ORGANIZATION OF THE CLASS I REGION OF THE BOVINE MAJOR HISTOCOMPATIBILITY COMPLEX (BOLA) AND THE CHARACTERIZATION OF A CLASS I FRAMESHIFT DELETION (BOLA-A_{del}) PREVALENT IN FERAL BOVIDS

A Dissertation

by

NICOLE RAMLACHAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Genetics

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December 2004

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ABSTRACT

Organization of the Class I Region of the Bovine Major Histocompatibility Complex (BoLA) and the Characterization of a Class I Frameshift Deletion (BoLA-A_{del}) Prevalent in Feral Bovids. (December 2004)

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Chair of Advisory Committee: Dr. Loren Skow

The major histocompatibility complex (MHC) is a genomic region containing genes of immunomodulatory importance. MHC class I genes encode cell-surface glycoproteins that present peptides to circulating T cells, playing a key role in recognition of self and non-self. Studies of MHC loci in vertebrates have examined levels of polymorphism and molecular evolutionary processes generating diversity. The bovine MHC (BoLA) has been associated with disease susceptibility, resistance and progression. To delineate mechanisms by which MHC class I genes evolved to function optimally in a species like cattle, it is necessary to study genomic organization of BoLA to define gene content, and investigate characteristics of expressed class I molecules.

This study describes development of a physical map of BoLA class I region derived from screening two BAC libraries, isolating positive clones and confirming gene content, order and chromosomal location through PCR, novel BAC end sequencing techniques, and selected BAC shotgun cloning and/or sequencing and FISH analysis. To date, this is the most complete ordered BAC array encompassing the BoLA class I region from the class III boundary to the extended class I region. Characterization of a frameshift allele exhibiting trans-species polymorphism in *Bos* and *Bison* by flow cytometry, real-time RT-PCR, 1D and 2D gel analysis is also described. This frameshift allele encodes an early termination signal within the antigen recognition site (ARS) of exon 3 of the BoLA BSA-A_{del} class I gene predicting a truncated class I protein that is

soluble. An ability to assess MHC diversity in populations and provision of animals with defined MHC haplotypes and genetic content for experimental research is necessary in developing a basis upon which to build functional studies to elucidate associations between haplotype and disease in bovids.

The BoLA class I region is immunologically important for disease association studies in an economically important species. This study provides knowledge of gene content and organization within the class I MHC region in cattle, providing a template for more detailed analysis and elucidation of complex disease associations through functional genomics and comparative analysis, as well as evolution of the MHC in bovids to optimize a population's immune response.

To my parents, brother, sister and nephew, extended family and friends worldwide who share my life's journey both in body and spirit; for always being there to fuel my passion to cast off my bowlines and explore the unknown, yet always providing a strong anchor and safe harbour for me to dock my heart and soul.

"Twenty years from now you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbour. Catch the trade winds in your sails. Explore. Dream. Discover."

\- *Mark Twain*

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The motto of my high school alma mater is "*per ardua ad astra*", little did I realize then that these words would be representative of my pursuit of a PhD! In my long and sometimes arduous journey to completion of my doctoral program, I have had the privilege to encounter many individuals who have impacted my life and shaped my views of the academic and professional world.

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Now, if only I could get that MBA I've always wanted……just kidding.

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CHAPTER I

INTRODUCTION

> The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.
>
> \- Sir William Bragg

The major histocompatibility complex (MHC)

The MHC was initially described by Peter Gorer (1937) in studies of tumour transplantation studies in mice. It was further defined by George Snell (1951; 1948) who found that transplantability was determined by major histocompatibility antigens (designated H) on the surface of the cell, controlled by genes within a limited area on a specific chromosome, that he termed the major histocompatibility complex (MHC). Minor histocompatibility (mH) antigens were also defined by Snell (1948) using congenic mice, and showed that in contrast to H antigens mH antigens accounted for a much slower and more chronic graft rejection (Barth et al. 1956). These are polymorphic, endogenously synthesized products that can be recognized by alloreactive T cells in context of major histocompatibility complex molecules.

Recent studies demonstrated that mHags are naturally processed peptides of intracellular proteins that associate with MHC molecules (Kotzampasaki et al. 2004). During transplants, although donor and recipient are genotypically identical at the major MHC (HLA) loci, mH antigens may trigger strong cellular immune responses if they vary between donor and recipient (Goulmy et al. 1996). Following characterization of the MHC in transplant rejection in humans and in recognizing 'self' from 'nonself' (Dausset 1958; Dausset et al. 1975), the MHC became one of the best studied regions in the genome of humans as well as mice.

This dissertation follows the style and format of Immunogenetics.

Unrelated compatibility systems have also been documented in various taxa of multicellular organisms such as the protozoan pheromone system, the self-incompatibility system in plants and the invertebrate allorecognition systems (Haring et al. 1990; Hiscock and Kues 1999; Hiscock et al. 1996; Scofield et al. 1982; Vallesi et al. 1995; Vallesi et al. 1998; Weissman et al. 1990). An allorecognition system has also been defined in a proto-chordate, *Botryllus schlosseri* where it is controlled by a single, highly polymorphic locus in which there is developmentally regulated heterozygote advantage (overdominance) that can explain both emergence and maintenance of extraordinary polymorphism that predates development of the functional MHC in vertebrates (De Tomaso 2004).

In addition to its role of the MHC in allo-recognition, it was later proposed that in vertebrates, the driving force for high polymorphism of MHC loci is the diversity of MHC-regulated immune responses to pathogens (Bodmer 1972). In addition to its role in immune function, the MHC is of evolutionary importance as it has been identified in all jawed vertebrates: including teleosts (Hansen et al. 1999; Matsuo et al. 2002), avians (Sato et al. 2001; Shiina et al. 2004) and numerous mammals (Anzai et al. 2003; Band et al. 1998; The MHC Consortium 1999; Miska et al. 2002; Wagner et al. 1999; Yuhki et al. 2003b).

Comparative studies of the gene dense MHC in species ranging from humans to amphioxus are elucidating early genomic events leading to the evolutionary success of vertebrates. In initial studies, the clustering of genes in the MHC of humans and rodents to a single genomic region was thought to be indicative of a “complex”. This terminology has been challenged, as MHC genes are not necessarily linked or contained in a complex on a single chromosome in some species. This is true in chickens, quail, and duck (Shiina et al. 2004) as well as in teleosts (Hansen et al. 1999). In humans, the MHC class I-like genes, such as those that are members of the *CD1* gene family, are found on a different chromosome to the MHC (Kulski et al. 2001a).

Close linkage of functionally related genes within the MHC was due to selection (Abi-Rached et al. 1999). Alternative explanations for clustering could be the

suppression of recombination or coordinated expression of alleles in close proximity. There seems to be some conservation within the basic genes, indicating that there may be an evolutionary advantage in conserving the MHC as a unit, which will be discussed later on. Semantics aside the MHC, whether it is a complex, region or mapped area of the genome, plays an important role in comparative analyses to define evolutionary significance or immunological importance of its molecular organization.

General organization and genetic content of the mammalian MHC

The genetic content of the MHC was first described in mice and humans, but has since been studied in other mammalian species and appears to be evolutionarily conserved (Beck et al. 2001; Chardon et al. 1999a; Di Palma et al. 2002; Ellis et al. 1995; Ellis et al. 1999; Gustafson et al. 2003; Holmes et al. 2003; Hurt et al. 2004; McShane et al. 2001; Yuhki et al. 2003b). Initial characterization of the MHC in mammals involved primarily cellular, serological, and biochemical analyses; however molecular analysis begun later has been seen to provide most accurate information on gene organization and content.

The typical mammalian MHC consists of three closely linked regions, I (most telomeric), III, and II (most centromeric), occupying a single chromosomal segment. Both class I and class II genes are embedded between well conserved framework genes, some of which are essential for adaptive immunity (e.g., genes involved in antigen-processing and peptide transport). Class I regions encode the classical I (class Ia) and nonclassical I (class Ib) molecules, that differ on the basis of expression and function. The class I loci in region I and the class II loci in region II are related in their structure, function, and evolution; specifically encoding class I and II antigen presenting molecules respectively. Region III, which is between regions I and II, contains loci unrelated to the class I and II loci, however it harbors a diverse array of structurally unrelated genes, among them several genes involved in innate immunity (e.g. cytokines, tumour necrosis factor family members, heat shock proteins, complement factors) (Klein and Sato 1998).

The associations of genes within the class III region with disease are well documented (Gruen and Weissman 2001; Yu 1998). The class III region is the most gene dense and evolutionary conserved of the three regions (Milner and Campbell 2001).

Also present in the MHC are genes involved in other cellular control processes like NOTCH4 (*Drosophila* homologue important in developmental cell signaling), RXRB (retinoid X receptor beta which is a MHC class I promoter binding protein), SC1 (TCF19, transcription factor 19) and GNL (or HSR1, encoding guanine nucleotide binding protein-like 1). Of 224 identified loci in the human MHC (excluding the extended class I) 128 are predicted to be expressed, but many are still of unknown function (Beck and Trowsdale 2000).

HLA has been used as the "gold standard" for comparative analysis for mammalian species, as it was the first sequenced MHC (The MHC Consortium 1999). Approximately 40% of expressed loci in the MHC are associated with the immune system (Trowsdale 2001). The complete 3.6 Mb HLA sequence (The MHC Consortium 1999) with its 224 genetic loci (128 expressed). Analyses of adjacent gene content and isochore boundaries revealed that sequence conservation and possibly linkage disequilibrium extended outside the traditional MHC; thus extending the core MHC from 3.6Mb to 8Mb to include an extended class II region (preceding class II) and a telomeric extended class I region (Herberg et al. 1998; Stephens et al. 1999; Totaro et al. 1998; Zemmour et al. 1990). The extended class I region of the HLA was found to have a cluster of olfactory receptor (OR) genes (Fan et al. 1995). Figure 1 shows an overview of the distribution of genes in HLA and its extended flanking regions.

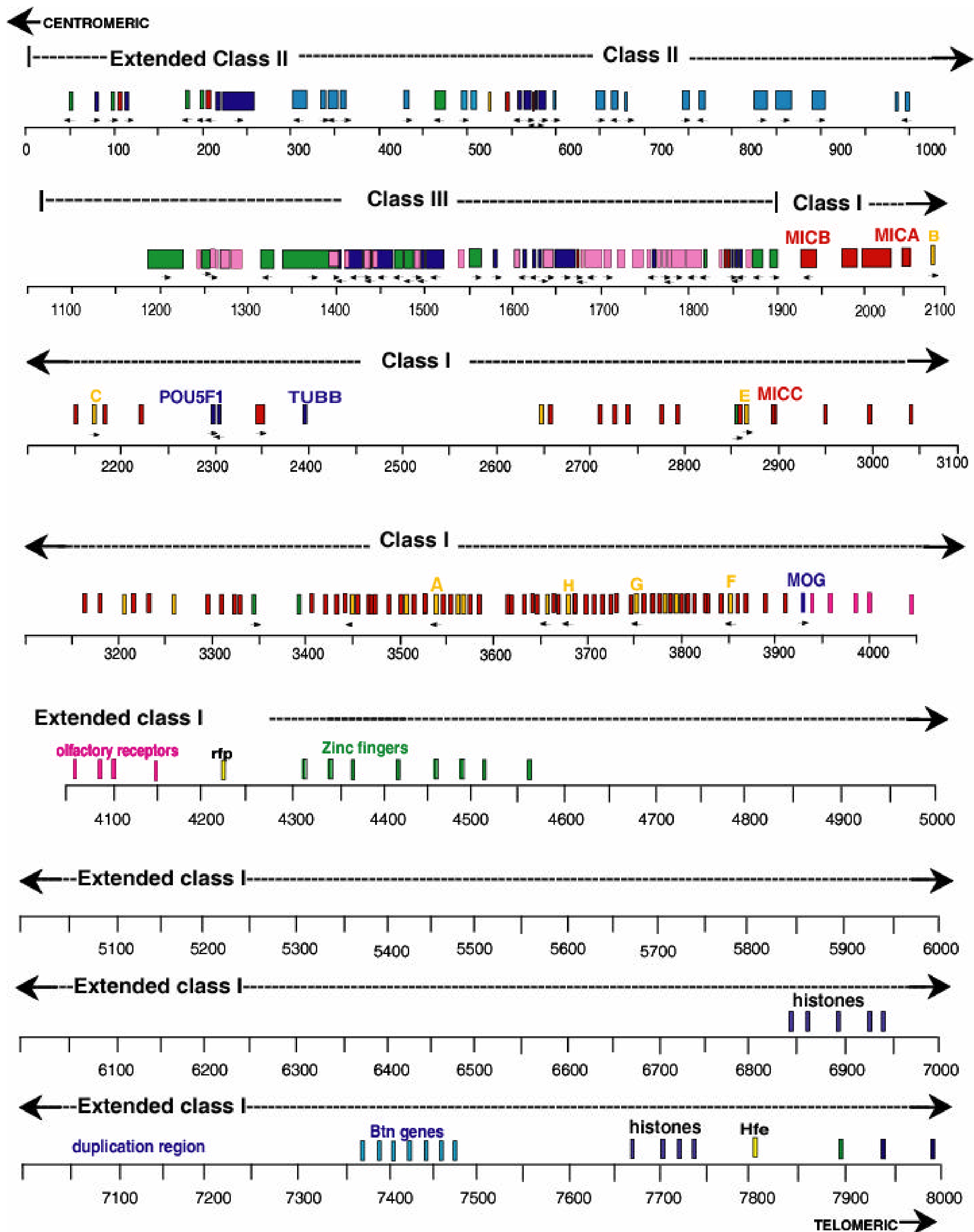


Figure 1. The HLA. Distribution of genes within the 8Mb extended HLA in the following order: extended class II, class II, class III, class I (MICB-MOG) and extended class I. Class I genes are illustrated in yellow (classical) and red (class I-like and pseudogenes) (Adapted from The MHC Consortium 1999).

Comparative analysis of the organization of the MHC across species

Overview

Homologous MHC regions have been discovered in all vertebrate species including shark (Flajnik et al. 1999; Kulski et al. 2002b; Trowsdale 1995). However, significant differences in the number and physical organization of the genes within the MHC have been demonstrated in several species (Band et al. 1998; Chardon et al. 1999a; Forbes and Trowsdale 1999; Hurt et al. 2004; McShane et al. 2001; Renard et al. 2001; Shigenari et al. 2004; Smith et al. 1995) (See figure 2). Of note is the chromosomal inversion and translocation of the class IIB region (20 Mb centromeric) within the bovine major histocompatibility (BoLA) complex (Band et al. 1998; McShane et al. 2001). Also, class I genes are found in "clusters" that tend to vary in location and number from species to species, depending on post-speciation duplication events that will be discussed later (Dawkins et al. 1999; Hurt et al. 2004; Kulski et al. 2002b; Renard et al. 2001; Shigenari et al. 2004; Shiina et al. 2004).

The human MHC (The MHC Consortium 1999; Shiina et al. 1999c) and the rat MHC (Hurt et al. 2004) are the only mammalian MHCs for which the complete sequence has been determined to date. Other mammals for which partial MHC sequences are available are the chimpanzee (class I region, complete), pig (class I region, partial), cat (class I and II region), and mouse (class II, III, and incomplete class I) (Chardon et al. 2001; Renard et al. 2001; Kumánovics et al. 2002; Anzai et al. 2003; Takada et al. 2003; Yuhki et al. 2003a, 2003b).

Comparisons reveal a complex MHC structure for mammals and a relatively simpler design for nonmammalian animals with a hypothetical prototypic structure for the shark. In the mammalian MHC, there are two (in pig, defined by Renard et al. 2001) to seven (in rat, defined by Hurt et al. 2004) different class I duplication blocks embedded within a framework of conserved nonclass I and/or nonclass II genes. With a

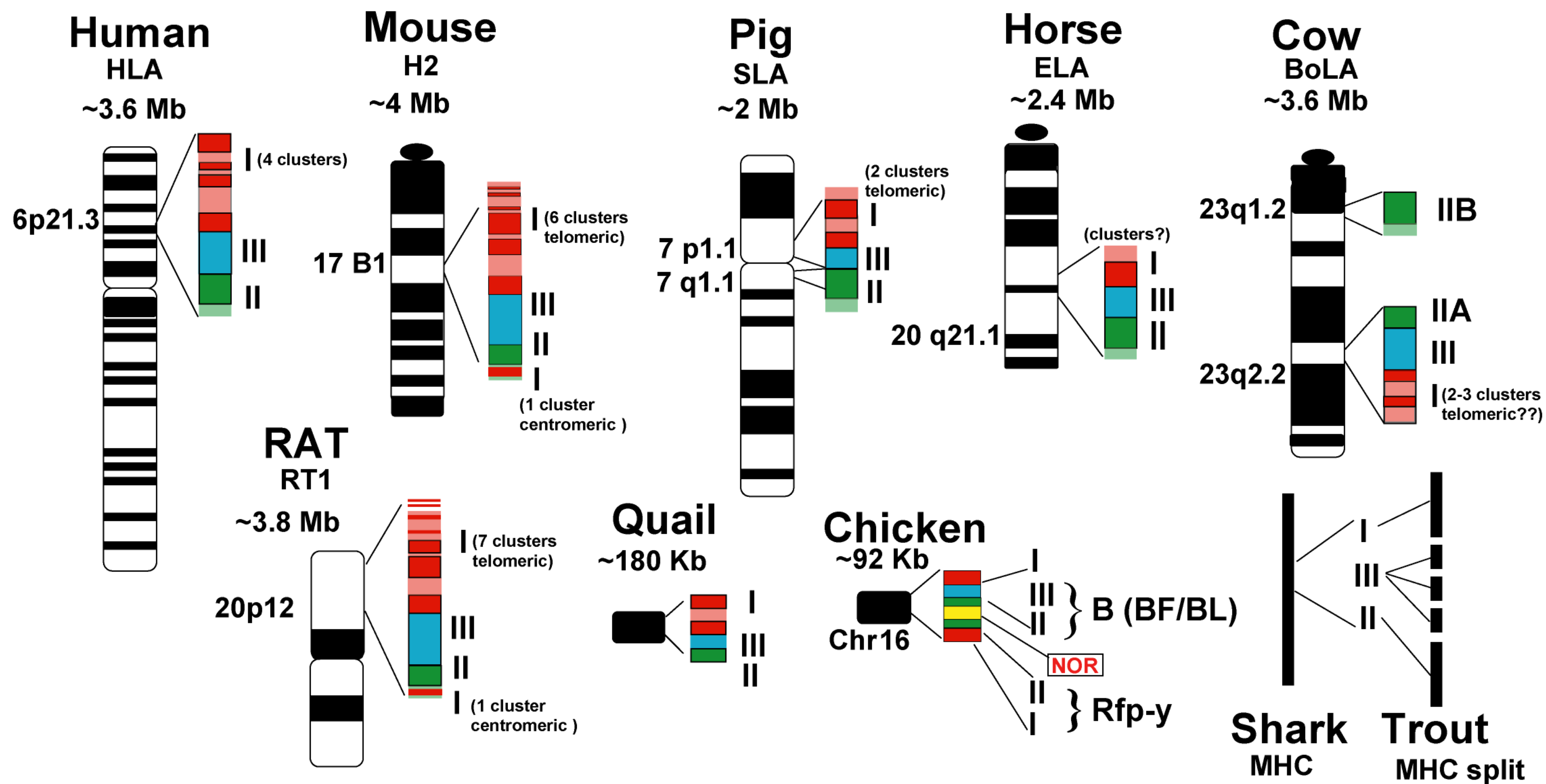


Figure 2. The organization of the MHC in several vertebrate species. The general organization of the MHC class I, II and III regions are conserved among jawed vertebrates, including avians, with the shark representing the prototypic vertebrate with the longest extant lineage. The number of clusters of class I sequences are indicated when known (red), with Rat having the most (8, including one telomeric cluster). This telomeric cluster is only seen in RT1 and H2 to date. The conserved framework genes in class I are illustrated in pink. The class III region (blue) is conserved in terms of gene content and location across most species. The class II region is in dark green, with the light green representing the extended class II's framework genes. Note the centromere splitting the SLA and the NOR (or nucleolar organizing region) separating the chicken MHC into two regions BF/BL and Rfp-Y. Also of note is the chromosomal inversion and translocation within the bovine MHC. The trout MHC is dispersed, with the three regions on different chromosomes. Adapted from Skow et al. 2004 unpublished.

few exceptions, the class I framework genes are absent from the MHC of birds, bony fish and sharks (Figure 2,3). Comparative genomics of the MHC reveal a highly plastic region with major structural differences between the mammalian and nonmammalian vertebrates. These striking differences suggest a major structural and evolutionary division between the MHC of mammalian and nonmammalian vertebrates. In order to better understand the diversity and complexity of the MHC genomic structure, comparative analysis of this region in different haplotypes and various representatives of the mammalian and nonmammalian vertebrate classes is necessary. A more detailed analysis of the MHC organization and gene content follows, and is described by class.

Class I

Mammalian vertebrates

The organization of the MHC class I region in nonmammalian vertebrates is different to that in mammalian vertebrates. The class I duplication blocks and conserved flanking framework genes observed in mammals are not evident in the nonmammalian vertebrates (Kulski et al. 2002b). The "framework hypothesis" proposed by Amadou (1999), explains the paradox where conserved framework genes whose alterations are deleterious, occupy homologous locations amidst non-orthologous class I sequences (Figures 2,3). In the class I region of HLA there are 18 *HLA* class I genes (6 coding and 12 pseudogenes) and 7 MHC class I-chain-related (*MIC*) genes (2 coding and 5 pseudogenes) spread throughout 1.8 Mb of the class I region (The MHC Consortium 1999).

The *HLA* class I and *MIC* (or PERB II) genes are organized together as a repeating unit within three duplication blocks designated as a, ß and ? (Figures 2, 3). The conserved framework genes are distributed between these blocks. Within these blocks there are varying degrees of polymorphism as well as a common ancestral and duplicative history that can be observed during comparative analysis (Kulski et al. 2002b) (Figure 3). In the SLA there are only two cluster of class I genes, while in humans the class I genes are found within three blocks, designated ß,a and ? (Renard

2001; The MHC consortium 1999) (See Figure 2,3). Analysis of the genomic DNA structure containing the MIC genes and the adjacent HLA-B and C genes shows that segmental duplication is a recurring phenomenon during the evolution of the MHC (Kulski et al. 1997; Mizuki et al. 1997; Shiina et al. 1998).

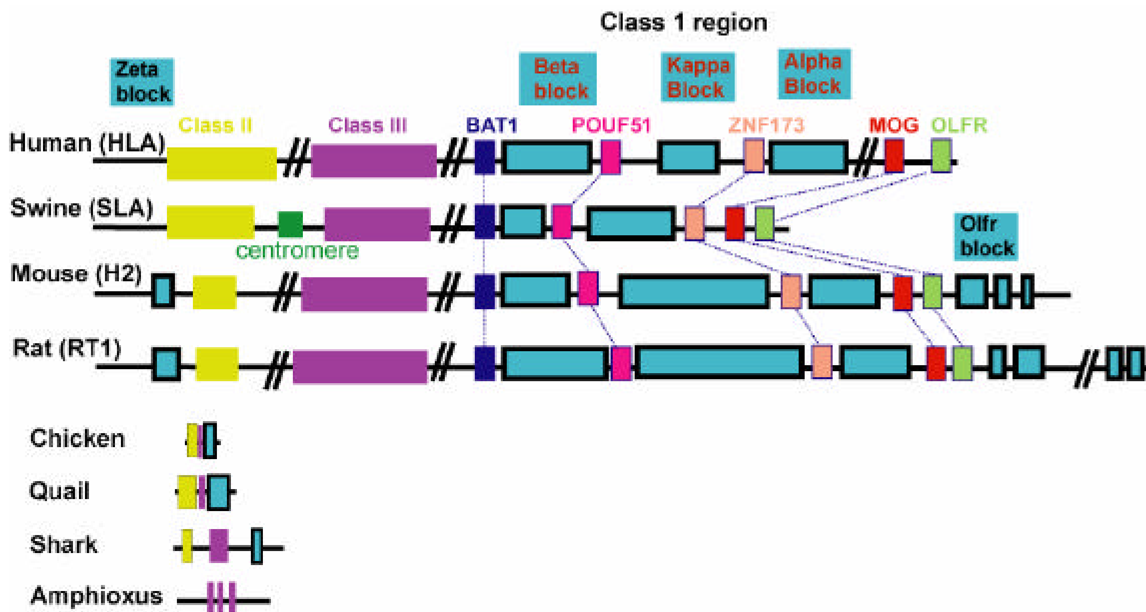


Figure 3. MHC class I duplication blocks. Class I duplication blocks (blue) are labeled as a, ß, ?, ? and Olfr. All mammals shown here have a, ß and ? blocks, except swine, which lacks the a block. Only mouse and rat have the ? and the OLFR blocks as indicated. Framework genes that flank the blocks are also shown. The orthologous relationships of class I duplication blocks and framework genes are indicated. In swine, the centromere separates the class II region from the class III and class I regions. Cass II gene region is indicated in yellow and class III genes in purple. Chicken, quail and shark lack framework genes, but maintain the order of class I and class II genomic structure as mammals. Amphioxus have no class I and class II genes but share framework genes, especially in the class III region. (Adapted from Kulski et al. 2002).

HLA class I molecules are diversified into three categories. The first is the classical genes (Ia) encoded by HLA-A, -B, and -C loci. These genes are expressed on the surface of most nucleated cells and are highly polymorphic. The second group is the nonclassical group (Ib) encoded by HLA-E, HLA-F and HLA-G, all of which have limited expression on particular cell types, little polymorphism and less is known in regards to their immunological function (Geraghty et al. 1987; Geraghty et al. 1990;

Koller et al. 1988; Shiina et al. 2004). The third group consists of class I pseudogenes such as HLA-H, HLA-J, HLA-K, and HLA-L (Geraghty et al. 1992; Messer et al. 1992; Zemmour et al. 1990).

In addition to the immune system genes, there are large families of olfactory-receptor (OR) genes, zinc-finger, RING-finger and transcription factor gene families in the extended class I (Gruen and Weissman 1997). The HLA-linked OR genes have been implicated in mate selection and maternal protection (Yamazaki et al. 2000) and addiction to tobacco smoking (Fust et al. 2004). HLA-associated sexual selection could also involve OR genes, not only participating in olfaction-guided mate choice, but also in selection processes within the testis (Ziegler et al. 2002). The location of OR genes was conserved in pig (Chardon et al. 1999a) and cat (Yuhki et al. 2003a). The number of OR loci was greatly expanded in mouse (Yoshino et al. 1997) and even more in rat (Hurt et al. 2004). The rat and mouse MHCs (RT1 and H-2 respectively), have extensive duplications of class I genes within the ß,a and ? equivalent blocks, as well as class I genes in the OR-linked extended class I region (Amadou et al. 2003; Hurt et al. 2004).

The class I region also contains families of loci involved in DNA repair and cell growth like TFIIH (also called GTF2H4, transcription factor II H polypeptide 4), DDR1 (discoidin domain receptor family), PRG1 (proteoglycan 1, secretory granule) and DHX16 (also called DDX16 or Dbp2, DEAH (Asp-Glu-Ala-His) box polypeptide 16) (Shiina 1999d). There are no apparent orthologous relationships between the three main functional class I genes in human and mice. There has also been a similar lack of orthology in class 1 sequences of the swine SLA and HLA and between H-2 and HLA (Renard et al. 2001). The comparison of the distal half of the HLA (continuous from HLA-E to 1000 kb telomeric of HLA-F) to the corresponding sequences in the mouse demonstrates similarity in the two species, showing a breakpoint in synteny between mouse and human, located in a point that is telomeric to the distal part of the H-2 region (The MHC consortium 1999). The region that is located telomeric to HLA on chromosome 6 in human is found on chromosome 2 in mouse which is different from that carrying H-2 (chromosome 17), however mapping analysis of paralogous genes

around the breakpoint shows that the human organization probably represents the putative human/mouse ancestral one (Amadou et al. 1995).

The gene order of the distal MHC class I region on mouse Chr 17 is conserved between mouse and human but not the physical distance, supporting the independent expansion of MHC class I genes in the so-called accordion model of MHC evolution (Klein et al. 1993a). Amadou et al. (2003) further defined this region with additional information from the mouse genome draft sequence, identifying 59 olfactory receptor (OR) loci (approximately 20% pseudogenes) in contrast to only 25 OR loci (approximately 50% pseudogenes) in the corresponding centromeric OR cluster that is part of the 'extended MHC class I region' on human chromosome 6. MHC-linked OR loci and MHC class I loci, have duplicated extensively and are polymorphic suggesting reciprocal influences act on evolution of the H2 region and the H2-linked OR loci in that the MHC-linked OR genes could be specifically involved in the detection of MHC diversity, thereby contributing to mate choice (Amadou et al. 2003).

More information can be gleaned from study of other mammalian species. The canine MHC, or dog leukocyte antigen (DLA) has at least four complete class I genes: DLA-88, DLA-12, DLA-64, and DLA-79. DLA-88 is highly polymorphic (at least 40 alleles) while the other class I loci are less polymorphic, with fewer than 12 alleles, but the specific order within the DLA region has yet to be defined (Wagner et al. 1999; Wagner et al. 2002). Estimates of as many as 20 class I loci have been described in the feline MHC (FLA) (Yuhki et al. 1989). Polymorphic single nucleotide polymorphisms (SNPs) detected in FLA class I alleles are, in most cases, in positions in codons which are also variable in humans and mice, whereas invariant positions with defined functional constraints were generally conserved and invariant between the three species as well (Yuhki et al. 1989). The horse genome contains at least four expressed class I genes (Chung et al. 2003) all located to two clusters equivalent to the ß and ? blocks within a class I region that spans approximately 1.2kb (Gustafson et al. 2003).

In contrast to rodents and primates, the class I region in the pig, is simpler. No orthologous relationships have been found between the MHC class I genes in man and

swine (Chardon et al. 1999b). In swine, the 12 SLA class I sequences constitute two distinct clusters. One cluster comprises six classical class I-related sequences, while the other comprises five class I-distantly related sequences including two swine homologues of the HLA MHC class I chain-related gene (MIC) sequence family. The positions of these two clusters are equivalent to rat RT1-CE and RT1-N clusters (Hurt et al. 2004). The number of functional SLA classical class I genes, as defined by serology, varies from one to four, depending on the haplotype (Chardon et al. 1999b; Renard et al. 2001).

The evolutionary history in the MHCs of primates also reveals a pattern of relative stability of loci within suborders, but is marked by episodic duplication, activation and expansion of loci coinciding with radiation of major taxonomic groups. The lack of orthology between mouse and human MHC class I genes is also seen in a comparison of primates (Platyrrhini and Catarrhini) (Parham 1989). It is generally believed that primate *MHC* class I genes (especially classical class I genes) experienced a relatively higher turnover rate through primate evolution than class II genes (Go et al. 2003). Class I genes in simian primates duplicated extensively in their common ancestral lineage and subsequent evolution in descendant species has been characterized mainly by independent loss of genes (Sawai et al. 2004).

There was initially 6 major clades of class I genes defined: New World primate HLA-G related loci, primate A and B loci, Carnivora (dog and cat), Ungulata (pig, sheep, cow and horse) and Rodentia; with each clade presenting a distinct set of MHC class I loci (Cadavid and Watkins 1997) (although this number is being expanded with more research). Phylogenetic analyses support the position of the gray whale MHC-1 loci within the ungulates and carnivores consistent with the common divergence of the Cetartiodactyls and Carnivores from the Ferungulates (Flores-Ramirez et al. 2000). Flugge (2002) used the tree shrew class I sequences for construction of a gene tree that includes class I genes of the orders Primates, Carnivora, Artiodactyla, Perissodactyla, Lagomorpha, and Rodentia. The tree shrew class I sequences cluster separately from Primates (human, rhesus macaque, cotton-top tamarin, marmoset), Carnivora (cat, dog),

Artiodactyla (cattle, sheep, pig), Perissodactyla (horse, rhinoceros) and Rodentia (mouse, rat, woodchuck, squirrel).

Phylogenetic analysis of classical class I genes, CD1 genes and non-classical genes in jawed vertebrates shows the non-classical MHC class I genes are much more divergent than the classical class I genes with more of a between species relationship (Cadavid and Watkins 1997). BoLA MHC class I groups with the other mammalian MHC class I in this analysis (Cadavid and Watkins 1997).

Non-mammalian vertebrates

Sequencing of the MHCs of several non-mammalian species has also been accomplished. The chicken *B* locus is considered to represent the minimal essential MHC within a nonmammalian vertebrate (Kaufman et al. 1999a) and is 92 kb in length with only 19 genes. The gene density of quail MHC (containing a total of 42 genes) (Shiina et al. 2004) is slightly higher than that in the chicken MHC B-F/B-L region with one gene per every 4.6 kb (Kaufman et al. 1999b), but four times higher than in the *HLA* region which has one gene per every 15.6 kb (The MHC consortium 1999).

There are indications that similar MHC organization exists in birds and amphibians. Evidence suggests that the quail class I region is physically separated from the class II region and characterized by a large number of the expressible class I loci (at least 14), class II loci (more than 10) and other duplicated genes (pseudogenes and gene fragments) in contrast to the chicken MHC, where the class I and class II regions are not clearly differentiated and only at most three expressed class I loci have been detected (Shiina et al. 2004 ; Shiina 1999b). In fact, it has been documented that a single dominantly expressed class I locus (B-FIV) determines the immune response to certain infectious pathogens in chickens (Kaufman 2000).

The chicken MHC is called the B-F/B-L region, and located in the B complex of the genome. It contains classical class I a-chain (B-F) and class II ß-chain (B-LB) genes, and determines rapid allograft rejection, mixed lymphocyte reactions and resistance to a variety of infectious pathogens (Rogers et al. 2003). The B-F/B-L region contains 19

genes in 92 kb of DNA, including two C-type lectin-like genes (Kaufman et al. 1999b). In contrast, the quail MHC contains 41 genes, corresponding to one gene per every 4.4 kb (Shiina et al. 2004). The proteins encoded by the NK and class I genes are known to interact as ligands and receptors, and unlike quail and the chicken, the genes encoding these proteins in mammals are found on different chromosomes (Shiina et al. 2004). This strongly suggests an evolutionary connection between the NK C-type lectin-like superfamily and the MHC.

Unlike the MHC of mammals, the particular haplotype of the B-F/B-L region of the chicken B locus clearly determines susceptibility to certain infectious pathogens as well as response to certain vaccines. The stronger association with immune response is thought to be due to the simple MHC of chickens. The B-F/B-L region is much smaller and simpler than the typical mammalian MHC, and expresses only a single class I gene at a high level of RNA and protein. The peptide-binding specificity of this dominantly expressed class I molecule in different haplotypes correlates with resistance to tumours caused by Rous sarcoma virus, while the cell-surface expression level correlates with susceptibility to tumours caused by Marek's disease virus (Kaufman 2000). Selection of single dominantly expressed class I and class II molecules may be due to co-evolution between genes within the compact chicken MHC.

Most of the framework genes (*BAT1*, *OCT3* (*POU5F1*), *HSR1* (*GNL1*), *GT257*, *ZNF173*, *ZNFB8*, *HCTEX4*, *MOG* and *UBD)* found in mammalian MHCs have not been found within the class I gene regions of birds and fish, but are located outside the MHC of non-mammals, as seen in Fugu (Sambrook et al. 2002). However, *FLOT* and *TUBB* are found in the region between the ß and ? blocks of mammals (Gunther and Walter 2001; Shiina 1999c; Shiina 1999d) and are also found in the class I gene region of the medaka fish ((Matsuo et al. 2002) and Fugu (Clark et al. 2001). Fugu genome studies have identified the presence of 67 of 106 human MHC orthologs, mostly outside the Fugu MHC region and located either alone or linked in pairs (Sambrook et al. 2002). Also, homologues of 11 out of 24 framework genes in the human class I region were not

identified in Fugu, including *KIAA01070*, *IEX-1*, *HCR*, *OTF3*, *DHFRP*, *MICA*, *MICB*, and the skin genes *CDSN*, *SEEK1* and *SPR1* (Sambrook et al. 2002).

Other class I-like homologues

MHC class I chain related (MIC) molecules show homology with classical HLA molecules, but do not combine with beta2 microglobulin, do not bind peptide and are not expressed on normal circulating lymphocytes. In response to stress, MIC proteins are expressed on the cell surface of freshly isolated gastric epithelium, endothelial cells and fibroblasts and engage the activating natural killer cell receptor NKG2D, which is found on many cells within the immune system (Collins 2004). Despite the highly polymorphic nature of MIC genes, only one polymorphic position has been identified that appears to affect the binding of NKG2D. Some MIC molecules which are expressed by tumours appear to shed and solubilize in plasma. This soluble form of MIC engages cells expressing NKG2D, rendering them inactive, and impairs tumour cytolysis. Similarly, a protein encoded by human cytomegalovirus (CMV) prevents MICB surface expression and subsequent NKG2D interaction (Collins 2004).

Some viruses also express MHC class I-like genes presumably in order to modulate and avoid the host's immune system (Wilson and Bjorkman 1998). The human and mouse cytomegaloviruses encode class I homologues (*gpUL18* and *m144*) (Chapman and Bjorkman 1998). Both viral class I proteins associate with ß2-microglobulin but only *gpUL18* can also bind endogenous peptides with the same characteristics as peptides eluted from class I molecules (Browne et al. 1990; Chapman and Bjorkman 1998; Fahnestock et al. 1995). The viral class I homolog gpUL18, with high affinity for the NK cell receptors LIR-1/ILT-2, uses the same binding interaction to recognize class I MHC molecules, and is known to serve as a natural killer cell (NK) decoy and to ligate NK inhibitory receptors to prevent lysis of an infected target cell (Kim et al. 2004). It has been postulated that UL18 can serve as an effective tool for the evasion of NK-mediated cytotoxicity and for the inhibition of IFN-gamma production during xenograft rejection (Kim et al. 2004).

Interestingly, there is a group of MHC-related CD1 proteins (encoded by a cluster of five *CD1* genes on chromosome 1), that present lipids and glycolipids rather than peptides to T cells in response to microbial infections (Porcelli et al. 1998; Sugita et al. 1998; Wilson and Bjorkman 1998). Phylogenetically, the CD1 proteins group between class I and II, suggesting that genomic organization of the human *CD1* gene cluster may have formed prior to evolution of the present organization of the *HLA* class I gene cluster (Kulski et al. 2001b; Kulski et al. 2002b). Also, the tapasin protein sequence was recently proposed to be a modified MHC class I-like gene on the basis of amino acid sequence similarity, predicted tertiary structure and domain organization, (Mayer and Klein 2001). The HFE gene, in which a single mutation causes most cases of hereditary hemochromatosis (Eijkelkamp et al. 2000), is also an unusual MHC-like molecule, located in an inverse duplication of the telomeric HLA called the omega block that includes the OLF and BT cluster about 4 megabases away from classical HLA.

Class III

The MHC class III region is located centrally between the class II and I regions. It is the most gene dense region in the human genome, with 62 genes containing more than 500 exons over 706 kb, or one gene per 11387 bp (Kulski et al. 2002b). This is observed in all the mammalian species studied to date (Chardon et al. 1999a; Hurt et al. 2004). Gene duplication in the class III is less extensive than in the rest of the MHC (Trowsdale and Parham 2004) and differences in the GC content (isochores) of the DNA point to a distinct origin of the class III region (Fukagawa et al. 1995). The jawless vertebrates (hagfish, lampreys) (Sato et al. 2000), cephalochordate, such as amphioxus (Abi-Rached et al. 2002), and echinoderms, such as the sea-urchin (Kulski et al. 2002b), do not appear to have any class I and class II genes, but they do have some components of the MHC class III paralogous anchor genes such as NOTCH, PBX, C3/4/5, Bf/C2, BAT1 and LMP/X. The protostomes (*D. melanogaster* and *C. elegans)* have orthologous NOTCH and PBX genes (Trachtulec et al. 1997).

Members of the complement cascade (C2, C4 and Bf) and tumour necrosis factor family members (TNF, LTA and LTB) are genes in class III that have characterized immunological function. There are several other genes with possible roles in the immune system. At the centromeric end, the G15 gene has homology to lysophosphatidic acid acyltransferase, suggestive of a role in intracellular signaling and inflammation (Aguado 1998). The IkappaB like gene is a transcription factor of the NFkB family (Albertella 1994) and is located next to BAT1. There have been groups that suggest a further subdivision of the MHC molecular genetic structure based on the class III/ class I boundary region (Gruen and Weissman 1997; 2001). This is due to the number of genes with a role in inflammation; (TNF, LFA, LST1/B144 and 1C7) that are grouped closely together spanning 300kb of the so-called class IV region (Gruen and Weissman 2001). Interestingly, an association between complement C4 protein polymorphism and smoking habits in Icelandic and Hungarian subjects has also been documented which was highly significant in females (Fust et al. 2004).

Class II

To date, all jawed vertebrates have been found to have class II genes and express a number of different class II molecules (or isotypes) (Flajnik and Kasahara 2001). Mammals express one or more of three classical isotypes (DR, DQ or DP) (Beck and Trowsdale 1999), however some ruminants like cattle also express a DY isotype (Wright 1994). Comparative analysis by Figueroa et al. (1994) showed DRB loci present in human populations diverged from one another before the prosimian and anthropoid primates split. Major allelic lineages of DRB1 locus, were established more than 85 million years ago, however the DRB6 gene was inactivated before separation of prosimians and anthropoids, remaining a pseudogene for more than 85 million years. Also the primate DRB region is evolutionarily structurally and functionally unstable, as some species have lost DRB genes and compensate functionally by substituting DOB and/or DPB. In *Otolemur garnetti*, for example, one chromosome carries at least three copies of the DRB3 pseudogene (Figueroa et al. 1994).

Bovine MHC (BoLA) studies also show variation in number of class II genes as well, with one paper by Davies et al. (1994) defining 38 class IIa (DR-DQ) and 5 class IIb (DYA-DOB-DIB) haplotypes showing that DYA, DOB and DIB genes are tightly linked; and DYA, DOB and DIB are found only in bovids. Ballingall et al. (2004a) suggests renaming BoLA-DIB to BoLA-DYB due to its characteristics. The class II region of BoLA has a number of unusual features, including separation of the DR and DQ genes from the LMP2, DOA and DOB genes by a large recombination distance (reviewed by Lewin et al. 1999). The region containing the LMP2, DOA and DOB genes also includes the DYA and DIB genes (Stone and Muggli-Cockett 1990; van der Poel et al. 1990).

In sheep, a class II B gene, designated DYB, physically separated from the DYA gene by 11 kb, has been described (Wright et al. 1994). Ballingall et al. (2004a) suggests the DY molecule is similar to the classical class II MHC molecules in structure; however, DY is distributed non-classically and is non-polymorphic. Analysis of DYA and DYB transcripts revealed open reading frames with potential to translate 253 and 259 amino acid proteins, respectively. Comparative sequence analysis between the DY polypeptides and classical cattle, human and mouse class II MHC a and ß polypeptide chains revealed 16 unique amino acid residues at positions predicted to be the putative peptide-binding region. Expression analysis shows *DY* genes of cattle are capable of translating distinctive class II MHC a and ß polypeptide chains. This limited polymorphism and restricted pattern of transcription (Ballingall et al. 2001) is seen in non-classical class II MHC-DO genes (Alfonso and Karlsson 2000) expressing MHC proteins with highly specialised functions (reviewed by O'Callaghan and Bell 1998; Alfonso and Karlsson 2000). *DY* genes may also express a unique class II MHC molecule with a specialised function in ruminant DC (Ballingall et al. 2004a).

Kriener et al. (2001) in a study of DRB genes in humans and new world monkeys demonstrated that molecular convergence had shaped exon 2 evolution in anthropoid MHC-DRB genes, thereby providing evidence that DRB genes will continue to diversify over time, however over 47 myr (divergence of humans and new world monkeys) these

genes generate and maintain characteristic motifs. The authors suggest that repeated *de novo* generation of such motifs in mammals as diverse as rodents, ungulates and primates indicates that there are common features in the targets of the immune response in which DRB genes are involved.

In the class II region of the MHC of the domestic cat, the gene order is similar to, but distinct from, that of human and mice; and unlike HLA and H-2, there are multiple duplications of the DRA and DRB genes and a deleted DQ region (Beck et al. 2001). DRB sequences isolated from three exotic cats demonstrated close association with a particular domestic cat DRB lineage, suggesting that these allelic lineages are derived from common ancestral alleles that existed prior to the divergence of these feline species about 10 to 15 million years ago (Yuhki and O'Brien 1997). In the dog class II region (DLA) there is one complete DRB gene called DLA-DRB1 with at least 24 alleles and one full-length DQB gene, DLA-DQB1, with 20 alleles characterized to date, whereas the DLA-DQA is less polymorphic with nine alleles and DLA-DRA appears monomorphic (Wagner et al. 1999).

The swine MHC (SLA) class II genes code for at least one functional SLA-DR and one SLA-DQ heterodimer product, but there are no DP encoding genes. The amino acid alignment of the variable domains of 33 SLA classical class I chains, and 62 DR beta and 20 DQ beta chains confirmed the exceptionally polymorphic pattern of these polypeptides. Among the class II genes, the genes are either monomorphic, like the DRA gene, or oligomorphic, like the DQA genes. In contrast, the DRB and DQB genes display considerable polymorphism, which seems greater in DRB than DQB genes (Chardon et al. 1999b).

In the sheep MHC, DR and DQ exhibits polymorphism as well (Amills et al. 1998). However in contrast to humans, high levels of polymorphism are observed in the DQ subregion (Escayg et al. 1997) and there is no functional DP subregion (Scott et al. 1991; Deverson et al. 1991), suggesting DQ is important for antigen (Ag) presentation (Escayg et al. 1997). In cattle, it has been shown that DQ molecules present Ag to $CD4^+$ T cells and the inter-haplotype pairing of DQA and DQB molecules forms functional

restriction elements (Glass et al. 2000). There is up to two DQA2 loci in sheep with expression of up to 23 alleles (Hickford et al. 2004). Phylogenetic analysis by Hickford et al. (2000; 2004) revealed that some of the DQA2 sequences were more closely related to cattle DQA3 or DQA4 than to the rest of the sheep DQA2 sequences. In cattle, variation in the number of DQA loci has also been described (Ellis and Ballingall 1999).

The clustering of sheep sequences with similar cattle sequences was observed for DQA2 and DQA2-like (Hickford et al. 2004) as well as DQA1 loci (Zhou and Hickford 2004). Partial genomic sequence analysis of exons 2, 3 and 4 of DYA and DIB genes suggests that no obvious orthologues are present within the human or mouse MHC (Stone and Muggli-Cockett 1990). However, orthologous genes have been described within the MHC of sheep (Wright et al. 1994) and goat (Mann et al. 1993), suggesting that they may be restricted to ruminants.

This supports the trans-species hypothesis (Klein 1987), suggesting trans-species evolution may be common for the MHC genes. Such sequences may be derived from primordial sequences that were present in a common ancestor and have persisted in the sheep and cattle populations since their divergence. This pattern of evolution suggests the action of prolonged natural selection on the DQA genes because neutral polymorphism is not expected to persist very long in a population (Hughes and Yeager 1998a). Pathogen recognition may provide selection pressure for maintaining particular MHC sequences, and the observation that ruminants share similar allelic sequences may be evidence of the need for a specific immune response to a common pathogen.

Orthologous class II genes in mammals are generally conserved, however the number of alpha and beta genes in the loci are variable dependent on species-species local duplication events. In a recent study of monotreme class II ß sequences, Belov et al. (2003) showed non-orthologous relationships with marsupial and eutherian ß chain clusters and through maximum likelihood analysis placed these as the ancestral status at the base of the mammalian clade. However, this too may be refuted, due to inconsistencies in methods for determining order of gene duplications that created present-day gene clusters.

The role of the major histocompatibility complex in immunity

In addition to its role in transplant rejection, more than 100 different autoimmune and infectious diseases have been associated with the MHC in different vertebrate species (da Silva 2003; Escayg et al. 1997; Gelder et al. 2003; Kaufman 2000; Keet et al. 1999; Kulski et al. 2002b; Pagany et al. 2003; Park et al. 2004b; Sharif et al. 1999). The MHC has also been reported to be linked to an organism's response to vaccination (Ballingall et al. 2004b; Gelder et al. 2002) and play a role in resistance to disease (Ballingall et al. 2004b; Escayg et al. 1997; Lewin 1989; Pagany et al. 2003). In chickens for example, a species with the minimum essential MHC, many studies show that the B region confers striking resistance or susceptibility to Marek's disease virus (MDV), an oncogenic a-herpesvirus (Schat 1987; Schat et al. 1994).

In humans, the MHC has been linked to most autoimmune conditions, including multiple sclerosis (O'Connor et al. 2001) and systemic lupus erythematosis (Truedsson et al. 1995) as well as non-immune diseases like narcolepsy and cancer, however it has been difficult to pinpoint relationships between disease conditions and specific alleles (Beck and Trowsdale 2000). In some cases, however, it is clear certain alleles are associated with resistance to disease like HLA-B*5301 and malaria (Hill 1996); or both like HLA-DQ*0602 conferring strong susceptibility to narcolepsy but dominant protection against type 1 diabetes (Siebold et al. 2004). Susceptibility or resistance to HIV infection is also seen in people with certain HLA alleles (Beck and Trowsdale 2000; Mallal et al. 2002; Moore et al. 2002).

An immune response is initiated by the mammalian MHC class I and class II-encoded cell surface proteins that function as antigen-presenting receptors for surveillance by cytotoxic and helper T lymphocytes, respectively. The interaction of T lymphocytes with appropriate antigen presenting cells represents the first step in clonal selection and differentiation of immune cells. Thus the genetic processes involved in antigen presentation are fundamental to understanding the role of genetics in animal

health and disease resistance. The major classes of peptide receptors encoded for by the MHC are the class I and class II molecules.

Class I molecules are recognized by CD8+ cytotoxic T cells (CTLs) and are expressed on the cell surface of nearly all nucleated mammalian cell types; while class II molecules are only expressed on the surface of antigen presenting cells (APC) and recognized by CD4+ helper (Th) cells. MHC class II molecules display exogenous antigens, and generally MHC class I molecules present endogenous peptides at the cell surface. However there is evidence of cross-priming as an alternative way for class I peptide loading, thus allowing for a phagosome to cytosol pathway for MHC class I-restricted presentation of exogenous antigens (Gromme and Neefjes 2002), thus the segregation between the two pathways is not absolute and is another means to provide protective immunity (See Figure 4). It has also been shown that class I molecules can also interact with NK cells to prevent NK mediated cell lysis (Mandelboim et al. 1997; Reyburn et al. 1997a; Reyburn et al. 1997b; Vales-Gomez et al. 2000).

The functions of some of the non-*HLA* framework genes in the class I region are related to cell growth, DNA replication and repair, and regulation of transcription (Shiina 1999c; Shiina 1999d). Using microsatellite markers within MHC, the *HLA* class I and non-HLA framework gene region around the ß block has been associated with Behçet's disease (Ota et al. 1999), psoriasis vulgaris (Oka et al. 1999), diffuse panbronchiolitis, rheumatoid arthritis (Ota et al. 2001) and nonmelanoma skin cancer (Oka et al. 2003). Conserved class I framework genes such as *S* (*CDSN*), *SPR1*, *SEEK1* and *STG*, are expressed specifically in skin tissue and may have important roles in psoriasis and skin cancer (Kulski et al. 2002a). Immunity-related functions of other HLA class I conserved framework genes remain somewhat uncharacterized.

In cattle, susceptibility to mastitis has been associated with major histocompatibility complex (MHC) haplotypes that have only a single set of DQ genes (Park et al. 2004b). However, in an alternative study by Sharif et al. (1998) BoLA associations with occurrence of disease in Holsteins showed a significant (P < or = 0.05) association between BoLA-DRB3.2*23 and occurrence of severe mastitis (of which

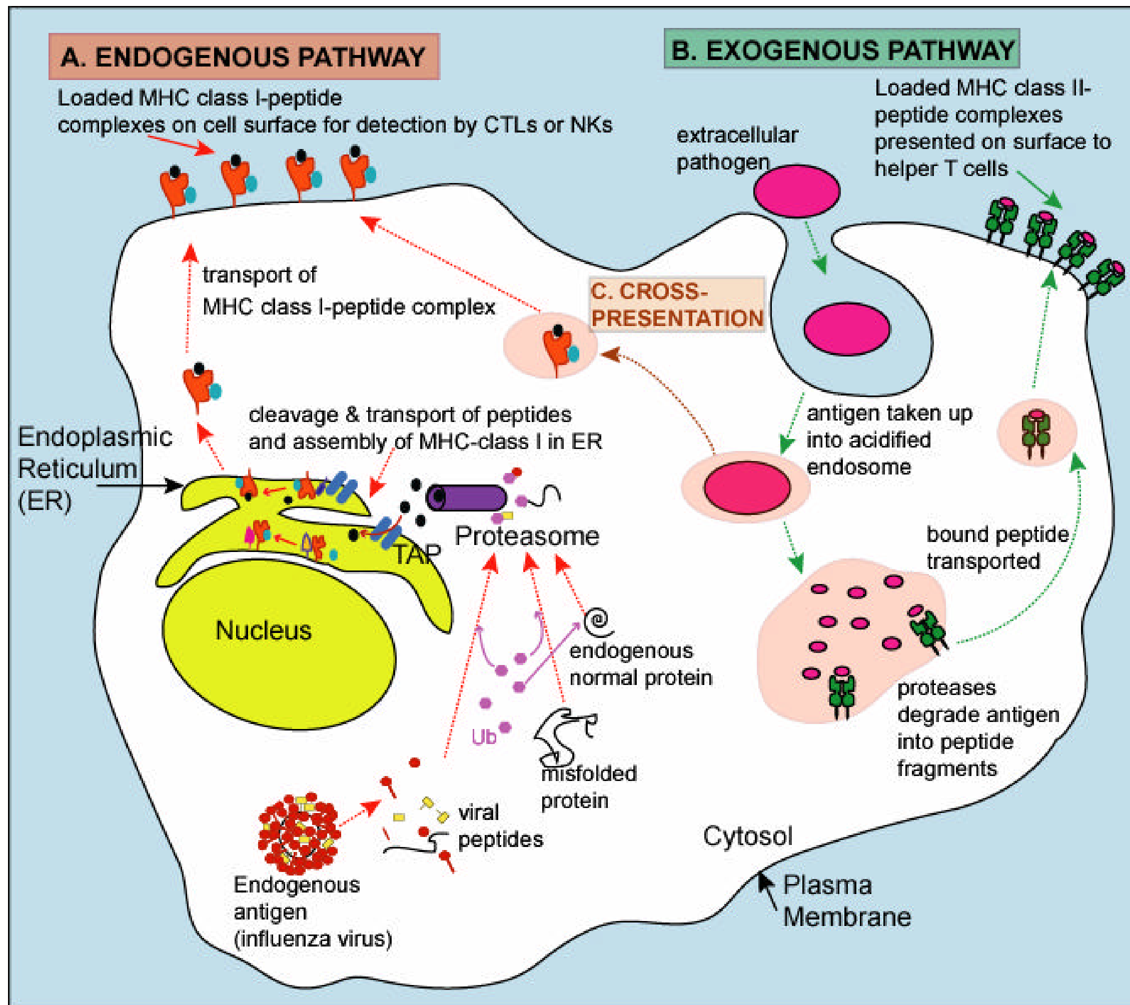


Figure 4. Pathways to antigen presentation by MHC molecules. A. Class I binds endogenous proteins (misfolded or normal self and/or foreign) that are ubiquinated (Ub), subsequently cleaved by the proteosome, enters via TAP into ER for assembly helped by chaperones, then presented at the cell surface to cytotoxic T lymphocytes (CTLs) or natural killer cells (NKs). **B.** Exogenous pathway allows MHC class II peptide complexes to be loaded. C. Cross-presentation can occur however, where exogenously derived proteins enclosed in endo/phagosomes can be accessed by MHC class I for antigen presentation.

coliforms was the most often isolated bacteria). The BoLA allele *3 was also associated with a lower risk of retained placenta (P < or = 0.05) and alleles *16 (P < or = 0.05) and *22 (P < or = 0.05) with a lower risk of cystic ovarian disease (Sharif et al. 1998). In a later study this group defined the presence of glutamine at position 74 of pocket 4 in the BoLA-DR antigen binding groove as associated with occurrence of clinical mastitis caused by Staphylococcus species (Sharif et al. 2000). BoLA DRB3*2703 and DRB3*1501 alleles have also been associated with variation in levels of protection

against *Theileria parva* challenge following immunization (Ballingall et al. 2004b). It is thus clear that a combination of DQ and/or DR alleles may offer protection against certain bacterial pathogens, dependent on the haplotype and disease state of the affected animal.

The evolution of MHC diversity and adaptive immunity is also related to fitness and survival of the host and its progeny in terms of mating preferences, chemosensory identity and reproductive mechanisms using both the MHC genes and a cluster of olfactory receptor (OR) genes that are in close proximity to the MHC (Potts 2002). Ziegler et al. (2002) proposed a MHC dependent selection of OR-bearing sperm adding another barrier in addition to a possible olfaction-driven mate choice, in favoring both avoidance of inbreeding and MHC heterozygosity.

Structure of the class I MHC molecule

Class Ia

Class Ia MHC molecules contain two separate polypeptide chains: a MHC-encoded alpha “heavy” chain (43 kDa) and a non-MHC-encoded beta chain ($ß_2$ microglobulin) (12 kDa). The antigen recognition sites (ARS) or peptide-binding region is a groove formed from the a_1 and a_2 regions which interact to form a "floor" of an 8-stranded, beta-pleated sheet with two opposite "walls" consisting of parallel strands of an alpha-helix (a_1 and a_2 each contribute 4 strands of beta-pleated sheet and one alpha-helix). A peptide about 8-10 amino acids long sits in the groove. Most of these peptides found on MHC class I molecules are of host cell origin generated from the cleavage of malfolded proteins or defective ribosomal products (DRiPs) (Princiotta et al. 2003). However, endogenous polypeptides from the host cell, viral and/or intracellular bacteria synthesized on the cell’s ribosomes are presented by MHC class I. Classically, endogenous peptides are ubiquinated and subject to proteasomal degradation (Figure 4), then transported into the lumen of the endoplasmic reticulum (ER) by the transporter

associated with antigen presentation or TAP. Loading of the resulting peptides involves a calnexin, calreticulin and tapasin loading complex, along with an association with $ß_2$m.

Following biosynthesis in the endoplasmic reticulum (ER) membrane, MHC class I heavy chains associate with the soluble, small subunit $ß_2$-microglobulin ($ß_2$m) and antigenic peptides of 8–11 amino acids. The assembly of these heterotrimeric complexes is facilitated by the lectin-like chaperones calnexin and calreticulin, the thiol-dependent oxidoreductase ERp57, and by the specialized chaperone tapasin that transiently bridges MHC-I to the TAP peptide transporter (reviewed by Cresswell et al. 1999; Momburg and Hengel 2002).

Cross-presentation of exogenous antigen is also facilitated by MHC class I molecules loaded in phagosome compartments or macropinocytic vesicles once the antigen is internalized through mechanisms of phagocytosis, receptor-mediated endocytosis, pinocytosis and macropinocytosis (Norbury et al. 1995; Fonteneau et al. 2003) (See Figure 5). It has been showed that exogenous proteins could escape from phagosomes and enter the cytosol (Guermonprez et al. 2003). Also cross-presentation involves transport of exogenous proteins from the phagosome into the cytosol, where these could join the pathway used to process endogenous antigens. This was supported by data showing cross-presentation depends on two components of the MHC class I pathway — the proteasome complex and the 'transporter associated with antigen presentation' (TAP). The proteasome complex degrades proteins in the cytosol and TAP shuttles the resulting peptide antigens into the ER for loading onto MHC class I molecules. After exogenous proteins are exported from the phagosome through the Sec61 channel, they are degraded by the proteasome and resulting peptide antigens are shuttled back into the phagosome by TAP and loaded onto MHC class I molecules on the inside of the phagosome membrane (Houde 2003).

Cross-presentation in dendritic cells occurs in a specialized, self-sufficient, ER–phagosome mix compartment (Guermonprez et al. 2003) confirming earlier observations that the phagosome is a fully competent antigen-processing compartment for the MHC class I pathway (Schmitz et al. 2000). If TAP is blocked in dendritic cells there is a 60%

decrease of MHC class I expression on the surface of dendritic cells (DCs) which is not seen in macrophages or lymphocytes. Gagnon et al. (2002) showed that phagosomes are wrapped in ER membrane, allowing ER-mediated phagocytosis and loading of MHC class I with normal machinery (TAP, Tapasin, Eh65) (Figure 5D).

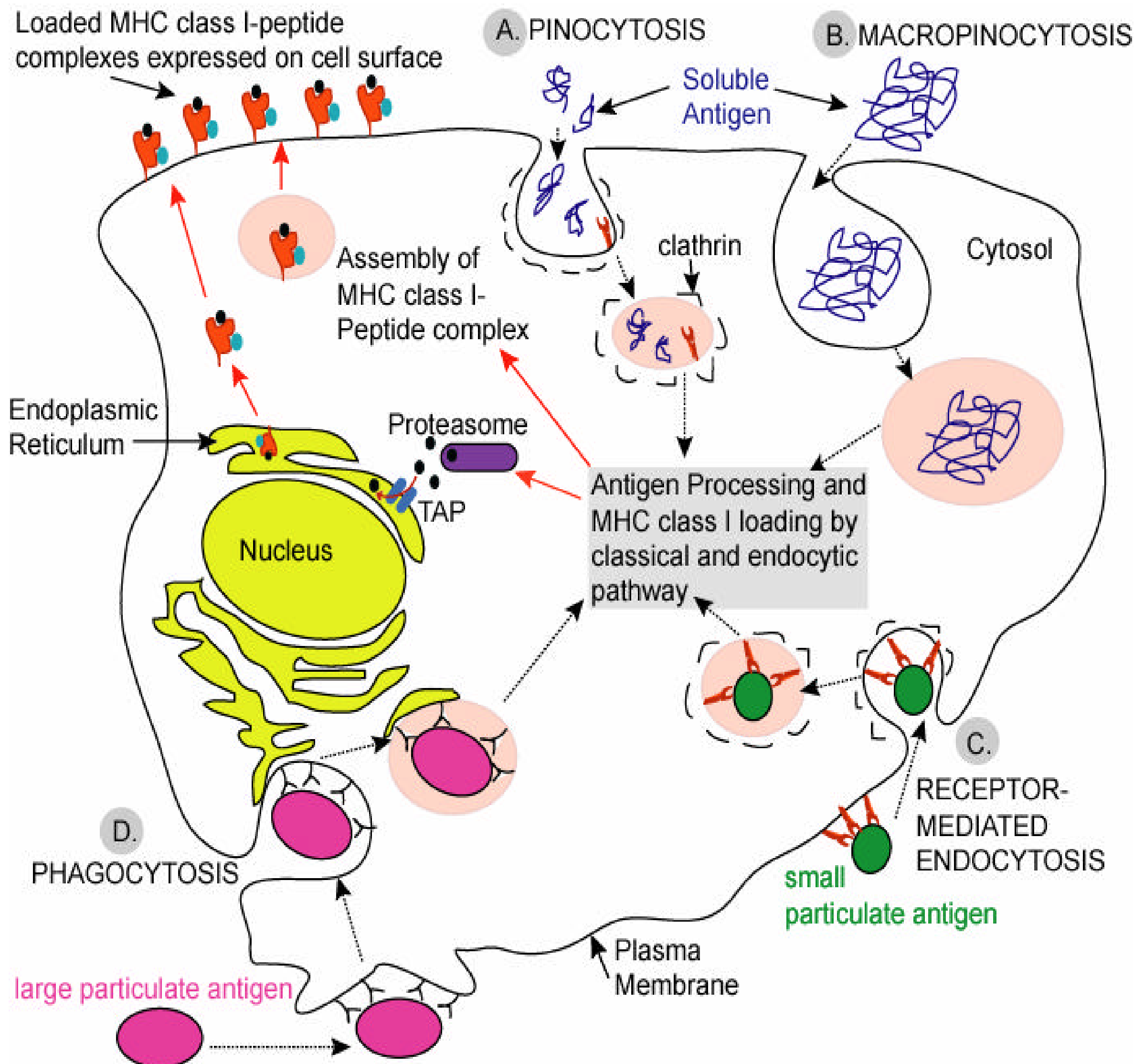


Figure 5. Additional mechanisms of exogenous antigen processing and MHC class I loading. A Pinocytosis and **B.** Macropinocytosis of soluble antigen; **C.** Receptor-mediated endocytosis of small particulate antigen; **D.** Phagocytosis of large particulate antigen allowing ER-mediated phagocytosis. These are all alternative mechanisms by which MHC class I molecules can access exogenous antigen for processing and loading by the classical and endocytic pathways.

The class Ia loci or classical class I loci are typically highly polymorphic; they are expressed in all nucleated somatic cells and function to present peptides to cytotoxic T cells. The class Ia loci are characterized by an enhanced rate of nonsynonymous nucleotide substitution in the ARS codons, indicating that polymorphism at these loci is selectively maintained. For any given MHC molecule, binding of a peptide usually requires the peptide to have one or more specific amino acids at a fixed position, frequently the terminal or penultimate amino acid of the peptide. Binding of the specific amino acid in the groove of the MHC molecule occurs in the anchor site(s). The other amino acids can be variable so that each MHC molecule can bind many different peptides. Other polymorphic residues of the MHC molecule are those in contact with the T cell receptor (TCR), which interacts with both peptide and the MHC molecule itself.

The a_3 segment that is highly conserved and is homologous to Ig constant domains is non-covalently bound to $ß_2$ microglobulin, an invariant molecule (non-encoded by MHC genes), also homologous to Ig constant domains. These two interact with a_1 and a_2 to maintain their proper conformation. The importance of the highly conserved region of a_3 is that CD8, a molecule expressed on cytolytic T cells that recognize class I MHC molecules, binds to this region. There is also a transmembrane region consisting of a stretch of ~25 hydrophobic amino acids and a cytoplasmic region (30aa) with a carboxyl terminal group containing phosphorylation sites and provides binding sites for cytoskeletal elements. (See Figure 6). The greatest variability in amino acids occurs in the a_1 and a_2 sequences forming the groove or cleft that interacts with amino acids in the peptide fragment (Bluestone et al. 1992). Thus, the polymorphism among class I MHC gene products creates variation in the chemical surface of the peptide-binding groove formed by the antigen-recognition sites (ARS) of a_1 and a_2 regions of the class I molecule.

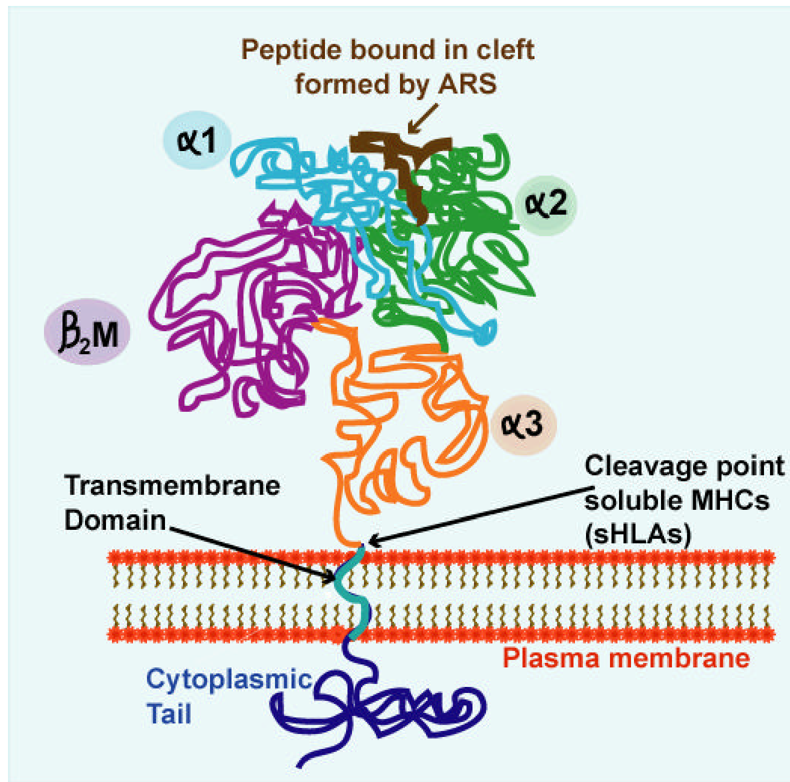


Figure 6. Structure of the MHC class I molecule. The alpha 1, alpha 2 and alpha 3 domains encoded by exons 2, 3 and 4 respectively, form a globular complex that is closely associated with the non-MHC encoded ß-2 microglobulin. The alloantigenic or antigen recognition sites (ARS) sites are found in the a1 and a2 domains, while the a3 domain is highly conserved. Exon 5 encodes the transmembrane region (TM) that is buried in the phospholipid bilayer of the plasma membrane. The cytoplasmic tail, sticks out into the polar environment is encoded by exons 6-8 of the MHC class I gene. Cleavage at the TM produces soluble MHC class I molecules (sHLAs).

Class Ib

The widely expressed, highly polymorphic class Ia molecules perform the well described antigen-presenting function to $CD8^+$ T-lymphocytes, but there are certain loci characterized by tissue-restricted expression, low polymorphism and proteic isoforms; known as class Ib or nonclassical class I loci (Hughes and Nei 1993). The function of class Ib genes has been debated due to their variation from Ia genes. The class Ib loci do not have an enhanced rate of nonsynonymous substitutions in the PBR codons; thus what little polymorphism is seen at these loci appears to be neutral polymorphism (Hughes

and Nei 1989). However, since they are less polymorphic they can associate with receptors that are less prone to genetic variation (Braud et al. 1999).

Natural killer (NK) cells are an essential component of the innate immunity toward tumors and virally infected cells. While the activity of NK cells is regulated by signals from activating and inhibitory receptors, the function of cytotoxic T cells requires engagement of the clonotype-specific TCR by cognate Ag (Lanier 2000). Despite this fundamental difference, NK cells and CTLs share receptor/ligand systems involved in initiation and regulation of cellular immunity. These signals are mediated by NK cell receptors that bind either classical MHC class I molecules or their structural relatives such as non-classical MICA, ULBP, RAE-1, and H-60. Two separate families of NK cell receptors have been identified: the immunoglobulin-like family (KIR, LIR) and C-type lectin-like family (Ly49, NKG2D, and CD94/NKG2). The latter, Ly49 C-type lectin-like proteins have been shown to interact with MHC class I molecules as determined by co-crystal structure of Ly49A/H-2Dd and Ly49C/H-2Kb (Dimasi et al. 2004) .

One mouse nonclassical class Ib gene encodes the protein Qa-1, the dominant ligand for CD94/NKG2 signaling receptors, which are expressed on a large fraction of NK cells and a subpopulation of $CD8^+$ T cells (Moser et al. 2002; Vance et al. 1998). Expression of Qa-1/Qdm complexes on target cells inhibits killing by $CD94/NKG2A^+$ NK cells. The CD94/NKG2 MHC class Ib recognition system appears to have an ancient origin in evolution because a homologous system is present in humans (Kambayashi et al. 2004). Human CD94/NKG2 receptors recognize the HLA class Ib molecule HLA-E (Braud et al. 1998), which although not a clear homologue of Qa-1 based on overall amino acid sequence comparison, share substitutions at the same residues that allow similar selective binding to MHC class Ia leader derived peptides.

Associations of several class Ib molecules with ligands that are less prone to variation has been well-documented. H2-M3 selectively binds to peptides with N-formyl-methionine residues and HLA-E and its functional homologues in mouse (H2-Qa1) and rat (RT-BM1) has been shown to associate with sequences derived from MHC class Ia leader peptides (reviewed by Lau et al. 2003). NK cells recognize several HLA

class Ib molecules employing both immunoglobulin-like (Ig-like) and C-type lectin receptors. The CD94/NKG2 and NKG2D lectin-like molecules, respectively, interact with HLA-E and MICA; CD94/NKG2A functions as an inhibitory receptor, while CD94/NKG2C and NKG2D trigger NK cell activity. HLA-E predominantly presents nonamers from the leader sequences of other class I molecules (Lopez-Botet et al. 2000).

The HLA class Ib gene product HLA-G regulates immune responses as it binds different receptors expressed on NK cells, T cells and myeloid cells. HLA-G1 can inhibit NK- and T-cell-mediated lysis of target cells by its interaction with the inhibitory receptors ILT2 and ILT4. The indirect recognition of HLA-G as peptide presented by HLA-E and recognized by the CD94/NKG2 receptor family might help the immune system battle tumor cells (Hofmeister and Weiss 2003). A peptide derived from HLA-G1 constitutes the highest affinity ligand for both CD94/NKG2 receptors. In addition to their classical antigen-presenting role, HLA class I proteins are recognised by members of the killer immunoglobulin receptor (KIR) and leukocyte immunoglobulin-like receptor (LILR/ILT/LIR) families. Members of the Ig-like transcript (ILT) or leucocyte Ig-like receptor (LIR) family (ILT2 or LIR-1 and ILT4 or LIR-2), expressed by other leucocyte lineages, interact with a broad spectrum of HLA class Ia molecules and HLA-G1. Among Ig-like KIRs, the KIR2DL4 (p49) receptor has been shown to specifically recognize HLA-G1; this molecule displays an unusual hybrid structure, sharing features with inhibitory and triggering KIRs (Lopez-Botet et al. 2000).

The expression of HLA-G by invading trophoblasts suggests a role for this molecule in embryo implantation but this issue is under continuous debate. Recently, van de Meer et al. (2004) showed that HLA-G1^{+} APCs are immunoinhibitory cells that by their local action, might be involved in the suppression of immune responses and by their long-term effects, inefficient immune escape or tolerance. Depending on physiopathological status, HLA-G^{+} APCs might act either as a help to boost or be a threat to eliminate. Similar function for class Ib encoded molecules have not been found in other species to date.

It is widely believed that HLA-G and Qa-2 (its mouse homolog) protect the fetus from rejection by maternal natural killer cells during implantation and placentation (Comiskey et al. 2003; Warner et al. 2002), and are thought to be functional homologs as a result of convergent evolution (Beck and Trowsdale 2000). Recently, the mRNA expression pattern of the *Ped* gene in cattle was associated with embryos of higher quality in bovine preimplantation development, and was associated with differential expression of an MHC class I gene (422.1) (Fair et al. 2004). Since *Ped* is the gene product of Qa-2, it was concluded that a homolog of the murine and human *Ped*, a non-classical class Ib, is functional in bovids.

Class Ib molecules are thus recognized by a wide variety of receptors, not only aß TCRs, but ?d TCRs and a variety of inhibitory or activatory receptors present on NK cells, NKT cells and cells of the monocytic/macrophagic lineage (Allan et al. 1999; Colonna et al. 1998). It seems that while class Ia molecules inhibit NK lysis, class Ib molecules may have a more complex role in either activating or inhibiting an immune response.

Soluble MHC class I molecules

Soluble MHC class I molecules (sHLA) can be produced from proteolytic cleavage or by alternative splicing of several MHC class I genes. Up to 3 species of sHLA has been found in serum from normal humans, with mw of 44,000, 40,000, and 35–37,000 kDa (McDonald and Adamashvili 1998). The sHLA recovered at mw 44,000 disassociated into a 33,000 mw component which contained the HLA specificity and an 11,000 mw component which was $ß_2$m. It has been postulated that these sHLAs can play an immunomodulary role (McDonald and Adamashvili 1998).

In transplantation studies, sHLAs released from hepatocytes and the passenger leukocyte population of the liver allograft have been considered as important contributors for spontaneous liver tolerance upon allogeneic transplantation into fully MHC-mismatched hosts (Dresske et al. 2002). The mechanisms of this natural liver-related tolerance phenomenon have not been completely evaluated in detail. These

include the production of donor-strain soluble MHC class I antigens by the transplanted liver (Sriwatanawongsa et al. 1995). In a related study, Piazza and associates found that kidney transplant recipients whose serum contained anti-HLA antibody obscured by donor sHLA had the same incidence of acute rejection as those who had no anti-HLA antibody, while those who had antibody excess had almost five times the incidence of acute rejection (Piazza et al. 1993). However, it is necessary to distinguish sHLA of donor origin from that of recipient origin and studies are largely observational, perhaps because of the difficulty in studying soluble allotypes without specific antibodies.

Because of the statistically significant association with clinical parameters, the level of sHLA-I antigens has been suggested to represent a useful marker to predict the evolution of viral infections and to monitor the clinical course of allografts. Elevated levels of functional sHLA-I and soluble Fas-ligand molecules have been detected in blood components and are postulated to play a role in the immunomodulatory effect of autologous and allogeneic transfusions (Zavazava 1998).

In humans, sHLA levels in autoimmune disease, cancer and viral infections have shown a correlation of elevated sHLA levels with disease activity in part due to its interference with NK and CTL activity (Bresciani et al. 1998; Contini et al. 2000; Filaci et al. 1997; Filaci et al. 1995; Ghio et al. 1999; Ghio et al. 2000; Migliaresi et al. 2000; Puppo et al. 2000; Puppo et al. 1998). Several soluble forms of HLA encoded molecules are also noted to have effects in viral and autoimmune disease. In patients with ankylosing spondylitis, there was a high level of surface expression of free heavy chains in monocytes and surface free heavy chains of HLA-B27 are considered as potential disease-causing molecules (Tsai et al. 2002). HLA-G harbors several structural characteristics, including the transcription of different membrane-bound and soluble isoforms, a unique promoter region and a truncated cytoplasmic tail. HLA-G molecules bind intracellular processed nonamer peptides (Diehl et al. 1996; Ishitani et al. 2003; Lee et al. 1995) and can serve as a restriction element for HCMV-derived peptides in transgenic mice (Lenfant et al. 2003), suggesting that HLA-G may play a role in the immune response against viral infections in the absence of classical MHC molecules.

HLA-G is also the ligand of several triggering or inhibitory receptors on natural killer (NK) cells (Lanier 1999) that could protect trophoblast cells from maternal NK lysis and/or also promote cytokine secretion important for the placentation (Croy et al. 2003). Secreted HLA-G5 can also bind CD8 and induces Fas/Fas ligand-mediated apoptosis in activated CD8+ lymphocytes (Hofmeister and Weiss 2003).

When the MHC class I heavy chains (HC) are unable to assemble properly (absence of $ß_2$m or peptides) misfolded HCs are removed from the ER by dislocation to the cytosol where they are subsequently degraded by the proteasome (Hughes and Yeager 1997). This physiological process is co-opted by the human cytomegaloviral proteins US2 and US11 that target HC for ER dislocation probably in order to subvert antiviral CTL responses (Wiertz et al. 1996a; Wiertz et al. 1996b). At this point the cleaved HC is treated like many other secreted or membrane-integrated ER proteins (reviewed by Tsai et al. 2002), and dislocation of misfolded HC involves the translocon as indicated by co-precipitation of HC en route to degradation with the translocon subunit Sec61 (Wiertz et al. 1996a).

For example, sera of leukemia patients, but not of healthy donors, contained elevated levels of soluble MICA (sMICA) and reduction of leukemia MIC surface expression by shedding may impair NKG2D-mediated immune surveillance of leukemias; so much so that determination of sMICA and sMICB levels may be implemented as a prognostic parameter in patients with hematopoietic malignancies (Salih et al. 2003) There is also a significant correlation of MIC heavy chain shedding and deficiency in NK cell function with the grade of disease in prostate cancer (Wu et al. 2004).

Unusual properties of HLA-B27 include an ability of free heavy chains (FHC) to reach the cell surface in the absence of $ß_2$m and to maintain their peptide-binding groove in vitro (Allen and Trowsdale 2004). The recognition of HLA-B27 in both the classical $ß_2$m- associated and $ß_2$m- independent forms by members of the KIR and LILR families could influence the function of cells from both innate and adaptive immune systems, and

may indicate a role for various leukocyte populations in HLA-B27-associated inflammatory disease.

There is a body of work which establishes that the MHC determines the unique odor of each individual. Kalmus (1955) observed that dogs had difficulty distinguishing odors of identical twins. Subsequently, the association between the MHC-linked OR region in determining body odour associated with mate selection,, familial relatedness and maternal protection was defined in mice and humans (Amadou et al. 2003; Eggert et al. 1998a; Eggert et al. 1998b; Hurst et al. 2001; Younger et al. 2001; Ziegler et al. 2002) It seems likely that these odortypes are determined by soluble MHC antigens or their catabolic products, but this remains unproven.

Pseudogenes

The class I and II regions contain many pseudogenes, with more than half of the genes in the class I thought to be nonfunctional. Novel gene families in both regions seemed to have been created by duplication events followed by divergence (Shiina 1999a). Pseudogenes may not be redundant if they played a role in generating new alleles by gene conversion. The HLA class III region is extremely gene dense with an expressed gene every 15 kb, and contains no pseudogenes except in haplotypes with C4 duplicated regions in its span of 800kb (The MHC Consortium 1999).

Although it was thought that pseudogenes are genomic fossils or defunct remnants of previously functional genes, a functional role for the *makorin*1 pseudogene "noncoding" RNA has been reported (Hirotsune 2003). Although 2-3% of human processed pseudogenes are expressed, interestingly, the mouse has a much smaller proportion of expressed pseudogenes (0.5-1%) (Yano et al. 2004). Since pseudogenes are functionally less constrained, and have accumulated more mutations than translated genes which allows for more rapid functional diversification than protein-coding genes in gene regulation.

Pseudogene sequences are not translated to protein as a result of the presence of stop codons, alterations in RNA splicing signals, or the loss of one or more exons. In the

HLA, the HLA-BEL and HLA-Y pseudogenes lack all of exon 3 because of a recombination event fusing introns 2 and 3 (Williams et al. 1999). HLA-COQ and HLA-DEL are two other HLA-A associated pseudogenes with sequence homology to both gorilla and human MHCs (Coquillard et al. 2004). This dual species sequence homology to human and other primates is an unusual genetic characteristic, indicating a trans-species polymorphism within pseudogenes that may be found in the MHC class I genes of other closely related species.

Evolution of the MHC

Origin of the MHC and putative ancestral "proto-MHC": evidence from paralogous and orthologous regions

The components of the adaptive immune system, including the MHC, appeared early in vertebrate development, but have not been found in any invertebrates. The exact order and time frame of events that shaped the MHC are still being debated, however the genomes of all jawed vertebrates contain MHC class I/II, TCR (T cell receptor) and Ig (immunoglobulin) genes leading to the assumption that the MHC developed after the evolutionary split of jawed and jawless vertebrates (Kumanovics et al. 2003). Many highly conserved anchor genes in the MHC class III region such as NOTCH 4, C4A and B, tenascin, HSP70, BAT2, TNF family (LTA, LTB, TNFa) have paralogous members on different chromosomes in humans (found on chromosomes 6, 1, 9, and 19 in humans) (Flajnik and Kasahara 2001), and synteny in mice, amphioxus and other species (Kulski et al. 2002b).

The core of conserved synteny in jawed vertebrates supports the theory of evolution by gene duplication first proposed by Susumu Ohno (1968) and later revisited (Ohno 1999; Wolfe 2001), supports the existence of a "proto-MHC" in two ways. First, there is evidence that several MHC genes (TUBB, TNXB, PBX2, NOTCH4, RXRB and RPS18) form syntenic conserved blocks (without any class I/II genes) in amphioxus (a protochordate) (Abi-Rached et al. 2002), as well as in *C. elegans* and *Drosophila*

(invertebrates) (Trachtulec et al. 1997). Secondly, Danchin et al. (2003) confirmed the existence of a region of conserved synteny between the human MHC and a paralogous region of 19 conserved genes in a Drosophila genomic region of less than 2 Mb.

These paralogous duplications may have resulted from either 'block' or genome duplication events prior to and after the emergence of cartilaginous fishes. Duplicates of VARS2, PBX2, NOTCH4, TAP2, PSMB X, RING3, COLIIA2 and RXRB can be found on both 6p21.3 and 9q33-34, while HSPA1, C4 and TNX are inversely duplicated within these same two chromosomal regions (Dawkins et al. 1999; Inoko et al. 1996; Kasahara 1997; Shiina 1999c). Some related regions are found at all four locations and to date, 30 gene families have been found in two, three or four of the locations (Kasahara 1999). MHC class I, II, tapasin, RAGE, BTN and RFB30 share common ancestors as evidence by a common IgC1 domain, and were probably derived from these ancestors by cis-duplication and shuffling (Abi-Rached et al. 1999).

The MHC paralogous regions have several framework genes as well as complement and cytokine genes and even a group of MHC class I-like genes with specific function (the CD1 cluster on chromosome 1) which are representative of the ancient set of genes present in the proto-MHC (Friedman and Hughes 2001). Abi-Rached et al. (2002) identified 9 anchor genes in the paralogous human MHC regions in *Amphioxus* . These syntenies were also reported for *Fugu*, zebrafish and trout (Clark et al. 2001; Gongora et al. 1998; Hansen et al. 1999; Takami et al. 1997). Of note is that one of the MHC paralogous regions (9q33-q34) retained the ancestral state in gene organization, while the other paralogous regions on chromosomes 1 and 19 seem to exhibit a substitution pattern that is under negative selection (Abi-Rached et al. 2002; Vienne et al. 2003).

It is possible that the full-fledged adaptive immune system emerged only after chromosomal duplication. Also, clustering of genes in an ancestral syntenic group and linkage of MHC-encoded genes with copies in the paralogous regions may have occurred by chance. It is also possible that linkage of such genes by chance could have later allowed for selective advantages (e.g. linkage of LMP/PSMB7 to the MHC). No

class II gene has been found to date outside the MHC thus, these genes may have arose out of a duplication event of class I genes within the MHC after chromosomal duplication. Alternatively, these could be either lost from the paralogous region while the class I paralogons were maintained, or alternatively not been located to date. Phylogenetic and genomic analysis of the cephalochordate, amphioxus (Abi-Rached et al. 2002), and other vertebrate species (Pennisi 2001) supports the latter view.

A different hypothesis proposes that the three major paralogous regions were not brought together instantaneously but occurred independently during different evolutionary periods by translocations, duplications and selective forces (Hughes and Yeager 1998b). Despite ongoing debate, the fact remains that by chance or not, study of these MHC paralogous regions would help elucidate aspects of genome organization and evolution.

Linkage of Class I, II and III regions in the ancestral MHC

In the zebrafish, a representative of the teleost fishes, the class II loci are divided between two linkage groups which are distinct from the class I loci. The beta2-microglobulin-encoding gene is loosely linked to one of the class II loci. The gene coding for complement factor B, which is one of the region III genes in mammals, is linked neither to the class I nor to the class II loci in the zebrafish (Murray et al. 1999). This data suggests that the class I and class II regions in another order of teleost fish are also in different linkage groups, indicating that close linkage of the two regions is not necessary either for regulation of expression or for co-evolution of the class I and class II loci. The separation of the class II from class I loci in teleosts is thought to have occurred by translocation rather than by genomic or chromosomal duplication (Kuroda et al. 2002).

More evidence of a core MHC region identifiable within the teleosts comes from zebrafish (Sultmann et al. 2000), fugu (Clark et al. 2001, Sambrook et al. 2002), medaka (Matsuo et al. 2002) and most recently catfish (Quiniou et al. 2003) and rainbow trout (Phillips et al. 2003) which have linked class Ia and Ib regions, an unlinked class II

region, and the class III genes dispersed on several chromosomes. In sharks, the most ancient investigated species with a complete MHC, the class I and II genes are linked (Ohta et al. 2000), so perhaps the separation seen in bony fish is a derived characteristic.

Evidence for ancient linkage can be seen in *Xenopus* where class I and II are linked to PSMB7 (LMP7), and also in chicken where the class I, II and III regions of the MHC resides in a microchromosome together with a nucleolar organizer (Ohno 1999). *C. elegans* has no MHC class I or II genes, but seems to have a region that resembles the class III (Trachtulec et al. 1997) which suggests, contrary to the view of Klein et al. (1993b) that genes in the class III were entrapped by chance between classes I and II, and that genes in the class III were the original inhabitants of the chromosomal region that became the MHC.

The shark is a cartilaginous fish and the oldest living animal lineage with an ancestral MHC/T-cell receptor recognition system (Flajnik et al. 1999). This adaptive immunity system includes insertion of a transposon leading to evolution of a repertoire of immunoglobulin and T-cell receptor genes through the transpositions mediated by the RAG1 and RAG2 recombinase enzymes. The 'jawed vertebrate' hypothesis proposes that the adaptive immune recognition system and the more specialized innate systems (NK cells, complement factors Bf and C2) co-evolved in the gastrointestinal region of primitive jawed vertebrates to defend against pathogens introduced by the predatory lifestyle (Matsunaga and Rahman 1998).

Recently a jawless vertebrate, the sea lamprey, has been found to have a new type of variable lymphocyte receptor (VLR) gene expressed in a monoallelic fashion on individual lymphocytes. This was probably an alternative evolutionary strategy generating diverse lymphocyte receptors to the rearrangement of immunoglobulin gene segments seen in jawed vertebrates (Pancer et al. 2004).

In swine, the major histocompatibility complex (MHC) or swine leukocyte antigen (SLA) is located on chromosome 7 and divided by the centromere (Chardon et al. 1999a), however the spatial relationships between the genes in the class II and class III regions, and between the well-conserved non-classical I genes of the class I region,

are similar to those found in the human, horse, rat, cat, mouse, cattle, chicken and quail MHC complex. This, along with phylogenetic and other data as described, supports the idea that the ancestral order is represented by class III intercalated between flanking class I and II regions.

Role of polymorphism within the MHC

The antigen recognition site (ARS) of the MHC molecules is usually the most polymorphic as it allows a population's immune response to be varied with animals having different antigen-binding sites available to a wide range of pathogens. Exons 2 (BoLA-class I, II) and 3 (BoLA-class I) are functionally important and encode amino acids associated with the ARS and account for a large part of the polymorphisms observed and a higher ratio of non-synonymous to synonymous substitutions in most species (Hughes and Nei 1989; Mikko et al. 1999; Sena et al. 2003). Polymorphism of the MHC in many species could be due to combinations of intraallelic and interallelic conversion, reciprocal recombination and mutation that was evolutionarily significant by positive selection of specific alleles for efficient presentation of pathogenic peptides to the immune system. For example, in the domestic cat, patterns of synonymous and nonsynonymous nucleotide substitution rates that occurred in ARSs and non-recognition (NAR) sites demonstrated a strong role of natural selection positive selection for ARSs and negative selection for NARs of feline DRB sequences, in the process of evolution of DR molecules (Yuhki and O'Brien 1997).

Protein sequences of the MHC molecules of different species seem to resemble one another more than the sequences coded by distinct alleles of the same species (Klein 1987). This is usually limited to the exons encoding the ARS region of the protein which is evolving under balancing selection (Hughes and Nei 1988; Jonsson et al. 1989) and in comparison of distantly related species (e.g. human, cattle and mice) the similarity is restricted to a few amino acid residues in the PBR (Andersson et al. 1991; Gustafsson et al. 1990; Kriener et al. 2000; Lundberg and McDevitt 1992). This trans-species polymorphism as mentioned previously has been documented in rodents (Figueroa et al.

1988), primates (Lawlor et al. 1988) and other vertebrates (Graser et al. 1996). This phenomenon has been suggested to be a result of either direct descent (Lundberg and McDevitt 1992), convergent evolution (Gustafsson and Andersson 1994) or by chance.

MHC polymorphism is generated initially by point mutations, then by gene conversion that shuffles short DNA sequences between alleles or loci (Parham 1989; Parham 1995; Ohta 1998). An important feature of the evolution of the MHC is that some allelic lineages are older than the species themselves as allelic lineages and loci are preserved during the evolutionary history of a particular taxon and may predate speciation (Figueroa et al. 1994; Kuhner et al. 1991; Lawlor et al. 1988; Lawlor et al. 1990; Zemmour et al. 1990). Duplication of functional genes may have allowed for mutations to take place in the non-functional duplicate at a rate consistent with evolutionary requirements based on selective pressures.

In humans, certain classic *MHC* genes (e.g., *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DR*, *HLA-DQ*) are among the most polymorphic in the genome (The MHC Sequencing Consortium 1999), and appear to be maintained by some form of balancing selection (Hughes and Nei 1988; Hughes and Nei 1989; Takahata and Nei 1990; Takahata et al. 1992). This elevated level of polymorphism is thought to be due to exposure to a large variety of pathogens, pathogenic mutability, and the result of co-evolutionary arms races between hosts and parasites.

The Red Queen Hypothesis (Penn and Potts 1999) proposes that an organism's immune defenses will have to constantly face evolving pathogens, thus the genetic diversity seen in the MHC was developed in part to deal with this constant race for domination. This is evident in studies on MHC class I evolution in great apes and Old World/New World primates and SIV, where the role of MHC class I alleles in selecting for new populations of viruses was seen (Vogel et al. 1999). The effect of rhesus MHC class I molecules on the evolution of SIV has also been described (O'Connor et al. 2003), and selection pressure conferred by HLA molecules is thought to be responsible for specific genetic variation in HIV-1 as reviewed by Carrington et al. (2002). These

studies provide evidence that pathogens co-evolve with the MHC class I molecules they encounter in a population.

A high diversity (2-7%) found in the classical class I region between the chimpanzee and human is due to balancing selection and a hitchhiking effect required for maintaining polymorphisms. Conversely, the low level of diversity (average nucleotide difference of 0.9%) found within the nonclassical *HLA-G* region (35 kb) for example, is due probably to functional constraints and negative selection acting to eliminate polymorphisms. The nonclassical *HLA-E* and *-F* regions were intermediate between *HLA-G* and the classical class I genes in sequence diversity (~ 2%). Human and chimpanzee MHC class I diversity profile resembles the peaks and troughs observed previously between different human genomic sequences of varying haplotypes (as reviewed by Kulski et al. 2002b).

Evolutionarily, class Ib loci are odd in that they do not show orthologous relationships between mammals of different orders, thus these loci may have arisen independently in different mammalian lineages (Hughes and Nei 1989). Class Ib loci may have evolved by duplication of class Ia loci followed by a change in expression pattern due to mutation in the promoter region, thus do not represent mutually exclusive groups over evolutionary time. This is apparent in that the class Ia genes of New World primates are homologous to the human class Ib locus HLA-G (Cadavid et al. 1997) and may be a locus in the common ancestor of New World monkeys and Old World monkeys, apes, and hominids, but in the former duplicated to form separate class Ia loci. In spite of the independent origin of class Ib loci in different mammals, there is evidence that they can evolve similar functions convergently. For example the class Ib HLA-E molecule of humans and other primates has convergently evolved features of the PBR that are similar to those of the mouse class Ib molecule H2-Qa-1a, and there is evidence that these molecules may bind similar peptides (Hughes and Yeager 1998b; Yeager et al. 1997).

Chardon et al. (2001) confirmed that all class I-related genes and two MIC genes between TNFA-BAT1 and POU5F1 genes in swine MHC (SLA), demonstrate an

organizational similarity to human and mouse MHC class I genes and class I-derived genes. In humans, this interval contained the classical functional HLA-B and HLA-C loci and the functional MICA and MICB loci (The MHC consortium 1999). In mice, this interval contained classical class Ia and class Ib genes but no MIC genes, which are not present in the mouse genome (Amadou et al. 1999). The SLA class Ia genes were located in a second orthologous swine segment which was also common to humans and mice and was located between the genes HSR1 and RBF30 (Chardon et al. 2001).

In humans, over 30 class I-related and MIC genes and fragments exist in a third segment situated between HTEX4 and MOG (kappa block). The corresponding orthologous region contains only 2 H2 class I-related genes in mice, and only 5 RT1 class I genes in rat (Hurt et al. 2004). No class I or MIC genes have been found in the interval between the TCTEX4 (orthologue of HTEX4) and MOG genes in pig (Renard et al. 2001) or in the preliminary data for the horse (Gustafson et al. 2003). The existence of class I and class I-related sequences in orthologous segments in distantly related species argues in favour of existence of common class I ancestors in these regions before mammal speciation. However, the differences in number of duplications and post-speciation recombination events clearly differs from species to species, with some class I genes heavily duplicated and others removed in the relative class I blocks.

More evidence comes from the introns flanking the polymorphic exons of the human MHC class I loci HLA-A, -B, and -C genes. These introns have been relatively conserved and have become locus-specific apparently as a result of recombination and subsequent genetic drift, leading to homogenization within loci over evolutionary time. Thus, HLA class I genes have been shaped by contrasting evolutionary forces maintaining polymorphism in the exons and leading to conservation in the introns (Cereb et al. 1997).

O'hUigin et al. (2000) postulated that balancing selection driving polymorphism at HLA-B is of sufficient magnitude to explain the nucleotide diversity found at the adjacent neutral regions. Evidence suggests that linkage to HLA-B is the main factor that maintains polymorphisms in adjacent regions and there is little or no evidence for any

strong influence of another locus under balancing selection in the interval. The extent of nucleotide diversity at these regions does not appear to be compatible with a high level of recombination at HLA-B (O'hUigin et al. 2000). The authors showed the effects of selection from HLA-B to TNF, some 200 kb away from HLA-B, where the diversity is double that found at other neutral loci.

The high degree of linkage disequilibrium seen in neutral polymorphisms appears to favor the view that HLA-B evolved in a manner reflecting "frozen haplotypes" rather than the recombination hotspot models (Klein 1991). This mode of evolution may be common among MHC loci. Preliminary data on class II polymorphisms indicate that at the HLA-DQB1 locus, as at the HLA-B locus, adjacent neutral regions show a higher than expected level of neutral polymorphism (Horton et al. 1998). According to this view, the MHC is evolving in a conserved fashion, without high levels of mutation or recombination, but simply by the accumulation of polymorphism over long time periods under balancing selection. It is more likely that various MHC loci are under different influences, the combination of which creates the diversity seen today.

Role of retroviral sequences

Human endogenous retroviral sequences (HERVs) are present in high density within polymorphic a and ß blocks of MHC (Kulski et al. 1999c). An ancient preduplication segment containing a HLA and PERB11 gene, an endogenous retrovirus (HERV-16), suggests that the latter have played a major role in duplication and indel events leading to the present organization of PERB11 and HLA class I genes. Examination of flanking sequences suggests that HERV-I and HERV-L had occurred by insertion into ancient L1 fragments. This study has revealed that the alpha- and beta-block region within the MHC is rich in HERV sequences occurring at a much higher ratio (10 to 1) than normally observed in the human genome (Kulski et al. 1999b). The P5-1 genomic sequence appears to be an example of an HERV within the MHC that expresses an antisense transcript with a possible role in immunity to retrovirus infection (Kulski et al. 1999a).

A coevolutionary model premised on tandem duplication of single and multipartite genomic segments has been used to explain the origins and genomic organization of retroelements, HERV-16, DNA transposons, PERB11, and HLA class I genes as distinct segmental combinations within the alpha- and beta-blocks of the human MHC (Kulski et al. 1999c). This model proposes that HERV's play a role in generating and localizing the process (Kulski et al. 1999b). This is functionally exemplified by large deletions within the HERV/PERV containing homologous HLA class 1 ß block in other species, for example there is only one single hybrid chimpanzee MIC gene (a mix of MICA and B HLA genes) in a segment of the MHC genetically linked to species-specific handling of several viral infections (HIV/SIV, hepatitis B and C) as well as susceptibility to various autoimmune diseases (Anzai et al. 2003).

Dawkins et al. (1999) proposes several models where homologous recombinations between two HERVs could result in deletion of intervening DNA and in the association of genes, or deletion/inversion of segments, or even reintegration of deleted segments and the HERV allowing for duplication. These models, although speculative, may corroborate with phenomena such as innate protection from re-infection with retroviruses, rapid development of protective polymorphism and even advantage/dominance of one species over the other due to its ability to survive infection.

Occurrence of MHC genes through "birth and death" process

These studies show that all jawed vertebrates possess MHC class I and II genes and that the function of the genes and conserved proteins seem to be conserved. However, this conservation remains despite the fact that there is no shared class I lineages among mammals for instance (except within the same orders) and that class II gene clusters (except DM genes) are not shared by mammals, birds, frogs and bony and cartilaginous fishes (Kumanovics et al. 2003). This is best explained by a single ancestral gene expanding through serial duplications or births, being changed by mutation and contracted by deletions or deaths; which is commonly called the "birth and death" process (Nei et al. 1997). The predecessor of a new lineage can be created by an

initial contraction leading to a single gene acting as an ancestor for subsequent expansions and contractions.

Birth and death, also called the accordion model of MHC evolution (Klein 1998), is supported by phylogenetic evidence of vertebrate class I and II genes as outlined in Nei et al. (1997). Birth and death evolution is a form of species-specific independent evolution and assumes that new genes are created by repeated gene duplication and divergence and that some duplicate genes may remain in the genome for a long time whereas others may be deleted or become non-functional pseudogenes. A number of authors have studied the evolution of multigene families from this perspective and have shown that this model of evolution can explain most multigene families concerned with immune and antigenic systems, including the MHC (Ota 1994).

Allelic lineages within the MHC

Insertions or deletions of DNA sequences are important genetic markers to define ancestral polymorphisms. Coupled with single nucleotide polymorphism (SNP) analysis, these tools have been used to assign allelic lineages and study evolutionary relationships. Chen et al. (1992) reported that the MHC class I (HLA) C locus is present only in humans and apes, generated by gene duplication in a common ancestor. While in the class II region of cattle and sheep, the gene cluster (DY/DI) is only observed in pecoran arteriodactyls (Trowsdale 1995). Also there is a chromosomal inversion within the class II region of the bovine MHC or BoLA (Band et al. 1998; McShane et al. 2001) (See figure 7). Kuroda et al. (2002) also reported a translocation event in Zebrafish as the hypothesized cause of the separation of class II from class I loci rather than genomic or chromosomal duplication.

The evolution of the MHC seems to be is subject to both trans-species polymorphism and convergent evolution. Some allelic lineages and loci are preserved during the evolutionary history of a particular taxon and may predate speciation (Figueroa et al. 1994; Lawlor et al. 1988; Zemmour et al. 1990). Duplication of functional genes may have allowed for mutations to take place in the non-functional

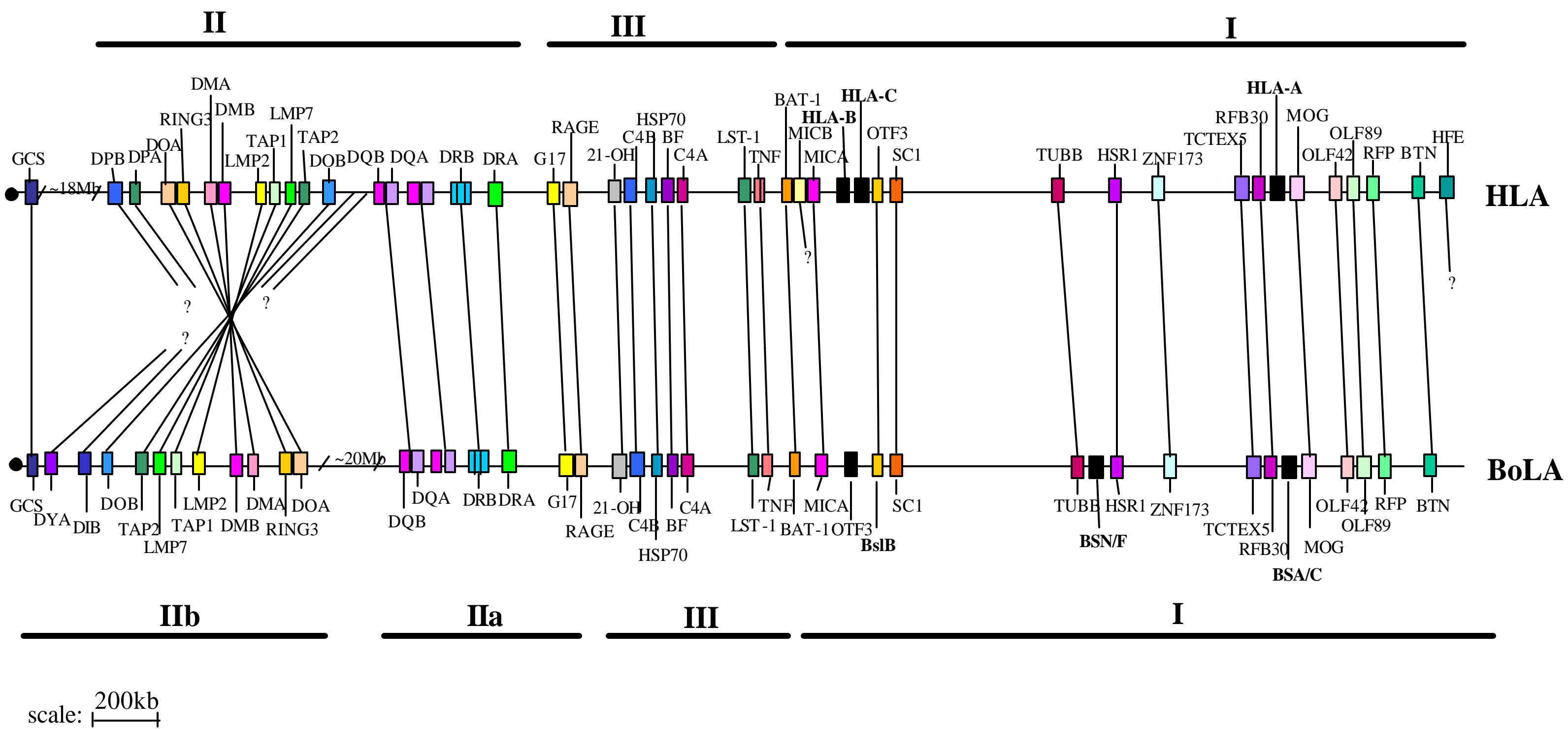


Figure 7. Preliminary Comparative Gene Maps of HLA and BoLA. This draft map of the gene content and organization is shown for both HLA and BoLA for major MHC Class I, II and III genes. It indicates that the ancestral ruminant MHC was disrupted by an inversion in the class II region and that the gene content seems to be generally conserved between species. Note the lack of loci showing gene content within the BoLA class I region.

duplicate at a rate consistent with evolutionary requirements based on selective pressures.

MHC polymorphism can also be generated initially by point mutations, then by gene conversion that shuffles short DNA sequences between alleles or loci (Ohta 1998; Parham 1989; Parham 1995). Recombination events may also contribute to the generation of polymorphism in MHC genes in an essentially random process. For example, phylogenetic trees for different regions of the ovine DQA2 gene by Hickford et al. (2004) suggests that genetic exchanges of segments may have occurred between ancestral alleles. This was supported by the sharing of common sequence between alleles, despite other significant sequence variation.

Evolutionarily, the MHC is under strong selective pressure from two opposing forces (negative and positive), enabling a balance between conservation and diversification. Patterns of nucleotide variation at the MHC class I and class II loci suggest evolution by overdominant (balancing) selection (Hughes and Nei 1988; Hughes and Yeager 1998b) and frequency selection (Klein and O'hUigin 1993). High levels of polymorphisms and heterozygosity within the MHC provides the immune system with a selective advantage against the diversity and variability of pathogens and help to increase the host's or population's fitness against infections (Potts 2002; Potts and Slev 1995).

The high level of polymorphisms and mutations in the MHC has the added risk however, of generating autoimmune diseases and other genetic disorders. O'Brien and Yuhki (1999) found reduced MHC variation contributing to uniform population sensitivity to emerging infectious pathogens in two cases of endangered Felidae (Florida panther and cheetah) due to species adaptation, mediated by MHC divergence. Organisms parasitic on vertebrates are presumably under strong selective pressure to evade host recognition systems and to render the host's immune attack ineffective. For instance, by analyzing DNA sequences from genes encoding surface proteins of the malaria parasite *Plasmodium falciparum*, Hughes and Verra (2002) provided evidence

for a balanced polymorphism which keeps the host from encountering a single form of the parasite surface protein in every infection.

While MHC class II and III genes seem to have direct orthologs in different species, it is clear that class I genes are subject to different evolutionary mechanisms having expanded and contracted in different species. The differences observed in class I gene composition presumably indicate a rapid evolutionary process. Like other gene families, the MHC class I gene family may have arisen by successive gene duplications which followed pathways differing with each species. Sequence analysis confirms the existence of a high degree of sequence similarity between the chimpanzees and human MHC class I region, however the 98.6% sequence identity drops to only 86.7% when the multiple insertions/deletions (indels) are considered (Anzai et al. 2003).

More analyses of MIC genes (encoding stress-inducible glycoprotein) in other species reveals a similar story. There are another five copies of the *MIC* gene family, *MICC* to *MICG*, distributed within the human MHC class I region that are highly fragmented in structure and appear to be nonfunctional (Kulski et al. 1999c). To date, the *MIC* genes have been detected in the pig (where MIC2 is a pseudogene) (Velten et al. 1999), cattle and primate species; but are not found in the mouse or rat (Gunther and Walter 2001). Taken together, these observations suggest that the *MIC* genes have undergone extraordinary selective pressure and genomic plasticity. The reason for the deletion and fragmentation of the *MIC* genes is not known, but may be due in part to the existence of other class I-like genes replacing the functions of the *MIC* genes (Radosavljevic et al. 2002). These data suggest that indels may be more important than the more subtle single-nucleotide substitutions for shaping allelic lineage in recently emerged primate species.

While the class III is considered the most conserved region of the MHC (as reviewed earlier), class I and II regions seem to have evolved via a series of duplicative events from a common ancestral gene. However, there is debate on which is ancestral, with one hypothesis supported by phylogenetic analysis proposing class II genes arose first (Hughes and Yeager 1998b; Klein et al. 1998). However, structural and functional

diversity of the itinerant class I-like genes and the restricted expression of the class II genes suggest that the class II genes may have evolved from an ancestral form of the class I genes. The debate of which came first, class I or class II, remains a contentious issue (Lawlor et al. 1990; Hughes and Nei 1993; Klein et al. 1998; Hughes and Yeager 1998a; Flajnik et al. 1999).

Parham et al. (1995) noted that the pattern of nucleotide differences in *HLA* class I genes shows strong locus specificity, suggesting that most newly arisen alleles result from a combination of point mutations and intralocus recombinations. Phylogenetic analysis in HLA class I genes produce defined sequence groupings based on loci (Lawlor et al. 1990). By contrast, mouse *H2-K*, *-D*, and *-L* class I genes show weaker locus specificity than *HLA* genes, indicating that interlocus gene conversion plays a major role in the generation of murine *MHC* class I gene diversity (Pease et al.1991, 1993; Pullen et al. 1992; Yun et al. 1997). Interlocus gene conversion may also predominate in cattle, where class I genes are difficult to assign to loci based on sequence (Ellis et al. 1999). Thus, it is essential that the evolutionary pattern of nucleotide diversity within and between loci in organisms phylogenetically intermediate between human and mouse, for instance cattle, be examined to comprehend the evolutionary histories of MHC class I genes.

Expression and phylogenetic analysis of BoLA class I loci

Early studies showed evidence for only one class I locus (al-Murrani et al. 1993). However, molecular studies later identified at least three transcribed class I loci in cattle (Garber 1994). Ennis et al. (1988) found at least four classical class I loci in cattle arranged in haplotypes that expressed any combination of one, two or three genes, although no one gene was expressed consistently. Holmes et al. (2003) used phylogenetic analysis to reveal that MHC class I sequences from examples of Cetartiodactyl species closely related to cattle were distributed among the main cattle gene "groups". Those from more distantly related species were scattered (sheep, deer), while others from more related taxa clustered in a species-specific manner (sitatunga,

giraffe); thus indicating that divergence of the MHC sequences had occurred independently from that of the hosts from which they were obtained.

Two clear instances of interlocus recombination among the cattle MHC class I sequences have been observed. Positive natural selection has been documented at positions throughout the a_1 and a_2 domains, primarily on those amino acids directly involved in peptide binding, although two positions in the a_3 domain, a region generally conserved in other species, were also shown to be undergoing adaptive evolution (Holmes et al. 2003). Ellis et al. (1999) reported that none of the cattle class I genes defined in the five haplotypes included in their study seemed to be consistently expressed. In the human, each haplotype has three classical MHC class I loci and alleles from all three loci are expressed, albeit at different levels. In fact, in contrast to human, cattle haplotypes differ from one another in both the number and composition of expressed classical class I genes. It was also found that phylogenetic analysis of available transcribed cattle MHC class I sequences revealed complex evolutionary relationships including possible evidence for recombination (Ellis et al. 1999).

In cattle, the number of expressed MHC class I genes varies from one to three in different BoLA haplotypes, suggesting many rearrangements or an unequal crossing-over of MHC class I haplotype components during their evolution (Ellis et al. 1999). Screening of a bacteria phage genomic library from an Angus Bull (#86 Texas Agricultural Experimental Station) with HLA B7 cDNA probe identified 18 class I loci, 5 non-classical class I genes and 12 pseudogenes or gene fragments (Hu 1996).

Problems with assignments of class I alleles to loci and determining number of loci and expression states are also seen in other species. Analysis of classical MHC class I genes of a homozygous thoroughbred horse defined two loci (Barbis et al. 1994; Carpenter et al. 2001); however, the number of loci and their expression states are unknown in horses. Chung et al. (2003) found that two or three loci could encode classical MHC class I molecules in the horse in certain haplotypes. Also, in the SLA, which represents the closest sequenced relative to BoLA by phylogeny, the number of

functional class I genes varies from 1-4 (depending on haplotype) and the class I genes are also too closely related to clearly assign alleles to loci (Chardon et al.1999a).

Haplotypic determination of differential expression (or silencing) in the cattle MHC class I genes would confer a high MHC diversity in cattle populations, which seem to be maintained despite human controlled breeding programs. A highly polymorphic MHC may have provided defense against infection from new pathogens that the animals would not have been exposed to in the wild, but would be exposed to during the process of domestication. Also, contributing to this MHC diversity would be multiple domestication events of more than one ancestral population of cattle with introgressions from wild Bovids over the course of cattle domestication to date (Ellis et al. 1999).

The diversity in genomic order and sequence variation within the MHC reflects that each species may harbour a certain degree of uniqueness. Comparative data obtained from characterization of the BoLA class I genes and its alleles is needed to provide us with a better understanding of mechanisms of immune diversity and its interactions with the environment and genetic adaptations to disease.

Hypothesis

Insight into the evolution of the MHC can be gained from comparative genomics in various mammalian species. Identification of conserved regions between the genomes of distant species is a crucial step in the reconstruction of the genomic organization of their last common ancestor. Using a comparative genomics approach to identify BAC clones containing bovine MHC genes will allow us to assemble the BAC clones into contigs of defined gene content and chromosomal orientation. This will allow us to develop an ordered BAC contig map of the BoLA class I region. The research proposed also describes the characterization of a frameshift deletion (A_{del}) at the predominant functional class I locus, BoLA-A (BSA) through transcriptional, genotyping, flow

cytometry, phylogenetic sequence analysis and proteomics from different species of feral and domestic bovids. Functionally, exons 2 and 3 in class I genes encode for amino acids that form the antigen presenting site; consequently, the hypothesis is that the bovine deletion allele, if functional, will be soluble whereas other alleles at this locus should encode membrane-bound proteins. This research will also address expression of the putative truncated allele.

Rationale and significance

The results of this research will provide a better understanding of the role of the bovine MHC in disease processes that will lead to the production of healthier cattle, and may identify a genetic basis for the anecdotal assertion that feral cattle and bison differ from domestic cattle in response to exposure of pathogens. The ability of an organism to identify and destroy foreign substances and to distinguish between self and non-self is regulated by genes in the MHC, and its polymorphism is a means of adaptive speciation to the environment.

The interaction of T lymphocytes with appropriate antigen presenting cells represents the first step in clonal selection and differentiation of immune cells; consequently, the genetic processes involved in antigen presentation are fundamental to understanding the role of genetics in animal health and disease resistance. Comparative study of the MHC in a wide range of species from humans to amphioxus will allow elucidation of early genomic events leading to the evolutionary successes of vertebrates. The goals of this research and the novel approaches proposed will gather data that will be valuable to the animal health research. Extensive polymorphisms of the MHC genes make them potential markers for the study of disease susceptibility. Information on sequence variation of BoLA genes can also be used to study the evolutionary history of the major histocompatibility complex. Such comparative studies in the MHC will be able to provide insight into its evolution and function in mammalian species.

Specific aims and objectives

1. Organization of the BoLA class I region

In order to better define the genomic structure of BoLA class I, a comparative genomics approach was used to identify BAC clones containing bovine class I MHC genes and then a rigourous analysis was performed including: end sequencing, PCR, fingerprinting, shotgun cloning, FISH and Fiber FISH to assemble the BAC clones into contigs of defined gene content and chromosomal orientation to produce a physical map of the class I BoLA region presented in Chapter II. Chapter III describes development of techniques used that were pivitol in obtaining good quality BAC end sequences, also called sequence tag connectors (STCs), used in the contig building and initial overlapping of BAC clones onto genomic sequence obtained from the two libraries for the class I region spanning the MHC class I region.

3. Phylogenetic analysis of frameshift deletion BoLA-A_{del} MHC class I gene

Genotyping of a frameshift deletion variant allele (BoLA-A_{del}) was used to investigate allele frequencies in feral populations of cattle and bison, as compared to production beef and dairy breeds. Phylogenetic analysis of A_{del} haplotypes was used to reveal whether haplotypes identified in bison are unique to bison but similar to the deletion haplotypes identified in cattle. It also allows verification of whether the A_{del} mutation predates the divergence of cattle and bison, as this suggests that natural resistance has played a role in maintaining this mutation over five million years of evolution. This will be presented in Chapter IV.

3. Transcriptional and proteomic characterization of frameshift deletion BoLA-A_{del}

For this objective, the expression of the truncated allele at the transcriptional level was examined by quantitative RT-PCR and flow cytometry to determine whether the mRNA transcript is present, and if so, whether it has been modified by alternate splicing, RNA editing or other types of epigenetic modification. We also examined the protein product using immunoprecipitation, 2D gel analysis, matrix associated laser desorptive ionization (MALDI-TOF) and tandem mass spectrometry to characterize the translated class I genes in genotypically defined cattle. This is presented in Chapter V.

Finally, Chapter VI summarizes the major points of this dissertation, and provides an overall analysis of the BoLA MHC class I region and summarizes details on the characterization frameshift deletion allele in feral cattle and Bison, and possible roles for an allelic form of a soluble BoLA MHC class I gene product.

CHAPTER II

GENOMIC ORGANIZATION OF THE BOVINE MAJOR HISTOCOMPATIBILITY (BoLA) CLASS I REGION

> What is wanted is not the will to believe, but the wish to find out, which is the exact opposite.
>
> - Bertrand Russell

Summary

The bovine major histocompatibility complex (BoLA) class I genes play an important role in the immune response facilitating antigen presentation and self-discrimination by acting as ligands for CD8+ cytotoxic T cells and NK cells. An ordered BAC array was developed for the BoLA MHC class I region from BACs isolated using gene specific overgo primers on the CHORI 240 Hereford bovine BAC library to complement already existing sequence data from BACS isolated from the TAMU Angus bull BAC library. A rigourous approach was used for confirmation including hybridization, PCR screening, DNA fingerprinting, comparison to existing bovine DNA fingerprint maps, BAC end sequencing and PCR, BAC sequencing, fluorescence in situ hybridization (FISH) and Fiber FISH. Two contigs containing 34 BAC clones, and 10 additional BACs identified *in silico,* span the BoLA class I and extended class I region from the BAT1 class III /I boundary framework gene to the histones in the telomeric end of BoLA. Dual-coloured FISH analysis localized selected BACs to BTA23q2.2, and fiber FISH allowed confirmation of order and distance between BACs to a resolution of 300 kb. Assembly of all class 1 sequences containing classical class I genes was not possible since sequence from animals of different haplotypes were pooled in this study. However, there was confirmation of at least 8 class I genes and at least 3 clusters of class I genes in the conserved BoLA class I region of all haplotypes studied.

Introduction

Nobel prize winners Peter Doherty and Rolf Zingernagel's pioneering research (reviewed by Ada 1994) defined the novel way that immune cells capable of killing virus-infected cells must recognise a part of the virus together with molecules of the host cell called major histocompatibility (MHC) antigens. The MHC was previously defined as an important genetic component for tissue transplantations in both mice and humans (Benacerraf and McDevitt 1972; Dausset 1981; Gorer 1937; Snell 1951). This requirement for antigen presentation in context of the MHC, has been termed "MHC-restriction". It is the basis of all cellular immune responses and has been critical for our understanding of infectious diseases, inflammatory diseases, such as rheumatoid diseases and diabetes, the design of vaccines and efficiency of organ transplantation.

The human MHC region, or HLA, encodes the most polymorphic human proteins known, some of which are encoded by more than 592 alleles (Forbes and Trowsdale 1999) (http://www.ebi.ac.uk/imgt/hla/stats.html). The consensus mammalian MHC consists of three closely linked regions, I, II, and III, occupying a single chromosomal segment of 2-4 Mb of DNA (Chardon et al. 1999a; The MHC Consortium 1999; Hurt et al. 2004; Shiina et al. 2004).

Classical MHC class I (Ia) molecules are highly polymorphic glycoproteins expressed on virtually all nucleated cells, with the notable exceptions of cells such as spermatozoa or cells in immunopriviliged sites, such as the central nervous system (CNS), placenta, hair follicles and eye (Niederkorn 2003). Class Ia molecules present cytosol-derived antigenic peptides bound within a cleft containing polymorphic antigen recognition sites (ARS), to cytotoxic T lymphocytes via the T cell receptor (TCR) facilitating destruction of infected cells and limiting spread of disease. Class Ia molecules are also recognized by natural killer (NK) cell receptors playing a role in inhibition of NK cell lysis (Kambayashi et al. 2001; Ljunggren and Karre 1990). Non-classical class I molecules (class Ib) are non-polymorphic, and have characteristically

low levels of expression and/or restricted tissue distribution (Allan et al. 2002; Lepin et al. 2000). The gene organization and structure/function relationship of the various exons comprising class I proteins are very similar among the class Ia and class Ib genes, however promoter structures among all class I genes are complex and only a few instances of conservation of class Ia promoter regulatory elements is seen among class Ib genes (Howcroft 2003).

The MHC has been defined as a complex located on a single chromosome in many species; including mammals, amphibians, reptiles, birds, and cartilaginous fish (reviewed by Kumanovics et al. 2003). However, significant differences in the number and physical organization of the genes within the MHC have been demonstrated in several species within the Artiodactyla (Band et al. 1998; Chardon et al. 1999a; Chardon et al. 2001; Forbes and Trowsdale 1999; McShane et al. 2001; Smith et al. 1995), within avian species like chicken and quail (Shiina et al. 2004) and within rodentia like rat and mouse (Hurt et al. 2004).

Comparative analyses illustrate that duplications, deletions and rearrangements, both inter- and intra- locus, are an important characteristics of MHC organization, especially class I genes, in most species (Hughes and Yeager 1998a; Takada et al. 2003; Yeager and Hughes 1999). Evidence for rearrangements can be seen in the inconsistent number of expressed class Ia genes between species; for example in humans, HLA-A, B and C class Ia alleles are always expressed, but in other species the number of expressed class Ia genes seems to be variable; for example ranging from 1 to 3 in horses, and 1 to 4 in cattle (Chung et al. 2003; Ellis and Ballingall 1999; Holmes et al. 2003). Also, there seems to be no orthologue for the human class Ia genes HLA-A, B and C and the class Ib genes E, F and G consistently found in other primates except for the shared allelic lineages for all six loci (but not alleles) between humans and African apes (Adams and Parham 2001). It has even been postulated that chimpanzees have experienced a severe repertoire reduction at their orthologues of the HLA-A, -B, and -C loci predating the (sub)speciation events, from an ancient selective sweep due to a SIV-like retroviral pandemic resulting in only AIDS resistant animals surviving (de Groot et al. 2002).

The bovine lymphocyte antigen (BoLA) complex was first described by Spooner et al. (1978). The organizational features, including a well documented disruption caused by a single large chromosomal inversion on BTA23, has been confirmed by several groups (Andersson et al. 1988; Andersson and Rask 1988; Band et al. 1998; Band et al. 2000; Leveziel 1984; McShane et al. 2001; Skow et al. 1996; Skow et al. 1988). This chromosomal inversion seems to predate the divergence of the advanced pecoran families from a common ancestor, as evidenced by conservation of chromosome banding patterns for BTA23 and its homologues among Bovidae, Cervidae, Giraffidae and Antilocapridae (Gallagher et al. 1992; Iannuzzi et al. 1993). McShane et al. (2001) used fluorescent in situ hybridization (FISH) of BoLA DYA and class I BAC clones to confirm that these genes map to discrete chromosomal locations on BTA23 and its homologue in white-tailed deer. Thus the order in the artiodactyl families of ruminants seems to indicate evolutionary conservation of gene order, and the organization of the BoLA is thought to be an ancestral condition for the advanced pecorans.

Evidence for evolution of the Arteriodactyl MHC can be seen in the more than 990 kb of the 1200 kb SLA class I region of the pig major histocompatibility complex (MHC) that has been sequenced. It is known that duplications gave rise to at least six SLA classical class I genes and other groups of non-classical class I genes and pseudogenes (Chardon et al. 1981; Chardon et al. 1999a; Renard et al. 2001; Shigenari et al. 2004). Human/pig sequence comparison also revealed that the presumably functional pig MIC2 gene was probably orthologous to the human functional MICA or MICB genes (Renard et al. 2003). When the region of the SLA corresponding to the junction of the class I and class III regions was sequenced, the order and molecular organization were exactly conserved in the SLA and HLA complexes, except for the SC1 gene which displayed a shift of the reading frame in swine. The cluster of the three SLA class I-related genes (Ib) and the MIC1 and MIC2 genes were located in the middle of the segment (Chardon et al. 2001), while another cluster of four classical class I genes were located between MOG and GNL (Renard et al. 2001). However, the third and fourth clusters of class I genes seem to be missing in the SLA. Earlier work in mapping the

BoLA region gave evidence of at least 4 clusters of class I genes (McShane et al. 2001; Newkirk 2000) which is more similar to the HLA (The MHC Consortium 1999).

Research into the immune systems of economically important species, such as cattle, is largely focused on disease control strategies. Existing vaccine failures often relate to poor induction of cellular immune responses, thus there is increasing interest in determining (or predicting) T cell epitopes. Thus, it is imperative to define genes of immunological importance, such as MHC class I genes, in terms of location and expression in cattle by comparative analysis. There is therefore a need to assess MHC diversity in populations of cattle, to allow provision of animals with known MHC genes and haplotypes, by relating serotyping to genotyping, for detailed analyses and to generate mapping data. The basic approach used in the construction of whole-genome fingerprint maps is to fingerprint a set of randomly selected clones that together represent in a redundant fashion the genome of interest, computationally identify overlapping clones based on shared restriction fragments, and assemble them into ordered arrays representing contiguous stretches of DNA ("contigs") (Schein 2004).

MHC class I genes in human and mouse have been well characterized, but little is known about the BoLA class I genes. Previous studies identified at least 20 class I loci by Southern blot analysis with a human cDNA probe (Lindberg and Andersson 1988). Screening of a bacteria phage genomic library from an Angus Bull (#86 Texas Agricultural Experimental Station) with a HLA B7 cDNA probe identified 18 class I loci, 5 non-classical class I genes and 12 pseudogenes or gene fragments (Hu 1996). Among these several classical class I genes that were previously identified by Garber et al.(1993) were isolated and later fully sequenced including BSA (AF396750) and BSC (AF396752) (McShane 1998). Also, the TAMU bovine BAC library (Cai et al. 1995) constructed from an Angus bull Y6 in addition to BAC clones from Longhorn, Brahman and Angus females, was screened and 16 class I containing BACs were isolated (McShane 1998; Newkirk 2000). Several of these BAC clones from the TAMU library were sequenced at the Baylor College of Medicine and sequence was available for inclusion in this study.

This study intends to build on previous data to produce a physical map of the BoLA class I region that can serve as a framework for complete sequencing of the area and allow identification of immunologically important genes that can elucidate the evolution of the MHC in bovids and Artiodactyl mammals, as well as providing us with tools for producing a more effective immune response against pathogens and better vaccine design. First a BAC anchor map was created to link the class I containing CHORI and TAMU library BACs to the genetic map of the BoLA class I region. Then these BACs were fingerprinted to be used in contig building, and FISH mapped to assign cytogenetic position of specific BACs. Finally, BAC end sequencing and select shotgun subclone sequencing of BACs in conjunction with fiber FISH was done to allow anchoring of BACs and shotgun-sequenced contigs to the physical map of BoLA.

Materials and Methods

Libraries

Sequences from class I genes used in this study were obtained from three libraries. The first is a bacteria phage genomic library from sperm DNA from an Angus Bull (Animal #86 Texas Agricultural Experimental Station) constructed from *Sau3A*I partial digests cloned into Lambda GEM11 by Xho I half-site fill-in reactions. This bull, B86, was used by Garber et al. (1994) to identify the BoLA BSA, BSC, BSX, BSN, BSF and Bs1b genes referred to in this study.

A bovine BAC library constructed by Cai et al. (1995) from *Hin*dIII and *Bam*H1 digested genomic DNA from white blood cells of an Angus Bull, Y6, cloned into the pBeloBAC11 vector (7.4kb), was previously screened by McShane (1998) and Newkirk (2000) for clones containing BoLA sequences. This Texas A&M (TAMU) library contains approximately 60,000 clones, representing approximately 3x genome coverage with an average insert size of 146 kb (Cai et al. 1995). Additional screening of DNA superpools from this BAC library by PCR typing was also done in this study.

Genomic DNA isolated from blood collected from Hereford bull L1 Domino 99375 in Dr. Michael MacNeil's laboratory, USDA-ARS, Miles City, MT was used to produce the Children's Hospital of Oakland Research Institute (CHORI-240) bovine BAC library (Osoegawa et al. 1998). Genomic DNA was isolated from Hereford bull L1 Domino 99375 and was partially digested with *Mbo*I. Size selected DNA was cloned into the pTARBAC1.3 vector between the *Bam*HI sites. Ligation products were transformed into DH10B electrocompetent cells (BRL Life Technologies) for segment 1 and DH10B T1 phage resistant electrocompetent cells (BRL Life Technologies) for segment 2, respectively. The library has been arrayed into 384-well microtiter dishes and also gridded onto 11 22x22cm nylon high density filters for screening by probe hybridization. Each hybridization membrane represents over 18,000 distinct bovine BAC clones, stamped in duplicate. This library represents 11x coverage of the genome with an average insert size of 167kb (http://bacpac.chori.org/bovine240.htm).

Design of overgo probes

A systematic approach was used to screen high-density BAC filters with probes generated from overlapping oligonucleotides (overgo) probes as described in Han et al. (2000). All available sequences of framework, classical and non-classical class I genes, ESTs, STSs, and sequenced class I MHC regions were examined. Conserved regions within orthologous genes in the MHC were identified using alignments and GenBank Pairwise BLAST® (Tatusova and Madden 1999). Repetitive regions were masked with RepeatMasker. Then BLAST® was used to identify 36-bp unique fragments of DNA for overgo probes, which were designed so that the forward and reverse primers were 20-bp each sharing an 8-bp overlap at their 3' ends. Overgo primers were designed to conserved regions using the Overgo Maker program and were screened against GenBank using *BLAST® (Altschul et al. 1990) to confirm specificity and exclude any additional repetitive elements (Table 1).

Table 1. Overgo primers. Sequence of overgo primers used in hybridization to CHORI 240 BAC DNA library filters and the gene or EST from which they were derived.

Gene	Accession Number	Forward Overgo Primer	Reverse Overgo Primer
BL3.7	M21043	GAGCTGTGATCTGGAGGAAGAAGC	CCTTTTTCACCTGAGCGCTTCTTC
BSA	AF396750	AGATCCTGAGCGGCTCGCCCACCA	CAAAGTCCCAGGTCCCTGGTGGGC
BTN1A	GI2253279	GAAAGGATTTGACCCCATGACTCC	CCAGAACCCATTCTCGGGAGTCAT
CDSN	AY328475	CATCTCTGAGGGCAAATACTTCTC	GATGATGGGGTTGCTGGAGAAGTA
DDR1	TC132972	TTCTACTGCTGCTGCTCTTGGTGG	TCAGCATCTCCAGTTGCCACCAAG
DDX16	TC132972	ACTTTGTACAGTTCAGATCGATGC	ACATCCCGGGCTCGGCGCATCGAT
GABBR1	GI29648318	TTTGAAAAGTGGGGCTGGAAGAAG	GCTGGATGGTAGCAATCTTCTTCC
GPX5	GI6754061	AAGGTCCATGACATCCGTTGGAAC	CCAGGAACTTTTCAAAGTTCCAAC
IER3	BF045468	ATGTGTCACTCTCGCAGCTCCCTC	GGACGGTCATGGTGGGGAGGGAGC
MIC1	AF329329	GTGCAGTCCAGGTTTTTTGCTGAG	GACGATCCAAGAATCCCTCAGCAA
MIC1-02	AF329107	GTGCAGTCCAGGTTTTTTGCTGAG	GACGATCCAAGAATCCCTCAGCAA
OR2H1	AL035542	TTAACCAAAGCTCCCCCATGGGCT	AAGCCCAGAAGGAGGAAGCCCATG
PP1R11	GI11386174	GTTCTCACTGCCAAACTGCCTGTC	GATAACTGGATCCCAGGACAGGCA
PRSS16	GI21396490	CCAGAAGTGTTTAAGCTTTTCCCG	TGCCACTGTCTCTGCTCGGGAAAA
RFP	GI7105396	TGAAGCTGTACTGCGAGGAGGACC	ACGCAGATGGGCATCTGGTCCTCC
TNF-a	AF348421	TACCTCATCTACTCACAGGTCCTC	AGCCTTGGCCCCTGAAGAGGACCT
TUBB	AY159127	CTCAACGTGCAGAACAAGAACAGC	ACTCCACGAAGTAGCTGCTGTTCT
SLA-ZNFB7	AJ251829	TTACTGCGAGGAGCATGGCGAGAA	GCAGAAGAAGTAGATCTTCTCGCC
ZNF192	XM_165759	TTCAGCATCTGAGAATTCACACAG	TGATAGGGTTTCTCCCCTGTGTGA
ZNRD1	AF024617	AGCTTTCAGTCGGACTTGGATTTC	AGCCGCAATCTGGACAGAAATCCA

Overgo labeling and BAC library screening by filter hybridization

High density filters from the USDA CHORI 240 bovine BAC library were probed with pools of radiolabelled overgos to identify clones containing BoLA classical and non-classical class I sequences. Primers were radioactively labeled either using a modification of the BACPAC hybridization protocol or a modified overgo protocol as described by Gustafson et al. (2003). Briefly, 10 uL labeling reaction containing 1 uM of forward primer, 1 uM reverse primer, 150 Ci/mmol each of ^{32}P dATP and ^{32}P dCTP (Amersham Biosciences, Piscataway, NJ), 2 U Klenow fragment DNA polymerase (Roche, Indianapolis, IN), and 1x DNA Polymerase Buffer, (Promega, Madison,WI) was incubated at 37°C for 30 mins. For each fill-in labeling reaction, 1 uL of a 250 uM dATP and dCTP mixture was added, at 37 ºC for 15 mins (Han et al. 2000). Unincorporated nucleotides were removed using Sephadex G-10 gravity flow columns. The labeled overgo probes were pooled and added to the hybridization solution (20X SSPE, 10% SDS, 5% milk, and 100X Denhardt's) containing 50% formamide, boiled for 10 mins, chilled and then hybridized onto filters at 42 ºC for 16 hours. Filters were washed three times at 55 ºC for 15 minutes in 2X SSPE. After overnight hybridization, filters were washed for 30 minutes with 1mM EDTA, 1%SDS, 40mM Na_2HPO_4, followed by 2X washes, 20 minutes each with 1.5x SSC, 0.1% SDS. A final 20 minute wash was done with 0.5X SSC, 0.1%SDS. The filters were then exposed to a Phosphoimager or film and positives identified. Positive clones were rearrayed on membranes and hybridized with individual probes to determine which BAC clones were positive for individual overgos.

DNA purification, fingerprinting, Southern blot analysis and contig assembly

BAC DNA was purified using a modified alkaline lysis protocol and exonuclease digestion step using the QIAGEN™ Large-Construct kit (QIAGEN, Valencia, CA) as per manufacturer's instructions. An additional polyethylene glycol (PEG) purification step was then performed to further purify DNA for downstream analysis with a 20 min incubation of BAC DNA (dissolved in milli-q water) with 0.16 volumes of 5M NaCl,

0.25 volumes of 13% PEG on ice, followed by a wash in 70% ethanol and resuspension in milli-q water to desired concentration.

BAC clones were fingerprinted using a modified protocol of Marra et al. (1997) using restriction enzyme digestion with *Bam*H1 or *Hin*dIII enzymes. Briefly, 500 µg of BAC DNA was digested to completion with 10 units enzyme in a volume of 30 µl at 37 °C from 3 hours to overnight. Then 10ul of each digest was diluted with 10ul of milli-q water, combined with 3ul of 6x Blue/Orange DNA loading dye (Promega, Madison, WI) and run for 22 hours at 50 volts on a 0.65% TBE agarose gel stained with ethidium bromide. Restriction fragment patterns identified overlapping BAC clones which were assembled into draft contigs as done previously (Gustafson et al. 2003; Marra et al. 1997). In addition, double digests and fill-in labeling using DNA polymerase I large (Klenow) Fragment kit (Promega, Madison, WI) with 250ng BAC DNA and 10 units each of Hae III and HindIII enzymes and a- ^{32}P-dATP in 20ul reactions, were run on 6% polyacrylamide gels as initial confirmation of DNA fingerprint patterns.

The gel images from agarose BAC DNA fingerprinted gels were digitally captured with the Alpha Innotech ChemiImager™ system and analyzed using IMAGE 3.0 software (Sulston et al. 1988). Preliminary contig assembly was performed using FPC V4.7.9 (Soderlund et al. 1997). For added robustness to the BAC order, internet contig explorer (iCE) (Fjell et al. 2003) was used to generate an *in silico* DNA fingerprint display of selected clones in the BoLA MHC class I region from BAC fingerprint maps generated from FPC processed gel images from another group (Marra et al. 1997). DNA fragments were Southern blotted onto nylon filters for subsequent hybridization with individual overgo probes to identify specific genes on particular BAC fragments as previously described above.

BAC DNA sequencing

End sequencing of BAC clones was performed on an ABI 3100 automated capillary sequencer using T7 and SP6 sequencing primers. This was done using a modified TaqDyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) with

Big Dye™ method described in Chapter III (Ramlachan et al. in preparation). Briefly, 10µl sequencing reactions were performed using 2µl BigDye™ V1.1 (Applied Biosystems), 2µl *half*BD™ and 0.5µl MasterAmp™ PCR Enhancer (Epicentre, Madison, WI), with approximately 1µg of BAC DNA and 10 pmol primer (T7 or SP6) per reaction. The thermal profile was 96 ºC for 5 m, then 8 cycles of 95ºC (0.25 s), 58ºC (0.25 s) (-1ºC/cycle), 65ºC (4 m); followed by 60 cycles of 96ºC for 25 s, 55ºC for 25 s and 60ºC for 4 m; and finally 4 holds of 96ºC (1 min), 50ºC (1 min) and 65ºC (1 min) then 4ºC until reactions are removed. Reactions were purified with G-50 sephadex columns (Biomax, Odenton, MD). Sequence fragments were separated and analyzed using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), and are available through GenBank.

Confirmation of overlapping BACs by PCR

PCR primers for specific genes or designed from the end sequences of selected BAC clones (Tables 2 and 3) were used on positive BAC clones to confirm gene content and the overlaps indicated by fingerprinting. PCR was carried out in 25 µL reactions containing 50 ng of BAC DNA from individual clones as the template, 0.25 units of JumpStart Red Taq Polymerase (Sigma, St.Louis, MI), 0.8 mM dNTPs, 0.4µM of each primer, Master Amp™ PCR Enhancer (Epicentre, Madison, WI) and 10x reaction buffer (100mM Tris-HCl, 500 mM KCl, 15mM $MgCl_2$, 0.01% gelatin). The thermal profile was as follows: 2 min at 95°C; 4 cycles X 30 sec at 95°C, 30 sec at 58°C (-1°C / cycle), 25 sec at 55°C; 30 cycles X 30 sec at 95°C, 30 sec at 54°C, 25 sec at 55°C; 10min at 65 °C.

Table 2. PCR primers. Sequence of PCR primers used in amplification of known genes class I classical or non-classical genes from CHORI 240 BAC clones. Primers were derived from alignments of human, mouse, swine or where available bovine gene or EST sequences.

Gene	Forward PCR Primer 5'-3'	Reverse PCR Primer 5'-3'	Annealing Temp ºC
ABCF1	TGGCTTCTTCAACCAGCAGTATG	CCGCTTGTAGTCTTCAAAGTCACC	59
AFP	ATGAACAAATACCAACACGGGC	ATCGCTTCCTCTTCCTCCTCTG	56
BSA	CCGCCCAACTCTGCTTCTTTC	GATACCTGGAGAACGGGAAGGA	63
BSC	AGTTATAAAGTCTCCACCGACCT	GATACCTGGAGAACGGGAAGGA	62
BTN1A	AATGGGTTCTGGGCTGTG	TTGGCTGGGCTGTAGAGG	59
DDR1	TGGCTGAAGGTGGGCTGG	TACAACACGGCAGGTGGG	59
DDX16	TGGCTGAAGGTGGGCTGG	AACACGGCAGGTGGGAGG	59
FLOT1	CAGATGCCCAGCCTCATA	GCCCATCCCTCAGTCTTG	57
GABBR1	TTCTGGGCTGATTTGGGT	GGGTTGCCGTGGTAACTA	56
GNL1	AGACCTACTTTCTCACTCCCTCCG	TTCACAGATGTCCCAGGCACAC	57
GPX5	TTGGGCACATTCAAATCT	TACGCCAGGATGTCTGTC	58
IER3	CGCAGCCGTAGGGTCCTC	TTTGGCAGGGTTCGGTTC	58
MIC1	TGAAAAACTGGCAGCAGAGACG	GCTCCCCATTGAAGTAGAGAAGC	56
MOG	ATCTCCAGGAAAGAACGCT	CACATTCCGGATCCTGAG GG	55
NRM	CTTCCTCTCCCGCTGAAT	GGAGTGGTCAGGGCAGAT	57
OR2B6	CGGCCTCTCCATTACTCAGTT	GCAGGTACACAGAGACGGCTG	57
OR2H1	CTGGTGGGCAACACACTC	AGGCCATCACTGTCAGGA	58
OR5V1	ATTCATCATCTTGGGATTC	ATTGCTGCCAGTAGGAGAC	55
POU5F1	TCAAGCAGTGACTACTCCCAACG	CACCTTCCCTCCAACCAGTTTC	55
PPP1R10	GATGATGGCGAACTCGTATC	AGTGAGCTGACTCCCAAAGG	57
PRSS16	TCAGCACCTGTTCCGCAC	CGAGGGCACCAGCATAAG	55
RFB-30	GGGATGTTGGGTTGGAAGAGTG	GCAGGAGTTTGGACTGTTTTCTCC	56
RFP (Trim 27)	GATAATCTGCGGCAAGTGCG	AGTCCAAGAAAATCCCCACCC	57
TCF19	CACAGCAGCGAGCAAATAGGTC	CAGAATGCCTTGGGTGATGC	56
TNFA	GAAGAGGTGAGTTTCTGGCCGGC	CCACCTGGGGACTGCTGGGGAGA	57
TRIM 15	CCTTTCTTTGCCGTCTGGA	GAAGCCGTCTGTGGTTCTG	60
TRIM 26	TGGATTATGAAGGGGGCACC	AGTGAAGCATCTGAGGGTTGGG	58
TRIM31	ATCACCACTCTTCAGCCCCATC	GCTCGCTCCTTGACCAGAAATC	55
SLA-ZNFB7	TACACTCGGCAGAAGCAGAACC	CCAGACAGCGAAGAAAGGAAACAC	60
TUBB	AGCAGATGTTTGACGCCAAG	TGCTGTTGCCGATGAAGGTG	61
ZNF173	CACCCCCAGCAGTTTGAATG	TCCTCATCCGTTCCCCAATC	60
ZNF184	GCCAAAGGAAATAAAGGT	ATGGCGAATAAGAGCTGA	55
ZNF187	CATTCTACGCAACCTTGG	AATCATCTCTTTGGGCAC	55
ZNF192	ATGAAGAAGCCCGAGACCT	TTTGCCACACTCCTTACAG	55
ZNF193	AGAGTGCGGGTCTTATCCAG	TCTTCTGATGGCGAATGAGG	57
ZNRD1	AGATTGCGGCTCGGTCCT	ACGGGTCCCTGGAACTCA	59

Table 3. List of PCR primers designed from BAC end sequences. These primers were used to confirm overlaps in adjacent BACs in CHORI 240 and TAMU libraries by PCR.

BAC END	Forward primer 5'-3'	Reverse primer 5'-3'	Annealing Temp (ºC)	Product Size (bp)
60_T7	GGTTCCCACAGACATAGGAGT	GACTTTGACATGGACGCTAGA	55	160
93C03_T7	CCCTCCCTCTATTTTGGTCTT	CGAAGGATATGGTTGACCTTG	55	150
126-T7	GTTCCTCAAGTCCTAGCAGCA	TGCAACCAATCAGTAGTCAGC	55	160
137-T7	GGAATAAAGCAGAGGAGAGCA	CAATCCCAGAATGTTTCATCTC	56	217
143-T7	GGTTTTAGAGGTGGATGTGGA	ATGTTTCACTACAGCCCAGTG	56	196
148-T7	ACATTTGGAGGGATACAGTGC	GCTTGTAGTCTGCTTGGGAGA	56	150
159-SP6	CCACATTTACAGTTTGGCCTAC	GGCGGACTTATATTCTGGTGA	56	213
166-T7	GTGAAGGAAGTAGGGCAAATG	CTAGAGCCTGGAAAAGCAAGA	55	228
176-SP6	GTTGTTGTCAGCTTCCTCCAC	GGTGTTCAGCCAAACGACTAT	55	112
176-T7	CTTCACTTCACTTCACCTGCTC	GGACTCTCCAAGACCATCAGA	55	182
192-SP6	ACTGGACAGAGATGGACAGATG	GCCCCATTCAAATATCCTTTC	56	235
192-T7	CACCTTTATTTCTCCCCACTG	GATGCTGATTTGGGACTGATT	56	452
222-T7	ACACATTTCACCTGGGTTCT	CCACCAAGCTAAGTCATCCTC	56	154
222-SP6	GCCTAGACATCCCTCTGACAC	GGTCCACTCATTACGCTCAAC	56	200
249-SP6	CACACCAACCCCTCCTGTG	GCACCCAGAGTCCTGCAA	55	572
251-T7	CTGGCTTTGGCTTTGCTT	GTATTGGCAGGTGCGTTCTT	55	150
257-T7	ACGCACACTCCCCACACA	AGGGTTCCCCCTTTGCAC	55	159
264-T7	TGTGTCTCGAATGGGACTAGA	CAATTCACTCAACAGGTATTTGG	55	196
304T-7	CACACTCCTAGACCAACGTGA	CATAAGGTGCAGACATGACAA	55	136
326-T7	CTGTGCAAATACAGCTTCCAG	AGCCCAGAGATAACCCACTTC	55	200

Table 3 continued.

BAC END	Forward primer 5'-3'	Reverse primer 5'-3'	Annealing Temp (°C)	Product Size (bp)
330-T7	CAACCCAGAGATCAAACCTGT	GCATTCATGTAGGAGGACACC	55	215
380-T7	GCATCATAACAGCCAAAATCA	CACACTGGAAGAGAATGCAGA	55	164
390-T7	CCCCTGGTTTACTCCCTTAGT	CATTCTTGAGCGTGGGTTC	55	470
413I22-T7	TAGAGAGGATGTGCCCTTCA	CAAGGAGAAAAGAGGCGTTC	55	157
421-SP6	GCTCATCTTTTTCTTGGCAGT	TACAAATCCACCAAACCAATG	55	166
438-T7	CCTAGATGTCCTATGAGCAGGA	GTGATGAATGTTTTCGCATTG	55	161
447-T7	TCCAAATCCCTTTCTTCATCTT	GCTAAGTCGCTTCAGTCGTGT	55	154
457-T7	TCAGTTAGTTCTCCCGGTCTG	CCCCACTCCCAAAATTCTAA	55	154
463-SP6	GCACTTCCACCACCTAAGAAC	GGTAAGGGATGAGGAGGAGAC	56	216
473-SP6	GGCTGGTGGAGTTAAGAATGA	GGCTCGATATGGTAGGCTCA	56	213
495D10-T7	TGACTGCTATCCTTTCGTCCT	ACACAGAAGGTGACGGTTCAT	56	151
499O08	AGTTGCTGGCACGGTTTG	CACAGGATACCTCCCCAGAA	55	105
500-T7	GATGGTGTGGGAACTTGAGAT	GTCAATGGATGCGTTACAATG	56	163
500-SP6	AGGCTGGCATGTGAATGG	CCTGCAAGTCAATCAGTGTGA	56	173
512-T7	ACAGGAGATGCAGGTTCAATC	AAGTAGGGGTTGACCATAGGC	55	232
588-T7	GGAGAGATGGAGTTGCTGCTA	GGCTGTGTCCTAAACCTTCCT	55	192

Shotgun cloning and sequencing of selected BACs

Selected BACs from the minimal tiling path were selected for shotgun cloning and sequencing from the CHORI 240 library to add to those from the TAMU library from which sequence was already available. Purified BAC DNA was further treated with Plasmid-Safe DNase (Epicenter Technologies, Madison, WI) overnight at 37°C, according to the instructions provided. Approximately 2 ug of DNA was then sheared to approximately 2 kb fragments using the Hydroshear™ Shearing Device (GeneMachines, SanCarlos, California). The TOPO® Shotgun Subcloning Kit was used to rapidly clone the blunt end-DNA for sequencing which was done using standard M13 and T7 plasmid primers.

Fluorescence in situ hybridization (FISH)

Cattle blood was used for both standard short-term pokeweed (Sigma) stimulated lymphocyte cultures to obtain interphase and metaphase chromosome preparations as well as agarose-embedded DNA plugs to obtain DNA fibers (Heiskanen et al. 1996; Raudsepp et al. 2004). Selected BAC clones were labeled with biotin-16-dUTP and/or digoxigenin-11-dUTP by nick translation using Biotin- or DIG-Nick Translation Mix (Roche Molecular Biochemicals, Indianapolis, IN), respectively, and hybridized to cattle metaphase chromosomes individually to confirm their location to BTA23. BAC clones were also differentially labeled and co-hybridized to metaphase chromosomes to determine relative positions of clones.

DNA from individual BAC clones was also used in hybridization to metaphase chromosomes to test specificity and signal intensity, then BACs with relatively clear signals were also hybridized onto mechanically stretched DNA fibers and performed as described previously (Raudsepp et al. 2004). Images were captured and analyzed for each experiment with a Zeiss Axioplan2 fluorescent microscope equipped with CYTOVISION/GENUS application software, Version 2.7 (Applied Imaging, Santa Clara, CA).

Results

BAC end sequencing and physical mapping of BoLA Class I region

For the construction of a physical map and assembly of contigs, BAC clones that gave an initial positive hybridization signal in the pooled screens were considered. Of the initial library screens with overgo probes, 355 of the CHORI 240 BACs were positive and picked from the glycerol stocks, and spotted onto secondary filters which were subsequently screened with individual probes. Table 4 summarizes the gene content of BACs isolated from both the TAMU and CHORI 240 libraries that were identified in this study and elsewhere (McShane 1998; McShane et al. 2001). All gene markers were confirmed by PCR, hybridization or sequencing in this study. From the secondary screen, approximately 100 were positive for individual probes. PCR screening was performed for individual gene markers to confirm gene content of selected BAC clones (See Figure 8).

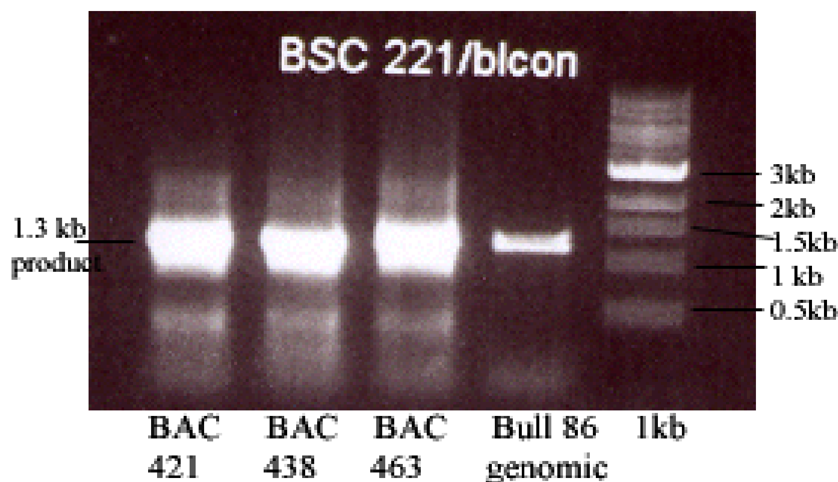


Figure 8. PCR screening of BAC clones. This shows the PCR product obtained from select class I containing BACs isolated from initial overgo hybridizations using BoLA-BSC locus specific primers (BSC 221 and BLCON). Bull 86 genomic DNA is the positive control from the animal for which the BSC sequence was derived.

Table 4. BAC library screens and selected sequencing results. Gene content confirmed by southern blot, PCR, overlap or sequencing of BACs from TAMU (Angus) and CHORI 240 (Hereford) libraries.

BAC	Library	Insert (kb)	Confirmed Gene Content
*283R4C1**	TAMU (A)	68	*TNFa, BAT1*
192E21	CHORI 240 (H)	235.8	*BL3.7, Bs1b, MIC1*
326F16	CHORI 240 (H)	164.9	*BL3.7, Bs1b, MIC1*
249J3	CHORI 240 (H)	167.5	*BL3.7, Bs1b, MIC1*
415K21	CHORI 240 (H)	194.8	*BL3.7, Bs1b, MIC1*
512N8	CHORI 240 (H)	195.7	*TNFa,BL3.7, Bs1b, MIC1*
63C13	CHORI 240 (H)	190.5	*BL3.7, Bs1b, MIC1*
176L2	CHORI 240 (H)	175	*BL3.7, Bs1b, MIC1, POU5F*
*187R1C3**	TAMU (A)	101.7	*Bs1b, MIC1*
*296R3C6**	TAMU (A)	101.7	*MIC1, POU5F1, TCF19*
*148R8C8**	TAMU (A)	74.7	*MIC1, POU5F1, TCF19, SPR1, CDSN, STG, RAGC*
*143R2C10**	TAMU (A)	75.7	*MIC1, POU5F1, TCF19, SPR1, CDSN, STG, RAGC*
*222R4C4**	TAMU (A)	75.7	*CDSN, STG, RAGC, VARS26*
499O2	CHORI 240 (H)	250.2	*RFP*
466B21	CHORI 240 (H)	182.9	*CDSN, DDR1, IER3, FLOT1, TUBB*
447A17	CHORI 240 (H)	154.9	*CDSN, DDR1, IER3, FLOT1, TUBB, DHX16*
487I9	CHORI 240 (H)	177.6	*DDR1*
358A21	CHORI 240 (H)	208.3	*DDR1*
383C15	CHORI 240 (H)	166	*DDR1*
500N12	CHORI 240 (H)	153.8	*DDX16, PPP1R10, ABCF1, PRR3, GNL1*
166F15	CHORI 240 (H)	203	*Class I*
93C03	CHORI 240 (H)	294	*Class I*
438O6	CHORI 240 (H)	102	*Class I*
390G9	CHORI 240 (H)	120	*Class I*
421J10	CHORI 240 (H)	151.4	*Class I*
*76R7C2**	TAMU (A)	156	*GNL1, Class I, Q9H63, RNF23 (Trim 39)*
*1402R4C8**	TAMU (A)	99	*Class I, ZNF173*
*159R5C3**	TAMU (A)	216.7	*ZNF173, RNF9 (Trim10), Trim 15, RNF39, PPP1R11*
*463M1**	CHORI 240 (H)	158.5	*Class I*

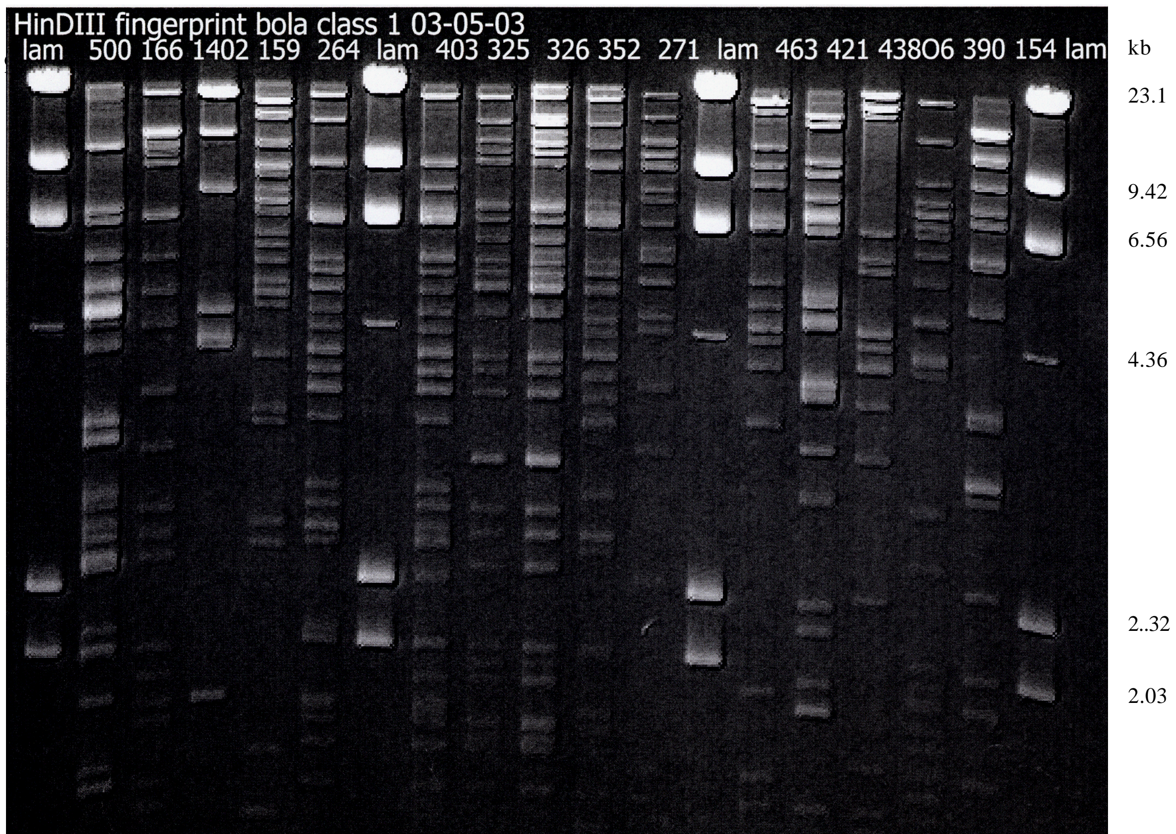
Table 4 continued.

BAC	Library	Insert (kb)	Confirmed Gene Content
165A09	CHORI 240 (H)	N/A	PPP1R11, ZNRD1,
264A9	CHORI 240 (H)	162.5	*PPP1R11, ZNRD1,*
403G2	CHORI 240 (H)	208.7	*PPP1R11, ZNRD1,*
352B12	CHORI 240 (H)	200	*PPP1R11, ZNRD1,*
271N5	CHORI 240 (H)	180	*CLASS 1(BSC-LIKE SEQUENCE)*
325N22	CHORI 240 (H)	164.5	*PPP1R11, ZNRD1*
326O9	CHORI 240 (H)	165.4	*PPP1R11, ZNRD1*
216F2	CHORI 240 (H)	N/A	PPP1R11, ZNRD1
75O23	CHORI 240 (H)	148.7	*PPP1R11, ZNRD1, GABBR1*
270M16	CHORI 240 (H)	154.5	*PPP1R11, ZNRD1, GABBR1*
203K5	CHORI 240 (H)	220.5	*PPP1R11, ZNRD1, GABBR1*
*137R3C10***	TAMU (A)	106.7	*MOG, GABBR1, OR2H3/H1/H2, UBD, OR6-36, OLF42-1,*
*60R2C11***	TAMU (A)	96.7	*GABBR1, OR2H3/H1/H2, UBD OR6-36, OLF42-1, CBRC7TM_528,*
*154R1C8***	TAMU (A)	107.7	*GABBR1, OR2H3, UBD*
*126R4C11**	TAMU (A)	59.7	*OR216, OR2WP, Tamls 113.3, Class I, OR2B7P, OR1F12, OLFR1535, ZNF 165*
*171R1C12**	TAMU (A)	203.7	*Class I, OR2B7P, OR1F12, ZNF 165, Wilms Tumour Assoc. Protein (Q15007)*
495D10	CHORI 240 (H)	138.3	*Prss 16*
251L24	CHORI 240 (H)	160.9	*BTN1A1*
477K22	CHORI 240 (H)	173	
257D08	CHORI 240 (H)	n/a	*BTN1A1*
457G05	CHORI 240 (H)	170	*BTN1A1, Histone (H5)*
*330R2C11***	TAMU (A)	41.7	*Class I*
588R6C12*	TAMU (A)	60.5	*Class I*
*386R7C9**	TAMU (A)	81.7	*Class I*
*235R8C5**	TAMU (A)	111.7	*Class I (BSX, PPP1R11)*

BACs in italics have been sequenced to min. 2X coverage.
**BACs isolated from McShane et al. (1997)*
***BACs isolated from Newkirk et al. (2000)*

Also, DNA fingerprinting (Figure 9A), Southern blotting (Figure 9B) were performed to confirm overlapping BACs, insert size and gene content.

A.



B.

Figure 9. DNA fingerprinting and Southern blotting. A. DNA fingerprint gel (0.6% agarose) showing *Bam*H1 digest fingerprint patterns for several class I classical and non-classical BACs from both CHORI 240 and TAMU libraries where overlaps are clearly visible. **B.** Hybridization of a class I overgo probe on to a nylon filter Southern blot of the same DNA fingerprint gel confirms the presence of class I genes on 8 separate fragments in 5 of the BoLA class I region BACs.

In addition to analysis of the iCE contigs (Marra et al. 1997) (Figure 10), and comparison of both the CHORI 240 library and TAMU library BACs, a minimum tiling path of 34 overlapping BACs (in addition to 10 BACs from the bovine fingerprint map on iCE) from both libraries for BoLA class I region was produced (Figure 11).

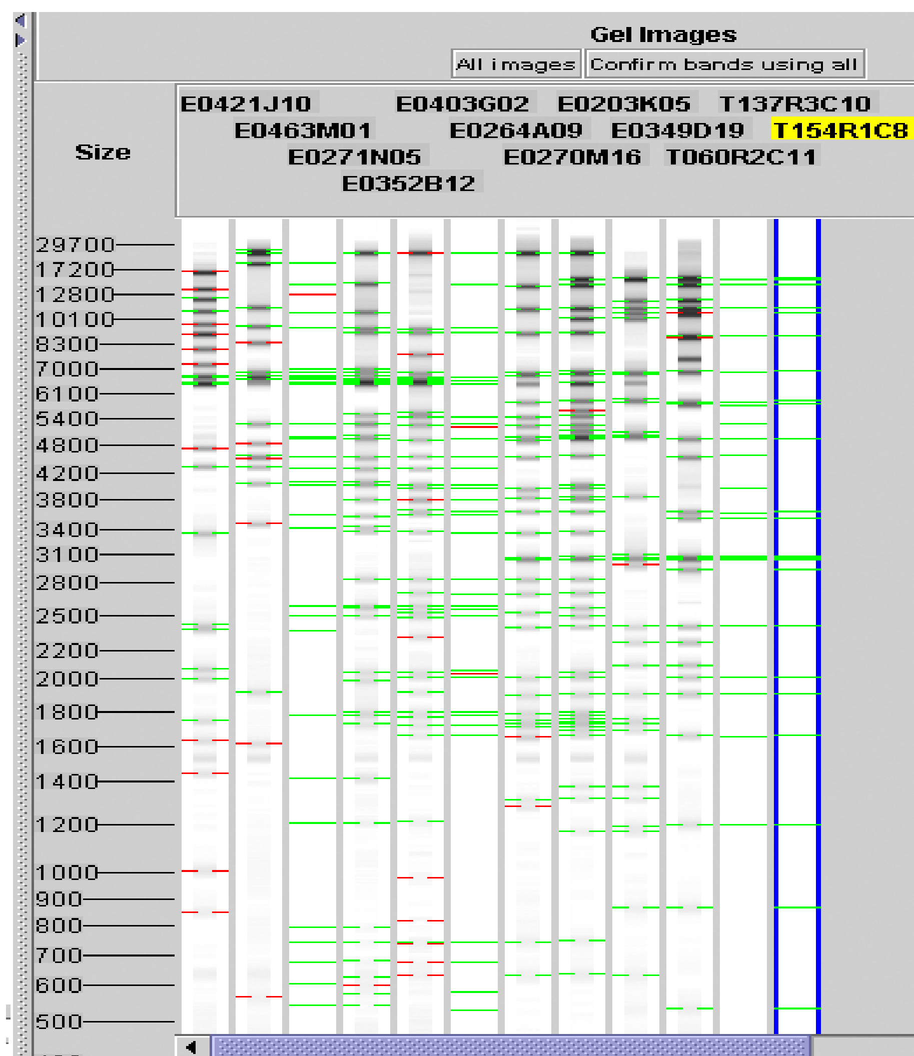


Figure 10. Image of *in silico* DNA fingerprint on iCE. Internet contig explorer (iCE) (Fjell et al. 2003) was used to generate virtual DNA fingerprint display of selected clones in the BoLA MHC class I map using fingerprint maps generated from FPC processed gel images (Marra et al. 1997). Red bands are fragments not shared by neighboring clones, and green bands are shared by at least two clones in the display. The ruler on the left indicates size of fragments. These maps were used to confirm overlaps on selected clones from CHORI 240 (E) and TAMU (T) using software lists of BAC clones available on iCE of clones localized to the BoLA MHC class I region.

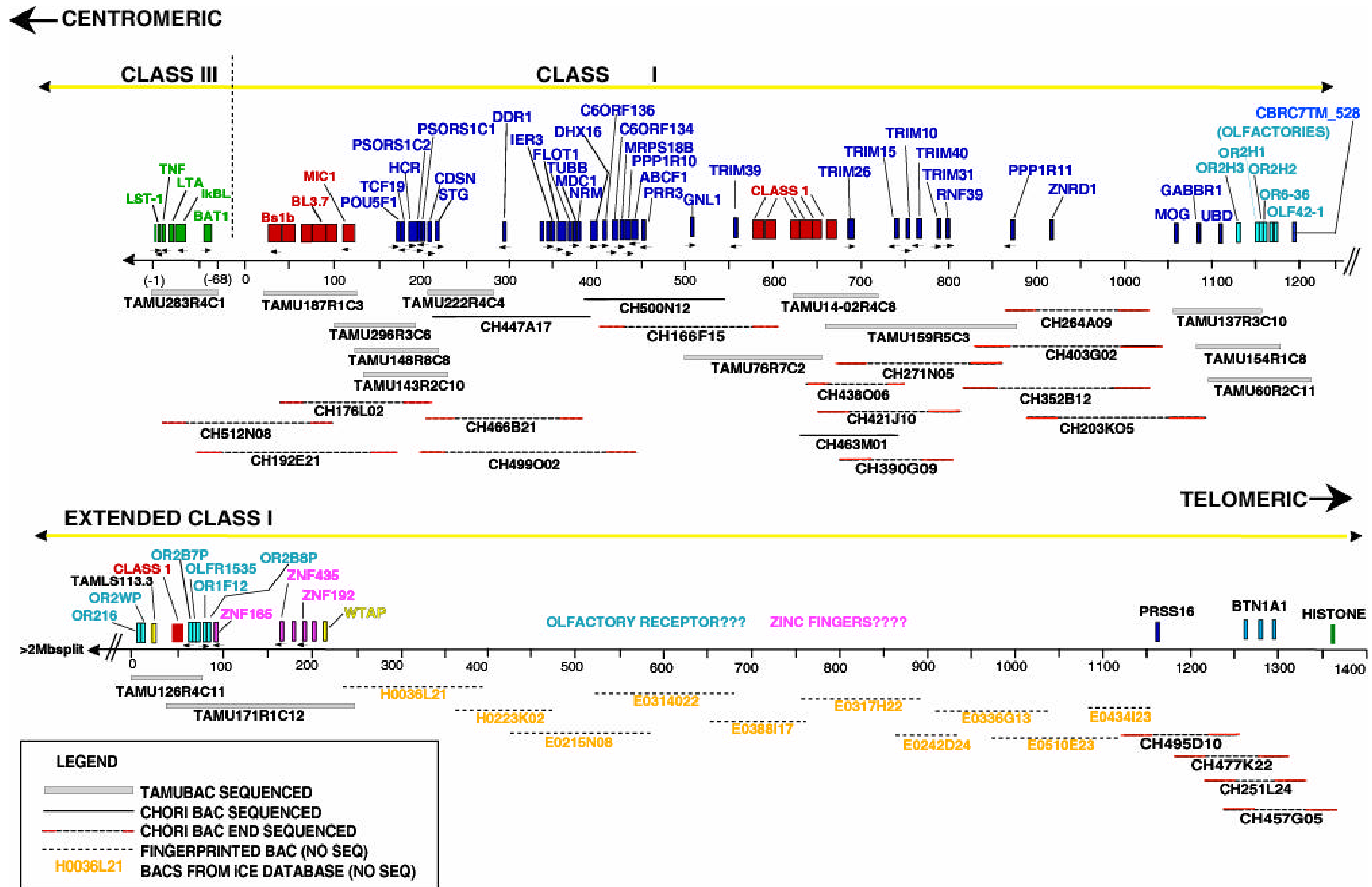


Figure 11. BAC contigs and gene content across BoLA class I region. Illustration of the physical organization of the BoLA class I clusters and framework genes for the minimal tiling path for the MHC class I region in cattle.

Fingerprint assemblies were performed using FPC software (Engler et al. 2003; Soderlund et al. 2000) and default parameters were used with the exception of the cutoff value for the Sulston score, which was selected to avoid false-positive clone overlaps. End sequencing had been completed and shotgun sequences have been obtained for selected clones across the BoLA class I region from two independent BAC libraries, prepared from an Angus bull (Y6- TAMU library) and a Hereford bull (L1 Domino-CHORI 240 library). End sequences were obtained from both the SP6 and T7 ends of 35 CHORI 240 and 14 TAMU library BAC clones in the BoLA class I region, for a total of 98 end sequences. PCR using end-sequence primers was used to identify overlapping BACs and assemble these into contigs to complement the repertoire of available sequences from the TAMU BAC library class I BACs that were already sub-cloned and sequenced. All BAC end sequences have been deposited in NCBI database.

Potential contigs were examined to evaluate consistency of the fingerprint data at potential merge points, and these merges were made only where supported by the fingerprint data. One technique used to assist with the contig merging was sequence similarity between the known HLA sequences and the BoLA sequence by BLAST (Altschul et al. 1990) and by comparisons on MapViewer to provide a more informative means of merging contigs.

The Java-based application iCE (Internet Contig Explorer) (Fjell et al. 2003) was designed to provide views of fingerprint maps and associated data online, including the bovine BAC fingerprint map. It was possible to search for and display individual clones, contigs, clone fingerprints, clone insert sizes and markers for both the TAMU and CHORI 241 library clones from available software lists and view available fingerprints to confirm or complement existing data.

Shotgun sequencing of 4 BAC clones from the CHORI 241 library (512N08, 447A17, 500N12, 463M01) was done to complement the 19 TAMU BAC library clones sequenced by Baylor, containing genetic marker sequences that map within the BoLA class I region. Sequence reads (ca. on average 3× coverage) were obtained for each BAC clone from random 2–3 kb plasmid subclone libraries and attempts were made to

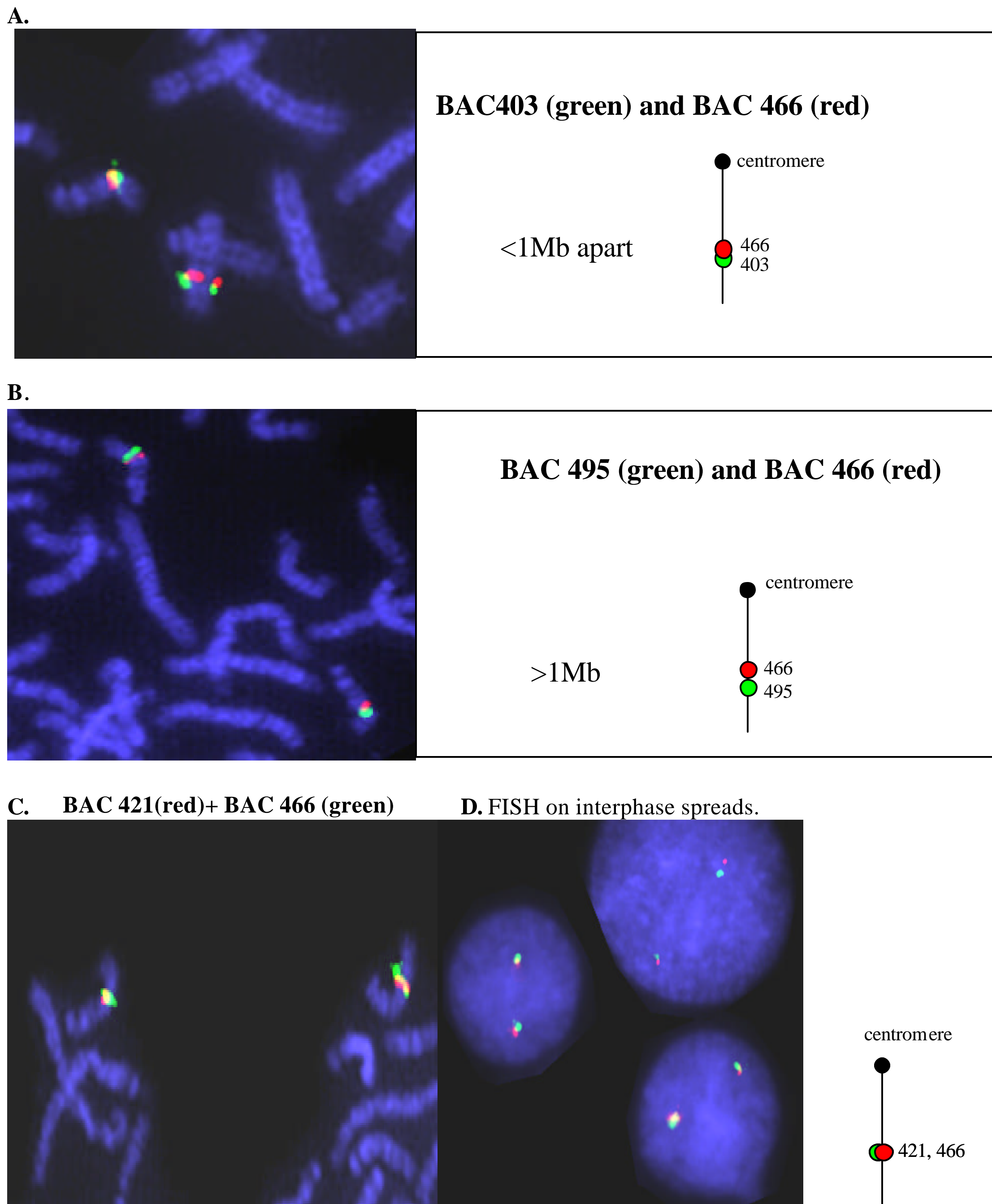


Figure 12. FISH on metaphase and interphase chromosomes with hybridization of BoLA class I BAC clones on BTA 23q21-22. A. FISH image of two CHORI 240 BACs that are less than 1 Mb apart. **B.** FISH image of one of the same CHORI 240 BACs (466, confirmed to have the TUBB gene) and another BAC 495 (confirmed to have PRSS16 gene sequence) located >1Mb away from BAC 466. **C.** FISH on metaphase chromosomes with differentially labeled BAC clones (421 in red and 466 in green) and **D**. FISH on interphase chromosomes with same BAC clones 421 and 466 estimated to be <1Mb apart.

assemble these BoLA class I BACs into a scaffold that would both assist with, and benefit from later whole genome sequencing efforts. Where supported by fingerprint data, gaps between adjacent contigs were removed and the clone groups joined. Additional merges between contig ends were also made based on fingerprint comparisons.

FISH analysis of overlapping BAC clones

FISH staining and analysis on metaphase spreads of bovine chromosomes confirmed the location of BACs in minimal tiling path throughout the BoLA class I region to be on BTA23 adjacent to the BoLA class III region. Figure 12 shows the initial FISH done with selected BACs and estimated distances across BoLA class I on BTA 23 q2.2-q2.3. Fiber FISH analysis was also done on several of the BACs in the minimum tiling path and it was possible to show overlap between BACs that were no more than 300kb apart. Visible overlaps between the class III-I bridging BAC 283 and BAC 512 (class I BAC) and 264I3 (class III BAC) are clearly illustrated in Figure 13.

Discussion

A minimum tiling path of 36 BACs from both the TAMU and CHORI 240 libraries that span the BoLA class I region has been produced. Using a rigorous methodology including fingerprint, hybridization and PCR data, these were assembled into 2 major contigs across the class I and extended class I regions. Since fingerprint fragment sizes and size of insert are known for each BAC, the largest BAC clones were selected for BAC end sequencing from a list of overlapping BACs. Using results from FPC, and *in silico* representations on iCE, it was possible to skip the second step and check the individual BAC directly. Marra et al. (1999) showed that the FPC program does a good job in putting overlapping BACs in the same contig, but their order within the contig has to be corrected manually. This technique was very useful to confirm and extend contigs using FPC data to supplement data obtained in this study.

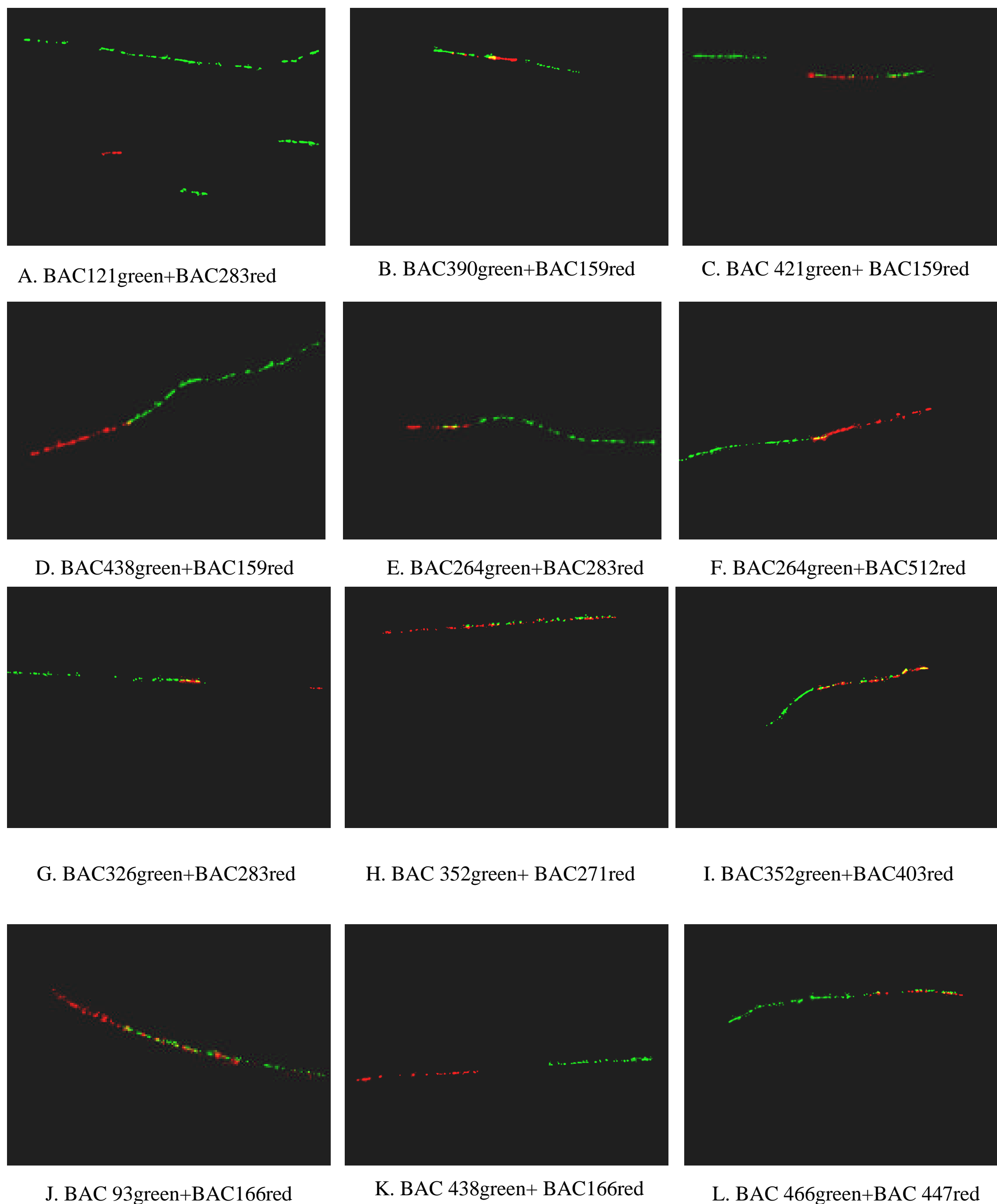


Figure 13. Fiber FISH for select BAC clones. DNA fibers hybridized with differentially labeled BAC clones (either dig (red) or biotin (green)) shows a range of overlapping signal (yellow) on panels B-J and L. A gap is clear between two BAC clones in A and K panels. Although BAC clones in the K panel do not overlap, they are at most 500 kb apart since these are seen on the same DNA fiber.

This chapter describes a nonradioactive, agarose gel-based, high-throughput DNA restriction digest fingerprinting methodology first described by Marra et al. *(1997)* for use in the construction of high-resolution physical maps from low-copy-number, large-insert clones. The procedure is robust and allows for the recovery of clone insert size information. Initially used to construct sequence tag site (STS)-based contigs, the methodology has also been applied to whole-genome, random-clone strategies that have resulted in the construction of high-resolution, sequence-ready physical maps of the genomes of *Arabidopsis thaliana*, human, *Caenorhabditis briggsae*, and *Cryptococcus neoformans* (Marra et al. 1997). The methodology is currently being employed in the construction of physical maps for several other large, mammalian genomes, such as those of mouse, rat and bovine.

Although end sequencing was used quite often to confirm overlaps, many of the individual BAC ends had repetitive sequence that made them unsuitable for use in contig merging. This was due to the CHORI library being created with *Mbo*1, which preferentially cuts at sites of repeat structures, so the ends of the inserts are enriched for repetitive sequence creating a biased distribution (D. Adelson, personal communication). There was also a very limited coverage of shotgun cloned BACs, averaging ca. 3x coverage of the TAMU library BACs which ranged in insert size from 59.6kb to 216.7kb and 2x coverage of the 4 CHORI 240 library BACs (average insert size 154kb). Thus, assembly into large scaffolds has not been possible, suggesting that repetitive sequences within clones are so abundant as to make it difficult on assembly of the contigs. One of the TAMU library BACs (159) that was critical for the merging of the class I containing contigs is currently itself split into 61 sub-contigs. An attempt was made to combine enough sequence reads from each of these BACs to represent ca. 3× coverage and to perform an assembly to determine if they would assemble individually or if repetitive sequences would interfere with proper assembly, but this was also not complete and mirrored the individual assemblies.

Another aim of the current project was to hybridize BoLA BAC clones to metaphase chromosomes using FISH, and to confirm overlapping BACs within 300kb of

each other by Fiber FISH. These data obtained for the BoLA class I region, will greatly assist with orienting scaffold assemblies generated from WGS currently underway at Baylor College of Medicine. A core set of clones containing class I or framework genetic marker sequences were identified based on BAC end sequence analysis and were given priority for FISH mapping. Fiber FISH analysis on the 34 clones of the minimum tiling path was more difficult as several of the BACs seemed to have repetitive sequence and were difficult to FISH, exhibiting non-specific staining in other chromosomal locations in addition to the BTA23q21-q22 region where one would expect BoLA I BAC clones to be located.

There are some confounding factors produced when using two libraries from a total of four different animals with varying class I genotypes and/or serotypes. Although class I sequence was obtained from both L1 (CHORI 240 library Hereford bull), Y6 (TAMU library Angus Bull), Longhorn and Angus females contributing BACs 14-02 and 7138-58 and even the phage library from Bull 86, assignment of allelic versus loci designation for these sequences was not possible, since these animals are all different haplotypes.

Assignment of location of BAC clones within the BoLA class I region was based on a rigorous methodology using a compilation of data including hybridization, PCR, end sequencing and FISH; which allowed greater confidence in contig merging and the ordered BAC array. For example, BSA and BSC are PCR amplified from CHORI BACs 463, 421 and 438 which confirms the original class I hybridization signals detected, and location of these CHORI 240 BACs fit in the tiling path. TAMU library BACs 588, 386, 7138-58 and 330 were all found to be positive by hybridization for BoLA class I (Newkirk 2000) and sequencing data corroborates class I sequence by nucleotide alignments within all these BACs, however we are unable to place these unto the current BAC array, indicating the haplotypic difference of class I sequence between animals may be a confounding factor. It is known that variable numbers of class I genes are expressed from MHC haplotype to haplotype in cattle, although serotypic data may suggest animals are homogeneous (Ellis et al. 1999; Garber et al. 1993; Garber et al.

1994; Holmes et al. 2003). Also, the A14 serotype of animals has up to three class I genes expressed while A11 has only one class I gene expressed.

There is more evidence for intralocus recombination in the MHC than for interlocus recombination. However, as in most multi-gene families, sequence analyses have suggested that such events have occurred in the past. For example the human class Ia HLA-A locus arose from a recombination between (1) a 5' region (exon 1 through exon 3) of a gene related to the other human class Ia loci HLA-B and –C; and (2) a 3' region (from intron 3 to the end of the gene) from a gene closely related to the human class I pseudogene HLA-70 (Hughes 1995). Similarly, we see evidence for interlocus recombination from the cattle BSA and BSC genes, where BSC arose from a recombination a segmental exhange between the 5' region and ARS region (exons 1-3) of a haplotypic cluster related to B13.7 and the 3' region (exons 3-UTR) from another phylogentic cluster containing BSA (Garber et al. 1993).

From the human, mouse and rat MHC complete sequence it is known that this region, between GNL (HSR1) and MOG contains up to 50 classical class I genes, pseudogenes and fragments (Amadou et al. 1999; Hurt et al. 2004; The MHC Consortium 1999) arranged in three clusters within what is defined as the "kappa" and "alpha" blocks within the frame work genes (Amadou 1999). Of note is that the BoLA MHC class I seems to have another cluster of class I sequence telomeric of MOG located within the olfactory receptor genes, UBD and TAML 113.3 microsatellite located on BACs 171 and 126; this is similar to a class I cluster found in both rat and mouse MHCs (Amadou et al. 2003; Hurt et al. 2004; Takada et al. 2003) in physical location, however the exact number of olfactory genes may not be as greatly duplicated as in the rat but this is unknown (See Figure 14).

Sequence comparisons show that the class I gene in this area is similar to classical class I sequence, however without fully characterizing it we are unable to determine if it is a functional gene, a pseudogene or a fragment. Cattle, swine, human, mouse and rat MHCs also share a cluster between BAT1 and POU5F1 that has non-classical and class I like sequence (MIC genes) and classical class I sequence in HLA

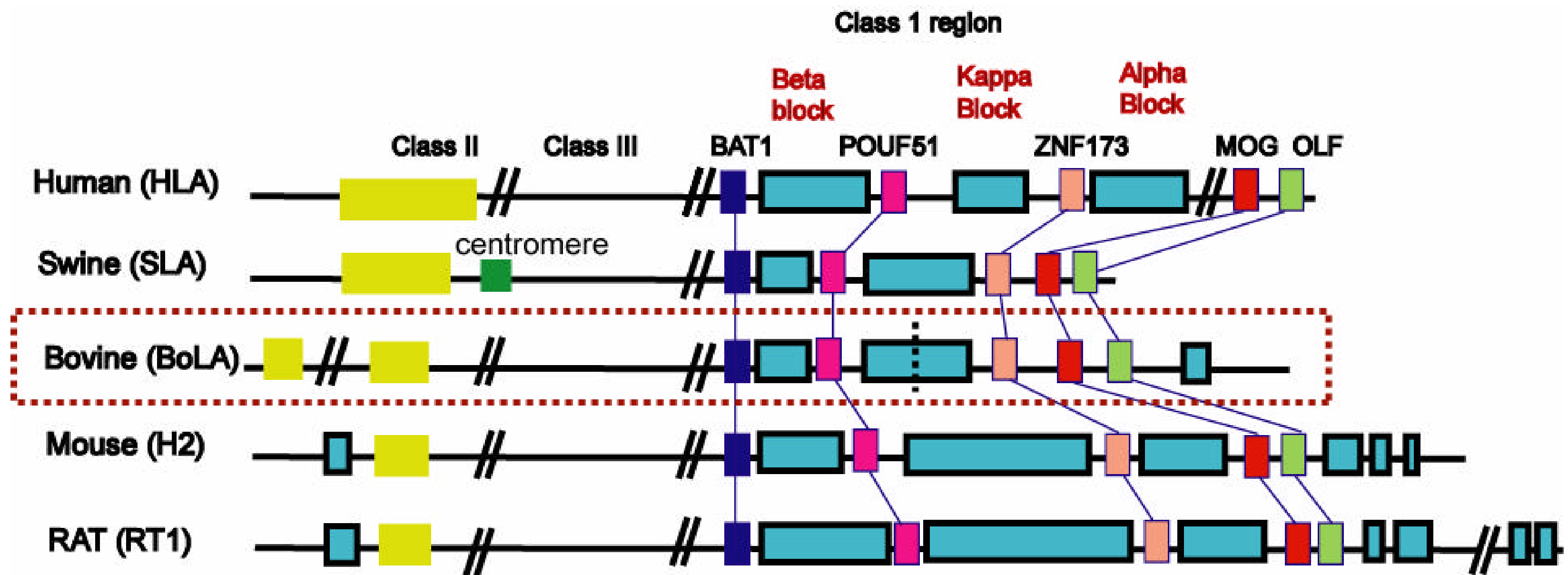


Figure 14. Clusters of MHC class I genes. Illustration of the comparative distribution of clusters of class 1 genes in human, swine, bovine (circled in red), rat and mouse MHC regions. The BoLA MHC class I region has 3 main clusters of class I genes with the second cluster, possibly divided into two but remains undefined (dotted black line). Of note is the third cluster on the telomeric end of BoLA distributed between olfactory genes, which are similar to a cluster that is highly duplicated in mouse and rat, but not found in swine and human. The framework genes defining the class I clusters are indicated as are the relative size of the class I and class II gene clusters.

called the "beta" duplication block. In BoLA, by sequence similarity analysis, this cluster was found to have BL3.7, Bs1b and MIC 1 sequence in the BACs from the CHORI 240 and TAMU libraries.

The data in this study supports previous data that there is at least 400kb of sequence that holds class I or class I-like genes and pseudogenes in BoLA between GNL and ZNF 173 (Di Palma et al. 2002; Holmes et al. 2003). It goes further to confirm at least three clusters of class I genes present in the BoLA class I region (Figure 16). The first cluster is adjacent to the class III region and holds the BL3.7 and MIC1 genes. The second cluster could possibly be separated into two clusters. The class I sequence represented on CHORI BACs 166F15, 390G9, 438O6 and TAMU BACs 76, 14-02 and 159 could possibly be in a separated cluster flanked by the Trim 39 (RNF23) and Trim 26 (ZNF173) framework genes. The possible third cluster shares TAMU BAC 159 with the second, but has some overlapping group of BACs that seem to be distinct from cluster 2. One of these problem BACs (CHORI 463M1) contains class I sequence (at least 2 genes) but seems to have very complex repeat structures that make it impossible to achieve clear FISH data that confirms end sequence and fingerprint data. This cluster also has CHORI library BACs 271N5 and 421J10 that have at least some fragment of class I sequence, that contig with BACs 264A9, 403G2, 352B12, 270M16 and are flanked by TRIM 15 (ZNFB7) and MOG telomerically. This contig also extends to the TAMU BACs 137, 60 and 154 that contain MOG, GABBR1, UBD and olfactory genes.

The estimated size of the class I BoLA region between the framework genes BAT1 to MOG is probably no more than 1.5Mb, then from MOG to the olfactories for 200kb. From MOG to the extended class I region is a little more difficult to estimate as the contigs do not overlap completely, especially beyond ZNF165 into the extended class I region with BTN1A1 and the histones. A small contig of two TAMU BACs is located telomeric of the olfactory genes, which are BAC126 (containing microsatellite TAML113.3 and zing finger genes including ZNF 187) and BAC171 (containing zinc finger genes 193, 165 and 184). From iCE and FISH analysis however, it would seem that there could be as much as 1Mb between BAC 171(containing ZNF165) and BAC

495D10 (containing PRSS16) and it is possible to join these two groups into one contig using the bovine DNA fingerprinting data available on iCE. The final group of the second contig is in the telomeric extended class I region, made of CHORI BACs 495D10, then 477K22 and 251L24 (containing BTN1A1) then lastly 457G05 (containing BTN1A1 and histone 5 sequence). BTN1A1 encoded butyrophilin which was originally purified from cow's milk and was subsequently identified as the predominant protein constituent of milk in other mammals (Jack and Mather 1990).

BTN1A1 has been mapped to the extended MHC region of chromosome 6, 400kb centromeric of the Hfe gene, which was sequenced revealing a cluster of at least 5 butyrophilin-like genes, which appear to be widely expressed (Banghart et al. 1998). Interestingly, the N-terminal domain of butyrophilin is similar to rat myelin/oligodendrocyte glycoprotein (MOG) and chicken B blood group system (B-G) protein (Pham-Dinh et al. 1993; Salomonsen et al. 1991). It is therefore possible that the butyrophilin gene is the product of an exon shuffling event which occurred between ancestors of the RFP and MOG genes, which is an example of the colocalization of a chimeric gene and its putative progenitors (Vernet et al. 1993).

Also of interest in this study is that attempts to localize a BAC clone containing RFP sequence resulted in only one positive BAC by hybridization and PCR (CHORI 499O2) which maps to the centromeric end of BoLA class I, overlapping BACs TAMU 143, 148, 222 and CHORI 447A17 and 466B21. This indicates that there might have been a possible translocation of RFP like sequence to the class III/I boundary in Bovidae. Evidence from RT1 and H2 show no indication of an RFP gene telomeric of MOG (Hurt et al. 2004). Interestingly, an additional butyrophilin-like sequence is found at the class II/III boundary in mouse, rat and human (Beck et al. 1999, Hurt et al. 2004). A strong duplicative nature is not uncommon in superfamilies of genes like butyrophilin genes, which are members of the immunoglobulin superfamily and related to the B7 family of co-stimulatory molecules (Linsley et al. 1994).

When the genomic sequences of a 433-kb segment located between the non-classical and classical SLA class I gene clusters in pig was compared in terms of

genomic organization and diversity with the orthologous region of the HLA complex (Shigenari et al. 2004) its length was 433 kb compared with 595 kb in the corresponding HLA class I region. This 162-kb difference in size between the swine and human genomic segments can be explained by indel activity, and the greater variety and density of repetitive sequences within the human MHC.

Twenty-one swine genes with strong sequence similarity to the corresponding human genes were identified, with the gene order from the centromere to telomere of HCR - CAT56. The human SEEK1 and DPCR1 genes are pseudogenes in swine. In this study, a single contig spanning the region between the non-classical BoLA class I MICA to the first cluster of Class I sequence is estimated to be 450-500kb based on insert size of BAC clones in the minimal tiling path. Within this region at least 18 genes from POU5F-TCF19-SPR1-CDSN-STG-RAGC-DDR1-IER3-FLOT1-TUBB-NR-DDX16-K1AA1949-PP1R10-ABCF1-GNL-Q9H63-RNF23 (Trim 39) have been confirmed to be in contiguous sequence contained within 3 overlapping BACs (TAMU 143, CHORI447A17 and 500N12. It can be concluded that the region is highly conserved in BoLA as well, and homologous to the corresponding region located between the HLA-C and HLA-E genes in the human and the non-classical and classical SLA genes.

The contig flanked by BL3.7 in the centromeric end at the Class III/I border till MOG is estimated to be approximately 1Mb which is equivalent in size to the homologous region in HLA (900kb) which is reduced in the swine (500kb) (Renard et al. 2001). In addition to this 1Mb, if the BACs containing MOG and several olfactories were added to this contig by overlapping STCs and FISH, the region is extended by an additional 300kb. A gap greater than 2Mb separates the next contig that is about 250kb in length containing TAML 113.3 and the zinc fingers. The next contig is separated by a region of 1Mb that has been defined by overlapping BACs in the Bovine fingerprint Map available on iCE in between the flanking BACs on the second contig, CHORI BAC 495D10 and TAMU BAC 171. The final group of BACs in the second contig is on the telomeric end ending at the histones but before HFE that was not included. In total, the extended BoLA class I seem to extend to a minimum of 6Mb of known overlapping

sequence, possibly extendable by several Mb in the unknown gap area between TAMU BACs 60 and 126.

There is evidence of at least 20 class I -like sequences (classical, non-classical, pseudogenes and gene fragments) in the cattle MHC (Ellis et al. 1999). It seems that at least 6 of these are expressed, but data relating serotype to genotype remains perplexing (Ellis and Ballingall 1999; Ellis et al. 1999) as individual haplotypes express anywhere between 1 and 3 of these in varying combinations. From this analysis, Southern blotting showed variable fragment numbers in various BACs fingerprinted from both TAMU and CHORI 240 libraries and amplification from genomic DNA with 'locus-specific' primers suggests genes not expressed are absent from that animal's haplotype which supports previous research.

The presence of more than 3 classical class I genes could increase MHC diversity within BoLA, and increase population fitness (as even inactivated genes could perform this function). The functional implications of variable class I haplotypes (including single class I locus haplotypes) in cattle, in terms of individual and population fitness, are at present unknown. These genes may vary in terms of: polymorphism, expression levels and even specific function. There appear to be at least 4 polymorphic loci (Ellis et al. 1995) and from serological analyses we know of at least 50 specificities (Davies and Antczak 1991a; Davies and Antczak 1991b; Davies et al. 1994; Garber et al. 1993; Garber et al. 1994). Since different parasites generate different antigenic fragments, and MHC molecules differ in the efficiency with which they bind particular sequences of amino acids in these fragments; therefore, some MHC products would be better than others at presenting parasite antigens to the immune system. It is conceivable that the high levels of what seems to be redundancy seen in cattle MHC class I is an evolutionary advantage in having a greater repertoire peptide binding capability with which to stave off infection and to have an overall optimization of immune response in a given population.

Evidence comes from studies on individuals infected with the human immunodeficiency virus (HIV) where possessing a particular haplotype (HLA-B*4405)

confers resistance to developing AIDS as compared with another HLA-B molecule (HLA-B*4402) (that differs by a single amino acid) (Zernich et al. 2004). This single amino acid polymorphism that distinguishes HLA-B*4402 (Asp116) from B*4405 (Tyr116) permits B*4405 to constitutively acquire peptides without any detectable incorporation into the transporter associated with Ag presentation (TAP)-associated peptide loading complex even under conditions of extreme peptide starvation. This mode of peptide capture is less susceptible to viral interference than the conventional loading pathway used by HLA-B*4402 that involves assembly of class I molecules within the peptide loading complex (Zernich et al. 2004). Also, when stickleback fish, which differed in number of class II alleles, were exposed to three types of parasites, fish with 5-6 alleles resisted infection significantly more fish with fewer (or more). So it may be the great diversity of class I and class II alleles in the population that has helped to reduce rate of pandemics and ensure that no single parasite or pathogen can decimate the population. Thus, resistance conferred by specific alleles to temporally variable pathogens may contribute to the observed polymorphism at MHC genes and other similar host defense loci in other species such as cattle.

Future systematic mapping to detect haplotypes that confer susceptibility to common diseases requires the construction of a fully informative polymorphism map. In humans, a 4.75 Mb of contiguous sequence for each of two common MHC haplotypes, to which susceptibility to >100 diseases has been mapped was sequenced using a BAC-based map (Stewart et al. 2004). There were > 18,000 SNPs detected in this region, with an average SNP densities ranging from less than one SNP per kilobase to >60. This may suggest that such detailed mapping to investigate disease association in cattle would require that every base pair of the genome would be sequenced in many individuals. This may not be necessary however, as using the comparative map produced here, polymorphic blocks within BoLA MHC can be isolated and selected for more detailed analysis based on disease associations known in cattle, as well as the vast information available from the studies on H2, HLA, SLA and RT1 and the MHC of other species. For instance, BoLA class I haplotypic structure may be defined using microsatellite

markers, and used to genotype animals to resolve loci and investigate susceptibility or resistance of haplotypes with disease associations as performed for the HLA (Li et al. 2004; Rubio et al. 2002; Rubio et al. 2004).

This study provides evidence to support the presence of at least 3 clusters of class I genes with a number of classical class I, non-classical, pseudogene and gene fragments distributed within these clusters. More questions arise as it is now known that all loci do not seem to be equally represented in various haplotypes as related to earlier expression data from other groups documenting expression of 1 to 6 class I loci in bovids (Ellis and Ballingall 1999; Ellis et al. 1999).

Paired BAC end sequence data are extremely important as sequence-tagged-connectors to assist in assembling scaffolds for WGS sequencing projects and specific-region sequencing projects, as with the BoLA class I region. These data, when paired with sequencing of BAC clones, can also provide valuable preliminary information on genome structure including, for example, repetitive element type, frequency and variability as well as putative open reading frame frequency.

The availability of both a fingerprint map and sequence assembly for the bovine genome from WGS sequencing would provide an opportunity to derive a direct link from the MHC class I BAC clones, and vice versa. This linkage is useful for identification of clones for use in functional studies of class I genes, determination of the sequence content of BAC clones of interest identified by BAC filter hybridizations, and access to sequencing substrates representing regions of interest within BoLA where current sequence coverage or quality is insufficient for analysis. Examination of the linkage between the map and the sequence is likely to identify BAC clones spanning gaps in the sequence assembly. Additionally, map contig overlaps can be identified by sequence where the extent of overlap is insufficient to detect with confidence using fingerprint similarity alone. Furthermore, any discrepancies in the fingerprint map and the sequence assembly locations would identify potential misassemblies in either the map or the sequence. Analysis and resolution of these discrepancies would serve to improve the quality of both the sequence assembly and the fingerprint map.

Comparative genomics is a powerful tool for utilization of the existing knowledge of well-studied species, such as humans and mice, to acquire genomic data in species like cattle, for which there is less information. BAC end sequencing of clones from three independent BAC libraries, limited contig assembly within sequenced BAC clones, coupled with fingerprint mapping and confirmation by FISH, allows for rigorous validation of the physical map of the BoLA class I region presented herein. In conjunction with bovine WGS sequencing and framework BAC sequencing currently being done at Baylor, completion of the sequence assembly of the BoLA class I region, using the physical map as a scaffold upon which to build, can be done elegantly and efficiently.

Web-based programs used:

IMGT: http://www.ebi.ac.uk/imgt/hla/stats.html
BLAST: http://blast.wustl.edu or http://www.ncbi.nlm.nih.gov/blast
Repeatmasker: http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker
Overgomaker: http://www.genome.wustl.edu/tools/?overgo.html
BAC CHORI library resources: http://bacpac.chori.org/bovine240.htm
Web-based FPC physical maps: http://www.genome.clemson.edu/fpc/
FPC home page: http://www.genome.arizona.edu/software/fpc/
Ensembl genome browser: http://www.ensembl.org
NCBI Map Viewer: http://www.ncbi.nlm.nih.gov/mapview/
iCE Home Page: http://ice.bcgsc.ca
Image—the fingerprint image analysis system: www.sanger.ac.uk/software/Image

CHAPTER III

A SIMPLE BAC END SEQUENCING METHOD USEFUL FOR BAC CONTIG ASSEMBLY

The real voyage of discovery consists not in seeking new landscapes but in seeing with new eyes.

- Marcal Proust

Summary

Genome-mapping efforts can require characterization of target DNA inserts by end sequencing of bacterial artificial chromosome (BAC) clones. Challenges to end-sequencing difficult templates have traditionally required costly modifications. The role of BAC end-sequencing is critical in whole genome or regional sequencing projects, as sequence tag connectors (STC) obtained allow production of a minimal tiling path of overlapping regions. These overlaps can be used to limit the number of clones to be sequenced and minimize overall sequencing costs. Problems can arise in end-sequencing of BAC clones due to complex template structure and size of insert. Here, we describe a simple, cost-effective technique to read through BAC end sequences with a robustness that consistently produces longer read lengths at a high quality (Phred 20 >700 bp).

Introduction

The initial foray into sequencing of the human genome required the use of several different chromosomal maps and honing of techniques to rapidly sequence three billion bases of DNA. One of the most useful of these resources, the BAC (bacterial artificial chromosome) maps allowed for megabase-scale assemblies of overlapping DNA clones into contigs. The BAC is a vector used to clone DNA fragments in *Escherichia coli* cells, based on naturally occurring F-factor plasmid found in the

bacterium *E. coli* (Shizuya 1992). BACs, typically contain 100- to 200-kb inserts of DNA, were produced as larger, more stable recombinant DNA clones than those previously available during the human genome sequencing project (HGS), representing the human genome more uniformly than previous systems (Poulsen 2004).

BAC-end sequences or sequence tag connectors (STCs) obtained from both ends of the BAC clone can be used in many applications to enrich whole genome sequencing (WGS) or other sequencing projects. Once any BAC or other large segment of DNA is sequenced to completion by conventional shotgun approaches, these STC tags can be used to identify a minimum tiling path of BAC clones overlapping known sequence for targeted sequence extension. In the case of the HGS using STCs as a means of identifying minimally overlapping clones represented only 1%-3% of the total cost of sequencing the human genome and provided resource to reduce total sequencing cost (Siegel et al. 1999).

STCs help researchers expand contigs and spacing helps to verify the contiguous sequence created by assembly software. STCs can also be used to physically define and "capture" gaps that occur when sequencing biochemistry is stalled by occasional difficult-to-read stretches of DNA sequence. STC reads can also be used for the design of sequence tagged sites (STSs) to be used as markers to construct the RH maps that complement contig building. STSs generated from BAC STC reads can also enrich RH maps. Conversely, a mapped STS can be used to isolate a BAC (or any DNA clone type) from a library of clones representing a genome. After maps are constructed, redundancy is unnecessary for a complete representation of the genome once the minimal tiling path has been developed from BAC contigs.

BACs libraries from the human, murine, rat, canine, and baboon genomes with average insert sizes ranging between 160 and 235 kb have been produced (Osoegawa et al. 1998). Additional libraries have been made for many other plant and animal species, including those with complex genomes like wheat (Safar et al. 2004). BAC-based fingerprint physical maps have been produced for human (IHGSConsortium 2001; Krzywinski et al. 2004a), mouse (Gregory et al. 2002), rat (Krzywinski et al. 2004b;

Schibler et al. 2004) to date, with projects in other species currently underway using high-throughput fingerprint technology (Marra et al. 1997).

Coupled with additional information from fingerprint maps and mapping of expressed sequence tags (ESTs) to chromosomal locations, enrichments of existing BAC maps would allow for more easy assembly. For a typical mammalian genome, assuming 200 kb clone inserts, approximately 21 000 clones are sufficient to represent the genome (Krzywinski et al. 2004a) which is a substantial reduction from the size of the parent library or libraries used to construct the map.

Ease of access of information from end sequence of BAC clones that are commonly stored on databases available online, can be used in a wide array of applications from genome sequencing to gene discovery. Researchers can readily access database information as well as individual BAC clones for analysis. Minimally overlapping clones can be identified and used as a source for shotgun-sequencing projects, to find clones for restriction fingerprints for building overlapping clone sets, to find appropriate clones for fluorescence *in situ* hybridization mapping, or to select a BAC clone that contains genes of interest (Poulsen 2004). BAC clone tiling sets have uses other than supporting sequencing efforts, ranging from focused studies on the structure and function of individual genes and gene families to fluorescence *in situ* hybridization (FISH) and BAC array comparative genomic hybridization (Gustafson et al. 2003; Pinkel et al. 1998).

Another use of BAC end-sequencing is in studies investigating genome rearrangements important in evolution (McShane et al. 2001), cancer and other diseases. Precise mapping of the rearrangements is essential for identification of pertinent genes. End-sequence profiling (ESP) is accomplished by constructing a BAC library from a test genome, measuring BAC end sequences, and mapping end-sequence pairs onto the normal genome sequence. Plots of BAC end-sequences density identify copy number abnormalities at high resolution and BACs spanning structural aberrations have end pairs that map abnormally far apart on the normal genome sequence. These pairs can be sequenced to determine the involved genes and breakpoint sequences. End sequencing of

approximately 8,000 clones (0.37-fold haploid genome clonal coverage) produced a comprehensive genome copy number map of the breast cancer cell line MCF-7 genome at better than 300-kb resolution and identified 381 genome breakpoints, and verified by FISH mapping and sequencing (Volik S 2003). These latter applications are particularly important for biomedical research.

There are many advantages to using *E. coli* as the model host in BAC libraries including rapid growth, high stability of the DNA fragment during growth, few chimeric clones, as well as easy and rapid purification of the BAC DNA (Poulsen 2004). The isolation of BAC DNA from *E. coli* is facilitated by using an alkaline method for purification of plasmids. To ensure that the BAC DNA is pure enough to be used for downstream application, many modifications on standard alkaline lysis preparations have been developed that achieve best quality BAC DNA. Plasmid prep kits have been produced from a variety of manufacturers to facilitate BAC DNA preparations in 96-well format or in large volume midi/maxi preps (Millipore, Qiagen) Good template quality of large-insert template or genomic DNA is of primary importance for generating consistent DNA sequencing results. BAC DNA presents are particularly challenging templates that traditionally require specialized and costly modifications to sequencing reaction chemistries or sequencing protocols and large amounts of template.

Routinely, large quantities of expensive sequencing reagents are used to overcome BAC template restrictions. In most instances as much as 8-16ul of BigDye™ has been used in higher volume sequencing reactions (40ul) containing as much as 3 ug DNA and primer in excess (80 pmol) in protocols in published literature (Crabtree et al. 2001; Schibler et al. 2000; Volik S 2003) or available online at http://www.genome.wustl.edu/tools/protocols/mapping/bacendsequencing.pdf).

For example, Crooijmans et al. (2001) used a BAC DNA isolation REAL Prep 96 plasmid kit (Qiagen) for DNA preparation, and 40ul BAC end sequencing reactions consisting of 16ul BAC DNA and 8ul BIG Dye™ and 8ul Half big dye 8ul of M13 F or R (10pmol/ul). This is a standard protocol that uses large quantities of expensive components.

In a recent survey of BAC end sequencing methods, primer and template quality was given as the determining factors in obtaining quality sequence, as ranges of conditions varied greatly from technique to technique (see Table 5) (https://dna.biotech.wisc.edu/documents/BAC_Sequencing_Information.htm).

Table 5. Summary of informal survey results on BAC end sequencing protocols. A large range of conditions are used by researchers to generate BAC end sequences some larger volume reactions requiring large amounts of DNA, primer and reagents. Adapted from https://dna.biotech.wisc.edu/documents/BAC_Sequencing_Information.htm.

Parameter	Range
Primer length:	18-24 nts
DNA amount:	0.2-5.0 ug
Primer amount:	10-30 pmol
Reaction volume:	10-40 ul
Denaturation	95-98° for 10-60 sec
Annealing	40-55° for 5-60 sec
Extension	60° for 4-5.5 min:
Cycles	25-99

Sequence read length and quality are of prime importance in BAC end sequence analysis to make it cost-effective as a tool for supplementing WGS , mapping and other analytical efforts. It is especially of importance when end sequencing difficult template like BAC DNA, especially when inserts contain genomic DNA from regions like the BoLA MHC class I region with high GC-rich areas and abundant repeat structures. Here, a simple technique for BAC end sequencing is described using DNA from a standard preparation by column filtration allowing for small volume sequencing reactions that consistently gives high quality reads for end sequences obtained from BoLA class I containing BACs.

Materials and Methods

BAC DNA isolation

A QIAGEN Plasmid Midi Kit (Qiagen, Valencia, CA) was used to prepare BAC DNA suitable for end sequencing, with slight modifications to the manufacturer's recommendations. Single colony BAC clones from the bovine CHORI 240 library were selectively isolated based on BoLA class I gene content, inoculated into 2ml starter cultures and incubated for 8 hours at 37°C. Subsequently, 500ul of this culture was used to inoculate 100 ml of LB broth with 12.5 mg/ml chloramphenicol which was incubated at 37°C shaking at 320rpm for 16 hrs. BAC DNA was isolated with 10 mls of each buffer P1, P2, P3, then SDS was discarded using filters following centrifugation. The elution step was performed using 5×1 ml of 65°C preheated buffer P5. After isopropanol precipitation, DNA pellet was then subjected to a second ethanol precipitation, followed by dissolution in milli-q water to a concentration of 250ng/ul. An additional polyethylene glycol (PEG) purification step was then performed to further purify DNA for downstream analysis. Briefly, BAC DNA (dissolved in milli-q water) was incubated with 0.16 volumes of 5M NaCl and 0.25 volumes of 13% PEG on ice, followed by a wash in 70% ethanol and resuspension in milli-q water to desired concentration. Typically, yields of 20-25 µg were obtained with this protocol. Purity was estimated by spectrometry and visualization on an agarose gel (0.75%) with ethidium.

BAC end sequencing

Three methods of BAC end sequencing were performed on the same DNA isolated from 43 individuals. For METHOD 1, sequencing reactions (10µl) were performed using 2µl BigDye™ (Applied Biosystems), 2µl *half*BD™ and 0.5µl MasterAmp™ PCR Enhancer (Epicentre, Madison, WI), with approximately 1µg of BAC DNA and 10 pmol of T7 (5'-ATTTAGGTGACACTATAG-3') or SP6 (5'-TAATACGACTCACTATAGGG-3') primer per reaction. The thermal profile was 96 ºC for 5 mins, then 8 cycles of 95ºC (0.25sec), 58ºC (0.25sec) with an increase of 1ºC per

cycle, 65ºC (4mins); followed by 60 cycles of 96ºC for 25 sec, 55ºC for 25sec and 60ºC for 4 mins; and finally 4 holds of 96ºC (1min), 50ºC (1min) and 65ºC (1min) then 4ºC until reactions are removed. Reactions were purified with G-50 sephadex columns (Biomax, Odenton, MD). Sequence fragments were separated and analyzed using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), and are available through GenBank.

A slight modification in thermocycling parameters was done in Method 2 as described in Goto et al. (2003). Sequencing reactions (10ul) were performed using a 2ul of BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) with approximately 1 ug of BAC DNA and 10 pmol primer (T7 or SP6) per reaction. The thermal profile was 96 ºC for 5 mins followed by 35 cycles of 96ºC for 30 sec, 55ºC for 5sec and 60ºC for 4 mins.

Method 3 used less BAC DNA (500ng) in a 20ul reaction volume containing 2ul BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) with 0.5ul of 5% DMSO and 10 pmol primer (T7 or SP6) per reaction. Thermocycling conditions were also modified as follows: initial denaturing at 98 ºC for 5 mins followed by 30 cycles of 98ºC for 30 sec, 55ºC for 20sec and 60ºC for 4 mins, with a final extension of 5 mins at 60ºC. Reactions were then purified with G-50 sephadex columns (Biomax, Odenton, MD) and immediately run on an ABI 3100 automated capillary sequencer.

Analysis

The data evaluation tool (DAEV) was used on raw sequence data to generate PHRED 20 quality information, trimmed length distribution, and read length distribution.

Results

In the initial analysis of BAC end sequence quality, Method 1 gave consistently higher numbers of high quality bases per read as defined as PHRED value >20, averaging 701.6 bp, with an average number of total trimmed bases per read at 728 bp. Method 2 fared slightly worse with the mean number of PHRED 20 bases at 614.5, and 644.7 for the mean number of trimmed bases per read. The method 3 variations produced a dismal 70.4 bp on average with a high quality Phred 20 score, and only 82.9bp estimated mean of trimmed bases per read.

In Figures 15-17, the distribution of reads is given for each method at a quality score of PHRED 20. The methods 1 and 2 both gave fairly high percentage of PHRED 20 quality reads, however method 2 resulted in a larger percentage of failed reactions (18.6%) compared to method 1 (4%). Method 3 in stark contrast had a failure rate of almost 76.7%. A reaction was considered to have failed if only 0-49 bp of high quality bases were obtained.

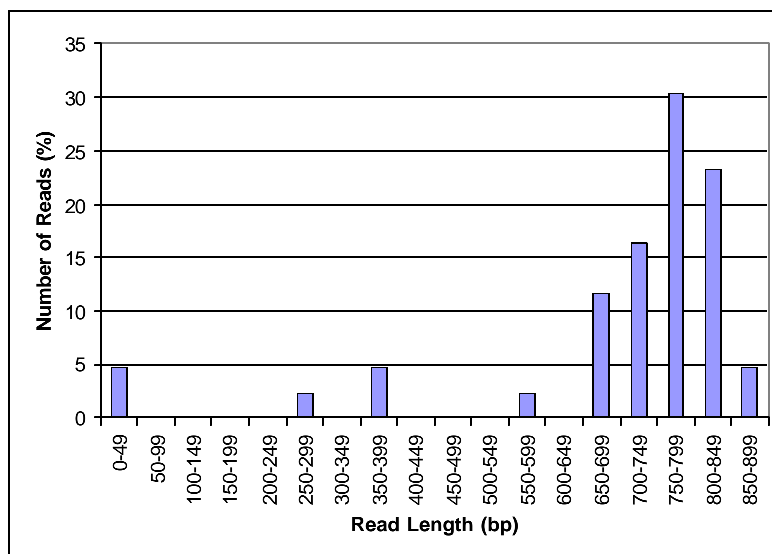


Figure 15. Histogram showing average read length obtained using METHOD 1. Many sequencing reads (>75%) had PHRED 20 values for at least 650 bp of the read length of total sequence obtained.

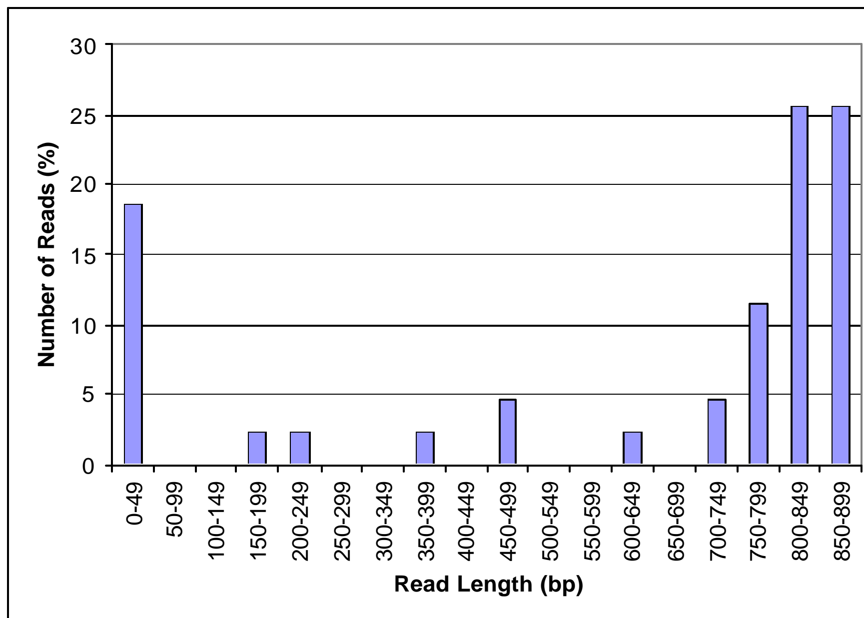


Figure 16. Histogram showing average read length obtained using METHOD 2. Almost 18% of the reads were total failures, however >60% of them were obtained with a PHRED 20 score on read lengths of more than 750 bps.

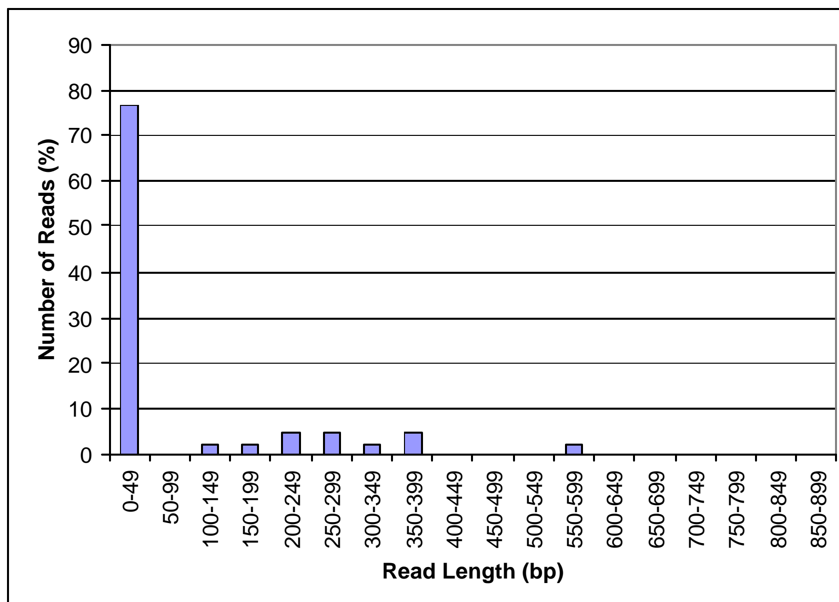


Figure 17. Histogram showing average read length obtained using METHOD 3. This method had the highest failure rate of all the methods, with > 75% of them considered total failures (<50bp).

Discussion

The standard established by the international publicly funded sequencing community for considering a sequence finished is that it be completely contiguous, with no gaps in the sequence, and that it have a final estimated error rate of <1 error in 10,000 bases which is measured by the PHRED 20 score. It is often difficult to obtain consistent reads of high quality from small volume DNA cultures or robot-prepped DNA with available kits. Although it is recommended that the DNA is prepared by column purification or specialized multi-well kits (Qiagen or Millipore) to reduce chromosomal contamination, it would seem the inconsistencies in DNA templates prepared robotically may be great enough to affect the cost efficiency of doing bulk preps for smaller end sequencing projects. In fact, in a recent larger-scale end sequencing project inconsistent template concentration accounted for an average of 32688 reads with less than 100 bases with phred 20 non-vector sequence out of a total 40,704 (D. Adelson, Personal Communication).

BACs have a low ratio of the length of region of interest to the length of the entire template molecule presenting steric and stoichiometric challenges to sequencing reaction chemistries. Standard chemistry protocols for BAC-end sequencing require up to 2.5-3.0 ug of purified BAC DNA template per reaction. Preparing a sufficient quantity of template DNA for these protocols requires the growth of large volumes of BAC clone cultures for subsequent DNA purification, adding considerable time and expense to the completion of BAC-end sequencing projects. The results of this study suggest that 1ug BAC DNA is enough to produce quality reads up to 700bp with no need to grow larger cultures.

Using the same template, primer concentrations (10pmol) and BigDye V1.1 (2ul) under the different parameters of the three methods, allows for direct comparison of concentration and cycling conditions. The results obtained from Method 3 illustrate that concentration (500ng template) and cycling conditions (only 30 cycles) is of prime importance. Methods 1 and 2 were similar in volume (10ul) and composition of the

sequencing reactions including template concentration (1ug), however showed significant differences in the amount of failed reactions. Although, method 2 produced a larger number of reads with PHRED 20 values greater than 800 bp (51%) than method 1 (30%), the failure rate were almost 3-fold greater. Overall, 95% of reads obtained by Method 1 had a >250bp with a PHRED 20 score compared to 76.7% in Method 2. This would indicate that DNA template quality and concentration while important, is not sufficient to guarantee the highest quality reads consistently. It would appear that the additional touchdown cycle, longer cycling times and additional extension step might help with hard to read templates as BACs vary in end sequence dependent on presence of repetitive sequence.

Also, the addition of MasterAmp™ enhancer and *half*BD™ helped to give a more consistently high quality read as both these reagents assist with sequencing hard to read templates. MasterAmp™ with betaine, in the PCR would eliminate the base-pair composition dependence of DNA melting, increases the enzyme's thermal stability, suppresses pauses of DNA polymerase, and reduces secondary DNA structure (Baskaran et al. 1996; Mytelka and Chamberlin 1996; Rees et al. 1993). As a result, long DNA templates, difficult DNA templates (e.g., those with a high GC content), and those with other complex structures like BAC DNA, can be amplified at higher denaturing temperatures, significantly reducing the effect of secondary structure without loss of enzyme activity. Addition of DMSO in method 3 was not enough to make a difference (although it is known to help in PCR reactions and sequencing difficult templates) as only half the concentration of template was used as in the other methods, and the reduction in cycling time during the PCR was not optimal to give longer reads.

It is intriguing that DNA template quality and/or concentration and primer concentration seems to be necessary, but not sufficient, to consistently give higher PHRED 20 read lengths. It would seem that high-throughput BAC end sequencing done in 96-well or even 384-well format, while starting with a lower quantity of DNA than used in these experiments, would benefit from slight changes in the chemistries and conditions of sequencing reactions. Also, researchers interested in BAC contig mapping

in smaller genomic segments or in individual BAC isolation and sequence analysis would gain from the knowledge that adding supplements and optimizing sequencing reactions can make a difference in producing PHRED 20 quality reads in difficult templates with hard-to-read sequence.

BACs are the central resource for insert-end sequencing, clone fingerprinting, high-throughput sequence analysis and as a source of mapped clones for diagnostic and functional studies. Efficient, cost-effective BAC end sequencing provides a greater number of available STC data sets for contig building in areas that had been hindered previously by the prohibitive cost of maintaining and processing libraries on the human genome scale. With the number of STC data sets now expanding, BACs can be screened computationally, then in-house with simple end-sequencing techniques so that scientists have ready access to BACs identified as candidates for use in contig extension, analysis of biologically significant gene content or evolutionary important rearrangements.

The techniques described in this chapter allowed for the development of a BAC DNA purification and end sequencing protocol that consistently gave >750bp of PHRED 20 quality end sequences for contig building and analysis during this study. It also allowed for end sequence to be obtained from BACs with significant quantities of repeat structures at the cloned insert sites, as was the case in the bovine CHORI genomic library which was made with *Mbo*I digested genomic fragments. In addition, the BoLA MHC class I region contains a significant number of repeat structures and GC-rich regions from which it is notoriously hard to obtain high-quality sequence. Thus, this protocol facilitated rapid production of BAC ends and STCs used in analysis of the BoLA MHC class I region described.

CHAPTER IV

ANALYSES OF BOVINE MAJOR HISTOCOMPATIBILITY (BOLA) CLASS I LOCI REVEAL A NOVEL FRAMESHIFT MUTATION IN A BOLA-A ALLELE PREVALENT IN WILD AND FERAL BOVIDS*

> The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I have found it) but 'That's funny…'
>
> \- Issac Asimov.

Summary

The significance of polymorphisms in genes of the MHC to disease association and pathogenesis has been well studied in several species. Polymorphisms in the bovine major histocompatibility complex (BoLA) class I molecules optimizes T-cell response to a variety of pathogens, thus optimizing a population's immune response. We describe an allelic form of a BoLA class I gene with a 2-base deletion in exon 2, resulting in a frameshift, diversity at the amino acid level and an early termination signal within the antigen recognition site (ARS) of exon 3. Genotyping of several cattle breeds and bison showed that the deletion allele was more common among feral cattle and bison populations. Phylogenetic analysis indicates the frameshift deletion predates the divergence of *Bos* and *Bison*, suggesting this allelic lineage is being maintained by selection, especially in feral and wild bovids.

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*Reprinted with permission from "Analyses of bovine major histocompatibility (BoLA) class I loci reveal a novel frameshift mutation in a BoLA-A allele prevalent in wild and feral bovids" by Ramlachan et al. (2004), Immunology 2004 pp51-62. Medimond S.r.l., Bologna, Italy. 2004 by Medimond S.r.l.

Introduction

The immune system of an organism functions to protect against pathogens and other foreign threats. An immune response is initiated by the MHC class I and class II-encoded cell surface proteins that function as antigen-presenting receptors for surveillance by cytotoxic ($CD8^+$) and helper ($CD4^+$) T lymphocytes, respectively. Thus, the genetic processes involved in antigen presentation are fundamental to understanding the role of genetics in animal health and disease resistance. Approximately 40% of expressed loci in the MHC are associated with the immune system (Trowsdale 2001). The human MHC, or HLA, encodes the most polymorphic human proteins known. HLA-B for instance has over 592 alleles (Forbes and Trowsdale 1999). Even more remarkable is that many of these alleles are ancient, and it is common that an allele from a particular HLA gene I more closely related to its orthologous chimpanzee than to a paralogous HLA allele (Anzai et al. 2003; Kulski et al. 2002b). This high level of polymorphism is also seen in BoLA, for which more than 100 different alleles from exon 2 of the BoLA-DRB3 gene have been deposited in GenBank (da Mota et al. 2004). In addition, their high variation resides primarily in amino acids known to be important in initiating the immune response, with some individual amino-acid sites having heterozygosities greater than 0.7 (Hedrick 2004). Polymorphisms in the MHC class I and II loci have been associated with autoimmune and infectious diseases (Gelder et al. 2003; Kaufman 2000; Keet et al. 1999; Pagany et al. 2003; Park et al. 2004b; Sharif et al. 1999; Xu et al. 1993), as well as to response to immunization in vertebrates (Ballingall et al. 2004b; Gelder et al. 2002).

Genetic polymorphism at the MHC locus is generated by nucleotide substitutions, insertions/deletions, gene conversion and inter or intra-allelic recombination (Ackerman and Cresswell 2004; Hughes and Nei 1989; Hughes and Nei 1993; Hughes and Pontarotti 2000). Most of these new mutations are eliminated by genetic drift or purifying (negative) selection, and only a few are incorporated into the

population by chance, diversifying or balancing (natural) selection. Balancing selection in the form of heterozygote advantage, frequency-dependent selection, or selection that varies in time and/or space, has been proposed to explain the high variation and diversity at major histocompatibility complex (MHC) genes. Balancing selection is a broad term identifying any kind of natural selection in which no single allele is absolutely most fit. Frequency dependent selection (the term given to an evolutionary process where the fitness of a phenotype is dependent on the relative frequency of other phenotypes in a given population) and heteroyzgote advantage (where the higher diverse genotype i.e. heterozygotes, achieve better overall health by doubling immunological repertoire to pathogenic peptides) are two types of balancing selection that have been suggested to explain MHC allelic diversity. One model suggests that, rather than the number of foreign antigens the system is protecting against, the diversity reflects the number of self antigens with which the immune system needs to avoid reactivity (De Boer RJ 1993).

Classical class Ia peptide complexes form ligands for CD8 positive T cells and natural killer (NK) cells, while class Ib, or non-classical class I molecules are recognized by receptors on NK, NKT and myelo-monocytic cells (Allan et al. 2002; Colonna et al. 1998). MHC class I heterodimers can be loaded within the endoplasmic reticulum (ER) with peptides derived from intracellular proteins. Alternatively, MHC class I molecules may be loaded with peptides derived from extracellular proteins in a less-defined pathway called MHC class I cross presentation, which can overlap those pathways operating in classical MHC class I presentation (Norbury et al. 2001; Wang et al. 2001; Yewdell et al. 1999).

Class Ib, or non-classical loci have little, if any, nucleotide variation, presumably due to their specialized functions or perhaps due to a lack of function and are usually neutral. However, sometimes non-classical genes appear to have increased amount of variation due to linkage, for example HLA-H has increased variation as a result of balancing selection acting on nearby loci such as HLA-A (Grimsley et al. 1998).

Insertions or deletions of DNA sequences are important genetic markers to define ancestral polymorphisms. Coupled with single nucleotide polymorphism (SNP) analysis, these tools can be used to assign allelic lineages and study evolutionary relationships. For example, the class I MHC (HLA) C locus is present only in humans and apes, generated by gene duplication in a common ancestor (Chen et al. 1992). Also, the gene cluster (DY/DI) in the class IIb region of cattle and sheep, is observed only in pecoran artiodactyls (Trowsdale 1995), as a consequence of a chromosomal inversion within the class II region of BoLA (Band et al. 1998; McShane et al. 2001).

The antigen recognition sites (ARS) of the MHC molecules are usually the most polymorphic allowing immune responses to be varied, with animals having different antigen-binding sites available to a wide range of pathogens. Exons 2 (BoLA-class I, II) and 3 (BoLA-class I) are functionally important encoding amino acids associated with the ARS, accounting for a large part of the polymorphisms and the higher ratio of non-synonymous to synonymous substitutions observed in these exons in most species (Hughes and Nei 1989; Mikko et al. 1999; Sena et al. 2003).

Nonsynonymous to synonymous substitution rate ration, ? = d_N/d_S, have been used to provide a measure of selective pressure at the protein level, with positive selection identifiable where ? exceeds 1. As described initially by Hughes and Nei (1989), the residues of MHC can be divided into ARS and non-ARS codons using two independent ω ratios to show diversifying selection in the ARS of classical class I MHC genes. More evidence of balancing selection operating at the MHC comes from many species ranging from humans to honeycreepers, supported by transpecies polymorphism and strikingly high d_N/d_S ratios at codons putatively involved in peptide interaction. In the i'iwi for example, natural selection may have maintained variation within the MHC, even in the face of what appears to a genetic bottleneck (Jarvi 2004).

Aguilar et al. (2004) found that MHC variation in the otherwise genetically depauperate population of San Nicolas Island foxes is inexplicably high, and suggest that their data support strong balancing selection. This was particularly interesting since this

population is genetically the most monomorphic sexually reproducing animal population yet reported with no variation in hypervariable genetic markers, but genetic variation at the MHC persists despite the extreme monomorphism shown by neutral markers. Also, in a study of South American Indians, Hedrick (2002) observed that distribution of allele frequencies and consequently heterozygosity is a function of autocorrelation of the presence of the pathogen in subsequent generations. When there is a positive autocorrelation over generations, the observed heterozygosity is reduced. In addition, when the effects of lower levels of selection and dominance and the influence of genetic drift were compared to the observed heterozygosity for two MHC genes, resistance conferred by specific alleles to temporally variable pathogens may contribute to the polymorphism at MHC genes and other similar host defense loci.

In a study of the MHC in domestic and wild species of Felidae, derivative gene duplications during the Felidae radiation, abundant persistent trans-species allele polymorphism, recombination-derived amino acid motifs, and inverted ratios of non-synonymous to silent substitutions in the ARS of the MHC was observed (consistent with overdominant selection) in class I and II genes (O'Brien and Yuhki 1999). MHC diversity quantified in population studies was used to examine historic demographic reduction in endangered species (Florida panther and cheetah) where reduced MHC variation contributes to uniform population sensitivity to emerging infectious pathogens.

Unlike the class I genes of humans where assigning loci based on sequence is simple, other mammalian species such as mice (Rada et al. 1990) and cattle (Ellis et al. 1999) appear to have a high degree of gene conversion in class I genes. This makes confirming sequence-based loci groupings difficult. In the past serological testing was used to assign haplotypes in cattle (Davies et al. 1992; Davies and Antczak 1991a; Davies et al. 1994; Nilsson et al. 1994), but molecular-based typing has shown discrepancies (Ellis et al. 1998; Garber et al. 1994). BoLA-A serologically typed animals can express at least three class I genes (Ellis et al. 1999), and it is thought that BoLA-A typing may not be allelic but rather haplotypic (Sawhney et al. 2001) since

more than one class I gene may be expressed. Using DNA-based methods for analyzing BoLA class I polymorphisms is imperative to elucidate such discrepancies. Polymorphic loci in the MHC provide useful tools for the study of evolution and to investigate the contribution of the MHC to genetic disease, particularly of the autoimmune type.

While developing a sequence-based BoLA-A haplotyping test using DNA sequencing analysis of the bovine MHC (BoLA) A locus, we identified a novel allele BoLA-A_{del} among different breeds of cattle and North American bison. This study describes the characterization of a frameshift deletion at the predominant functional class I locus, BoLA-A BSA, by genotyping and phylogenetic sequence analysis of feral and domestic bovids.

Materials and Methods

DNA collection and cell culture

Blood obtained from multiple cattle breeds and *Bison* was collected on Whatman^{R} (Clifton, New Jersey) FTA^{TM} cards. Wild and feral bovid samples were obtained from 172 Yellowstone and 52 Texas *Bison*; 70 Florida Scrub and 450 Texas Longhorn feral cattle. Also samples were obtained from 322 pooled *Bos Taurus* and *Bos Indicus* domestic breeds. These breeds included Holstein, Hereford, Black Angus, native Columbian cattle and Simmental. The mixed breeds are designated by their herd names: McGregor (Red Angus), Uvalde (Brahman/Angus), Nebraska (Holstein and Hereford), Granada (Brahman/Angus), Hetzel (Simmental, Brahman, Angus and/or Zebu) and Vogt (native Columbian cattle).

Genotyping polymerase chain reaction (PCR) amplification and sequencing

Initially, locus A-specific primers were designed to amplify BoLA A from genomic DNA for sequencing (Figure 18A). The disc of DNA was used directly in an initial PCR amplification using locus specific primers at the 5' UTR (Bucon New BSA 5'-CCGCCCAACTCTGCTTCTTTC-3') and exon 3 (Blcon:5'-GATACCTGGAGA-ACGGGAAGGA-3') of BoLA A to amplify a 1329 bp product. Primary reactions (25 µl) contained 100 ng of DNA, 0.5 µM of each primer, 200 µM dNTPs and 1U of *Taq* DNA polymerase (Roche diagnostics, Indianopolis, IN) with these conditions: 3 mins at 95°C, 35 cycles x 95°C for 45s, 63°C for 45 s, 72°C for 1 min; 72°C for 10 mins. A QIAQuick^R PCR purification kit (QIAGEN, Valencia, CA) was used to purify PCR reactions, and 2 ul was sequenced using the ABI dye terminator method as outlined below. Sequence from this original PCR product was used to design the BINDEL primers for the nested PCR.

Genotyping was performed via a nested PCR on 1.5ul of PCR product with a M13 sequencing primer using standard labeled-primer protocol (Oetting et al. 1995). Thermal cycling conditions were: 5min at 95 °C , 5 cycles x 30s at 94°C, 30s at 60°C (-1°C/cycle), 30s at 72°C; 30 cycles x 30s at 94°C, 30s at 56°C, 30s at 72 °C; 15 mins at 72°C.

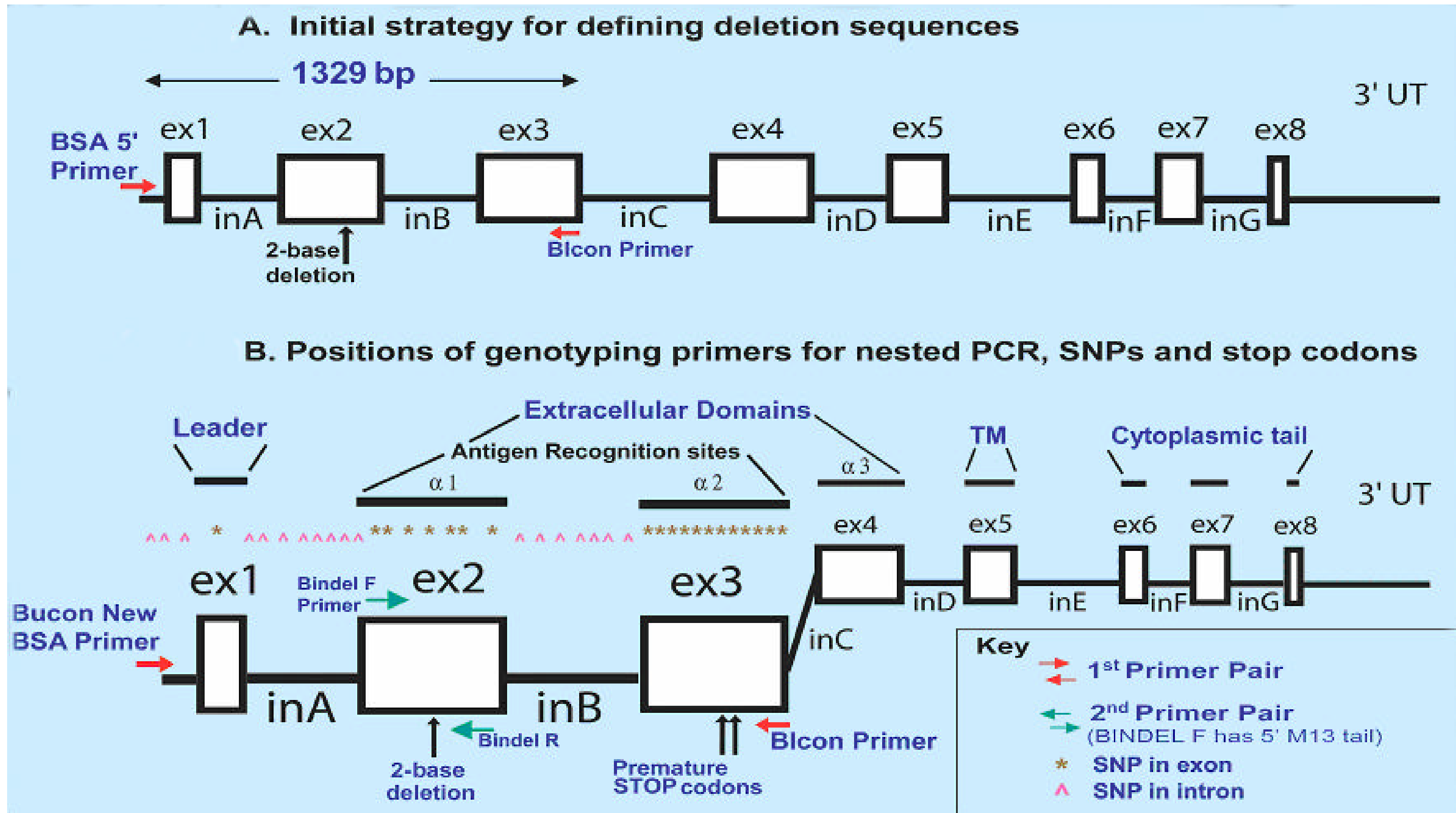


Figure 18. Genotyping and sequencing strategies. **A.** Locations of locus-specific primers used to amplify BoLA -A BSA from *Bison*, domestic and feral cattle. Sequencing of this PCR product initially revealed the frameshift 2-base deletion. **B.** Position of nested PCR primer pairs (red arrows 1st primer set, green arrows internal primer set) used in genotyping strategy, position of stop codons as a result of the deletion, and the location of 34 SNPs found in the 35 animals used.

Fluorescence-based detection was facilitated by using 0.33µM of a FAM-tailed primer and 0.5µM of a forward primer with a 19-bp extension identical to a M13 sequencing primer on the 5' end (BINDEL F 5'-M13 38 forward-GGCCAAAGTATTG-GGAT-3'), in conjunction with 0.5µM BINDEL R (5'-GGCCTCGCTCTGGTTGTA-3') a gene specific primer downstream of the deletion used in the PCR reaction (Figure 18B). The BoLA-A_{del} allele was distinguishable from the BoLA-A BSA by a 2 bp difference in peak height when run on an automated ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA) and analyzed using Genotyper 3.6™. Heterozygous animals were initially identified by the presence of 2 detectable peaks at position 118 and 120 in the chromatogram (Figure 19). Sequencing products were cloned and sequenced as described below to confirm presence of the deletion.

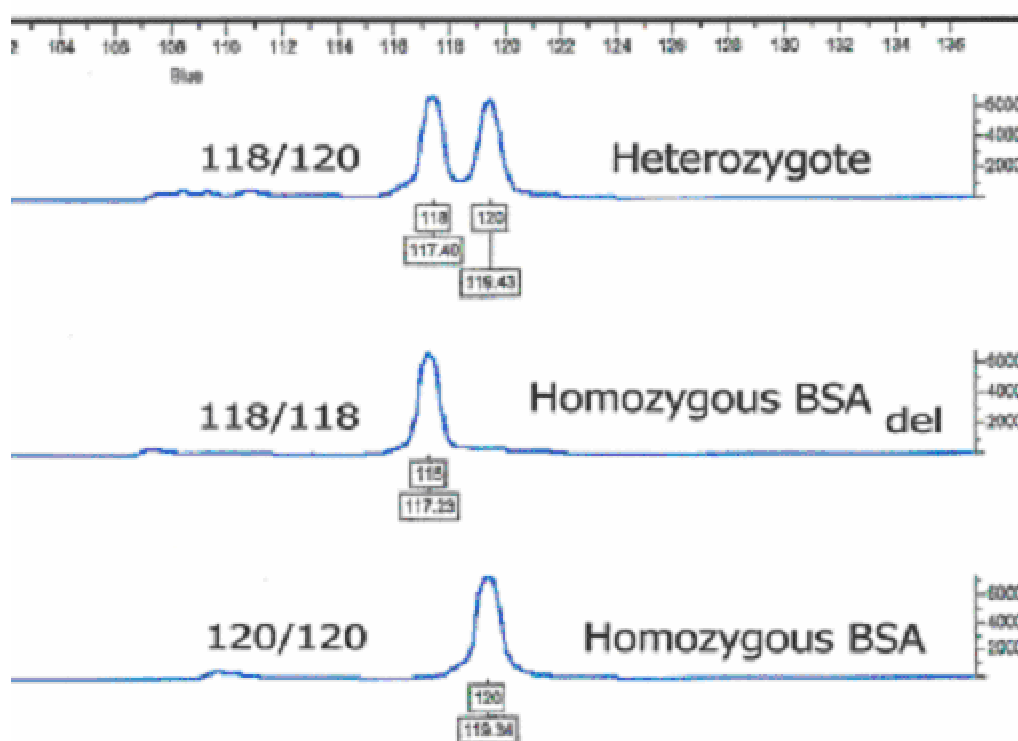


Figure 19. Chromatograph produced by Genotyper™ during genotyping analysis. This chromatograph illustrates the differentiation between the two peaks (118/120) detected in the heterozygous animals (BSA-Adel/BSA), and typical profiles obtained for the homozygous BoLA BSA Adel (118/118) and the homozygous non-deletion (BoLA BSA/BSA) animals when this genotyping assay was performed.

Cloning and sequencing of PCR products

Amplification products were purified and either cloned into plasmid pCR4-TOPO (TOPO TA Cloning Kit; Invitrogen) or sequenced directly using internal primers. Plasmids containing inserts were confirmed prior to sequencing. A modified protocol from the TaqDyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) with Big Dye™ was used with these parameters: 2 mins at 96°C; 40 cycles x 30 s at 96°C, 20s at 50°C, 1 min at 60°C; 5 min at 60°C. Each 10 ul sequencing reaction included: 50 ng purified PCR product, 2ul Big Dye, 1:M primer, 0.5x MasterAmp™ PCR enhancer, and was run on an automated ABI 3100 capillary sequencer (Applied Biosystems). A total of 34 SNPs were detected in BoLA-A BSA_{del} allele in 35 homozygous animals (Figure 18B).

Analysis of nucleotide substitution rates and allelic frequencies

Modified Nei-Gojobori distances with the Jukes-Cantor correction in MEGA™ (Kumar et al. 2001; Zhang et al. 1998) was used to analyze genomic sequence from BoLA-A and BoLA-A_{del} alleles. Statistical significance of the difference between d_N and d_S was tested by a Z-test provided by MEGA2 (Kumar et al. 2001). Allelic frequencies were calculated using GENEPOP™ (Raymond 1995).

Phylogenetic and sequence analysis

Phylogenetic relationships were inferred from analyses of nucleotide sequences from 29 homozygous deletion and 5 non-deletion BoLA-A animals following alignment with homologues (GenBank) using MacVector v7.0 (Genetics Computer Group, Madison, Wis). A total of 1159 nucleotide positions, excluding gaps, from *Bos* and *Bison* were utilized in the phylogenetic analyses. Maximum parsimony analysis was performed using PAUP 4.0™ (Swofford 2002) to produce phylogenetic trees derived from both nucleotide and coding sequences as it allows reversibility of character states. All bootstrap values were obtained from 1000 replicates of the original data set and MEGA 2.1™ was used for all other statistical analyses.

Results

Genotyping and sequencing of BoLA-A alleles

DNA sequencing of the PCR products of the bovine MHC (BoLA) A locus among deletion homozygotes found in breeds of cattle and North American bison yielded 32 positions of single nucleotide polymorphisms (SNPs) in the BoLA A_{del} sequences. Additionally, a 2-base deletion at position 917 in exon 2 was identified, resulting in a downstream frameshift mutation producing either one of two premature translation stop codons in exon 3, (codon 141 and codon 175; Figures 20). The YNP1860 is a Yellowstone *Bison* deletion homozygous animal that has the earlier of the two termination signals produced by the frameshift deletion as illustrated in Figure 22B. Animal Neb4810 is a homozygous deletion animal (domestic cattle breed), with the second termination signal, resulting in a stop codon that terminates later in exon 3.

Figure 20. Alignment of deletion BoLA-A_{del} and non-deletion BSA sequences. A. Nucleic acid sequence alignment for BoLA-A gene BSA and BSA_{del} allele with a 2-base deletion**. B.** Predicted amino acid sequence alignments are illustrated for a non-deletion BSA normal translated product, followed by two sequences from deletion homozygotes showing the frameshift (arrow) and downstream stop codons in exon 3 (asterisks). The YNP 1850 sequence is a Yellowstone buffalo allele with a stop at residue 142, and the Neb4810 sequence is a Nebraska cattle allele with the stop codon at residue 174. If translated without modifications, these stop codons would truncate the transmembrane region and cytoplasmic tail encoded by downstream exons, predicting a soluble MHC class I.

Frequency of deletion allele

The BoLA-A_{del} allele was identified in all species of feral (Longhorn) and wild (*Bison*) (744) and domestic (322) (mixed *Bos taurus/indicus* bovids) tested. However, the frequency of the BoLA-A_{del} allele was greater in feral cattle (0.422 for Texas Longhorn, 0.329 for Florida Scrub) and *Bison* (0.453 for Yellowstone and 0.365 for Texas) than domestic cattle (*Bos taurus/indicus*) (0.115 for pooled domestic cattle breeds) (See Table 6). The genotypic frequencies of the deletion allele in populations of feral and wild bovids deviated significantly from Hardy-Weinberg equilibrium (HWE) ($p<0.01$) with higher observed frequencies of deletion homozygous alleles causing a shift in H_O (Table 6). Notably, domestic cattle also deviated significantly from HWE ($p<0.05$), but this was due to a higher than expected frequency of BoLA-A BSA non-deletion alleles (0.885) versus BoLA-A_{del} (0.115) in this population. When allelic frequencies between males and females was subjected to a Fisher's T-test, no significant difference was observed ($p=0.07$).

Table 6. Frequency of BoLA-BSA and BoLA-A_{del} alleles in feral and domestic bovids. H_E = number of expected heterozygotes and H_O = number of observed heterozygotes. This table was generated using genotyping data from a large number of domestic cattle pooled from several herds (322 animals), in addition to *Bison* and feral cattle (744 animals), and using the GENEPOP™ program for computation (Raymond 1995).

Species or Breed	Total	Allelic Frequencies		Population Statistics		
	N	BoLA-A (120)	BoLA-A_{del} (118)	H_E	H_O	P
Bos taurus/indicus						
Domestic (pooled)	322	0.885	0.115	32.8505	27	0.0366
Feral						
Longhorn	450	0.578	0.422	110.022	56	0.0000**
Florida Scrub	70	0.671	0.329	15.667	7	0.0018**
Bison bison						
Yellowstone	172	0.547	0.453	42.877	56	0.0050**
Texas	52	0.635	0.365	12.2941	17	0.0869

* * P-value showing significance of the deviation from Hardy-Weinberg for that population.

Analysis of nucleotide substitutions indicates diversifying selection in ARS of BSA allele and neutral selection in ARS of BSA$_{del}$

Within the non-ARS sites of exons 1, 2 and 3 of both the non-deletion BoLA-A BSA allele and the BoLA-A$_{del}$ allele, $d_N = d_S$, therefore ? = 1. However, ARS sites in the non-deletion BSA allele, show significant evidence for overdominant selection ($d_N > d_S$, ?>1; p<0.01). Table 7 shows d_N in the ARS region is lower in the BoLA-A$_{del}$ allele (d_N = 1.00) compared to the non-deletion BoLA-A BSA (d_N=14.27; p<0.01), providing evidence that A$_{del}$ may not be transcribed as a functional classical class I molecule.

Table 7. Analysis of 1200 bp of genomic sequence of BoLA-A locus deletion (BSA$_{del}$) and non-deletion (BSA) genes showing nucleotide distance (d) and proportion of non-synonymous (d_N) and synonymous (d_S) substitutions in 28 bovids. d = Kimura 2-parameter of average distances among sequences. Modified Nei-Gojobori distances with the Jukes-Cantor correction in MEGA™ was used to calculate the following: d_N and d_S which are average non-synonymous and synonymous distances in the overall domain and within the non-antigen recognition sites or (non-peptide binding) and the antigen recognition sites (or peptide binding) within exons 1, 2 and 3 (Kumar et al. 2001; Zhang et al. 1998).

NAME	NO. OF TAXA	OVERALL DOMAIN			NON-ANTIGEN RECOGNITION SITES		ANTIGEN RECOGNITION SITES	
		d ± SE	d_N ± SE	d_S ± SE	d_N ± SE	d_S ± SE	d_N ±SE	d_S ± SE
BoLA-A BSA	15	12.34 ± 1.77	7.15 ± 1.01	5.59 ± 1.14	4.58 ± 1.26	3.94 ± 0.73	14.27* ± 2.84	7.76 ± 2.48
BoLA-A BSA$_{del}$	27	1.04 ± 0.24	0.08 ± 0.04	1.00 ± 0.03	0.9 ± 0.3	0.7 ± 0.4	1.00 ± 0.01	1.30 ± 0.6

* p<0.01

Phylogenetic analysis reveals Bison haplotypes of BoLA-A similar to, but distinct from cattle

There were 16 haplotypes detected in the 32 BoLA BSA A$_{del}$ homozygous animals in this analysis as confirmed by Arlequin™ (Schneider 2000). The sequence of a related BoLA class I gene, BSC (Accession # AF396752) was used as an outgroup in this maximum parsimony analysis. Deletion alleles from *Bison* and *Bos* segregated into similar, but distinct haplotype groups based on DNA sequence comparisons. An

unrooted dendrogram generated from peptide sequences produced a smaller number of haplotype groups, but the relationship of the *Bison* to *Bos* alleles (see Figure 21) still reveals a trans-species ancestral polymorphism.

Figure 21. Phylogenetic and haplotypic analyses of BSA_{del} and BSA homozygous animals of the family Bovidae. A. Rooted consensus tree of 32 animals showing maximum parsimony analysis of genomic sequence (exon1 –exon3) using a heuristic approach with majority rule (Sourdis and Nei 1988) and a related BoLA class I gene defined as an outgroup. The bison sequences (red), non-deletion sequences (circled in blue), reference BSA sequence (yellow) and class I gene BSC used as an outgroup are shown. Note the segregation of the animals into 16 haplotypes confirmed by analysis with Arlequin™ (Schneider 2000). **B.** Unrooted dendrogram shows phylogenetic relationship of coding sequences for 32 bovids. Both figures, illustrate the distribution of bison (red), non-deletion (circled in blue) and deletion animals into haplotypes that are similar to, but distinct from other breeds. Bootstrap values equal to or higher than 60% are shown. For MP bootstrapping analysis, a full heuristic algorithm and PAUP*4.0™ (Swofford 2002; Swofford et al. 2001) were used to search for the best trees from 1,000 replicates. Sequences were added randomly, and tree-bisection reconnection was applied for branch swapping during the MP tree searches.

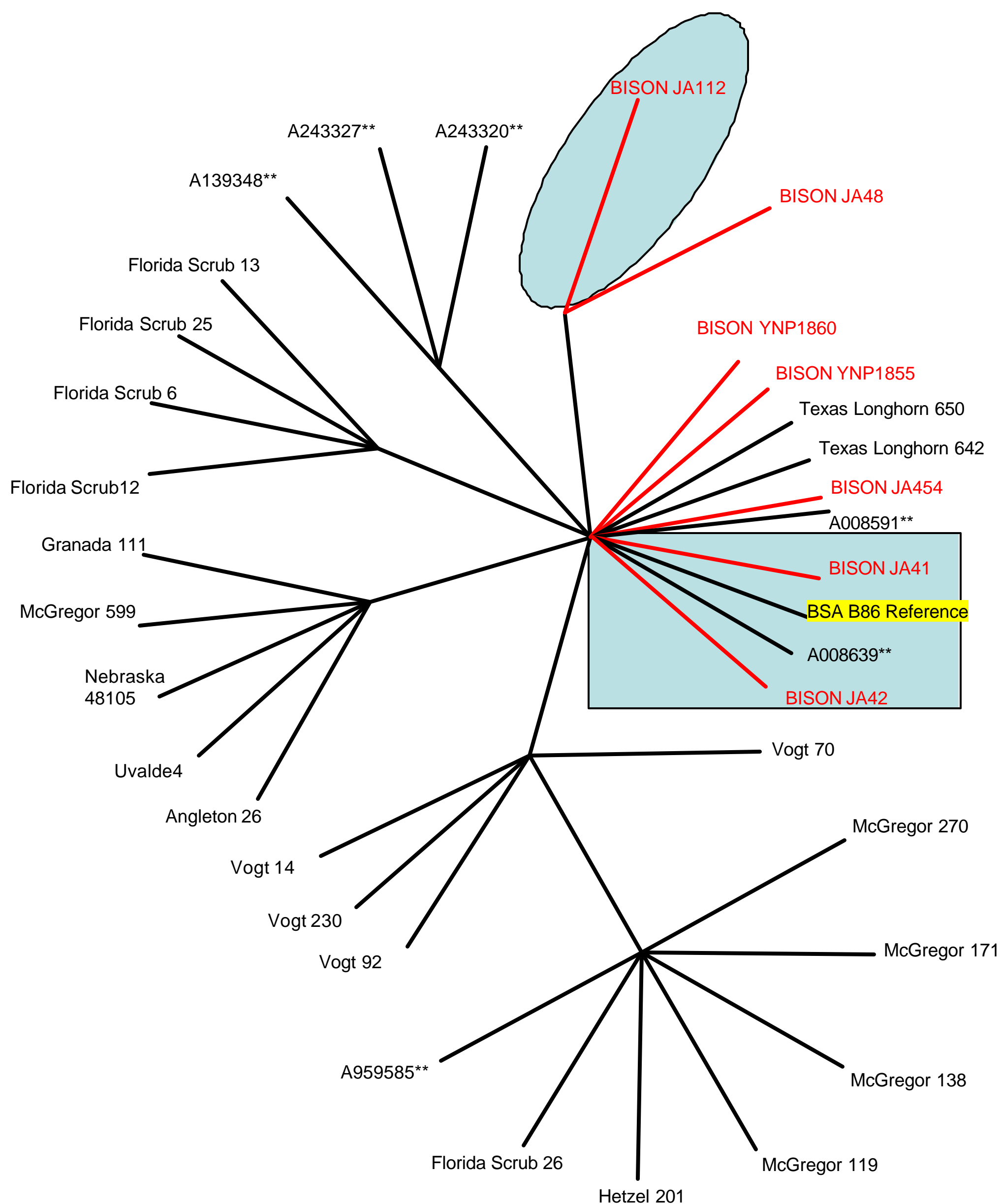


Figure 22. Additional phylogenetic analysis of BSA_{del} and BSA homozygous animals of the family Bovidae. Unrooted dendrogram showing relationship between coding sequence from 32 bovids derived from this study and additional BSA-like sequences from GenBank denoted by **. Note that there was only one previously identified BSA_{del} sequence (A008591) that groups with the Bison and Texas Longhorn deletion animals.

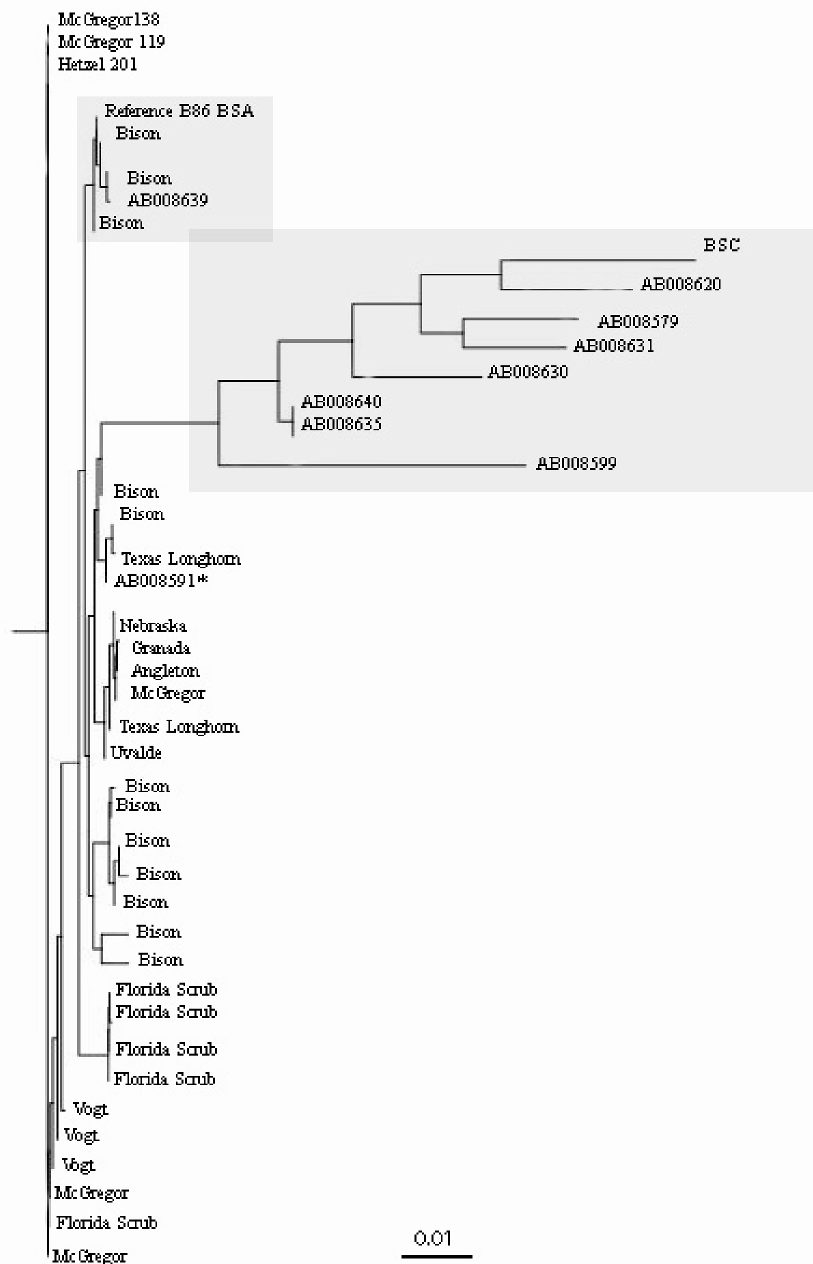


Figure 23. Maximum likelihood tree of nonsynonymous substitutions of coding sequences of BSA_{del} and BSA from homozygous animals of the family Bovidae. The non-deletion BSA sequences and related BSA sequences from Genbank are grouped in highlighted boxes. The GenBank sequence AB008591 (*) is the only deletion sequence reported other than those in this study.

When additional deletion sequences from the EST database and GenBank were added (Accession numbers for ESTs: A9595858, A13934850, A24332712 and A008591 for GenBank deletion sequence; A008639 for non-deletion BSA-like sequence) Ab to the analysis there was less resolution of some of the deletion sequences but nevertheless the trans-species polymorphism was still evident (Figure 22). An additional analysis using neighbour joining to elucidate the relationships of nonsynonymous substitutions per site (disregarding the deletion as a character state) showed a similar haplotype grouping (Figure 23), but branch lengths between the deletion sequences were very short due to level of relatedness.

Discussion

In this article we describe a new allele, BoLA-A_{del}, detected during haplotype analysis of feral, wild and domestic bovids, and identify 34 different polymorphic sites in the BoLA A_{del} sequences. The 2-base deletion causes a stop codon in the putative translated product that, if not subject to RNA decay or modification events, will produce a soluble MHC molecule. Since the leader peptide encoded by exon 1, and the a1 and a2 domains of the antigen recognition sites encoded by exons 2 and 3 may be unaffected, it is possible that the antigen recognition mechanism of the putative soluble molecule is functional. However, since ?=1 for the ARS sites of the BSA_{del} allele, it may not function as a classical class I molecule if translated, since classical class I alleles of all species examined reveal a pattern of overdominant selection within the ARS (Hughes and Nei 1989; Yuhki and O'Brien 1997). Analysis of all available BoLA class I sequences revealed a very low number of A_{del} sequences amongst BoLA class I entries which are mostly from domestic breeds of bovids (only one in GenBank and three in EST databases). Coupled with analysis from this study, it can be concluded that the deletion allele exists in low frequencies in domestic cattle. The prevalence of this allele in feral cattle and *Bison*, predating the evolutionary split, may identify a genetic basis for

the anecdotal assertion that feral cattle and *Bison* differ from domestic cattle in response to exposure of pathogens.

Serologically typed animals can express various combinations of four or more class I genes (Ellis et al. 1999), but allelic versus locus assignment of expressed genes remains a difficult task due to high gene conversion rates in the BoLA class I genes. The low value of d_S in the non-ARS codons of the BoLA-A BSA allele indicates a pattern of nucleotide substitutions characteristic of a gene that has undergone homogenization through gene conversion as documented for most MHC genes (Gregory et al. 2002). In the ARS region of the non-deletion BoLA-A BSA gene, the ratio (?) of nonsynonymous substations (d_N) to synonymous substitutions (d_S) is greater than 1, where positive selection is identifiable when ? exceeds 1. This would indicate positive selection and implies that there may be a fitness advantage to the nonsynonymous mutations in this gene so that these are retained in the population at a higher rate than the synonymous mutations.

The pattern of nucleotide substitution in nonclassical genes is different from that in classical genes, as the rate of nonsynonymous substitution is higher in the ARS than in other gene regions for classical genes. However, unlike the case of classical genes, the nonsynonymous rate does not always exceed the synonymous rate in the antigen recognition site. Nonclassical MHC class I proteins further differ from classical proteins in having amino acid replacements in conserved antigen recognition site positions, consistent with the hypothesis that nonclassical genes have originated from classical genes but have lost classical class I function because of deleterious mutation (Hughes and Yeager 1998a; Yeager and Hughes 1999). The BoLA-A_{del} allele may be demonstrating such a mutation, and if functional could behave within the definition of a nonclassical class I molecule with its limited polymorphism.

It is, however, important to recognise that within the allelic polymorphism at the DNA level which seems endless, there are ancient lineages which predate speciation and maintain themselves in closely related species. This is the basis of the trans-species polymorphism theory proposed by Jan Klein and has found widespread support (Klein et

al. 1993c). Allelic lineages may be shared by related species, such as human and apes or cattle and bison, having been found on a common ancestor.

There have been trans-species polymorphisms documented in bony fish (Kruiswijk et al. 2002) (the *ZE* lineage) that have been maintained for up to 100 million years and the unusual conservation of the peptide binding domains not only within species, but also across species highlight the importance of their function. Although these domains show an unusually high conservation at the amino acid level, each domain exhibited a high degree of nucleotide diversity as shown by divergence time estimates based on the level of synonymous substitutions. The conservation of the 1 and 2 domains may relate to recognition of highly conserved molecular patterns derived from common pathogens and the molecular structures might be the driving force to conserve the 1 and 2 domains in different species.

The trans-species polymorphism between the BoLA-A BSA and BSA_{del} alleles seem to be ancient and related to a history of domestication. Although persistence of MHC alleles after a speciation event could be maintained by overdominant selection in response to pathogens (Klein 1987), it is unusual that the deletion allele is maintained in both populations as evidence shows in analysis of its ARS d_N/d_S ratio (?= 1), it is a neutral polymorphism. It may be maintained by linkage to a class I gene that is selected, especially due to the close proximity of class I genes in BoLA. Alternatively, the non-deletion allele may be subject to diversifying selection indirectly, due to linkage with economically important traits that are heavily selected via human manipulation (e.g. milk yield in Holstein) during domestication of the species independent of environmental pathogens. The latter would explain the Hardy-Weinberg disequilibrium ($p<0.05$) observed in domestic cattle, which had more non-deletion BoLA-A BSA homozygous animals than any other population, tested (See Table 2). However, this is probably not likely, as to date no quantitative trait loci of economic importance to the dairy or beef industry has been linked to the MHC region.

Ongoing sequence analysis will provide more information to elucidate the mechanisms of MHC class I expression at the BoLA A locus. Determining if $BoLA\text{-}A_{del}$

is transcribed and translated in its predicted form will help to relate sequence structure to functional genomics within the MHC class I of bovids, and garner a better understanding of the role of the bovine MHC in disease processes leading to the production of healthier cattle.

CHAPTER V

CHARACTERIZATION OF AN ALLELIC VARIANT OF BOLA-A BSA (A_{del}) WITH A FRAMESHIFT DELETION SHOWS TRANSCRIPTION OF SOLUBLE GENE PRODUCT

> The seeds of great discoveries are constantly floating around, but they only take root in minds well prepared to receive them.
>
> – Joseph Henry

Summary

Research into the immune systems of economically important species, such as cattle, is largely focused on disease control strategies. Existing vaccine failures often relate to poor induction of cellular immune responses, understanding of which requires detailed analyses of MHC genes and their expression. BoLA class I genes have been associated with disease susceptibility and resistance due to their role in antigen presentation. Soluble MHC class I molecules are produced by alternative splicing and proteolytic cleavage of both classical and non-classical class 1 gene products in several mammalian species. This study describes the first soluble form of a MHC class I protein that is allelic to a classical class I gene in any species. The BoLA BSA A_{del} product has a frameshift deletion resulting in a truncated protein, and found in *Bos* and *Bison* with higher frequencies than domestic cattle. Homozygous deletion animals do not show differential expression of overall class I expression by flow cytometry or real-time RT-PCR analysis. Models are suggested by which a soluble form of MHC class I gene, the first described for the bovine MHC (BoLA), could provide immunoregulatory function.

Introduction

Products of genes within the MHC play critical roles in the immune response, especially in immune recognition binding self and foreign peptide fragments for

presentation to T lymphocytes. A large amount of genetic diversity needs to be generated within the MHC to deal with the constant evolution of pathogens to defeat its defense responses. This diversity can be generated through overdominant selection (heterozygote-advantage) or through preferential selection of rare genotypes that confer benefits (negative frequency-dependent selection) or some combination of both (Hughes and Nei 1988; Potts and Wakeland 1990).

This class I molecule is a heterodimer consisting of a heavy chain and a light chain (beta-2 microglobulin). The heavy chain is anchored in the membrane. Class I molecules play a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen. They are expressed in nearly all cells. The heavy chain is approximately 45 kDa and its gene contains 8 exons. Exon one encodes the leader peptide, exons 2 and 3 encode the a1 and a2 domains, which both bind the peptide, exon 4 encodes the a3 domain, exon 5 encodes the transmembrane region, and exons 6 and 7 encode the cytoplasmic tail. Polymorphisms within exon 2 and exon 3 are responsible for the peptide binding specificity of each class I molecule as it changes amino acids in the antigen recognition sites (ARS). Typing for these polymorphisms is routinely done for bone marrow and kidney transplantation in humans, and to date 1,114 HLA class I alleles have been identified (Table 8).

HLA A, B and C genes are expressed by all individuals, but this is different in cattle, where there is expression of a large number of class I genes and a variable number of haplotypes (Table 8B). In humans, HLA-A and –B encode for protein products that present peptides, while HLA-C encoded molecules interact with natural-killer-cytotoxic T lymphocytes (NK-CTLs), which are a subset of $CD8^+$ CTLs that express HLA (human leukocyte antigen) class I-specific inhibitory receptors. HLA-E, F and G encoded molecules are non-classical, demonstrating limited polymorphism, tissue restriction and alternatively spliced forms producing soluble isoforms. HLA-E molecules (CD94/NKG2A) are recognized by NK-CTLs and has the ability to bind peptides derived from the leader sequence of various HLA class I alleles as well as from several viral proteins (Moretta et al. 2003).

Table 8. Number of alleles at the classical class I loci defined in human and cattle to date. A. The number of alleles recognized at the class I loci and B. their serological equivalents as defined for human and cattle to date. Source http://www.ebi.ac.uk/imgt/hla/stats.html. B. Number of MHC class I expressed genes, known alleles and serological types characterized in humans and cattle.

A.

Species	Allele		Number of alleles
Human	HLA class I		1114
		HLA-A	325
		HLA-B	592
		HLA-C	172
		HLA-E	5
		HLA-F	2
		HLA-G	15
Cattle	BoLA class I		
	At least 6 expressed class I genes		?

B.

Species	Expressed genes	DNA-level Alleles	Serological Equivalents
Human	HLA-A	119	40
	HLA-B	245	88
	HLA-C	74	9
Cattle	HD6	6	A18
	JSP.1,	3	A10
	HD, HD7,	(1) (2)	A31
	D18.2, D18.3	(3) (2)	A11
	E223.1, E223.2	(1) (4)	KN12
	D18.1, D18.4, D18.5	(4) (1) (2)	A14
	BSA,BSC,BSN,BSX	?	
	Bs1B	?	

In addition to being expressed on nucleated cells, classical and nonclassical HLA class I molecules are present in serum in soluble form (sHLA-I) due to being alternatively spliced or cleaved post-translationally. Lines of evidence suggest that sHLA-I molecules are immunologically functional and may play an immunoregulatory role, as they elicit antibodies in both allogeneic and xenogeneic combination and inhibit the activity of alloreactive cytotoxic T lymphocytes (CTL)(Puppo 2002). However assigning functional roles of these sHLA-I molecules remain controversial, as stable expression of these isoforms has not been completely proven (Bainbridge et al. 2001).

Soluble class I molecules have been studied in primates (Kubens et al. 1995; van der Ven et al. 2000; Villar et al. 1989) and rodents (Tompkins et al. 1998) but have not

been characterized in the Artiodactyl family to date. HLA-G harbors several structural characteristics, including the transcription of different membrane-bound and soluble isoforms, a unique promoter region and a truncated cytoplasmic tail. HLA-G molecules bind intracellular processed nonamer peptides (Diehl et al. 1996; Lee et al. 1995 and Ishitani et al. 2003) and can serve as a restriction element for HCMV-derived peptides in transgenic mice (Lenfant et al. 2003), suggesting that HLA-G may play a role in the immune response against viral infections in the absence of classical MHC molecules. HLA-G is also the ligand of several triggering or inhibitory receptors on natural killer (NK) cells (Lanier 1999) that could protect trophoblast cells from maternal NK lysis and/or also promote cytokine secretion important for the placentation (Croy et al. 2003).

The MHC class I chain-related molecules (MICs) have previously been shown to be induced on most epithelial tumor cells. Engagement of MIC by the activating immune receptor NKG2D triggers NK cells and augments antigen-specific CTL anti-tumor immunity. The MIC-NKG2D system was proposed to participate in epithelial tumor immune surveillance. Paradoxically, studies suggest that tumors may evade MIC-NKG2D-mediated immunity by MIC shedding-induced impairment of effector cell function. A significant correlation of MIC shedding and deficiency in NK cell function with grade of disease in prostate cancer has been seen (Wu et al. 2004). The presence of surface target MIC is counteracted by shedding, so that a significant increase in serum levels of soluble MIC (sMIC) and a correlated deficiency in NK cell function as shown in patients with advanced cancer (Wu et al. 2004).

There appears to be specific MHC class I molecules, including those with soluble forms, which play a particular role in certain biological functions in many species, including cattle. In humans, HLA-G is expressed specifically on invasive, cytotrophoblast cells and may protect these cells from attack by CD4+ T lymphocytes and/or natural killer (NK) cells (Ellis et al. 1986; Le Bouteiller et al. 1999; Bainbridge et al. 2001; Park et al. 2004). In viviparous animals, regulation of expression of MHC class I antigens by the trophoblast cells, which constitute the outermost layer of the placenta, seems to be critical for maternal immunological acceptance of an allogeneic

fetus. Cattle are unusual in this regard, since the bovine trophoblast cells, in specific regions of the uterine/placental interface, normally express MHC class I antigens during the third trimester of gestation. This expression appears to be biologically relevant as MHC class I compatibility between a cow and her fetus has been associated with an increased incidence of placental retention. This suggests that maternal immunological recognition of fetal MHC class I proteins triggers an immune/inflammatory response that contributes to placental separation at parturition in cattle. Early in pregnancy, a complete shutdown of MHC class I expression by trophoblast cells appears to be critical for normal placental development and fetal survival (Park et al. 2004b).

Also, in a review of recent evidence of the evolutionary adaptation of HIV-1 to the specific immune system in SIV-infected rhesus macaques, experiments show unequivocally that amino acid replacements in CTL epitopes are the result of positive selection and that these escape mutants have a lower class I MHC binding affinity or are less likely to be recognized by CTLs than non-escape variants (da Silva 2003). These analyses show that positive selection of MHC class I polymorphism may be related to immunological function or susceptibility/resistance to a disease state, thus such polymorphisms in MHC class I molecules seen in other species may be maintained due to positive selection to some function or environmental pathogen.

Haplotype and phylogenetic analyses suggest there may be 6 or more cattle class I genes that can be expressed and that individual haplotypes express between 1 and 3 of these genes, in a number of different combinations (Di Palma et al. 2002; Ellis and Ballingall 1999; Garber 1994; Holmes et al. 2003). Also in cattle, no single locus seems to be consistently expressed.

The very high degree of polymorphism seen makes it possible for each individual to possess a different set of MHC alleles, however, within this high level of allelic polymorphism at the DNA level there are allelic lineages maintained within closely related species. Some of these allelic lineages exhibit trans-species polymorphism and are ancient lineages that predate speciation (Ayala et al. 1994; Klein et al. 1993c). This

is also especially seen in the MHC of primates (Anzai et al. 2003; Kulski et al. 2004; Kulski et al. 2002b; Lawlor et al. 1988; Mayer et al. 1992)

Hughes and Yeager (1998b) suggests four independent lines of evidence to support the hypothesis that MHC polymorphisms are selectively maintained: (a) distribution of allelic frequencies does not fit the neutral expectation, (b) rate of nonsynonymous nucleotide substitution significantly exceeds the rate of synonymous substitution in the codons encoding the peptide-binding region of the molecule, (c) polymorphisms have been maintained for long periods of time ("trans-species polymorphism") and (d) introns have been homogenized relative to exons over evolutionary time, suggesting that balancing selection acts to maintain diversity in the latter, in contrast to the former.

BoLA has been estimated to contain at least 20 class I loci by southern blot analysis with a human cDNA probe (Lindberg and Andersson 1988). Biochemical techniques have also been used to detect class I protein from at least two loci in immunoprecipitates and one dimensional isoelectric focusing (1D-IEF) and peptide mapping also indicate a minimum of at least three class I loci expressed at the protein level (al-Murrani et al. 1994; al-Murrani et al. 1993; Garber et al. 1994; Sawhney et al. 1995).

At least six classical class I genes, as well as several other non-classical class I genes and pseudogenes, have been found by sequence and transcription analyses in the pig MHC (or SLA) (Chardon et al. 1981; Chardon et al. 1999a; Renard et al. 2001; Shigenari et al. 2004). In the equine MHC (ELA), 30 class I loci have been detected by southern blot analysis, however only four of these are thought to be transcribed (Ellis et al. 1995). Improved typing of classical MHC class I molecules is required to more accurately define these molecules and to extend the number identified as an alternative to serological assays. Also, defining classical MHC class I allelic polymorphism is important in evaluating cytotoxic T lymphocyte (CTL) responses in many species. Like cattle, horses sharing ELA-A haplotypes defined by serotyping do not always share cDNA sequences, suggesting subhaplotypic variations within serologically defined

ELA-A haplotypes; as well, 13 horses in this study had two to five classical MHC class I sequences, indicating that multiple loci code for these genes (Chung et al. 2003) similar to the situation in cattle. Thus the situation in cattle is not unique as it is seen in rat, mouse and horse where gene conversion plays an important role in MHC allelic lineages (Amadou et al. 2003; Holmes et al. 2003; Hurt et al. 2004; Kulski et al. 2002a; Shiina et al. 2004).

Intra- and inter-locus recombination in MHC class I genes have been observed in the evolution of the MHC in several species, although the evolutionary mechanisms that possibly generated this diversity has been controversial (Amadou et al. 1999; Amadou et al. 2003; Andersson et al. 1991; Cadavid et al. 1997; Garber et al. 1994; Hughes 1991; Hughes and Nei 1988; Hughes and Yeager 1997; Hughes and Yeager 1998a; Jones et al. 1999; Kumanovics et al. 2003; Pamer and Cresswell 1998; Takada et al. 2003).

The use of phylogenetic trees to test hypotheses of recombination and gene conversion giving rise to inter-allelic recombination is useful as is estimating numbers of nucleotide substitutions per site in a particular gene region (Hughes and Yeager 1998b; Yeager and Hughes 1999; Yeager et al. 1997). However, it is difficult to assign a sequence on the basis of allele versus loci using phylogenetics, especially in cattle MHC analyses (Di Palma et al. 2002; Ellis et al. 1986; Holmes et al. 2003). Sequencing clones from RT-PCR with classical MHC class I-specific primers should also be useful for selection of haplotype matched and mismatched cattle for CTL or class I expression studies, and could be useful in providing information needed to develop simpler and more stringent typing procedures by determining DNA sequence.

The functional implications of variable class I haplotypes (including single class I locus haplotypes) in cattle, in terms of individual and population fitness, are at present unknown. BoLA class I genes may vary in terms of polymorphism, expression levels and specific function. Recently, we described a frameshift deletion in a BoLA class I-BSA allele in both feral cattle and bison, which if transcribed, would produce a putative soluble class I molecule, of a 12kDa molecular weight and an isolectric point (pI) of 9.9, without a transmembrane domain or cytoplasmic tail (Ramlachan 2004). This trans-

species polymorphism may have shared an alleleic lineage due to response to shared environmental pathogens. Also, if translated, its soluble form could function as cleaved sHLAs or alternatively spliced soluble MHC class I molecules and be secreted into the cytosol. If translated as predicted by sequence analysis, this BoLA BSA-A_{del} allele would be the first alleleic form of a soluble MHC class I molecule to be charactertized.

Cell lines were obtained from homozygous animals with and without the BoLA BSA-A_{del} and characterization of this frameshift deletion mutant allele of BoLA Class I-BSA was performed in this study through quantitative RT-PCR and flow cytometry analysis to determine differential expression of MHC class I in deletion and non-deletion homozygous animals. Also, attempts were made to determine the structure of the putative soluble MHC class I predicted by the DNA analysis of the BoLA-A_{del} and use this information in 1D-IEF and 2D gel analysis and peptide mapping to isolate the peptide and/or amino acid sequence by mass spectrometry analysis.

Materials and Methods

DNA collection and cell culture

Blood samples from various domestic (Angus, Holstein, Hereford, Jersey, Simmental) and feral (Texas Longhorn and Florida Scrub) cattle and bison breeds were collected on Whatman^{R} (Clifton, New Jersey) FTA^{TM} cards at time of bleeding (125ul per card) and stored at room temperature. A 1.2 mm disc was excised using a coring device and DNA was prepared for downstream PCR according to the protocol recommended by the manufacturer. Venous blood from the 100 Wichita Mountain Refuge Texas Longhorns was also collected in sodium citrate CPT tubes (BD Vacutainer, Franklin Lakes, NJ). CPT tubes were centrifuged as directed by the manufacturer to obtain peripheral blood mononuclear cells (PBMC). Plasma was removed for downstream analysis and PBMCs were either frozen at -80 ºC or cultured in RPMI 1640 (Gibco BRL, Grand Island, N.Y.), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO), 50 ug/ml of gentamicin

(Gibco BRL, Grand Island, N.Y.), 5x 10^{-5} M 2-mercaptoethanol (Gibco BRL, Grand Island, N.Y.), 2mM L-glutamine (Gibco BRL, Grand Island, N.Y.) and 100 units of human recombinant IL-2 (Gibco BRL, Grand Island, N.Y.) per ml. Cells were grown at 36ºC in a humidified atmosphere of 5% CO_2 and stimulated with concavalin A (5ug/ml) (Sigma, St. Louis, MO) every three days then collected, washed with PBS, then frozen at -80 ºC or stained for flow cytometry.

Genotyping, polymerase chain reaction (PCR) amplification and sequencing

This was done as described in Ramlachan et al. (2004). Briefly, locus A-specific primers were designed to amplify BoLA BSA from genomic DNA for sequencing, then this sequence was used to design internal primers used in genotyping. Genotyping was performed via a nested PCR on 1.5ul of PCR product with a sequencing primer using a standard labeled-primer protocol (Oetting et al. 1995), run on an automated ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA) and analyzed using Genotyper 3.6^{TM}. All genotyping products were run on a 1% agarose gel, excised, cloned and sequenced as detailed below for confirmation.

Cloning and sequencing of PCR products

PCR was performed in 25µl with the following components: 1-2µl of cDNA or genomic DNA, 1.5µM MgCl2, 1µM of each primer, 200µM of dNTPs, and 0.6U Taq polymerase (Perkin Elmer). Amplification products were purified (QIAquick™ PCR Purification kit, QIAGEN) and run on the ABI 3100 directly for genotyping analysis or cloned into plasmid pCR4-TOPO™ (TOPO TA Cloning Kit; Invitrogen). Plasmids containing inserts were confirmed prior to sequencing by restriction enzyme digestion with SalI and/or HindIII and agarose gel electrophoresis. Clones (up to 12 per animal) were selected and DNA was prepared from single colony isolates inoculated into 2 ml cultures (with 2XLB broth) grown in 96-deep-well plates with appropriate antibiotic (Kanamycin or Ampicillin) at 37ºC on a shaker at a 320 r.p.m. for 20-24 hours. A Montage™ Plasmid Miniprep$_{96}$ kit (Millipore, Bedford, MA) with a vaccum manifold

was used as per manufacturer's instructions to prepare sequence-quality DNA templates from clones.

Sequencing with internal primers in both orientations in a 96-well format was done using a modified protocol from the TaqDyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) with Big Dye™ and run on an automated ABI 3100 capillary sequencer (Applied Biosystems) as described in Chapter II. Sequencing reactions were subjected to a clean-up set prior to being run on the capillary sequencer to remove dye terminators, salts, dNTPS, primers, buffers, and labeled nucleotides. Briefly, 40 ul of a precipitation mix (624 ul of 3M sodium acetate (pH 5.2), 6 mls water, 26 mls 95% ethanol) was added to each 10ul sequencing reaction in a 96-well plate, which was then covered in foil, vortexed and left to incubate for 15 mins at room temperature. The plate was then centrifuged at 3250 rpm at 4°C for 45-60 mins. Supernatants were removed then 50 ul of chilled 70% ethanol was added to each well followed by an additional centrifugation at 4000 rpm at 4°C for 5 mins. Plates were then shaken and spun upside-down on a tissue at 250 rpm for 30-60 secs and allowed to dry before loading.

RT-PCR of MHC class I gene transcripts

Accurate analysis of *in vivo* gene expression might be complicated by unintended *ex vivo* gene expression or degradation of gene transcripts. To reduce incidence of this, we collected blood to be used for RNA extraction in a sampling system (PAXgene™ Blood RNA tubes; PreAnalytiX) that includes a stabilizing additive in the blood collection tube (Rainen et al. 2002). Total RNA was extracted from animals genotyped as homozygous or heterozygous for BoLA-A and BoLA A_{del} alleles using the recommended protocols of the PAXgene™ kit (PreAnalytiX, Qiagen, Valencia, California). This was followed by reverse transcription PCR (RT-PCR) using a forward and reverse primer specific for the 5' UTR (BSA 5': 5'-TGTCTCCCCAAGTTTCAC-3') and the end of exon 3 (Blcon: 5'-GATACCTGGAGAACGGGAAGGA-3') regions of BoLA-A BSA, respectively.

Generation of cDNA for use in Real-Time quantitative RT-PCR

To selectively enrich for the BoLA A_{del} cDNA, a deletion-specific reverse primer was used in the RT reaction to produce source cDNA template to detect deletion transcripts in downstream PCR from known homozygous or heterozygous animals. Two forward primers were used in separate reactions with the same deletion specific reverse primer to reduce amplification bias. Turbo DNA-free™ (Ambion, Austin, TX) was used remove residual genomic DNA before RT-PCR analysis. Anchored oligo $(dT)_{23}$ or specific primers were used with Enhanced Avian Reverse Transcriptase (Sigma, Saint Louis, Missouri) to generate first strand cDNA. Briefly, 0.2- 5µg of purified RNA was added to 1ul dNTP mix (500µM each) and 1 uM of either 3' antisense specific primers (BLCON for BoLA-BSA or 295DELREVPCR for BoLA-BSA_{del}) or anchored oligo $(dT)_{23}$ were used in 10µl reactions and incubated for 70ºC for 10 mins to denature RNA secondary structure.

Following this, the reaction mix was placed on ice, centrifuged and the following components were added to a 20 µl volume reaction: 2µl of 10x Buffer for AMV-RT, 1µl of enhanced AMV-RT enzyme (1U/µl) and 1µl RNAse Inhibitor (20U/ul) and placed in a GeneAmp® thermocyler at 63ºC for 50 mins. Subsequently, 1ul of this first-strand cDNA was used in an Advantage-GC™ cDNA PCR kit in 50ul reactions containing 10ul 5X GC cDNA PCR Reaction Buffer, 10ul GC Melt (5M), 2ul primer mix (10µM each), 1ul dNTP mix (10mM each) and 1ul of Advantage-GC cDNA polymerase Mix. Primers specific for bovine glucose-6-phosphate dehydrogenase (GAPDH) were used as a control. Amplification conditions was the same as for amplification from genomic DNA or cDNA using Taq polymerase with only quantities of components differing (94°C for 1 min; followed by 35 cycles of 30 s at 95°C, 30 s at 63°C, 1 min at 72°C, and final extension at 72°C for 10 mins). Generated PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining. Cloning and sequencing confirmed the identity of products generated with different primer pairs.

Quantitative Real-Time PCR of BoLA BSA transcripts

Polymerase chain reaction (PCR) was performed using a GeneAmp 5700 Sequence Detector (Applied Biosystems) and SYBR Green (Molecular Probes, Eugene, OR) as a double-stranded DNA-specific fluorescent dye. The PCR reaction mixture (25 µl) contained 0.625ul (10µM stock) of each primer and SYBR Green I Master Mix (12.5ul) along with 0.5ul of cDNA (~0.1ug). Product identity was confirmed by ethidium-bromide-stained 2% agarose gel electrophoresis. The PCR cycling parameters were: initial denaturation for 4 min at 94 °C, 35 cycles of denaturation (94 °C, 1 min), annealing (60°C s) and extension (72 °C, 2 min), followed by one cycle of final extension (72 °C, 5 min). Fluorescent data were acquired at 85°C. The melting protocol consisted of holding at 40°C for 60 sec and then heating from 50 to 94°C, holding at each temperature for 5 sec while monitoring fluorescence. As negative controls, tubes were always prepared in which template or reverse transcriptase was omitted during the reverse transcription reaction.

The comparative C_T method was used for quantification of expression levels (ABI Prism Sequence Detection System User Bulletin No. 2, PE Applied Biosystems, Foster City, CA). The quantification was normalized to the endogenous control bovine GAPDH. Fluorescence was acquired in each cycle in order to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence rose above background for each sample. Within this region of the amplification curve, each difference of one cycle is equivalent to a doubling of the amplified product of the PCR. According to the comparative C_T method, the $?C_T$ value was determined by subtracting the GAPDH C_T value for each sample from the BoLA A or A_{del} C_T value of the sample. Calculation of $??C_T$ involved using the highest sample $?C_T$ value (i.e., the sample with the lower target expression) as an arbitrary constant to subtract from all other $?C_T$ sample values. Relative mRNA expression of target was determined by using the formula $2^{-??CT}$ (ABI Prism Sequence Detection System User Bulletin No. 2 [PE Applied Biosystems]). Primer sequences, annealing temperature, the approximate sizes of the amplified fragments, and the GenBank accession number are shown in Table 9.

Standard curves using a plot of log of input amount (ng) versus ?C_T were generated to validate the target and reference PCR reactions as equal. The melting peaks (mp) of all samples were routinely determined by melting curve analysis in order to ascertain that only the expected products had been generated. Additionally, molecular sizes of PCR products from randomly selected samples were monitored by agarose gel electrophoresis analysis (2% agarose, ethidium bromide stained). All samples were measured in triplicate.

Table 9. Sequence and annealing temperatures for primer pairs used in quantitative real-time RT-PCR analysis. Once primers were optimized in RT-PCR, the most efficient deletion specific and gene specific primer pair was used in the quantitative analysis.

Gene	Primer Sequence (5'-3')	Annealing Temp (°C)	Fragment Size (bp)	GenBank Accession no.
GAPDH	**GAPDHFor:** TTCTGGCAAAGTGGACCATCGT **GAPDHRev:** GCCTTGACTGTGCCGTTGA	60	113	AJ000039
Class I (BSA)	**BUCONFor:** GCTACGTGGACGACACGCA **BLCONRev:** CCTTCCCGTTCTCCAGGTATCT	60	452	AF396750
	Or **126DELFOR:** GTACCTGGAAGTCGGCTACG **288EX1*2REV:** ACTCGGAAACTGTGCGTTG	60	162	
BSA_{del}	**65DELFORPCR:** GCTCCCACTCCCTGAGGTAT or	60	230	
	126DELFOR: GTACCTGGAAGTCGGCTACG with **295DELREVPCR:** CAGGTTCACTCGGAAACTGT.	60	169	

Flow cytometry

We used the QuantiBRITE^{TM} flow cytometry system (BD Biosciences, San Jose, CA) which yields an absolute antigen expression value (antibodies bound per cell) useful in standardizing surface antigen expression analysis. This system makes use of a highly purified phycoerythrin (PE)-labelled antibody with a 1:1 fluorochrome to protein (F : P) ratio, and multi-level calibrated beads with known absolute PE fluorescence. PBMCs were collected by centrifugation and/or histopaque purification either fresh, or after 3, 6, or 12 days of culture. Cells were washed twice in phosphate-buffered saline (PBS), then class I surface expression was determined by flow cytometry on 1 x 10^6 cells. The following monoclonal antibodies, conjugated with phycoerythrin (PE) were used: mouse anti-human HLA-ABC W6/32 (Catalog #MCA81PE, Serotec, Raleigh) and goat anti-mouse IgG2a (Serotec, Raleigh) for labeling class I molecules and IgG2A as an isotype control respectively. Following blocking with goat anti-IgG (2mg/ml) (Sigma, St. Louis, MO), cultured cells were incubated on ice for 30 min with 10 µg/ml of the relevant antibodies in PBS containing 1% FCS, 1 mmol/l EDTA and 0·1% NaN_3, then washed once with cold PBS-0.1% sodium azide and centrifuged at 1200rpm for 10 minutes. Negative controls consisted of isotype-matched controls and unstained cells. After washing, cells were fixed in 2% formaldehyde. All samples were analysed on a FACS Calibur^{TM} using the software CellQuest^{TM} (BD Biosciences, San Jose, CA, USA). A minimum of 10 000 gated events was counted. The PE fluorescence within the gates was measured as specific geometric mean fluorescence intensity of the whole population of cells and converted into antibodies bound per cell (ABC) and graphs produced with Microsoft Excel^{TM}-spreadsheet. ABC values represent MHC class I expression for the different cell populations.

Preparation of cell lysate and separation of membrane-bound and soluble fractions

Cultured PBMCs from genotypically defined animals were used for immunoprecipitation of BoLA class I protein. Triton X-114 phase fractionation of integral membrane surface proteins of the PBMCs was necessary to separate membrane-bound from soluble BoLA class I protein and performed by using a modified protocol from Garber et al. 1994. Approximately $5x10^6$ - $1x 10^7$ cells was solubilized in cold lysis buffer (pre-condensed 5% Triton X-114, 150mM NaCl, 10 mM Tris pH 7.4, 1mM EDTA, 0.2 mM sodium ortho-vanadate, protease inhibitor cocktail (Boehringer Mannheim), 0.5% IGEPAL CA-630) and incubated on ice for 30 min. Cell debris was removed by a 1.5 mins spin in a microfuge at 14,000rpm. The insoluble, membrane-bound protein component was recovered in the supernatant, and then the extract was warmed for 5 mins at 37ºC, and following several centrifugation steps the detergent phase was recovered and used in downstream immunoprecipitation applications. The aqueous phase was also kept and analyzed for presence of soluble MHC BoLA class I protein. When used in immunoprecipitation with HC10 (a monoclonal antibody detected denatured heavy chain), lysates were denatured by boiling in 1% SDS for 5 mins.

SDS-PAGE and Western blot analysis

Initially, SDS-PAGE was performed on approximately 50µg of protein per sample in the NuPAGE Tris-Glycine system (Invitrogen, Carlsbad, CA) run at 125V for 90 minutes. Gels were stained with comassie blue and/or silver stain, and sometimes run in parallel so as to ascertain the identity of protein bands excised for mass spectrometry by western blot analysis. Immunoblotting was performed using a panel of monoclonal antibodies (MAbs) directed against MHC class I/ $ß_2$M complexes for detection (see Table 9). The protein concentration of the lysates was determined using the Bradford or BCA assay. Following denaturation, equal quantities of protein extracts were loaded on to a 12% SDS-polyacrylamide gel. The proteins were transferred on to nitrocellulose 0.2 mM (Millipore) using the Invitrogen XCell^{TM} Blot module (Invitrogen, Carlsbad, CA) using transfer buffer (20 mM Tris-HCL pH 8.5, 150 mM glycine, 0.01% SDS, and 20%

methanol) at 200 V for 1 h at 4°C. The blot was subsequently blocked with 1× TBS (0.25%Tween) containing 5% milk protein for 1 h at room temperature.

The hybridizations were carried out using a 1:1000 dilution of a monoclonal MHC class I /$ß_2$M complex specific antibody (PT85A or H58A, VMRD, Pullman Washington; HC10, $ß_2$M and or W6/32 from Dr. Michael Edidin). The hybridized filters were washed with TBS Tween (25 mM Tris-HCL pH 7.6, 150 mM NaCl, and 0.5% Tween 20) followed by a final wash in milli-q H_2O, then hybridized with polyclonal donkey anti-mouse IgG (Santa Cruz, California) coupled with horseradish peroxidase using a dilution of 1:10000 at room temperature for 1h. The proteins were detected by enhanced chemiluminescence (1:1 luminol enhancer solution:stable peroxidase buffer solution, Western Lightning^{TM}; Perkin-Elmer, Boston, MA) and exposed to film.

Immunoprecipitation of MHC class I BoLA A proteins and SDS-PAGE

Antibody-conjugated Protein G agarose beads (Sigma, St. Louis, MO) were used to precipitate BoLA A MHC class I protein from the cell lysates as prepared above. Unless specified all incubations were done in at 4ºC on a rotator. Pre-clearing by adding 50µl of Protein G:Agarose beads (Sigma, St. Louis, MO) to 500µl of PBMC cell lysate (from 50 ul either denatured or undenatured detergent phase of the cell lysate dilute 1:2 in lysis buffer), incubated for 2 hrs followed by removal of agarose beads by centrifugation. Monoclonal antibody was added at a ratio of 1:500 to total pre-cleared lysate and incubated overnight. Next, 25µl of a 50% suspension Protein G Agarose (Sigma) will be added to 500µl of total lysate:MAb and incubated overnight. The precipitants were washed 3 times in 1x lysis buffer, twice more with autoclaved ddH_2O and either eluted with 50µl of 0.1 M Glycine, pH 2.5 or boiled 2 times with 25 ul Laemmli 6x loading buffer for 5 mins. If being eluted, after 30 mins incubation, the supernatant will be removed by centrifugation and used as the IP sample.

To neutralize the pH, 5µl of 1M Tris pH 8.0 will be added prior to adding 10µl of 5X concentrated electrophoresis Laemmli sample buffer (125mM Tris pH 6.8, 4% SDS, 10% Glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol) with iodoacetamide (Sigma, St. Louis, MO) to each sample and boiled for 5 mins. Samples were used for

Western Blotting, 1D or 2D gel analysis. Some antibodies were also cross-linked then immunoprecipitated using the Seize X Protein G Immunoprecipitation Kit (Pierce, Rockford IL) to avoid competing proteins of similar molecular weight masking MHC class I (e.g. beta actin, heavy chain IgG, Bovine Serum Albumin) on SDS-PAGE gels. Table 10 gives a list of all antibodies used in this study and if they were cross-linked or used in batch IPs.

Table 10. Antibodies used in immunoprecipitation (IP) and western blotting. These monoclonal antibodies with varying specificities were characterized previously as reactive to bovine MHC class I. During IP the antibody was used as a batch and/or cross-linked form, however during western blotting incubations were done in batch only.

Monoclonal Antibody	Specificity	Source	Batch/Cross-linked IP
HT58A	Bovine MHC class I	VMRD, CA	Batch
PT85A	Bovine MHC class I	VMRD, CA	Batch/Cross-linked
HC10	Denatured Human MHC class I	Dr. Michael Edidin	Batch/Cross-linked
W6/32	Human MHC class I	Dr. Michael Edidin	Batch/Cross-linked
$ß_2M$	Human $ß_2M$	Dr. Michael Edidin	Batch
Ke2	Mouse MHC class I	Dr. Michael Edidin	Batch

Isoelectric Focusing (IEF)

The proteins of the transmembrane and soluble fractions were resolved by pI using a ZOOM IPGRunner^{TM} system for isoelectric focusing on ZOOM^{TM} strips (3-10 or 4-7) Gel System (Invitrogen, Carlsbad CA), with final resolution by size on a 12% SDS-PAGE gel (the latter as described by Garber et al. 1994). The predicted molecular weight and pI of BoLA BSA from sequence analysis is 43.3 kDa and 4.5 respectively; while that of the putative soluble protein product of BoLA A_{del} is 12.9 kDa and 9.9 respectively. Prior to IEF, each sample (~100ug protein) was concentrated using the Perfect-FOCUS^{TM} (GenoTechnology, St. Louis, MO) to prepare lower conductivity protein samples free from agents known to interfere with net protein charge and allowed precipitation of the samples quantitatively into a small volume. For specific IPs, prior to

elution or boiling, beads were treated overnight at 37ºC with either 20ul of 20U/ml neuraminidase (type VIII, Sigma, St. Louis, MO) in buffer (100mM Na acetate, 5mM CaCl2, pH 5.5) or buffer alone as a negative control. A non-interfering Protein Assay™ was then used to quantify protein prior to loading unto IEF gels.

Approximately 20µg of total protein was loaded unto a ZOOM™ Strip (pI 3-10 or 4-7 depending on sample's predicted pI) and incubated for 16 hours for rehydration. IEF was then performed as per manufacturer's instructions in a step voltage protocol as follows: 200 V for 20 mins, 450 V for 20 mins, 750 V for 20 mins 1500 V for 15 mins and finally 2000V for 45 mins. Strips were then equilibrated in NuPAGE LDS Sample buffer, followed by alkylation of the sulfhydryl groups of the proteins to reduce vertical streaking in a second equilibration step using idodoacetamide. The equilibrated ZOOM strip was then loaded unto a NuPAGE® Novex 4-12% Bis-Tris ZOOM® gel immediately for the 2D SDS-PAGE gel analysis and run at 125V for 90 minutes in MOPS SDS running buffer. The gels were either stained as per manufacturer's recommendations with zinc (GelCode E-zinc reversible stain, PIERCE), Commassie blue (Sigma., St. Louis, MO) or silver (SilverQuest™, Invitrogen, Carlsbad, CA). Bands approximate in size to a either predicted pI on the IEF 1st dimension gels or kDa on the 2nd dimension were excised and used immediately (or frozen at -80ºC) for in-gel analysis by mass spectrometry and peptide sequencing. A range of 42-48 kDa was used for gel excision of the BoLA A product, and 10-15 kDa for the predicted soluble BoLA-A_{del} product.

In-gel and MALDI-TOF mass spectrometry analysis

Proteins were isolated using polyacrylamide gels as described above. The protein spot of interest was gel excised (or thawed), and washed repeatedly by alternating solutions of ammonium bicarbonate and 70% acetonitrile in ammonium bicarbonate then completely dehydrated under vacuum. The gel was rehydrated using a solution containing a trypsin protease. Enough ammonium bicarbonate solution was added to completely cover the gel piece and the sample incubated at 37ºC. After 3-12hrs, peptides

eluted from the gel were analyzed by mass spectrometry. The samples were prepared by first coating the stainless steel target plate with a-cyano-4-hydroxycinnamic acid (0.15M). Once the matrix dried, approximately 1µL of the peptide mixture was mixed with 1µL of matrix and loaded on top of the matrix underlayer (Murphy et al. 1999). The matrix/peptide crystals were washed twice with cold ddH_2O to remove residual buffer and salts.

Peptide mass mapping data was obtained using an Applied Biosystems Voyager STR equipped with a MALDI source and delayed extraction, in order to obtain high resolution and high mass accuracy (< 50ppm). The peptide mass fragment data collected was analyzed using database-searching programs as described by Park and Russell, 2001. Identification of proteins using peptide mass fingerprints was done using databases such as the non-redundant protein sequence data base maintained at the European Bioinformatics Institute (EBI, Hinxton, UK; ftp://ftp.ebi.ac.uk/pub/data bases/ PeptideSearch).

When necessary, sequencing of the peptides was done using tandem MS. Sequencing of peptides was completed on a PE-Sciex QSTAR Pulsar equipped with a nanospray ion source. The QSTAR combines the ion selectivity of a quadrupole ion guide with the high resolution and mass accuracy of a time-of-flight (TOF) mass spectrometer. Initially all ions are allowed to pass through the quadrupoles into the TOF portion of the mass spectrometer, where the intact masses are determined. In order to sequence the peptides, the first quadrupole is tuned to filter out all but the target mass. A buffer gas is introduced into the second quadrupole, which fragments peptides upon collision, and then fragment ion masses are determined in the TOF region. Fragment masses were then analyzed and the intact peptide sequence determined. This process was repeated for all masses found in the mixture. The experimental sequence data was then interrogated using a web based searching algorithms available, such as MASCOT (http://www.matrixscience.com), PepSea (http://195.41.108.38/PepSeaIntro.html), or Protein prospector (http://prospector.ucsf.edu/).

Modelling

Identification of function of a protein is possible by performing a CDD search which uses a database of motifs to characterize 'conserved-domains' in a protein sequence (Marchler-Bauer et al. 2002). A CDD search was performed with the standard protein–protein search option against the consensus of the deletion allele translated amino acid sequences to match its predicted structure with known structures. The structure of the predicted alignment was visualized with the Cn3D program (Wang et al. 2000).

Statistical analysis

Statistical analysis was performed to determine the statistical significance of differences between the two groups of animals (deletion homozygotes versus non-deletion homozygotes). For the quantitative real-time RT-PCR samples of three different animals were analyzed in duplicate, and data expressed as means ± standard deviation. Because the data were not distributed normally in flow cytometry data analysis, geometric means and medians were used to describe the distributions of continuous variables. The nonparametric Mann–Whitney *U* test was used to determine the statistical significance of differences in median values. The chi-square test was used to test the association between categorical variables and the presence and absence of deletion. Differences were considered significant if $P < 0.05$.

Results

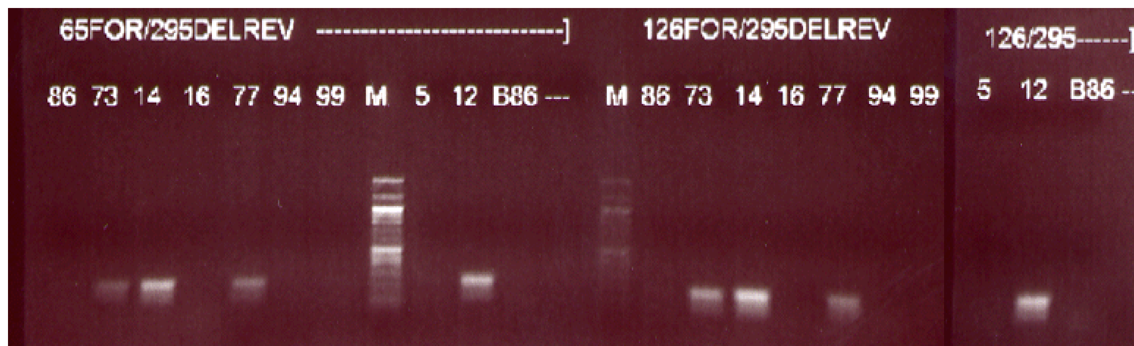
Genotyping and sequencing of BoLA A and A_{del} in Wichita Longhorns

Through genotyping and sequencing analysis of the Wichita Longhorn animals, 8 homozygous BoLA A_{del}, 32 homozygous BoLA A BSA, and 3 heterozygous animals were detected. When PCR products of BoLA A or A_{del} genes were cloned and sequenced (up to 12 clones for each of the deletion animals), no more than two sequences were obtained confirming their alleleic status and presence at the same loci. The genotyping products that were also cloned and sequenced to confirm the assignment of genotype in all the animals tested.

RT-PCR of A_{del} transcripts

Unique RT-PCR products were obtained from homozygous A_{del} and BSA/ A_{del} heterozygous animals using specific primers, which were not seen in non-deletion BSA homozygous animals (Figure 24A). These animals were all genotyped previously to confirm which were to be used for the RT-PCR analysis. Samples from two of each of these groups were selected for further analysis. Figure 24B shows results of an initial RT-PCR screen with deletion specific primers (A), including the RT-PCR analysis done with conserved MHC class I primers (B) that gave two products (600bps and 450bps) that were subsequently subcloned and sequenced. Sequences from these clones matched several other BoLA class I genes when analyzed by BLAST and compared to available ESTs and GenBank sequences.

A.



B.

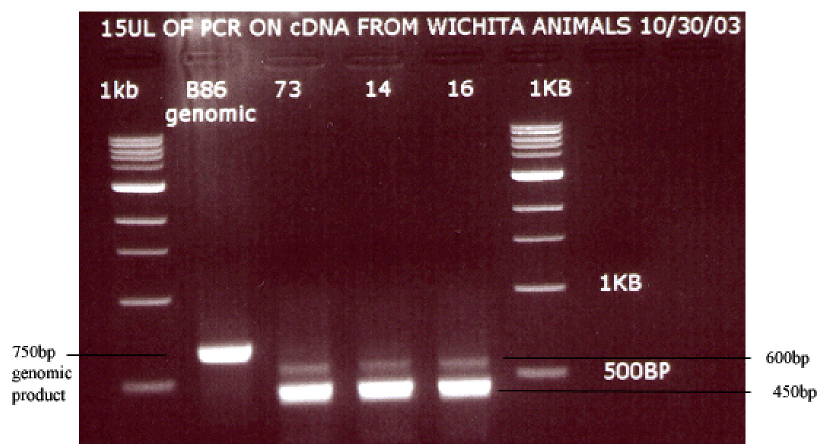


Figure 24 Electrophoresis of PCR product derived from cDNA from several homozygous deletion, homozygous non-deletion and heterozygous Longhorn and control animals. Bull 86 squared cloned (Angus) is labeled 86; animals 73 and 77 are heterozygous; animals 14 and 12 are homozygous A_{del}; animals 77, 94, 99 and 5 are homozygous BoLA A BSA; B86 is bull 86 from whom BSA was originally characterized. A single band (~ 230bp for 65FOR/295DELREV or 169bp for 126FOR/295DELREV deletion specific primers) is visible in each of the A_{del} homozygous animals (77,14, 12) and one heterozygous animal (73). **B.** Initial PCR screen result with Bucon/Blcon primers that amplified two bands from each of the heterozygous (73), A_{del} homozygous (14) and BSA-A homozygous (16) animals. When these were sub-cloned and sequenced, mRNA from several MHC class I genes were identified, leading us to develop deletion specific primers for RT-PCR as done in A.

Quantitative real-time RT-PCR analysis of BoLA BSA-A and A_{del} expression

Quantification of mRNA expression was done using the comparative C_T methods as RT-PCR was done in separate tubes and not in a multiplex due to possible primer competition as well as the additional confounding effect of the sequence similarity between the MHC class I alleles. This analysis was done following a validation experiment for all PCR primers used to show relative efficiency of the target and reference sequence was equal between the two systems.

BoLA BSA-A_{del} and BSA expression levels showed no significant difference irrespective of the primer sets used. When the deletion specific primers were used in real-time analysis to quantify relative mRNA expression levels in heterozygous BoLA BSA-A_{del} /-A and homozygous A_{del} animals, there was a 1.35-fold greater expression of A_{del} in the heterozygous animal (Figure 25), however this was a significant difference.

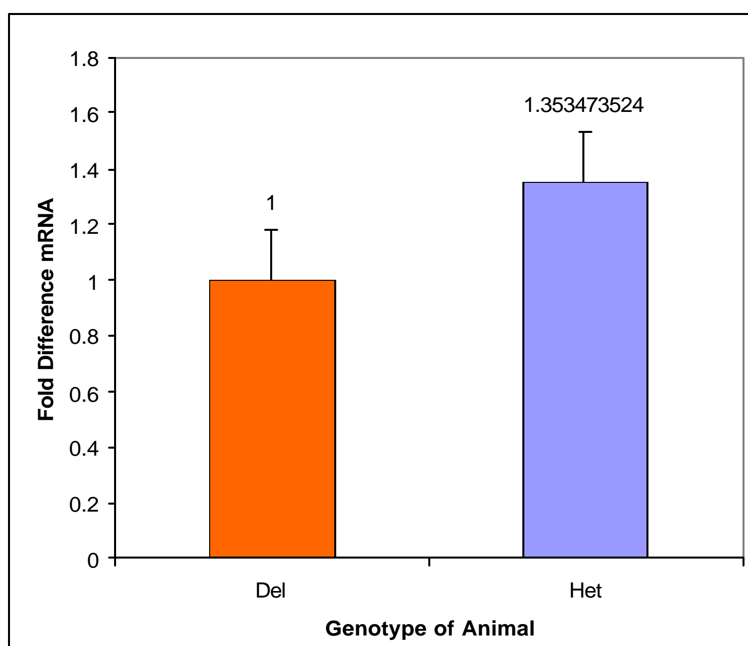


Figure 25. Relative abundance in gene expression of BoLA A_{del} allele. Fold differences in gene expression of the BoLA BSA- A_{del} deletion in animals homozygous for BoLA BSA- A_{del} (DEL) and heterozygous for BoLA-A/BoLA-A_{del} (HET) in real-time RT-PCR analysis with deletion specific primers illustrate no significant difference from each other. Relative transcript abundance value is given above each bar.

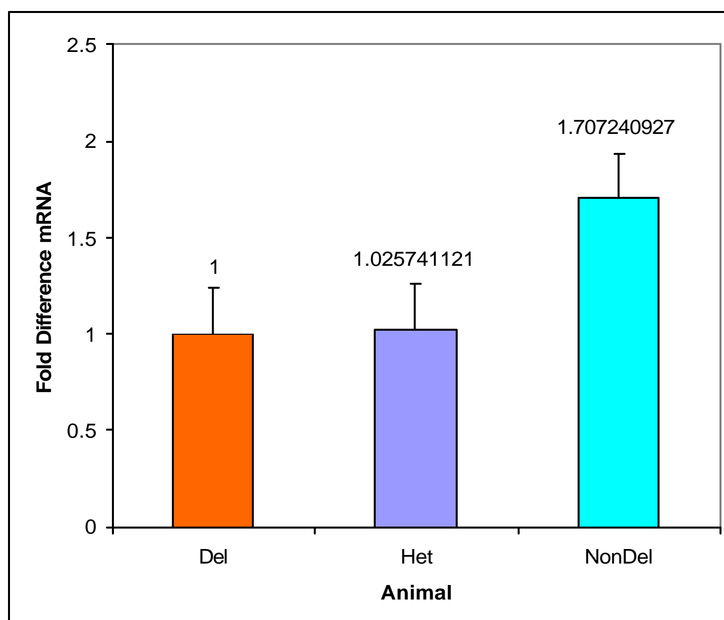


Figure 26. Relative abundance in gene expression of BoLA-A. Fold differences in gene expression of the BoLA BSA- A in animals homozygous for BoLA BSA- A_{del} (DEL), heterozygous BoLA -A/BoLA -A_{del} (HET) and homozygous for BoLA BSA-A in real-time RT-PCR analysis with BSA specific primers show no significant difference from each other. Relative transcript abundance value is given above each bar.

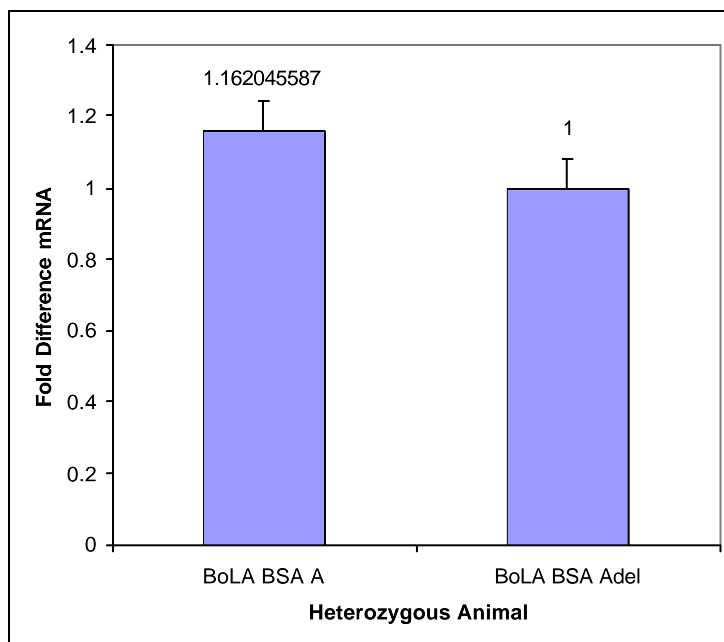


Figure 27. Relative abundance in gene expression of BoLA-A and BoLA-A_{del}. Fold differences in gene expression of the BoLA BSA- A in a heterozygous BoLA -A/BoLA -A_{del} (HET) animal of each allele amplified by different specific primers show no significant difference from each other. Relative transcript abundance value is given above each bar.

Comparison of the expression levels of the two alleles in animals homozygous for BoLA BSA- A_{del} (DEL), heterozygous BoLA-A/BoLA-A_{del} (HET) and homozygous for BoLA BSA-A was done using two sets of primers and this data was pooled and standardized to show no significant difference in relative gene expression (Figure 26). The difference in relative abundance was just 1.7 fold between the non-deletion homozygote animal and deletion homozygote animal. Figure 27 shows that within the heterozygous animal, there was no significant difference observed in the expression of BoLA BSA-A_{del} and BSA alleles using deletion and non-deletion specific primers.

Flow cytometry analysis of expression profiles for MHC class I in both deletion and non-deletion animals

The HLA-ABC PE conjugated antibody allowed quantitative values to be assigned to each sample so that geometric means, medians and the conversions to antibodies bound per cell (or ABC) could be compared between experiments. When used in conjunction with 1:1 conjugates of PE-to-monoclonal antibody, the QuantiBRITE PE beads provide a simple yet robust means of quantifying expression levels in terms of ABC. This was necessary since cell lines from 50 animals could not be maintained simultaneously prior to assignments of genotypes. Once animals were typed, PBMCs from homozygous A_{del} (12, 14) and non-deletion (BoLA BSA-A) animals were cultured and used for analysis over a time course. PBMCs from L1 domino and his daughter (1449) were also used in this analysis. Fibroblasts cultures were begun from four deletion animals (14, 12, 2, 21) as well as from L1 domino. Frozen fibroblasts cultures from B86 (reference animal homozygous for the BSA gene sequence) and a newly-isolated fibroblast culture from L1 were used as a positive control as no skin tags were available for the non-deletion Longhorns tested in this study. Interestingly, since B86 was used in a cloning experiment here at Texas A&M University, his clone B86 squared was available for sampling. It was assumed since B86 was a clone, and his genotype was the same, it would be equally as effective as analyzing class I expression from cultured PBMCs.

Figure 28 shows the initial time course experiment, where PBMCs from deletion and non-deletion animals were pooled and samples taken at 6, 9, 12, 15, >18 days post-culture, stained with antibody and used in the flow cytometric analysis. There was no significant difference between homozygous deletion and non-deletion groups. As expected, there was an overall linear decrease over time of MHC class I expression with an almost 2-fold reduction in expression seen between the 6 days to 18 days samples.

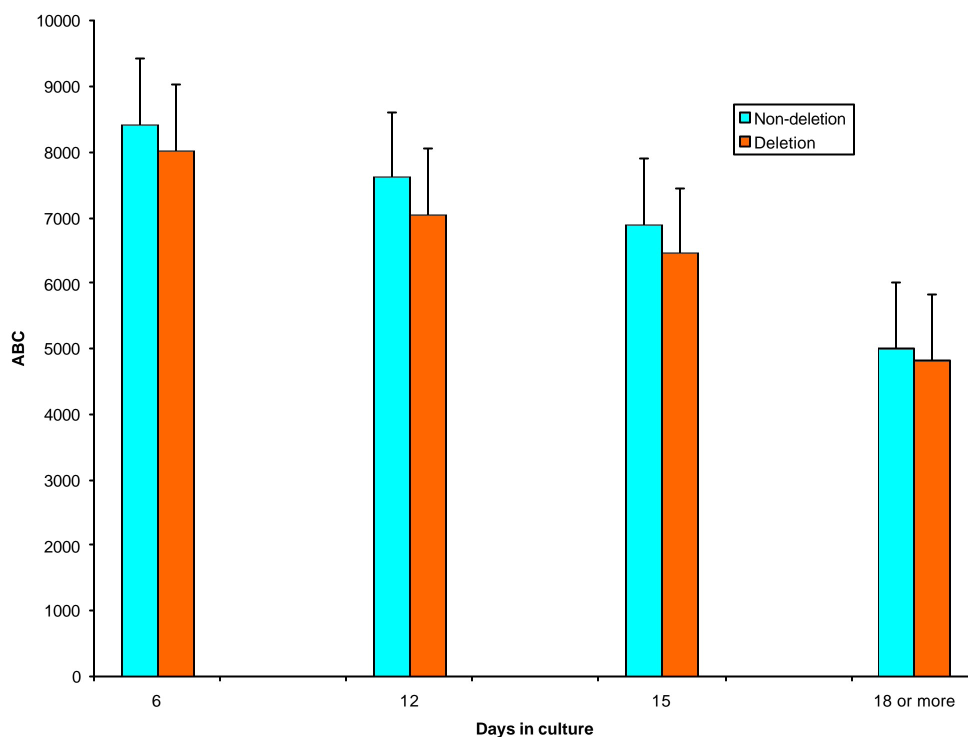


Figure 28. Flow cytometry data. A. Test of expression of MHC class I molecules on surface of stimulated cultured PBMCs from deletion and non-deletion animals taken at 6, 9, 12, 15 and >18 days time points. B. Representative histograms are shown in bottom panel. A summary of at least four experiments are shown for at least 2 animals in each category.

During individual analysis, as illustrated by the flow illustrated in Figure 29, it was clear from that there was no significant difference between deletion (1335) and non-deletion homozygotes, however differences were observed between Bull 86 fibroblasts and fibroblasts of L1 domino in MHC class I expression (Figure 30).

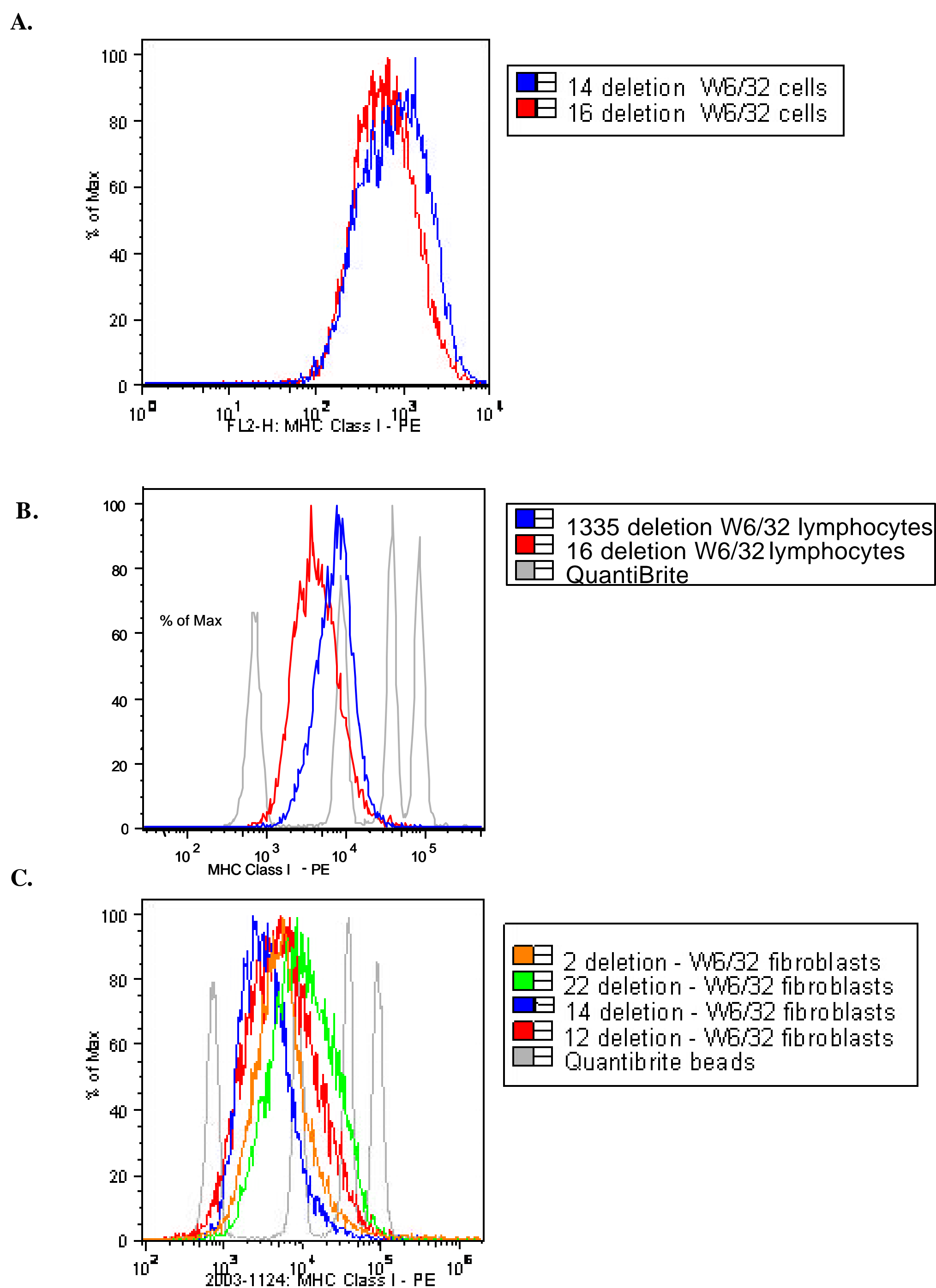


Figure 29. Percentage of MHC class I positive molecules detected in Longhorn homozygous animals. Raw profiles from flow cytometric analyses A and B Cultured PBMCs from deletion (1335, 14L) and non-deletion homozygous (16) animals. All cells were incubated with PE conjugated IgG2a to set an isotype control in every experiment. C. represents a sample experiment on fibroblasts of deletion homozygous animals (2,22,14,12) to illustrate the lower levels of MHC class I expression observed in fibroblasts overall as compared to PBMCs.

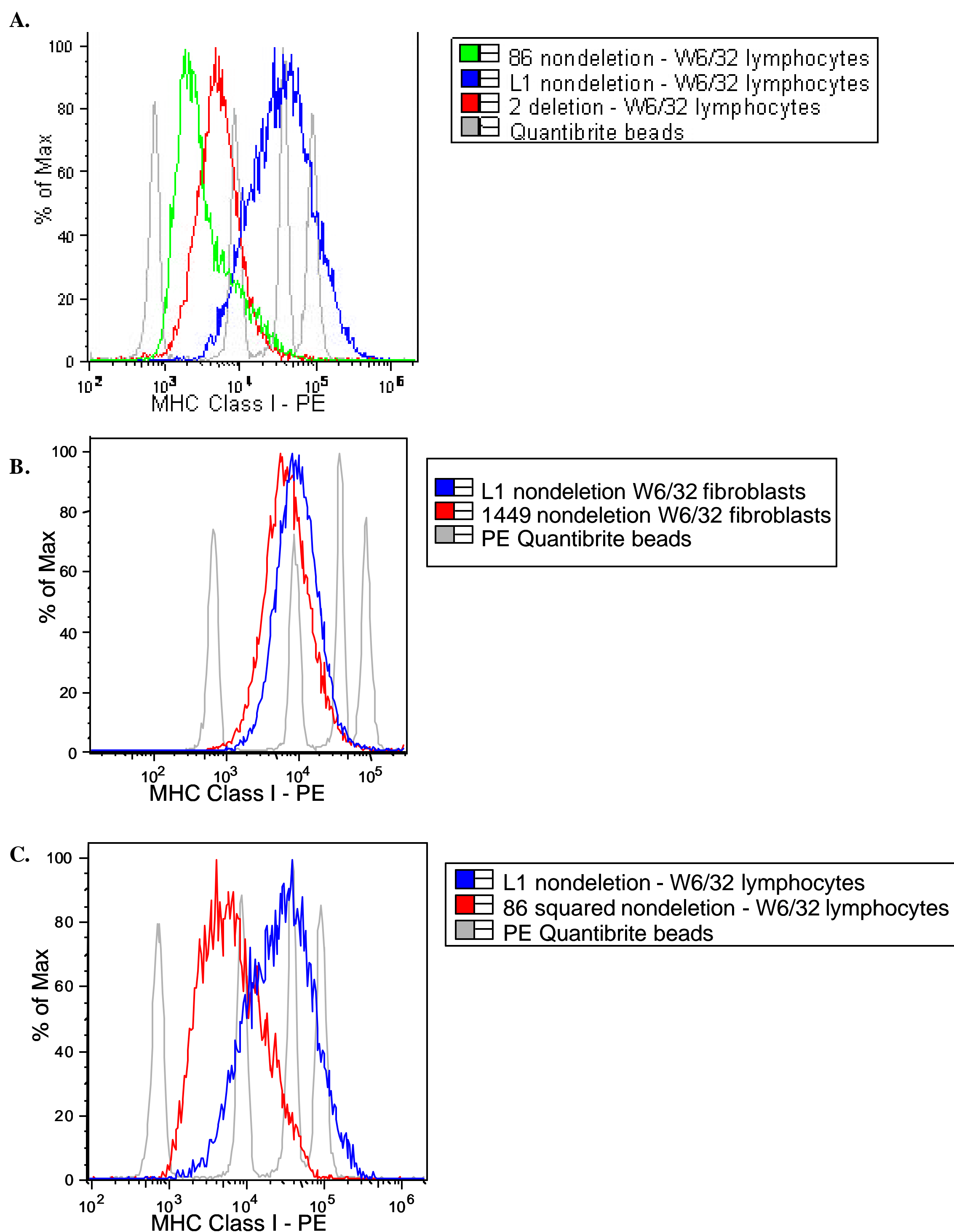


Figure 30. Percentage of MHC class I molecules detected. A. B86 fibroblasts show lower levels of class I expression than deletion (2) and non-deletion homozygous (L1) animals. **B.** L1 and his daughter 1449 show no difference in MHC class I expression. **C.** B86 squared PBMCs also show lower percentage of MHC class I cells expressing MHC class I. Isotype and bead (grey) controls were also performed.

When experiments were repeated (three times for each sample), it was observed that there was a significant difference between L1 and the deletion homozygotes. However, there was also significant difference between L1 and B86 MHC class I expression on fibroblasts ($p<0.01$) (Figure 31). Also, there was significant difference between the MHC class I expression on the cultured PBMCs of B86 squared, L1 and his daughter 1449 (Figure 32). To eliminate the possibility of confounding factors from culture conditions and mitogenic stimulation (Concavalin A was added during culture), the experiment was repeated using freshly isolated PBMCs, but the same results were obtained.

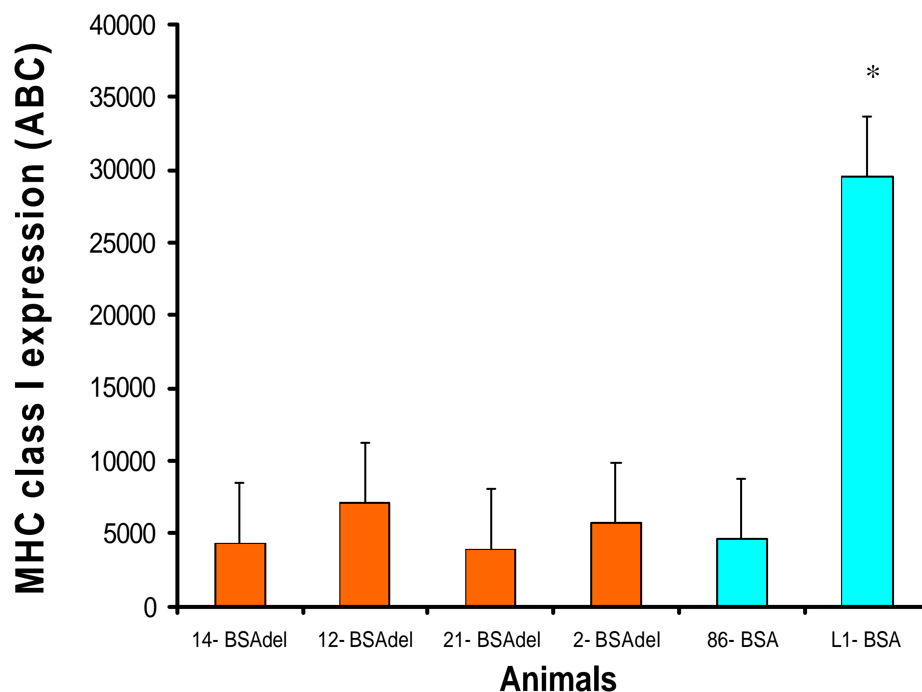


Figure 31. Flow cytometric analyses of MHC class I expression on fibroblasts of BoLA-BSA and A_{del} animals. MHC class I expression as measured by ABC on unstimulated 3 day cultured fibroblasts of homozygous BSA-A_{del} and BSA-A animals, including Bull 86 (86-BSA). Statistically significant differences between deletion and non-deletion animals are shown as *$p<0.05$.

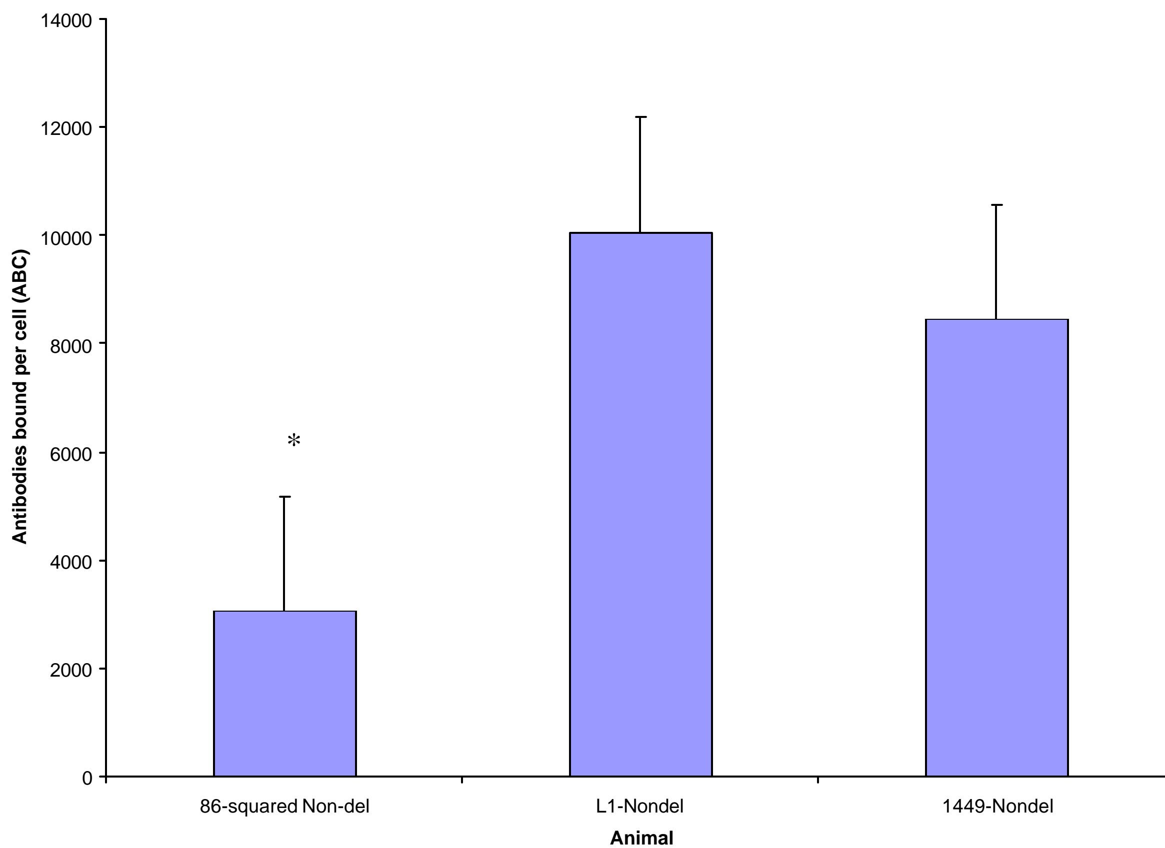


Figure 32. Flow cytometry data for unstimulated PBMCs from non-deletion BoLA-BSA animals. PBMCs from Bull 86-squared, the clone of Bull 86 the source of the BSA sequence and phage library, was used as the reference sample in comparison with PBMCs from L1 domino and his daughter 1449 to compare MHC class I expression as a function of antibodies bound per cell (ABC). L1 Domino is the Chori-240 BAC library source bull and 1449 is one of his daughters. Statistically significant differences between deletion and non-deletion animals are shown as *$p<0.05$.

Modeling of BoLA BSA-A_{del} predicted structure and 3D alignments

The NCBI visualization program Cn3D was used to predict the model structure for the top hit, a HLA-E molecule and peptide. The visualization of the 3D alignments can be seen in Figure 33. Note the conserved residues fit the pattern of the MHC antigen binding cleft, with a few substitutions within the cleft that do not predict a change in conformation (Figure 34). Also the non-conserved residues seen in grey belong to the peptide bound inside the antigen cleft, the transmembrane region of HLA-E and the ß$_2$m molecule that HLA-E is complexed with in this particular model.

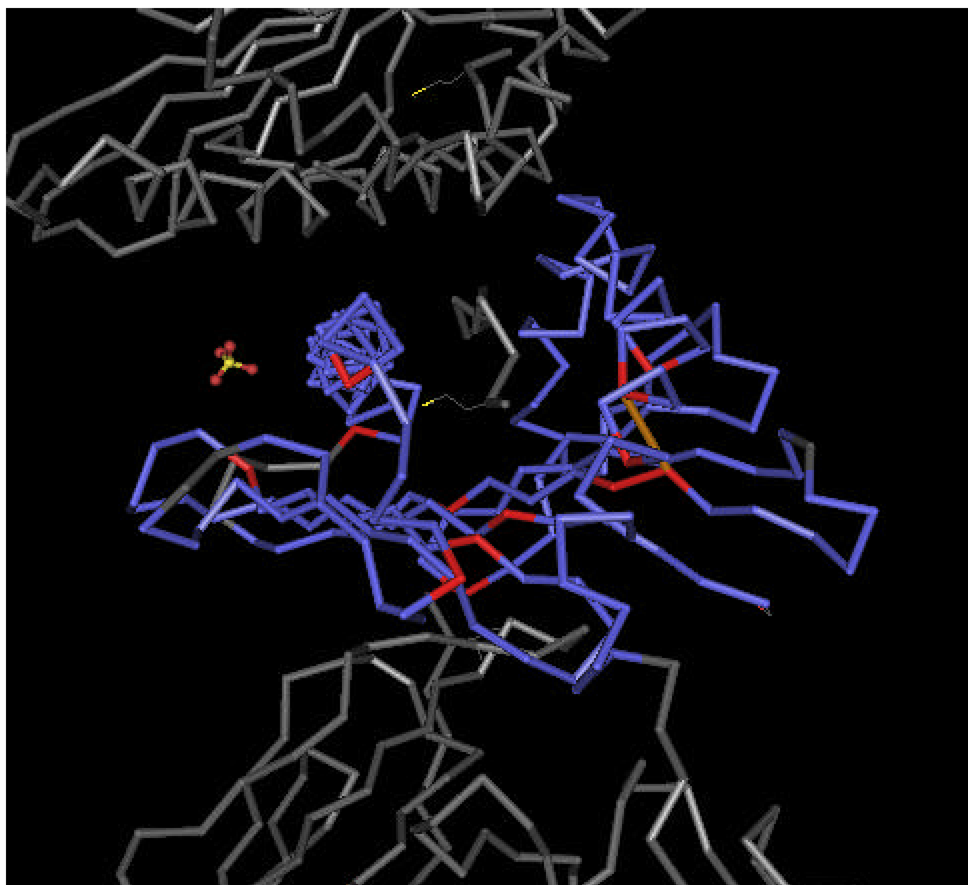


Figure 33. Alignments of consensus of A_{del} sequence and HLA-E complexed with ß$_2$m and peptide. Grey structure on top is ß$_2$M. The grey structure on the bottom represents residues of the transmembrane domain and cytoplasmic tail encoded by exons 4-8 of HLA-E. The grey structure above the blue and red coloured aligned residues is the ß$_2$M molecule. The grey colour represents non-aligned sequence in the HLA-E, ß$_2$M and peptide complex not shared by the predicted structure of A_{del} from its nucleotide sequence in this model.

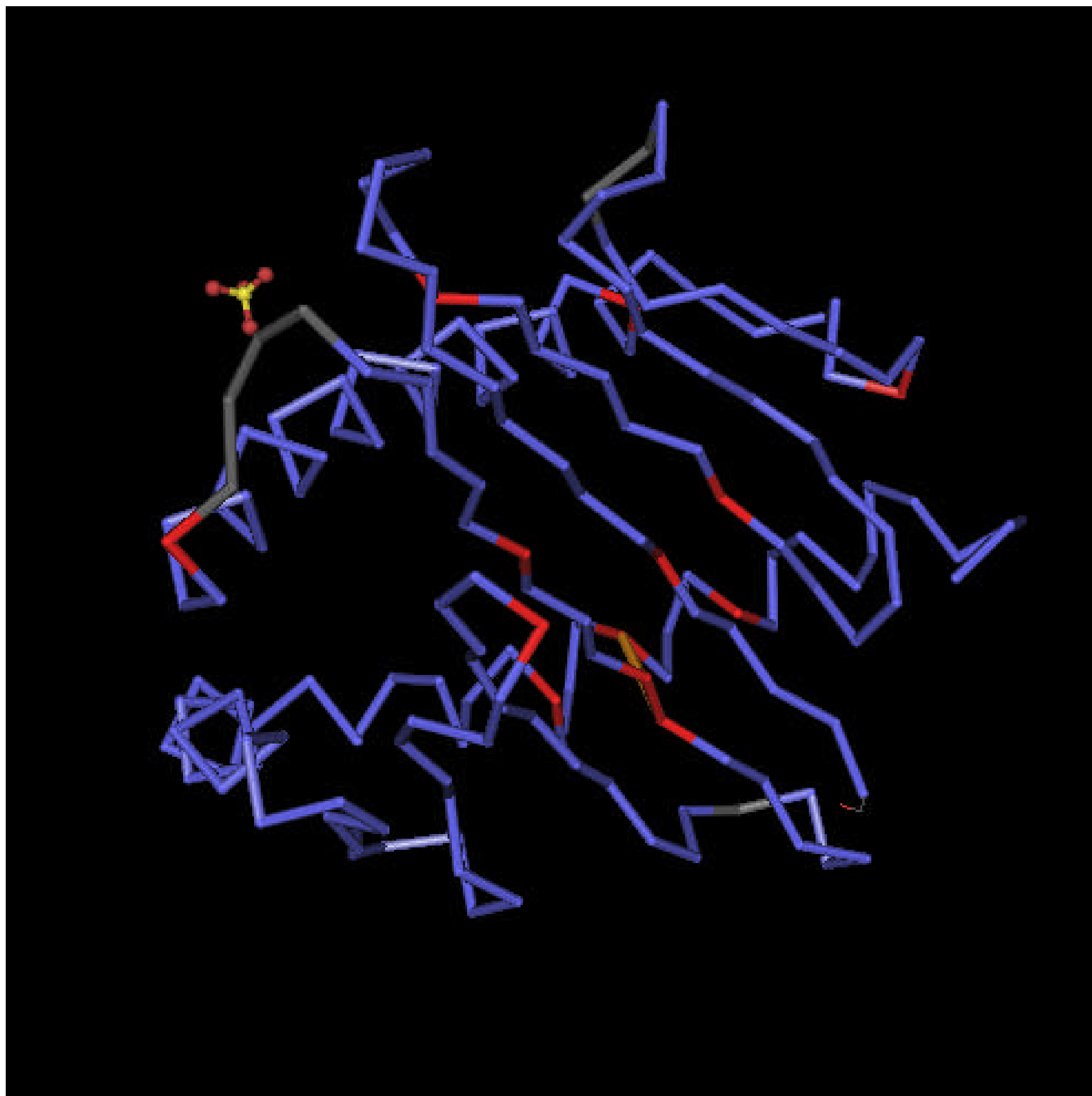


Figure 34. Predicted 3D structure of the BoLA-A_{del} encoded molecule. Alignment of a BoLA BSA A_{del} predicted structure with a structure of a partial HLA -E molecule encoded by exons 1-3 forming the antigen binding cleft of the class Ib molecule. The blue represents conserved sequence alignments, the red indicates synonymous nucleotide substitutions in the alignments and the grey regions are unaligned residues.

Immunoprecipitation, detection and isolation of BoLA class I for mass spectrometry analysis

SDS-PAGE gel analysis was performed using immunoprecipitated cell lysates from deletion and non-deletion homozygous animals to detect any difference in banding patterns. If any differences were seen, these bands were excised and sent for mass spectrometry analysis to detect peptide sequence of MHC class I protein produced from a deletion homozygous animal versus that from a non-deletion animal. Figure 35 illustrates one such polyacrylamide gel picture showing the resulting western blot profile using HC10 Ab which binds to denatured heavy chain class I from an IP of cell lysates using W6/32 (binds to MHC class I-β_2M complex). Several antibodies were used in this analysis and this represents a sample profile of a gel section.

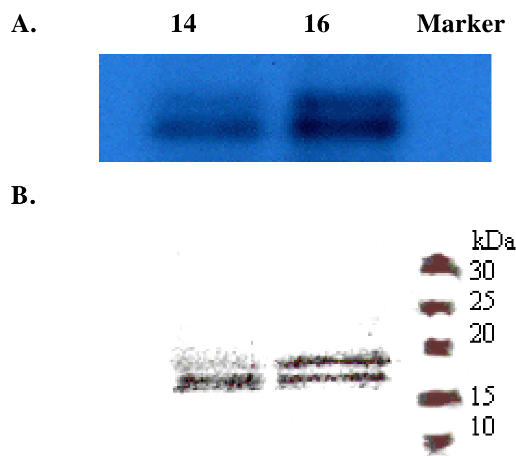


Figure 35 Image of representative SDS-PAGE analysis of immunoprecipitated MHC class I protein from cell lysates of deletion (BoLA BSA-A_{del}) and non-deletion (BoLA-BSA) homozygous animals. A. Immunoblot of 10% Bis-Tris gel (with HC10 Ab) of detergent extracted PBMC cell lysates from homozygous deletion (14) and non-deletion (16) animals immunoprecipitated with W6/32 Ab and neuraminidase treated. B. Photograph of gel stained with Comassie Blue for visualization of banding difference in immunoprecipitation with MHC class I. Two products were visible for the non-deletion animal between a 15-20kDa range, however the deletion homozygous animal showed a decreased level of the 19kDa peptide.

One problem encountered during this study was the contamination of the band of interest (BoLA MHC class I runs at 46 kDa) with many cellular proteins that also have a similar molecular weight (bovine serum albumin, Beta-actin, and even heavy chain of the antibody used in the immunoprecipitation). Figure 36 illustrates the difference in background obtained from boiling the beads and bound product prior to running on the gel versus elution of the bound product off the beads with 1M glycine (pH 2.9). A much cleaner sample was obtained and processed for mass spectrometry analysis.

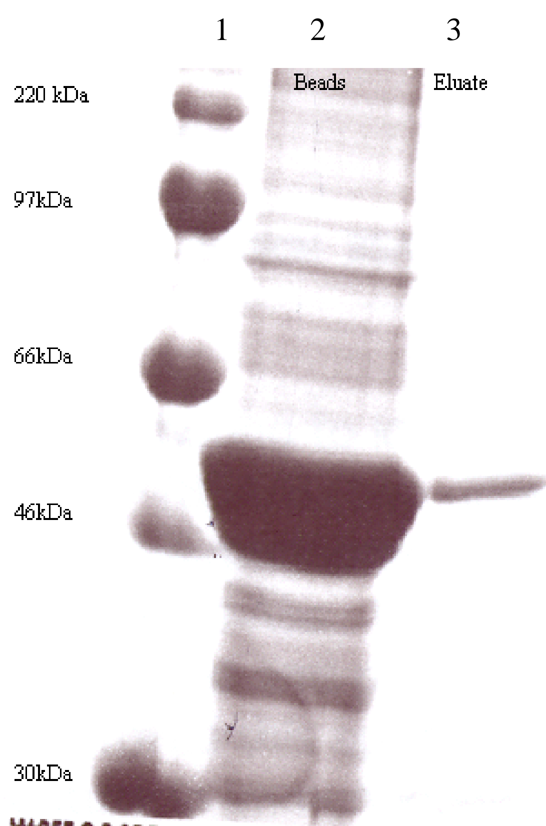


Figure 36. Image of SDS-PAGE gel of immunoprecipitated MHC class I protein from cell lysates after boiling and elution of the protein G beads. Lane one is the molecular weight marker, lane 2 is the profile of the peptides obtained after boiling and no neuraminidase treatment, and lane 3 is the eluate of bound MHC class I using a low pH glycine buffer from the Protein G beads.

Culture supernatants from homozygous deletion and homozygous non-deletions animals were used in immunoprecipitation experiments with HC-10 antibody, as this binds free MHC class I heavy chain to facilitate the restrictions of this study whereby the interactions with ß$_2$M have not been characterized. Detectable bands possibly representing soluble products were observed on the immunoblot of the immunoprecipitates of supernatants from the deletion homozygotes (14, 12, 2, 21) that was not seen in the non-deletions (L1, 22, B86^2) (Figure 37). Interestingly, the supernatant from the only heterozygote animal showed a soluble product but was detectable in lower quantities.

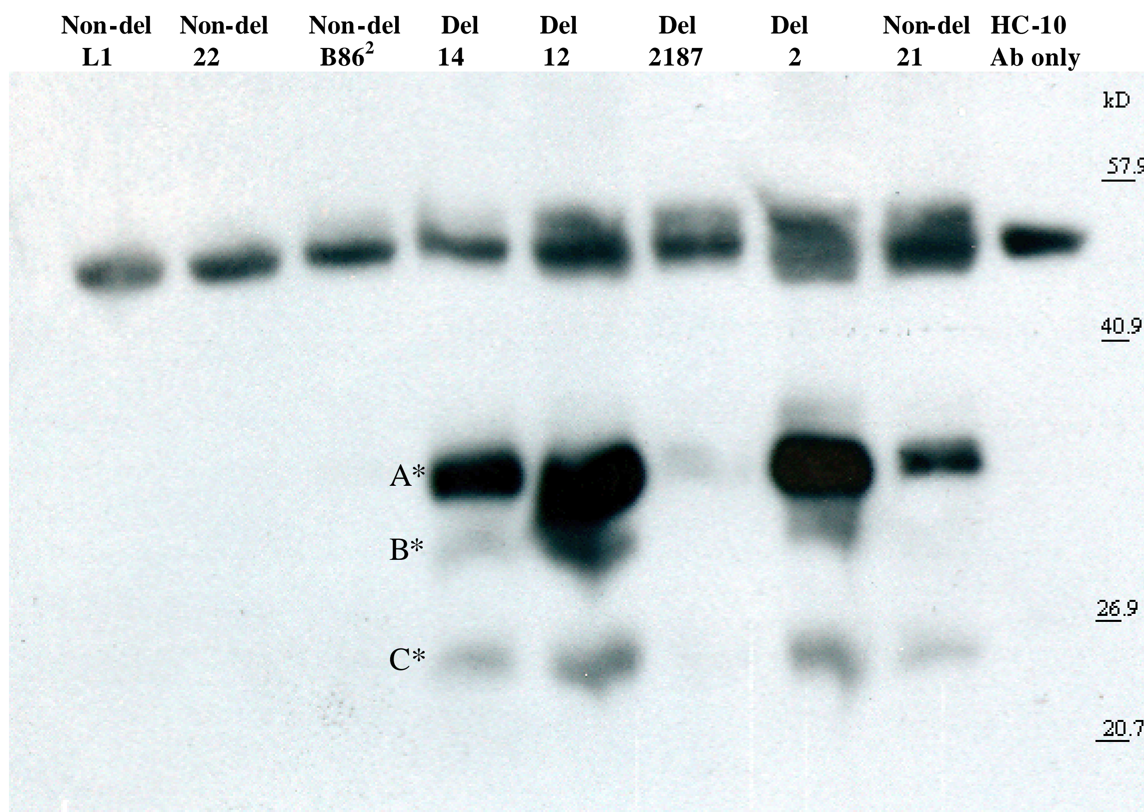


Figure 37. Immunoblot of SDS-PAGE gel separated BoLA class I immunoprecipitated protein from several animals. PBMC supernatants taken at 4 days post culture were subjected to immunoprecipitation with HC-10 mAb, run on a 10% Bis-Tris gel and immunoblotted with HC-10 for detection of soluble BoLA class I heavy chain. Detected bands represented by * that were not seen in the non-deletion BSA animals. Note that band A* was also detected in the heterozygote animal 2187.

A parallel experiment was run with HC-10 immunoprecipitates from the sample 4-day culture supernatant samples. The immunoprecipitated protein was run on a 10% SDS-PAGE Bis-Tris gel, commassie stained and photographed as a loading control and for isolation of the positive band for mass spectrometry analysis. Figure 38 shows the duplicate SDS-PAGE gel image, and that the equivalent band from the immunoblot is too faint for detection to be excised for mass spectrometry. Parallel gels were run for every sample used in the study so Western blot profiles could be compared to gel excised bands sent for mass spectrometry analysis.

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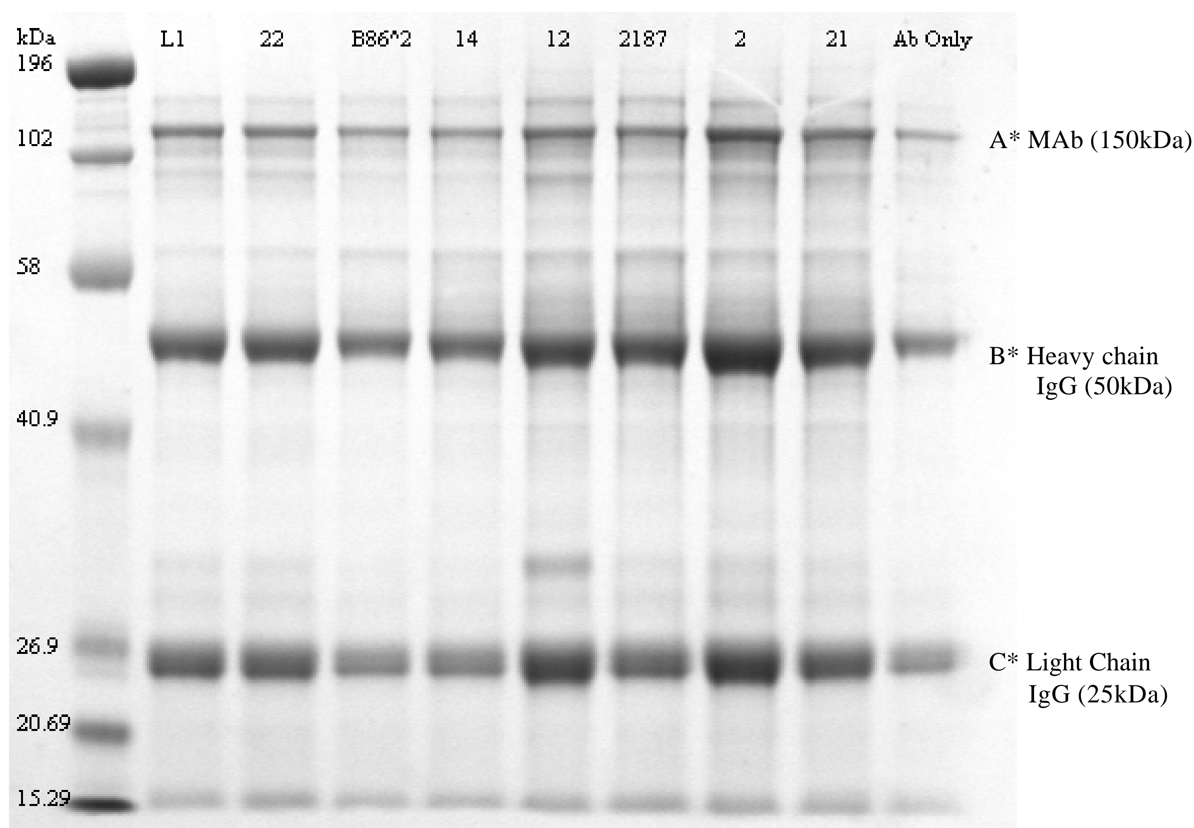

Figure 38. Image of SDS-PAGE gel of immunoprecipiates from cultured PBMC supernatants from several animals. Duplicate gel of immunoprecipitated samples from non-deletion homozygous (L1, 22, B862), deletion homozygous (14, 12, 2, 21) and heterozygous (2187) animals. The three darkest bands are forms of the HC-10 antibody used in the IP and for detection on the immublot.

Isoelectric and 2D analysis of BoLA MHC class I for mass spectrometry analysis

Isolation of the soluble MHC class I protein using 1D isoelectric focusing gel followed by a 2nd dimension 4-12% Bis-Tris SDS-PAGE gel was done for several lysate and supernatant samples from homozygous deletion and non-deletion animals. Staining of the SDS PAGE gel gave very clean visualization of the immunoprecipitation product on silver stain (see sample in Figure 39). However, mass spectrometry analysis of the excised band (pI 9-10, <15 kDa) which fit the criteria for the predicted soluble BoLA-A_{del} gave no conclusive peptide sequence.

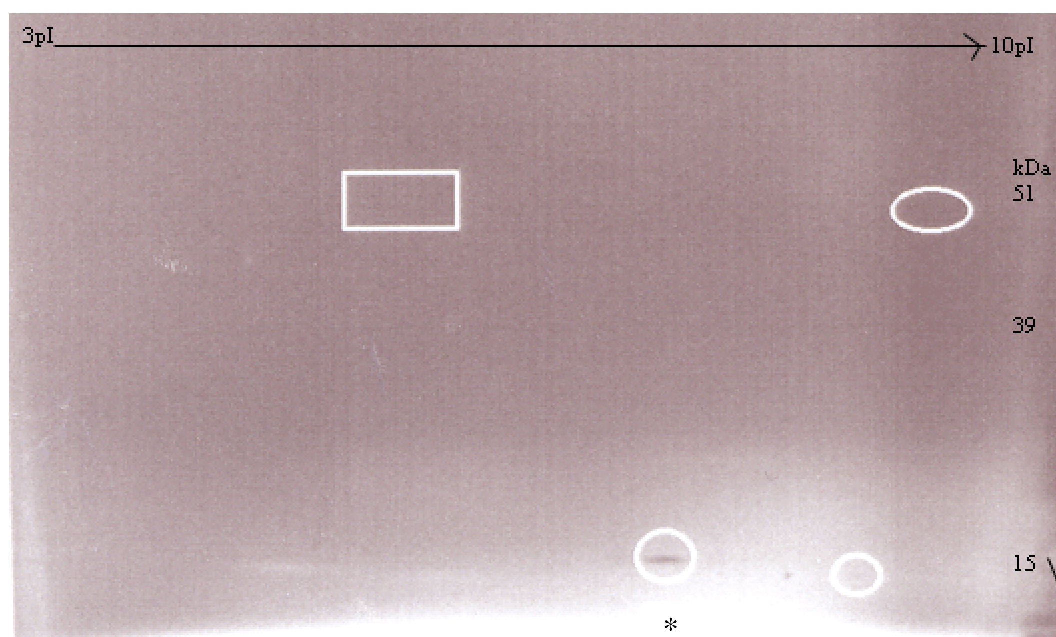

Figure 39. 2D gel analysis of immunoprecipitated class I protein from PBMCs from a BoLA-A_{del} homozygous animal. Image of gel showing direction of isoelectric strip on top and kDa molecular marker size down the right side. Visible spots were excised, trypsin digested and analyzed by mass spectrometry to ascertain peptide and/or amino acid sequence. The spot indicated by * is the within the range of the predicted molecular weight and pI for the truncated soluble protein product of the A_{del} and was excised for analysis. The lack of spots on this gel at around 51 kDa and pI 5 (region indicated by rectangle) shows that no product was obtained for the deletion homozygous sample in this range which the expected range for normal BoLA -A product.

Discussion

In the previous chapter, a BoLA MHC class I frameshift allele BSA A_{del} was isolated, cloned and described in several species of cattle (feral and domestic) and bison. This chapter characterizes the transcription of a BoLA MHC class I allele BSA A_{del}, common in wild and feral bovids, which may produce a soluble class I molecule. To date, no allelic form of MHC class I in any species has been found that produces a soluble class I molecule. Soluble MHC class I (sMHCs) defined to date, are produced by proteolytic cleavage or by alternative splicing (Bouvier et al. 1998; Fournel et al. 2000; Mallet et al. 1999). Characterization of the A_{del} product was attempted through a rigorous analysis of expression encompassing a variety of transcription and translation assays, including real-time RT-PR, flow cytometry, 1D and 2D gel analysis and mass spectrometry analysis.

The real-time PCR methods which monitor accumulation of PCR products can be adapted for the validation of differentially expressed genes as or as in this instance, to detect alleleic differences. One of the major advantages of this method is that it enables simple and rapid measurement of accumulation of PCR products during the log-linear reaction phase. Thus, this procedure has the advantage of greater precision and more objectivity than other quantification methods (like northern blots) and can be used cases such as this, where there is only a 2-base deletion difference in both alleles being quantified. Selection of quantitative RT-PCR primers that span an intron to reduce genomic DNA contamination was not possible, due to need to design deletion specific primers within a highly conserved region of a gene that is part of a large MHC gene family. However, bias was avoided by using different primers to amplify both the deletion and non-deletion alleles and standard curves were constructed to confirm the relative efficiency of targets and reference PCR amplification. Also, performing the data acquisition step at an elevated temperature was helpful in minimizing interference of possible primer-dimers with the quantification. Thus, a robust method was used to detect potential differences in expression of the BoLA BSA and A_{del} alleles, and no

significant difference was found between levels of expression in deletion and non-deletion homozygous animals. Also, no significant difference was also observed between alleles in the heterozygous animals (BSA-A/BSA-A_{del}).

The expression of MHC class I on the cell surface quantified during flow cytometry analysis using the antibodies bound per cell (ABC) assuming a PE to mAb ration of 1:1, also showed no significant difference between deletion and non-deletion animals. There were significant differences between L1 and Bull 86 MHC class I expression level in fibroblast culture, which was thought to be possibly erroneous since Bull 86 frozen stocks were used. Since it is not possible to get fresh unstimulated lymphocytes from Bull 86 (culled), these were obtained from Bull 86 squared (the viable genetic clone of Bull 86) with the same genotypic profile. When unstimulated fresh lymphocytes from L1, his daughter 1449 and Bull 86 squared were analyzed by flow cytometry, there appeared to be significant differences in expression of MHC class I between L1, 1449 and Bull 86 Squared. Possible discrepancies could be due to detection with an antibody (HLA-ABC) quantifying overall MHC class I expression level by measuring all BoLA class I molecules bound to antibody on the cell surface, which may be different in these non-deletion animals. Expression levels of BoLA class I on unstimulated fibroblasts were generally lower (2-fold) than expression in stimulated lymphocytes for both non-deletion and deletion animals. Overall however, when samples were pooled, there did not seem to be a significant difference in the MHC class I expression of the deletion animals versus the non-deletion animals. The high expression of L1 was negated by the low expression of B86 Squared in the non-deletion homozygous animal group once samples were pooled for analysis, and there was no detectable significant difference from the deletion homozygous animals.

Thus, the expression data taken from quantitative RT-PCR and flow cytometric analysis does not show a significant difference in expression of BoLA BSA A and BSA A_{del} alleles. Therefore, there is no evidence to suggest differential expression of the putative soluble or membrane-bound alleles of the BoLA class I gene.

There were however, several confounding factors to be considered. The mAb W6/32 recognizes only class I/ß$_2$m complexes but not free class I heavy chains, whereas the mAb HC10 recognizes free heavy chain. This was useful for discrimination of protein bands when doing immunoprecipitations and western blotting of cell lysates and supernatants. However, no specific antibody is available to the BoLA A_{del} protein, so inferences were made that HC10 would bind at least an intact heavy chain encoded by exons 1-3. The W6/32 mAb is a pan-MHC class I and therefore can detect all forms of HLA-E, -F, and -C in addition to HLA-G, as well as BoLA class I that. Since there are several BoLA class I genes expressed on PBMCs and fibroblasts, it cannot be excluded that the staining of these different BoLA class I molecules may have masked the specific labeling of BoLA A_{del}. Problems in detection of the soluble MHC class I, in addition to generating quantities of protein necessary for detection by MALDI-TOF or tandem mass spectrometry could not be solved by radioactive labeling as this affected the ability of detection in mass spectrometry analysis of the final product. Also, cross-linking of antibodies and elution of bound product to reduce contaminating cellular proteins in the immunoprecipitations may have not been optimal, thereby restricting the amount of protein obtained in eluates and in turn limiting the amount subject to trypsin digest and mass spectrometry analysis.

Another limiting factor of this study was the limited supply of product, as the genotyped Longhorns from which PBMCs and fibroblasts were obtained for this study are at the Wichita Mountain Refuge in Oklahoma and only rounded up once per year, then culled from the herd. Thus, obtaining uncultured PBMCs with which to do comparative analyses from deletion and non-deletion homozygous animals is just not possible. This is of particular concern as mitogenic stimulation of PBMCs may cause induction of genes not induced *in vivo*, and primary cell cultures of PBMCs necessitates addition of cytokine IL-2 and mitogen Concavalin A for cell growth/survival. There are additional confounding factors in that there are different cleaved forms of soluble protein observed, and without a specific antibody, it is not possible to confirm detection of the specific BoLA A_{del} form by immunoblot with W6/32, HC10 or any of the other BoLA

class I monoclonal antibodies that recognize either any BoLA class I heavy chain epitope that is free or in complex with $ß_2$m.

The alignments of the predicted crystal structures of BoLA-A_{del} and HLA-E showed some similarity in the a1 and a2 encoded domains, including conserved substitutions in the ARS-specific binding sites. This may indicate a similar function, in that BoLA-A_{del} may function as a soluble non-classical class I BoLA. The soluble form of HLA-E fails to bind CD8 (Braud et al. 1999) however, secreted HLA-G5 can bind CD8 and induces Fas/Fas ligand-mediated apoptosis in activated CD8+ lymphocytes (Hofmeister 2003).

A role for soluble class I molecules has been defined in maternal/fetal recognition and immune regulation and also allo-reactivity in organ transplantation (Behrens et al. 2001 ; Pickl et al. 1996; Zavazava 1998; Zavazava and Kronke 1996; Zavazava and Muller-Ruchholtz 1994). Recent studies have revealed that sHLA molecules can induce antigen-specific apoptosis or anergy in autoimmune diseases (Nicolle et al. 1994), suggesting that sHLA molecules might be used as a novel therapeutic strategy for selective elimination of unwanted T-cell responses in autoimmune disease (Casares et al. 2002). The serum level of sHLA-I molecules is significantly increased in a variety of physiological and pathological conditions, such as pregnancy, acute rejection episodes following organ allografts, acute graft-versus-host-disease (GVHD) following bone marrow transplantation, autoimmune diseases, viral infections, and malignant melanoma(Zavazava 1998; Zavazava and Kronke 1996).

In addition to their classical antigen-presenting role, HLA class I proteins are recognised by members of the killer immunoglobulin receptor (KIR) and leukocyte immunoglobulin-like receptor (LILR/ILT/LIR) families. Unusual properties of HLA-B27 include an ability of free heavy chains (FHC) to reach the cell surface in the absence of $ß_2$m and to maintain their peptide-binding groove in vitro. The recognition of HLA-B27 in both the classical $ß_2$m- associated and $ß_2$m- independent forms by members of the KIR and LILR families could influence the function of cells from both innate and

adaptive immune systems, and may indicate a role for various leukocyte populations in HLA-B27-associated inflammatory disease(Allen and Trowsdale 2004).

It is possible that this particular frameshift is being maintained as a trans-species polymorphism due to a specific function of this deletion mutant MHC class I molecule in responding to an ancient environmental pathogen under feral conditions in homozygous bovids. However, it is equally likely that it is being lost in domesticated bovids due to linkage to loci that are not economically important quantitative trait loci (QTL).

Humans express killer cell immunoglobulin-like receptors (KIR) (functionally equivalent to C-type lectin-like Ly49 receptors in mice) that interact with restricted subsets of classical class I molecules, and to regulate natural killer (NK) cell function. Cattle appear unusual in having putative functional KIR and Ly49 genes, (Ellis SA, personal communication) which means the MHC class I specificity of NK receptors is much broader in cattle possibly being facilitated by the presence of variable class I haplotypes, such a haplotype expressing an allelic soluble BoLA class I.

Classical genes are apparently duplicated with a high frequency in the evolutionary process, and many of the duplicated genes seem to degenerate into nonclassical genes as a result of deleterious mutation (Hughes and Nei 1989). The nonclassical class I Qa genes in the mouse, that encode soluble molecules, have sequences homologous to regulatory sequences involved in the universal expression of classical class I genes, but they have accumulated numerous nucleotide substitutions in these sequences (Das et al. 2000; Vance et al. 1998; Yamauchi et al. 1981).

The cytoplasmic tail (CT) region of MHC class I possesses important structural features related to functional capability of the molecule, for instance, specific sorting to endolysosomal compartments is mediated by a highly conserved tyrosine motif within the CT (Brode and Macary 2004). Also the transmembrane domain is important for localization of the MHC class I molecule on the cell surface. Since both of these structures are missing due to nucleotide deletions from the predicted molecular form the

BoLA A_{del} frameshift allele, an alternative function other than the classical cell-surface antigen presentation is likely.

Another alternative is that the soluble form acts like MICA, allowing for promiscuous receptor binding. NKG2D is an activating receptor that is expressed on most NK cells, CD8 aß T cells, and ?d T cells. Among its ligands is MICA, which has no function in antigen presentation but is induced by cellular stress. Moreover, NKG2D stably interacted with surface molecules encoded by three newly identified cDNA sequences (N2DL-1, -2, and -3), which are identical to the human ULBP proteins and may represent homologs of the mouse retinoic acid-early inducible family of NKG2D ligands (Steinle et al. 2001). Because of the substantial sequence divergence among these molecules, these results indicated promiscuous modes of receptor binding. Comparison of allelic variants of MICA revealed large differences in NKG2D binding that were associated with a single amino acid substitution at position 129 in the alpha2 domain. Varying affinities of MICA alleles for NKG2D may affect thresholds of NK-cell triggering and T-cell modulation (Steinle et al. 2001).

Also the role of shedding of soluble MICA has been defined as a potential immune escape mechanism of human tumors in vivo In the case of MICA, reduction of surface expression on tumor cells lowers the levels of NKG2DL capable of inducing a cellular antitumor response by cytotoxic lymphocytes and may provide a mechanism for the cells to escape local immune surveillance by limiting activating signals to the host (Salih et al. 2003; Salih et al. 2002). Thus, there could be a similar role in tumour surveillance for the gene product of the BoLA A_{del}. However, if the BoLA-A_{del} soluble form is inhibiting CTL function, it would most likely be subject to purifying selection, but this is not the case (Ramlachan 2004).

Evasion of host immune responses is well documented for viruses and may also occur during tumor immunosurveillance. The mechanisms involve alterations in MHC class I expression, Ag processing and presentation, chemokine and cytokine production, and lymphocyte receptor expression. HCMV for instance expresses at least five proteins that interfere with the MHC class I assembly pathways in a coordinated fashion. These

proteins inhibit the generation of HCMV-specific T-cell epitopes, block the transporter associated with antigen presentation (TAP), retain MHC class I molecules in the endoplasmic reticulum (ER), and redirect nascent class I heavy chains back to the cytosol for subsequent degradation all of which inhibit lysis by T cells (Gewurz et al. 2001; Ploegh 1998).

In addition to T lymphocytes, NK cells play a pivotal role in the control of viral replication. In humans, it has been reported that NK cell deficiency leads to an extreme susceptibility to viral infections, however, HCMV has evolved strategies to inhibit NK lysis (Biron et al. 1989; Ciccone et al. 1994; Lorenzo et al. 2001). However, HCMV gpUS6 protein, which has been demonstrated to downregulate the expression of human leukocyte antigen (HLA) class I and the presentation of cytotoxic T lymphocyte epitopes by blocking transporter associated with antigen presentation (TAP function), does not affect the ability of HLA-E to inhibit NK cell mediated lysis of K-562 cells by interaction with CD94/NKG2A expressed on NK cells (Ulbrecht et al. 2003). Moreover, it is possible that TAP dependence of HLA-E ligands varies between different cell types as has been demonstrated for Qa-1, the mouse homologue of HLA-E (Bai et al. 2000).

In trophoblast cells, HLA-G mRNA is alternatively spliced into a variety of forms which are predicted to encode a full-length membrane-bound form, three short membrane-bound isoforms and two soluble isoforms but only full length HLA-G, but not short HLA-G isoforms can be expressed on the surface of human cells while soluble HLA-G is rapidly secreted (Bainbridge et al. 2000b). Thus, it is likely that the full-length membrane-bound and soluble forms of HLA-G are the only biologically active forms to which the mother is exposed during pregnancy.

Soluble forms of HLA-G produced by placental cells induce apoptosis of activated CD8+ cells (Fournel et al. 2000). In mice, Qa2 also exists in a soluble form, and has to capacity to activate alloreactive CTLs (Bainbridge et al. 2000a). RT1-E2 in rats have been found both in placenta and neurospheres, which are *in vitro* cultures of neural stem cells (McLaren et al. 2001; Park et al. 2002). It may be feasible to speculate that the soluble BoLA A_{del} allele product has a role in maintaining tolerance and immune

response to viral infection during pregnancy at cells in the placental boundary. Such functional analysis is beyond the limits of this study however, as this would require defining expression levels in placental and cord tissue from homozygous and heterozygous BoLA A_{del} animals which were not available for analysis.

It remains unanswered why mammalian cells generate soluble non-classical class I gene products, like HLA-G (humans), RT1-E2 (rat) and H2-Qa2 (mouse), by dual mechanisms: alternative splicing and proteolytic shedding. For example, biological function of the HLA-G5 soluble protein that is an alternative splice variant compared to soluble HLA-G1, is seemingly redundant. One possible explanation might be that the proteolytic shedding of membrane-bound HLA-G1 is favored in pathological situations in which mutations in HLA-G gene splicing sites are introduced. In this case, it was believed that HLA-G evolved into proteolytic shedding as a backup to alternative splicing to control specific immunomodulatory functions. Alternatively, soluble HLA-G1 and HLA-G5 might be qualitatively different from each other in mediating immune responses if they bind different spectra of peptides. Soluble HLA-G1 molecules generated by the cleavage of membrane-bound HLA-G1 associate with beta2-microglobulin and contain bound peptides that are stable at physiological conditions. Also, soluble HLA-G1 is able to protect HLA class I-negative K562 cells from NK lysis, suggesting that soluble HLA-G could act as an immunoregulator in NK cell recognition and possibly in other immune responses (Park et al. 2004a)

One could postulate that it could act as a soluble BoLA class I immunomodulator in a similar fashion to soluble HLA-G. Also, it could be that structural differences in the soluble form allows it to be resistant to certain infection, similar as well to HLA-G, which has evolved harboring a short cytoplasmic tail making it more resistant to HCMV infection than other classical class I, including HLA-2 or -A3. This structural characteristic probably delayed the time and efficiency of HLA-G down-modulation by HCMV l compared to HLA-A2 (Pizzato et al. 2004; Sawai et al. 2004).

Thus BoLA BSA-A_{del} could be maintained due to trans-species polymorphism in cattle and bison, because of an advantage in peptide presentation and resistance to

viral/pathogenic infection due to its soluble form and structural difference. It is possible as well that this allele represents a redundant form of another alternatively spliced BoLA class I that is soluble. This is seen in HLA-G, which has a large abundance of redundant forms produced from alternative splicing and proteolytic cleavage as described previously.

An alternative path is that the BoLA-A_{del} allele can process antigens via cross presentation. It is now defined that ER–phagosome fusion defines a compartment for proteasome- and TAP-dependent cross-presentation in phagosomes (Guermonprez et al. 2003). By doing so, antigen-presenting cells cope with the scarce number of exogenous antigens gaining access to the proteasome for degradation, the low efficiency of antigenic peptide generation by proteasomes and the short half-life of these antigenic peptides in the cytosol (Reits et al. 2003; Rock 2002). By limiting the competition with endogenous antigens, dendritic cells may focus all the cross-presentation machinery on the antigens relevant for the initiation of most immune responses acquired by phagocytosis (Gil-Torregrosa et al. 2004; Guermonprez et al. 2003).

The constant sampling of self and foreign molecules by the immune system is part of a complex quality control process ensuring the recognition and clearance of aberrant cell forms during development, tumorigenesis and infection by microbial pathogens. What could be the biological importance of recovery of extracellular peptides free from processing: recognition of microbial peptides; recognition of partially proteolized autologous proteins in inflammation; or a component of auto-immunity?

Conventionally, MHC class I-restricted antigen processing requires the action of the multimolecular peptide-loading complex within the ER. It has been shown that early phagosomes from human dendritic cells contain the peptide-loading complex, incorporating MHC class I, $ß_2$m, transporter associated with Ag processing (TAP), calreticulin, tapasin, and ERp57 (Ackerman and Cresswell 2004; Ackerman et al. 2003). Antigenic peptides could be translocated into purified phagosomes by TAP and loaded onto cognate class I molecules, inducing their specific dissociation from the loading complex.

However it is known that certain soluble viral proteins, like cytomegalovirus US6 and ICP47 (IE12) encoded by the herpes simplex virus, interfere with antigen presentation via MHC class I molecules selecting TAP as target for immune evasion, blocking TAP and retrotranslocation to the ER and import of peptides (Bauer and Tampe 2002; Hewitt et al. 2001; Kyritsis et al. 2001). Therefore it may be advantageous to have soluble MHC class I, like the gene product of BoLA-A_{del} capable of binding such peptides without restrictions of a transmembrane region or localizations signals found on a cytoplasmic, and access ER and ER-like compartments to bind such free antigenic peptides in the cytosol. Evidence for HLA polymorphism controlling the choice of antigen presentation pathway can be seen in HLA-B, where a single amino acid polymorphism that distinguishes HLA-B*4402 (Asp116) from B*4405 (Tyr116) permits B*4405 to constitutively acquire peptides without any detectable incorporation into the TAP-associated peptide loading complex (Zernich et al. 2004). This mode of peptide capture gives the host an advantage as it is less susceptible to viral interference than the conventional loading pathway used by HLA-B*4402 that involves assembly of class I molecules within the peptide loading complex.

Therefore, a model is proposed where the soluble gene product of BoLA BSA A_{del} is inhibitory acting in a TAP-independent manner to either 1) inhibit NK mediated lysis as is the case of HLA-E inhibiting NK cell mediated lysis of K-562 cells by interaction with CD94/NKG2A expressed on NK cells (Ulbrecht et al. 2003) or Qa-1 in mice (Bai et al. 2000); 2) induce apoptosis of activated CD8+cells in immunopriviledged sites like placenta and neural stem cells in immunoprivileged sites, to induce apoptosis of activated CD8+ cells and induce tolerance during pregnancy like soluble HLA-G (Fournel et al. 2000) and reduce autoimmune-like disease and killing of neural stem cells like RT1-E in rats (Lau et al. 2003); or 3) activate immune response through cross-presentation pathway without competing with endogenously-derived peptides in dendritic cells. If functional, it is most likely not TAP-dependent, so the capability to bind to an antigenic peptide shared by cattle and bison in an ancestral environment can provides a means of antigenic presentation of viral peptides that possibly hijack the TAP

pathway like US16 in HCMV (Lorenzo et al. 2001) and confer the ability to circumvent such pathogenic evasionary tactics. This last option would have the evolutionary trade-off in selection for optimal HLA class I loading versus effective pathogen evasion as the peptide loading complex may have been susceptible to viral interference during evolution of the bovids, and a soluble allelic form in absence of an alternatively spliced variant would have much needed immunomodulatory effects.

Many MHC non-classical and classical molecules that have alternatively spliced forms and upregulation of expression in specific tissues or cell types have been the focus of an ever-increasing amount of interest investigating the potential for multiple roles in antigen presentation and immunoregulation. There is also a high degree of redundancy seen in soluble MHC class I molecules through alternatively spliced variants and cleaved forms being produced. This study represents the first study of an allelic variant of a classical MHC class I gene that has a predicted soluble gene product. It provides information with which functional studies can be performed to further define the pathways, if any, by which BoLA soluble MHC class I molecules provide antigenic response or suppression and/or tolerance in bovids. It also provides insight into the maintenance of trans-species polymorphism between *Bos Taurus* and *Biso*n, similar to that seen for several MHC class I genes within other related species, like primates or rodentia. There is great potential for future study to elucidate the functional role of soluble BoLA class I, especially relating to a immunogenic shared function in both *Bos* and *Bison.* Such studies will be of evolutionary, as well as economic importance, in defining immune evasion strategies possibly used in an ancestral species that could assist in selection and design of vaccines and disease reduction strategies in modern cattle breeding practices.

CHAPTER VI

FINAL SUMMARY AND DISCUSSION

> One does not discover new lands without consenting to lose sight of the shore for a very long time.
>
> – Andree Gide

The profitability of the cattle industry is deleteriously affected by the occurrence of disease, causing millions of dollars in losses worldwide per year. Research using molecular biology is critical to supplement traditional breeding programs to select animals for disease-resistance and develop novel vaccines in the control of disease in livestock populations. The role of genes within the MHC in antigen presentation and characterization in mouse and human, make it a prime target for using comparative genomics to gain a better understanding of genetics and function of the host immune system in other species, like cattle.

Over 200 genes have been identified in the MHC region of humans of which most has either a role in antigen presentation (class I and II) or are involved in the processing of foreign antigens for immune recognition or involved in other immune responses like inflammation or complement (class III). Most of these genes in the HLA have been found to have orthologues and a general conserved gene framework and order (classI, III, II) in other mammalian species, including those with already sequenced MHCs like mouse, rat and swine. In cattle, the MHC has been mapped to BTA23 and has a similar gene order, however there is a major division of class II and a translocation of class IIb separating it by 20 Mb from class IIa, III and I. As well, there are some orthologues not present (DP) and other genes that are shared only in cattle, sheep and goats (DYA and DIB). It is possible that more of these differences are present, but until complete sequencing and assembly is complete, this is not known.

Duplication of MHC genes has occurred many times during the process of evolution and evidence of a "proto-MHC" can even be seen in early chordates. This is

especially apparent in the class I clusters which are seen in human (4 clusters), mouse (6 clusters), rat (7 clusters) and swine (2 clusters) which contain duplicated classical class I genes, pseudogenes, fragments and non-classical class I genes. As discussed in Chapter II, the BoLA class I region appears to have at least 3 and possibly 4 class I clusters that share similarities with known MHCs of other mammalian species. For instance the first class I cluster mapped in BoLA telomeric of class II is between BAT1 and POU5F1 is equivalent to the ß block in humans, mouse, rat and swine. This is followed by one or possibly two clusters between GNL (HSR1) and ZNF173 (Trim 26), which is present in mouse, rat and human as two clusters, the first between GNL and FLJ22638, and the second between RNF23 (Trim 39) and ZNF173 (Trim26). There is also one cluster between the GNL and ZNF173 framework genes also present in the swine MHC. Preliminary evidence from the horse MHC also shows class I genes in these two major blocks (Gustafson et al. 2003). In BoLA however, there is evidence of another cluster of MHC class I genes telomeric of MOG and GABBR1 within the olfactory region, which is found in rat and mouse MHCs but not in human. It can be postulated that the extended duplicated olfactory region was coupled with post-speciation class I gene duplications in these species, and can be related to the increased role of olfactory genes in mate selection.

The heterozygote advantage of the high polymorphism seen in the MHC genes, helps to overcome the fallacies predicted by the Red Queen hypothesis as discussed in Chapter I, by the fact that an offspring is unlikely to inherit the same allele from both parents, and thus has double the MHC recognition capability for antigen detection from pathogens regardless of the rate of a pathogen's evolutionary evasion tactics. The large number of MHC alleles documented in the human population (>1000 to date) would contribute to overall population fitness as it is not likely that a single pathogen would evade such diversity entirely, providing a barrier to pandemics of potential disease.

Generally "classical" class I genes like HLA-A, B and C are expressed in all humans, and this seems shared by other primates like chimps and gorillas. However closely related primates like orangatuns and rhesus monkeys have variable expression of

A, B and C orthologues, with C not even present in rhesus monkeys. This illustrates variation in numbers and expression of MHC genes even in the more related species. In ruminants like cattle and sheep, as well as in pig and horse, there seems to be a large number of expressed class I genes and a variable number of haplotypes. The variability seen in class I genes of cattle however, is unique since no class I locus is consistently expressed, there are variable haplotypes with even single class I locus haplotypes, and extensive class I interlocus recombination. This has made complete assembly of DNA sequencing data from class I BACs within BoLA very difficult.

The fact that several animals have been used to date for assembly of BoLA and no single haplotype has been used to provide a complete BoLA map provides additional confounding factors. The presence of so many MHC class I & II alleles that are null, or contain synonymous substitutions, also adds another problem so that the diversity of MHC molecules that can be identified by antibodies serologically, is considerably fewer than that by DNA sequencing. The presence of pseudogenes, gene fragments and non-classical class I sequences spread liberally throughout class I clusters make it more difficult to assign alleles to loci by DNA sequence analysis. For instance, Bull 86 was thought to be heterozygous for BSA and BSC alleles thought to be at the same locus. However, when molecular and serological data were aligned, it was realized that Bull 86 was heterozygous for BSC and homozygous for BSA; previous “alleles” which are now known to be clearly not alleleic as BSC was formed from an interlocus recombination event between BSA and another class I gene.

The FISH and fibre FISH analyses were very important in confirming the overlapping BACs in the preliminary contigs and to map them to BTA23 q2.2. This was especially needed when class I sequence and BACs were obtained from up to five different animals, and DNA sequence analysis was not enough to order them.

The use of phylogenetic trees to test hypotheses of recombination and gene conversion giving rise to inter-allelic recombination is useful as is estimating numbers of nucleotide substitutions per site in a particular gene region (Hughes and Yeager 1998b; Yeager and Hughes 1999; Yeager et al. 1997). However, it is difficult to assign a

sequence on the basis of allele versus loci using phylogenetics, especially in cattle MHC analyses (Di Palma et al. 2002; Ellis et al. 1986; Holmes et al. 2003) due to the high levels of gene conversion. It was possible to however, characterize the BoLA A_{del} as a trans-species polymorphism seen in *Bison* and *Bos* predating speciation (Chapter IV).

The nested PCR primer pairs used for genotyping analysis of the BSA-A and BSA-A_{del} alleles however were specific and consistent, as cloning and sequencing of genotyping products matched predicted genotypes for all animals in the analysis. Also, no more than two sequences were obtained from sequenced clones of BSA "locus-specific" PCR products from each animal, indicating that they were allelic and not from different loci.

The A_{del} allele of the BSA gene was consistently amplified by using the deletion specific primers in several homozygous and heterozygous animals, including a large number of Texas Longhorn animals used for expression analysis. In the experiments described in Chapter V, flow cytometric and quantitative RT-PCR analysis showed no significant difference in overall MHC class I expression between BoLA MHC class I and A_{del} in homozygous deletion and non-deletion animals. Although 1D-IEF, 2D gel analysis and western blotting showed several bands to be BoLA class I specific from immunoprecipitations of homozygous deletion PBMCs, analysis by mass spectrometry was not clear enough to determine the peptide sequence of these protein bands. This was in part due to the lack of product obtained from cell lysates and supernatants during these experiments. Since it was not possible to re-bleed the animals used in this study, there was a limitation to the amount of material available dependent on the last collection at Wichita Mountain Refuge in September 2003.

It is possible that functional data can be obtained using BoLA-A_{del} transfectants (e.g.CHO cells) to produce larger quantities of class I protein which can also be used for downstream mass spectrometry analysis and further functional assays. Also, production of a monoclonal antibody specific for BoLA-A_{del} would also provide an invaluable tool, as all antibodies used in this study were either developed for humans and reactive in cattle, or developed for other cattle class I genes. The caveat in this study is that it is

unknown whether or not lack of confirmation of detection of a soluble product by mass spectrometry is due to it not being translated, or because of failure to obtain enough of a detectable product.

William of Ockham's philosophical idea states that the best hypothesis to explain a process is the one that requires the smallest number of assumptions. This may be the most important technique in comparative genomics of the MHC, as it is important to not make any more assumptions than absolutely necessary in analyzing genetic and functional aspects of the BoLA class I region. Thus, in conclusion, there is no evidence to suggest that BoLA class I A_{del} is differentially expressed from any other BoLA class I gene, nor that the soluble product is non-functional. The BoLA class I region does share some similarity in gene order, structure and duplicated clusters as detailed in this study, with other mammalian species. Also, the maintenance of the A_{del} allele as a trans-species polymorphism in feral and wild *Bos* and *Bison* could be 1) pure chance, or 2) be possibly lost from certain highly genetically-manipulated domestic breeds as a by-product of domestication and linkage to negatively selected loci, or alternatively, 3) serving a functional purpose by recognizing an environmental pathogen common to both species or providing immunological tolerance and thus facilitating either the initiation or suppression of an immune response, respectively.

The molecular genetic structure of the MHC evolved possibly as a result of selective pressure imposed by infectious microorganisms. Acquisition of complete and accurate sequence data over polymorphic regions such as the BoLA using a BAC-based approach, provides a definitive resource for the construction of informative genetic maps, and avoids the limitation of chromosome regions that are refractory to PCR amplification or difficult to sequence by other approaches. Construction of such genetic maps, especially including haplotypic structure, has the potential of detecting variants with high susceptibility or resistance to disease.

The present organisation of the MHC represents a snapshot in its evolution from its ancestral form. Many of the molecular structures described above have been shown to be targets in the various strategies devised by infectious organisms, in particular viruses,

to evade immune detection. Diverse evolutionary pressures may have directed the evolution of the MHC differently in other organisms and further study of the comparative genomic organisation of MHC genes in cattle, mouse, chicken, rat, teleosts as well as more primitive members of the chordate family (eg. the lamprey), may help to answer the riddle set by the genetic structure of the MHC as we see it today.

Also, further analysis of the BoLA class I region, including studies on defining a functional role for the soluble allelic form of the BoLA BSA A-del gene product, would allow for a greater understanding of the immunological role of BoLA MHC class I genes. This is of prime importance in the development of vaccines, the design of animal breeding programs to maximize resistance to pathogens, and to allow for evaluation and optimization of a population's immune response in one of the most economically important species worldwide.

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