by Goddeeris *et al.,* (1986b) who showed that lysis of target cells by CTL clones carrying the same class I specificity could be blocked using the monoclonal antibody W6/32 (Barnstable *et al.*, 1978) which sees a non-polymorphic region on MHC class I molecules. Differences in the ability of MHC class I haplotypes to restrict the immune response to *T. parva* became evident following studies involving animals carrying a range of specificities. In most cases restimulated CTL (both bulk cultures and CTL clones) from immune heterozygous animals were found to be restricted by one haplotype over the other when tested for cytotoxicity against a panel of targets (Goddeeris *et al.*, 1990, Morrison *et al.*, 1987). Comparison between animals identified a continual bias amongst haplotypes with for example CTL from most A6/KN104 animals killing targets expressing the A6 specificity in preference to KN104 targets. This resulted in the definition of a so-called 'hierarchy of dominance' amongst the specificities studied with A6 being the most dominant.

Most of this groundbreaking work involved African Boran (*Bos indicus*) cattle, which can be distinguished from European (*Bos taurus*) cattle by the presence of a hump. While the disease occurs in Boran cattle it is found to be particularly severe in European breeds introduced to Africa for milk and meat production and so it is important to study the CTL response in European cattle in an attempt to identify any dominant class I alleles which may provide some protection against *T. parva*.

All animals included in previous studies had been typed by serology using specific alloantisera in microlymphocytotoxicity assays. This method of typing has since been shown to be extremely limited, with specificities assigned in no way indicating the full range of MHC class I molecules expressed on bovine cells. Extensive characterisation of cattle at IAH, Compton using a combination of serology, 1D-isoelectric focusing and PCR-SSP has allowed us to determine the number of classical class I genes and their corresponding alleles found in common haplotypes. Using this information we aimed to examine MHC restriction in cattle (*Bos taurus*) expressing a combination of haplotypes, namely A14/A10 and A18/A31. These haplotypes are variable in the number of classical I genes expressed, with A14 expressing three, A31 two and A18 expressing one. Although A10 was also previously believed to express only one class I gene, recent experiments (see chapter 3) indicate this haplotype may express another class I gene. Further investigations are currently taking place to characterise this gene in more detail. Initially CTL would be tested against cell lines expressing one or other haplotypes to determine if one haplotype restricted the response more than the other as previously observed. Following on from this various methods would be employed to look at restriction

through individual genes (see chapter 6). In this way we hoped to be able to identify those genes important in restricting the response to *T. parva* which could eventually aid vaccine design through identification of parasite epitopes presented. This information would also be important when considering future breeding plans for European cattle in areas affected by *T. parva*.

5.2 Materials and methods

All cell culture methods were carried out as described by Goddeeris and Morrison (1988) with minor adaptations. All materials were obtained from Invitrogen (Paisley, Scotland) unless stated. Incubations took place at 37 °C in a humidified incubator (Jouan Ltd., Derby) with 5% CO_2 in air.

5.2.1 Materials

5.2.1.1 Media

Tissue culture media consisted of RPMI 1640 medium supplemented with $5x10^4$ units penicillin, 50mg streptomycin and 146mg glutamine (in final volume of 500ml), 10% foetal bovine serum (FBS, Australian origin) and 0.05M 2-mercaptoethanol (2-ME). Cytotoxicity media was made up of RPMI 1640 media as supplemented above with 5% FBS added.

5.2.1.2 Cattle

3 Friesian animals, 1 heifer and 2 castrated males, were obtained from the IAH farm, Compton. These animals ranged from 3 to 10 months, were healthy and had not previously been used for any scientific investigation. The animals were chosen based on their MHC haplotypes, which had been assigned by parentage studies, i.e. A14/A10 (animals 495 and 506) and A18/A31 (animal 313). 2 A14/A10 animals

were used in order to compare their results to give an indication of animal to animal variability. Following infection with *T. parva* (section 5.2.2.1) the animals were housed in MAFF category 2 medium security units for 3 weeks p.i. to ensure clearance of all piroplasms. Following this they were moved to low security units.

5.2.1.3 Flow cytometry materials

PBS obtained from tissue culture department, IAH, Compton was supplemented with 1% w/v bovine serum albumin (BSA, BDH, Poole) and 0.1% w/v sodium azide (NaN_3, Sigma) for use as a washing and dilution buffer.

Cells were labelled with a secondary goat anti-mouse immunoglobulin (H+L) FITC conjugated antibody (Southern Biotechnology Associates Inc., Alabama, USA) to allow detection.

All primary antibodies used in flow cytometric analysis were supplied by the monoclonal antibody department, Compton (see table 5.1), with the exception of the class I antibody ILA-88 which was supplied by Dr N.D MacHugh, University of Edinburgh.

Table 5.1: Monoclonal antibodies used for phenotyping cell lines

Antibody name	Epitope recognised	Isotype	Source, Antibody medium
ILA-88 (Toye *et al.*, 1990)	Bovine MHC class I molecules, conformation independent	IgG2a	ILRI, ascitic fluid
MM1A (Davis *et al.*, 1993)	CD3 marker on T lymphocytes	IgG1	IAH: tissue culture supernatant
ILA-58 (Mukwedeya *et al.*, 1993)	B cell marker (Ig light chain)	IgG2a	IAH: tissue culture supernatant
CC8 (Howard *et al.*, 1989)	CD4 marker on CD4+ T cells	IgG2a	IAH: tissue culture supernatant
CC63 (MacHugh *et al.*, 1991)	CD8 marker on CD8+ T cells	IgG2a	IAH: tissue culture supernatant
CC15 (Howard *et al.*, 1989)	WC1 marker for bovine γδ T cell subset	IgG2a	IAH: tissue culture supernatant
ILA-21 (Taylor *et al.*, 1993)	MHC class II	IgG2a	IAH: tissue culture supernatant
TRT1 (Cook *et al.*, 1993)	Ab against Turkey Rhinotracheitis Virus (isotype control)	IgG1	IAH: tissue culture supernatant
TRT6 (Cook *et al.*, 1993)	Ab against Turkey Rhinotracheitis Virus (isotype control)	IgG2a	IAH: tissue culture supernatant

5.2.2 Methods

5.2.2.1 Infection and treatment

This method, which allows the animal to develop a protective immune response and become resistant to the immunising strain of parasite, was carried out essentially as Radley *et al.* (1975) with some alterations. Animals were given a subcutaneous

injection of 1ml of tick stabilate (Muguga strain, the kind gift of Dr. Alan Walker, Centre for Tropical Veterinary Medicine, Edinburgh) above the prescapular lymph node followed by intramuscular treatment with 1ml/10kg oxytetrin 20LA (Schering-Plough Ltd.). The stabilate was stored in liquid nitrogen at a concentration of 2.5 tick equivalents (te)/ml with 1ml per vial. Before use, it was thawed quickly and made up to 5ml with Eagles Minimal Essential Medium (EMEM, tissue culture department, Compton), 3.5% BSA and 7.5% glycerol (BDH) giving a final concentration of 0.5 te/ml.

Animals were inspected every day until 18 days p.i. for clinical symptoms including fever and enlargement of prescapular lymph nodes. Following onset of fever, lymph node biospies were taken every second day by aspirating the lymph node using an 18-gauge needle attached to a 1ml syringe. Samples of these were smeared onto glass slides, air dried then tested with a *T. parva*-specific antibody for presence of macroschizonts.

5.2.2.2 Establishing homozygous and heterozygous *T. parva* lines

T. parva-infected cell lines were produced from PBM from the three heterozygous animals mentioned above. In addition lines were also made from animals homozygous for the A14, A10, A31 and A18 MHC haplotypes.

PBM were isolated from each of the animals as described in section 2.2.1, resuspended at $1x10^6$ cells/ml in culture medium and 2ml added to each well of a 24

well plate. Concanavalin A (ConA) was then added to a final concentration of 5μg/ml and the plates incubated for 5 days. Cells were then harvested and counted as in section 2.2.2. $5x10^6$ viable cells were collected into a universal and pelleted by centrifugation at 400xg for 5 min. The supernatant was removed and stored until further use. 1 vial of tick stabilate (1ml) was quickly thawed in a 37°C water bath and resuspended using a pipette. 200μl was then added slowly to the ConA blasts, the tube was gently shaken, then incubated at 37°C for 2 hours with shaking every 15 minutes. A small amount of the stabilate was added to 2ml of media and incubated to check for fungal growth. Following this the cells were washed twice in tissue culture media at 400xg for 5 min, resuspended in 18ml fresh media (15% FBS) and 18ml of the old supernatant, and 1.5ml distributed into each well of a 24 well plate. To prevent contamination plates were incubated at 37°C in ethanol-washed boxes with the lids were kept slightly ajar to allow for CO_2 flow. The plates were checked every day for contamination and the appearance of infected lymphoblasts, which generally began to grow between 7 and 10 days. Following this cells were harvested by aspiration using a pipette, spun down at 400xg, then resuspended in 15ml and incubated at 37°C in T25 tissue culture flasks. Once cell lines were growing well they were maintained by passaging with fresh media every 4 days (4 parts fresh media to 1 part cell culture).

5.2.2.3 CTL assays – CTL production

PBM, isolated from 60ml blood, were counted and resuspended at $4x10^6$ cells/ml in media, then distributed into 24 well plates, 1ml per well. Autologous *T. parva*

infected cells were resuspended at $2x10^5$ cells/ml and irradiated with 5000 rads of gamma radiation using a ^{137}Caesium Gammacell (Mordion International Inc., Ontario). These were added to the PBM at 1ml per well and the plates incubated at 37°C for 7 days. Following this, the cells were harvested, counted, then made up to $2x10^6$ cells/ml and distributed at 1ml/well into 24 well plates. *T. parva* cells were irradiated as previously, resuspended at $4x10^5$ cells/ml and 1ml added to each of the wells. Autologous PBM were obtained from the animals, irradiated as above and added to the wells at $2x10^6$ cells/well to act as filler cells. All further rounds of stimulation were carried out in the same way.

5.2.2.4 CTL assays – target labelling

All CTL assays were carried out at day 6 post stimulation. *T. parva*-infected targets were passaged the day before and were considered to be healthy and growing well at the time of the assay. $1x10^6$ target cells were labelled with 100μCi sodium chromate (^{51}Cr, Amersham, New Jersey) at 37°C for 90 min, washed 3 times in cytotoxicity medium and resuspended at $5x10^5$ cells/ml.

5.2.2.5 CTL assays – effector (CTL) preparation

Cells from the restimulated cultures were harvested and their viability determined by Trypan blue exclusion. Cells were then made up to $1x10^7$cells/ml in cytotoxicity medium and distributed into a round bottom 96 well plate in triplicate at the following densities, 7.5, 5, 2.5, 1.25 and $0.625x10^5$ cells per well (100μl volume).

5.2.2.6 CTL assays – assay

50µl of the target cells were added to each of the wells containing effector cells giving final effector to target ratios of 30:1, 20:1, 10:1, 5:1 and 2.5:1. Targets were also added to wells containing medium alone in order to determine background ^{51}Cr release. Plates were then incubated for 4 hours at 37°C. In order to measure maximum release, 50µl of labelled targets were added to 100µl water in 1.5ml eppendorf tubes and subjected to 3 rounds of rapid freezing in dry ice followed by slow thawing at room temperature, which effectively lyses cells. These samples were then added to the test plates.

After 4 hours incubation plates were removed and the samples mixed on a plate shaker for 5 min to facilitate release of all ^{51}Cr into the medium. The cells were then pelleted at 400xg for 5 min. 25µl of supernatant was harvested from each using a finnpipette, loaded onto a betaplate filter mat (Wallac, Finland) and allowed to dry overnight. The next morning mats were sealed in plastic bags with scintillant and placed in a Wallac 1205 Betaplate to be read.

Percentage cytotoxicity was calculated as follows:

% cytotoxicity = (test release-spontaneous release)/(maximum release-spontaneous release) x 100.

Percentage spontaneous lysis was calculated by:

(spontaneous release/maximum release)/100.

The coefficient of variation was calculated by (standard deviation/mean) x 100 for each set of triplicates, including those of spontaneous and maximum release.

5.2.2.7 Flow cytometric analysis - one colour (indirect) staining

Single cell suspensions of between 0.5 and $1x10^6$ cells were added to round bottom wells of a 96 well plate and pelleted for 2 min at 400xg. Supernatant was removed by inverting and gently flicking the plate. 25µl of primary antibody diluted in buffer was added (neat if culture fluid, 1/500 dilution in buffer – 5.2.1.3 - if ascities) to the cells and mixed by pipetting. The plate was then incubated at 4°C for 30 minutes to allow binding of the antibody. Following this the cells were washed twice by adding 100µl of the wash buffer and pelleted as above. 25µl of the secondary, FITC-conjugated antibody was then added (1/250 dilution) and the cells incubated for a further 30 min in darkness at 4°C. Following two rounds of washing the cells were made up to 100µl with washing buffer and added to FACS tubes (Micronic systems, Holland) containing 400µl of PBS. Samples were then analysed on a FACScan Flow Cytometer (Becton Dickinson, California) using the FACScan program. Further analysis was carried out using the FCSexpress program. Initially cells were gated on forward and side scatter followed by plotting of the data on a histogram of fluorescent intensity detected by the FL-1 channel. Isotype controls (see antibody list) were included to indicate positive from negative staining.

5.2.2.8 PCR

Typing of cell lines was carried out using primers that have previously been shown to be allele-specific, with generic primers Bov 7 and 11 also included as positive controls (Ellis *et al.*, 1998, see table 5.2). PCR from cDNA was set up as described

in section 2.2.8.2 and run under the following conditions; 5 cycles of 95°C for 1 minute (min), 65°C for 1 min, 72°C for 2 min followed by 25 cycles of 95°C for 1 min, 65°C for 1 min and 72°C for 2 min. Following this 5µl of PCR product was added to 1µl 6x loading buffer and loaded onto a 1% agarose gel. Electrophoresis conditions were as previously described (section 2.2.9).

Table 5.2 Primers used for typing cell lines

The allele-specific primers shown below were used to type the *T. parva* cell lines (Ellis *et al.*, 1998). Bov7 and Bov11 are generic primers which amplify all bovine class I genes. These were included as positive controls.

Target	Primer name	Sequence (5' – 3')	Tm (°C)
D18.1	F D18.1A	CCGGCCCGGCCTCGA	56
	R D18.4B	CAAAGACTCAGCATAACCTT	56
D18.4	F D18.4'	CCGTGGATAGAGAAGGAA	54
	R D18.4B		
D18.5	F D18.5A	GGACGACACGCAGTTCACA	60
	R D18.5B	TCCTCTCGCCCTCCGCAGC	62
HD1	F HD1A	ACGACACGCAGTTTCGTGT	58
	R HD1B	CGCACTCGCCCTCCAC	56
HD7	F HD7A'	GAGCCGCGCTTCATCTCT	58
	R HD7B	CCCTCCAGGTAGTTCCTT	56
HD6	F HD6A'	CCGGGATCCGAGGACT	54
	R HD6B*	CTCCATCTTGCGTTTGGA	54
A10	F A10A	CTCCCACTCGATGAGGTAT	54
(JSP1)	R A10B	ATCTGAGCCATCGTCTCCA	54
Class I	F Bov 7	GGC TAC GTG GAC GAC ACG	55
	R Bov 11	CCC TCC AGG TAG TTC CT	49

5.2.2.9 Freezing cell stocks

Prior to freezing cells 1ml of FBS with 10% DMSO per $5x10^6$ cells was prepared and kept on ice. Cells were pelleted by centrifugation at 300xg for 5 min, supernatant removed, resuspended in 1ml of the freezing solution and rapidly aliquotted into 1ml

Cryovials (Greiner, labortechnik). Vials were frozen at -80°C for short term storage or in liquid Nitrogen (-110°C) for storage of longer than one month.

5.3 Results

5.3.1 Confirmation of expressed Class I genes on cell lines

Prior to attempting CTL assays it was necessary to confirm the typing of the animals and the cell lines made from these to ensure all further results in this study were valid. cDNA was produced from each of the lines as described in sections 2.2.5 and 2.2.6 and the genes amplified using PCR-SSP with primers which have previously been shown to be allele-specific (Ellis *et al.*, 1998). In addition the generic primer pair Bov 7 and 11 which amplify exons 2 and 3 of all class I alleles were included as a positive control. As can be seen in fig.5.1 the primers amplified products of the correct size for each cell line. Since the specificity of these primers has already been determined no sequencing of products was required. Amplification using the D18.5 and Bov primers from 495, 506 and A14 gave rise to two bands, one of the correct size and one approximately 200bp larger. Sequencing of this larger band showed the inclusion of intron sequence indicating the presence of genomic DNA contamination in these cDNA preparations.

5.3.2 Phenotyping cell lines

Theileria-infected cell lines established for use in this study were phenotyped using a range of monoclonal antibodies described in table 5.1. The results of this phenotyping are summarised in table 5.3 and the histogram profiles in appendix b.

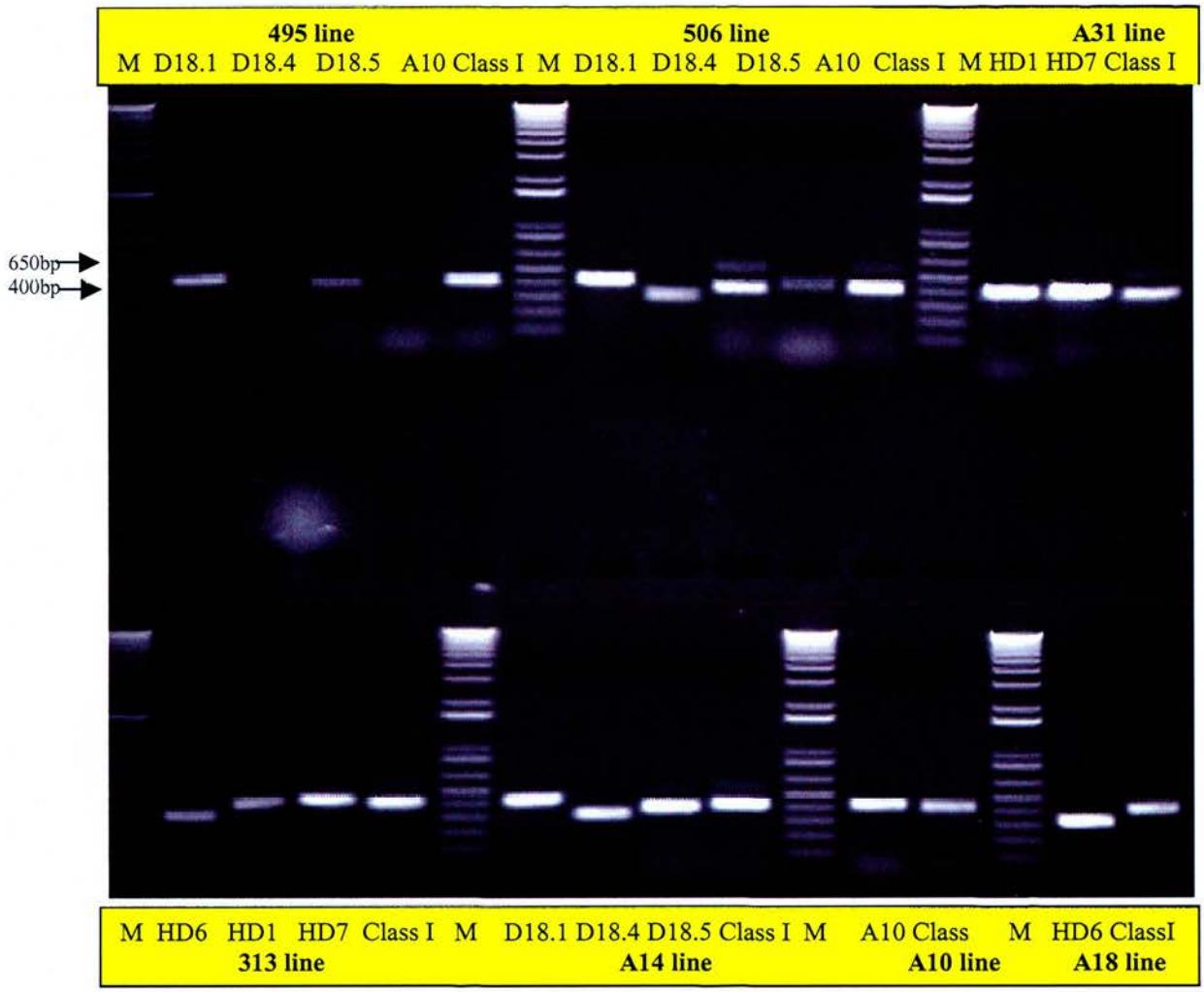


Figure 5.1: Confirmation of MHC class I typing by PCR-SSP

Standard PCR reactions using allele-specific primers (section 5.2.2.8, table 5.2) were carried out on cDNA from seven *Theileria*-infected cell lines to ensure correct typing of their MHC class I alleles.

Table 5.3: Summary of phenotyping of *T. parva*- infected cell lines by flow cytometry

Specific monoclonal antibodies (mAbs) for cell markers were used to phenotype the *T. parva* cell lines used in this study (section 5.2.2.7). Levels of staining are shown on a scale from +++ for very positive, to – for negative. The histogram profiles of these results are shown in appendix b.

Marker	A18 Tp line	A31 Tp line	A14 Tp line	A10 Tp line	495 Tp line	506 Tp line	313 Tp line
CD3	++	+	+	+++	+	++	+++
B cells	-	-	-	-	-	-	-
CD4	-	-	+++	+++	-	-	+++
CD8	-	++	+	++	+++	++	-
γδ	++	+	+	-	-	++	++
MHC class I	+++	+++	+++	+++	+++	+++	+++
MHC class II	+	++	+++	+++	++	++	+

We found that all of the cell lines were positive for the T cell marker CD3 but only two, A10 and 313, appeared to be expressing CD3 on all cells. Staining for surface immunoglobulin (Ig) was negative for all lines but this was not surprising considering the previous observation that B cells lose this over extended culture (Morrison *et al.*, 1989). Staining against an alternative B cell marker BoWC4 (antibody CC55, Naessens and Howard, 1991) was also negative which suggests that all the lines were T cells but some had lost CD3 expression. Alternatively there may be natural killer cells or null cells present which have no surface Ig and do not express any T cell markers but can be infected with *T. parva* (Baldwin *et al.*, 1988). These data fit in with previous studies which have shown that while *T. parva* sporozoites can infect T cells and B cells with similar efficiency *in vitro*, over time the T cell population greatly outgrows the B cells (Morrison *et al.*, 1996).

Staining for CD4 and CD8 markers showed that three of the lines contained CD8 T cells, while one, 313, contained CD4 T cells. Two of the lines, A14 and A10, expressed both. Previous observations have shown that CD4+ infected cells can gain expression of CD8+ but not the reverse (Baldwin *et al.*, 1988), suggesting that those cells expressing both markers were originally CD4+ CD8-. Alternatively these may be mixed populations with both CD4+ and CD8+ T cells present. This could be determined by double staining the lines with both antibodies but unfortunately time restrictions prevented this.

Only two of the lines, A10 and 495, were negative when tested for the γδ T cell marker, with the remainder staining positive. A18 was negative for CD4 and CD8

indicating that this line may be a pure γδ T cell line since they do not express either of these co-receptors (Clevers *et al.*, 1990). As γδ T cells represent a large proportion of T cells in ruminants (Hein and Mackay, 1991) the presence of them in the lines here is not surprising.

The most important result from this phenotyping was the positive staining of all cell lines for MHC class I since this is essential for use of the cells as stimulators and targets in CTL assays. MHC class II staining was slightly more variable with some cell lines only being partially positive e.g. A18 and 313. It is not known whether this will affect the cells stimulatory capacity.

5.3.3 CTL assays

PBM were isolated from three animals immune to *T. parva* and stimulated at weekly intervals using autologous *T. parva* infected cell lines for one to three weeks. Following each stimulation cytotoxicity against a range of cell lines was assayed in order to determine if MHC restricted killing was occurring. Targets included autologous cells, homozygous cells expressing either the maternal or paternal haplotype (partially matched) and total mismatches i.e. not sharing either MHC haplotype. While cells were numerous enough to carry out assays up to three stimulations it was generally found that they were too depleted to do a fourth since such a large amount of cells were required for each assay. Unfortunately Home Office limits with the amount of blood that could be sampled meant that larger stimulations could not be carried out.

Two factors were used to indicate how well the assays had worked: spontaneous lysis and the coefficient of variation. Spontaneous release of less than 30% indicates good labelling on healthy cells which we found with the majority of assays shown here. Although this value is incorporated into each of the assays to remove background counts we have chosen to show them to indicate those targets where the value is more than 30% and the data is considered unreliable. The coefficient of variation gives an indication of the level of variation between the triplicates of each data point with less than 10% considered a good assay (Siliciano *et al.*, 1985). These data are summarised in appendix c. In general most triplicates fell below 15% although there were some spurious results which could be due to pipetting errors.

5.3.3.1 Animal 313 (A18/A31) results

Assay results using CTL derived from animal 313 are shown in fig. 5.2 (A-D) with the corresponding number of times stimulated and spontaneous release from targets indicated. Only charts A and C are with the same CTL population, the others represent different sets of CTL. Although these CTL assays are not quantitative it should be possible to detect trends with the assumption that more stimulations should lead to an increase in specific CTL and levels of killing. This was hampered however due to problems encountered with attempting sequential assays using the same CTL. Comparing A and C there appears to be an increase in levels of cytotoxicity following another round of stimulation which is most obvious at low effector to target ratios. Levels of killing appeared to increase most against autologous targets (note different effector to target ratios on charts). Levels of

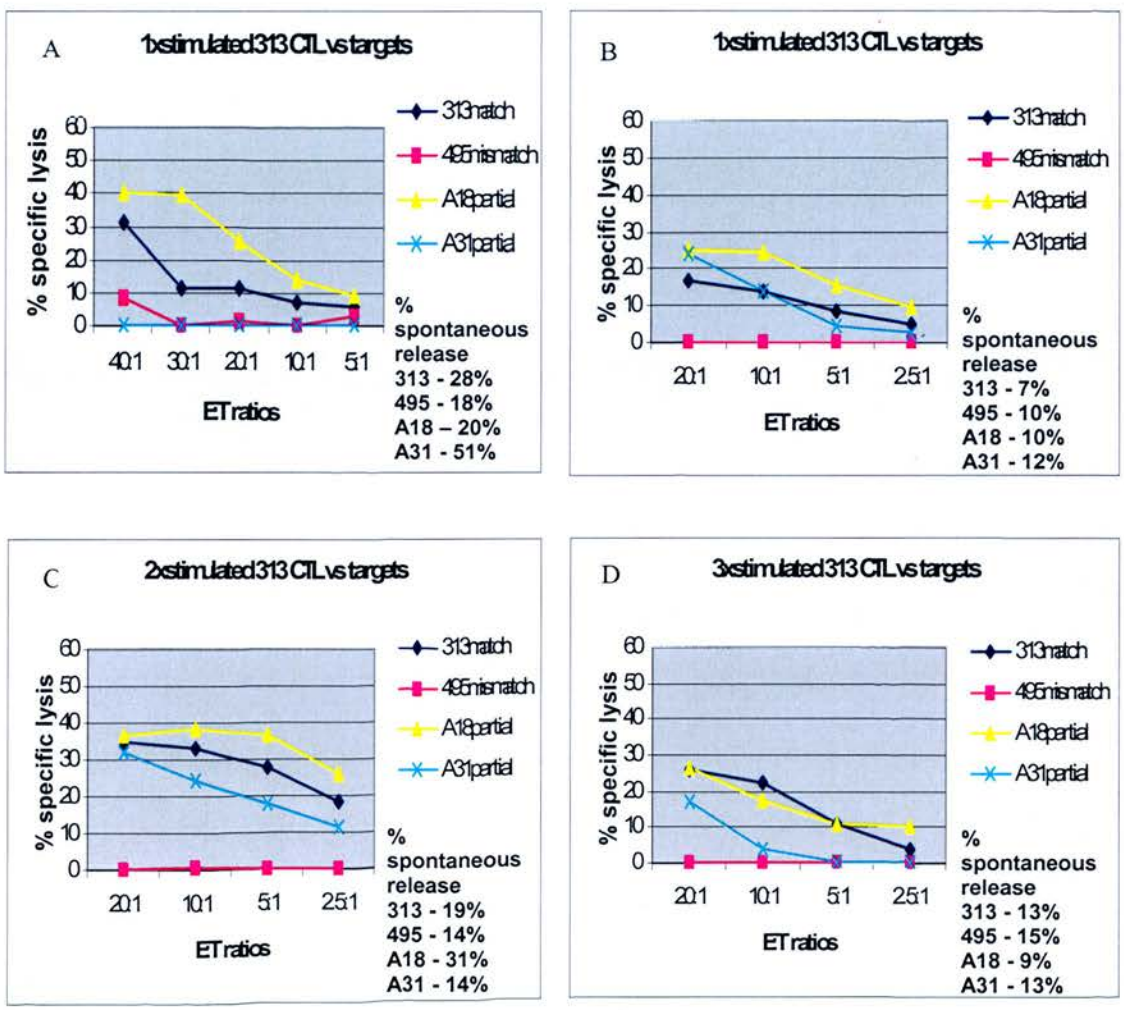
killing against A31 also increased suggesting that there is an increase in a population of A31-specific CTL. Since this population will also recognise the A31 class I molecules on the autologous cells they may explain the increase in killing against autologous targets. However it is important to note the high level of spontaneous release from the A31 targets in panel A. Overall the general trend seems to indicate that A18 is the dominant haplotype with a larger proportion of CTL generated being restricted by this thus explaining the higher levels of killing seen against A18 than A31 targets.

This is surprising considering that this haplotype carries only one classical class I gene, HD6. In addition it is surprising to see that in most cases higher levels of killing are seen against A18 than autologous targets. This may be due to the number of HD6 class I molecules present on the surface of the different targets. A previous PhD student attempted to quantify the number of different class I molecules on the cell surface in cattle using a quantitative indirect immunofluorescence kit in conjunction with allele-specific antibodies (Smith, 2000). She found that cells from A31 and A18 homozygotes carried the same total number of class I molecules as A18/A31 heterozygotes. Taking into consideration that the A31 homozygote has four class I genes expressed while A18 has two this suggests that half the molecules on the A18/A31 cells are HD6, with the other half being a combination of HD1 and HD7. Thus A18/A31 cells express half the amount of HD6 that A18 homozygous cells do and will not be recognised as efficiently by A18-restricted CTL.

HD6 is similar to FJ101, an allele carried on the African haplotype A6 which was found to be the most dominant haplotype in restricting the CTL response to *T. parva* in African animals studied (Taracha *et al.*, 1995). This indicates that HD6 and other similar alleles are forming immunodominant complexes with antigenic peptides.

Figure 5.2: CTL assay results from animal 313 (A18/A31)

CTL generated from animal 313 (A18/A31) were tested for cytotoxicity against a panel of *Theileria*-infected cell lines including autologous cells (match), cells with no shared MHC class I haplotypes (mismatch) and targets homozygous for either the maternal or paternal haplotypes (partial). The results for four different assays A-D are shown. Note the different range of effector to target (E:T) ratios used in panel A. Levels of cytotoxicity are shown as % specific lysis and % spontaneous release indicates the background amount of ^{51}Cr chromium release. These were calculated as described in section 5.2.2.6.



5.3.3.2 Animal 506 (A14/A10) results

The CTL results from animal 506 are shown in fig. 5.3. As can be seen the amount of spontaneous release of these targets was much lower than in the 313 assays. In all cases 506 autologous targets were recognised and killed, however killing on A18 mismatch targets was quite high in three of the assays shown. cDNA was produced from the 506 *T. parva* line and checked with HD6 primers to ensure the correct typing of this animal but no amplicon was detected which confirms the presence of both MHC-restricted and unrestricted CTL. Comparison of the levels of cytotoxicity against A14 and A10 targets shows that values are approximately the same in 3 of the 4 charts with no obvious dominant haplotype.

5.3.3.3 Animal 495 (A14/A10) results

The CTL results from animal 495 show a marked contrast to those from 506 with only MHC restricted CTL being generated during stimulation (fig 5.4). Highest levels of killing were seen against the autologous cell line in each assay although it is clear that the assays are highly variable as indicated by the vast changes in lysis from approximately 20% (assay A) up to 70% (assays C and E). In three out of the five assays, C, D and E, levels of killing against A14 targets were higher than A10 targets. Interestingly these were the assays where good levels of killing (greater than 40%) were seen against the autologous line, suggesting that this is a valid result.

Figure 5.3: CTL assay results from animal 506 (A14/A10)

CTL generated from animal 506 (A14/A10) were tested for cytotoxicity against a panel of *Theileria*-infected cell lines including autologous cells (match), cells with no shared MHC class I haplotypes (mismatch) and targets homozygous for either the maternal or paternal haplotypes (partial). The results for four different assays A-D are shown. Note the different range of effector to target (E:T) ratios used. Levels of cytotoxicity are shown as % specific lysis and % spontaneous indicates the background amount of ^{51}Cr chromium release. These were calculated as described in section 5.2.2.6.

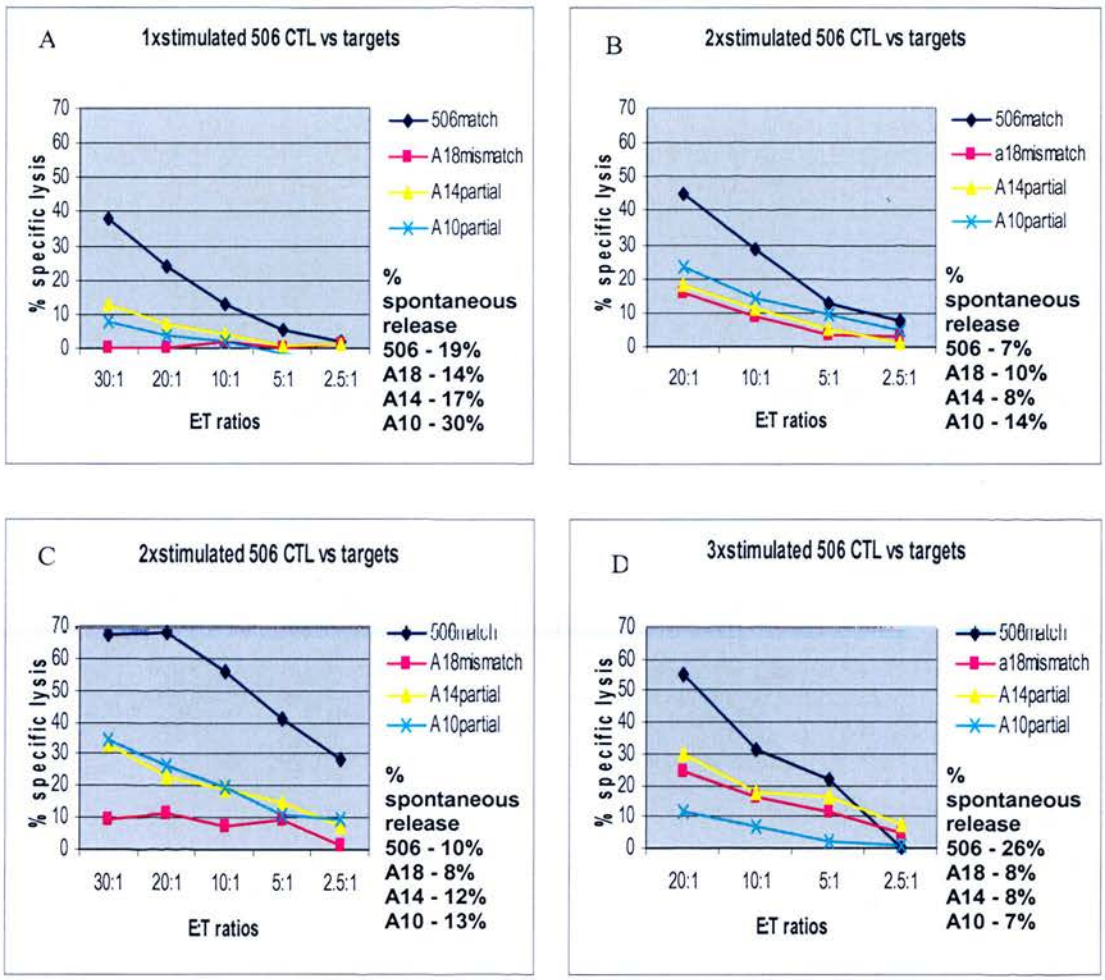
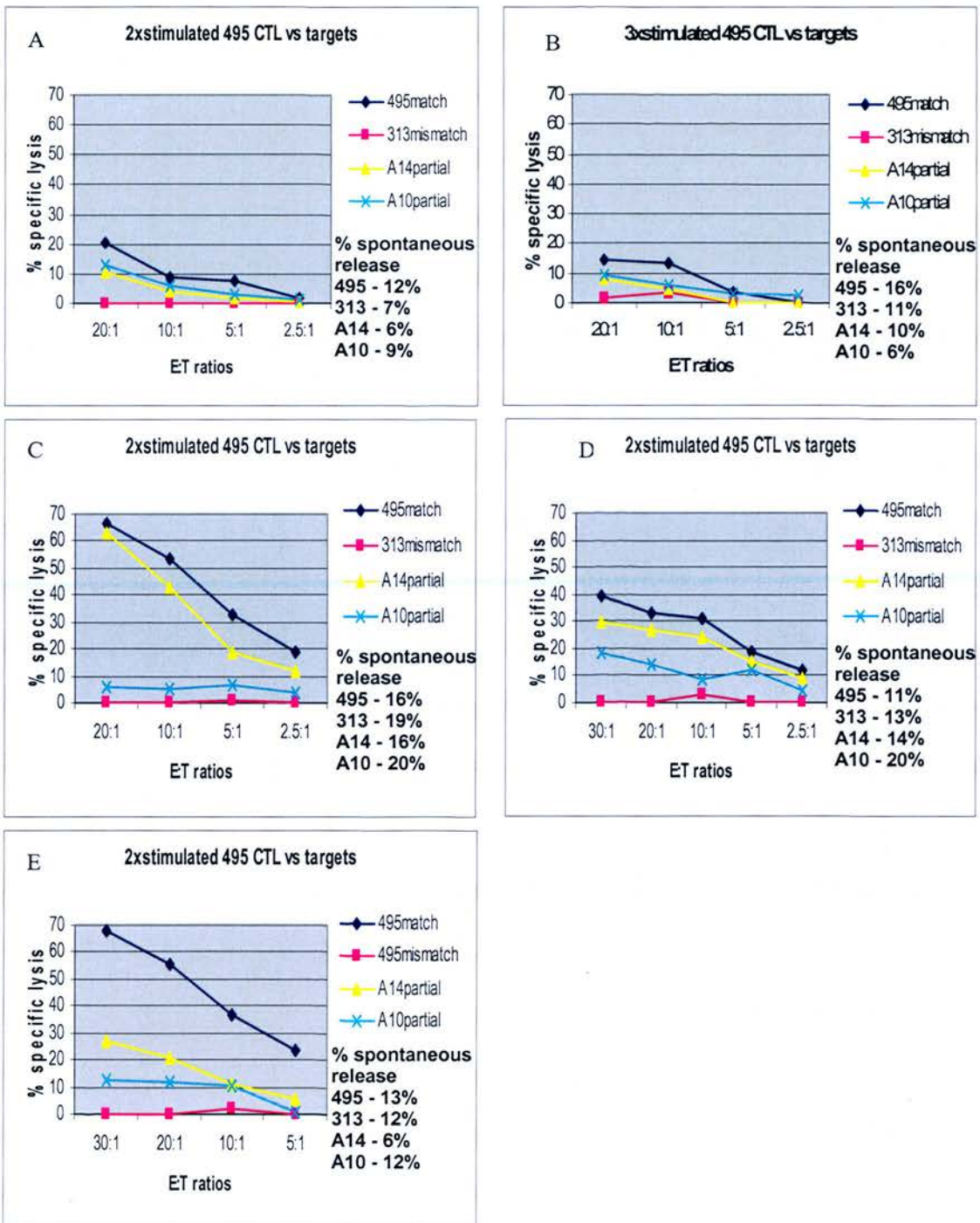


Figure 5.4: CTL assay results from animal 495 (A14/A10)

CTL generated from animal 495 (A14/A10) were tested for cytotoxicity against a panel of *Theileria*-infected cell lines including autologous cells (match), cells with no shared MHC class I haplotypes (mismatch) and targets homozygous for either the maternal or paternal haplotypes (partial). The results for five different assays A-E are shown. Note the different range of effector to target (E:T) ratios used in panels D and E. Levels of cytotoxicity are shown as % specific lysis and % spontaneous indicates the background amount of ^{51}Cr chromium release. These were calculated as described in section 5.2.2.6.



5.4 Discussion

The aim of this set of experiments was to determine if there was a bias in MHC restriction of *T. parva*-specific CTL derived from heterozygous animals to either the maternal or paternal haplotype. This work differs to that previously carried out by various groups (Goddeeris *et al.*, 1986, Taracha *et al.*, 1995) in that we are studying animals whose MHC haplotypes have been fully characterised, with all the classical class I molecules identified. In addition we have chosen to look at bulk CTL responses rather than CTL clones since this more accurately reflects what is happening within the animal unlike CTL clones which represent a highly selected part of the CTL population (Morrison *et al.*, 1987).

Three animals were chosen for this study, one A18/A31 animal and two A14/A10 animals. Following infection of the animals with *T. parva* and treatment with oxytetracycline CTL were generated by *in vitro* re-stimulation with autologous infected cells and assayed for cytotoxicity against a panel of targets. CTL from each animal were found to be genetically restricted with killing evident against autologous cells but not against those with different haplotypes. Only CTL from animal 506 contained a non-specific element as shown by killing against a total mismatch.

Comparing the levels of cytotoxicity against targets homozygous for each parental haplotype allowed us to determine if there was any bias in MHC restriction occurring. Levels of killing were generally 5 to 10% higher on A18 targets than on A31 targets indicating this to be the dominant haplotype in the A18/A31 animal.

Assays involving the two A14/A10 animals were less clear-cut, with their CTL appearing to differ in terms of MHC restriction. CTL from animal 495 appeared to be restricted by A14 while there was no discernible difference in restriction between A10 and A14 with CTL from 506.

One major problem with this set of experiments was the variation in levels of cytotoxicity observed between assays, even those using CTL that had undergone the same number of stimulations. Although these assays were all executed in the same manner a number of factors such as the quality of the stimulator cells and labelling efficiency of the targets could cause assay to assay variation. It is most likely however that CTL were not proliferating sufficiently due to the small number of stimulations carried out as a result of limitations on the starting volume of blood. Previous investigations encountered problems with the presence of non-specific cytotoxic cells when stimulating CTL in a mixed lymphocyte reaction after one or two rounds of stimulation (Pearson *et al.*, 1979, Pearson *et al.*, 1982, Emery and Kar, 1983). Goddeeris *et al.* (1986b) found these could be removed by increasing the rounds with an increase in the levels of cytotoxicity observed between each round of stimulation. This suggests that the cells in our assays should have been repeatedly stimulated at least three or four times prior to use rather than assaying at an early stage. It may also be the case that at one year p.i. the number of circulating memory cells may have rapidly decreased although it has been documented that *T. parva* specific CD8+ CTL can be detected three years p.i. by *in vitro* stimulation (Burridge *et al.*, 1972, Taracha *et al.* 1992).

The quality of the stimulations would also have a major influence on assays with the state of the infected cells and the CTL being stimulated affecting this, although the viability of these cells was tested prior to use and found to be good. It may be that another cell type e.g. γδ T cells are proliferating rather than CD8+ cells which has been shown to be a problem when trying to generate CTL against RSV (Roy Cook, IAH, personal communication). In most of the assays attempted it was found that CTL were killing autologous targets but not total mismatches indicating that the killing is specific and MHC restricted. This suggests that γδ contamination is not a problem although they may be present and potentially masking a much higher level of killing by interfering with cell to cell contact. In order to confirm this, phenotyping of the CTL following stimulation is required.

The animal to animal variation observed could be due to different numbers of lymphocyte subpopulations present in the blood from which CTL are generated or alternatively due to phenotypic differences between the autologous stimulators. Analysis of the 506 *Theileria* line showed it to be a combination of CD8+ and γδ T cells compared to the 495 line that was mostly CD8+ T cells. Although both cell lines had similar levels of class I expression it may be that the γδ cells confer some disadvantage to CTL proliferation.

CTL were not cleaned up between each assay and stimulation to remove dead cells and debris which may also have affected the assays by reducing cell to cell contact and CTL to target ratios. Filler cells were included in stimulations based on observations by Goddeeris *et al.* (1986b) who found them to be beneficial in aiding

good proliferation. In contrast Taracha *et al.* (1991) found no benefit in their use when stimulating CTL so it may be that they were having a negative effect by preventing CTL from interacting with the stimulators.

While it is difficult to draw any definite conclusions from this work the results seem to indicate that a bias in MHC restriction is occurring, with A18 and A14 appearing to be dominant haplotypes. Ideally this work should be repeated using larger volumes of blood with only effector to target ratios of 40:1 or more being tested. In addition the CTL should be stimulated three or more times prior to assay as it has previously been shown that at 95 weeks post immunization no cytotoxicity is observed but following five stimulations *in vitro* levels of 95% are obtained at effector to target ratio of 15:1 (Goddeeris *et al.*, 1986). IL-2 or 7 could also be included to ensure the presence of a specific CD8+ population.

Inclusion of animals with a wider range of MHC haplotypes and animals heterozygous for A18/A14 would also provide useful information potentially allowing the detection of a hierarchy of dominance amongst the A14, A10, A31 and A18 haplotypes. Further analysis of the A14 haplotype is described in chapter 6.

Chapter 6

Further analysis of MHC restriction involving the A14 haplotype

6.1: Introduction

Based on the work described in chapter 5 for animal 495 it appears that the haplotype A14 may be dominant over A10 in its ability to restrict the CTL response. The A14 haplotype is known to express three classical class I genes, D18.1, D18.4 and D18.5. It is possible that the dominant restriction observed is due to the CTL recognising the products of one of these genes presenting antigenic peptides (i.e. is immunodominant) or alternatively it may be due to a cumulative effect with CTL generated against all three. In this chapter we aimed to examine this restriction further through the use of *T. parva*-infected transfectants as targets for an A14-specific CTL clone produced from the animal 495. As no D18.5-specific antibody is available at present we chose to exclude this gene from the study. Alternative methods of screening for positive transfectants with D18.5 are discussed later in this chapter but unfortunately time restrictions prevented these being tested.

Previous attempts to transfect genes, particularly MHC class I genes, into *T. parva* cell lines have proved difficult (Prof. D. McKeever, personal communication), with optimisation of the electroporation conditions required for each individual recipient cell type in order for the transfection to be successful. Eichorn and Dobbelaere (1995) were successfully able to permanently transfect the IL-2 receptor into a *T. parva*-infected T cell line using G418 to select for positive transfectants. Within our laboratory optimisation of this method has overcome the problems associated with transfecting class I genes allowing analysis of the role of individual class I genes in restricting the CTL response.

6.2 Methods

6.2.1 Production of transfectants

D18.1 and D18.4-containing plasmids (prepared by Dr S. Ellis IAH, Compton) were transfected into A31 homozygous *T. parva*-infected cell lines by electroporation. $2x10^6$ actively growing healthy cells were washed and resuspended in 0.5ml FBS-free RPMI medium. 20µg of the pcDNA3 plasmid containing either D18.1 or D18.4 was added and the cells mixed gently. They were then transferred to a sterile 0.4cm cuvette and electroporated at 270V, 500µFD using a Biorad Gene pulser. The cells were then placed on ice for 30 min to recover. Following this the cell suspension was added slowly to 24ml warm media containing 10% FBS, the tube was gently tilted to disperse the cells, and 1ml of the cell suspension was added to each well of a 24 well plate, and incubated for 24 hours at 37°C. 1ml of media containing 8mg/ml G418 sulphate (Sigma) was then added to each well to select for positive transfectants and the plate further incubated at 37°C. The cells were observed daily for signs of growth. Any wells that appeared to grow were allowed to expand in media containing 2mg/ml G418 and the cells then tested for expression by flow cytometry (section 5.2.2.7) using the mAbs ILA-35 (Ellis *et al.*, 1999) and CC218 (unpublished), which see D18.4 and D18.1 respectively (IAH tissue culture department, Compton). Positive wells were then cloned in 96 well flat bottom microtitre plates at densities of 100, 10, 1 and 0.5 cells per well. Any clones which grew were picked and transferred to 24 well plates for expansion. These were again tested by flow cytometry to confirm expression. Cells were then maintained in

media containing 10% FBS and 2mg/ml G418 and passaged with fresh media every 3 to 4 days.

6.2.2 CTL clone

A *T. parva*-specific A14-restricted CTL clone derived from animal 495 was kindly provided by D.Ngugi, University of Edinburgh. This was produced based on the method described by Goddeeris and Morrison, 1988. MHC restriction of the clone had been determined by testing in CTL assays with A14 and A10 homozygous *T. parva*-infected cells as targets. The CTL clone was stimulated every 7 to 21 days by plating at $1x10^3$ cells per well of a 96 well plate and adding $1x10^4$ irradiated autologous *T. parva*-infected cells and 10U/ml recombinant human interleukin-2 (IL-2, Sigma) in a final volume of 200µl. For larger restimulations $1x10^5$ cloned cells were mixed with $1x10^6$ stimulators and 10U/ml IL-2 in 24 well plates with a final volume of 2ml per well. All CTL assays were carried out as described in chapter 5, however duplicates rather than triplicates of each effector to target ratio were used. Ratios from 20:1 to 2.5:1 were tested in the assays.

6.3 Results

6.3.1 Screening transfectants

D18.1 and D18.4 transfectants were screened for positive expression both before and after cloning. Three D18.4 (A3, A4.1, A4.2) and two D18.1 transfectants (B4 and B1) were chosen for cloning which was required to ensure homogenous cultures containing only one population of cells were obtained. From these four positive clones showing good expression were obtained, D18.1(B1)E8, D18.1(B4)F10, D18.4(A4.2)F7 and D18.4(A4.2)D8. These results are illustrated in fig. 6.1.

6.3.2 CTL assays with a CTL clone

CTL assay results from animal 495, typed as A14/A10, indicated that there is a bias in the MHC restriction of the CTL produced in response to *T. parva* infection to the A14 haplotype. Following on from this we chose to use *Theileria*-infected cells transfected with either D18.1 or D18.4 as targets for an A14-restricted CTL clone derived from animal 495 to determine if either of these genes were restricting the CTL response. Two separate assays were carried out, the results of which are shown in fig. 6.2 and 6.3. As in chapter 5 two factors were used as indicators of assay quality, spontaneous lysis of the targets and coefficient of variation. Less than 30% spontaneous release and less than 10% variation between results indicates a good assay.

Figure 6.1: Screening of transfectants and clones for D18.1 and D18.4 expression

The monoclonal antibody CC218 was used to screen transfectants and clones for D18.1 and D18.4. Staining with the specific mAb is shown in black, staining with an isotype control, L180/1, a mouse anti-sheep mAb specific for an erythrocyte marker (Hunig, 1985), is shown in red.

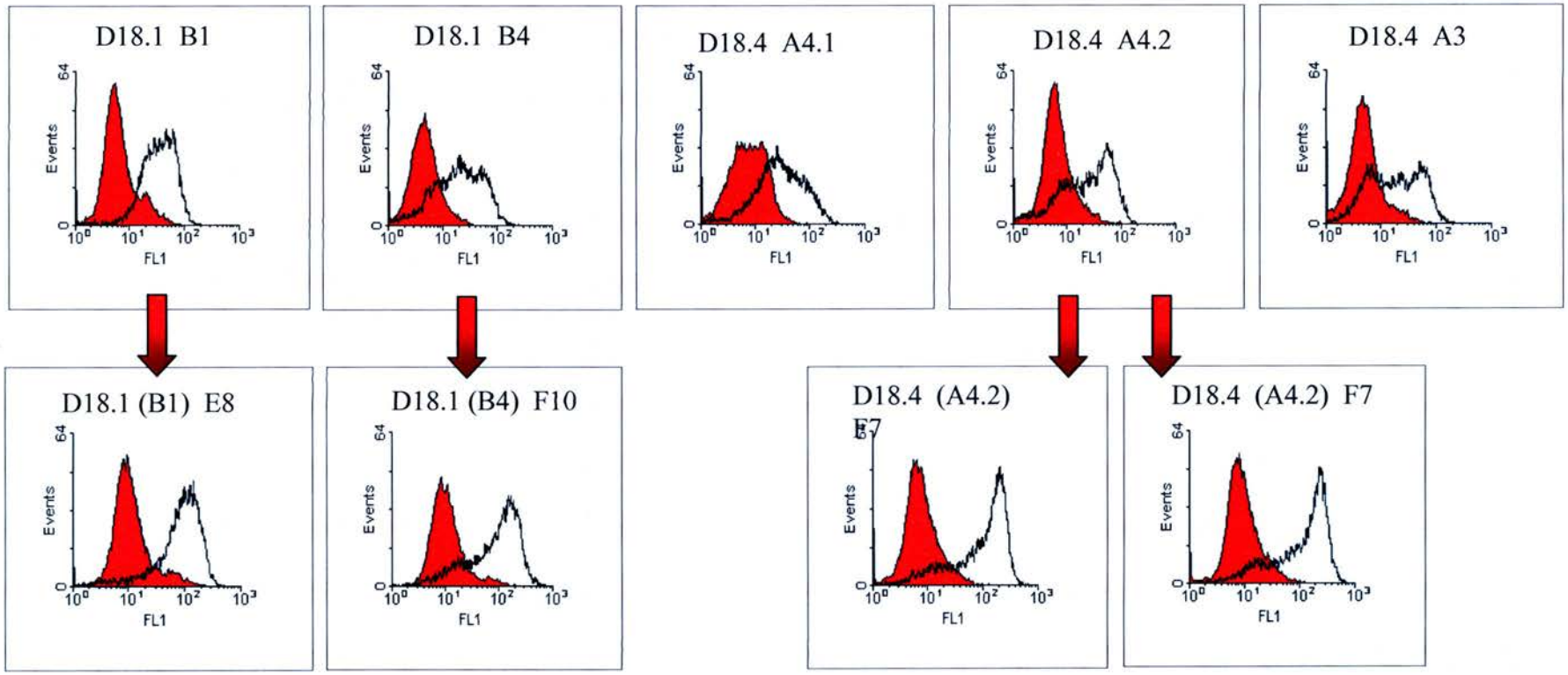
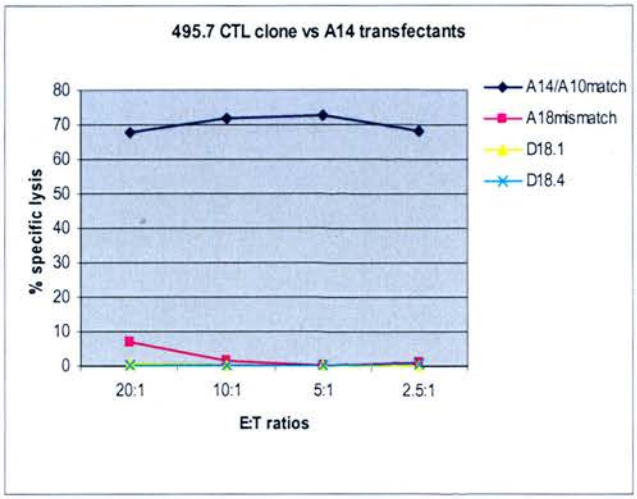


Figure 6.2: CTL assay 1

An A14-restricted clone was tested for cytotoxicity in a ^{51}Cr release assay against an autologous *T. parva*-infected cell line, a mismatched line, and two lines transfected with D18.1or D18.4 both of which are found on the A14 haplotype. Effector to target ratios from 20:1 to 2.5:1 were tested.

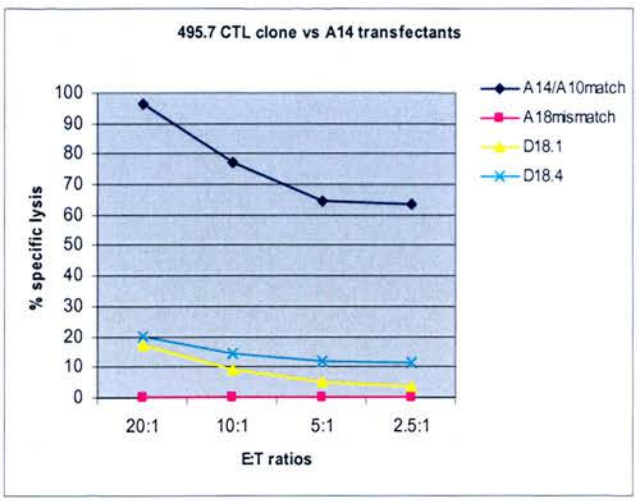


Coefficient of Variation				
	A14/A10	A18	D18.1	D18.4
min	4.8	6.0	0.4	4.8
20:1	1.9	0.8	3.2	4.8
10:1	2.9	8.3	5.1	0.3
5:1	0.4	1.6	2.6	8.3
2.5:1	1.0	2.2	3.6	2.0
max	7.3	3.8	2.7	23.0

% spontaneous release
A14/A10 – 10%
A18 – 20%
D18.1 – 28%
D18.4 – 35%

Figure 6.3: CTL assay 2

See figure 6.2 for explanation



Coefficient of Variation				
	A14/A10	A18	D18.1	D18.4
min	8.9	10.5	18.5	20.8
20:1	14.3	3.3	17.9	13.9
10:1	13.4	16.7	21.6	10.5
5:1	10.7	0.34	14.6	5.3
2.5:1	25.5	1.0	13.4	15.3
max	27.0	13.3	17.3	0.2

% spontaneous release
A14/A10 – 20%
A18 – 24%
D18.1 – 17%
D18.4 – 12%

In both assays the MHC restriction of the clone 495.7 was proven, with high levels of killing observed against the MHC matched target A14/A10 cell line but little to no cytotoxicity against the A18 mismatch. With assay 1 no killing of either the D18.1 or D18.4 transfectants occurred. In contrast in the second assay these transfectants were recognised and killed, albeit at low levels, with more cytotoxicity observed against D18.4. By comparing the % spontaneous release it can be seen that the transfectants were clearly in better condition for the second assay however the coefficient of variation values were higher than in the first assay. As only duplicates rather than triplicates of each effector to target ratio were included this suggests the data may be unreliable. In addition the recognition of both D18.1 and D18.4 transfectants indicates that the clone may not be a homogenous population of cells all expressing the same TCR.

6.4 Discussion

It is obvious from these preliminary experiments that more work needs to be carried out in order to investigate the A14 haplotype more fully. While the differences observed between the two assays make it difficult to interpret this data further, the results in assay two could be discarded. This is based on the fact that the CTL clone should in theory have only one type of TCR and therefore not be able to recognise two different MHC molecules. However the recognition of both could be possible if the two molecules were very similar in $\alpha 1$ and $\alpha 2$ and presented similar peptides since these are the major points of interactions with the TCR (Bjorkman *et al.*, 1987a). Comparison of the D18.1 and D18.4 sequences identified 18 aa differences

in α1 but only two in α2 (see fig. 6.4) while HD1, which appears to be an allele of the same gene as D18.4, has 21 aa differences most of which are in α2. When D18.1 is modelled based on a human MHC class I molecule with the Swiss PDB package (Guex and Peitsch, 1997) most of the aa differences in α1 are located in the β sheet of the binding groove of the molecule. This could affect the peptide binding motif resulting in D18.1 presenting very different peptides to D18.4, with different side chains pointing out of the groove preventing the same TCR from binding. Alternatively the TCR may have more interactions with the α helices as seen with the mouse H-2Kb molecule when modelled with the dEV8 self-peptide and 2C TCR (Garcia *et al.*, 1996, 1998). Thus the clone may be capable of recognising both D18.1 and D18.4 molecules resulting in the similar levels of cytotoxicity seen in assay two.

In order to study this further the use of either bulk CTL or a panel of CTL clones should be considered. In addition a better mismatch target would be an A31 *T. parva* infected cell line since this is the parent line into which D18.1 and D18.4 are transfected. The inclusion of this would then allow the detection of any specific killing against either transfectant as a result of CTL recognition of either gene product. If the results from these assays were similar to assay one it suggests that the restricting element is something other than D18.1 or 4 i.e. D18.5. Ideally D18.5 should also be included to allow a full study of this haplotype. While no specific mAb has been raised against this molecule various methods could be employed to test transfectants. A blocking assay, whereby the class I molecules on the parent line are blocked with a specific mAb then the transfectants tested by flow cytometry with

Figure 6.4: Amino acid sequence of α1 and α2 domains of D18.4, D18.1 and HD1

The amino acid sequence of D18.1 and HD1 are shown aligned to D18.4. Dots (.) indicate that the sequence is the same to D18.4. The division between α1 and α2 is shown as a black line.

```
                                        α1
                 *        20         *        40         *        60         *        80
D18.4 : GSHSLRYFYTAVSRPGLGEPRFISVGYVDDTQFVRFDSDAPNPREEPRAPWIEKEGPEYWDRETRISKENTLVYRESLNN
D18.1 : ........S........E.....I..............S....A........Q.......E....V.DTAQT..AN..T
HD1   : ................D..................W.........K...T.........................G....

                                        α2
                 *|       100         *       120         *       140         *       160
D18.4 : LRGYYNQSEAGSHNIQAMYGCDVGSDGSFLRGYSQDAYDGRDYIALNEDLRSWTAADTAAQITKRKWEAEGYAESLRNYL
D18.1 : AL........|.....................................................................
HD1   : ..........|...TF.Q.......P..RL.G..R.Y................................A.D..RF.... :
                  |

                 *       180
D18.4 : EGRCVEWLRRYLENGKDALLRA
D18.1 : ..T..............T....
HD1   : ..E...G..........T....
```

either ILA-88 or W6/32 which recognise all bovine class I molecules could be used. By comparing the results to untransfected parent cells which had also been blocked it would be hoped that the reactivity of the class I antibodies would be less in the untransfected cells indicating that the antibodies are still recognising class I molecules on the surface which do not belong to the parent cells i.e. the transfected gene products. This approach however relies on the specific mAb and the class I Ab recognising the same epitope on the parent class I molecules. This may be unlikely since the mAb is recognising a specific element on the parent MHC molecules while both ILA-88 and W6/32 recognise an epitope found on all bovine MHC class I molecules.

Alternatively cDNA from transfected cells could be tested using D18.5-specific primers. While this does not prove expression of D18.5 it should be indicative. It may also be possible to screen for positive transfectants by incorporating a tag into the D18.5 construct.

Analysis of the A14 haplotype identified two genes which are thought to be non-classical, X and Z (Di Palma *et al.*, 2002). While little is known about Z, X has been shown to be expressed and is thought to be present on γδ T cells which are found in the A14 *T. parva* line, albeit at low levels (Dr. S. Ellis, personal communication, appendix b). It may be that the clone used here is restricted by this and so does not recognise either D18.1 or D18.4.

While G418 is included to ensure selection of cells containing the transfected plasmids it can be quite toxic to the cells and cause them to become quite leaky and poor in quality. As a result transfectants were removed from G418-containing media 24 hours prior to assay. It is possible that this time is not long enough to allow the cells to recover, so they label poorly with ^{51}Cr. Also long term culture of the transfectants may cause them to lose expression of the transfected genes over time although these cells were regularly tested for expression as in 6.3.1 and did not show any reduction. G418 is an aminoglycoside which attaches to the ribosome binding site and prevents transcription in cells without neomycin resistance genes. The amounts used here were four times the amount normally required for transfection into *T. parva* cells. It is possible that the G418 may be killing the parasite since it does not have the resistant gene which could result in no antigen being available for presentation by the class I molecules. This could be tested by checking for parasite viability through either the use of a specific mAb or giemsa staining or by RT-PCR of the polymorphic immunodominant molecule found on the surface of the parasite (Toye *et al.*, 1991). However it should be noted that previous investigations have shown that by killing the parasite infected cells are unable to continue replicating and resort to a resting state or die by apoptosis unless exogenous growth factor is added (Dobbelaere and Heussler, 1999).

Chapter 7

General Discussion

7.1 Summary of achievements

We have successfully identified locus-specific primers for four out of the six putative bovine class I genes. These primers allowed us to test the potential of RSCA as an effective method for typing the class I MHC. We were successful in typing animals for HD1, D18.5, D18.3, A10 and MAN2 and identified 19 new alleles, 6 for group 1, 3 for group 3 and 10 for group 3. The results obtained from this study indicate RSCA, with further optimisation, will be a formidable method for class I typing in cattle and will have a significant impact on future studies of the class I genes.

In the second component of this study we have been able to detect a bias in the MHC restriction of CTL by different class I haplotypes and have further investigated this at the individual gene level for the A14 haplotype.

7.2 Study of introns 1 and 3 in class I genes

Investigations of the MHC class I in cattle have identified more than 50 serological specificities in a range of breeds indicating that polymorphism is a feature of these genes however only 40 alleles have actually been sequenced. Lack of locus-specific characteristics in the coding region has prevented accurate assignment of these sequences to individual loci. Previous work in humans identified bases in introns 1 and 3 of class I gene sequences which were locus-specific. Primers designed around these then allowed locus-specific amplification of products used for RSCA. We sequenced

introns 1 and 3 from ten class I genes found within the herd at IAH, Compton and identified conserved bases for four of the six putative bovine class I loci. Primers were designed around the bases and tested for specificity against a panel of previously typed DNA samples. From this we were able to choose a pair for each locus for use in RSCA.

The locus-specific nature of the introns in humans appears to be due to homogenisation as a result of recombination and genetic drift over time (Cereb *et al.*, 1997). In addition the formation of new alleles by intra-locus recombination with little inter-locus recombination occurring ensures little or no sequence from other loci is inherited (Hughes *et al.*, 1993, Parham *et al.*, 1995). In cattle however inter-locus recombination appears to be playing a significant part in creating diversity (Holmes *et al.*, 2003). As a result it may be expected that locus-specific bases are absent in the introns or are present with reduced frequency compared to the human sequences. This did not seem to be the case however as conserved bases between alleles of the same group were found in both introns 1 and 3. HD7 was the main exception identified here. This allele, which is classified as group 2, appears to be a result of recombination between groups 2 and 4 since it has group 4-specific nucleotides in intron 1 and group 2-specific bases in intron 3. As a result the group 2 primers cannot amplify HD7. It is highly likely that there are more alleles like this. These could be detected either by amplification of the α1 and α2 domains from cDNA with generic primers followed by sequencing or by using different combinations of the forward and reverse group-specific primers. When further alleles for each of the groups become available it should be possible to align the sequences, including introns, and identify points at which recombination between genes has

occurred. This will then allow us to determine if any of the other locus-specific primers are likely to be affected in their ability to detect all alleles of a gene.

As stated in chapter 3 priority was given to ensure the primer pairs amplified the alleles identified in the Compton herd rather than the BS alleles previously identified since little information other than coding sequence is available for these. However they have been amplified from cDNA and so some are likely to represent classical alleles (Garber *et al.*, 1993, 1994). Comparison of their sequences to the specific primers shows that the group 2 primers will amplify BSN, while the group 1 sequences do not amplify BSX (fig. 3.3 and 3.4). The group 3 forward primer has three nucleotide differences from BSA, but does share the specific base at the 3' end, while the reverse primer is identical. Thus the primer pair may amplify BSA if the annealing temperature is low in the PCR reaction, although this would encourage non-specific binding to other genes present. Based on these findings some effort should be made to attempt to optimise the other previously designed primers which do have conserved bases in the BS sequences to allow amplification of the BS alleles. It is likely however that even by including these alleles we will miss others.

Human class I and class II introns have been studied in an attempt to understand the evolution and diversification mechanisms of the MHC genes. Links between intron polymorphism and allelic lineages were identified, with low levels of sequence diversity between alleles of the same lineages (Kotsch *et al.*, 1997, Gomez-Casado *et al.*, 1997,

Blasczyk *et al.*, 1998). Based on this it would be interesting to sequence the introns of any new alleles identified in cattle in an attempt to define alleles of the same lineage.

7.3 Establishing RSCA and future work

Using the group-specific primers we were successful in establishing RSCA as a method for typing bovine class I genes. We assigned mobility values to known alleles and were able to type random animals which proved positive for these alleles. In addition, we have identified a number of new alleles for each group by sequencing products with different mobility values to those previously found. These can now be included in future typing.

From the results presented here the majority of samples when amplified by group-specific primers and analysed with RSCA have only one heteroduplex. This would normally indicate either a sample homozygous for that gene or alternatively heterozygous with one allele matching the FLR (although this would be confirmed by the use of a second FLR). In cattle there is the added alternative of gene deletion which is the most likely option when single heteroduplexes are seen with this frequency.

RSCA has been shown to be a useful tool for typing MHC genes (both class I and II) in a number of species with the ability to manipulate duplex formation and assign a unique mobility value allowing, in theory, the detection of all alleles. By continuing the development of RSCA the scope for analysis of the bovine MHC is wide. For example,

incorporation of the remaining groups and wide-scale comparison of different breeds will likely lead to the identification of differences between breeds as found with the cat and dog studies (Kennedy *et al.*, 2002, 2003) and will allow us to determine the frequency of different genes and alleles. Haplotypes with different gene configurations to those previously observed may be found which will give a better idea of how the number of genes varies between haplotypes. These configurations may help in the detection of alleles missed by the group-specific primers. For example if it is found that the group 1 gene is always on the same haplotype as group 4 then the presence of an amplicon with group 1 primers but absence with the others indicates that a group 4 allele has been missed. RSCA could also easily be adapted for typing the MHC class II genes in cattle since much more information about these is available.

From the sequencing results in chapter 4 lineages of alleles are already becoming apparent. It is likely that by identifying and sequencing new alleles we will find additional lineages of alleles within each group. It may then be possible to define supertypes for each gene as in humans (Sidney *et al.*, 1995, Sette and Sidney, 1999). These supertypes incorporate families of alleles with overlapping peptide-binding motifs and are potentially useful for the design of peptide-based vaccines, overcoming the issue of polymorphism affecting the use of such vaccines (discussed below).

Until more alleles have been found it is necessary to continue the approach used here by combining RSCA and sequencing to define new alleles, with all sequencing incorporating the entire α1 and α2 regions to ensure accurate identification of alleles.

Once the method has been optimised and the error limits defined for each allele's mobility value a database of mobility values can be established allowing typing by RSCA alone.

7.4 MHC restriction of CTL responses to *T. parva*

Analysis of the CTL response to *T. parva* in *Bos taurus* and *Bos indicus* animals found it to be almost entirely restricted by either the maternal or paternal haplotype (Goddeeris *et al.*, 1990). In this study we chose to compare the A14 haplotype to A10 and A18 to A31 by infecting animals heterozygous for these with *T. parva* and then testing CTL derived from them against homozygous infected cell lines *in vitro*. We were unable to determine any clear dominance of the haplotypes studied here but did detect some differences in their ability to restrict the CTL response to with A18 appearing to be partially dominant over A31 while there was some evidence for A14 being dominant over A10 in one animal but not another. Further analysis of the A14 haplotype using *T. parva* cells transfected with either D18.1 or D18.4 as targets for an A14 restricted CTL clone provided inconclusive results, with killing of the targets in one assay but not another.

7.5 Alternative methods for measuring cytotoxicity

From the results obtained in both sets of CTL experiments it is obvious that our methods need refining, while it is imperative that a shorter amount of time lapses between immunisation of animals and the assays taking place. The ^{51}Cr release assay used in

these studies is the traditional approach for quantitating cell-mediated cytotoxicity. While this method is sensitive and acts as a direct indicator of killing activity problems including the isotopes' long half life and restriction of usage, the labour-intensive nature of the method, difficulty with cell labelling and high spontaneous release and the need for a relatively large number of fresh cells in good condition has led to the development of a range of alternative techniques which could be used in future. Examples of these include flow cytometric assays, ELISpot, and the use of tetramers.

A large number of flow cytometric assays have been devised which tend to involve measuring fluorochrome release from or retention by either pre-labelled effectors or targets. Examples of these labels include carboxyfluorescein diacetate and calcein (McGinnes *et al.*, 1986, Papadopoulos *et al.*, 1994). These labels do not affect the morphology or function of the labelled cells, have low toxicity, allow the user to distinguish between effectors and targets (thus giving a more accurate effector to target ratio) and also allow the exclusion of cell debris and dead cells. However problems with poor labelling and high spontaneous release have been identified as well as a need for complex analysis with the flow cytometry data. Attempts have been made to improve these methods through the testing of alternative fluorochromes such as carboxyfluorescein diacetate succinimidyl ester), PKH-26 and 7-amino actinomycin D (Sheehy *et al.*, 2001, Lecoeur *et al.*, 2001) to identify those that do not have labelling or leakage problems. When compared to the ^{51}Cr release assay these assays are found to have similar or higher specificity and with the routine use of flow cytometers in research represent a good potential alternative for measuring cytotoxicity.

ELISpot measures another function of effector CD8+ T cells, namely cytokine secretion in a system based on the ELISA methodology. PBMC or *in-vitro* stimulated CTL are added along with infected cells or antigenic peptides to a plate coated with anti-cytokine antibodies then following incubation a second enzyme-linked cytokine-specific antibody and the enzyme substrate are added. This results in the formation of a coloured (or fluorescent) spot, with each spot corresponding to a cytokine producing CTL and the total number of spots indicating the number of CTL present in the effector population. The advantages of ELISpot include its high sensitivity and easy usage, while automation of the spot counting reduces problems associated with manual counting and enhances reproducibility. However there are still some problems with high background in some which can make detecting effector cells at low numbers difficult (Miyahira *et al.*, 1995, Hickling, 1998). Despite this ELISpot offers the most likely alternative to the ^{51}Cr release assay and is becoming an increasingly popular method.

Tetramer staining uses flow cytometry to assay directly the number of CD8+ T cells present in PBMC that can recognise a specific antigenic peptide. Tetramers consist of four identical biotinylated MHC-peptide complexes which are bound together by streptavidin. When the tetramer is mixed with cells, any T cells with a TCR specific for the MHC-peptide complex is bound with high affinity. The inclusion of a fluorochrome attached to the streptavidin then allows the binding to T cells to be monitored by flow cytometry. The main advantages of this method is that it allows direct measurement of T cells with TCR specific for particular epitopes without the need for prior stimulation, is highly sensitive and quantitative (Altman *et al.*, 1996, Gallimore *et al.*, 1998).

However prior knowledge of peptide sequence and MHC type is required and so this method will only be of use when further data on *T. parva* antigens and dominant MHC class I molecules is available.

7.6 MHC and disease associations

One of the major stimuli for studying MHC in farm animals is to improve knowledge regarding the association between alleles and potential resistance to disease, thereby allowing development of effective breeding strategies and vaccines. The MHC has been found to be more associated with disease than any other genetic region and is linked to all or most autoimmune diseases. Pathogens are believed to be the driving force behind selection of MHC polymorphism and heterozygosity which helps to increase the hosts' ability to fight infection against diverse pathogens and those capable of antigenic variation. However there is little actual evidence to support this theory. The most often quoted study is that by Hill *et al.*, (1991) with the finding that the allele HLA-B*5301 is associated with lowered susceptibility to malaria in West African communities. However this association has not been found in East Africa. It may be that the human MHC is too complicated to allow large scale detection of such associations with strong linkage disequilibrium between genes making it difficult to assign susceptibility or resistance to particular class I or II genes and to rule out the involvement of other genes here (Goldsworthy *et al.*, 2000). In addition because there is effectively no wild type of class I and II alleles it is generally found that those alleles linked to disease naturally occur at high frequency in the population but for some reason e.g. contributory factors

such as the environment disease only occurs in a fraction of the population. For example HLA-B27 is found to be associated with ankylosing spondylitis and while it occurs in 95% of the population only 3% of Caucasians with this allele actually develop the disease (Edwards *et al.*, 2000).

In chickens the MHC is much smaller and simpler than mammals with many common haplotypes having only one dominantly expressed class I molecule and the level of class I surface expression varying between haplotypes. As a result it is much easier to establish connections between the MHC and disease. An association between the B21 haplotype which has a low level of expression and resistance to Marek's disease virus has been identified. In contrast chickens with the B19 haplotype which is expressed at high levels are highly susceptible to this virus (Kaufman and Salomonsen, 1997). In addition an alternative class I molecule with reduced peptide binding ability has been shown to be linked to susceptibility to Rous sarcoma virus (Kaufman, Volk and Wallny, 1995).

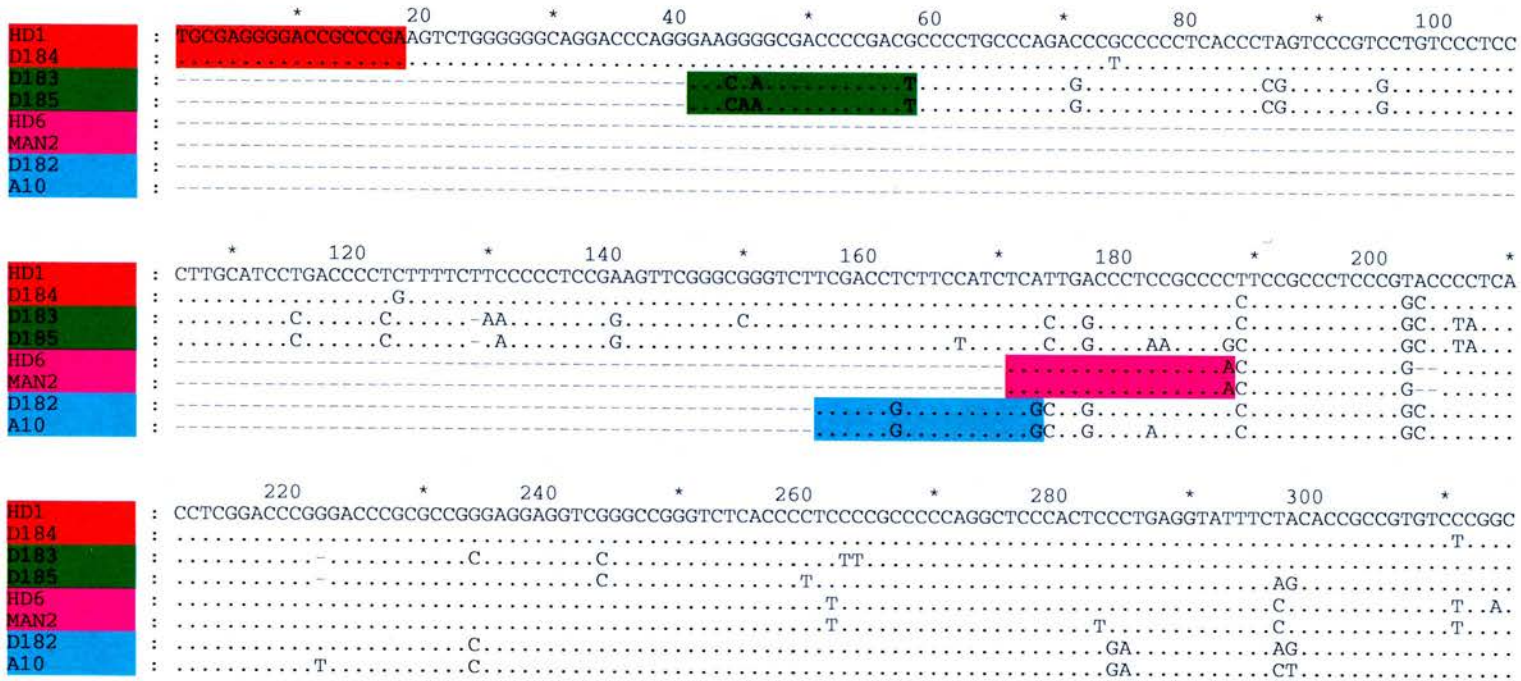
Cattle herds potentially provide a good population structure for studying the role of alleles in disease resistance since a single bull can give rise to a large number of offspring allowing the effects of a single allele or haplotypes to be traced. However, as with the human MHC, proving linkage may be difficult due to the large number of genes within this region and their tight linkage (Di Palma *et al.*, 2002). The existence of single gene MHC class I haplotypes such as A18 in cattle populations may however allow associations to be detected. Possible future studies linking RSCA and *T. parva* could

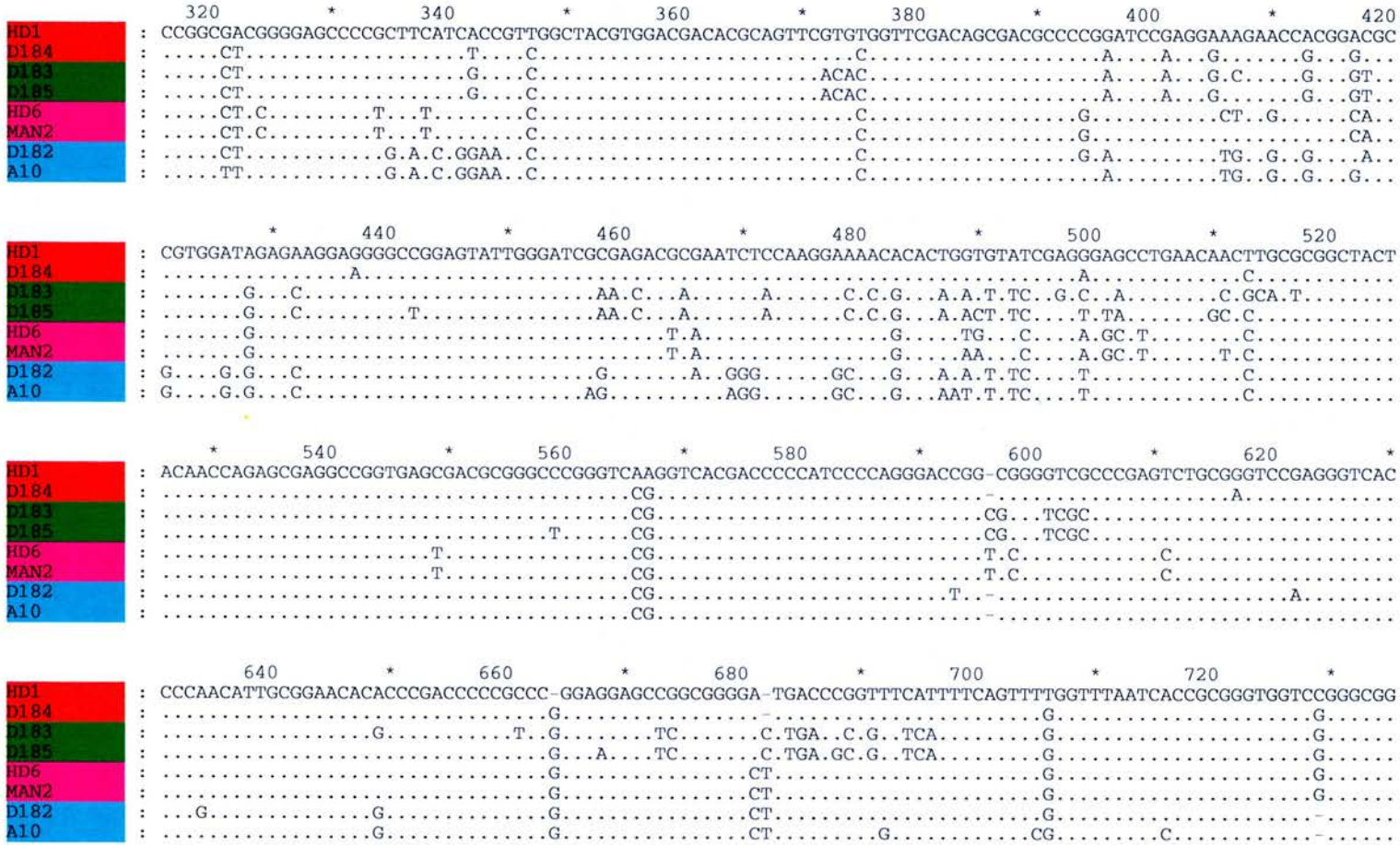
involve typing the class I genes in cattle from an area endemic for Theileriosis to examine the frequency of different class I alleles. Through this it may be possible to identify a link between expressed genes and resistance to disease. It may also be interesting to type buffalo which are carriers of *T. parva* but are resistant to the parasite to determine if they have unusual class I alleles which may be involved in resistance.

Determination of the sequence of antigenic peptides which elicit a strong CTL response when presented by MHC class I molecules allows for the development of potentially highly efficacious vaccines. In addition, the definition of HLA supertypes aids vaccine design by allowing good population coverage without the need for a particular allele being present at a high frequency. A huge effort has gone into defining peptides from various human pathogens such as HIV and Epstein-Barr virus (Hollsberg, 2002, Day *et al.*, 2001) and attempts have also been made to identify antigenic peptides from a number of infectious agents affecting cattle including respiratory syncytial virus and bovine herpesvirus1 (BHV1, Gaddum *et al.*, 1996, Hegde *et al.*, 1999). Thus by developing a method for typing MHC class I genes with wide-scale application in cattle this will allow us to determine the frequency of the class I alleles these epitopes are specific for and potentially allow the definition of supertypes through the identification of new alleles.

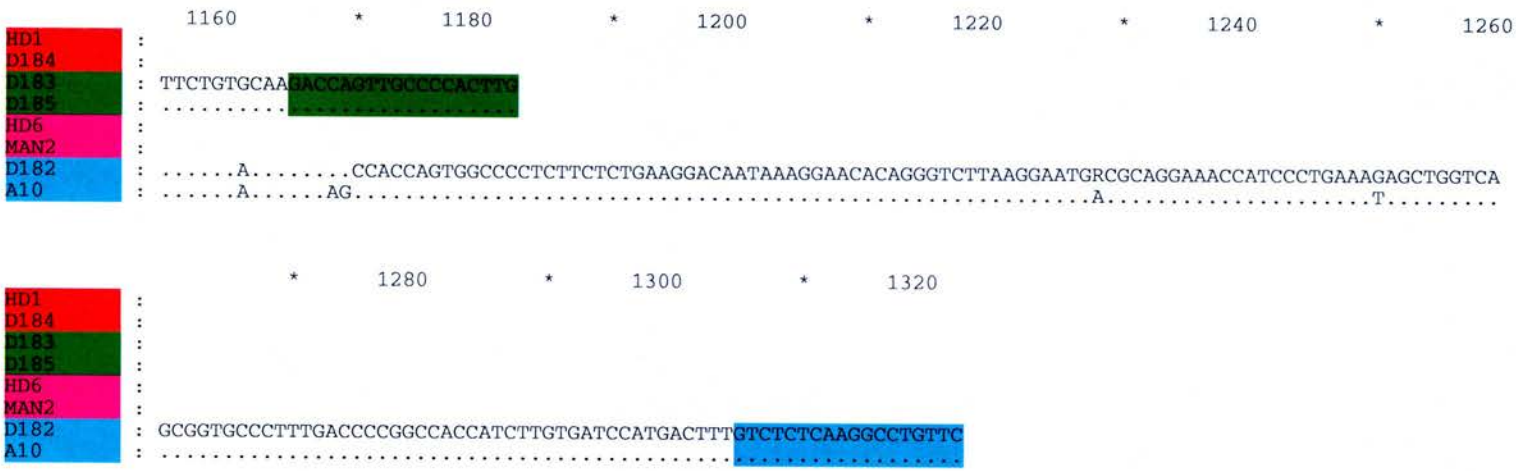
Appendix A: Complete sequence of the reference strands used in RSCA

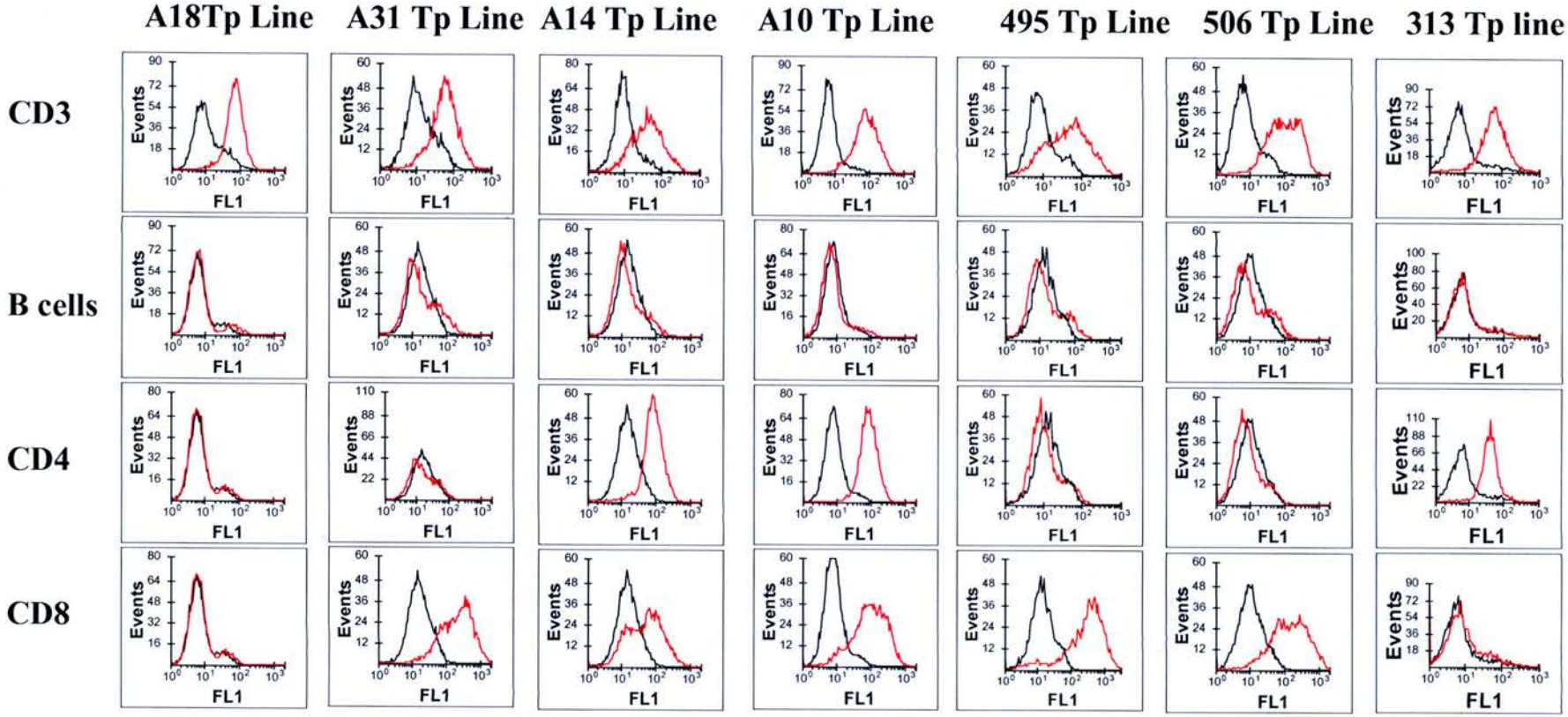
This alignment shows the complete sequence of the reference strands chosen for each of the groups. The names of the alleles are colour coded to indicate those from the same groups with the forward and reverse primers also highlighted.



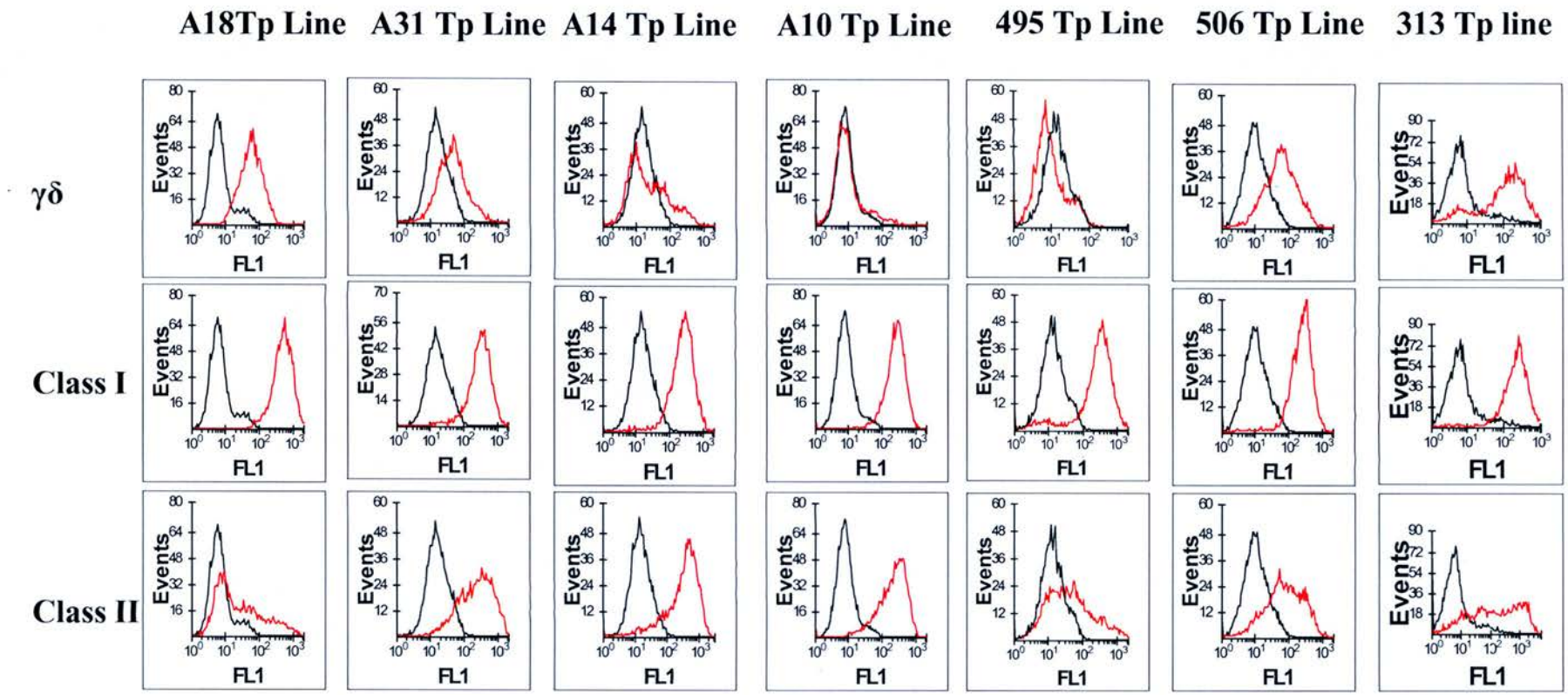

320 * 340 * 360 * 380 * 400 * 420
HD1 : CCGGCGACGGGGAGCCCCGCTTCATCACCGTTGGCTACGTGGACGACACGCAGTTCGTGTGGTTCGACAGCGACGCCCCGGATCCGAGGAAAGAACCACGGACGC
D184 : .....CT.....T....C.....C.....A....A...G.....G...G...
D183 : .....CT.....G....C.....ACAC.....A....A...G.C.....G...GT..
D185 : .....CT.....G....C.....ACAC.....A....A...G.....G...GT..
HD6 : .....CT.C.....T...T.....C.....C.....G.....CT..G.....CA..
MAN2 : .....CT.C.....T...T.....C.....C.....G.....CA..
D182 : .....CT.....G.A.C.GGAA..C.....C.....G.A.....TG..G..G....A..
A10 : .....TT.....G.A.C.GGAA..C.....C.....A.....TG..G..G...G...
* 440 * 460 * 480 * 500 * 520
HD1 : CGTGGATAGAGAAGGAGGGGCCGGAGTATTGGGATCGCGAGACGCGAATCTCCAAGGAAAACACACTGGTGTATCGAGGGAGCCTGAACAACTTGCGCGGCTACT
D184 : .....A.....A.....C.....
D183 : .....G...C.....AA.C...A.....A.....C.C.G...A.A.T.TC..G.C..A.....C.GCA.T.....
D185 : .....G...C.....T.....AA.C...A.....A.....C.C.G...A.ACT.TC....T.TA.....GC.C.....
HD6 : .....G.....T.A.....G.....TG...C....A.GC.T.....C.....
MAN2 : .....G.....T.A.....G.....AA...C....A.GC.T.....T.C.....
D182 : G....G.G...C.....G.....A..GGG.....GC...G...A.A.T.TC....T.....C.....
A10 : G....G.G...C.....AG.....AGG.....GC...G...AAT.T.TC....T.....C.....
* 540 * 560 * 580 * 600 * 620 *
HD1 : ACAACCAGAGCGAGGCCGGTGAGCGACGCGGGCCCGGGTCAAGGTCACGACCCCCATCCCCAGGGACCGG-CGGGGTCGCCCGAGTCTGCGGGTCCGAGGGTCAC
D184 : .....CG.....-.....A.....
D183 : .....CG.....CG...TCGC.....
D185 : .....T.....CG.....CG...TCGC.....
HD6 : .....T.....CG.....T.C.....C.....
MAN2 : .....T.....CG.....T.C.....C.....
D182 : .....CG.....T..-.....A.....
A10 : .....CG.....-.....
640 * 660 * 680 * 700 * 720 *
HD1 : CCCAACATTGCGGAACACACCCGACCCCCGCCC-GGAGGAGCCGGCGGGGA-TGACCCGGTTTCATTTTCAGTTTTGGTTTAATCACCGCGGGTGGTCCGGGCGG
D184 : .....G.....-.....G.....G.....
D183 : .....G.....T..G.....TC.....C.TGA..C.G..TCA.....G.....G.....
D185 : .....G...A....TC.....C.TGA.GC.G..TCA.....G.....G.....
HD6 : .....G.....CT.....G.....G.....
MAN2 : .....G.....CT.....G.....G.....
D182 : ...G.....G.....G.....CT.....G.....-.....
A10 : .....G.....G.....CT.....G.....CG.....C.....-.....


740 * 760 * 780 * 800 * 820 * 840
HD1 : GTCAGGGTCTCACACCTTCCAGCAGATGTTCGGCTGCGACGTGGGGCCGGACGGGCGCCTCCTCGGCGGGTACAGGCAGTACGCCTACGACGGCAGAGATTACAT
D184 : .....ATA.....GC.....A.....T.....A.TT.....C.....T...G.....
D183 : .....G.....A.....T.....CT.....T..T.....T.....
D185 : .....TG.....C.....T.....C.....TTT.....T..G.....A.....
HD6 : .....C.....G.....A.....T.....T....G.C.....TGA.....G.....C.
MAN2 : .....AT....G.....A.....A.....T..C.....T..G.....
D182 : .....TG.....A.....TGA.....G.....
A10 : .....C.....T.....C.....T.....T....G.C.....T..T.....T..G.....C.
* 860 * 880 * 900 * 920 * 940
HD1 : CGCCCTGAACGAGGACCTGCGCT
D184 : .....
D183 : .....CCTGGACCGCGGCGGACACGGCGGCTCAGATCACCAAGCGCAAGTGGGAGGCGGCAGGTGAGGCGGAGAGACAGAGGAACTA
D185 : .....CT.....G.CG.....
HD6 : .....T....A.....AT.....C....CT.....TTC.....
MAN2 : .....C.....TTC.....
D182 : .....G...G....A.....CT.....
A10 : .....T...G....T.....T....A.....AT.....C.....A..T...GT.....
* 960 * 980 * 1000 * 1020 * 1040 *
HD1 :
D184 :
D183 : CCTGGAGGGCCGGTGCGTGGAGGGGCTCCGCAGATACCTGGAGAACGGGAAGGACACGCTGCTGCGCGCAGGTACGAGGGGCGCGGGGCCGCCCTGATCTCCCCT
D185 : .....A.....G.....G.....
HD6 : .....AC.....G.....A.....C.....
MAN2 : .....G.....G.....T.....T.....
D182 : .....G.....A.....
A10 : .....G.....A.....
1060 * 1080 * 1100 * 1120 * 1140 *
HD1 :
D184 :
D183 : CGGGCTGGAGCTGGCTTCCCACGAGGAGAGGAAAATGGGGTCCCTGTGGAAACAGCGCCCCAGGTTCTTGTCAGGAGAGGGAGGAAATCCGCCCGGGTTTTCATA
D185 : .....C.....G.....
HD6 : .....G.....
MAN2 : .C.....G.....
D182 : T.....A.....A.....G.....C.....A.....T.....
A10 : T.....A.....A.....A.....G.....C.....A.....T.....


1160 * 1180 * 1200 * 1220 * 1240 * 1260
HD1
D184
D183
D185
HD6
MAN2
D182
A10
TTCTGTGCAAGACCAGTTGCCCCACTTG
.....A.....CCACCAGTGGCCCCTCTTCTCTGAAGGACAATAAAGGAACACAGGGTCTTAAGGAATGRCGCAGGAAACCATCCCTGAAAGAGCTGGTCA
.....A.....AG.....A.....T.....
* 1280 * 1300 * 1320
GCGGTGCCCTTTGACCCCGGCCACCATCTTGTGATCCATGACTTTGTCTCTCAAGGCCTGTTC



Appendix B: Phenotyping of *T. parva* infected cell lines by flow cytometry.
Specific monoclonal antibodies (mAbs) for cell surface markers were used to phenotype the *T. parva* cell lines used in this study (section 5.2.2.7). Staining with the specific mAbs are shown in red while black lines indicate isotype controls.



Appendix B: Continued from previous page

Appendix C

Coefficient of variation results from Chromium release assays in chapter 5.
The coefficient of variation was calculated by (standard deviation/mean)x100 for the minimum, maximum and test samples in each assay. In most cases standard deviation was deduced for triplicate repeats. The letter above each indicates which graph they refer to.

313 Cr assay results coefficient of variations

A

	313match	495mismatch	A18partial	A31partial
min	3.1	1.7	11.6	7.4
40:1	15.1	25.8	6.4	6.6
30:1	6.5	2.6	30.0	9.9
20:1	22.3	31.2	7.6	15.8
10:1	6.9	22.1	6.6	8.0
5:1	25.2	29.6	22.6	1.0
max	22.2	8.8	2.3	5.2

B

	313match	495mismatch	A18partial	A31partial
min	5.2	6.5	8.7	15.7
20:1	4.9	2.1	5.7	5.4
10:1	20.1	4.7	7.6	6.3
5:1	5.5	14.5	6.5	11.9
2.5:1	19.6	7.1	1.7	10.2
max	16.9	3.0	0.6	24.7

C

	313match	495mismatch	A18partial	A31partial
min	5.7	3.1	55.9	3.2
20:1	3.5	9.9	10.9	3.3
10:1	6.1	10.4	15.9	9.8
5:1	11.7	10.4	13.2	8.2
2.5:1	5.2	3.0	5.5	14.0
max	3.9	11.7	11.8	5.7

D

	313match	495mismatch	A18partial	A31partial
min	11.2	11.1	8.2	6.7
20:1	12.9	14.2	4.7	8.1
10:1	48.0	8.2	18.4	14.2
5:1	20.7	12.0	19.4	20.3
2.5:1	8.2	3.4	5.2	18.5
max	15.5	33.8	6.5	24.9

506 Cr assay coefficient of variations

A

	506match	A18mismatch	A14partial	A10partial
min	8.2	4.6	11.4	14.3
30:1	1.9	9.3	5.7	11.5
20:1	3.6	73.4	0.5	9.1
10:1	2.7	45.5	4.5	3.5
5:1	4.1	19.3	5.2	6.8
2.5:1	0.5	12.1	6.0	14.6
max	15.0	24.5	20.1	13.2

B

	506match	a18mismatch	A14partial	A10partial
min	19.7	10.2	36.2	30.0
20:1	3.9	13.3	18.6	9.4
10:1	1.9	10.8	3.4	15.2
5:1	2.0	2.0	12.9	7.5
2.5:1	18.8	6.6	7.1	13.5
max	6.7	0.6	24.6	4.1

C

	506match	A18mismatch	A14partial	A10partial
min	11.1	4.0	2.9	12.0
30:1	4.8	3.7	3.8	2.6
20:1	1.5	5.6	8.1	11.2
10:1	5.0	8.4	2.4	5.7
5:1	3.5	0.0	14.5	9.8
2.5:1	3.4	1.7	8.9	24.2
max	10.8	23.3	8.9	3.1

D

	506match	a18mismatch	A14partial	A31partial
min	11.1	12.5	23.1	7.2
20:1	6.3	11.7	1.9	6.0
10:1	17.5	11.2	5.9	6.6
5:1	13.5	7.7	8.8	11.9
2.5:1	2.5	1.3	2.6	9.5
max	6.8	11.8	6.4	1.5

495 Cr assay results – coefficients of variation

A

	495match	313mismatch	A14partial	A10partial
min	13.6	8.0	8.9	8.6
20:1	3.4	5.8	13.7	10.7
10:1	3.9	17.8	7.6	8.5
5:1	13.8	8.2	10.4	11.0
2.5:1	12.3	7.2	4.7	15.3
max	3.0	16.9	24.6	4.1

B

	495match	313mismatch	A14partial	A10partial
min	18.5	18.0	31.7	13.6
20:1	11.3	57.2	12.3	9.4
10:1	21.5	56.7	8.4	22.2
5:1	8.4	5.0	11.9	12.1
2.5:1	21.5	30.2	12.2	20.6
max	33.8	15.5	8.7	6.7

C

	495match	313mismatch	A14partial	A10partial
min	4.5	1.2	7.5	4.0
20:1	6.5	4.9	17.3	1.5
10:1	12.3	7.3	18.7	1.1
5:1	2.1	8.9	13.1	14.7
2.5:1	4.7	5.7	2.7	5.5
max	11.7	3.9	6.4	1.5

D

	495match	313mismatch	A14partial	A10partial
min	27.9	9.2	13.4	12.9
30:1	3.5	4.2	19.0	21.9
20:1	9.3	19.7	14.5	13.8
10:1	22.9	14.6	9.4	6.5
5:1	14.1	20.9	20.0	11.6
2.5:1	57.3	7.6	13.2	6.7
max	4.4	13.5	8.9	3.1

E

	495match	495mismatch	A14partial	A10partial
min	4.3	5.4	4.6	7.1
30:1	10.6	17.0	3.9	5.0
20:1	7.9	14.1	0.8	6.5
10:1	8.7	21.8	6.9	8.3
5:1	10.0	16.9	13.2	21.7
max	2.2	3.0	6.2	1.9

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