ANALYSIS OF CLASS I MHC GENES IN CATTLE

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DECLARATION

I declare that the work presented in this thesis is my own and that where others have assisted me, their contributions have been clearly indicated.

ABSTRACT

The aim of this project was to isolate a functional BoLA class I gene, to transfect this into L cells and to investigate the possibility of a second BoLA class I locus.

Attempts to isolate a functional BoLA class I gene from a cosmid library was unsuccessful, but clones encoding complete BoLA genes were successfully identified from a bacteriophage library. This library was made from animal 10769 which has BoLA type w10/w11 and the genes were identified with a class I cDNA probe, pBoLA-1. These clones were transfected into mouse L cells to look for expression. One of the clones, 19.1, expressed class I molecules when analysed by indirect immunofluoresence on the FACScan and by fluorescence microscopy. The Northern blot analysis confirmed class I transcription products when probed with pBoLA-1. Clone 19.1 was identified as encoding a w11 gene by a microlymphocytotoxicity test. Also in a T cell cytotoxicity assay, Ltk 19.1 cells were killed by alloreactive anti w11 cytotoxic T cell clones but not by anti w10 clones. Nucleotide sequencing of gene encoded in 19.1 demonstrated homology between this and BoLA cDNA clones and HLA. Therefore a functional BoLA class I gene with a BoLA type w11 was isolated and is a stepping stone to many other investigations.

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Though I am excited to return home, I am sad to leave all my friends in Edinburgh. TERIMA KASIH (a Malaysian thank-you).

DEDICATION

WITH LOVE

To my mother and late father for always believing in me that one day I would do a Ph.D. study.

To my husband, Zul and daughter, Atikah for all the sacrifices made.

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CHAPTER 1

THE MAJOR HISTOCOMPATIBILITY COMPLEX - GENERAL INTRODUCTION

1.1. WHAT IS THE MHC?

The major histocompatibility complex or the MHC as it is commonly known is a cluster of genes encoding transmembrane proteins that occupies some 2000 to 4000 kilobase pairs of DNA (Klein, 1986; Dunham et. al., 1987 and Lawrance et. al., 1987). The MHC has been given different names in different species (Klein, 1986). It is designated H-2 in the mouse, HLA in humans, B in the domestic fowl, RT1 in the rat, BoLA in cattle, SLA in swine and different appropriate names for all the species studied.

There are three clusters of genes in the MHC complex; the class I, II and III regions. In the class I region there are the class I genes which code for the classical transplantation antigens, and these are known as HLA-A, -B and -C in man and H-2K, -D and -L in the mouse, only one locus in the rat, RT1 and one well defined locus, BoLA-A in cattle with evidences for a second (Lindberg & Andersson, 1988; Joosten et. al. 1988; Bensaid et. al. 1988; Ennis et. al. 1988; Toye et. al. 1990; Bensaid et. al. 1991; Joosten et. al. 1992 and Al-Murrani et. al. manuscript in prep). The related functional genes or nonclassical class I genes are called HLA-E, -F, -G, -H and -X in man (Shimizu et. al., 1988) and the Qa and Tl genes in the mouse (Flaherty et. al., 1980). In cattle, the counterpart of these genes have been inferred by Brown, 1989b from Southern blot studies.

In the class II region, there are the genes HLA-DR, -DQ, -DP, -DNA, -DOB, RING 6 (-DMA), RING 7 (-DMB) in man (Trowsdale et. al., 1991), in the mouse I-A and I-E are equivalent to -DR and -DQ (Kaufman et. al., 1984) and in cattle -DR, -DQ, -DNA, -DOB, and -DY homologues have been found (Andersson et al.,

1986a, 1986b, 1988a, 1988b & Sigurdardottir, et al., 1988). There are also non class II genes in this region, i.e. the recently discovered genes, Tap 1 and Tap 2 thought to be involved in class I antigen processing and transport of peptides (Livingstone et. al. 1989; Deverson et. al. 1990; Spies et. al., 1990, Monaco et. al. 1990; and Trowsdale et. al., 1990).

In the class III region, genes encoding proteins involved in the immune system include the complement components C2, C4 and factor B (Carroll et. al., 1984) as well as the steroid 21-hydroxylase gene (Carroll et. al., 1985). Two more genes that map between the class III and class I regions as determined by pulsed field gel electrophoresis (Dunham et. al., 1987) or field-inversion gel electrophoresis (Ragoussis et. al., 1988) are the loci for tumour necrosis factor (TNF A) and lymphotoxin (TNF B).

One of the most interesting features of the genes coding for class I and class II MHC glycoproteins is that they are the most polymorphic loci known in eukaryotes. The degree to which alleles can differ is remarkable. Alleles at the same locus can differ by as few as one or as many as a hundred nucleotide substitutions (Klein, 1986 and Parham et. al., 1988). The high degree of genetic polymorphism is related to their function as primary recognition elements in the immune response.

The following sections of this introduction will focus on the classical and non-classical class I molecules of the MHC.

1.2 DISCOVERY OF MHC CLASS I

The MHC was first identified by Gorer through its role in graft rejection in mice. He showed in 1936 that a rabbit anti-mouse serum contained an antibody

which reacted with red blood cells from only some mouse strains. He designated the determinant detected by this antibody as 'antigen-II'. It was controlled by a dominant autosomal locus. Subsequent studies by Gorer in 1937 showed that the locus controlling 'antigen-II' and a locus controlling resistance or susceptibility to tumour transplants not only segregated together but the tumour resistant animals were producing antibody against 'antigen-II' (alloantibody). He concluded that the same locus controlled 'antigen-II' and tumour resistance or susceptibility. He hypothesised that the incompatibility for 'antigen-II' between the host and tumour donor produced an immune response which caused the destruction of the graft. This discovery was the basis for the current understanding of tissue transplantation and the MHC of the mouse.

In 1948, G.D. Snell became interested in tumour transplantation and his approach was different from that of Gorer's. His work was based on cellular immunity, measuring *in vivo* compatibility of tissues from different individuals. His construction of congenic mouse strains also allowed tremendous progress in the understanding of the immune system. Congenic strains are genetically identical, except for a limited segment of chromosome carrying a selected marker locus and closely linked loci. These strains permit analysis of allelic products at a single locus or closely linked loci on a standard genetic background.

Studies by Gorer, Lyman and Snell showed that the locus controlling 'antigen-II' was in linkage group IX which is now designated as chromosome 17. They named this locus the histocompatibility 2 or H-2 of the mouse (Gorer, Lyman & Snell, 1948).

In humans, the first indication of a system controlling histocompatibility was provided in 1943 by Gibson and Medawar. While working at a hospital, they

observed a graft rejection process where the host became sensitized by the first graft and therefore rejects the second graft more rapidly. Medawar continued to investigate this rejection process in mice. He found there was a distinct difference of response between an autograft (grafts taken from one part of the body and placed on another part) and an allograft (grafts exchanged between genetically different individuals of the same species). He concluded that the antigens that cause the allograft reaction were similar to those discovered by Gorer in mice.

Although the existence of an MHC in the mouse was quickly established, it was not until the beginning of the sixties that the human MHC begun to be described. Three human systems of leukocyte antigens were defined: first, MAC, designation given to the antigen from the first letters of the nonagglutinator's surnames (Dausett, 1958); second, 4a/4b, a leukocyte antigen group 4, that was controlled by a single locus with at least two alleles, 4a and 4b (van Rood and van Leeuwen, 1963) and third, LA, a single leukocyte - antigen locus with at least three alleles : LA1, LA2 and a blank when both antigens are absent (Payne et al., 1964). Finally, a nomenclature was agreed by a committee for the human MHC to be HLA - human leukocyte antigen.

Most antibodies for the analysis of the HLA system were derived from multi-parous women who had been immunised by paternal antigens present on foetal cells in the maternal circulation. Depending on the differences in HLA type and the degree of antigenic stimulation, these reagents can be of a sufficiently high titer and specificity to be used as tissue typing reagents. Their analysis led to the classification of the three classical loci, the HLA-A, HLA-B and HLA-C (van Rood et. al. 1958). The molecules in these loci are very polymorphic (Dausset 1981) and the complex was mapped to chromosome 6 (van Someren et. al., 1974).

In cattle, transplantation studies were initiated to differentiate between monozygotic and dizygotic twins (Anderson, et al., 1951). However, dizygotic twins can tolerate skin grafts from each other. Anderson suggested that this was acquired tolerance resulting from exchange of erythrocytes and leukocytes precursors between such twins during foetal development (cf. Owen, 1945). Further studies on cattle were limited until Spooner et al. (1978) looked for association between a putative bovine MHC and disease resistance in cattle. In their studies, they provided evidence for the presence of cattle MHC, BLA (BoLA) on the basis of skin grafting experiments and serology. At the same time, Amorena & Stone (1978) described an SD (serological determining) locus as BoLA-A. The BoLA complex is described in section 1.9.

1.3 THE MAJOR HISTOCOMPATIBILITY COMPLEX IN MOUSE AND HUMAN

The use of DNA cloning and sequencing techniques has allowed rapid progress in studies of the MHC. The most extensively characterised MHCs are the human leukocyte antigen (HLA) and the mouse H-2 complex. Therefore to have a better general understanding of the mammalian MHC system, the HLA and the H-2 complexes will be reviewed. Figure 1 shows the genetic maps of the two complexes.

The general organisation of class I genes within the respective MHCs differs between mouse and human. In the mouse, the class II loci are intercalated between the class I loci (Brorson et al., 1989), while in man, the class II loci are centromeric to the class I loci (Trowsdale et. al., 1991). The human organisation seems to be most common since it is found in every other mammalian species so far examined (Klein, 1986). The mouse type of arrangement has only been found in the mouse

FIGURE 1. *HLA AND H-2 COMPLEXES*

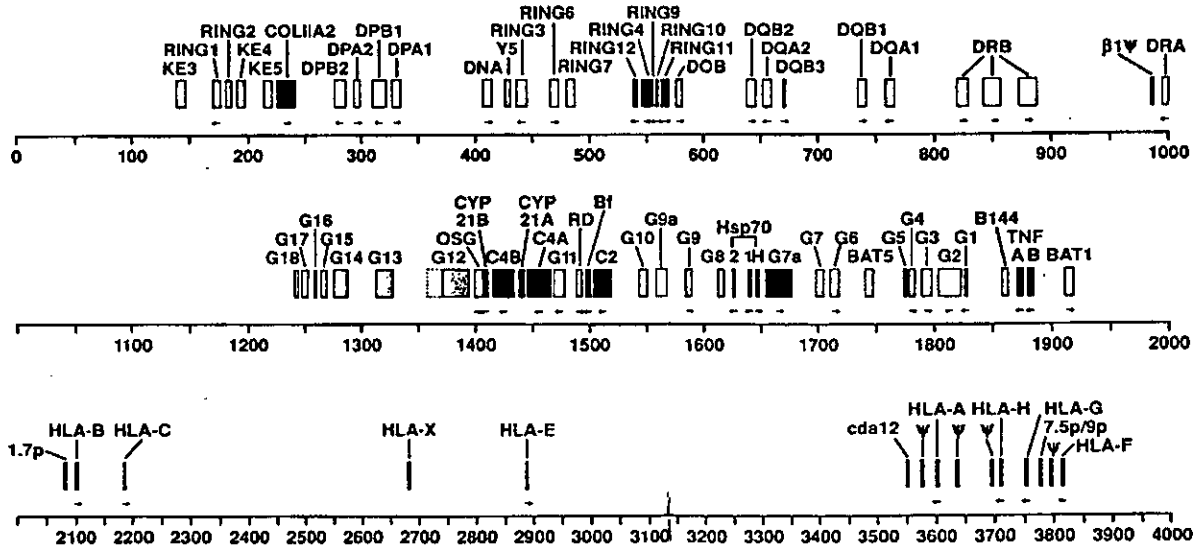
Figure 1a

Genetic map of the HLA complex. The map was compiled from physical mapping and cloning data from various laboratories. (After Trowsdale, Ragoussis and Campbell, 1991).

Figure 1b

Genetic map of H-2 complex in the BALB/c mouse. Breaks in the DNA map indicate that these regions have not been linked by overlapping genomic clones. (After Brorson et. al., 1989.

a.


RING2 COLIIA2 DPB1
RING1 KE4 DPA2 DPA1
KE3 KE5 DPB2
RING6
RING3
Y5
DNA
RING9
RING4 RING10
RING12 RING11
RING7 DOB
DQB2
DQA2
DQB3
DQB1
DQA1
DRB
β1Ψ DRA
0 100 200 300 400 500 600 700 800 900 1000
G16
G17 G15
G18 G14 G13
CYP 21B
OSG
G12 C4B
CYP 21A
C4A
G11
RD
Bf
C2
G10
G9a
G9
Hsp70
G8 2 1H G7a
G7
G6
BAT5
G5
G4
G3
G2
G1
B144
TNF
A B BAT1
1100 1200 1300 1400 1500 1600 1700 1800 1900 2000
HLA-B HLA-C
1.7p
HLA-X
HLA-E
HLA-A HLA-H HLA-G
cda12
7.5p/9p
HLA-F
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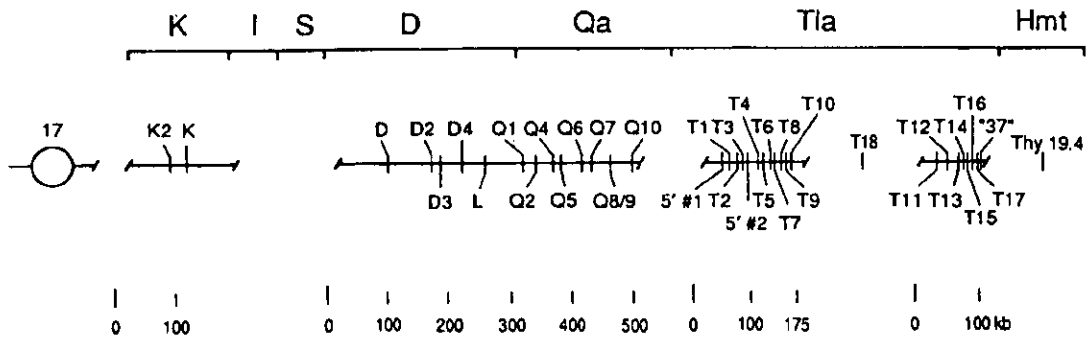
b.


K I S D Qa Tla Hmt
17
K2 K
D D2 D4 Q1 Q4 Q6 Q7 Q10
D3 L Q2 Q5 Q8/9
T4 T10
T1 T3 T6 T8
5' #1 T2 T5 T9
5' #2 T7
T18
T16
T12 T14 "37"
T11 T13 T17
T15
Thy 19.4
0 100
0 100 200 300 400 500
0 100 175
0 100 kb

and rat (Kunz, 1982).

1.3.1 The H-2 Complex

The H-2 complex is located on chromosome 17, the third smallest mouse chromosome and occupies a chromosomal segment of 2cM. The H-2K genes which are at the centromeric end of the H-2 region are separated from the remaining class I genes by class II and by loci coding for complement components (Hood et. al., 1983).

The availability of inbred strains of mice congenic for MHC haplotypes (e.g. BALB/c and C57BL lines which are classified as H-2^d and H-2^b haplotypes respectively) has facilitated extensive genetic analysis of this system. In 1959, Peter A. Gorer and Z.B. Mikulska tested progeny from the cross (A x C57 BL)F_1 x C57BL for two class I antigenic determinants H-2.4 and H-2.11, carried by the A strain. A and C57BL are two inbred mouse strains and 4 and 11 are determinants which were assigned consecutively in the order of determinant discovery. From this cross, they observed the 4 possible genotypes with most being of the parental genotypes and only two mice with the recombinant genotypes. They suggested that the determinants 4 and 11 are on the same chromosome and are closely linked but encoded by separate loci. At that time, H-2 antigenic determinants were still referred to by capital letters rather than by numbers, so determinant 4 was then called D and determinant 11 was K (the fourth and eleventh letters of the alphabet, respectively). Another locus, L was proposed in 1974 by Snell and his coworkers to explain anomalous serological typing results in different strains of mice. The genes in the K,D and L loci are the major transplantation antigens and commonly known as the classical class I molecules.

The genetic nomenclature of the H-2 complex is complicated and confusing. A particular gene is designated by a capital letter (e.g. K,D or L) for the locus and each locus has alleles designated by small-letter superscripts in combination with the H-2 symbol (e.g. K^a, K^b, D^a, etc). The distinct set of alleles of MHC genes located on a single chromosome contained in each mouse strain is referred to as a haplotype.

There are 2 other groups of loci in the H-2 complex, the Qa and Tl loci. They are known as the non-classical class I molecules. The non-classical antigens are structurally homologous to their classical counterparts. They only differ from one another in two respects: (1) The K and D loci are extremely polymorphic with 92 alleles at the K and 63 alleles at the D locus. The L locus appears to be less polymorphic with only 2 alleles (Klein, 1986). In contrast the Qa and Tl antigens are less polymorphic than K and D antigens (Michaelson et. al., 1977 and Flaherty, 1980). As a result of this lack of polymorphism they do not serve as restriction elements for CTL as do their classical counterparts (Kastner et. al., 1979a & b). They also do not seem to elicit a major graft rejection response (Flaherty, 1980). (2) Only the K, D and L transplantation antigens are expressed on all nucleated somatic cells (Klein, 1979). The Qa antigens are expressed mainly on subsets of lymphoid cells and possibly act as lymphoid differentiation antigens (Harris et al., 1984), while the Tl antigens are found on some T cells but are strongly induced in certain T cell leukeamias (Michaelson et al., 1986).

There is considerable divergence in the number of class I genes located in the region H-2D/H-2L loci of the BALB/c ($H\text{-}2^d$) and B10 ($H\text{-}2^b$) mice (Weiss et. al., 1984 and Stephan et. al., 1986). Five class I genes map to the D/L region of the BALB/c mice, i.e. D^d, $D2^d$, $D3^d$, $D4^d$ and L^d. The L^d gene is most distal in this region and is separated by 60kb of DNA from the most proximal gene of the Qa

region, Q1. Only 2 class I genes map to this region in the B10 mice. The D^b gene is proximal to the L^b gene. However, the chromosomal location relative to the Qa region is similar in both inbred strains. The organisation of the H-2K region is similar in BALB/c and B10 mice. Both these inbred strains have 2 genes in the H-2K region, K and K2. Only the K gene appears to be expressed.

Ten class I genes are located within the Qa region in both strains and they are designated Q1-10. In transfection experiments, even though the Q6, 7, 8 and 9 genes of both strains appear to encode Qa-serological determinants, none was found to encode molecules expressed on the cell surface. From sequencing studies of Q7 and Q8 (Devlin et al., 1985) and Q10 (Mellor et al., 1984) it was found that each Qa gene had an in-frame termination codon in exon 5 that disrupted translation of the cytoplasmic and transmembrane region encoding exons. The exact position of the termination codon in exon 5 varies among the Qa genes sequenced (Steinmetz et. al., 1981b; Mellor et. al., 1984 and Devlin et. al., 1985).

In mouse the Tl region is approximately 1.0cM ($\sim$ 1000kb) from H-2D (Flaherty, 1980). The Tl region contains the largest number of class I genes. In terms of gene number it also contains the greatest differences between the class I gene families of the two strains. In B10, there are 17 class I genes, designated $T1^b$ - $T15^b$, $T22^b$ and $T23^b$ whereas in BALB/C, there are 20 class I genes, designated $T1^d$ - $T11^d$, $T16^d$ - $T24^d$ (Klein et. al., 1990).

Recently the Hmt locus, which encodes genes required for the expression of Mta (maternally transmitted antigen of the mouse) was identified as a member of the MHC (Fischer Lindahl et. al., 1983 and Richards et. al., 1989). The genes at the Hmt locus are considered to be class I genes because (a) Mta-specific cytotoxic T cells are not H-2 restricted, (b) an active $ß_2m$ is necessary for Mta expression and (c)

Hmt maps at the distal end of class I genes on chromosome 17 (Fischer Lindahl et. al., 1983).

1.3.2 The HLA Complex

The HLA complex was assigned to chromosome 6 through its linkage to the phosphoglucomutase-3 locus (PGM-3) by Lamm et. al., 1971. This linkage suggested that the HLA complex was located on the shorter (the p arm) of the two arms of chromosome 6.

Within-family segregation and population analysis provide two ways to study the human MHC. In family studies it was observed that some determinants encoded by one chromosome were transmitted together to the progeny and some were transmitted as a combination of unlinked determinants. Recombination was inferred as a result of crossing-over between loci controlling these two determinants. Population studies led to a similar conclusion. When a large number of individuals were typed with a large battery of antisera, there was a distinct regularity in the distribution of positive and negative reactions of some of the antisera. It was found that certain groups of the antisera seemed to detect different alleles. At first only two segregant series of antigens were designated, HLA-A and HLA-B, but the discovery of antisera with inconsistent reaction patterns showed the existence of another locus, HLA-C. These three classical loci were shown to be closely linked. A genetic map of chromosome 6, based on recombination frequencies places HLA-B 0.2cM proximal to HLA-C, and HLA-A 0.8cM distal to HLA-C (Bronson et al., 1991). Compared with the K, D and L loci in mice, these three loci are similarly polymorphic. There are 23 HLA-A alleles, 47 HLA-B alleles and 8 HLA-C alleles (Bodmer and Bodmer, 1984).

Southern blot analysis (Orr et. al., 1982) and genomic cloning (Malissen et. al., 1982 and Boncinelli et. al., 1984) showed that the genetic complexity of the HLA class I loci was greater than that indicated by the three loci. Orr and his coworkers constructed a cosmid library from a human lymphoblastoid cell line and screened it for class I-like sequences. On mapping and sequencing these genes, they succeeded in characterising 17 class I genes. Of these, 11 were found to be pseudogenes or fragments and three corresponded to HLA-A, -B and -C.

The remaining three genes on HindIII fragments of 6.0, 6.2 and 5.4 kb, were found to encode functional HLA class I antigens (Geraghty et. al., 1987; Koller et. al., 1988 and Geraghty et. al., 1990) respectively. They were renamed HLA-G, HLA-E and HLA-F by the HLA nomenclature committee (WHO) in 1990. All 3 genes contain in their 5' flanking region, a variant of the consensus TATAAA sequence (TCTAAA) found in the promoter region of HLA-A, -B and -C genes. The three chains are closely enough related to HLA-A, -B and -C α chains to share with them determinants that are recognised by a monoclonal antibody (mAb) w6/32 (Shimizu et. al., 1988) which recognises determinants formed by association of HLA-A, -B, or -C α chains with $ß_2$m (Barnstable et. al., 1978).

The tissue distribution, cell-surface expression and function of the nonclassical HLA-E, F and G genes are poorly understood (Shimizu et. al., 1988 and Orr et. al., 1989). Each gene has intact exons 1-5, i.e. the leader peptide through the transmembrane region. The HLA-G (6.0kb) gene has an in-frame stop codon at the beginning of the first cytoplasmic exon, exon 6. This prevents translation of a majority of the cytoplasmic region of the HLA-G polypeptide. The promoter region was found to resemble the promoter region of a Qa region gene. As a result of these structural similarities Geraghty et. al., (1987) suggest that this gene is a structural

homolog of a murine Qa region class I gene.

Another new class I gene, HLA-AR (HLA-H) was found to be closely related to HLA-A, probably by a gene duplication (Zemmour et. al., 1990). The difference between them is that HLA-H is not a functional Ag-presenting locus because of a deleterious single nucleotide mutation present in its alleles. Compared with the human class I sequences so far characterised HLA-H genes most closely resemble the classical class I genes HLA-A, B, C (Koller et. al., 1987). However, HLA-12.4, an allele of HLA-H was described by Malissen et. al., 1982 as a pseudogene because its predicted protein sequence lacked the invariant cysteine at position 164, which forms the critical disulfide bond of the α_2 domain, and transfection did not result in cell surface expression of a protein product. There are five other HLA-H alleles, all of which have the same mutation as HLA-12.4 which converts codon 164 from cysteine to phenylalanine. As a result of this substitution, the structural rigidity of the second domain of the protein encoded by this gene is in question (Malissen et. al., 1982). These five HLA-H alleles also share a second deleterious mutation not found in HLA-12.4. Deletion of a single nucleotide in codon 227, in the centre of the Ig-like α_3 domain, causes a shift in reading frame, producing a non-Ig-like sequence for the carboxyl-terminal half of α_3 and premature termination at codon 272 near its end. Therefore the proteins made from any of the HLA-H alleles are highly unlikely to associate with $ß_2$m or function as Ag-presenting molecules.

1.4 STRUCTURE OF MHC CLASS I

The overall structure of MHC class I genes and their products is highly conserved within and between species. Structurally the class I proteins are heterodimers consisting of a highly polymorphic 45kd integral membrane

glycoprotein. In the formation of a class I molecule the α-chain non-covalently associates with $ß_2$-microglobulin, a non-polymorphic, non-glycosylated 12kd polypeptide encoded outside of the MHC. (Grey et al., 1973 and Silver & Hood, 1974). In the mouse the $ß_2$-microglobulin is located on chromosome 2 (Michaelson 1981) and in man, on chromosome 15. (Goodfellow et. al., 1975).

Amino acid sequence analyses of both human and mouse class I molecules show that the heavy chain can be divided into three functional regions - extracellular, transmembrane and cytoplasmic. (Springer & Strominger, 1976; Tragardh et al., 1980 and Coligan et al., 1981).

At the cell membrane, the class I molecule is characterised by the presence of four extracellular regions, three (α_1, α_2, α_3) for HLA and H-2 (N, C_1 and C_2) from the MHC encoded α chain and one from $ß_2$-microglobulin.

Springer & Strominger, 1976 showed that papain cleaved the HLA heavy chain 13 residues before the transmembrane region, yielding a soluble heterodimeric fragment that retained all the serologically detected epitopes (antigenic determinants) of the native cell-surface molecule.

The resolution of the crystal structure of the human class I molecule, HLA-A2 gave an insight into the structure of an MHC class I molecule and showed how an antigen could be presented to the TcR by the MHC molecules. (Bjorkman et. al., 1987a; 1987b). The HLA-A2 molecule consists of two pairs of domains, with the α_3 and $ß_2$m domains being close to the membrane and the α_1 and α_2 domains being further away. The α_1 and α_2 domains are responsible for peptide binding and MHC-restricted recognition by TcRs while the α_3 domain comprises the major site of interaction with $ß_2$m. In the mutant human cell, Daudi, that lacks $ß_2$m, the class I heavy chain is incorrectly folded, is never exported from the ER, and is rapidly

degraded (Arce-Gomez et al., 1978). Therefore, the association between the heavy chain and the $ß_2$m is required for class I folding and expression at the cell surface. The two subunits are found to interact in a manner similar to the association of immunoglobulin constant regions (Townsend et. al., 1989).

The α_1 and α_2 domains are each composed of a four-stranded antiparallel ß-pleated sheet that forms the amino terminal half of the domain, followed by a long α-helical region at the carboxyl terminal. The four ß strands of each domain combine to produce a planar structure that forms the floor of the peptide-combining site (Bjorkman et. al., 1987b). There are two α-helices in the helical regions of the α_1 and α_2 domains, a short helix and a long helix. The short helices form the means by which the floor of the domain (i.e. the ß-pleated sheet) is linked with the top of the domain (i.e. the long helices). Figures 2 & 3 illustrate the structure of the MHC class I molecule. There is an obvious gap between the two long helices. This is one of the many indications that suggest that the binding site for antigenic peptides is the deep groove located between the two α helices (Bjorkman et. al., 1987a). For reviews of other evidence, see Chothia (1984) and Livingstone & Fathman (1987).

Bjorkman et. al., 1987b observed the very intimate contact between the bound peptide, the class I heavy chain and $ß_2$m. The binding of peptides and $ß_2$m to heavy chains is co-operative and therefore it was suggested that the process of peptide binding and class I assembly are linked phenomena. The studies of Townsend et. al., 1989 on mutant mouse cells, RMA-S confirmed the role of peptide in stabilising the class I structure. The RMA-S cells express reduced levels of class I molecules and cannot present peptides derived from antigen. In a healthy cell the class I molecules will bind peptides that are derived from self proteins. This association would not provoke a cytotoxic T cell response as a result of self tolerance. In a viral

FIGURE 2. *STRUCTURE OF MHC CLASS I MOLECULE*

Schematic drawing of the structure of a human class I MHC glycoprotein from crystallographic studies. Most of the polymorphic amino acid residues are located around the antigen-binding groove. (After Bjorkman et. al., 1987a).

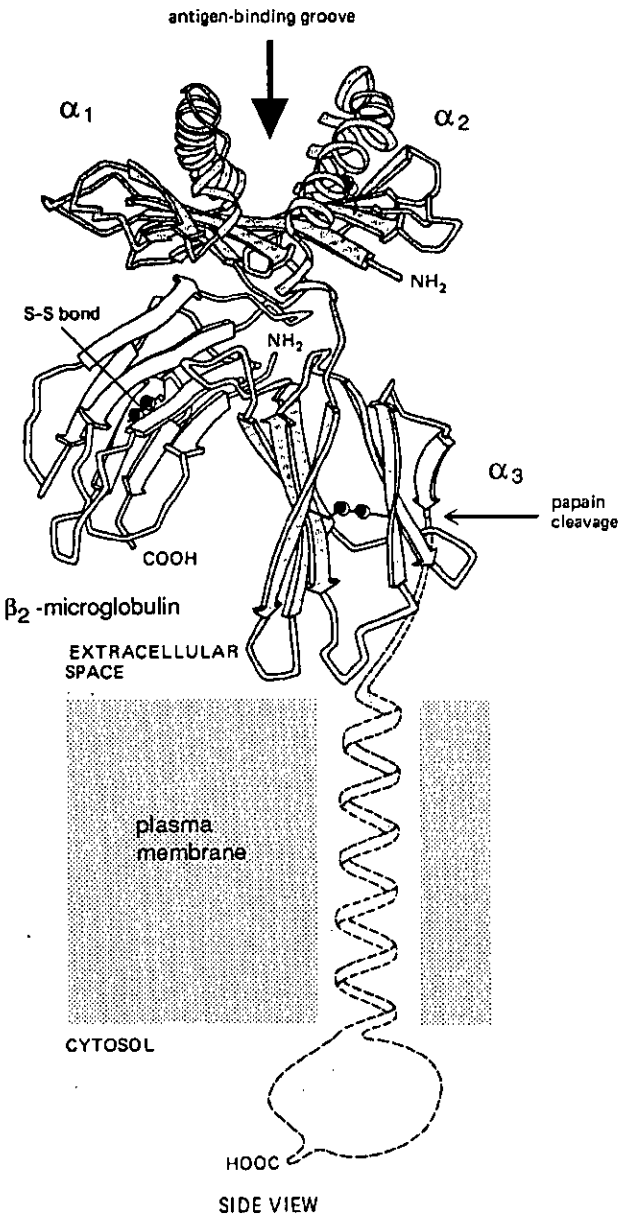
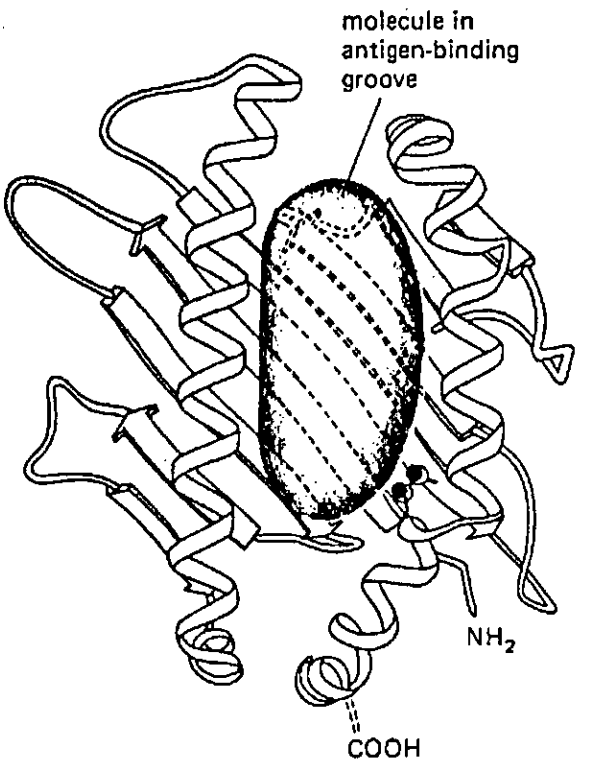

antigen-binding groove
α1
α2
NH2
S-S bond
NH2
α3
papain
cleavage
COOH
β2 -microglobulin
EXTRACELLULAR
SPACE
plasma
membrane
CYTOSOL
HOOC
SIDE VIEW

FIGURE 3. *ANTIGEN-BINDING GROOVE*

Schematic drawing of a top view of an antigen-binding groove. The walls of the groove are constituted by 2α helices and the bottom by a ß pleated sheet. The groove may contain either a self or non-self peptide. (After Bjorkman et. al., 1987a).



TOP VIEW

infection, viral peptides will compete with self peptides for the peptide-binding site of the class I molecule. When viral peptides are presented at the cell surface a TcR on $CD8^+$ cytotoxic T cells would recognise the foreign peptide - MHC complex and a cytotoxic T cell response would occur.

The transmembrane region contains a stretch of hydrophobic residues and Nathenson et. al., (1981), by a process of elimination, localised this region to 284 - 307 in H-$2K^b$. Generally, the membrane binding portion is approximately 24 amino acids long in HLA (Robb et. al., 1978) and H-2 (Uehara et. al., 1981) class I molecules. This length of polypeptide in an α-helical conformation is capable of spanning a lipid bilayer 35Å deep (Guidotti, 1977). The end of the transmembrane domain contains a cluster of basic amino acids (e.g. arginine and lysine) which probably help to anchor the polypeptide chain in the membrane by interacting with the negatively charged phospholipid headgroups of the cytoplasmic surface of the membrane. (von Heijne and Blomberg, 1979).

The class I protein terminates with the cytoplasmic region, which is hydrophilic at its carboxy terminus (Ploegh et. al., 1981b). There are about 31 residues in the human (Robb, 1978) and 39 in the mouse (Nathenson et. al., 1981) cytoplasmic region with approximately 50% being polar amino acids, particularly serine. Some of these serine residues may be phosphorylated (Rothbard et. al., 1980 and Pober & Strominger, 1981). It has been suggested that such phosphorylation is involved in transmitting signals from the MHC molecules to appropriate intracytoplasmic mediators.

The basic structure of the extracellular portion of the class I molecules is conserved for all species (Maloy, 1987). In the 3 extracellular domains, the 90 amino acids have a similar amino acid sequence and there is also conservation of the

position of disulphide loops. Most sites of glycosylation are also conserved. Due to these similarities, the identity of the genetic locus encoding the class I molecule often cannot be determined from the sequence of the extracellular domains of the molecule.

In contrast to the conservation of structure in the extracellular portion, there is great variability in the transmembrane and cytoplasmic domains of the class I molecule between loci and species (Ploegh et. al., 1981b and Kimball and Coligan, 1983). Considerable diversity is seen in the carboxy-terminal portion of class I molecules, especially where molecules encoded by different regions of the MHC are compared. Therefore a particular class I molecule can often be assigned to a locus based on its carboxy-terminal sequence. However no functional significance of these locus - specific sequences has been determined.

The lack of conservation in the transmembrane and cytoplasmic regions between different class I molecules would also explain the expression of a cloned, truncated H-2L^d gene (Evans et. al., 1982a). This gene was found to lack exons coding for the cytoplasmic region. It is shown to be expressed in the membrane and to function normally, implying that the interactions between cytoplasmic proteins and class I molecules are not required for antigen presentation function.

The class I light chain, $ß_2$m, is a non-polymorphic protein in humans, but it is dimorphic in mice, with alleles exhibiting a single amino acid change at position 85 (Michaelson et. al., 1980). The complete amino acid sequences for $ß_2$m from a variety of species have been determined. Homology of $ß_2$m among species ranges between 61% and 74%, except for mouse and rat, which, as might be expected, show greater homology (86%). This high degree of sequence homology among species implies evolutionary and functional conservation of the light chain molecule.

1.5 *ORGANISATION OF MHC CLASS I GENES*

The organisation of the class I genes in the animal species so far studied is remarkably similar. The genes are encoded by up to eight exons (Steinmetz et al., 1981b).

The first exon encodes a 24 amino acid signal peptide in man (Srivastava et. al., 1985) and a 21 amino acid sequence in mice (Lalanne et. al., 1982). However, in HLA-A3, the leader peptide is longer (29 amino acids) (Strachan et. al., 1984). The leader peptide is hydrophobic (von Heijne, 1981) and is removed during translation into the endoplasmic reticulum (Koller et. al., 1987).

The three extracellular domains, α_1, α_2 and α_3 are encoded by exons 2, 3 and 4 respectively. These appear to be very well conserved in length across species, at about 90 amino acid long (Maloy, 1987). Exon 5 codes for the transmembrane region of the protein.

The cytoplasmic and 3' untranslated regions are encoded by up to 3 exons whose number depends on the particular class I gene. Therefore the total number of exons within a class I gene can vary between 6 and 8. Variations in length and number of exons in this region are due to alternate splicing of exons (Steinmetz et al., 1981a; Archibald et al., 1986) and the position of in-frame termination codons (Steinmetz et al., 1981b; Mellor et al., 1984; Waneck et al., 1987; Geraghty et al., 1987 and Gussow et al., 1987).

The 5' flanking sequence of a gene includes the promoter region and its distance upstream from the initiation codon AUG varies between genes. There are 2 sequences in the promoter region, the CCAAT and TATAAA sequence, which are the sites at which RNA polymerase II and associated transcription factors bind to initiate the transcription of RNA. The mouse class I genes show a conventional

TATAAA sequence 30 nucleotides upstream from the transcriptional initiation site (-30). The HLA class I genes contain a variant TATAAA sequence, TCTAAA, located in a position corresponding to the TATAAA sequence of other eukaryotic genes (Strachan, 1987). There is, however, a conventional CCAAT sequence at position -50 for all class I genes with the exception of HLA Cw3.

In HLA-Cw3 (Sodoyer et. al., 1984), the sequence of both these promoter elements is altered. The CCAAT sequence is replaced by CCGGT and the TCTAAA sequence by TCTGAA. These two alterations in the promoter region of an HLA-C gene provide an explanation for the relatively low levels of HLA-C detectable on the surface of cells. Due to these base substitutions, the HLA-C promoter may be relatively inefficient in its ability to direct RNA polymerase II binding and transcription (Kelly and Darlington, 1985).

$ß_2$ microglobulin ($ß_2$m) is 99 amino acids long and the $ß_2$m gene is found as a single copy gene in the haploid genome. It consists of four exons and three introns, with the second exon being the longest (encoding amino acids 3-95). The first exon which codes for the leader peptide is separated from the rest of the coding sequences by a long intron (2.8 kb) (Parnes and Seidman, 1982).

1.6 FUNCTION OF THE MHC CLASS I

One of the functions of an MHC molecule is to bind and present antigenic peptides at the surface of the majority of cell types in the body. This process is essential for the induction of a specific immune response. (Babbitt et. al., 1985; Guillet et. al., 1986 and Buus et. al., 1987).

The express role of the MHC molecules was not discovered until 1974 when Zinkernagel and Doherty, while looking at infections caused by lymphocyte

choriomeningitis virus (LCMV) in mice, observed that the cytotoxic activity of the immune lymphocytes of the infected mice had dual specificity for viral antigens and self-surface antigen determined by class I MHC loci. Mice of different H-2 types were injected intracerebrally with the virus. After a week, some were killed and the cytotoxic activity of their spleen cells was tested on virus-infected L cells ($H\text{-}2^k$). Only spleen cells from $H\text{-}2^k$ haplotypes which were comparable lysed the cells. Zinkernagel and Doherty in 1974 termed this phenomenon MHC restriction.

MHC restriction is due to processes which involve the selection and control of the TcR repertoire against the MHC-peptide complex. These processes take place mainly in the thymus of each individual during early development and there is evidence for distinct positive and negative selection during which the TcRs of immature T cells are exposed to MHC molecules complexed with peptides from endogenous (self) proteins. Cortical epithelial cells are the main cell type which mediate positive selection while haematopoietic and probably medullary epithelial cells mediate negative selection (Sprent et. al., 1988; Lo & Sprent, 1986 and Benoist & Mathis, 1989). Potentially autoreactive cells with receptors that interact too strongly with MHC-self antigen complexes are eliminated (Marrack & Kappler, 1987; Sprent et. al., 1988 and Kappler et. al., 1989). On the other hand, cells which are not autoreactive would be stimulated by the MHC-peptide complex and are positively selected (Sha et. al., 1988 and Teh et. al., 1988). Therefore, if a thymocyte TcR fits the MHC peptide complex expressed by the cortical epithelium it will be positively selected.

Kappler et. al. 1987 observed that the elimination of autoreactive cells by clonal deletion was one of the mechanisms of tolerance to self-MHC - self-antigen complex in an unmanipulated animal. Cell death as a result of negative selection

occurs by a programmed process known as apoptosis (Smith et. al., 1989).

An alternative mechanism of peripheral tolerance was shown in studies involving transgenic mice. Morahan et. al. (1989) showed that tissue-specific class I restricted T cells may not be eliminated but instead remain dormant or inactivated. If interleukin-2 (IL-2) was provided under abnormal conditions a cascade of autoimmune reactions may be initiated and these results have suggested a basis for the emergence of autoimmune diseases.

The end result of the maturation of T cells is that they will only interact with self MHC-foreign peptide complexes that are different from those that have been presented in their development process and thus, MHC molecules play a major role in the selection and elimination or inactivation of T cells.

On the cell surface, MHC-peptide complexes act as markers by which the cell can be identified by T cells of the immune system (Townsend et. al., 1985). The MHC molecule binds small unfolded peptide epitopes, approximately 9 amino acids long, between the two helices of the α_1 domain (Bjorkman et. al., 1987a, 1987b) and carries them to the cell surface where interaction with a TcR occurs. Although these receptors are structurally very similar to immunoglobulins they are far less versatile. They only bind to MHC-peptide complexes and not to the great range of peptide epitopes recognised by immunoglobulins (Marrack and Kappler, 1987 and Rothbard and Taylor, 1988).

The TcR is composed of either an $\alpha\beta$ (Saito et. al., 1984) or a $\gamma\delta$ (Borst et. al., 1987; Moingeon et. al., 1987) heterodimer. TcR $\alpha\beta$ is responsible for the recognition of antigens presented on MHC molecules (Hood et. al., 1985) while the most probable function of the $\gamma\delta$ T cells is the protection of epithelial surfaces (Janeway et. al., 1988 and Hein and MacKay, 1991).

Janeway and colleagues speculated that the γδ receptors may be largely specific for class I MHC antigens different from those expressed on most somatic cells. They may recognise a less polymorphic ligand which could bind antigen. Matis et. al., 1987 were able to identify a T-cell line expressing the γδ receptor which had MHC linked recognition specificity and cytotoxicity. The target cells map this cytotoxic activity to H-2D, Qa or Tl regions of the $H\text{-}2^b$ haplotype. The T cells also lyse $H\text{-}2^q$ target cells. Thus, these TcR γδ T cells display a broad pattern of cross-reactivity for allogenic target cells. This degree of cross-reactivity is in contrast to that expected from MHC class I specific TcR αß alloreactive T cells and suggests the recognition of relatively nonpolymorphic antigenic determinants.

The γδ T cells occur in only very small numbers in the conventional T-cell domains of spleen and lymph nodes (MacKay et. al., 1989 and McClure, 1989). There is, however, a larger number of γδ T cells in the lymphoid system of sheep and cattle (ruminants) as compared with those of humans and mice (Hein and MacKay, 1991) but the reasons for these differences are unclear (Hein et. al., 1990). However the fact that not only ruminants but also chickens (Sowder et. al., 1988) and pigs (Hirt et. al., 1990) have a large number of γδ T cells suggests that the γδ T cells might play a significant role in surveillance for invasion of antigenic substances derived from microorganisms of the epithelium, and in the elimination of damaged epithelial cells (Takeuchi et. al., 1992).

There are two structurally distinct, but related, classes of MHC molecules which present peptides to two subsets of T cells. Class I MHC molecules present antigens (peptides) to cytotoxic T cells that express the CD8 cell surface glycoprotein while class II MHC molecules present antigens to helper T cells that express the CD4 cell surface glycoprotein (Parnes 1989 and Teh et. al., 1988).

CD4 and CD8 antigens are coexpressed in immature thymocytes. However, on maturation, they are expressed in mutually exclusive subpopulations of T cells (Reinherz & Schlossman, 1980), hence the two subsets of T cells. The natural ligand for CD8 and CD4 is the non-polymorphic determinant on the MHC class I molecules and class II molecules, respectively. These antigens were previously considered to be accessory molecules but there is evidence that they contribute to T cell activation by delivering intracellular signals (Blue et. al., 1987 and Carrel et. al., 1988). Therefore, the TcR - CD3 complex associated with CD4 or CD8 forms a multimeric complex through which physiological T cell activation occurs (Emmrich, 1988).

The functions of class I and class II MHC molecules have been thought to be separate, but recent evidence suggests that they may overlap. Townsend et. al., (1989) in their studies, discovered that if a cell is bathed with a concentrated solution of an extrinsic peptide, they found both class I and class II molecules carrying the peptide, on the cell surface.

1.7 ANTIGEN PROCESSING AND PRESENTATION

Now it is generally accepted that MHC molecules present fragments of antigens, in the form of peptides, to T cells. MHC class I molecules, which are expressed on the surface of all nucleated cells, normally present peptides derived from proteins that are produced endogenously in the cell. Infected cells can thus be eliminated by cytotoxic T lymphocytes, which recognise antigenic peptides bound to MHC class I molecules.

Townsend et. al. (1988) showed that rapid degradation of cytoplasmic antigens was required for efficient presentation. This suggested that the degradation process

may occur in the cytosol. Evidence of this was obtained when short hydrophilic peptides expressed in the cytosol were presented efficiently to T cells (Gould et. al., 1989, Sweetser et. al., 1989 and Whitton & Oldstone, 1989).

In vitro experiments suggested that all MHC class I molecules can bind many different peptides, but this is not so. Falk et. al. (1990) showed that MHC molecules are involved in the determination of which peptides occur intracellularly. In their experiment with normal mouse spleen cells identical for all genes except MHC class I, they found different patterns of peptides were presented from endogenous protein. There is also evidence of this in studies with naturally processed viral peptides. Rötzschke et. al. (1990) extracted peptides from virus-infected cells by the process of acid elution and showed that the processing of viral peptides are MHC class I restricted because infected H-2^d cells did not contain the H-$2D^b$ restricted peptide. Similarly, the infected H-2^b cells did not contain the H-$2K^d$-restricted peptide.

In another experiment, van Bleek & Nathenson (1990), isolated an endogenously processed allele-specific peptide, bound to MHC class I molecules. The antigenic peptide, which was K^b restricted, bound only to the H-$2K^b$ cells and not H-$2D^b$ cells. This suggested a high degree of binding specificity for allelic class I molecules.

How and where these processed antigens crossed the membrane of the vacuolar system and bound to the class I molecule was a question posed by Nuchtern et. al. (1989). They proposed the endoplasmic recticulum as an obvious organelle for the assembly of class I α and ß subunits along with the binding of the processed peptide. To test their hypothesis, they used brefeldin A, a fungal antibiotic which blocks the movement of proteins out of the ER. Consistent with their hypothesis,

they provided evidence for the inability of brefeldinA-treated cells to present endogenously synthesised antigens at the cell surface.

The studies of Townsend et. al. (1989) suggested that peptide binding may be required for efficient assembly and intracellular transport of MHC class I molecules. Recent work on the murine lymphoma mutant cell line, RMA-S, in the assembly of these molecules, suggested that in the absence of peptides, class I molecules could assemble but were only stable at reduced temperature (19-33°C) and dissociated at body temperature (Ljunggren et. al., 1990).

Another interesting study on the process of antigen presentation was reported by Cox et. al. (1990). They showed that presentation occured only when MHC class I molecules were transported from the ER. They used the E19 glycoprotein which both blocks the presentation of vaccinia and influenza virus proteins to CTLs and inhibits MHC class I transport from the ER, in an MHC class I allele-specific manner. When they transfected cells with the E19 adenovirus protein, newly synthesised class I molecules in the ER were retained, inhibiting class I - mediated antigen presentation. Therefore it seems to be necessary for a class I - peptide complex to go out from the ER for presentation at the cell surface. After leaving the ER, class I molecules move through the Golgi apparatus, receiving modification to their carbohydrate side chains and arrive at the cell surface with an average half-time of 30 mins (Neefjes et. al., 1990). There is no direct evidence for a class I chaperonin equivalent to the invariant chains found in class II assembly (Burgess et. al., 1987).

There is genetic evidence for the transport of peptide fragments from the cytoplasm into the ER containing newly synthesised MHC molecules. Livingstone et. al. (1989), Deverson et. al. (1990), Monaco et. al. (1990), Spies et. al. (1990)

and Trowsdale et. al. (1990) described a gene or genes in the MHC complex class II region controlling the class I presentation pathway in rat, mouse and human. These genes have sequence similarities to a superfamily of genes involved in the ATP-dependent transport of a variety of substrates across cell membranes.

These genes were first known as Mtp-1 and Mtp-2 in rat (Livingstone et. al., 1989; Deverson et. al., 1990), HAM-1 and HAM-2 in mouse (Monaco et. al., 1990) and RING 4/PSF 1 and RING 11/PSF 2 (Trowsdale et. al., 1990; Spies et. al., 1990) in man. Monaco, (1992) in his review, explained that to avoid confusion, the HLA nomenclature committee at a recent meeting in November 1991, chose a standardised name for these 2 genes, Tap-1 and Tap-2 for transporter associated with antigen processing.

Bikoff et. al. (1991) provided evidence that MHC-linked genes encoding a peptide transporter are necessary for MHC class I assembly and stability at the cell surface. They investigated the possibility that defective MHC class I assembly in embryonic cells was associated with a lack of expression of HAM-1 mRNA. They induced HAM-1 mRNA expression by treatment with interferon and surface expression of class I molecules was obtained. Their results also suggested that HAM-1 transcription was developmentally regulated.

Similar results were obtained when mutant human and mouse cell lines (with a defective transporter gene) were transfected with a complementary DNA (cDNA) of the transporter gene and levels of cell surface expression were restored (Spies & DeMars, 1991 and Powis et. al., 1991). But the same cloned gene (cDNA corresponding to the HAM-1 gene) did not restore class I expression to the mutant line 721.174 where both transporter genes were deleted (Spies et. al., 1990).

Attaya et. al. (1992) showed that expression of a cloned copy of the HAM-2

gene in RMA-S cells resulted in recovery of the ability to process and present class I restricted antigens to CTL. The HAM-2 gene apparently relieved the block in processing of endogenous antigen in RMA-S cells. The HAM-1 gene did not correct the defect in these cells. Thus a defect in either HAM-1 alone or in HAM-2 alone prevents the processing and presenting of antigen to class I-restricted T cells. This suggests that a functional peptide transporter requires the presence of both genes.

In man there appears to be a similar situation (Kelly et. al., 1992 and Spies et. al., 1992) with the protein products of both genes (RING 4/PSF 1 and RING 11/PSF 2) being required to form a functional complex.

These results are consistent with the hypothesis that the ABC transporter genes consist of two transporter polypeptides that form a heterodimer and both are necessary for delivering peptide across the membrane of the ER (Higgins et. al., 1990). Recently substantial evidence for the role of the putative transporter genes in antigen processing and presentation was shown by Powis et al. (1992). They demonstrated that polymorphism in Mtp-2 determines the peptide bound by the class I molecules.

1.8 MHC POLYMORPHISM

The MHC polymorphism in most species is extensive and has remained so through time (Klein, 1987). Many questions remain about the origins of the polymorphism and its functional significance. Gene conversion or non-homologous interlocus recombination has been suggested as a mechanism for the generation of polymorphism in the MHC. For example, a mutant allele H-2K^{bm1} of the H-2K^{b} gene was cloned by Weiss et. al. (1983a), and shown to be a result of gene conversion. Mellor et. al. (1983) provided evidence for Q10 as the potential donor

gene for the H-2K^b to H-2K^{bm1} gene conversion event. Weiss et. al., 1983b also showed that several of the differences in nucleotide sequence between the H-2K^b and the H-2K^d alleles could be explained by a similar gene conversion event.

When Pease et. al. (1983) compared the amino acid sequence of mutant and normal H-2 genes, the polymorphism observed could also be explained by gene conversion. More evidence for gene-conversion is the generation of the 2 HLA-B27 subtypes in the human class I genes (Seemann et. al., 1986).

There is also accumulating evidence for intra-allelic reciprocal recombination or *in vivo* exon shuffling as an alternative mechanism for the generation of polymorphism. Holmes and Parham (1985) observed such recombination in the HLA-Aw 69, which they suggested was due to *in vivo* exon shuffling between the α_1 domain of HLA-Aw 68 and the α_2 and α_3 domains of HLA-A2. Parham et. al. (1988) when comparing 39 HLA-A, -B and -C sequences showed that genetic exchange between alleles at the same locus has been a more important mechanism in the generation of MHC diversity than genetic exchange events between alleles of different loci. He also observed the positions of high variability were directly involved in the peptide-binding site as defined by the structure of HLA-A2 (Bjorkman et. al., 1987a). The regions of the α_1, α_2 and α_3 domains that do not line the peptide binding site were less variable. The patterns of polymorphism at the HLA-A, -B and -C loci of the human class I are different and this suggests that there may be functional diversification between these loci products to bind structurally distinct groups of peptides or TcRs.

Hughes and Nei (1988) proposed another explanation for the extensive polymorphism. They found that the ratio of replacement to silent substitutions of amino acid at positions in the antigen combining site was significantly higher than

amino acid further away from the site. They suggested that polymorphism in class I MHC molecules was as a result of natural selection and could be explained in terms of heterozygote advantage. The possession of two different alleles at a locus would have the advantage of allowing a response to more pathogens, and thus heterozygotes would have a higher fitness than homozygotes. A novel ability to respond to an antigen could be as a result of the improvement in either presentation of peptides or in thymic selection of relevant TcRs. Therefore almost all of the peptide and TcR interacting residues within the peptide binding site would be expected to show some sequence variation.

Heterozygous advantage has been postulated, on theoretical grounds, as the mechanism maintaining MHC polymorphism in natural populations (Takahata and Nei, 1990). Ritte et. al. (1991) provided direct experimental evidence for the effect of overdominant selection on the H-2 complex when they studied wild populations of the house mouse. They recommended that the only way of learning how MHC polymorphisms are maintained is to study populations in their natural settings.

Although polymorphism is predominantly found in the α_1 and α_2 domains, variation in the α_3 domains may also be of importance, especially with respect to CD8 interactions. Two molecules, HLA-Aw68.1 and HLA-Aw68.2, which do not bind CD8, have a valine residue at position 245 instead of alanine as in other HLA-A, -B and -C molecules (Salter et. al. 1989). They also showed that CD8 binds to the α_3 domain in both human and mice class I molecules.

Klein (1980) was the first to point out that HLA alleles appear to have persisted for significant evolutionary periods of time, in many cases predating the divergence of present-day species. He called this the trans-species hypothesis (retention of ancestral polymorphism). This hypothesis assumes that a group of

ancestral major genes were present before speciation. When a new species evolved, the whole set of alleles would be passed on when there are no drastic environmental changes. Additional polymorphisms would result only from random mutations. A truly new polymorphism would only be generated by drastic changes in the mode of life (Klein & Figueroa, 1986; Lawlor et. al., 1988; and Mayer et. al., 1988).

Evidence for this theory is seen in the studies of Mayer et. al. (1988), who compared chimpanzee class I sequences with their human counterparts. Their data supported the transmission of alleles from one species to another.

Lawlor et. al. (1988) compared class I MHC alleles of humans and chimpanzee. They found individual HLA-A or B alleles to be more closely related to their chimpanzee counterparts than to other HLA-A or B alleles. Therefore their results are in agreement with the theory of trans-species evolution that there was polymorphism in HLA-A and -B before divergence of the chimpanzee and human lines.

It is apparent from the evidence given above that the maintenance of these polymorphisms is the result of a composite of the different hypotheses.

1.9 THE BOVINE MHC

The existence of the cattle MHC was independently reported in 1978 by Spooner et. al., and Amorena and Stone on the basis of skin grafting experiments and serology. Evidence for a bovine class II region was obtained by Usinger et. al. (1977) from the genetic control of mixed lymphocyte reactions (MLR).

The MHC of cattle is denoted BoLA for bovine lymphocyte antigens. The class I molecules have been shown to be controlled by alleles at a single locus, the BoLA-A locus (Oliver et. al., 1981) while the class II region is given the designation

BoLA-D. There is close linkage between BoLA-A and BoLA-D (Usinger et. al., 1981).

The BoLA class I glycoproteins were shown to have the conventional basic structure of a class I molecule. Hoang-Xuan et. al. (1982) observed the association of the detergent-solubilised 44,000 mol. wt. alloantigen with a smaller polypeptide with a mol. wt. of 12000 as seen in HLA and H-2 class I molecules. In the absence of a reducing agent, SDS-PAGE of immunoprecipitates also revealed the smaller polypeptide peak, indicating it was not bound to the larger polypeptide via a disulfide bridge.

In 1986 Fries and coworkers provided preliminary evidence for the location of the bovine MHC on chromosome 23 using *in situ* hybridisation with a cloned DNA sequence specific for a class I gene of the pig MHC (SLA).

MHC class I polymorphism was first studied using serology. Specific alloantisera were obtained by skin grafting between dam and calf pairs or from multi-parous cows. Further evidence supporting the BoLA-A locus as an MHC locus in cattle was observed by Spooner & Oliver (unpublished observation) when skin grafting was performed between unrelated cattle heterozygous for the same two BoLA alleles and individuals which carry non identical BoLA types. They found that skin grafts between matched MHC individuals survived considerably longer than between mismatched animals.

In the first international BoLA workshop in 1978 (Spooner et. al. 1979), it was observed that the phenotypes of the test panel of lymphocytes were consistent with the hypothesis that the clusters detecting antigens were controlled by multiple alleles at a single autosomal locus.

In serological analysis, the high frequency of null alleles (undefined) in many

cattle breeds indicate that a considerable number of BoLA class I gene products have not been identified. There has been international collaboration to identify these gene products. In the 2nd international workshop, 17 specificities were confirmed and given the designation w. There was only evidence that these antigens were controlled by a series of codominant alleles at a single autosomal locus (Anon, 1982).

In a population study using serological techniques, Spooner et. al. (1987a) obtained results consistent with the hypothesis of a single BoLA class I locus in West Africa as well as Europe.

The number of recognised specificities was increased to 30 by the 3rd International Workshop (Bull et. al., 1989). In this Workshop, the w25 specificity was described as the best serological evidence for the existence of a polymorphic second BoLA class I locus. At the recent 4th International Workshop, 50 specificities were characterised (Bernoco et. al., 1991).

One of the specificities defined by alloantisera at the first Workshop was BoLA w6 and Spooner and coworkers in 1979 observed evidence for subgroups of w6. Subsequently, serum absorption and immunization studies demonstrated that there were at least four subgroups of w6 (Spooner and Morgan, 1981) and two of these were given Workshop designations in the second BoLA Workshop (Anon, 1982).

The question of why cattle, in contrast to man and mouse, have only one expressed class I locus has not yet been answered by serology. Possible hypotheses for the difficulty in identifying more than one locus have been suggested by various researchers. Spooner et. al., in 1988, suggested that the tight linkage of class I loci probably result in isolation of sera identifying haplotypes and not individual products. The sera produced may also identify only the most antigenic locus. For example anti

w6 has been identified in most laboratories.

Other techniques which might subdivide serological specificities have been looked into. One-dimensional isoelectric focussing (1D-IEF) was shown to improve and extend the results obtained by serology (Joosten et. al., 1988). IEF generally agrees with the serological definition of antigens and detects antigens not yet defined by serology and ID-IEF is useful for BoLA typing but depends on the mAb used. Joosten et. al. (1988) used two mAb, antihuman w6/32 and anti $ß_2$m B1.1G6 in their IEF studies. They suggested that the different bands seen in some haplotypes indicated the expression of more than one class I locus.

Monoclonal antibodies have an advantage over alloantisera in that they bind to individual epitopes. An alloantiserum is assumed monospecific when it reacts with only one antigen after absorption. Another advantage of mAbs is that they can be produced in large quantities. In 1983 Chardon and coworkers and in 1984, Spooner and Ferrone showed the cross reactivity of anti-HLA mAbs with bovine lymphocytes.

In Southern blot analysis, when genomic DNA was digested and probed with a human class I cDNA, Lindberg and Andersson in 1988 observed 10 to 20 restriction fragments with only five non polymorphic fragments. They concluded that there are several bovine MHC class I loci. In a similar analysis using a bovine class I cDNA as a probe, 4 to 5 polymorphic fragments and up to 10 non-polymorphic fragments were obtained with several restriction enzymes (Brown, 1989b). It was concluded that the BoLA class I region consists of multiple loci, and the polymorphic fragments may code for classical class I genes while the non-polymorphic fragments code for genes similar to the conserved Qa or Tl genes of the mouse.

Further evidence for BoLA as a multilocus complex was provided by the

sequential immunoprecipitation of class I molecules in a heterozygous animal with alloantisera and mAb. There was evidence for at least five different class I molecules on the cells of this animal (Bensaid et. al., 1988).

Class I MHC products of various species (Alter et. al., 1973; Eijsvoogel et. al., 1973, Thistlewaite et. al., 1984 and Teale et. al., 1985) have been shown to be the principal recognition structures for alloimmune CTL.

The function of BoLA MHC in alloreactive and MHC restricted T cell cytotoxicity and antigen presentation has been shown. With all BoLA serological specificities studied, there has been very close correlation between CTL specificities and serology (Teale et. al., 1985, Goddeeris et. al., 1986, Spooner et. al., 1987b and Innes et. al., 1989).

In a recent experiment in which murine L cells (Ltk$^-$) were transfected with genomic DNA from a w10, KN104 (ILRAD designated specificity) homozygous animal, two clones were isolated which express either w10 (L10) or KN104 (L4) (Toye et. al., 1990). Alloreactive CTL also distinguished the two populations. Based on this experiment, they suggested that KN104 and w10 were encoded by separate class I loci on the same haplotype.

Bensaid and coworkers in 1991 produced two cDNA clones, 2.1 and 5.1 which differed at both the 5' and 3' termini. When DNA from L4 and L10 (Toye et. al., 1990) were used as probes, it hybridised to the 3'UT regions of clones 2.1 and 5.1 respectively. This also supported the hypothesis of a second BoLA class I locus.

Three other cDNA copies of genes encoding class I molecules have been isolated (Ennis et. al., 1988 and Brown et. al., 1989a). Two of the clones, BL3-6 and BL3-7 were isolated by Ennis et. al. while one, pBoLA-1 was cloned by Brown

et. al. Ennis et al. suggested that because of the low level of similarity between the 3' untranslated regions of the two sequences, the clones were derived from different loci. However, as the class I BoLA type of the B cell line, BL3 has not been established, it is difficult to know whether either or both genes encode functional class I MHC molecules expressed on the surface of bovine cells.

When the nucleotide sequence of the three above cDNA clones were compared, BL3-6 and BL3-7 have 92.8% and 89.2% nucleotide similarity with pBoLA-1 (Brown, 1989a). Comparison of only the 3'UT regions of these three clones reveals similarity of 89% and 88.8% respectively to pBoLA-1 and 86.2% to each other. The high level of similarity displayed by the bovine clones is expected, but the values obtained for the 3'UT comparison are probably too low to assign them as alleles, since values of 93.7% to 98.3% are found in man (Ennis et. al., 1988).

In this project, pBoLA-1 was used as the probe in the screening of a genomic library, and also in Southern and Northern blot analysis. pBoLA-1 is 1263 bp in size and encodes a complete mature class I protein. When compared with other class I proteins from man, rabbit, mouse and rat, pBoLA-1 has all the features attributed to functional class I proteins. The only feature unique to pBoLA-1 is an amino acid substitution at position 158 where a conserved alanine is replaced by an asparagine. The functional significance of this is unclear, but it may simply represent a species specific residue (Brown et. al., 1989a). This cDNA clone is incomplete, lacking the 5'UT sequence, an ATG start codon, and some 3'UT sequence. There is, however, a signal peptide, first, second and third protein domains, transmembrane region and a cytoplasmic domain. Therefore pBoLA-1 is truncated at both the 5' and 3' ends.

AIM

The aims of the work described in this thesis were to isolate and characterise a functional BoLA class I gene from an animal of a known BoLA serotype. Cosmid and bacteriophage libraries from such animals were screened with a cDNA sequence (pBoLA-1) and the genes isolated transfected into a mouse L cell system. Expression of the genes was assessed by FACS analysis of cell surface proteins and Northern blotting analysis. Further characterisation of the isolated genes was by functional assays and sequence analysis.

CHAPTER 2

ANALYSIS OF A w10/KN104 COSMID LIBRARY

2.1 INTRODUCTION

Cosmids are plasmid vectors which combine the advantage of a plasmid to accept larger fragments of foreign DNA with the high efficiency of transfection of packaged lambda phages. Their ability to accept fragments of up to 52kb compared to the 18-21kb of a phage vector make them an obvious choice in studies involving cloning of large continuous segments of DNA (Embden, 1985). A general introduction to cosmids and genomic libraries can be found in Chapter 3.

2.2 MATERIALS AND METHODS

2.2.1 Cosmid Library

At the beginning of this Ph.D. study, a genomic cosmid library prepared by Dr. John Williams was available. The genomic DNA was from an animal with a genotype w10/KN104 (w10 - international specificity 10; KN104 - ILRAD designated genotype). This genotype was selected because Bensaid and coworkers (1988) observed that w10 and KN104 were encoded by different bovine class I loci. The cosmid vector was cneox (Bates, 1987) and the selectable markers on this vector were ampicillin and neomycin (the neomycin gene also confers resistance to the neomycin analogue G418).

The library was screened with pBoLA-1 (Brown et al., 1989a) and 45 positive clones were isolated after 3 rounds of screening. A dot blot assay was carried on the 45 clones and the blot was probed with pBoLA-1. Six clones : 1.1, 2.1, 5.1, 6.1, 7.1 and 10.1 were selected because they had the strongest signal with pBoLA-1.

Also available was a restriction enzyme pattern of the six cosmid clones with the restriction enzymes BamHI, EcoRI, HindIII and PvuII (figure 4a).

2.2.2 *Preparation of Cosmid DNA*

The cneox vector before insertion of foreign DNA had ampicillin (Amp) and kanamycin (Kan) resistance. The Kan gene between the 2 cos sites, was removed during packaging. The recombinant cosmid was selected in the presence of ampicillin.

The preparation of cosmid DNA is as written in appendix 1 and the strain of E.coli used was NM554.

2.2.3 *DNA - mediated gene transfer*

As mentioned earlier, the cneox vector used for cloning carries an endogenous selectable neomycin gene and thus a foreign selectable marker is not required during transfection (cf. thymidine kinase gene described in section 4.2.2). The same transfection technique was used, as described in chapter 4, with the exception that pTk DNA was not added. Transfected cells would be resistant to selection in G418 (Geneticin - GIBCO).

An additional preliminary experiment necessary in this case was the titration of G418 on Ltk$^-$ cells to determine the concentration required for selection. The experiment was carried out as follows:

Cells were seeded at 5 x 10^5 cells/flask and kept in culture under normal conditions for 3 days (as described in section 4.2.1). The concentrations of G418 titrated were 0.1mg/ml, 0.2mg/ml, 0.4mg/ml and 0.8mg/ml. In a control flask, no G418 was added. Each titration was carried out in duplicate flasks. On the fourth day, the appropriate concentrations of G418 were added to the medium. The killing effect of G418 was observed after 24 hours. In the flask with 0.1mg/ml and 0.2mg/ml of G418, there were surviving cells while in the control flask, all the cells

were alive. 0.4mg/ml was found to be the minimum concentration which gave good selection, i.e. no survivors. Therefore from the G418 titration experiment, 0.4mg/ml of G418 would be used in the medium (appendix 3) to select for transformed Ltk$^-$ cells.

2.2.4 Indirect immunofluorescence test

The indirect immunofluorescence test was as described in section 4.2.3. The same monoclonal antibody and FITC-conjugated rabbit anti-mouse Ig was used.

2.2.5 Extraction of DNA from transfected cells

DNA was extracted from transfected cells as described by Kavathas & Herzenberg, 1986. The cells were removed from the surface of the flask using 1ml of 0.2% EDTA solution and counted (section 4.2.1). The cells were centrifuged, the supernatant removed and resuspended in about 5mls of TEN for 10^8 cells (appendix 3). An equal volume of proteinase K solution (appendix 3) was added to lyse the cells. The mixture was incubated for 15 mins at 65°C and then at 37°C overnight. A solution of 2 M Tris pH8 was added to the mixture to a final concentration of 100 mM. A phenol/chloroform extraction was carried out (appendix 1). The DNA was then precipitated with 2.5 volumes 100% ethanol and 0.3 volumes 3 M sodium acetate (appendix 1). The DNA precipitate formed immediately and was hooked out with a pasteur pipette. The precipitate was washed by dipping in 75% ethanol and was allowed to dry before dissolving in TE pH8. RNAase was added to 20μg/ml and incubated at 37°C for at least 1 hour. The DNA was again phenol/chloroform extracted and precipitated with ethanol. A small aliquot was run on a 0.75% gel to check the quality of the DNA and the concentration of DNA was determined by

spectrophotometry (appendix 1).

2.2.6 *Restriction enzyme analysis*

(a) The six cosmids (~ 1μg) were digested with BamHI, EcoRI, HindIII and PvuII in a total volume of 20μl at 37°C for 1½ hours. (b) Genomic DNA from the transfected cells (~ 10μg) were digested with 30-40 units of BamHI and EcoRI in a total volume of 200μl at 37°C for 4-5 hours. The completeness of digestion was checked by running a small aliquot of each digest on mini agarose gels. On complete digestion, the digested cosmids were run on a 0.75% agarose gel in 1 x TBE buffer at 20-30 volts overnight. Phage lambda DNA digested with HindIII was included as the size marker. The gels were stained in ethidium bromide and photographed using UV transillumination (section 3.2.3 and appendix 1).

2.2.7 *Southern blot analysis*

Southern blots of the digested genomic DNA from transfected cells was performed as described in section 3.2.3. Hybridisation of the filters with the pBoLA-1 insert is as described in 3.2.3. The filters were washed at low stringency (appendix 1).

2.3 *RESULTS*

2.3.1 *DNA-mediated gene transfer*

Since the cosmid vector carries the neomycin gene, Ltk$^-$ cells transfected with cosmid DNA would survive G418 selection. Transfection of the 6 cosmids into the Ltk$^-$ cell lines resulted in transfected cells that survive G418 selection. FACS analysis of the transfected cells showed that none of the 6 cosmid clones showed any

appreciable level of expression. Even though the more-fluorescent cells were sorted and grown, in a second round of analysis, there was no increase in the proportion of cells expressing fluorescence.

2.3.2 Southern blot analysis

When DNA from the transfected cells were digested with BamHI and EcoRI and probed with pBoLA-1 insert, there was no hybridisation signal indicating the absence of a BoLA class I gene in the transfected cells or the copy number of BoLA class I gene is too low to be detected.

2.3.3 Restriction enzyme analysis

A different restriction enzyme pattern was observed with two of the cosmids after a second preparation of the cosmid DNA. The cosmids, 5.1 and 6.1, when digested with BamHI, EcoRI, HindIII and PvuII, gave different patterns from the initial preparation (figure 4). The approximate size of cosmid 6.1 from the restriction patterns in figure 4b was 25kb which suggest a deletion has occurred. Cosmid 5.1 appear to have a broad band of approximately 3.5kb. This could be a supercoil form of the plasmid with no sites for the 4 restriction enzymes and since the same band is seen with all 4 enzymes, it suggests that there was complete deletion of the inserted fragment.

2.4 DISCUSSION

The negative result obtained from the Southern blot analysis indicated the absence or low level of BoLA class I DNA in the transfected cells. The former could be due to non integration of the cosmid DNA into the mouse genome (Ltk$^-$

FIGURE 4. *RESTRICTION DIGEST OF COSMIDS*

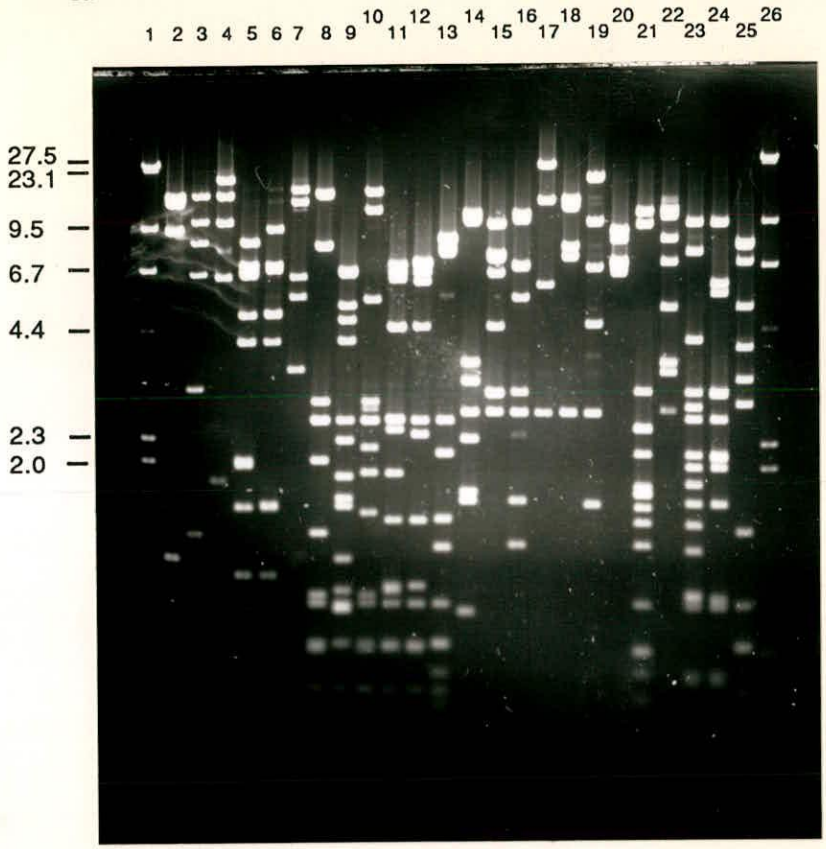
Figure 4a

Restriction digests of cosmids 1.1, 2.1, 5.1, 6.1, 7.1 and 10.1 with BamHI, PvuII, HindIII and EcoRI. Tracks 1 and 26 are the HindIII digested λ DNA molecular weight markers (sizes are shown in the figure). Tracks 2-7 are BamHI digestions; tracks 8-13 are PvuII digestions; tracks 14-19 are HindIII digestions and tracks 20-25 are EcoRI digestions. (Restriction enzyme digestion and gel carried out by Dr. Alan Teale, ILRAD).

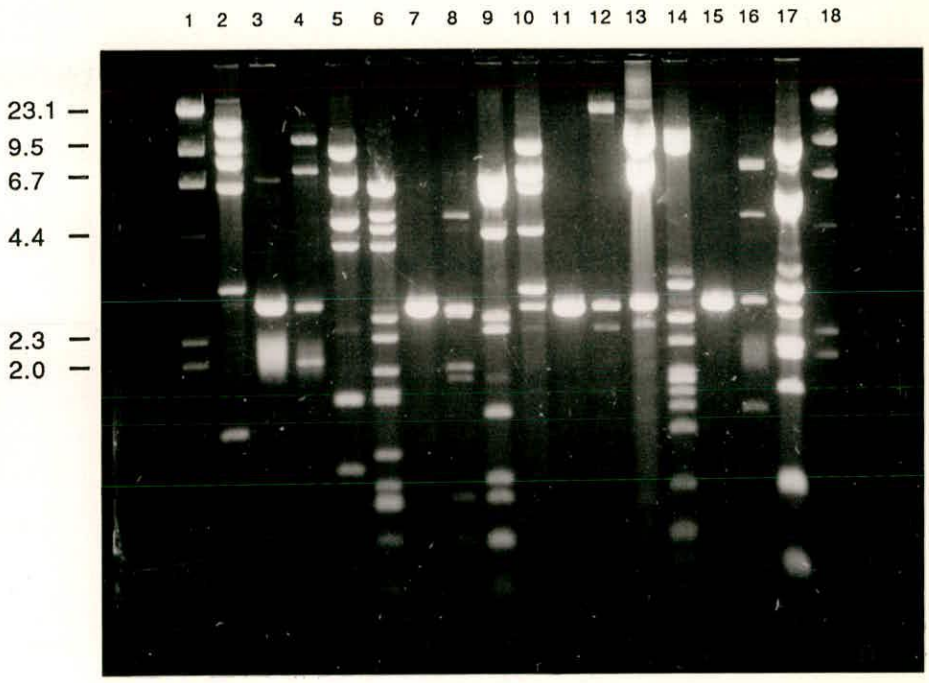
Figure 4b

Restriction digests of cosmids 2.1, 51., 6.1 and 7.1 with BamHI, PvuII, HindIII and EcoRI. In each case 1μg of the cosmid DNA was digested and run on a 0.75% agarose gel at 20V overnight. Tracks 1 and 18 are the HindIII digested λ DNA molecular weight markers (sizes are shown in the figure). Tracks 2-5 are BamHI digests; tracks 6-9 with PvuII; tracks 10-13 with HindIII and tracks 14-17 with EcoRI. All cosmids (including 1.1 and 10.1, not in the figure) gave a similar restriction pattern as seen in figure 4a except cosmids 5.1 and 6.1 which appear to be deleted (section 2.3.3).

a.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
27.5
23.1
9.5
6.7
4.4
2.3
2.0

b.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
23.1
9.5
6.7
4.4
2.3
2.0

cells). Insufficient amounts of bovine class I DNA in the digestion that could not be detected by hybridisation may be another explanation for the negative result. This could indicate only a small proportion - if any - of the transfectant cells contain cosmid DNA. Northern blot analyses were not performed.

At this point a decision was taken to construct a new genomic library (see chapter 3) since:-

1. two of the cosmids, 5.1 and 6.1, showed deletion. Recombinant cosmids have been shown to have a tendency to be unstable. The recombinant cosmids are normally lost as a result of deletion during propagation (Grosveld et al., 1981).
2. the animal the library was made from had a w10/KN104 haplotype. As KN104 is not an internationally recognised allele, it was decided to choose an animal with an internationally recognised haplotype.

CHAPTER 3

PREPARATION OF GENOMIC LIBRARY FROM AN ANIMAL WITH BoLA TYPE w10/w11

3.1 INTRODUCTION

In studies of gene organisation and expression, the initial step is to isolate a functional gene within one recombinant molecule in a DNA library. This process involves the establishment and screening of libraries which comprise millions of independent clones.

There are two types of DNA library, genomic and cDNA. Both types of library serve specific purposes.

In a genomic library, total DNA is extracted from the donor and the entire genome is cleaved at random. These fragments are then inserted into either a phage or a cosmid vector and introduced into *E. coli*. Recombinant bacteriophages can then be recovered as plaques and stored for further analysis.

In a cDNA library, only the DNA sequences that are transcribed into RNA are selected, that is, the mRNA is extracted. A DNA chain is synthesised on this RNA template using a reverse transcriptase and the single stranded cDNA converted into double stranded cDNA by DNA polymerase. This double stranded cDNA is then inserted into a plasmid or phage vector (the size of a cDNA is suitable for insertion into plasmids or conventional phage vectors), and *E. coli* is transformed with these recombinant molecules. The resulting bacterial clones, each containing a single cDNA, make up the cDNA library.

One disadvantage of constructing a genomic library is that only some of the inserted fragments in the library will contain complete genes, but many will contain a portion of a gene. Therefore the number of clones which must be constructed and screened to obtain a complete gene from a genomic library is at least 100 fold more

than that required for a cDNA library.

However, the choice of a library depends on the aim of the study and not on the work involved in setting it up.

The genomic library would enable a study of the organisation of the entire gene including introns, the promoter regions and the flanking sequences. A cDNA clone would lack introns and sequences which flank the transcribed region of the structural gene. However, a cDNA library is an obvious choice to determine the primary structure of a protein as nucleotide sequencing of cDNA clone takes less time than that of a genomic clone.

The primary structures of different MHC genes of the same class are similar to one another, even between unrelated species. cDNA clones or locus specific genomic clones have been used successfully to probe DNA libraries (Barbosa et. al., 1982, Holmes and Parham, 1985, Singer et. al., 1988 and Chimini et. al., 1988). Malissen et. al. (1982) used a mouse H-2 cDNA to probe a human genomic library to study the exon/intron organisation of an HLA gene.

To date, there are no known genomic BoLA class I clones and there are only 5 BoLA class I cDNA clones (Ennis et. al., 1988, Brown et al., 1989a and Bensaid et. al., 1991). In this study a genomic library was constructed and was screened using a BoLA class I cDNA probe (pBoLA-1, Brown et. al., 1989a). One of the main aims in constructing this genomic library was to look into the question - is there more than one class I BoLA locus? A library from a known genotype would enable not only the isolation of complete genes but the identity and function of BoLA class I genes can also be studied. The identity in terms of genotype and sequence data would provide an insight into the MHC system.

There are two common types of vector for the construction of genomic

libraries, cosmids and bacteriophages. Cosmids are hybrid plasmid-bacteriophage vectors (Collins & Hohn, 1978). They were constructed to allow cloning of large fragments of DNA and they have combined many advantages of plasmid and bacteriophage vectors.

As a result of deletions in 2 of the 6 initial cosmid clones analysed (chapter 2), the genomic library prepared here was constructed in a bacteriophage vector. Since class I genes from other species are less than 6kb in length, it was decided that a bacteriophage vector would suffice in this study.

Bacteriophage lambda has been developed successfully for use as a vector in molecular cloning. About 40% (20kb) of the λ genome is not essential for productive infection and can be replaced by foreign DNA. To maintain the viability of the recombinant phages, the lengths of their genomes must not be more than 105% or less than 78% of wild-type lambda. The vector selected for cloning in this study was EMBL3 (Frischauf et. al., 1983). The BamHI site in the polylinker sequence is especially useful in cloning Sau3A partial digests. In this vector, two genes involved in recombination, *red* and *gam* are located in the non-essential area (stuffer fragment) and are removed before cloning. The product of the *gam* gene is involved in the inactivation of the potent exonuclease V encoded by the host's *rec*BC genes. In *gam*⁻ recombinants, the concatenated linear λ DNA produced by rolling-circle replication is degraded by the *rec*BC exonuclease. The product of the *red* gene is involved in the recombination events that occurs in the early lytic cycle, that is, to convert viral DNA replicating in the θ form into closed circular progeny molecules. These molecules are the templates for the rolling-circle replication which produces linear oligomer. The linear oligomers of viral DNA are the preferred template for packaging. Bacteriophages lacking the *red* and *gam* genes were found to grow well

in P2 lysogens. These were termed Spi⁻ in comparison with the wild type (Spi⁺) which did not grow well in P2 lysogens (sensitive to P2 interference). Thus the first genetic selection for the *red⁻ gam⁻* EMBL3 recombinants is to grow them in a P2 lysogenic strain of *E. coli*.

Another genetic factor in the vector is the *chi* site (a specialised octanucleotide substrate in which the directional recombination catalysed by the *rec* BC system in the bacteriophage λ is promoted). As mentioned earlier, the usual substrates for encapsidation are not available in *red⁻ gam⁻* phages. Therefore an alternative concatemeric substrate is required. In phages which have a *chi* site, recombination gives rise to closed circular dimers which can be used for encapsidation. Therefore, the plaques formed are almost as large as those of *red⁺ gam⁺* bacteriophages. The EMBL vectors have *chi* sites incorporated in the arms and will give rise to recombinant plaques of relatively uniform size (Murray, 1983).

The host strain of *E. coli* used for amplifying and screening of the bacteriophage λ libraries is also important. The host used in this study was NM646, a P2 lysogenic derivative of K802. The K802 strain is *mcr⁻*. The *mcr* (modified cytosine restriction) systems (*mcr*A and *mcr*B) of *E. coli* restrict incoming DNA containing methylcytosine (Raleigh et. al., 1988). This could interfere with cloning experiments if the DNA is heavily methylated, as is normally the case with mammalian DNA. Therefore NM646, an *mcr⁻* P2 lysogenic strain, is suitable for the recovery of recombinant DNA (Whittaker, 1988).

3.2 MATERIALS AND METHODS

3.2.1 λ Genomic Library

(a) *Preparation of vector arms of EMBL3*

In λ EMBL3, the central fragment (or the stuffer) is flanked by the two essential arms. The left arm is approximately 19.9kb in length and the right arm is 8.8kb in length. The central fragment is bounded by polylinkers in inverted orientation (SalI-BamHI-EcoRI - central fragment - EcoRI-BamHI-SalI). These are the only sites for the 3 enzymes (except for two additional SalI sites in the central fragment), so any of them could be used for cloning. However, EMBL3 was designed for use with BamHI since the core of the BamHI recognition sequence contains the recognition site for the enzymes Sau3A and MboI, enzymes often used to generate partially-digested genomic fragments for cloning. Cleavage with both BamHI and EcoRI makes the central fragment of EMBL3 unable to compete with the donor DNA insert. This strategy was used in the preparation of this genomic library. The EMBL3 vector DNA was first cut with BamHI and when cleavage was complete, the reaction buffer was adjusted to EcoRI conditions and a 3-fold excess of EcoRI was added to the digestion mix. This was to ensure complete cleavage of the central fragment by the EcoRI. A small aliquot of the digestion mix was run on a gel to verify complete digestion by the two enzymes.

(b) *Preparation of genomic DNA*

DNA was prepared from bovine peripheral blood monocytes (PBM) separated from whole blood of animal 10769 (with BoLA type w10/w11) by spinning

at 3000rpm for 30 minutes at room temperature. The buffy coat was removed to a fresh tube. The contaminating red cells in the buffy coat were osmotically lysed by adding sterile H_2O. The mixture was then made isosmotic by the addition of an equal volume of 1.8% saline equal to the amount of water added. The suspension was centrifuged at 1500 rpm for 10 minutes, the supernatant was decanted and the cells lysed by addition of 0.5mls of 10% SDS. The volume was adjusted to 5mls by adding 4.5mls of digestion buffer (0.15 M NaCl, 10 mM Tris HCl pH8;. 10 mM EDTA/Na pH8) with 200μg/ml proteinase K. The preparation was incubated at 37°C for at least 3 hours before extraction with phenol twice, phenol/chloroform once and chloroform once (appendix 1). The DNA was then precipitated by addition of two volumes of absolute ethanol. The precipitated DNA was spooled on a glass rod and left to air dry before it was resuspended in 1ml of TE pH8. The DNA was stored at 4°C. The concentration of the DNA was determined by spectrophotometry (appendix 1).

(c) *Preparation of 20-kb fragment of insert DNA*

Preliminary expt : The DNA was partially digested with MboI which recognises the 4-bp sequence GATC. A reaction mixture of 10μg of genomic DNA and restriction enzyme buffer in a total volume of 150μl was prepared. Eppendorf tubes were labelled 1-9. 30μl of the reaction mixture was dispensed into tube 1 and 15μl into tubes 2-9. The tubes were chilled on ice. 4 units of MboI was added to tube 1 and mixed with the Gilson. The concentration of enzyme was thus 2 units/μg DNA. 15μl of the reaction mixture in tube 1 was transferred to tube 2. The enzyme concentration was

1 unit/μg DNA in tube 2. The mixture was again mixed with the Gilson and the twofold serial dilution was carried on until tube 8. No enzyme was added in tube 9. Tubes 1-8 were then placed in the 37°C waterbath for 1 hour. The reactions were terminated by chilling the reactions to 0°C and adding 1μl of 0.5 M EDTA pH8. 5μl of gel-loading dye (appendix 1) was added to each sample and analysis on a 0.4% agarose gel was carried out with λ HindIII size markers (appendix 1). The electrophoresis was carried out at 20V overnight. The gel was stained in ethidium bromide and photographed. With the help of the size markers, the digest containing the maximum intensity of fluorescence in the 15-20kb region of the gel was found to be 0.0125units/μg DNA. 250μg DNA was then digested by scaling up of the optimised conditions. After digestion the DNA was gently extracted with phenol, phenol/chloroform and chloroform, precipitated with ethanol, and washed once in 70% ethanol. The DNA was then vacuum dried briefly before dissolving in 500μl TE pH8. 18ml of 40% and 18ml of 10% sucrose was prepared to make a sucrose density gradient. The sucrose solutions were made in a buffer containing 1 M NaCl, 20 mM Tris HCl pH8 and 5 mM EDTA pH8 and a 10-40% sucrose density gradient was prepared in a Beckman polyallomer tube using a peristaltic pump. The DNA was heated for 10 minutes at 68°C, and cooled to 20°C to minimize annealing of the cohesive ends and loaded on the sucrose density gradient. The tubes were centrifuged at 26,000rpm and 20°C for 24 hours in the SW71 rotor.

The bottom of the centrifuge tube was punctured and 68 0.5ml fractions were collected into eppendorf tubes. 5μl samples were pooled in groups of 3 and analysed on a O.3% agarose gel. Fractions containing fragments of 15-20kb

were pooled and then were dialysed against 4 litres of TE pH7.8 at 4°C for 12-16 hours with one buffer change. The dialysed DNA was repeatedly extracted with equal volumes of 2-butanol until the volume was reduced to about 5-8ml. The DNA was then precipitated with ethanol, washed with 70% ethanol, vacuum dried and dissolved in 20μl TE pH8. 1μl of the sample was analysed on 0.4% agarose gel to check the range of fragment sizes.

(d) *Ligation of the selected insert to the vector*

A molar ratio of 3:1 (arms : inserts) was used in the ligation of the 15-20kb fragments with the arms of EMBL3. The ligation mixture included:

5μl insert DNA (0.5μg)

5μl vector DNA (3μg)

2μl of 10 x ligase buffer (appendix 1)

7μl H_2O

1μl (2 units) of T4 DNA ligase (GIBCO-BRL)

The ligation reaction was left at 15°C overnight. 1μl of the ligation reactions was analysed on a 0.4% agarose gel with λ HindIII and intact λ DNA as markers.

(e) *In vitro packaging of the ligated DNA into phage heads*

In vitro packaging involves the use of cell extracts obtained from two induced λ lysogens whose prophages have different complementing mutations in their genes which are essential for the assembly of a mature phage particle. When these extracts are mixed together with DNA of appropriate size, 38.5-50kb, and of appropriate form, linear or circular multimers (concatamers), they are

packaged into infectious 'phage particles' (Hohn and Murray, 1977 and Kaiser and Murray, 1985). 3μl (~0.5μg DNA) of the ligation reaction was removed and packaged. The packaging mixes were obtained from Amersham and packaging was carried out as instructed by the manufacturer. At the end of the packaging reaction, 0.5mls of phage buffer (appendix 2) was added to the tube and followed immediately by 10μl chloroform. This mixture was mixed gently and stored at 4°C as a normal phage stock.

(f) *Preparation of plating bacteria*

The recommended host strain for EMBL3 vectors is NM646. 50ml of L Broth (appendix 2) supplemented with 0.2% maltose was inoculated with a single bacterial colony. The culture was grown with shaking at 37°C until its optical density at 600nm was 0.5. The cells were then harvested by centrifugation at 3000 rpm for 10 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended in 20ml sterile 0.01 M $MgSO_4$. The preparation of plating cells may be stored at 4°C for up to 3 weeks.

(g) *Infection and plating of packaged DNA*

Assuming that there is random chance of any sequence being represented, the size of the library can be calculated. The size of the library that gives a high probability of containing the DNA sequence of interest can be calculated from the size of the insert and the size of the haploid genome (both in base pairs). For a mammal, the number of independent recombinant DNA molecules required in a lambda replacement vector is approximately 8 x 10^5 to get a

99% probability of having cloned a particular DNA sequence (Kaiser and Murray, 1985).

Ten-fold serial dilutions of the phage stock was prepared in phage buffer and 0.1ml of each dilution was dispensed into 5ml sterile tubes. 0.1ml of the plating bacteria was added to each tube and mixed by vortexing. The tubes were incubated at 37°C for 20 minutes to allow the bacteriophage particles to adsorb onto the bacteria. 3ml of medium containing 0.7% agarose kept at 47°C (appendix 2) was added to the tubes, vortexed gently and poured immediately onto 90mm labelled petri dishes containing 25-30ml of bottom agar medium (appendix 2). The dishes were swirled gently to ensure an even distribution of the top agarose, and left at room temperature for 5 minutes to allow the agarose to harden. They were then transferred to the 37°C incubator overnight with the plates inverted. The resultant plaques were counted and from these numbers, the phage titre in the original stock was calculated. The total library size estimated from plating out of samples was 1×10^7 plaque forming units.

(h) *Amplification and screening of the genomic library*

The library was plated out on 14 square (24cm x 24cm) petri dishes at a plaque density of 60,000 per plate. This meant that the plates were nearly confluent. Agarose was used instead of agar in the top layer to enable filter replicas to be lifted off easily. Duplicate filter (hybond) replicas of the plate were made. Each filter replica was treated as follows:-

(i) 3 minutes on 3MM Whatman wetted with 10% SDS, to limit the diffusion of DNA.

(ii) 5 minutes on 3MM Whatman wetted with 0.5 M NaOH/1.5 M NaCl to denature the DNA.

(iii) 5 minutes on 3MM Whatman wetted with 1 M Tris pH7.4/1.5 M NaCl to neutralise the filter.

The hybond filters were left to air dry for 5 minutes before they were UV irradiated to irreversibly fix the DNA to the filter. Screening was carried out by hybridisation (appendix 1) using pBoLA-1. The filter was prehybridised in hybridisation buffer (appendix 1) to saturate non-specific DNA binding capacity and was probed by hybridisation with a radioactively labelled pBoLA-1 fragment overnight at 65°C. The probe should bind to the regions of the filter that carry DNA sequences homologous with pBoLA-1, i.e. class I BoLA genes. The filters were washed in low stringency hybridisation buffer (appendix 1). Hybridisation was detected by autoradiography (appendix 1). Three rounds of screening were carried out:

(i) Primary screening - where there was a positive signal, a plug of agar was removed from the square plates using a pasteur pipette and placed in 1ml of phage buffer + 20μl of chloroform. The phages were allowed to diffuse out of the agar plug overnight at 4°C. This was then diluted 1/100 and 10μl of the dilution was plated on small petri dishes. Duplicate filter replicates were made and probed with pBoLA-1.

(ii) Secondary screening - where there was a positive signal from the primary screening products, 1-3 plaques were picked from each plate into 0.5ml of phage buffer + chloroform. Isolates with more than one plaque were further diluted 1/100 and 30μl of this dilution was

plated out to produce single plaques.

(iii) Tertiary screening - where there was a positive signal from the secondary screening products, single plaques were picked and placed into 0.5ml of phage buffer + chloroform.

From the three screenings 49 putative BoLA class I clones were obtained.

(i) *Phage growth and purification of DNA*

To analyse the 49 recombinants it was first necessary to purify the DNA from them. The method used in this investigation to prepare stocks from the single plaques after the 3° screening was by plate lysate, in which the bacteriophages from the picked plaques were propagated in bacteria grown in soft agar.

(i) *Plate lysates*

25μl of the bacteriophage (10^5pfu ~ 1/20 of a resuspended plaque, Sambrook et al., 1989) were incubated with 100μl of plating cells at 37°C for 20 minutes in a 5ml sterile test tube. 3ml of molten top agarose (47°C) was mixed and quickly poured into a labelled 90-mm plate with 25-30ml of hardened bottom agar. This was left to stand for 5 minutes then incubated at 37°C overnight. Next day the confluently lysed top agarose layer was gently scraped into a sterile centrifuge tube. 5ml of phage buffer was added to the tube and the phage were left to diffuse on a gentle shaker at 4°C overnight. The tube was centrifuged at 3000rpm for 10 minutes at 4°C and the supernatant was placed into a fresh sterile tube. One drop of chloroform (~ 10-20μl) was added and the stock (10^{10}-10^{11} pfu/ml) was stored at 4°C.

(ii) *Preparation of phage DNA*

To 100ml aliquots of L Broth in 250ml conical flasks, 0.01 M $MgSO_4$ and 0.2% (w/v) maltose were added. They were then inoculated with 1ml of overnight culture of NM646. The flasks were incubated at 37°C with vigorous shaking until the O.D. 600 = 0.5. They were then infected with 10^{10} pfu of phage stock and incubated for a further 3-5 hours until lysis occurred. 2ml of chloroform was added to each flask and the incubation was carried on for 10 minutes with shaking at 37°C.

(iii) *Purification of bacteriophage* λ

The lysed culture was cooled to room temperature and DNAase and RNAase was each added to a final concentration of 1μg/ml. The flask was left at room temperature for 30 minutes before the bacterial debris was removed by centrifuging at 10,000rpm for 10 minutes at room temperature. PEG6000 was added to 10% and NaCl was added to 1 M to the phage supernatant and left at 4°C for at least 2 hours. The precipitated phage were pelleted by centrifugation at 10,000rpm for 15 minutes at 4°C. The phage pellet was then dissolved in 1.5ml of phage buffer. Residual debris was removed by mixing vigorously with an equal volume of chloroform and brief centrifugation at 3000rpm for 5 minutes. The top phase was removed and layered onto a CsCl step gradient comprising 1ml of 1.7g/ml CsCl, 1ml of 1.5g/ml CsCl and 0.75ml of 1.3g/ml CsCl (appendix 2). The gradient was centrifuged in a SW50 rotor for 2 hours at 38,000rpm at a temperature of 4°C. The bluish phage band was collected by puncturing the side of the tube with a wide gauge needle and sucking into a 1ml syringe. The phage was

dialysed for 2 hours against two changes of 10 mM NaCl, 50 mM Tris HCl pH8 and 10 mM $MgCl_2$ at room temperature. The dialysed phage was then phenol, phenol/chloroform and chloroform extracted. After ethanol precipitation, the phage DNA was pelleted at 8000rpm for 10 minutes and dissolved in an appropriate volume of TE pH8. It was then analysed on a 0.7% agarose gel to check the size and quality of the phage. The concentration of the DNA was determined by spectrophotometry (appendix 1).

3.2.2 Dot blot assay

The fastest way to check if the 49 clones had both 5' and 3' ends of a class I gene was to probe dot-blotted phage DNA with the 5' and 3' ends of pBoLA-1 (appendix 1).

The dot blot membrane was presoaked in 6 x SSC and placed between the frames of the manifold. The diluted phage DNA (100ng in 400μl TE pH8) was dotted on the hybond membrane under vacuum. It was then washed with 400μl of 6 x SSC containing 4% bromophenol blue. The hybond was then removed from the manifold, denatured, neutralised, air dried and UV cross-linked as in (3.2.1). 4 controls were included in the assay, 3 positives (pBoLA-1, 5' end of pBoLA-1 and 3' end of pBoLA-1) and 1 negative (pPolyI, the vector of pBoLA-1). The dot blot was done in duplicate. The blots were then hybridised with the 5' and 3' ends of pBoLA-1 individually. The filters were washed at a high stringency (20 mM phosphate buffer pH7.2 and 0.1% SDS - appendix 1) at 65°C and hybridisation was visualised by autoradiography (appendix 1).

3.2.3. Restriction enzyme analysis of recombinant bacteriophages

(a) *Restriction enzyme digests of phage DNA*

1μg of phage DNA was digested in a volume of 20μl with the appropriate enzyme concentration. The digestions were generally carried out at 37°C for 1½-2 hours. The completeness of digestion was checked by running a small aliquot of each digest on mini agarose gels. On complete digestion, 5μl DNA loading dye (appendix 1) was added and the digest was heated at 65°C for 2 minutes before loading on an agarose gel.

(b) *Agarose gel electrophoresis*

The efficient range of separation of linear (digested) DNA molecules depends on the percentage of agarose in a gel (Maniatis et. al., 1982). 0.7% gels were used most frequently as these resolve DNA fragments over a range (0.8-10kb) which is sufficient for most restriction digestions. When separation was required in the range of 10-20kb, 0.3% to 0.5% gels were used and 1.5% agarose was used for separating fragments in the size of 0.2-4kb. The buffers used in preparing and running the gels were TBE and TAE electrophoresis buffers (appendix 1).

Rapid/mini agarose gels were run at 50-100 volts while restriction digest gels were run at 20-30 volts overnight to get better resolution of the fragments. The gels were stained with ethidium bromide at a final concentration of 0.5μg/ml (appendix 1) either during or after the run. The DNA fragments were viewed and photographed using UV transillumination (appendix 1). The size of the DNA fragments was determined by comparison with known standards generated from lambda phage DNA digested with HindIII (appendix 1).

(c) *Southern Blot*

DNA fractionated on an agarose gel was transferred to Hybond-N (Amersham) using the capillary blotting method of Southern (1975). The gels were pretreated before transfer as described by Wahl et. al., (1979) as follows:

The gels were immersed in 0.22 M HCl (this was omitted if the fragments were less than 5kb in size) and agitated for 2 x 15 minutes. The solution was aspirated off. The denaturing solution (0.5 M NaOH, 1.5 M NaCl) was next added and agitated for 2 x 15 minutes. Finally, the neutralisation solution (1.5 M NaCl, 0.5 M Tris.HCl pH7.4) was added and agitated for 2 x 15 minutes. The solution used for transfer was 20 x SSC (appendix 1). The apparatus for transfer consisted of a sandwich box reservoir containing a raised glass plate, with 3MM chromatography paper as the wick. 3 sheets of 3MM paper cut to the same size as the gel were placed on the wicks followed by the gel. The hybond membrane, presoaked in 2 x SSC, was laid on top of the gel followed by 3 sheets of 3MM paper presoaked in 2 x SSC. Layers of towels were placed on top of this to draw the solution up through the gel. Once transfer was completed (normally left overnight), the hybond-N membranes were UV fixed and saran wrapped.

(d) *Radioactive labelling of DNA*

The Amersham multiprime DNA labelling system (RPN 1601) uses the method of Feinberg and Vogelstein (1983) in which hexanucleotides of random sequence prime DNA synthesis at numerous sites on a denatured DNA template.

The DNA (10-20ng) was denatured for 2 minutes by boiling and chilling on ice quickly. The following was added to the denatured DNA. 10μl of the multiprime buffer solution (dATP, dGTP and dTTP in a concentrated buffer solution

containing Tris.HCl, pH7.8, magnesium chloride and 2-mercaptoethanol); 5μl of the primer solution (random hexanucleotides in an aqueous solution containing nuclease-free BSA); 5μl α^{32} P dCTP (50μCi) and 1 unit of Klenow fragment DNA polymerase 1. The reaction was incubated at room temperature for a minimum of 5 hours.

The incorporation was assayed by precipitation with trichloroacetic acid (TCA). 1μl of the reaction mixture was transferred to a sterile disposable tube containing 200μl of water and 50μl carrier DNA solution (provided in the labelling kit). 2ml of ice cold 10% TCA solution was added, vortexed and allowed to stand on ice for 15 minutes. The labelled and carrier DNAs should co-precipitate. The precipitated DNA was collected by vacuum filtration on a glass fibre disc (Whatman) and the cerenkov [^{32}P] counted. Incorporation was routinely in excess of 1 x 10^9 dpm/μg DNA.

(e) *Hybridisation of hybond filters to radioactive probes for DNA and RNA*

The technique of Church and Gilbert, 1984 was used in the hybridisation procedure. The UV-fixed membranes were prehybridised in hybridisation buffer containing 100mgs of herring sperm DNA for ½-1 hour in a perspex hybridisation chamber. The labelled probe was denatured by adding 0.25 volumes of fresh 2 M NaOH for 5 minutes before adding to the prehybridised membrane and incubating at 65°C overnight with agitation.

(f) *Washing of the hybridised filters*

Washing conditions of the hybridised filters should be as stringent as possible. Stringency is controlled by a combination of salt concentration and temperature. In Southern and Northern blots, a low stringency (appendix 1) wash at 65°C was

sufficient. The DNA in a Southern blot is size-fractionated and a low stringency wash was sufficient to remove non-specific binding. In a Northern blot, a very small amount of mRNA of the total RNA extracted are detected by a probe and a low stringency wash would be sufficient. However in the dot blot analysis, it is important to identify truly positive signals as the DNA was concentrated to a spot. A high stringency wash (appendix 1) was used for the dot blots as there was sufficient cloned DNA present to give a clear hybridisation signal and to assess the amount of similarity between the probe and the DNA.

The filters were removed from the perspex hybridisation chamber and placed in a sandwich box. The washing buffer (appendix 1) was added and agitated for 30 minutes at 65°C. This was decanted and the wash was repeated. The filters were then wrapped in Saran Wrap before autoradiography (appendix 1).

3.3 RESULTS

3.3.1 Dot blot assay

The 49 phage clones isolated after the third screening of the library with pBoLA-1 were analysed by a dot blot assay. The dot blot was probed with

(i) the 5' EcoRI/BglI fragment of pBoLA-1 (which corresponds to the leader peptide and first two domains).

(ii) the 3' EcoRI/PstI fragment of pBoLA-1 (which corresponds to the 3'UT region).

The dot blots were washed at high stringency (appendix 1). The results are shown in figure 5 and are tabulated below.

FIGURE 5. *DOT BLOT ASSAY*

c.

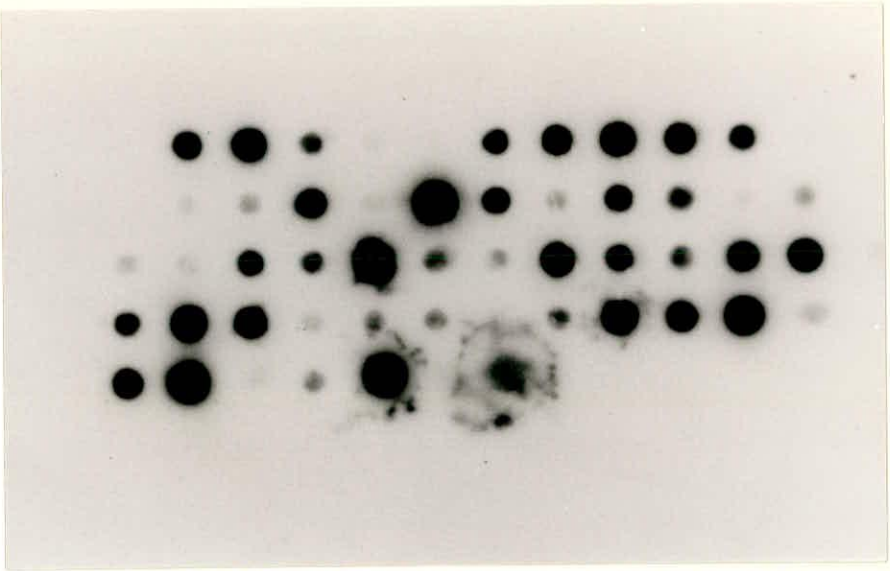
1.1	1.2	1.3	2.1	3.1	3.2	3.3	4.2	4.3	5.2	5.3	5.3x
5.3y	6.1	6.2	6.3	7.1	7.2	7.3	8.2	8.3	9.1	9.2x	10.1x
10.1y	10.2	10.3	11.1	11.2	11.3a	11.3b	14.1	14.2x	14.2y	14.2	14.3a
14.3b	15.2	15.3	16.1	16.3	16.4	17.1	17.2	17.3x	17.3y	18.1	18.3a
18.3b	19.1	19.3	14.2y	pBoLA-1	3' pBoLA-1	5' pBoLA-1	pPoly1				
1	2	3	4	5	6	7	8	9	10	11	12

10ng of the 4 controls and 100ng of each phage DNA were dotted in duplicate onto nylon membrane (section 3.2.2) as shown above (Figure 5c).

Figure 5a shows the hybridisation pattern with a probe to the 5' end of pBoLA-1 while Figure 5b is probed with 3'probe. 15 clones were selected on the basis of strongest hybridisation intensity after a high stringency wash. They are 1.3, 4.2, 4.3, 5.2, 6.3, 7.2, 7.3, 14.2, 14.3a, 15.2, 17.3x, 17.3y, 18.1, 18.3b and 19.1.

Figure 5c is the clone number key for the dot blots.

a.



b.

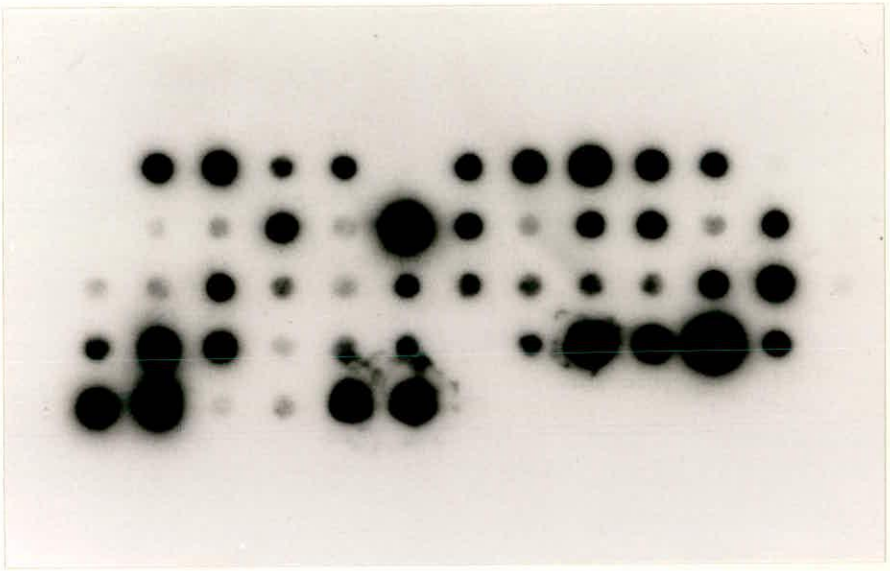


TABLE 3.1 Hybridisation signals of 49 phage clones with the appropriate probe

Hybridisation to			
5' and 3' ends of pBoLA-1	only the 5' end of pBoLA-1	only the 3' end of pBoLA-1	neither ends
1.3, 4.2, 4.3, 5.2, 6.3, 7.2, 7.3, 14.2, 14.3a, 15.2, 17.3x, 17.3y, 18.1, 18.3b, 19.1 pBoLA-1	11.2, pBoLA-1 5' end	3.1, 10.1x, 14.1 18.3a, pBoLA-1 3' end	1.1, 3.2, 5.3x, 5.3y, 17.1, pPoly1

In the numbering of the recombinant phage, the first number indicates the plate and the second number the phage number, i.e. 1.3 would be the third plaque isolated from plate 1.

When the controls in the dot blot were probed with the 5' end of pBoLA-1, only pBoLA-1 and 5' end controls hybridised. In that blot probed with the 3' end of pBoLA-1, only pBoLA-1 and 3' end controls hybridised to the probe. In both cases there was no hybridisation with pPoly1.

From this result, the 15 clones that hybridise with the 5' and 3' ends of pBoLA-1 were selected for further experiments.

3.3.2 Restriction enzyme analysis

Preliminary restriction enzyme analysis was carried out on the 15 clones. When digested with BamHI, these clones all exhibited a similar restriction pattern. To check the result, the gel was blotted and hybridised with pBoLA-1. All 15 clones showed a hybridisation signal confirming the presence of a BoLA class I gene (figure 6). Seven clones had hybridising bands of about 15kb, two clones had identical 15 and 9kb hybridising bands and six clones had unique patterns (table 3.2).

FIGURE 6. *HYBRIDISATION SIGNALS OF THE 15 PHAGE CLONES*

Hybridisation of pBoLA-1 to BamHI digested recombinant phages. 1μg of phage DNA was digested with 2-3 units of BamHI and run in a 1.5% agarose gel at 20V overnight. DNA was transferred to nylon membranes and hybridisation was performed as described in section 3.3.3. Tracks 1-15 are the clones 1.3, 4.2, 4.3, 5.2, 6.3, 7.2, 7.3, 14.2, 14.3a, 15.2, 17.3x, 17.3y, 18.1, 18.3b and 19.1 respectively. The positions of the size markers (HindIII digested λ DNA) are shown at the side of the figure.

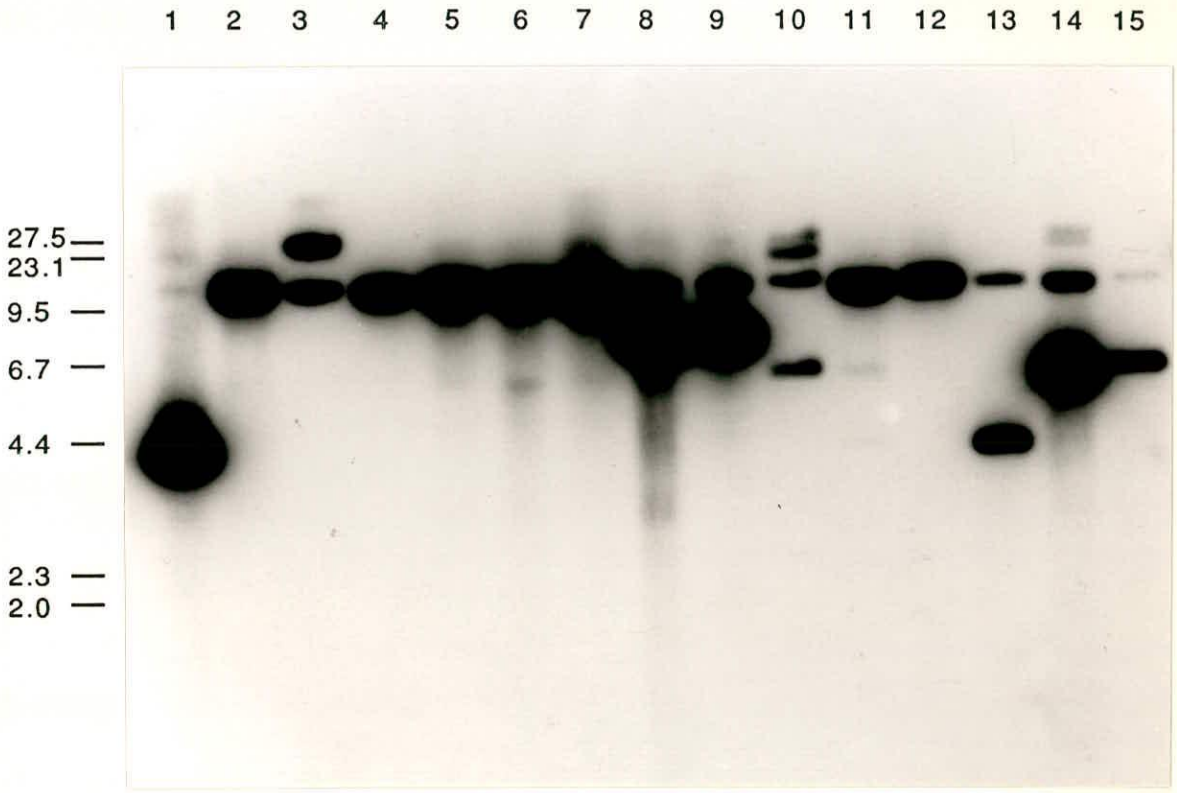

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
27.5
23.1
9.5
6.7
4.4
2.3
2.0

TABLE 3.2. Hybridsation pattern of the 15 phage clones with pBoLA-1

Phage clones	Approximate size of hybridising fragment (kb)
4.2, 5.2, 6.3, 7.2, 7.3, 17.3x, 17.3y	15
1.3	4.4
4.3	37, 15
14.2, 14.3a	15, 9
15.2	27.6, 15, 7
18.1	15, 4.4
18.3b	15, 7
19.1	7

Several deductions could be made from the hybridisation patterns of the 15 clones digested with BamHI:-

1. the 15kb hybridising band in seven of the clones could be the gene/gene fragment attached to the 8.8kb right arm, or if the BamHI cloning site was conserved at both ends of the arms, the 15kb could be the inserted fragment containing the class I gene or gene fragment. The possibility of the gene or gene fragment attached to the left arm can be ruled out as the size of the left arm alone is 19.9kb.
2. in clone 19.1, the 7kb fragment was later shown to contain a class I gene in a Southern blot analysis of the clone. Clones 15.2 and 18.3b have a similar 7kb hybridization signal. There is also a 15kb fragment which could be the right arm attached to part of the class I gene. There is an additional 27kb fragment in 15.2 which could be the result of a partial digestion (incomplete digestion). If so, these 2 clones may be similar. The 27kb fragment could

be the left arm attached to the inserted class I gene fragment.

3. clones 14.2 and 14.3a are similar with a 15kb and 9kb bands. The 15kb would most probably be the right arm attached to the gene fragment while the 9kb could be the inserted fragment containing the class I gene.

4. there is a 4.4kb hybridizing band in 18.1 and 1.3. The complete class I gene could be encoded in such a fragment because the sizes of the genomic class I clones in mouse and man are between 3700bp and 5000bp (Strachan et al., 1984, Weiss et al., 1985, Watts et al., 1987 and Isamat et al., 1990). The additional 15kb fragment in 18.1, as in the other clones, could be the right arm attached to part of the gene.

5. in clone 4.3, there are two hybridizing bands of 37kb and 15kb. The 37kb most probably would be the left arm attached to a gene fragment, while the 15kb would be the shorter right arm attached to part of the class I gene. This suggest that the BamHI cloning sites at both arms were not conserved and a single BamHI site in the inserted fragment results in 37 and 15kb fragments.

Though some of the ethidium bromide-stained bands in the gel corresponded to the hybridisation signals, most of them did not seem to match. These complicated results were worrying. All 15 phage clones cleaved with BamHI gave the same 5 strongly ethidium bromide-stained bands but closer inspection of the gel photograph revealed other faint bands in some lanes. This was the first hint of contamination, i.e. there was DNA from more than one phage clone present in the samples. This suggested that the hybridizing DNA was only a small fraction of the total phage DNA present indicating that the phage were probably contaminated to a high degree. The result of the restriction digests suggest that if contamination is the problem, then the

source of contaminant must be in something used universally.

One possible source of the contaminant phage was the host strain (NM646). A plaque assay of the NM646 stock showed confluent lysis even without prior infection of any phage. This indicated the presence of a lytic phage in the NM646 used in preparation of the phage DNA. When a plaque lift was made of the contaminant and the hybond membrane probed with pBoLA-1, there were no hybridisation signals indicating that the contaminating phage did not contain any BoLA class I sequences. The NM646 must have been contaminated during the preparation of the 49 phages for DNA extraction, after the initial screening and amplification stages.

A new stock of NM646 was obtained which was free of phage contamination. Infection with phage 19.1, which at this time showed evidence of encoding a functional gene, was carried out on the uncontaminated NM646. Plaque lifts of the plates were made and probed with pBoLA-1. 7 strongly hybridising, well-separated plaques were picked into phage buffer. A second round of screening was carried out to ensure that the plaques were free of contaminant phage. A large scale preparation of phage 19.1 was made to purify DNA for restriction enzyme mapping.

The DNA from the uncontaminated 19.1 was digested with six enzymes, i.e. BamHI, EcoRI, HindIII, SacI, SalI and SmaI. SalI should give the upper limit of the insert as these sites flank the cloning site (Frischauf et. al., 1983). Also because the genomic DNA was inserted as Sau3A fragments into the BamHI site, digestion of the recombinant 19.1 with BamHI would provide information as to whether the BamHI sites were conserved at both ends. SmaI has a single site in both arms whilst HindIII has only one site in the right arm. There are no EcoRI or SacI sites in the arms (Frischauf et. al., 1983 and Sambrook et. al., 1989). The restriction enzymes

were selected on the basis of having none or very few sites in the arms to allow easier restriction enzyme mapping.

Single and double digestions with these six enzymes were made (figure 7a). The gels were blotted and the hybond membrane probed with pBoLA-1 (figure 7b), 5' end of pBoLA-1 and 3' end of pBoLA-1. A tabulated reading of the fragment sizes of both patterns was obtained using a digitiser with the MAPGEL program (prepared by John Coadwell, IAPGR, Cambridge). A restriction map of 19.1, based on the digestion and hybridisation patterns, is shown in figure 7c.

The class I gene in 19.1 is in a 7kb Bam HI fragment with the 5' end on the side of the right arm of EMBL3. There is approximately 2kb between the right arm and the 5' end of the gene and 12-13kb between the left arm and the 3' end of the gene.

FIGURE 7. *SOUTHERN BLOT ANALYSIS OF CLONE 19.1*

1μg of clone 19.1 DNA was digested with 2-3 units of the appropriate enzymes and run in a 1.5% agarose gel at 10V for 48 hours. DNA was transferred to nylon membranes and hybridisation was performed as described in section 3.2.3.

Figure 7a

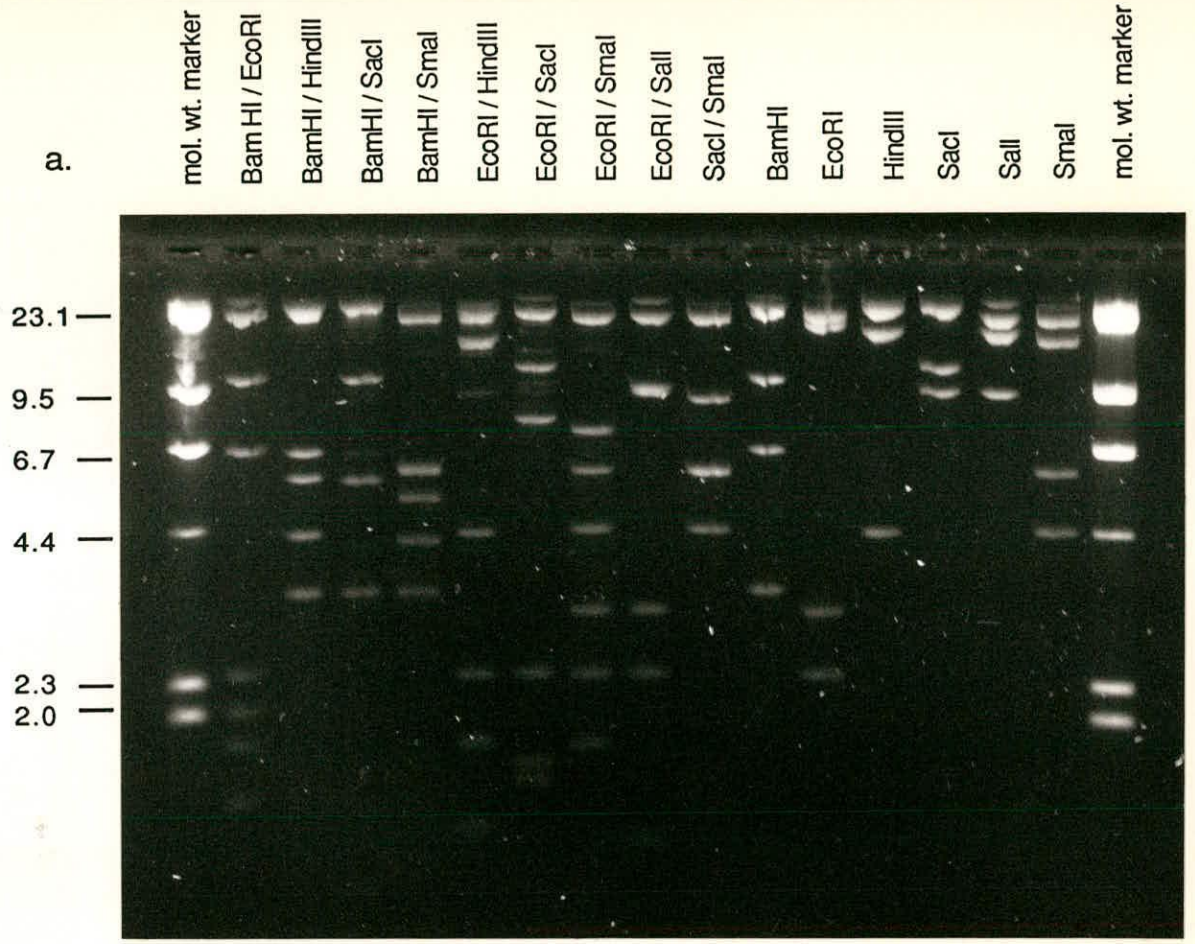
Restriction patterns of 19.1 with the six restriction enzymes stained with Et.Br.

Figure 7b

Hybridisation patterns of the figure 7a gel.

The molecular weight markers (HindIII digested λ DNA) are at both ends of the gel and the sizes are shown at the sides of the figures.

a.


mol. wt. marker
Bam HI / EcoRI
BamHI / HindIII
BamHI / SacI
BamHI / SmaI
EcoRI / HindIII
EcoRI / SacI
EcoRI / SmaI
EcoRI / SalI
SacI / SmaI
BamHI
EcoRI
HindIII
SacI
SalI
SmaI
mol. wt. marker
23.1
9.5
6.7
4.4
2.3
2.0

b.

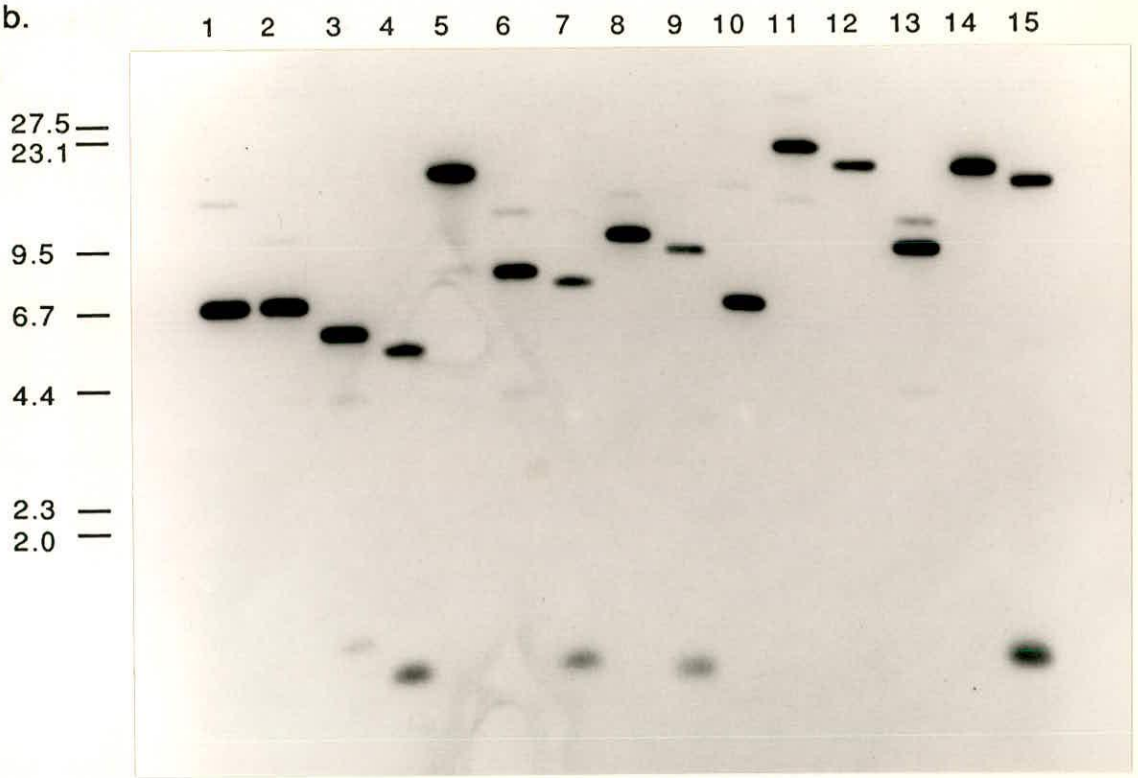
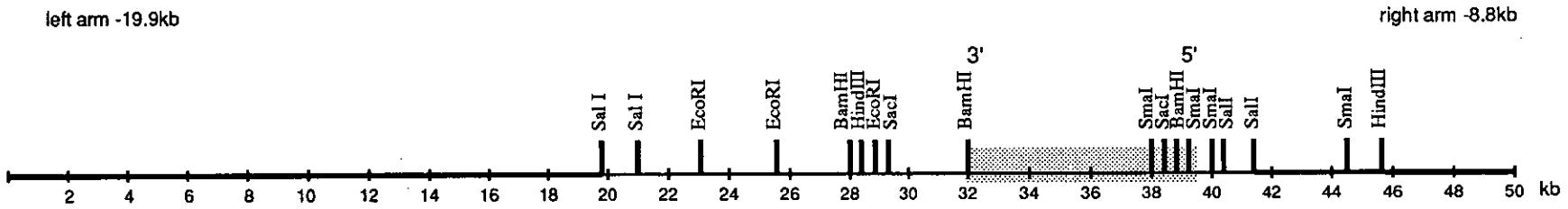

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
27.5
23.1
9.5
6.7
4.4
2.3
2.0

FIGURE 7c. *RESTRICTION MAP OF 19.1*

Clone 19.1 contains 21kb insert fragment. The 7kb BamHI fragment which hybridised to pBoLA-1 is shaded. The orientation of the gene encoded in 19.1 is as shown in the figure. EMBL3 vector is shown as a thick line while the insert is shown as a thin line.


left arm -19.9kb
right arm -8.8kb
3'
5'
Sal I
Sal I
EcoRI
EcoRI
BamHI
HindIII
EcoRI
SacI
BamHI
SmaI
SacI
BamHI
SmaI
SmaI
SalI
SalI
SmaI
HindIII
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50
kb

3.4 *DISCUSSION*

The contamination of the phage clones was not detected in the dot blot analysis as there was sufficient cloned DNA present to give a clear hybridization signal. After the dot blot analysis, the 15 clones were transfected individually into L cells for detection of expression (chapter 4).

Clearly, it would be useful to purify the other 14 phage clones from the contaminated stocks. Restriction enzyme analysis would allow comparison of these clones with 19.1. These results could then be used in a sequencing strategy to identify the gene or genes encoded by the 14 uncharacterised clones. If there is a w10 gene in one of these 14 clones, it could be used to compare with the w11 in clone 19.1. As mentioned earlier there is circumstantial evidence for both these genes to be at different loci (Al-Murrani et al., manuscript in prep). The sequences of the w11 and w10 genes would enforce the evidence for a second BoLA class I locus.

The class I gene in 19.1 is located in an ideal position not too near the left arm or the right arm. It would most probably have the necessary flanking sequences to allow regulated transcription and translation, i.e. the promoter sequences, 5' and 3' untranslated regions. Direct sequencing of the gene could begin with the 1kb SmaI fragment that hybridises strongly with the 5' end of pBoLA-1. Subcloning the 7kb BamHI fragment which probably contains the whole class I gene would be the initial goal. Restriction analysis of this fragment when cloned in the polylinker of pBS would provide information to direct the sequencing strategy. In the sequencing experiment in Chapter 6, a shotgun subcloning of Sau3A fragments which hybridised with pBoLA-1 was chosen, whilst the contamination problem was resolved in the light of lack of time available. Complete sequencing of this gene would be informative

on the organisation of the BoLA class I gene, i.e. an insight into class I gene structure and regulation.

CHAPTER 4

GENERATION OF MOUSE FIBROBLAST CELL LINE LTK⁻ EXPRESSING BOVINE MHC CLASS I GLYCOPROTEINS

4.1 INTRODUCTION

DNA-mediated gene transfer or transfection has proved valuable in the study of the structure and function of eukaryotic cell surface antigens and the genes that encode them. Barbosa and co-workers in 1982 first characterised functioning genes for HLA through the use of transfection, indirect immunofluorescence and fluorescence activated cell sorter (FACS).

Many methods of transferring genes into mammalian cells have been described. These include:

i) addition of DNA as a calcium phosphate precipitate (Graham and van der Eb, 1973; Wigler et. al., 1979a & b and Graham et. al., 1980)

ii) addition of DNA via electroporesis (Neumann et. al., 1982)

iii) membrane vesicle fusion with protoplasts (Schaffner, 1980 and Rassoulzadegan et al., 1982) or with liposomes (Straubinger et al., 1983)

iv) transfection with viral vectors (Sugden et. al. 1985)

v) micro-injection (Capecchi, 1980)

vi) DEAE-dextran (McCutchan & Pagano, 1968).

4.1.1 The calcium phosphate precipitation technique

The most common method for transfection with DNA is its addition as a calcium phosphate precipitate.

In 1973, Graham & van der Eb found that naked adenovirus DNA could be taken up much more efficiently by mammalian cells as a calcium phosphate precipitate. This technique was expanded when Wigler and co-workers in 1978

showed that the transfer, stable integration and expression of single copy eukaryotic genes was regularly possible using total cellular DNA as donor. They also demonstrated the possibility of cotransforming cells with two physically unlinked genes in 1979b. The co-transformed cells can be identified and isolated when one of the genes codes for a selectable marker, for example, the thymidine kinase gene (tk).

A mouse Ltk^- (fibroblast cells derived from a C3H mouse, $H2^k$) was obtained from J. Lang (Beatson Institute, Glasgow). These cells do not make the enzyme thymidine kinase. Ltk^- cells will therefore not survive in HAT medium since aminopterine inhibits *de novo* synthesis of DNA and the absence of thymidine kinase does not allow utilisation of hypoxanthine and thymidine in the salvage pathway. The transfection protocol is based on cotranfection of Ltk^- cells with a mixed calcium phosphate precipitate of DNA containing the gene of interest and a plasmid containing the thymidine kinase gene.

The transformants will be selected by their ability to grow in the selective medium, HAT (medium containing hypoxanthine, aminopterine and thymidine) (Szybalski et. al., 1962).

Many parameters affect the possibility and efficiency of the calcium phosphate precipitation technique.

Graham & van der Eb in 1973 showed the two essential roles of the calcium ions in the technique. First, in forming the DNA-calcium phosphate coprecipitate and second, in the uptake of DNA into the cells during incubation at 37°C.

Graham and co-workers in 1980 showed that the method used to form the DNA-calcium phosphate coprecipitate also influenced the yield of transformants. They found that the addition of DNA in 250 mM $CaCl_2$ dropwise to 2 x HBS with

agitation by bubbling as described by Wigler and co-workers in 1979a was about 10 fold more effective than simply mixing the two solutions.

Other important parameters that have to be determined before a transfection are the pH of the buffer in which the DNA is diluted, the DNA concentration, the density of the recipient cells, the length of exposure of cells to the DNA-calcium phosphate precipitate and carrier DNA size.

The DNA-calcium phosphate coprecipitate binds to the recipient cells and the DNA is taken up. Most of the DNA is rapidly degraded without ever reaching the nucleus (Loyter et. al., 1982). DNA which reaches the nucleus may be transcribed so that transient expression maybe observed for certain genes (Barbosa et. al., 1982) usually after 48 hours. Some of the DNA fragments reaching the nucleus may also undergo recombination (Anderson et. al., 1982). For stable expression to occur the DNA must be integrated into a chromosome. Integration appears to occur after ligation of fragments into an apparently random chromosome site (Perucho et. al., 1980 and Robins et. al., 1981).

Transfection is combined with flow cytometry to study genes which code for cell surface antigens.

4.1.2 Gene expression studies

Cell surface antigens can be labelled by indirect immunofluorescence with a monoclonal antibody and a fluorescent conjugated second antibody. The labelled cells are detected and isolated on the fluorescence activated cell sorter (FACS). Levels of expression can be enriched through various sorting procedures on the FACS until a 100% expression is obtained.

In a transfection experiment, only the class I heavy chain is introduced into

the recipient cell. It has been shown that heterologous association between mouse $ß_2$m and HLA class I heavy chains is sufficient for cell-surface expression (Brodsky & Parham, 1982, Barbosa et. al., 1982 and Lemonnier et. al., 1982).

It has also been established that free $ß_2$m may exchange with $ß_2$m associated with class I heavy chains expressed on the cell surface (Hyafil and Strominger, 1979, Schmidt et. al., 1981, Kefford et. al., 1984 and Bernabeu et. al., 1984).

In an experiment to study the altered structure of HLA class I heavy chains when associated with the mouse $ß_2$m, transfected cells were adapted to grow in either bovine, murine or human serum-supplemented medium prior to analysis with polymorphic and monomorphic HLA class I-specific mAbs (Ferrier et. al., 1985). They found a decrease or loss of expression of these molecules when the heavy chains were associated with mouse $ß_2$m but there was restoration of expression when it was associated with the human $ß_2$m. If an increase is as a result of the $ß_2$m exchanges, this would tie in with the amino acid sequence analysis of $ß_2$m of the three species. The human $ß_2$m is more homologous to the cow $ß_2$m than to the mouse $ß_2$m (Groves and Greenberg, 1982).

In subsequent studies (Kahn-Perles et. al., 1987 and Mierau et. al., 1987), the w6/32 antibody (anti-human class I mAb) was found to cross-react in a polymorphic fashion with cultured murine cells. These reactions are entirely due to murine class I molecules which have exchanged their autologous $ß_2$m for bovine $ß_2$m that is present in the culture medium.

Exon shuffling (i.e. *in vitro* construction of hybrid class I genes containing exons from two genes) coupled with transfection experiments enable the mapping of functionally important areas of the class I glycoproteins. Evans et al. (1982b) found in their experiments involving exon shuffling between H-2D^d and H-2L^d, the

polymorphic serological determinants recognised by mAb are located on the N, C1 and C2 domains of these H-2 antigens. While Murre et al. (1984) in a similar experiment, found that the determinants recognised by allospecific cytotoxic T lymphocytes are located on the N and C1 domains.

Sodoyer et. al., in 1986 created hybrid class I genes by exon shuffling to study the individual contributions of the first two external domains of the HLA-B7 heavy chain to the expression of allele specific (B7) and locus specific (B) antigenic determinants. They showed the first domain of the HLA-B7 heavy chain controls the expression of most of the allele-specific determinants whereas the second domain is responsible for the expression of the HLA-B locus specific antigenic determinants.

Another interesting question in gene expression is the role of the intervening sequences or the introns. There are evidences of regulatory elements in the introns, i.e. sequence elements that direct specific DNA rearrangements (Tonegawa, 1983), that regulate transcription (Queen & Baltimore, 1983) or improve transcription efficiency in transgenic mice (Brinster et al., 1988). Buchman and Berg (1988) observed the dependence of rabbit ß-globin cDNA on introns for expression. Therefore it appears from the literature that stable expression is enhanced with the presence of introns.

4.2 TISSUE CULTURE MATERIALS AND METHODS

4.2.1 Cells and culture media

(a) *Recipient cells*

Ltk$^-$ cells were the recipient cells for the DNA-mediated gene transfer of the bovine class I genes (see 4.1.1) and the cells were maintained in culture in 1 x Dulbecco's modified Eagles medium (DMEM 10 x, Gibco-BRL Ltd.,

Paisley, Scotland) supplemented with 10% heat-inactivated foetal calf serum (Globepharm Ltd., Surrey); L-glutamine at 2 mM final concentration (200 mM or 100 x), Gibco-BRL Ltd.), 5mls of sodium bicarbonate (7.5%, Gibco-BRL Ltd.) was added to the medium at a pH of 6.8 to 7.2 and gentamycin at 50 μg/ml final concentration (10mg/ml, Gibco BRL Ltd.). Ltk^- cells were passaged using a 1:20 split ratio just prior to confluence (every 3 days). The adherent Ltk^- cells were harvested from the flask by incubating with 1ml 0.02% EDTA solution (Sigma Chemical Company, USA) in a 75cm^2 flask and 0.5ml in a 25cm^2 flask for 5 minutes at 37°C followed by a sharp tap. Cells were reseeded in fresh medium in a 75cm^2 flask (Nunc, Gibco-BRL Ltd). The flasks were left in the CO_2 incubator at 37°C with the cap loosely on. The selection medium was as above but 1 x HAT was added to select for the transformants (HAT Supplement 50 x, Gibco-BRL Ltd.).

(b) *Bovine peripheral blood mononuclear cells (PBM)*

These cells were required for the testing of the mAb and the second-stage FITC-conjugated rabbit anti-mouse IgG reagent (RAMIg; Dakopats Glostrup, Denmark) in the indirect immunofluoresence test.

20ml of blood was collected in ACD siliconised tubes (80 mM disodium hydrogen citrate and 140 mM D Glucose) to prevent clotting. The blood was layered onto 15ml of lymphoprep (Nycomed, Birmingham, England) in a sterile siliconised tube. The cells were centrifuged at 3000rpm for 25 mins at room temperature. Most of the plasma were removed and the buffy coat was taken into a sterile 30ml universal. The cells were washed three times with PBS at 1500 rpm for 10 mins. The live cells were counted using a

haemocytometer(e).

(c) *Preparation of frozen L cells*

Cells were removed from $75cm^3$ flask culture flask just prior to confluence and pelleted by centrifugation at 1000rpm for 5 minutes at 4°C. The cells were counted using a haemocytometer(e). The pellet was resuspended in ice cold freezing solution A (containing 50% FCS, 50% DMEM). An equal volume of ice cold freezing solution B (containing 20% DMSO in FCS) was added dropwise with gentle swirling to a final concentration of 1×10^7 cells. The cells were aliquoted into freezing vials and stored at -70°C overnight before placing under liquid nitrogen.

(d) *Thawing frozen L cells*

The freezing vials were removed from liquid nitrogen and thawed rapidly in the waterbath at 37°C. The cells were placed in warm medium pelleted by centrifugation and resuspended in 1ml warm medium. They were then seeded into a $75mm^2$ flask containing 10ml of medium and left in the 37°C CO_2 incubator with the cap loose.

(e) *Counting cells using a haemocytometer*

The haemocytometer (Weber Scientific Ltd, Teddington, England) enables the cells within a defined volume to be counted. This count in the presence of trypan blue (Sigma Chemical Co., USA) enables live cells to be distinguished from dead cells. The haemocytometer is a glass microscope slide with a central 5 x 5 grid engraved into it. When a glass coverslip is positioned over

the grid and the cells added a predetermined volume lies over the grid. By counting the cells and multiplying by the dilution factor and 1 x 10^4, the number of cells per ml can be determined.

4.2.2 DNA-mediated gene transfer and HAT selection

Phage-clones bovine class I genes were introduced into Ltk$^-$ cells using the calcium phosphate precipitation method. This technique involves the formation of a complex consisting of DNA (phage DNA carrying the gene of interest, pTk, the plasmid carrying the thymidine kinase gene as the selection marker and a high molecular weight mouse embryo DNA) bound to a calcium phosphate microprecipitate.

The phage DNA was prepared as described in 3.2.1. Before transfection, the phage DNA was ethanol-precipitated under aseptic conditions and resuspended in sterile TE pH8. pTk1 plasmid DNA (Lang et. al., 1983) and high molecular weight mouse embryo DNA from C3H mice was kindly provided by Keith Ballingall, Moredun Institute. The pTk1 plasmid was ethanol-precipitated prior to transfection.

A flask of rapidly growing Ltk$^-$ cells was split into 25cm^3 flasks at a density of 5 x 10^5 cells per flask 24 hours before the transfection experiment. The flask were left in the CO_2 incubator at 37°C with loose caps.

In one set of sterile bijoux vials:

a) 403μl sterile distilled water (calculated to give a final volume of 500μl).

b) 30μl mouse embryo carrier DNA (1μg/3μl).

c) 100ng of thymidine kinase plasmid pTk1.

d) 6μg of phage DNA containing bovine class I genes was added and each vial was vortexed. Then 60μl of $CaCl_2$ (appendix 3) was added and the vial was

flicked to ensure mixing. The vial was left for 2-3 hours at room temperature to ensure complete mixing.

In another set of sterile bijoux vials, 0.5ml of sterile 2 x HBS (appendix 3) was added. To these HBS vials, the DNA-$CaCl_2$ solution was added dropwise with agitation by bubbling. The tubes were left at room temperature for 20-30 minutes for the microprecipitate to form. A good precipitate is one that is not obvious under 10 x magnification. The precipitate was added directly to the culture media of the previously prepared Ltk^- cells. The flasks were left in the CO_2 incubator with the caps loose for 15 hours.

The precipitate was removed by washing the cells gently in warm phosphate-buffered saline (PBS) twice and fresh medium was added. The flasks were again left at 37°C for 24 hours. At this stage the cells should be at near confluence. The medium was again removed and replaced by the HAT selective medium. The media was replaced every 2 days initially and every 3-4 days subsequently as the colonies developed.

Colonies began to appear after 4-7 days and these were left to develop for two weeks before they were harvested to be kept as a stock in $25cm^3$ flask and to be sorted for class I expression in $75cm^3$ flask. . When the cells in the $75cm^3$ flask are near confluence, they are labelled with the mAb and second-stage reagent in the indirect immunofluoresence assay.

The cotransfection protocol is:

Day 0

Seeding of Ltk$^-$ cells at 5 x 10^5 cells/flask and each transfection is done in duplicate

Day 1

1. DNAs (mouse embryo DNA + DNA phage clones + tk plasmid) + $CaCl_2$, mixed and incubated for 2-3 hours.
2. Add to 2 x HBS and incubate for 30 minutes. Transfect for 15 hours.
3. PBS wash and change of media.

Day 2

Observe

Day 3

Apply selection pressure i.e. in 1 x HAT, only cells with the tk gene will grow

After 14 days

Treat with EDTA and seed into flasks:

1. Reserve stock
2. Sort on the FACS

4.2.3 *Indirect immunofluorescence technique*

The HAT surviving colonies in each flask were pooled for the indirect immunofluorescence technique to get as many cells as possible for the sorting.

Only one mAb, IL-A88 (monomorphic mouse anti-bovine class I mAb, ILRAD, Nairobi) was used to select for class I cell surface antigen from the transformed cells. This mAb has been used successfully in detecting class I expression in ILRAD (Toye et. al., 1990). The fluorescein-isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulin is used as the second stage reagent. As a result of the preliminary experiments on bovine PBM, it was decided that IL-A88 was used at 1/500 and the second-stage at 1/160.

The transfected cells were removed from $25cm^3$ tissue culture flasks using 0.5ml of 0.02% EDTA solution. They were washed once in Earles balanced salt solution (appendix 3). The cells were pelleted by centrifugation at 1000rpm for 5 mins at 4°C. They were resuspended 100μl of IL-A88 for 40 minutes at 4°C with occasional agitation. Unbound antibody was removed by washing three times in EBS. After the final wash, the pellet was resuspended in 100μl of FITC conjugated rabbit anti-mouse IgG. These were again incubated for 40 minutes at 4°C with occasional agitation. Unbound antibody was washed off three times with EBS and the cells resuspended in 500μl EBS.

Before FACS analysis and sorting, 100μl propidium iodide (PI) (200μg/ml, Sigma Chemical Co. Ltd., Poole, England) was added in order to differentiate the dead cells. Dead cells were shown to pick up fluorescent proteins non-specifically. Such cells are eliminated from analysis and sorting by staining with PI, which penetrates only cells with damaged membranes and intercalates in DNA rendering the cells brightly red fluorescent (Yeh et. al., 1981). Therefore the red PI-stained cells

can be gated out.

Another parameter to consider is autofluorescence - the inherent fluorescence of a cell. Cultured cells tend to be more autofluorescent than 'fresh' cells (Parks et. al., 1979). The autofluorescent molecules are normal cell constitutes like flavins (Benson et. al., 1979) and cytochromes which tend to be found in greater quantity in larger cells. The effects of autofluorescence can be minimised by changing to longer wavelength excitation (Parks et. al., 1986).

4.2.4 Analysis by flow cytometry (Becton Dickinson FACscan)

One of the uses of flow cytometry is to detect, quantitate and isolate surface molecules coded by specific genes.

The flow cytometer utilises an argon-ion laser beam (15mA, 488nm) through which a single cell suspension is directed. As cells pass through the laser beam, scattered light is detected. This is electronically coverted by the 'consort 30' software package (Becton Dickinson) to provide information on five parameters necessary for analysing the cells. They are forward scatter (FSC) which is a measure of cell size, side scatter (SSC) which is a measure of the internal complexity of a cell, fluorescence 1, 2 and 3 (FL1, FL2 and FL3) which are three measures of the amount of fluorescent dye in cells (when a dye is coupled to a specific antibody, it indicates the concentration of an antigen). Routinely FL1 measures blue-green fluorescence, FL2 measures orange fluorescence and FL3 measures red fluorescence.

The second-stage is conjugated to fluorescein isothiocyanate (FITC) and fluorescein, with an absorption maximum at 495nm, is matched well to the 488nm line obtained from the argon ion laser. Therefore the use of an FITC-conjugated second antibody allows the primary antibody to be identified and this enables the

MHC class I expressing transformants to be separated from the population of pooled transformed cells on the basis of surface fluorescence

The FACScan requires calibration to define acquisition gates or live gates for the population. Untransformed Ltk$^-$ cells labelled with IL-A88 and RAMIg were used to calibrate the cytometer. Once the gates are defined, it is possible to collect a large amount of data for analysis.

Once information on all five parameters was accumulated by cytometer for a set number of cells (in this case 10,000 cells), it was analysed immediately on consort 30 or stored to disc for subsequent analysis using the Becton Dickinson 'lysis' software. The data can be analysed graphically in the form of dot plots or histograms with logarithmic displays. It has been shown that any statistical test that assumes normal distributions is better performed on the log transformed data (Heath, 1967).

The isolation or sorting of the L cells expressing bovine MHC class I molecules was achieved using a fluorescent activated cell sorter (Becton Dickinson, FACS IV) and it operates on the same principal as the FACScan.

In a sterile sort, analysis of the parameters is done immediately. The sorting gates were arranged to define both positive and negative populations.

When the fluorescent stained cells in aqueous suspension are introduced into the sorter chamber and are illuminated by the laser beam, the instrument breaks up the cell suspension into individual droplets each containing a single cell. Drops containing cells to be sorted are charged as they separate from the cell suspension. Empty drops or drops containing unwanted cells are not charged. As the drops pass between two charged deflection plates, they will be deflected into appropriate collection tubes containing culture media.

These positive cells were grown till near confluence in a $25cm^3$ flask to be sorted a second time. This process of resorting is repeated until a population of cells that expresses ~100% of bovine MHC class I molecules is obtained.

4.2.5 Cytospin preparation

Cytocentrifugation sediments cells from suspension onto a vertical microscope slide as the specimen's suspension medium is absorbed by a filter card. Cytocentrifugation is performed in a specially designed bench-top centrifuge with a unique rotor and sample chamber that together constitute a cytocentrifuge (Shandon cytospin 2, Shandon Scientific Ltd.).

Cell suspensions are centrifuged in special plastic sample chambers. Each sample chamber assembly comprises a sample chamber, special filter card and glass microscope slide held in position by special stainless steel cytoclips.

Slides are precleaned in ethanol and filters are prewetted with 100μl of EBS by spinning at 800rpm for 4 minutes.

Prior to this cells were labelled as in the indirect immunofluorescence technique but using a higher concentration of IL-A88 and RAMIg (IL-A88 at 1/200 and RAMIg 1/100).

100μl of labelled cells (5 x 100^5 cells) are put into the sample chamber and spun for 6 minutes at 350rpm. Slides are then removed to air dry in the dark for 10 minutes. The slides are then mounted in UV inert mounting medium (BDH) and examined using a Leitz ortholux microscope with the UV light and an appropriate barrier filter.

4.2.6 *Northern blot analysis*

This technique involves the identification of specific messenger RNA sequences immobilised on a hybond filter by hybridisation to ^{32}P-radiolabelled DNA probe. This technique was used to identify mRNA transcribed in BoLA class I - expressing cells.

(a) *Preparation of total RNA by RNAzol (Biogenesis Ltd., 1987).*

The transfected cells were removed from flasks with 0.02% EDTA solution. They were washed once in PBS. The cells were pelleted by centrifugation at 1000rpm for 5 minutes at 4°C and counted using the haemocytometer (4.2.1). 200μl of RNAzol was used to lyse 10^6 cells in a sterile eppendorf tube. The lysate was pipetted a few times to ensure solubilisation of RNA. To the lysate 1/10 volume of chloroform was added and each tube was shaken vigorously for 15 seconds. The tubes were then placed on ice for 15 minutes. The suspension was then centrifuged for 15 minutes at 4°C. The RNA is in the upper aqueous phase, the lower phenol-chloroform phase is discarded. The upper aqueous phase is transferred to a new sterile eppendorf and an equal volume of isopropanol is added. The samples are incubated between 45 - 60 minutes at -20°C. They are then centrifuged for 15 minutes at 4°C, 12000rpm. The white RNA pellet is visible at this stage. The pellet is washed twice with 1ml of ice cold 75% ethanol by vortexing and subsequent centrifugation for 8 minutes at 4°C, 12000rpm. The pellet is then dried down under vacuum for 5 minutes. It is important not to let the RNA dry out completely as this will greatly decrease its solubility. The RNA pellet is dissolved in TE pH8 and left overnight at 4°C. The OD 260/280 ratio

should be greater than 1.9, for a pure RNA solution. The RNA samples are stable when stored at -70°C.

(b) *Electrophoresis of RNA*

The total RNA prepared as described above was then separated by electrophoresis on a denaturing 1.5% agarose gel. 4.5 gram of agarose was dissolved in 210ml of autoclaved water by heating. The agarose solution was left to cool to 60°C and 30ml of 10 x MOPS (appendix 1) and 60ml of 38% formaldehyde (FSA Laboratory Supplies, England) was added. After mixing, the solution was poured into a gel tray and left to set for at least an hour. 10-30μg of total RNA was dissolved in 12μl of TE pH8 and added to 25μl deionised formamide, 5μl 10 x MOPS, 8μl 38% formaldehyde. The sample mixture was heated at 65°C for 10 minutes with 3μl of sterile loading buffer (appendix 1) and 2μl of ethidium bromide (1mg/ml) was added prior to introducing the samples into the gel. Electrophoresis was carried out in 1 x MOPS electrophoresis buffer at 30V (constant voltage) at room temperature for approximately 18 hours.

The RNA on the gel can be transferred directly to the hybond membrane without any pretreatment but the gel could be soaked in autoclaved distilled water to remove any remaining formaldehyde. The transfer is as described for the Southern transfer (section 3.2.3). The filter was air-dried and RNA covalently bound in the UV transilluminator. Labelling of the filter is as described in section 3.2.3.

4.3 PRELIMINARY EXPERIMENTS

A series of preliminary experiments were carried out to obtain efficiency of transfection. These were:

1. to check the quality of precipitate of the two stock solutions, $CaCl_2$ and HBS at the various pH's.
2. to determine the density of seeding, i.e. to achieve confluence just before applying selection pressure.
3. to determine the duration of transfection.
4. to titrate the plasmid pTk containing the thymidine kinase selection gene.
5. to titrate the mAb and the second stage FITC reagent for the indirect immunofluoresence analysis.

4.4 RESULTS

4.4.1 Preliminary experiments

(1) *To check the quality of precipitate of the two stock solutions at the various pH.*

One of the crucial factors for obtaining efficient transformation is the pH of buffer used for the calcium phosphate precipitation. Three levels of pH of the 2 x Hepes Buffered Saline (HBS) were tested : pH6.95, pH7 and pH7.1. The experiment was carried out as follows. In a bijoux vial, 440μl H_2O and 60μl $CaCl_2$ were mixed. This was left for 2-3 hours to allow complete mixing. This was done in triplicate. In 3 separate bijoux vials, 0.5ml of the three different pH 2 x HBS was added. To each of the 2 x HBS solution, the DNA-$CaCl_2$ solution was added dropwise to 2 x HBS with agitation by bubbling (as described by Wigler & co-workers in 1979a). The tubes were

left for 30 minutes for the microprecipitate to form. It was observed that the 2 x HBS at pH6.95 gave the finest precipitate after 30 minutes, 6 hours and 15 hours. Therefore 2 x HBS at pH6.95 was selected as the stock solution.

(2) *To determine the density of seeding, i.e. to achieve confluence just before applying selection pressure.*

It is important to determine the density of seeding as the cells grow at a different rate in the presence of calcium phosphate precipitate and when there is foreign uptake in the cells. It is ideal to have as many cells (near confluence or at confluence) as possible before applying selection pressure. This is to increase the number of the transformed cells. Various densities of cells were seeded 1 x 10^5 cells/flask, 5 x 10^5 cells/flask, 1 x 10^6 cells/flask and 5 x 10^6 cells/flask. The transfection experiment is as described (4.2.2) and it was found that 5 x 10^5 cells/flask was the ideal density of seeding.

(3) *To determine the duration of transfection*

This is another important parameter. In the literature, various durations of transfection have been used, 24 hours (Cole et. al., 1989 and Kavathas and Herzenberg, 1986), 4 hours (Wigler et. al., 1979a). It is intended to leave the precipitate as long as possible to allow as many of the cells to take up the precipitate but it is also necessary to check the density of the precipitate. If the precipitate is too dense, it is difficult to remove all of it and also the cells round up if the precipitate is too dense. A titration of 4 hours, 6 hours, 15 hours and 24 hours was carried out, and 15 hours was the best as the precipitate was easily washed off with warm PBS and most, if not all of the cells are still adhering to the surface of the flask.

(4) *To titrate the plasmid pTk1 containing the thymidine kinase selection gene*

Various amounts of pTk1 were used to determine the concentration of the plasmid required to form 50 - 100 colonies in a $25cm^2$ tissue culture flask seeded initially with 5×10^5 Ltk^- cells. Wigler et. al., in 1979b described the possibility of cotransfecting a selectable gene with a non-selectable gene present in excess. In that case transfectants expressing the selected marker frequently expressed the non-selected gene as well. The aim of this titration is to use as a low concentration of pTk but enough to form 50-100 HAT stable colonies. Stock pTk1 plasmid DNA at $1\mu g/\mu l$ was diluted in TE buffer and transfection was carried out with different amounts 10ng, 50ng, 100ng, 250ng, 500ng and $1\mu g$. Colonies were counted after 14 days of HAT selection. When there was more than 250ng of pTk1, the cells were at confluence and 100ng resulted in the formation of 78 colonies, which is ideal. The 50ng and 10ng amounts both gave 20 and 3 colonies respectively. Therefore a pTk1 concentration of 100ng per transfection reaction was used.

(5) *To titrate the monoclonal antibody and the second stage FITC reagent for the indirect immunofluorescence analysis*

Bovine PBM (4.2.1) cells were used to titrate the mAb, IL-A88. IL-A88 (IgG2a) reacts with a monomorphic determinant on bovine class I MHC molecules and it does not recognise mouse class I antigens expressed by L cells (Toye et. al., 1990). The second stage antibody used is the FITC-conjugated rabbit anti-mouse IgG reagent. IL-A88 was used at a dilution of 1/500, 1/1000 and 1/2000 and the second stage used was 1/40, 1/80, 1/160, 1/320 and 1/640. The various possible combinations of IL-A88 with the 2nd stage were analysed on the FACScan. The best results were obtained when

IL-A88 was at a dilution of 1/500 and RAMIg at a dilution of 1/160. There was excellent specific binding of the 2nd stage to the mAb with little detectable non specific binding properties. Therefore throughout the sorting for 100% expression, IL-A88 was used at 1/500 and RAMIg at 1/160 in the indirect immunofluorescence test.

4.4.2 Generation of mouse Ltk⁻cell expressing bovine class I glycoprotein - Transfection of the 15 lambda genomic clones

The 15 lambda genomic clones which hybridised with both the 5' and 3' ends of pBoLA-1 presumably would encode complete genes. They were selected for transfection experiments to generate L-cell lines expressing bovine MHC class I glycoproteins. They were transfected individually (as described in section 4.2.2) and after the 14 days HAT selection, the colonies were harvested and grown to near confluence in $75cm^3$ flask. They were labelled with IL-A88 and RAMIg for the FACS analysis and sorting.

Clone 19.1 gave a 5.4% expression level while clones 18.1, 7.2, 6.3 and 4.2 showed a 1-2% expression level. The other 10 clones gave an expression level of below 1%. Ltk^- cells transfected with only the carrier DNA and pTk1 was used as the negative control in sorting for the class I glycoproteins in the transformants. This is called Ltk^+ (figures 8a & 9a).

The 5 sorted clones were grown in $75cm^3$ flasks till near confluence and sorted a second time. There was an increase in expression only for clone 19.1. There was no increase in expression for the other 4 sorted clones. Therefore purification of the bovine class I glycoprotein was carried out for clone 19.1. A total of 3 rounds of sorting resulted in a 100% expression (figures 8 & 9). In the initial sort, 5.4% of

FIGURE 8. *FACS ANALYSIS OF PROGRESSIVE SORTING OF THE TRANSFECTED MOUSE Ltk⁻ CELLS WITH DNA FROM PHAGE 19.1*

The figures show relative fluorescence of transfected cell populations stained with the following antibodies.

8a. Control Ltk^+ cells stained with mAb ILA-88 and FITC conjugated RAMIg.

8b. Transfected Ltk^- cells similarly stained. 5.4% of the cells fell within the gates indicated and were sorted and cultured to subconfluence.

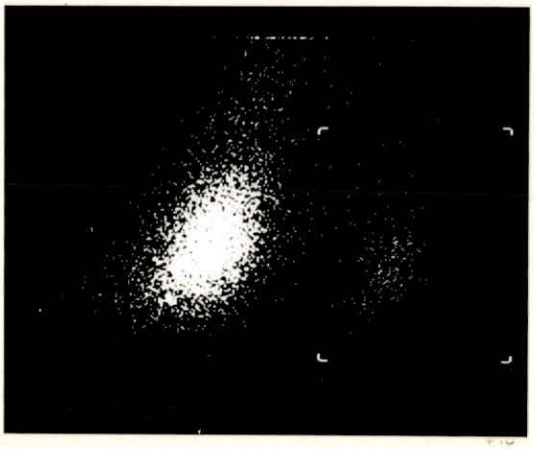
8c. Transfected cells after one round of sorting. 51.9% of the cells fell within the gates indicated and were similarly sorted and cultured.

8d. Transfected cells after two rounds of sorting. 80% were positively stained.

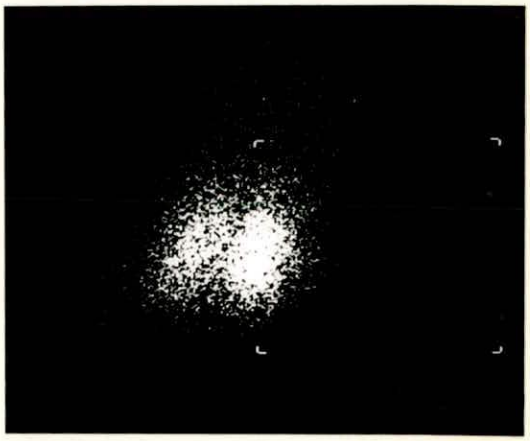
8e. Transfected cells after the final (third) round of sorting. 100% were positively stained.

a.

b.



c.



d.

e.

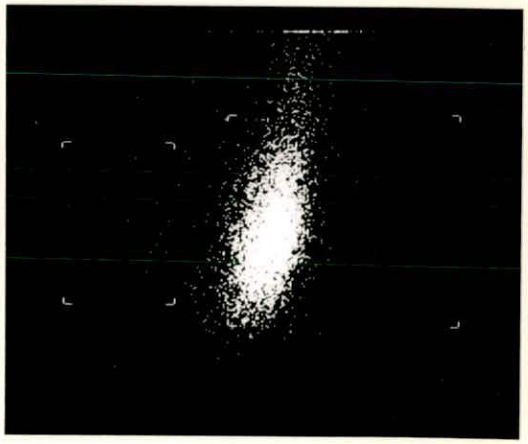
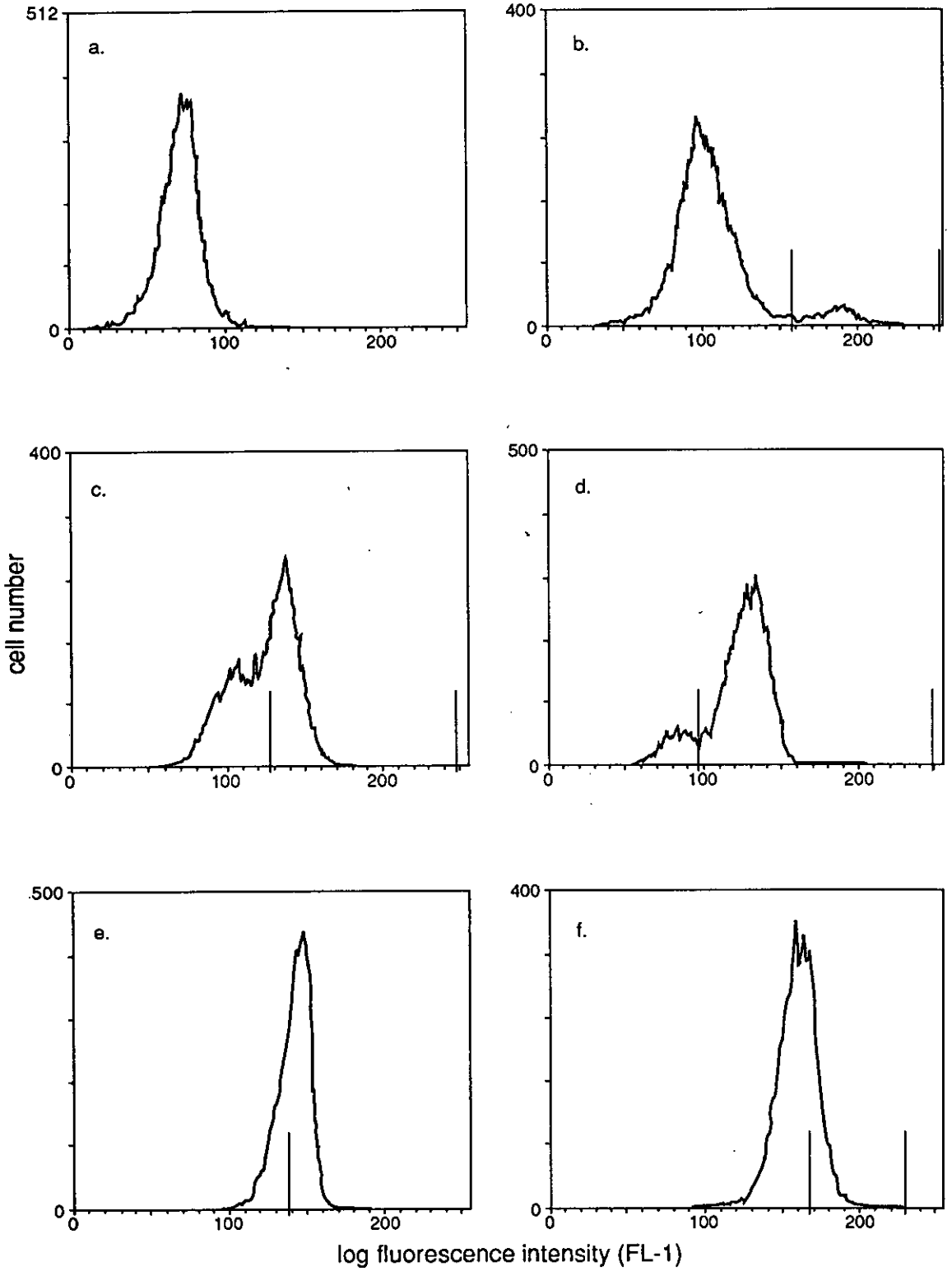


FIGURE 9. *HISTOGRAMS OF FACS ANALYSIS OF THE STAGES OF SORTING THE CLONE 19.1 TRANSFECTED POPULATION*

a - e in this figure correspond to a - e in figure 8. Figure 9f shows the distribution of positive staining on brightest cells sorted from Figure 9e. The brightest of these cells were further sorted and the cells maintained as a stock of transfectants. The population selected at each sorting was that between the vertical bars of the histograms.


512
a.
0
0
100
200
400
b.
0
0
100
200
400
c.
0
0
100
200
500
d.
0
0
100
200
500
e.
0
0
100
200
400
f.
0
0
100
200
cell number
log fluorescence intensity (FL-1)

the cells were BoLA class I positive (figures 8b & 9b). These cells represented approximately 60,000 cells. They were sorted and grown for 10-14 days in culture. The percentage of class I positive cells was increased to 51.9% in the second sort (figures 8c & 9c). The cells represented approximately 144,000 cells and was again grown till near confluence in culture. In the third sort, the percentage had increased to 80% and this represented approximately 250,000 cells (figures 8d & 9d). After growing in culture again, the cells were approximately 100% positive in the fourth sort. The brightest 50% (figures 8e & 9e) were sorted for the stock (figure 9f). The L-cell line carrying the expressed bovine class I gene from clone 19.1 is called Ltk 19.1. Ltk 19.1 was grown in various $75cm^3$ flask and frozen immediately as a stock. Ltk 19.1 was also kept in culture for further experiments and, during various intervals, it was analysed by the indirect immunofluorescence assay. To date it maintains the 100% expression. Therefore Ltk 19.1 is a stable transfectant.

4.4.3 Cytospin of Ltk 19.1

A cytospin preparation (section 4.2.5) of Ltk 19.1 and Ltk^+ provided a visual proof of the class I surface molecules on Ltk 19.1 (figure 10). There are some non-specific binding with Ltk^+ but this is acceptable as background level because a high dilution of both first and second reagent were used to label the cells.

4.4.4 Northern blot analysis

Northern analysis (section 4.2.6) of 19.1 at the four stages of sorting and clones 18.1, 7.2, 6.3 and 4.2 after the first sort was carried out (figures 11 and 12). Total RNA from bovine PBM was used as the positive control and total RNA from Ltk^- and Ltk^+ as the negative controls. The probe used in the Northern blot was

FIGURE 10. *FLUORESCENT LABELLING OF CYTOSPIN PREPARATIONS OF CONTROL AND TRANSFECTED CELLS*

Figure 10a

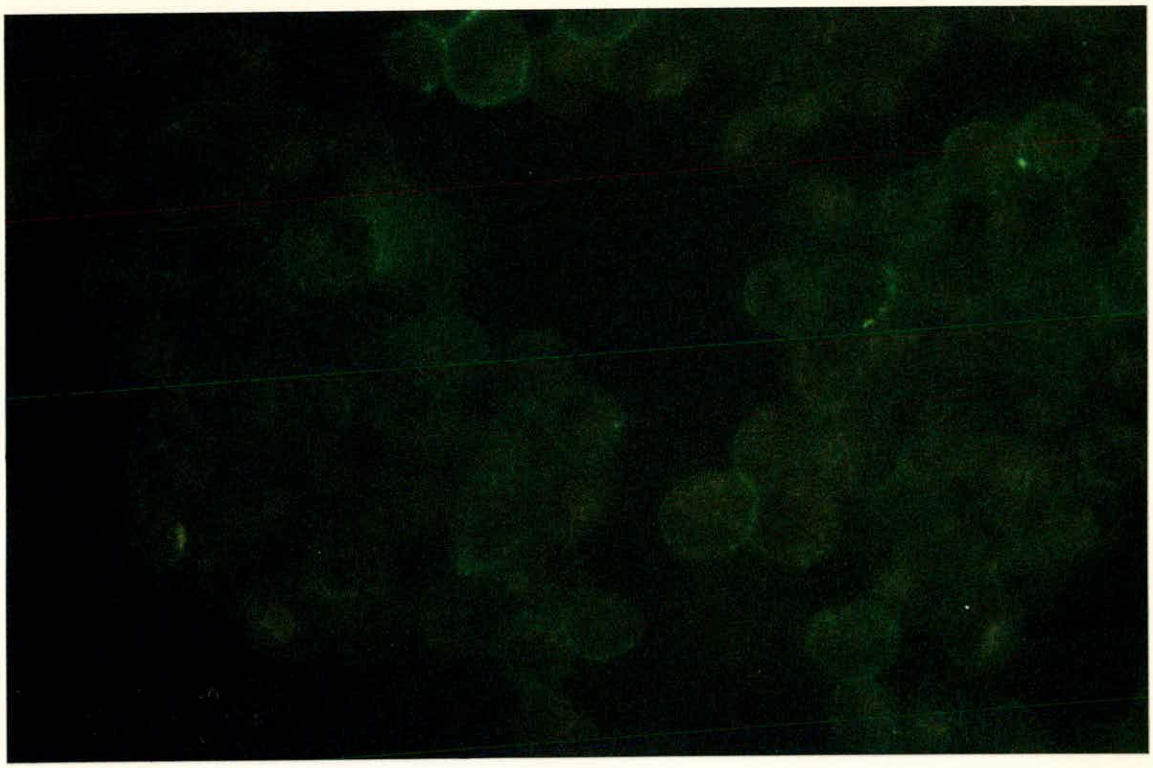
Cytospin preparation of a control Ltk$^-$ L cell line.

Figure 10b

Cytospin preparation of the transfected cell line, Ltk 19.1.

The Ltk$^-$ and Ltk 19.1 cells were labelled with ILA-A88 (1/200 dilution) and RAMIg FITC (1/100 dilution) and observed by fluorescence microscopy.

a.



b.

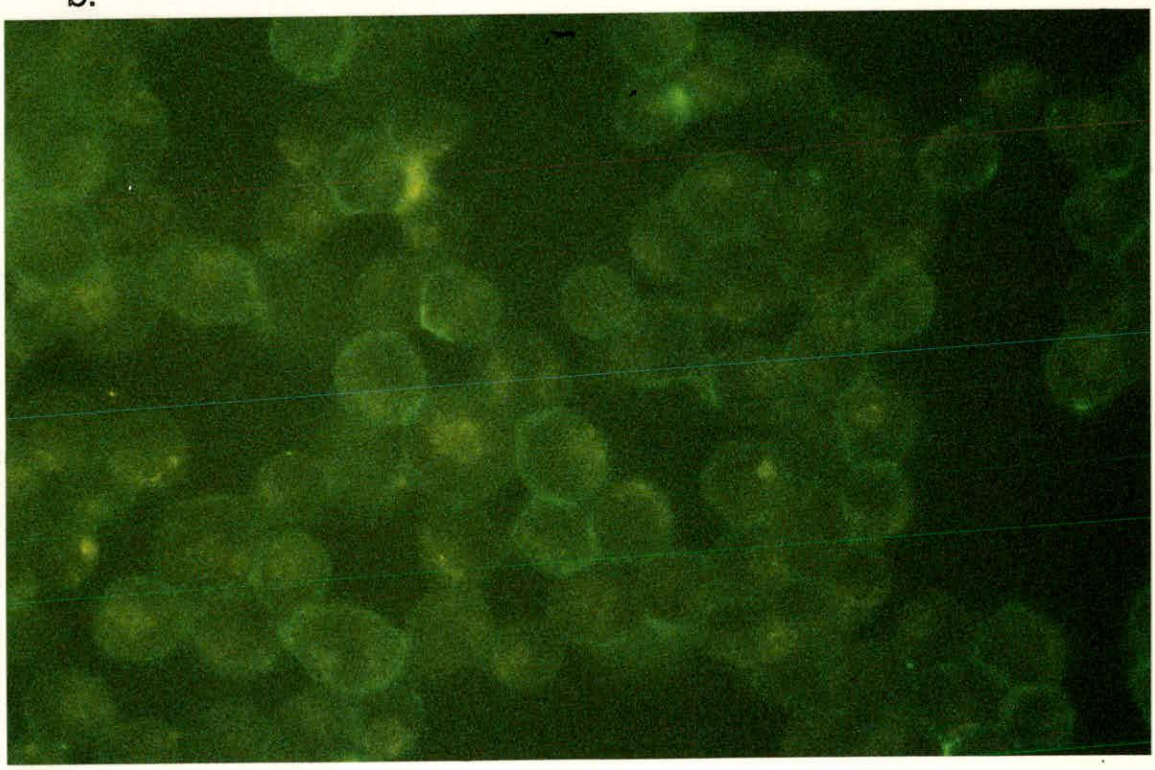


FIGURE 11. NORTHERN BLOT ANALYSIS OF TRANSFECTED CELLS

10-30μg of RNA was separated by electrophoresis on a denaturing 1.5% agarose gel as described in section 4.2.6. RNA was transferred to nylon membrane and hybridisation was performed as described in section 3.2.3.

Figure 11a

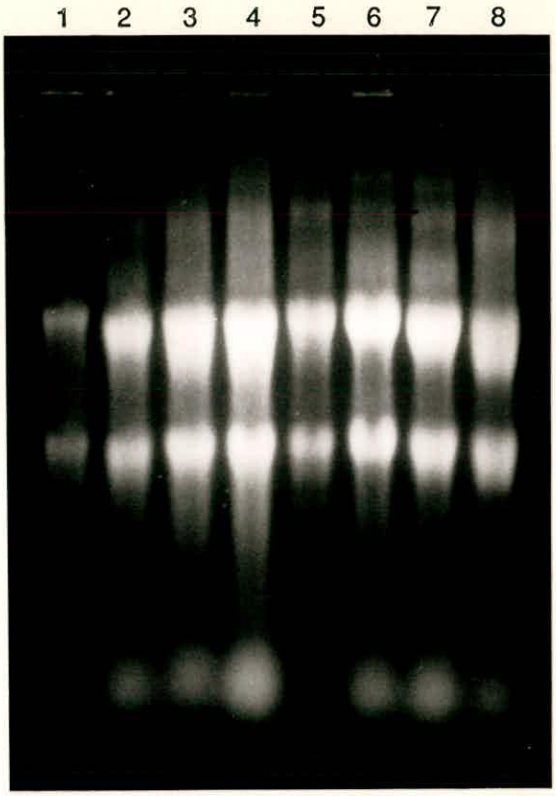
ET.Br. stained gel.

Figure 11b

Hybridisation pattern with the pBoLA-1 probe.

The positive control is bovine PBM (track 1) while the negative controls are Ltk^- and Ltk^+ (tracks 2 and 3 respectively). The four transfected lines besides 19.1 (track 6) analysed on the gel were 18.1, 7.2, 4.2 and 6.3 (tracks 4, 5, 7, 8 respectively). These 4 transfected cells lines were less than 2% positive for class I expression by FACS analysis. The ribosomal 28S (roughly equivalent to 5kb in size) and 18S (roughly equivalent to 2kb in size) bands are indicated as reference size markers.

a.



b.

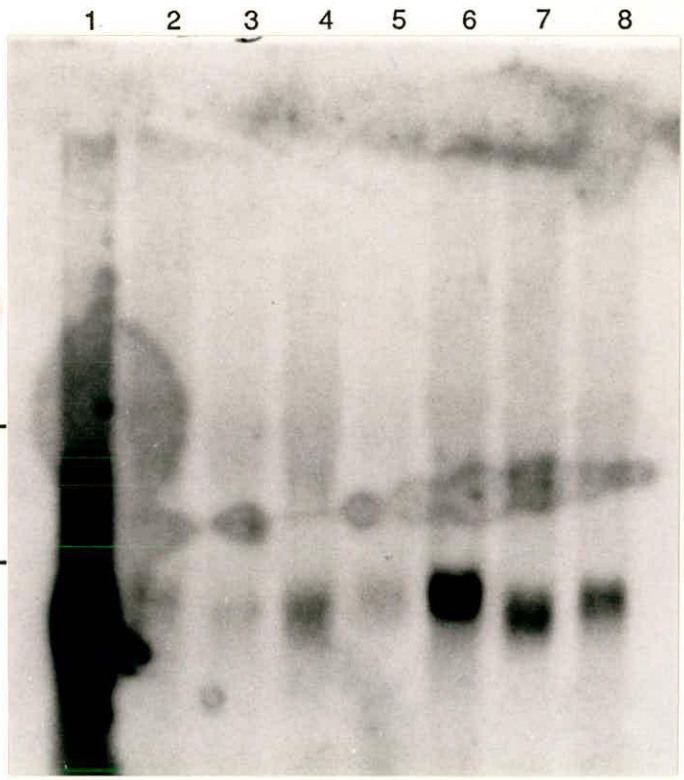


FIGURE 12. *NORTHERN BLOT ANALYSIS OF TRANSFECTED CELLS FROM VARIOUS STAGES OF SORTING*

RNA was extracted as described in section 4.2.6 from transfected cells at various stages in the sorting procedures (see figure 8 for stages).

10-30μg of RNA was separated by electrophoresis on a denaturing 1.5% agarose gel as described in section 4.2.6. RNA was transferred to nylon membrane and hybridisation was performed as described in section 3.2.3.

Figure 12a

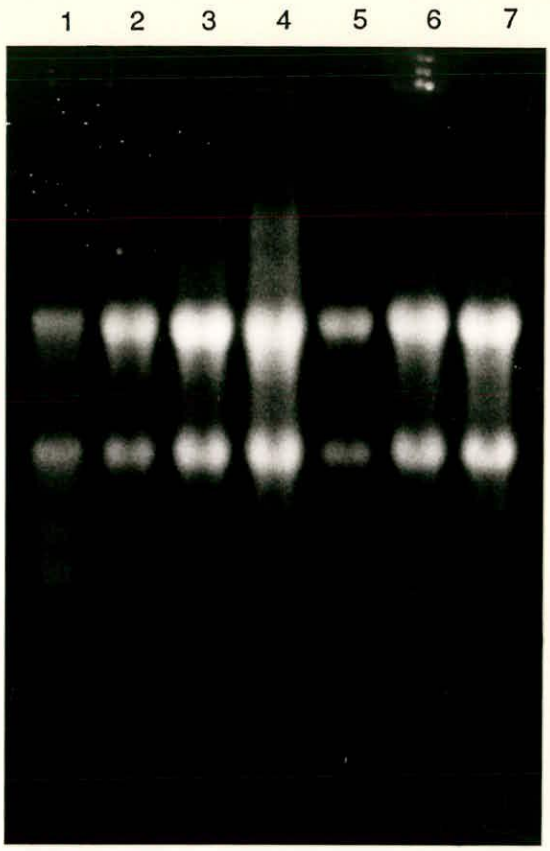
Et.Br. stained gel

Figure 12b

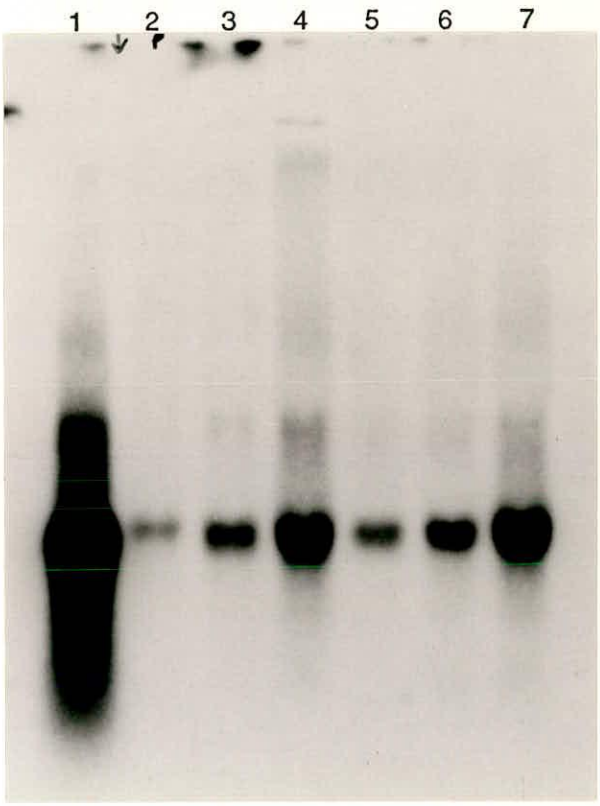
Hybridisation pattern with the pBoLA-1 probe

The positive control is PBM (track 1). Tracks 2-4 are RNA extracts from the first 3 rounds of sorting. Track 7 is RNA from the final round of sorting. Tracks 5 and 6 are the RNA's from cells at the left and right hand ends respectively of a staining distribution of cells growing after the third sort and suboptimally stained with ILA-88. The ribosomal 28S (~5kb) and 18S (~2kb) are indicated as reference size markers.

a.



b.



pBoLA-1.

The evidence showed an increased intensity of class I mRNA with the four sorts (figure 12). While in the Northern blot analysis of RNA from clones 18.1, 7.2, 6.3 and 4.2 (figure 11), there was a weak positive signal in the 4 clones with pBoLA-1. This indicates a low level of transcription in these three clones. However the level of expression did not increase after the first round of sorting on the FACS.

4.5 DISCUSSION

The Ltk$^-$ cell line (Lang et. al., 1983) and the transfection technique described above is routinely used at the Moredun Institute, therefore these were not checked. Toye et al. (1990) showed it was possible to 'shot gun' transfect sheared genomic DNA into Ltk$^-$ cells but there is a disadvantage to this method as the genes involved are not available for further characterisation. They were only able to characterise the genes on the basis of interaction with alloantisera. In this study, a cloned gene was used in the transfection to enable further structural and functional characterisation of these genes. There is, however, a disadvantage in this method, in the process of preparing the lambda genomic library, it is possible that all the specificities were not isolated and thus would not be detected in a transfection experiment.

The probe used in selecting the clones from the dot blot assay (chapter 3) was pBoLA-1 which is a cDNA clone, with truncated 5' and 3' ends. The dot blot assay therefore gave no information about whether the selected clones have promoter sequences or other necessary elements for transcription and translation. This can be studied by identifying the location of the gene/gene fragment in the recombinant vector. If the inserted DNA is one continuous fragment and is located centrally, i.e.

not situated immediately to either arms, the possibility of it being complete is higher. This was not investigated before the transfection experiment. Therefore perhaps selection of the 15 clones with the 5' and 3' end of pBoLA-1 would result in selection of incomplete genes. This may possibly explain why most of the clones did not express cell surface glycoproteins. A Southern blot was performed on the DNA of the transfected cells but upon hybridisation with pBoLA-1 and pTk, no positive signals were obtained not only for the 14 clones but also for clone 19.1. Perhaps the genomic DNA ($\sim 10\mu g$) used was not sufficient to enable detection of class I and thymidine kinase genes. However, FACS analysis and sorting resulted in a transfectant cell line, Ltk 19.1 that was expressing a bovine class I on the basis of staining with IL-A88. The data suggested that fluorescence intensity is directly related to increased transcription and translation levels of class I molecules. Although this was not followed up it suggests that further selection of cells with IL-A88 might yield a stable highly expressing cell line.

There are only a few anti-BoLA class I mAb available and the precise specificities of the ones available are not known. The mAb used in selecting for class I expression is IL-A88 which shows a broad specificity on the basis of IEF analysis of precipitated antigens (Oliver, pers.comm.) whether it recognises products of all BoLA class I loci is not known. Therefore the failure of IL-A88 to detect the expression of the other 14 clones is another possible explanation in obtaining only 1 clone expressing BoLA class I molecules. The other monomorphic anti-BoLA class I mAb, IL-A19 could not be used as it was found to cross-react with mouse class I products.

Another possibility is a pseudogene, i.e. a gene which is non functional. Various criteria have been proposed for a gene to be a pseudogene. When a gene

contains a termination codon at an inappropriate location, or it contains a deletion or insertion that changes the coding sequence (frameshift mutation) or many other possible mutations, it can be classed a pseudogene (Klein, 1986). Perhaps there are some pseudogenes among the clones selected for transfection. Nathenson et al. (1986) suggest pseudogenes as a potential source of donor sequences involved in the gene conversion events which maintain the extensive polymorphism in the class I genes of the MHC.

An interesting observation is the reduced level of expression when the transfectants are at confluence. Therefore, before a sorting or any functional tests, to get a good expression level, the cells have to be at sub confluence.

As mentioned in section 4.1, the interchange of $ß_2m$ does not affect the result in this case because the possible source of free $ß_2m$ is from foetal calf serum which in fact would be ideal as the heavy chain is of the same species.

In the Northern blot analysis, evidence that 19.1 is transcriptionally active substantiates the results of the indirect immunofluorescence assay while the positive results obtained from the cytospin preparation provided visual evidence of the cell surface glycoproteins.

Therefore 19.1 must be a complete active gene with sufficient elements necessary for transcription and translation to occur. The next obvious step would be to find out which BoLA class I gene, 19.1 encodes.

CHAPTER 5

IDENTIFICATION OF THE GENE PRODUCTS OF CLONE 19.1

5.1 INTRODUCTION

The expression of bovine class I histocompatibility genes was obtained after DNA-mediated gene transfer. The next essential step was the identification of BoLA surface antigens expressed by transformed Ltk$^-$ cells - Ltk 19.1.

Two tests were carried out to identify the antigens on Ltk 19.1. They comprised microlymphocytotoxicity and alloreactive cytotoxic T cells. In these tests the panel of alloantisera available in this laboratory were used as the reagents. These battery of alloantisera, most of which were prepared by skin grafting between dam and offspring, defined 28 alleles at a single locus in international comparison tests (Bull et. al. 1989).

(a) *Microlymphocytotoxicity*

A microlymphocytotoxicity test requires three components : the target cells, antibodies and complement. It is carried out as a two stage test. In the first stage, the target cells are incubated with the antiserum and in the second stage, the target cell - antiserum complex is incubated with the complement. In this experiment, the specificity of the antigen present on the surface of Ltk 19.1 was identified with the battery of alloantisera.

(b) *T cell cytotoxicity*

As in other species, the class I MHC products of cattle have been shown to be the major recognition structures for alloimmune cytotoxic T lymphocytes (Teale et al. 1985). Close correlation between serologically defined BoLA specificities and allospecific cytotoxic T cells were found (Teale et al. 1985 and Spooner et. al. 1987b).

This experiment involved:-

i) generation of alloreactive cytotoxic T cells (CTL)

ii) cytotoxicity assay (^{51}Cr release)

Cloned CTL specific for w10 and w11 of the two BoLA specificities in 10769 were used to identify products on Ltk 19.1.

5.2 MATERIALS AND METHODS

5.2.1 Microlymphocytotoxicity

(a) *Target cells*

The adherent Ltk^-, Ltk^+ and Ltk 19.1 cells were harvested from the flasks and counted using a haemocytometer (4.2.1). They were resuspended in Hank's BSS (appendix 4) at a concentration of 2.5 x 10^6 cells/ml. Ltk^- and Ltk^+ cells were the negative controls and PBM from cow 10769 (with genotype w10/w11) provided the positive control (preparation of PBM - section 4.2.1).

(b) *Alloantisera (antibody)*

The alloantisera used in this test are those that are routinely used in the typing service in this laboratory and in all the international BoLA Workshops. The identification codes are those used at the workshop. A total of 55 antisera defining 28 specificities were used in this test at the appropriate dilution (1/4 - 1/32). These alloantisera were shown to clearly define w10 and w11 on PBM of 10769 and the same alloantisera were used on Ltk^-, Ltk^+ and Ltk 19.1.

(c) *Complement*

Normal pooled rabbit serum.

(d) *Method*

Testing was performed as described by Spooner et. al., (1979). 50μl of liquid paraffin was dispensed into the Terasaki plates using a Histo Oiler (Dynatech Laboratories, Virginia, USA). 1μl quantities of antisera diluted at the appropriate dilution in Hank's BSS were distributed under the liquid paraffin. The plates were spun to allow the antisera to settle at the bottom of the wells. 1μl of the various cell suspensions at 2.5 x 10^6 cells/ml were added. These were incubated at room temperature for 30 minutes and 5μl of undiluted rabbit complement was added. These were again incubated at room temperature for an hour. After incubation, 2μl of 5% eosin stain and then 5μl of fixing solution (appendix 4) was added. The plates were read on a phase contrast microscope and the standard scoring system for the test was:

Code	Meaning
1	negative - same viability as normal serum control (0 to 9% lysis)
2	negative (10 to 19% lysis)
4	weak positive (20 to 39% lysis)
6	positive (40 to 79% lysis)
8	strong positive (80 to 100% lysis)
0	not readable invalid

The control was non-reactive bovine serum.

5.2.2 *T cell cytotoxicity assay*

(a) *Generation of alloreactive cytotoxic T lymphocytes*

Skin from the donor cow (10769, w10/w11) was implanted into a potential responder cow (w3/w10). Grafting was found to accelerate the development of alloreactive CTL in the *in vitro* mixed lymphocyte culture (Teale et. al. 1985 and Teale et. al. 1986).

The alloreactive CTL lines were generated *in vitro* by stimulation with irradiated PBM from the donor in an allogenic mixed lymphocyte culture. Four rounds of restimulation were performed. The T helper ($CD4^+$) cells were removed from the population by complement mediated lysis with mAb cc8 (Howard et. al. 1991). The remaining T cytotoxic ($CD8^+$) cells were enriched by restimulation with irradiated donor cells. IL-2 (Boehringer Mannheim, BCL) was also added to stimulate the growth of Tc. Cells were restimulated every week and maintained as a continuously growing cell line. FACS analysis with mAb IL-A51 (Morrison et. al. 1989) as first reagent and RAMIg as second was performed to confirm the presence of $CD8^+$ cells in the generated anti-w11 CTL line. A similar procedure was carried out to produce an anti-w10 CTL line.

(b) *Target cells*

Spooner & Brown, 1980 and Teale et. al. 1983 showed that the theileria-infected lymphoblastoid cell lines maintain the same class I phenotype as the animals from which they were derived. Teale et al. 1985 showed that these cells performed identically to PBM in their uptake and release of ^{51}Cr in the cytotoxicity assay. Therefore these cell lines from matched and mismatched

specificities were used as positive and negative control targets respectively in the cytotoxicity assay. Ltk 19.1 were the test target cells and Ltk$^-$ provided another negative control target cell.

(c) *Cytotoxicity assay*

The target cells were labelled with 100μCi ^{51}Cr sodium chromate at 37°C for 60 mins with periodic mixing. A standard 4 hour chromium release assay was performed involving the effector (CTL) and target cell in different effector : target ratio (Teale et. al. 1985 and Spooner et. al. 1987b). Modifications were made when using the adherent Ltk$^-$ and Ltk 19.1. These cells were harvested with EDTA 16 hours before performing the cytotoxicity assay. They were resuspended in medium and left in an untreated polystyrene tissue culture petri dishes (Sterilin or Corning) to which they do not adhere. The cells were washed twice with medium immediately prior to use in the assay (Townsend, et. al., 1984). This modification was made because cell death caused by EDTA was interfering in the results. The cells would also be able to recover from EDTA. The chromium release assay performed at 6 hours instead of 4 hours gave a better result as fibroblast are much less sensitive to cytotoxic T cell killing than PBM (Simpson and Chandler, 1986). The percentage specific ^{51}Cr release was used as a measure of target cell lysis and was calculated using the formula.

% specific ^{51}Cr release =

$$\frac{\text{test release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$$

5.3 *RESULTS*

5.3.1 *Microlymphocytotoxicity*

Animal 10769 was the source of DNA from which 19.1 was cloned for the genomic library. Its BoLA type was w10/w11. PBM, Ltk$^-$, Ltk$^+$ and Ltk 19.1 cells were tested with 55 antisera defining 28 international recognising BoLA class I specificities.

The only positive reactions are shown in Table 5.1. There are three sera defining w11, Ed73, Ed76 and Ed110 while only two, Ed69 and Ed71 defines w10.

TABLE 5.1 Results from microlymphocytoxicity

Cells	Specificities				
	w11			w10	
	Ed73	Ed76	Ed110	Ed69	ED71
PBM	+	+	+	+	+
Ltk$^-$	-	-	-	-	-
Ltk$^+$	-	-	-	-	-
Ltk 19.1	+	-	+	-	-

This shows that Ltk 19.1 is expressing w11 but detected by only two of the three sera seeing w11 on PBM. To study further the difference between the three w11 sera, they were titrated and tested on PBM and Ltk 19.1. The results are shown in Table 5.2.

TABLE 5.2. Titration of anti-w11 with PBM and Ltk 19.1

		Alloantisera Dilution					
Alloantisera	Target cells	Neat	1/2	1/4	1/8	1/16	1/32
Ed73	19.1	+	+	±	±	-	-
	PBM	+	+	+	+	+	±
Ed76	19.1	-	-	-	-	-	-
	PBM	+	+	+	+	-	-
Ed110	19.1	+	+	+	±	-	-
	PBM	+	+	+	+	+	±

This shows that Ed76 is a lower titre than Ed73 and Ed110. Thus the difference in reaction between PBM and Ltk 19.1 with Ed76 appear to be quantitative. Ltk 19.1 being more resistant to lysis is not killed by the lower titre reagent, Ed76.

5.3.2 Cytotoxicity assay

The results of the T cell cytotoxicity are shown in figure 13. BoLA w11 specific CTL clones kill w11 positive TaH infected cells and Ltk 19.1 at similar effector : target ratios. While BoLA w10 specific CTL clones only kill appropriate w10 positive TaH cells and not Ltk 19.1. Ltk$^-$ and non w10 or non w11 TaH cells are not killed by either CTL clones. These results further support the findings that Ltk 19.1 express BoLA w11. The results also show conclusively that CTL are not reacting with mouse class I.

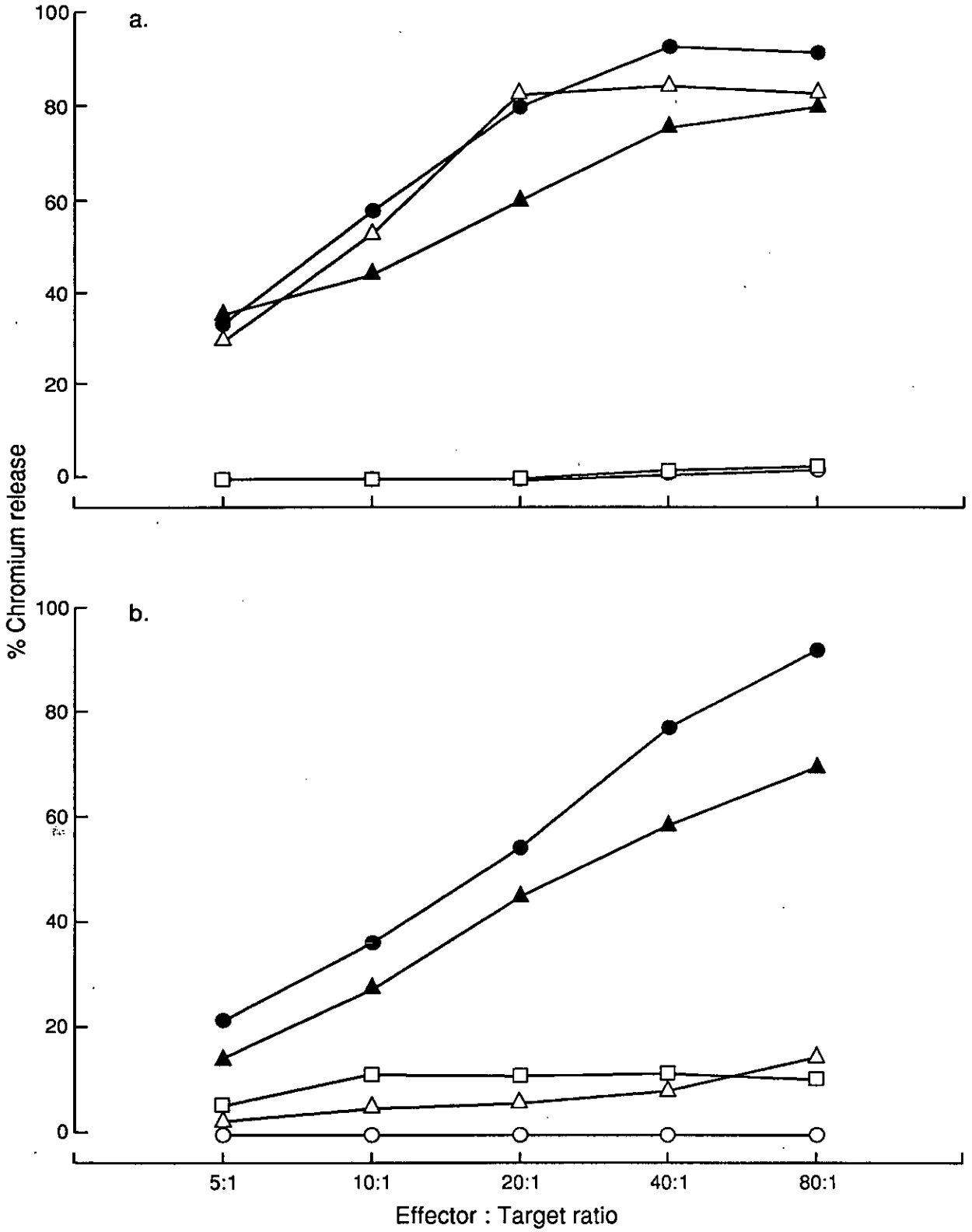
FIGURE 13. CYTOTOXICITY ASSAY

Cytotoxicity assays using:

a) anti-w11 CTL and

b) anti-w10 CTL

of a panel of cells as follows: 10779 TaH with BoLA type w10/w11 (●); 10557 TaH with BoLA type w11/- (▲); 2836 TaH with BoLA type w6/Ed99 (○); Ltk⁻ (□) and Ltk 19.1 (△). The assays were performed as described in section 5.2.2.


a.
b.
100
80
60
40
20
0
% Chromium release
5:1
10:1
20:1
40:1
80:1
Effector : Target ratio

5.4 *DISCUSSION*

The microlymphocytotoxicity strongly suggests that Ltk 19.1 was expressing a w11 specificity. PBM and Ltk 19.1 were killed at the same effector:target ratio, however, with both tests the Ltk 19.1 cells required significantly longer incubation. Lemonnier et. al. (1983) and Sodoyer et. al. (1986) showed that the successful identification of Ltk$^-$ cells transformed with HLA class I genes was possible using microlymphocytotixicity. In BoLA class I isoelectric focusing analysis, the mAbs used to immunoprecipitate the antigen were shown to play an important role in characterising them, i.e. some mAbs were shown to precipitate more bands than others (Joosten et. al. 1988), while immunoprecipitation with alloantisera has revealed antigens that are identified serologically (Al-Murrani et. al. manuscript in prep).

In this analysis, BoLA class I antigens from PBM, Ltk$^-$ and Ltk 19.1 (refer to draft of paper in appendix 5), the bands seen from immunoprecipitation of Ltk 19.1 with w6/32, IL-A88 and Ed102 corresponded to some bands from immunoprecipitation of PBM with the 2 mAbs and alloantiserum. These would be the products of w11 as Ltk 19.1 was shown to encode a w11 gene from the first two assays. The different banding patterns seen between Ltk 19.1 with the mAbs and alloantiserum suggest that these antibodies recognise different epitopes of the product on Ltk 19.1. An interesting observation is the cross reaction of alloantiserum Ed102 with Ltk$^-$.

The Ltk 19.1 was found to express class I antigens at a low level (Al-Murrani, pers. comm.). This observation was made on the basis of the finding that an increased number of cells were required for immunoprecipitation as compared to PBM in the IEF studies.

FACS analysis was extensively used in isolating Ltk 19.1 (chapter 4). Attempts were made to identify w11 using alloantisera in indirect immunofluorescence using FACS analysis. The results however were confusing in that Ltk$^-$ reacted with non-reactive bovine serum as well as alloantisera. In fact Ltk 19.1 cell showed less non-specific binding. This taken together with the cross reaction of Ed102 with Ltk$^-$ in IEF analysis (Al-Murrani, pers. comm.) implies that although alloantisera appear to be monospecific in the microlymphocytotoxicity, it is not so in IEF and indirect immunofluorescence.

CHAPTER 6

SEQUENCE ANALYSIS OF GENE ENCODED IN CLONE 19.1

6.1 INTRODUCTION

DNA sequencing is important in relating the structure to the function of a gene. It has been used to identify functional gene in studies involving analysis of MHC genes, that is, whether the sequences have the features expected of an expressed class I molecule that presents peptide antigen.

The characteristic features of a functional class I gene necessary for expression at the cell surface are the leader peptide, α_1, α_2, α_3, transmembrane and cytoplasmic domains.

There are other conserved features in the class I molecules of which the significance of only some are known. The specific cysteine residues which are required for the formation of internal disulphide bridges (Kimball and Coligan, 1983), are one of the conserved features. There is considerable evidence for the importance of disulphide cysteine residues. In the pseudogene HLA 12.4, the presence of phenylalanine instead of cysteine at position 259 has been postulated to be the cause of the gene being non-functional (Malissen et. al., 1982) due to loss of structural rigidity. In *in vitro* HLA studies, Shiroshi et. al., 1984 showed that when cysteine is changed to serine, the protein structure is not recognised by a majority of mAb tested.

In man, mice and cattle, the disulphide cysteine residues are present at amino acids 101 and 164 in the second protein domain and at amino acids 203 and 259 in the third domain (Kimball and Coligan, 1983; Ennis et al., 1988; Brown et al., 1989a and Bensaid et al., 1991). The disulphide loops are of approximately the same size as those in immunoglobulins (Igs) and when the amino acid sequence of the second disulphide loop in HLA-B7 is compared to that of Igs, there is a high level

of homology (Orr et al., 1979). Perhaps the conservation of cysteine at the expected positions is a result of the evolutionary relationship between histocompatibility antigens and Igs.

Similarly, there is a conserved site for N-linked glycosylation at position 86 for all species including cattle (Lew et al., 1986; Ennis et al., 1988; Brown et al., 1989a and Bensaid et al., 1991), even though there are many potential sites in the mouse; at positions 86, 90, 176 and 256 (Lew et al., 1986). This residue is in a loop connecting α_1 to α_2 (Bjorkman et. al., 1987a). Santos-Aguado et al. (1987) studied the role of this conserved carbohydrate in the HLA-A2 gene. They suspected that certain residues in the glycosylation sequence (asn-X-ser) are necessary in maintaining the general structure of the glycoprotein. Substitution at position 86 and 88 were carried out using site-directed mutagenesis to create new molecules devoid of carbohydrate to enable them to study the effects of a nonglycosylated HLA-A2 molecule. This eliminates the secondary effects on protein synthesis when tunicamycin was used to inhibit N-linked glycosylation, which they suggest is the reason of the negative result that Ploegh et al. (1981a) obtained with respect to rate of expression. Their results suggest that glycosylation is not essential for cell surface expression and interaction of HLA-A2/$ß_2$m but the asparagine residue at position 86 may be important for the conformation of the HLA heavy chain and its association with $ß_2$m.

Another conserved feature in class I molecules is the site for phosphorylation in the sequence (ser-asp/glu-X-ser(P)-leu). In an analysis of HLA-B7, the site for phosphorylation is serine 335 (Guild & Strominger 1984). When they aligned the protein and DNA sequences of human and mouse class I antigens, they found the same conserved phosphorylation site in mouse. Ennis et al. (1988), Brown et al.

(1989a) and Bensaid et al. (1991) observed a similar possible conserved phosphorylation site (ser-asp-val-ser(P)-leu) in bovine class I molecules. The preservation of this sequence and site suggests a functional significance but this has yet to be shown.

In class I molecules, when comparing the sizes of the domains only the three α domains are preserved while different lengths are observed for the leader peptide, transmembrane and cytoplasmic domains.

The leader peptide of HLA is mostly twenty four amino acids long while that of H-2 is shorter with twenty one amino acids. The five BoLA cDNA clones have variable leader peptide length:

BL3-7	-	24 amino acid
BL3-6	-	21 amino acid
pBoLA-1	-	23 amino acid
BoLA-AW10	-	14 amino acid
BoLA-BKN104	-	18 amino acid

When compared to other class I molecules the 4 BoLA cDNA (except BL3-7) might have lost several amino acids in the process of cloning (Ennis et al., 1988; Brown et al., 1989a and Bensaid et al., 1991).

The transmembrane domains of class I molecules also vary in size, but there seem to be uniformity in size within a locus, i.e. 39 amino acids for HLA-C; 37 amino acids for BoLA-A (Parham et. al., 1988, Ennis et. al., 1988, Brown et. al., 1989a and Bensaid et. al., 1991).

The cytoplasmic domain of class I molecules is encoded by exons 6, 7 and

sometimes 8, and this domain also seems to have locus-specific features. In HLA-B genes, exon 8 is not used as there is a termination codon in exon 7 and this results in a cytoplasmic domain with 27 amino acids. Whereas HLA-A and -C molecules do use exon 8 to produce cytoplasmic domains with 28 amino acids. There are also 28 amino acids in the cytoplasmic domains of BoLA-A molecules.

In comparisons of the HLA-A, -B and -C sequences it was found that there appears to be selection for polymorphism in the α_1 and α_2 domains and against polymorphism in the α_3 domain (Parham et al., 1988).

Polymorphism at some amino acid positions in the α_1 and α_2 domains of HLA is responsible for the specificity of recognition by T cells, and for the variation in responsiveness to particular foreign antigens (Bjorkman et al., 1987b). In contrast, the conserved amino acids are involved in maintenance of the class I structure and recognition by constant features on T cells. In the X-ray structure of the HLA-A2 molecule (Bjorkman et al., 1987a) 15 of the 17 polymorphic amino acids were found to be located in the binding site region for processed antigens, i.e. in the α_1 and α_2 domains. In these domains, the conserved amino acids were found to be pointing away from the antigen-recognition site (Bjorkman et. al., 1987b).

A similar situation was observed for BoLA class I sequences. In the α_1 domain, which is the most polymorphic domain, there was great divergence between the BoLA sequences (Ennis et al., 1988, Brown et al., 1989a and Bensaid et al., 1991). Another similar situation between BoLA and HLA molecules is that the polymorphism in this domain is concentrated in the α helix and not in the ß sheet of the peptide binding site, while in the α_2 domain, the polymorphism is found in the ß sheet and not the α helix (Ennis et al., 1988 and Parham et al., 1988). Yokoyama and Nathenson (1983) provided evidence in mice for the α_1 and α_2 domains to be

recognition elements for the immune system and for α_3 to be involved in binding to $ß_2m$.

Although polymorphism is predominantly found in the α_1 and α_2 domains, variations in the α_3 domain in HLA and H-2 have been shown to be important with respect to interaction with the accessory molecule, CD8 (Potter et al., 1987; Norment et al., 1988; Connolly et al., 1988; Garrett et al., 1989 and Salter et. al., 1989). Residues of α_3 that are implicated in binding CD8 from *in vitro* mutagenesis experiments in human are 223, 226, 227, 229, 233 and 245 (Bjorkman and Parham 1990). Residue 227 of class I molecules is highly conserved in mice, human and rat (Klein & Figueroa, 1986) and from the X-ray structure of HLA-A2, this residue was found on the surface but at a considerable distance from the peptide binding site which involves residues of the α_1 and α_2 domains (Connolly et al., 1988). This supports the other evidence that residue 227 may be involved in CTL function by binding to the CD8 glycoprotein (Potter et al., 1987).

In the α_3 domain of HLA-Aw68.1 and HLA-Aw68.2, there is a valine instead of an alanine residue at position 245, and these molecules do not bind CD8. This single substitution in the α_3 domain also affects recognition by alloreactive and influenza-specific CTL (Salter et. al., 1989). Similarly, Castano and coworkers in 1988 observed that an amino acid substitution at position 236 (ala→glu) in the α_3 region of an HLA-A2 variant influenced the specificity of alloreactive CTL recognition. They commented, based on crystallographic analysis of HLA-A2 (Bjorkman et al., 1987a) that position 236 is close to residues involved in interdomain contacts of α_3 with both α_1 and $ß_2m$, but is not involved in the antigen-binding site. Therefore Castano and coworkers proposed to raise specific CTL against this naturally occurring variant to provide an insight in the restriction properties of the

HLA-A2 molecule.

There is a low frequency of coding substitutions in the transmembrane and cytoplasmic domain in class I molecules. In the transmembrane domain, the lack of polymorphism is a result of selection for its function of being hydrophobic, enabling the protein to be anchored in the cell membrane. In the cytoplasmic domain, however selection operates to prevent the carboxyl terminus from entering the bilayer (Ploegh et al., 1981b).

Diversity in class I molecules is due solely to amino acid substitutions in the heavy chains and not from the $ß_2$m except in the mouse (Michaelson et al., 1980). There are 2 alleles of $ß_2$m in mice which differ by a single amino acid at position 85 (Gates et al., 1981).

Examination of the HLA-A2 structure identified the inter-domain contacts between the domains of the heavy chain and $ß_2$m (Bjorkman et. al., 1987a). The residues involved in contacts between α_1 and α_3, α_2 and α_3, and α_3 and $ß_2$ are as shown below. The other inter-domain contacts are in Bjorkman et. al., 1987a.

TABLE 6.1. Residues involved in inter-domain contacts (from Bjorkman et. al., 1987a).

α_1 - α_3		$ß_2$m - α_3	
29,30	209	8	231, 244
30	211	10	234, 242
27, 33	234	11	242
α_2 - α_3		12	238
176, 177, 179	181	13	188, 206
180	182	22	237
179	209	26	233, 234
		65	237
		97	204

Comparison of $ß_2$m sequences from seven species, that is, bovine, human, mouse, rabbit, rat, pig, dog and guinea pig reveals that these $ß_2$m-α_3 contacts are highly conserved. A similar conservation of $ß_2$m contact residues is found in the heavy chain between species (Ennis et. al., 1988).

The BoLA class I protein sequences seem to be more similar to HLA than to H-2 molecules (Ennis et. al., 1988). In both BL3-6 and BL3-7, there was a remarkable similarity between HLA-B and BoLA-A in the α_2 and α_3 domains. Ennis et al. (1988) suggested that this similarity could explain the serologic crossreactivity between HLA and BoLA class I molecules.

In HLA, certain features of the classical class I gene sequences can assign them to a particular locus. There are 31 positions in the amino acid sequence where only products of a single locus show conservation. These conserved residues are found in all domains but the majority are in the transmembrane and cytoplasmic domains (Bjorkman & Parham, 1990). For example, a significant locus-specific difference in α_3 is the presence of arginine at position 239 in all HLA-B molecules and of glycine in all HLA-A and -C molecules.

Therefore in BoLA, it is necessary to sequence known class I genes to have a better insight into whether BoLA is a single locus or a multilocus complex. If it is multilocus, identification of locus-specific residues would assist in assigning new alleles to their respective loci.

6.2 SUBCLONING 19.1 DNA FRAGMENTS INTO PLASMID VECTORS

6.2.1 Introduction

The 7kb BamHI fragment of clone 19.1, encoding the whole class I gene (as discussed in section 3.3.2), was digested with Sau3A. These fragments were

shotgun subcloned into the BamHI site of pBS (Stratagene, Northumbria Biologicals Ltd.). The recombinant plasmid was transformed into the *E. coli* host strain JM109 and 27 recombinant colourless colonies were found. Identification of recombinant class I insert was by hybridisation with pBoLA-1 (as described in section 6.2.2).

Discrimination between recombinant and non-recombinant vector-transformed cells used a simple chromogenic method. The phenomenon of α-complementation between the N-terminally truncated ß-galactosidase encoded by the F' episome of the bacterial strain JM109 and the N-terminal ß-gal fragment encoded by the pBS vector regenerates ß-galactosidase activity which converts the colourless substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) (Northumbria Biologicals) into a blue chromophore which is visible in the bacterial colonies. When non-recombinant plasmids are transformed into the host bacterial strain JM109 in the presence of X-gal and the inducer isopropyl-ß-D-thiogalactoside (IPTG) (Northumbria Biologicals) blue bacterial colonies are formed. The insertion of foreign DNA into the multiple cloning site of the pBS vector prevents α-complementation and results in colourless colonies. The plasmid vector also contains the ampicillin resistance gene which allows the transformed cells to be selected for. Therefore on ampicillin plates in the presence of X-gal and IPTG only JM109 cells transformed with a recombinant plasmid should result in colourless colonies.

The two sequencing techniques in current use are the enzymatic method of Sanger et. al. (1977) and the chemical degradation method of Maxam and Gilbert (1977). In the Maxam and Gilbert chemical cleavage method, the DNA is specifically cleaved by reagents that modify and then remove a base from its sugar. Before cleavage, the DNA strand is labelled at one end with ^{32}P. These fragments are then separated by polyacrylamide gel electrophoresis, which can resolve DNA

molecules differing in length by just one nucleotide and this will be displayed by autoradiography of the gel. The method of Sanger involves the use of 2',3'-dideoxynucleotide triphosphates (ddNTPs) which act as specific chain-terminating inhibitors of DNA polymerase. This method was used for sequencing the gene in 19.1 and will be discussed in more detail (6.2.3).

6.2.2 Materials and Methods

(a) *Preparation of competent JM109 bacterial cells.*

A working stock of JM109 bacteria was maintained on glucose minimal agar stored at 4°C in order to ensure retention of the F' episome required for the detection of recombinants. A single colony was picked for inoculation into 10ml of LB broth and grown with vigorous agitation overnight. 1ml of this culture was used to inoculate 500ml of LB broth which was grown to an optical density of 0.9 at 600nm. The cells were then centrifuged at 5000rpm for 10 minutes and gently resuspended in 250ml of sterile ice cold 100mM $CaCl_2$. They were left on ice for 30 minutes and harvested by centrifugation. The cells were then resuspended in 5ml of 100mM $CaCl_2$ containing 10% glycerol, and were aliquoted into 200μl volumes for storage at -70°C.

(b) *Preparation of the cloning vector*

As a stock for ligation to a restriction fragment, 5μg of pBS DNA were digested with the restriction enzyme BamHI. An aliquot was analysed by agarose gel electrophoresis to ensure complete digestion. The vector was purified by phenol extraction and precipitated in ethanol. The dried pellet was resuspended in 25μl of dephosophorylation buffer (appendix 1) and 1 unit

of calf intestinal phosphatase (Northumbria Biologicals) was added. The reaction proceeded at 37°C for 30 minutes after which the DNA was again purified by phenol extraction and ethanol precipitation. The DNA pellet was resuspended to give a final concentration of 50ng/μl.

(c) *Ligation of vector DNA to insert DNA*

In this investigation the strategy for ligation was the shotgun cloning of Sau3A fragments of clone 19.1 into BamHI digested pBS. 2μl (~1μg) of the 7kb BamHI fragment of 19.1 was digested with Sau3A to completion and the DNA was then precipitated with ethanol for at least 15 minutes at -70°C. The precipitate was centrifuged at 13000rpm for 5 minutes and washed in 70% ethanol twice. After the final wash, the excess ethanol was removed and the pellet was air dried for 5 minutes. It was then resuspended in 5μl of H_2O. The ligation mixture contained:

1. 3μl pBS vector stock (cut with BamHI).
2. 5μl insert DNA (7kb BamHI of 19.1/Sau3A fragment).
3. 1μl ligase buffer (appendix 1).
4. 1μl T4 DNA ligase (Gibco-BRL).

The ligation reaction was left overnight at 12°C and was stopped by the addition of 1μl 0.5 M EDTA.

(d) *Transformation of JM109*

A 200μl aliquot of the frozen stock of competent bacterial cells prepared as in 6.2.2 (a) was removed and slowly thawed on ice. 5μl of the ligation reaction mixture was added to 60μl of cells in duplicate. The mixture was

incubated on ice for 30 minutes after which it was heat-shocked by incubation at 42°C for 2 minutes. L Broth was added to a total volume of 200μl and incubated at 37°C for 1 hour. The transformation mix was spread evenly on fresh L agar plates (appendix 2) which contains ampicillin (50μg/ml), X-gal (40μg/ml) and IPTG (8μg/ml). The plates were incubated at 37°C and colourless recombinant and blue non-recombinant colonies were visible after 15 hours. The plates were left at 4°C for a further hour to allow the blue colour to develop clearly.

(e) *Identification of recombinant with class I insert*

A plate lift with hybond (Amersham) filters was done to make a filter bound replica of the bacterial colonies. The filters were lifted and placed on a fresh L agar plate with ampicillin at room temperature to allow the colonies to grow overnight. The filters were then placed on 3MM Whatman paper soaked with:

1. 10% SDS for 3 minutes to limit the diffusion of the DNA.
2. 0.5 M NaOH/1.5 M NaCl for 5 minutes to denature the DNA.
3. 1 M Tris pH7.4/1.5 M NaCl for 5 minutes to neutralise the DNA.

The filters were left to air dry on 3MM Whatman paper for 15 minutes and then were immersed in 2 x SSC for 5 minutes and the colonies were physically wiped off. The filters were again dried and the DNA was fixed to the hybond membrane by UV crosslinking. The filters were then probed with the 500bp 5' half of pBoLA-1 (section 3.2.3) and from the hybridisation signals, two positive colonies were identified on the original transformation plate. The colonies were picked using a sterile loop and inoculated into 10ml

of LB broth with ampicillin. They were grown for 15 hours and a frozen stock prepared (1.5ml aliquot and 10% glycerol kept at -70°C). The plasmids were then purified using the small scale plasmid preparation technique and analysed by restriction enzyme digestion and agarose gel electrophoresis. The plasmids DNA was stored at -20°C for sequence analysis.

(f) *Small scale preparation of plasmid DNA*

10ml LB + ampicillin was inoculated with the recombinant colony and incubated at 37°C with constant shaking overnight. The suspension was centrifuged at 3000rpm for 15 minutes. The supernatant was discarded and the pellet was resuspended in 1ml of ice cold glucose buffer (appendix 1). This was transferred to a 1.5ml microfuge tube. The cells were pelleted by centrifugation at 13000rpm for 20 seconds and resuspended in 200μl of ice cold glucose buffer. 400μl of SDS/NaOH solution (alkaline lysis solution) was added and cells were left on ice for 5 minutes. This was followed by the addition of 300μl of high salt solution (appendix 1) and the mixture left on ice for 30 minutes. After centrifugation for 5 minutes at 13000rpm the supernatant was collected and plasmid DNA precipitated by the addition of 0.75ml of isopropanol followed by incubation at -20°C for 2 hours. The DNA was pelleted by centrifugation, washed in 70% ethanol, dried and resuspended in 100μl TE. RNA was digested by the addition of RNAase (Sigma Chemical Co.) to a final concentration of 30μg/ml for 15 minutes at 37°C. The remaining protein was removed by phenol extraction and DNA precipitated in ethanol, washed in 70% ethanol and dissolved in 20μl TE.

6.2.3 Nucleotide sequence analysis

This analysis was carried out to characterise the w11 gene in 19.1 using the dideoxy chain termination developed by Sanger et. al., (1977).

All sequencing carried out in this investigation used the Pharmacia T7 sequencing kit (Pharmacia, Milton-Keynes), which uses the enzyme T7 DNA polymerase to catalyse the chain extension. The source of template was the 3.2kb double-stranded recombinant pBS+/- phagemid (Stratagene) (figure 14). The sequence was determined from both ends (5' and 3') of the cloned DNA insert using the 17 base universal primer and reverse primer.

(a) *Annealing of the primers to double stranded PBS template*

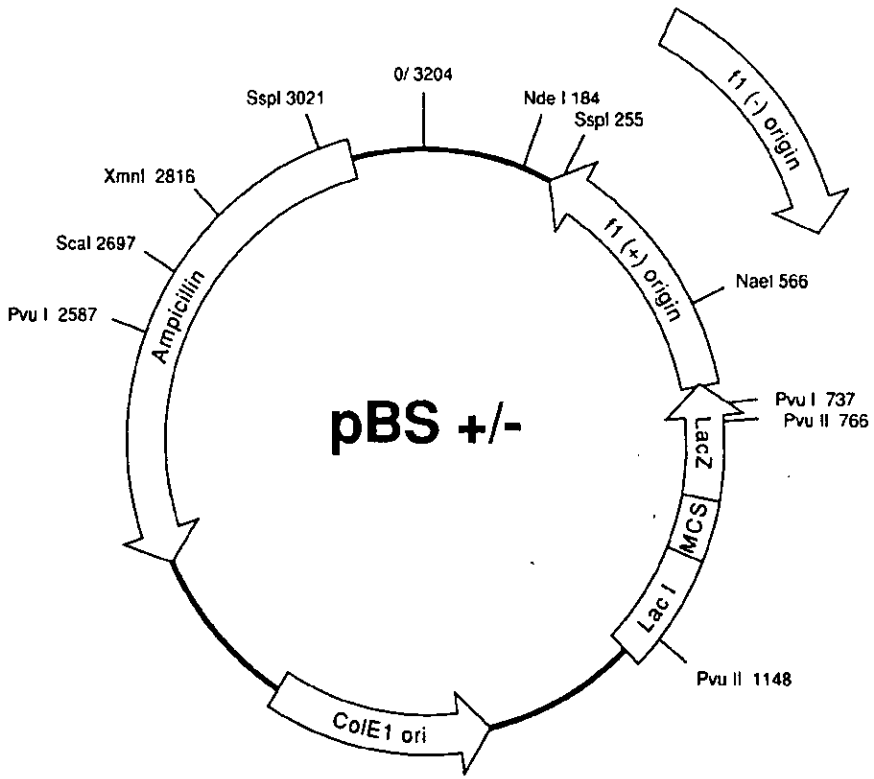
The primer was annealed to 1.5-2μg of double stranded plasmid DNA which had been denatured by addition of 2μl of 2 M NaOH to 8-10μl of plasmid DNA. The DNA was incubated at room temperature for 10 minutes after which it was precipitated by adding 3μl of 3 M sodium acetate pH4.5, 7μl of distilled water and 60μl of ethanol at -70°C for 30 minutes. The precipitated DNA was collected by centrifugation at 13000rpm for 10 minutes. The pellet was then washed with ice cold 70% ethanol. The mixture was recentrifuged for 10 minutes and the DNA pellet was resuspended in 10μl of distilled H_2O. The annealing reaction involves adding 2μl of the annealing buffer and 2μl of the universal primer or reverse primer.

(b) *Sequencing Reactions*

To the primed template 3μl of labelling solution was added (dCTP, dGTP and dTTP) plus 10μCi of α^{35}S dATP and 2μl (3 units) of diluted T7 DNA

FIGURE 14. SCHEMATIC DRAWING OF pBS (+/-) PHAGEMID

The plasmid pBS is 3204bp. It carries the ampicillin resistance gene and the 5' end of the beta-glactosidase gene which is controlled by the inducible lac promoter. The positions of the reverse primer, universal primer and the polycloning sites are as shown below the figure. (Reproduced from Stratagene Catalogue, 1991).



Reverse Primer
5' AACAGCTATGACCATG 3'

T3 Primer
5' ATTAACCCTCACTAAAG 3'

MET T3 Promoter +1 Hind III Sph I Pst I Hinc II Acc I Sa·I Xba I BamH I Xma I Sma I Kpn I Sac I Eco RI

5' GGAAACAGCTATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTAC 3'
3' CCTTTGTCGATACTGGTACTAATGCGGTTCGAGCTTTAATTGGGAGTGATTTCCCTTGTTTTCGAACGTACGGACGTCCAGCTGAGATCTCCTAGGGGCCCATGGCTCGAGCTTAAGCGGGATATCACTCAGCATAATGTTAAGTGACCGGCAGCAAAATG 5'

Beta-Galactosidase

←-1 T7 Promoter

3' GATATCACTCAGCATAA 5'
T7 Primer

3' TGACCGGCAGCAAAATG 5'
M13 -20 Primer

polymerase. Following incubation at room temperature for 5 minutes, 4.5μl aliquots were added to each of 4 labelled tubes, containing 2.5μl of the relevant base-specific termination nucleotide mix. The reaction was allowed to proceed at 37°C for 5 minutes and was terminated by the addition of 5μl of stop solution (deionised formamide containing EDTA, xylene cyanol and bromophenol blue). 3μl aliquots of each reaction mixture were denatured by heating at 75-80°C for 2 minutes and were loaded onto a polyacrylamide sequencing gel.

(c) *Sequencing gel electrophoresis*

Six percent (w/v) denaturing polyacrylamide gels were formed between two 42cm x 37cm glass plates (with one side coated with "Sigmacote", Sigma Chemcals Co. to prevent sticking of the acrylamide) which were separated by 0.4mm spacers. Gels were prepared by dissolving 42g of ultrapure urea (Gibco-BRL) in 14.5ml of 40% acrylamide stock solution (appendix 1), 10ml 10 x TBE buffer (appendix 1) and water to 100ml. The polymerisation reaction was initiated by the addition of 0.8ml of 10% w/v ammonium persulphate (APS, Bio-Rad) and 40μl of N.N.N. N-tetramethylethelenediamine (TEMED Bio-Rad). A sharkstooth comb was positioned so that the teeth just touched the surface of the gel to provide the sample loading wells once the gel was immersed in 1 x TBE electrophoresis buffer within the BRL model S2 electrophoresis apparatus. The samples were denatured by heating for 2 minutes at 75-80°C immediately prior to loading. 3μl aliquots of each sample were loaded in the order, G, A, T and C. Samples were electrophoresed at 1500V until the bromophenol blue (BPB) marker reached the base of the

plates. A second loading of the same four samples were applied to the gel and electrophoresis continued until the BPB dye fronts of these samples reached the base. A third loading of samples was then applied and electrophoresis continued until the BPB dye fronts of the last samples reached the base of the plate. The plates were separated and the gel fixed in a mixture of 10% acetic acid and 10% methanol for 20 minutes. After removal from the fixative, the gel, still attached to one plate, was transferred to a supporting sheet of 3MM Whatman chromatography paper and covered with plastic wrap. The gel was dried, and then autoradiographed for 15 hours at room temperature.

(d) *Analysis of autoradiographs*

The nucleotide sequence was translated from the pattern of bands on the autoradiograph. Comparative computer analysis of the nucleotide sequence, determination of exon boundaries and conversion to amino acid sequence was performed using the University of Wisconsin Genetics Computer Group (UWGCG) Programmes (Devereux et. al., 1984).

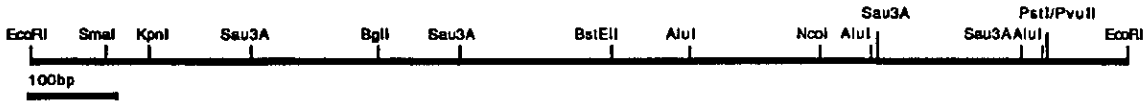
6.3 RESULTS

The probe used in selecting the recombinant plasmids was the 500bp SmaI-BstEII fragment of pBoLA-1. This fragment encodes the α_1, α_2 and a little of the α_3 domain (exons 2-4) (figure 15). The 300bp EcoRI-BglI fragment of pBoLA-1 which encodes the leader peptide and the first two domains could not be used as this probe cross-hybridised with pBS vector. This cross-reaction probably results from some contamination of the 3' sequences of pPoly1 vector DNA (Lathe et. al., 1987)

FIGURE 15. RESTRICTION MAP OF pBoLA-1 INSERT

The figure shows the positions of restriction sites used to produce bovine class I gene probes. The 5' end probe used was the EcoRI - Bgl1 fragment of about 400bp which corresponds to the leader peptide and the first two domains of the pBoLA-1 protein. The 3' end probe was the PstI - EcoRI fragment of about 100bp which corresponds to the 3' UT region of the protein. The SmaI - BstEII fragment of about 500bp which corresponds to the first two domains and part of the third domain was used for selection of the recombinants in pBS for sequencing. (After Brown, 1989b).

Restriction Map of pBoLA-1



by the 5' cDNA fragment during gel isolation procedure.

The probe hybridised to only two recombinant colonies, and when these plasmid DNAs were digested with EcoRI and HindIII, which should cleave the vector from the insert, an approximately 500bp insert fragment were found in addition to the 3.2kb pBS fragment. The recombinant colonies were designated pNH1 and pNH2. Upon sequencing the insert fragments, pNH1 and pNH2 were found to be identical. The sequence of the entire 494bp fragment was determined using universal and reverse primer. From pNH1, 307 bases of sequence were obtained with the universal primer and 198 with the reverse primer. There was an overlap of 11 bases between the two sequences. This sequence is referred to as NH1 (figures 16 & 17).

Alignment of NH1 with pBoLA-1 (BTMHLA, Brown et. al., 1989a), BL3-6 & BL3-7 (BTBoLAA and BTBoLAB Ennis et. al., 1988) and BoLA-BKN104 and BoLA-Aw10 (BIMHAWA and BIMHBKNA, Bensaid et. al., 1991) was performed using BESTFIT and results are as shown in the table below.

BESTFIT is a program from the UWGCG package which produces an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximise the number of matches.

TABLE 6.2. Alignment of NH1 with the 5 cDNA

					Bases	
NH1 with	No. of bases compared	% similarity	No. of substitutes	Gaps	Start	End
BTBoLAA	99	94.9%	5	0	606	704
BTBoLAB	99	96.9%	3	0	629	727
BIMHAWA	99	92.9%	7	0	587	685
BIMHBKNA	99	96.9%	3	0	587	685
BTMHLA	99	91.9%	8	0	613	711

FIGURE 16. *NUCLEOTIDE SEQUENCING GEL AUTORADIOGRAPH*

The nucleotide sequence of the plasmid, pNH1 containing a 500bp Sau3A fragment that hybridises with the pBoLA-1 SmaI - BstEII fragment was determined by dideoxysequencing using universal and reverse primers. The figure shows a representative autoradiograph and sequence ladder.

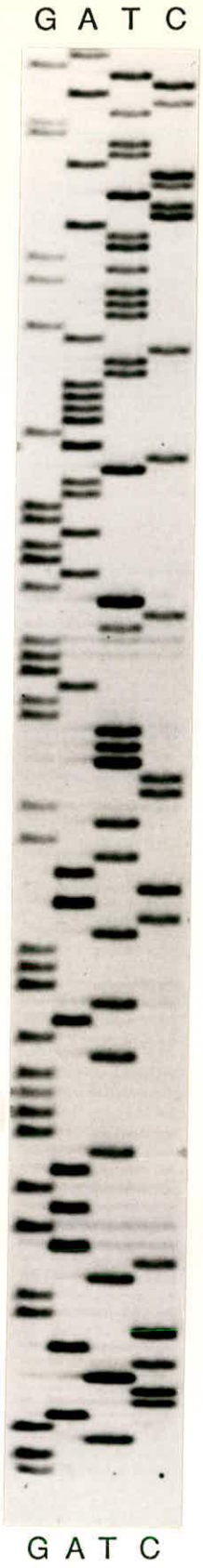
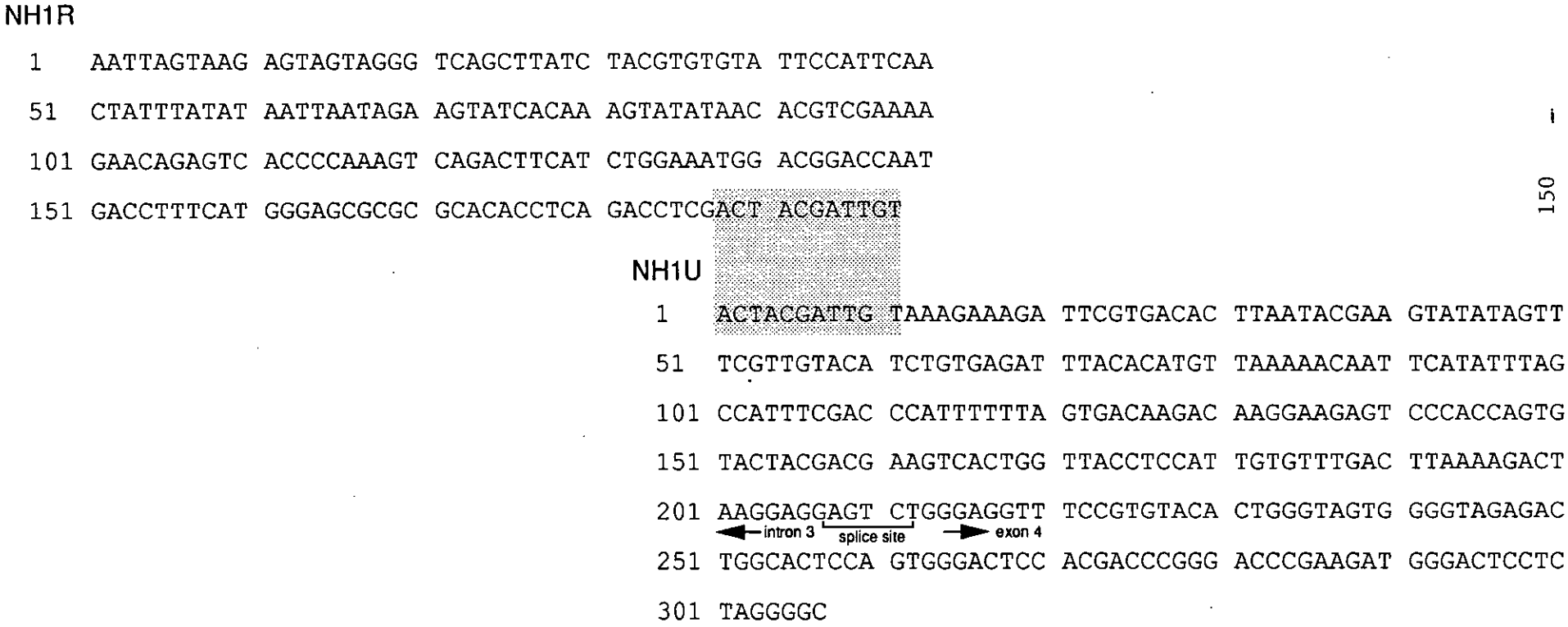

G A T C
G A T C

FIGURE 17. *NUCLEOTIDE SEQUENCE OF pNH1 OBTAINED FROM* THE *UNIVERSAL AND REVERSE PRIMER*

The area of overlap is shaded and the intron-exon boundary shown in determined by analogy with the human sequence.


NH1R
1 AATTAGTAAG AGTAGTAGGG TCAGCTTATC TACGTGTGTA TTCCATTCAA
51 CTATTTATAT AATTAATAGA AGTATCACAA AGTATATAAC ACGTCGAAAA
101 GAACAGAGTC ACCCCAAAGT CAGACTTCAT CTGGAAATGG ACGGACCAAT
151 GACCTTTCAT GGGAGCGCGC GCACACCTCA GACCTCGACT ACGATTGT
150
NH1U
1 ACTACGATTG TAAAGAAAGA TTCGTGACAC TTAATACGAA GTATATAGTT
51 TCGTTGTACA TCTGTGAGAT TTACACATGT TAAAAACAAT TCATATTTAG
101 CCATTTCGAC CCATTTTTTA GTGACAAGAC AAGGAAGAGT CCCACCAGTG
151 TACTACGACG AAGTCACTGG TTACCTCCAT TGTGTTTGAC TTAAAAGACT
201 AAGGAGGAGT CTGGGAGGTT TCCGTGTACA CTGGGTAGTG GGGTAGAGAC
intron 3
splice site
exon 4
251 TGGCACTCCA GTGGGACTCC ACGACCCGGG ACCCGAAGAT GGGACTCCTC
301 TAGGGGC

To date, these cDNAs are the only available BoLA class I sequences. From this alignment, NH1 appears to contain coding sequences of the third domain (exon 4) because the cDNA clones are similar to NH1 only in this domain. Only 99 bases of NH1 out of a total of 494 bases were aligned. This implies that the other 395 bases must be noncoding sequence (intron).

Since NH1 contains 99 bases encoding the start of the α_3 domain, it suggests that the intron sequences must be from intron 3 between the α_2 domain and the α_3 domain. No genomic BoLA class I sequences are available, so comparisons with known HLA genomic sequences was carried out. Alignment of NH1 with HLA-A, HLA-B and HLA-C showed that NH1 contains part of intron 3 and part of exon 4 (figure 18).

The NH1 sequence was translated into an amino acid sequence of 31 residues which was well conserved (figure 19) when compared to the other five BoLA clones. There was one amino acid different from BTBoLAA, BTBoLAB and BIMHBKNA; two amino acids different from BIMHAWA and 5 amino acids different from BTMHLA (which was used as the probe).

Ennis et. al. (1988) when comparing their two cDNA bovine class I clones, BTBoLAA (BL3-6) and BTBoLAB (BL3-7), found these 2 clones to be 95% identical to each other in the α_3 domain. There were five substitutions, four of which (S →P193, R → H219, E → D220 and E → D253) represented conservative replacements, while one (G → D196) was nonconservative. When they compared their two clones with known HLA sequences, they found most similarity between HLA-B and BoLA-A in the α_2 and α_3 domains. However, with the H-2 sequences compared, there was little similarity with both their BoLA clones.

When comparing NH1 with BTBoLAA, a single nonconservative replacement

FIGURE 18. *ALIGNMENT OF NH1 WITH HUMAN CLASS I GENE*

NH1 sequence was aligned with the human HLA-B27 allele using the UWGCG program BESTFIT. The sequence in the lower case is that of NH1 and the sequence in the caps is that of HLA-B27. The splice site (shaded) is determined by analogy with HLA-B27.

```
184  tgttctgttccttctcagggtggtcacat.gatgctgcttcagtgaccaa  136
1989 TGTCCTGTCCATTCTCAGGCTGGTCACATGGGTGGTCCTAGGGTGTCCCA 2038
135  tggaggtaacaca..aactgaattttctgattcctcc..tcagaccctcc   90
2039 TGAGAGATGCAAAGCGCCTGAATTTTCTGACTCTTCCCATCAGACCCCCC 2088
                                  intron 3  splice site  exon 4
89   aaaggcacatgtgacccatcaccccatctctgaccgtgaggtcaccctga   40
2089 AAAGACACACGTGACCCACCACCCCATCTCTGACCATGAGGCCACCCTGA 2138
39   ggtgctgggccctgggcttctaccctgaggagatccc    3
2139 GGTGCTGGGCCCTGGGCTTCTACCCTGCGGAGATCAC 2175
```

was found, the same as to that seen between BTBoLAA and BTBoLAB, that is, G → D196. Between NH1 and BTBoLAB, there is also only one substitution, S → P193. Ennis et. al. (1988) may have considered this to be a conservative replacement on the basis that residue 193 was not implicated in any functional aspect of the class I molecule. However, this substitution could also be considered to be nonconservative as serine has an uncharged polar side chain while proline has a non polar side chain. Whether the substitution is in reality conservative would depend on the function of residue 193.

TABLE 6.3. Comparisons of NH1 with the 5 cDNA (see also figure 19)

	cDNA clones	Nonconservative replacement	Conservative replacement
NH1 with	BTBoLAA BTBoLAB	D→G196 -	- P→S193
	BIMHBKNA BIMHAWA	- D→G196	D→E196 H→R191
	BTMHLA	D→G196	P→S193 E→D212 R→H197

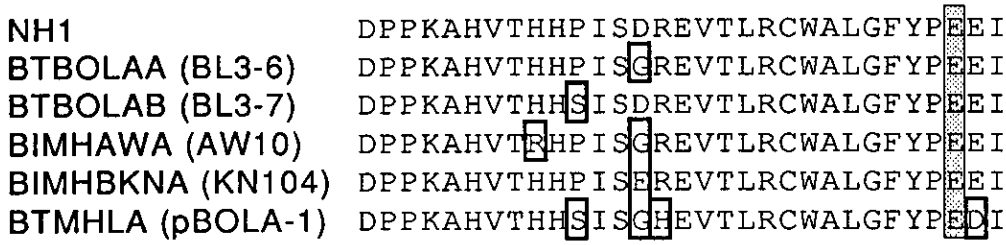
Interestingly the greatest number of substitutions seen between NH1 and the other five cDNA class I is with BTMHLA, which was used as the probe. Of the total 31 amino acid sequence, residue 196 is the most polymorphic residue with non conversative replacements in three out of five comparisons.

The amino acids involved in interaction with $ß_2$m, H(188), W(204) and L(206) are conserved in all six sequences. The cysteine at position 203 that presumably forms the internal disulphide bridge is also conserved in all six BoLA class I clones. The glutamate residue at position 211 is found in all six BoLA class I sequences but

FIGURE 19. *COMPARISON OF THE AMINO ACID TRANSLATION OF THE FOURTH EXON OF NH1 WITH THE SAME REGION OF THE 5 BoLA cDNA CLONES*

Potential bovine-specific conserved residues are shaded. Residues which differ from the NH1 sequence are boxed.

Third Domain (Exon 4)



is not found in the class I sequences of other species. Therefore this supports the view that NH1 encodes the α_3 domain of a class I BoLA molecule.

When NH1 was compared with 4 HLA clones, namely

HSHLIA	-	HLA-A3	(Strachan et. al., 1984)
HSHLABW	-	HLA-Bw57	(Girdlestone, J. 1990)
HSHLAB27	-	HLA-B27	(Weiss et. al., 1985)
HSHLIC	-	HLA-Cw3	(Sodoyer et. al., 1984)

NH1 was confirmed to have sequences of the third intron and the fourth exon (α_3 domain) (figure 18) and the exon-intron boundary, the 3' splice site (NCAGA) was conserved.

Comparison using BESTFIT between NH1 and the 4 HLA genomic clones gave an approximate 82% similarity. When only the exons of the 4 HLA clones were compared, NH1 was found to be more like the two HLA-B sequences than HLA-A or HLA-C (Table 6.4).

TABLE 6.4. Comparison of NH1 with the 4 HLA genomic clones

NH1 with	% similarity exons & introns	length of bases	% similarity exons	length of bases
HSHLIA	82.9%	187	90.8%	98
HSHLABW	82.9%	187	92.8%	98
HSHLAB27	82.9%	187	91.8%	98
HSHLIC	82.4%	187	87.7%	98

NH1 is 494 bases but only 187 bases were aligned with the four HLA sequences as the sequence of NH1 outside this segment did not align well. This was

very interesting because Lew et al. (1986) when comparing between loci or species in mouse and man found that the length of exons and introns were similar with the exception of introns 3 and 5 between mouse and man. Not only is intron 3 longer in mouse than in man but it is also variable between mouse class I loci. In contrast intron 5 is longer in man than in mouse but is similar between the HLA loci. This implies that the length of the introns between cattle and man is also variable. As shown in the table, the length of the exon is 98 bases. Therefore out of a total of 396 bases of intron sequence, only 89 bases could be compared between these two species. When NH1 was compared with two genomic mouse clones (Mmmhh2dk and Mmmhh2bd, Watts et. al. 1987), only 23 bases of intron could be aligned. A similar situation was observed between the 4 HLA genomic clones and the 2 genomic mouse clones (23 bases of intron).

Another possibility is that the length of intron 3 is similar between cattle and man, but the introns are only well-conserved near to the exon, and further from the exons there seems to be less sequence conservation, implying that selection for intron function here only affects the ends of the introns.

A comparison of the translated product is as shown in Table 6.5 and figure 20.

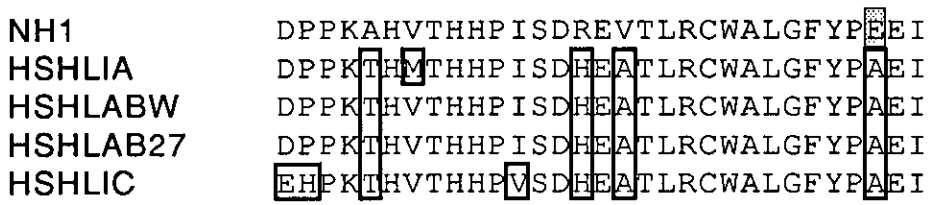
TABLE 6.5. Comparisons of translated products of NH1 with the 4 HLA clones

			REPLACEMENT		
NH1 with	% similarity	% identity	Total	conservative	Non conservative
HSHLIA	90.3%	83.8%	5	3	2
HSHLABW	90.3%	87.1%	4	2	2
HSHLAB27	90.3%	87.1%	4	2	2
HSHLIC	87.1%	77.4%	7	4	3

FIGURE 20. *COMPARISON OF AMINO ACID TRANSLATION OF THE FOURTH EXON OF NH1 WITH THE SAME REGION OF THE 4 GENOMIC HLA CLONES.*

The potential bovine-specific glutamate residue is shaded and differences from NH1 are boxed.

Third Domain (Exon 4)



A similar result is seen to the comparison of the nucleotide sequences. NH1 is most similar to HLA-B, followed by HLA-A and is least homologous with HLA-C. Out of the four amino acid substitutions between NH1 and HLA-B, three were at positions which are conserved for human class I genes (Table 6.6). The other difference between NH1 and HLA-B is at position 197 and is probably a conservative substitution (R → H). This suggests that the major differences between NH1 and HLA-B are at the species-conserved amino acid residues 187, 199 and 211.

TABLE 6.6. Comparison of 6 BoLA clones with HLA-B and other species

		Conserved in		
Position	Replacement	Bovine	Man	Defined by Klein and Figueroa (1986)
187	A → T	A	T	A - shared residue between rat, rabbit and some mouse.
				T - ancestral residue, conserved in man and found in some mouse
199	V → A	V	A	V - mouse/rat specific residue
				A - human/rabbit specific residue
211	E → A	E	A	E - not found in the 4 investigated species
				A - shared residue between mouse, rat, human and rabbit

In their evolutionary studies involving 4 species : mouse, rat, rabbit and human, Klein and Figueroa (1986) observed that shared residues were found throughout the α domains with α_3 being the most conserved domain (figure 21). This indicates that there was limited remodelling of the class I genes in the 4 species.

FIGURE 21. *COMPARISON OF AMINO ACID TRANSLATION OF THE FOURTH EXON OF THE 6 BoLA CLONES WITH THE SAME REGION OF GENOMIC AMINO ACID SEQUENCES OF MOUSE, RAT, HUMAN AND RABBIT*

The human, mouse, rat & rabbit alignment are from Klein and Figueroa, 1986.

Potentially species-conserved residues 187, 198, 199 and 211 are arrowed.

Class 1 α3 - 3rd Domain

a.

	185 190 195 200 205 210
NH1	DPPKAHVTHHPISDREVTLRCWALGFYPEEI
BTBOLAA (BL3-6)	DPPKAHVTHHPISGREVTLRCWALGFYPEEI
BTBOLAB (BL3-7)	DPPKAHVTHHSISDREVTLRCWALGFYPEEI
BIMHAWA (AW10)	DPPKAHVTRHPISGREVTLRCWALGFYPEEI
BIMHBKNA (KN104)	DPPKAHVTHHPISEREVTLRCWALGFYPEEI
BTMHLA (pBOLA-1)	DPPKAHVTHHSISGHEVTLRCWALGFYPEDI

b.

H-2K^{b}	DSPKAHVTHHSRPEDKVTLRCWALGFYPADI
H-2K^{d}	DSPKAHVTYHPRSQVDVTLRCWALGFYPADI
H-2K^{k}	DSPKAHVTRHSRPEDKVTLRCWALGFYPADI
H-2K$^{w29.7}$	DSPKAHVTHHSRPEDKVTLRCWALGFYPADI
H-2D^{b}	DSPKAHVTHHPRSKGEVTLRCWALGFYPADI
H-2D^{d}	DPPKAHVTHHRRPEGDVTLRCWALGFYPADI
H-2L^{d}	DSPKAHVTHHPRSKGEVTLRCWALGFYPADI
H-2Q7^{d}	DPPKAHVTHHPRSYGAVTLRCWALGFYPADI
H-2Q10^{b}	DPPKTHVTHHPGSEGDVTLRCWALGFYPADI
H-2Q10^{q}	DPPKTHVTHHPGSEGDVTLRCWALGFYPADI
H-2T1a^{b}	DPPKTHVTHHPRPEGYVTLRCWALGFYPADI
H-2T1a^{c}	DPPKTHVTHHARPEGDVTLRCWALGFYPAHI
RT1.1	DPPKAHVTLHPRPEGDVTLRCWALGFYPADI
HLA-A2	DAPKTHMTHHAVSDHEATLRCWALGFYPAEI
HLA-A3	DPPKTHMTHHPISDHEATLRCWALGFYPAEI
HLA-Aw24	DPPKTHMTHHPISDHEATLRCWALGFYPAEI
HLA-A28	DAPK THHAVSDHEATLRCWALGFYPAEI
HLA-B7	DPPKTHVTHHPISDHEATLRCWALGFYPAEI
HLA-Cw3	EHPKTHVTHHPVSDHEATLRCWALGFYPAEI
HLA-12.4	DPPKTHMTHHPISDHEATLRCWALGFYPAEI
HLA-328	NPPKTHVTHHPLSDHEATLRCWALGFYPAEI
RLA-1	DPPKAHVTHHPASDREATLRCWALGFYPAEI
RLA-2	DPPKAHVTHHPASDKEATLRCWALGFYPAEI

6.4 *DISCUSSION*

It was intended to determine the nucleotide sequence of 19.1 corresponding to the 5' half of pBoLA-1 which encodes exons 2-4. Since 19.1 has been shown to encode a w11 gene, it would be useful to know the nucleotide sequence and the amino acid sequence (translated products) of a w11 gene.

To date there are 5 BoLA class I sequences, of which only two are of known haplotypes - w10 and KN104. w10 is an internationally recognised specificity whilst KN104 is a specificity observed in a cattle breed (Ndama) in Kenya (Bensaid et. al., 1991). The other three clones, BL3-6 & BL3-7 (Ennis et. al., 1988) and pBoLA-1 (Brown et. al., 1989a) are of unknown haplotypes.

Unfortunately as a result of limitation of time, only part of the third intron and part of the fourth exon was sequenced. If complete sequencing of 19.1 was done, this would have been the first sequenced genomic class I DNA.

However with the limited sequencing result obtained it has been shown that 19.1 is a class I molecule as there is more than 85% similarity with the other 5 BoLA class I molecules (Ennis et al., 1988).

It was not possible to determine whether these six bovine clones were allelic to one another because the α_3 domain is highly conserved. However, because part of the α_3 has a little polymorphism, some comparison between species and within species was possible.

Since only BoLA class I cDNA sequences were available, no comparison of the intron sequences in NH1 was possible. As there is less conservation of intron sequences between species than within species, the comparison with human class I introns did not yield much useful data.

An interesting observation was found when comparing the six bovine clones

with the human, rabbit, mouse and rat sequences compared by Klein & Figueroa (1986), Ennis et. al. (1988) and Brown et. al. (1989a) and is shown in Table 6.7 and figure 21.

TABLE 6.7. Comparison of cow, human, rabbit, mouse and rat sequences in the 99 bases of the α_3 domain

Position	Amino Acid	Species
198	E	rabbit, bovine & human
	K/E/D/A/Y	mouse & rat
199	V	mouse, rat & bovine
	A	rabbit & human
211	E	bovine
	A	mouse, rat, rabbit & human

The conservation of residues among the 5 species does not follow any obvious evolutionary path. Another interesting observation is the unique glutamate residue in position 211 for bovine. From this comparison E211 seems to be a bovine-specific residue.

If the whole gene was sequenced,

i) it would have been the first serologically characterised bovine class I gene (w11).

ii) comparison in the conservation and polymorphism of the w11 sequences with the BoLA cDNA clones and other species can be made.

iii) identification of species-specific residues would be possible when combined with the sequences of the 5 other BoLA clones.

The evidence of BoLA having a second class I locus remain circumstantial (Lindberg & Andersson, 1988, Ennis et al. 1988, Toye et al. 1990, Bensaid et al. 1991, Joosten et al. 1992 and Al-Murrani et al., manuscript in prep). If there are indeed two or more class I loci, sequencing of these class I genes would provide evidence. The complete sequence of the w11 gene from this study could be compared with the five known BoLA class I cDNA clones (two are of known identity, w10 and KN104, Bensaid et al. 1991). Comparison with the w10 sequence would be most interesting as Al-Murrani et al. (manuscript in prep) have circumstantial evidence for w10 and w11 to be on different loci.

CHAPTER 7

GENERAL DISCUSSION

Although BoLA was designated the cattle equivalent of HLA and H-2 in 1978 (Spooner et. al. and Amorena et. al.), little is known about it as compared to the other 2 complexes.

In the 4 international serological comparison tests conducted to study the BoLA MHC, only 1 locus with multiple alleles was detected. All the alloantisera available detect the products of only one locus and no recombination events have been seen in population studies as measured by the microlymphocytotoxicity test. One hypothesis commonly accepted in serology for not obtaining more than 2 BoLA types in an animal is linkage disequilibrium. If there was more than 1 locus, they must be very close to one another to reduce recombination events. The alleles cannot assort independently but segregate together and are therefore in linkage disequilibrium. This may explain the absence of alloantisera that will identify different loci.

There is however circumstantial evidence in RFLP, IEF, sequencing and gene expression studies to suggest the possibility of more than one BoLA locus (Lindberg & Andersson, 1988, Ennis et al., 1988, Joosten et al., 1988, Toye et al.,. 1990, Bensaid et al., 1991, Joosten, et al., 1992 and A-Murrani, et al., manuscript in prep). Clearly, having several class I loci, with each locus having multiple alleles would provide the extensive polymorphism that seems to be a characteristic feature of a class I molecule.

This study was aimed to analyse the BoLA class I MHC genes. The first step taken was to construct a genomic library from an animal with a known BoLA type and to isolate class I genes which were potentially complete (chapter 3). Identifying a clone with a functioning gene as opposed to pseudogene was an obvious priority

(chapter 4). The functional and structural experiments performed after the isolation of an active, cloned BoLA class I gene of known type are described in chapters 5 and 6.

This study resulted in the isolation of an active BoLA class I gene with the w11 specificity which is expressed and performs as a class I molecule in an alloreactive cytotoxicity T cell assay and which in the future will be used as a model for studying antigen presentation by class I molecules. Fourteen other clones which potentially encode complete class I genes were also isolated and a genomic library from a well characterised animal has been constructed. These may all be of use in extending investigations of the bovine MHC.

Further interesting investigations will include:

i) Complete sequencing of the w11 gene in clone 19.1

ii) Purification of the other 14 clones from the contaminant and the identification of complete class I genes which are either w10 or perhaps an unidentified BoLA type. There are 3 locus-specific regions identified in mouse and man, i.e. transmembrane, cytoplasmic and 3' untranslated regions. The availability of complete sequences of w11, w10 or the unidentified BoLA type would allow comparison in these regions to identify them as one or different locus products in the BoLA class I region. Comparison could also be made with the w10 cDNA sequence (Bensaid et. al. 1991).

iii) Transfection studies which would then provide information on these complete genes being active (as performed in chapter 4). An active isolated gene is essential for functional studies involving antigen presentation. It is also useful to use a cocktail of class I monoclonal

antibodies in identifying expression of gene products.

iv) Cytotoxic T cell assays would identify the gene products as functioning class I molecules. Flyer et. al. (1983) in their studies involving class-I restricted antigen presentation using a BALB/c - 3T3 cell line showed that these fibroblast cells could function as an antigen presenting cell that expresses both antigen and MHC molecules. The Ltk 19.1 was shown to function as a antigen presenting cell in the alloreactive cytotoxicity T cell assay. It may also function similarly in a specific cytotoxic T cell assay. The Ltk 19.1 could be transfected with an antigen linked to a selectable marker and expression of the antigen could be detected by an indirect immunoflourescence test on the FACS (as in chapter 3). The effector cells (anti-w11 CTLs) could be generated against the specific antigen and the effects shown in a ^{51}Cr-release assay (as in chapter 5). Data from these experiments would suggest whether the w11 gene in Ltk 19.1 could function as a class I molecule.

v) Analysis of the gene products of w10 or other unidentified BoLA type with 1D-IEF. 1D-IEF results of class I molecules from animals with BoLA type w10/w11 showed evidence of the gene products of w10 and w11 being at two different loci (Al-Murrani et. al. manuscript in prep). However, there is the possibility that the IEF assigned w10 and w11 bands are a composite of w10 and w11 with another unidentified BoLA type. If these BoLA types, as a result of linkage disequilibrium, are always detected with either the w10 or w11 products, identification of other specific gene products may perhaps be

impossible. However, analysis of clones encoding a particular gene would specifically show the products of the gene. This study would provide clones encoding not only w10 but perhaps other unidentified BoLA types for isoelectric focusing studies.

This thesis described the first successful *in vitro* expression of a complete BoLA class I gene in a transfection system. The application of this approach should give some insights into the structure and function of the bovine MHC.

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APPENDIX 1
BUFFERS, REAGENTS AND TECHNIQUES IN MOLECULAR CLONING

(A) *Buffers and reagents*

1. TE buffer
   10 mM Tris. pH8
   0.1 mM EDTA pH8

2. *Electrophoresis buffer*

   a) TBE - STOCK 10 x TBE
   Per liter : 108g Tris
   55g Boric acid
   40ml 0.5 M EDTA pH8
   used at 1 x TBE

   b) TAE - Stock 50 x TAE
   Per liter : 242g Tris
   57.1ml glacial acetic acid
   100ml 0.5 M EDTA pH8
   used at 1 x TAE

3. *MOPS/EDTA electrophoresis buffer (10x) ph7*

   0.2 M MOPS (3 - (N - morpholino propanesulphonic acid) (Sigma, Chemical Co.,Poole, England).
   0.5 M sodium acetate
   0.01 M EDTA
   used at 1 x MOPS/EDTA

4. *Formamide (Bio Rad analytical grade)*

   The formamide was deionized by stirring on a magnetic stirrer with a mixed bed resin AG - 501 - X8 (D) for 8 hours, filtering through Whatman No. 1 filter paper into eppendorf tubes and stored at -20°C.

5. *SSC*

   STOCK 20 x SSC
   Per liter : 175.3g NaCl
   88.2g sodium citrate
   used at 20 x SSC and 2 x SSC

6. *Loading buffer*

i) For DNA on agarose gels per 15ml

30% Ficoll 400
0.1% SDS
40 mM EDTA/Na pH8
1.2mg/ml bromophenol blue

ii) For RNA on MOPS/FORMALDEHYDE gels

50% v/v glycerol
0.1mg/ml bromophenol blue
autoclave before use

7. *Hybridisation buffer*

0.5 M Na_2HPO_4 pH7.2
1% SDS
1 mM EDTA at 65°C

8. *Hybridisation wash*

Low stringency wash at 65°C	40 mM Na_2HPO_4 pH7.2 1% SDS
High stringency wash at 65°C	20 mM Na_2HPO_4 pH7.2 0.1% SDS

9. *Ligase buffer*

0.2 M Tris HCl pH7.6
0.05 M $MgCl_2$
0.05 M Dithiothreitol (DTT)
500μg/ml BSA
ATP to a final concentration of 1 mM in the final reaction

10. *Dephosphorylation buffer*

0.01 M $ZnCl_2$
0.01 M $MgCl_2$
0.1 M Tris HCl pH7

11. *Glucose buffer*

50 mM glucose
25 mM Tris HCl pH8
10 mM EDTA pH8

12. *High salt solution*

3 M potassium acetate
1.8 M formic acid

13. *Acrylamide stock solution*

95g acrylamide (Sigma Chemical Co. Poole, England)
5g N, N-methylenebisacrylamide
made to 250ml with autoclaved distilled water

14. *Lambda DNA molecular-weight marker* (Boehringer Mannheim GmbH, W. Germany)

Lambda DNA digested with Hind III were used as standard in DNA agarose gels. The marker included fragments at 23.1, 9.5, 6.7. 4.4. 2.3, 2 and 0.6kb.

B. *TECHNIQUES*

Purification of DNA with phenol/chloroform extraction

Proteins are removed from nucleic acid solutions using phenol, phenol:chloroform and chloroform extractions. On the day of extraction, phenol is equilibrated to a pH of 8.0 by mixing phenol and 0.1 M Tris.HCl pH8 several times. The equilibrated phenol can be stored with an equal volume of 0.01 M Tris HCl pH8 at 4°C. It is important to equilibrate the pH of phenol because nucleic acids tend to be in the lower organic phase and not the upper aqueous phase when the pH of phenol is not between 7.8-8.

An equal volume of phenol is added to the DNA solution, mixing thoroughly and centrifuging to separate the organic from the aqueous phase. The aqueous phase (DNA containing phase) was then transferred to a clean tube and extracted with an equal volume of phenol:chloroform mixture (1:1, mixing them thoroughly and centrifuging to separate the two phases. The aqueous phase is then transferred to a fresh tube and an equal volume of chloroform is added. Mixing and centrifugation was carried on as earlier and the aqueous phase was removed to a clean tube for subsequent procedures.

2. *Ethanol precipitation of DNA*

Ethanol is used to concentrate DNA. 2.5 volumes of 100% ethanol with 0.3 volumes of Na Acetate pH7 were added to the DNA solution and incubated at either -20°C or -70°C for a minimum period of 3 hours or 30 minutes respectively. The DNA was recovered by centrifugation at 11000rpm for 30 minutes and the supernatant decanted. It was then washed with 70% ethanol, centrifuged and excess ethanol was evaporated in a vacuum drier for 5 minutes. It was resuspended in an

appropriate volume of TE pH8 and stored at 4°C or -20°C.

3. *Spectrophotometric analysis of DNA and RNA samples*

The concentration and purity of DNA and RNA samples was determined on the spectrophotometer with a deuterium light source at 260 and 280nm. An optical density reading of 1 at 260nm equals 50μg DNA/ml and 40μg RNA/ml. The 260/280nm ratio gives the base content, that is, 1.8 for DNA and 2.0 for RNA.

4. *Isolation of DNA from agarose gels*

A derived fragment of DNA to be used as a probe would have to be recovered from a gel. The gel section containing the fragment was exercised with the help of a UV transilluminator. Dialysis tubing was boiled with 1-2 mM EDTA pH8 for 10 minutes, allowed to cool and stored at 5°C. The tubes were washed thoroughly with distilled water before use. Approximately 0.5-1ml of 1 x TAE was filled into a dialysis bag and the slice of agarose transferred into the bag. The bag was immersed in a shallow layer of 1 x TAE in an electrophoresis tank. An electric current 4-5v/cm for 2-3 hours was passed through the bag. When the DNA is electroeluted out of the gel and onto the inner wall of the bag (this process can be confirm when viewed on the UV transilluminator), the polarity of the current was reversed for one minute to release DNA from the inner wall of the bag. The buffer was then transferred to a fresh disposable plastic tube. The dialysis bag was rinsed a few times with 1 x TAE and the wash added to the tube. The gel slice could be immersed in 1 x TAE containing ethidium bromide (0.5μg/ml) for 30-45 minutes to confirm that all the DNA was eluted. The DNA in the tube was purified using the DE52 column.

5. *Purification of DNA*

In this method, an anionic matrix is used to bind the negatively charged DNA passing through the column. The contaminants are washed though the column. The DNA is then eluted from the matrix by increasing the ionic strength of the elution buffer. The DE 52 column was constructed in a disposable pasteur pipette plugged with a siliconised glass wool plug. Approximately 500-600μl of DE 52 was added to the pipette slowly avoiding any air bubbles. The column was rinsed with TE pH7.6. The DNA sample is passed through a few times to ensure all DNA gets attached in the column. The column was washed twice with 1.5ml of TE pH7.6 containing 0.1 M NaCl. The DNA was eluted with three 0.5ml washes of TE pH7.6 containing 0.6 M NaCl. The elute was extracted with phenol:chloroform and precipitated with ethanol (appendix 1). The DNA was collected by centrifugation and its concentration determined by an aliquot on an agarose gel or through the spectrophotometer.

Alternatively, the DNA was removed from the agarose by 'gene-clean' (Geneclean II Kit, Stratech Scientific Ltd., Luton, England). In this technique, DNA is isolated using a slice matrix that binds the DNA but not to the agarose. The procedure was carried out as instructed by the manufacturer. Briefly, the fragment of DNA was excised from the gel and placed in an eppendorf tube. Three volumes of 6 M sodium iodide (NaI) were added. The mixture was incubated at 55°C to dissolve the agarose. The silica matrix was added to bind the DNA and the eppendorf tube was centrifuged. The supernatant containing the agarose was removed. The silica pellet was washed three times with the wash solution and the DNA recovered from the matrix by incubation at 55°C for 2 minutes in 10-20μl of water.

6. *Preparation of plasmid/cosmid DNA*

An overnight culture of *E.coli* containing plasmid was set up in 10mls of LB with antibiotic. This is used to inoculate 2 litres of LB and grown to stationary phase overnight. The cells were harvested by centrifugation at 8000rpm for 10 minutes and the supernatant discarded. The pellet was resuspended in 25mls of lysis buffer (50 mM glucose, 25 mM Tris HCl pH8, 10 mM EDTA/Na pH8 with 2mg/ml lysozyme freshly added). The centrifuge pots were left on ice for 30 minutes and were lysed by addition of 60mls of 9:1 0.22 M NaOH/10% SDS. The pots were incubated on ice for a further 5 minutes before adding 30mls 5 M K acetate pH5. Incubation was carried on for a further 15 minutes to ensure precipitation of protein and high molecular weight DNA. They were centrifuged at 8000rpm for 30 minutes at 4°C to remove the debris. The supernatant was carefully powered into fresh pots and the plasmid DNA was precipitated with 0.65 volumes of isopropanol for 30 minutes at room temperature.

7. *Autoradiography*

Autoradiography were done with X-ray film (Agfa-gevaert film RPI). Films were exposed at -70°C with Dupont intensifying screens for filters probed with ^{32}P.

8. *Photography*

The agarose gels were stained with 0.5μg/ml of ethidium bromide. Ethidium bromide is a fluorescent dye which contains a planar group that intercalates between the stacked bases of DNA. When illuminated with a UV transilluminator at a wavelength of 302nm, the bound DNA can be visualised. Ethidium bromide is used to detect both DNA and RNA, but the affinity for RNA is relatively low and the

fluorescent yield is poorer than for DNA. The gel is photographed using a polaroid camera whilst illumination from below with a uv transilluminator.

APPENDIX 2

BACTERIAL GROWTH MEDIA AND ANTIBIOTICS

1. *Phage buffer.*

This buffer is used for storage and dilution of bacteriophage λ stocks.

10 mM Tris HCl pH7.5
10 mM Mg804
0.01% gelatin
The phage buffer was autoclaved

2. *Caesium chloride solutions for step gradients prepared in phage buffer*

Density	CsCl	Phage buffer (ml)
1.3	23.43	51.57
1.5	34.05	40.92
1.7	42.18	32.84

3. *LB medium*

Per liter :	Bacto-tryptone	10g
	Bacto-yeast extract	5g
	NaCl	10g

Adjust pH tp 7.5 with sodium hydroxide and autoclaved as 500ml stocks.

4. *LB plates*

The LB media is made up according to the above formula and before autoclaving, one of the following was added (per liter) :

Bacto-Agar 15g (for plates)
Agarose 7g (for top agarose)
For JM109

The agar was dissolved by heating to boiling point and once cooled, ampicillin (50μg/ml) was added and 25-30ml poured into 90mm plastic petri-dishes to set.

5. *Minimal medium*

Distilled H_2O	750ml		
5 x M9 salts	200ml		
Glucose 20%	20ml		
5 x M9 salts per liter:		Na_2HP0_4 - $7H_2O$	64g
		KH_2PO_4	15g
		NaCl	2.5g
		NH_4Cl	5g

Minimal agar plates

The minimal agar plates were prepared by the addition of 15g/l of bacto-agar to minimal medium before autoclaving.

6. *Preparation of frozen stocks of E. coli*

To 0.85ml of a bacterial culture, 0.15ml of glycerol was added and vortexed for 10 seconds to ensure mixing. They were then transferred to sterile eppendorf tubes and frozen at -70°C. Bacterial strains were revived by using a sterile pipette tip to scrape the surface of the frozen stock and transferring a small sample to a 10ml sterile universal containing L-broth.

7. *Maltose*

A stock solution of 20% maltose is prepared and autoclaved. Maltose (0.2%) is added to the medium during growth because the sugar induces the maltose operon, which contains the gene (lamB) that codes for the bacteriophage λ receptor. Therefore bacteria grown in the presence of maltose adsorb bacteriophage λ more efficiently.

8. *MgSO4*

A stock solution of 1 M MgSO4 is prepared and autoclaved. MgSO4 is used

at a concentration of 10 mM in the bacteria growing medium or resuspending the cells.

9. *Ampicillin* (Sigma Chemical Co. Poole, England)

A stock solution of 50mg/ml is prepared in water.

APPENDIX 3

TISSUE CULTURE MATERIALS

1. *L cell growth medium*

   i) 10mls of 10 x Dulbecco's modified eagles medium
   5-5.2mls sodium bicarbonate 7.5% (to get pH6.8 to 7.2)
   2mls L Glutamine
   10mls foetal calf serum
   0.5mls gentamycin
   to 100mls with autoclaved distilled water

   ii) *Selection medium*

   The recipe is as above but 2mls of 50 x HAT is added to select for the transformants.

2. *Phosphate buffered saline (PBS)*

Per 5 litres:	NaCl	42.5g
	Na_2HPO_4	5.35g
	NaH_2PO_4	2.55g

made up in distilled water and autoclaved.

3. *TEN buffer*

150 mM NaCl
10 mM Tris HCl pH7.8
1 mM EDTA

4. *Proteinase K solution*

0.4mg/ml proteinase K (Sigma) in TEN with 0.4% SDS. Incubate at 37°C for at least 10 mins to predigest contaminating RNAase and DNAase.

5. *Earle's balanced salt solution (EBS)*

10 x EBS (Gibco)

Used at 1 x EBS supplemented with

5ml 1 M HEPES buffer pH7.2 - 7.4
0.1% (w/v) sodium azide
3% foetal calf serum
to 500ml with autoclaved distilled water

6. *Calcium phosphate transfection reagents*

All stocks were filter sterilised and stored at -20°C.

i) Calcium chloride ($CaCl_2$) : 2 M stock solution (Sigma Tissue Culture Tested)

ii) Disodium phosphate (Na_2HP0_4) : 0.2 M stock solution (Sigma Anhydrous Tissue Culture Tested)

iii) Sodium chloride (NaCl) : 1 M stock solution (Sigma Tissue Culture Tested)

iv) HEPES : 1 M stock solution (Northumbria Biologicals Ltd)

v) 2 x HEPES buffered saline (2 x HBS)

5ml of 1M HEPES
0.75ml of 1 M Na_2HPO_4
28.8ml of 1 M NaCl
made up to 100ml with autoclaved distilled water and the pH adjusted to 6.95 with HCl.

APPENDIX 4

MICROLYMPHOCYTOTOXICITY MATERIALS

1. *Hank's balanced salts (HBSS) (Gibco BRL)*

   10 x STOCK

   1 tub of dry HBSS
   100ml H_2O
   a few drops of $NaHCO_3$ until colour just changes
   0.4 units heparin solution (5000 units/ml)

   Stored at 4°C

   used at 1 x HBSS
   2 x HBSS

2. *Eosin stain (2', 4', 5', 7' - tetrabromofluorescein) (Sigma).*

   5% eosin in 1 x HBSS

   Stored at 4°C. Stable for 1 week.

3. *Fixing solution*

   40ml formaldehyde
   60ml 0.9% NaCl
   5ml 0.15 M Na_2HPO_4

APPENDIX 5

THE TRANSFECTION AND EXPRESSION OF A BOVINE CLASS I MHC GENE

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INTRODUCTION

The MHC class I molecules are polymorphic membrane glycoproteins expressed on most cell types, which play an important role in immune responsiveness (Zinkernagel and Doherty 1974, Nathenson et al 1986, Koller et al 1987). The bovine MHC (BoLA) class I antigens have been identified by serology, and the majority of sera accepted by 4 international workshops apparently identify alleles of a single codominantly expressed locus (Spooner et al 1979, Anon 1982, Bull et al 1989, Bull et al 1992). With all BoLA specificities that have been examined there has been close correlation between the serological definition and specificity of cytotoxic T cells (Preston et al 1983, Teale et al 1985, Goddeeris et al 1986, Spooner et al 1987 Innes et al 1989).

Extensive efforts have been made to identify the products of additional BoLA class I loci serologically, however, tight linkage has resulted in sera being predominantly against the products of one locus. Isoelectric focusing of BoLA class I antigens immunoprecipitated with non-polymorphic monoclonal antibodies has revealed the products of additional BoLA loci (Joosten et al 1988) and immunoprecipitation with allo-antisera has revealed the bands that arise from the antigens that are identified by the sera (Al-Murrani et al manuscript in prep). To date 5 BoLA class I cDNA clones have been reported (Ennis et al 1988, Brown et al 1989, Bensaid et al 1991), comparison of their sequences data suggest that the clones arise

from two genetic loci. Bensaid et al (1991) has used field inversion electrophoresis to demonstrate that cDNA clones coding for the w10 and KN104 antigens arise from distinct, but closely linked loci.

In order to more fully characterise the BoLA class I antigens and to provide tools for addressing the question of how parasite antigen is processed and presented in association with MHC class I antigens, we have identified BoLA class I clones from a genomic library prepared from an animal with a well characterised BoLA serological phenotype. These were transfected into murine L-cells and one expressed clone was identified which codes for the w11 antigen. This was identified on the surface of transfected L-cells by serology, cytotoxic T cells and isoelectric focusing.

METHODS

Cloning

A genomic library was prepared in EMBL-3 from peripheral blood mononuclear cells (PBM) of a cow expressing w10 and w11 antigens. The library was screened with a bovine class I MHC cDNA clone, pBoLA -1 (Brown et al 1989), which contained the entire coding sequence. Forty nine positive plaques were selected, these were further screened for those that contained the entire coding region by successive rounds of screening using the 5' then the 3' regions of pBoLA-1.

DNA mediated gene transfer

Calcium phosphate mediated co-transfection with the thymidine kinase (tk) gene into Ltk^- cells was carried out as described by Wigler et al (1979). DNA from lambdaphage containing BoLA class I clones was mixed with a tk containing plasmid pTk (Lang et al 1983) and high molecular weight mouse embryo carrier DNA. The

DNA was precipitated onto Ltk$^-$ cells and left for 15 hr before washing in PBS. The cells were then grown in DMEM medium (Gibco) for 24 hr before applying selection in hypoxanthine-aminopterine-thymidine (HAT) containing medium. The cells were then grown for 2-3 weeks in HAT medium before analysing them for the expression of the bovine MHC product.

FACS Analysis

This was performed essentially as described for PBM (Glass and Spooner 1990). Cells were grown to high density avoiding confluence, removed from tissue culture flasks with EDTA and labelled with either monomorphic mouse anti-bovine class I monoclonal antibody (IL-A88 ILRAD Nairobi). The second stage anti-mouse in the indirect immunofluorescence assay was FITC labelled (Nordic). Labelled cells were analysed on a FACScan (Becton Dickinson). In populations of cells in which expression was observed the expressing cells were purified by sorting on a FACS IV (Becton Dickinson). Three rounds of growth and purification were required to achieve a population of 100% expressing cells.

Microlymphocytotoxicity Test

The micro-lymphocytotoxicity test was performed as described by Spooner et al (1979).

Mapping clones

Single and double restriction enzyme digestions were carried out with six enzymes, BamHI, EcoRI, HindIII, SacI, SalI and SmaI. Fragments were separated on 1.5% agarose gels in TBE buffer (Sambrook et al 1989) and transferred to

Hybond-N membranes (Amersham International). Membranes were probed with either the 3' or 5' regions of pBoLA-1, to reveal the location and orientation of the class I gene. The 7kbp BamHI fragment from clone 19.1 that contained the BoLA class I gene was digested with Sau3A and subcloned into the BamHI site of pBS (Stratagene). The subclones were screened with the 5' region of pBoLA-1 and positive clones sequenced by di-deoxy chain termination using a T7 DNA polymerase kit (Pharmacia).

BIOCHEMICAL ANALYSIS OF MHC EXPRESSION

Metabolic Radiolabelling

Transfected and Ltk$^-$ (H-2K^k) cells were starved for 30 min in methionine free medium MEM (Gibco) supplemented with 10% foetal calf serum (10^8 were used for one immunoprecipitation). Three hundred μCi of ^{35}S labelled methionine (Amersham) were then added and the cells incubated at 37°C for 6 hr. As a control 10^7 peripheral blood mononuclear cells (PBM) were labelled as above but using 50 μCi of radiolabelled methionine and incubation was extended overnight.

Immunoprecipitation

Labelled L-cells were removed from the culture flasks by 2% EDTA and washed once with PBS. They were then processed for analysis by isoelectric focusing as described by Joosten et al (1988). MHC class I antigens were precipitated with 5μl mAb recognising either BoLA class I antigens (IL- A88 ILRAD, Nairobi), mouse H-2K^k (MCA172, Serotec), or an anti-HLA class I mAb that recognised the class I products of mice and cattle (w6/32, Serotec), alternatively 50μl bovine anti-BoLA w11 alloantiserum (Ed 102) were used. Pansorbin (Calbiochem) was used to

precipitate mAbs, while Gammabind-G Sepharose (Pharmacia), which has a higher affinity for bovine immunoglobulin, was used for alloantisera.

Isoelectric focusing

MHC antigens were separated by IEF as described by Joosten et al 1988. Focusing was performed overnight and the gels were then fluorographed, dried and exposed to X-ray film (Kodak X-OMAT) for 7 days at -70°C.

Generation of cytotoxic T lymphocytes (CTL)

Alloreactive cytotoxic T cell lines specific for the BoLA w11 class I antigen of the donor cow, from which genomic library was prepared, were generated *in vitro* from a responder animal with class I MHC phenotypes w3/w10 in an allogeneic mixed lymphocyte culture as described by Teale et al.(1985) and Spooner et al. (1987). The responder cells were restimulated every week with irradiated peripheral blood mononuclear cells (PBM) from the donor animal. After four rounds of restimulation, T helper ($CD4^+$) cells were removed by complement mediated lysis using the mAb cc8 (Howard, 1991) to enrich the cell population for T cytotoxic ($CD8^+$) cells. This resulted in killing of 32% cells. The remaining Tc cells were restimulated in 6 well plates by addition of 5 ml aliquots of responder and irradiated donor cell suspensions at $1x10^6$ cells per ml and $2.5x10^6$ cells per ml, respectively. IL-2 (Boehringer Mannheim, BCL) was added to each well at a concentration of 10 i.u. per ml. The cells were restimulated every week and maintained as continuously growing cell lines. FACS analysis on these cells revealed that about 97% cells were CD8 positive using the mAb IL-A51 (Morrison et al 1989).

Cytotoxicity assay

A standard 4 hr chromium release assay was performed as described previously by Teale et al. (1985) and Spooner et al. (1987). *Theileria annulata* Hissar (Tah) infected lymphoblastoid cell lines from the donor animal, animals sharing one BoLA specificity with the donor and BoLA mismatched animals were used as target cells for establishing specificity of effector cells. The procedure was slightly modified when using adherent transfected L-cells and control Ltk$^-$ cells as the target cells. These cells were harvested with EDTA 16 hours before performing the cytotoxicity assay, resuspended in medium and incubated overnight in tissue culture untreated polystyrene petri dishes (Sterilin or Corning) to which they do not adhere. The cells were washed twice with RPMI 1640 medium (Gibco) immediately prior to use in the assay (Townsend et al.,1984). Since L-cells are more resistant to lysis than PBM they were incubated with effector cells for 6 hours (Simpson & Chandler,1986).

RESULTS

A total of 49 independent clones were selected following screening with pBoLA-1, of these 15 hybridised with both 3' and 5' ends of the cDNA coding sequence and were considered candidates for full length genes. These 15 clones were therefore tested for expression by transfection into murine L-cells and analysed by flow cytometry (FACS) using a monomorphic anti-bovine class I MHC antibody, IL-A88. Out of the 15 clones only one, 19.1, showed significant levels of expression and was further characterised.

The 19.1 clone contains a 21kbp DNA insert with the BoLA gene contained within a 7kbp BamHI fragment. This fragment has 2kbp of flanking DNA at the 5'

end, with respect to the orientation of the gene and 12kbp at the 3' end. A 500 bp Sau3A subclone from the BamHI fragment which contained regions that hybridised with the α_1, α_2 and some of the α_3 regions (exons 2 - 4) from pBoLA-I was sequenced (NH1). NH1 covers 99 bp of coding region of exon 4 running into intron 3. The sequence (NH1) was compared with the 5 available BoLA class I cDNA sequences and human genomic class I sequences representing A, B and C locus genes. There is 92 - 97% sequence homology between NH1 and the other BoLA sequences. Translation of the NH1 sequence gives 31 amino acids of the α_3 domain. This sequence shows a single amino acid substitution when compared with the sequences derived from the published BoLA cDNA clones, BTBOLA A and B (Ennis et al 1988) and with BIMHBKNA which codes for the BoLA KN104 antigen (Bensaid et al 1991). The clone BIMHAWA, which codes for the w10 antigen (Bensaid et al 1991) has two amino acid substitutions as compared with NH1. Interestingly NH1 has 4 amino acid substitutions in comparison with pBoLA-1 which was used as the probe (figure 1a).

Amino acids at positions 118 (H), 204 (W) and 206 (L) are involved in the interaction with $ß_2$ microglobulin and are conserved between the 6 BoLA cDNA sequences. The cysteine residue at position 203 that is involved in the formation of the internal disulphide bond is conserved in all 6 BoLA sequences. The consensus 3' splice site (NCAGA) is present in NH1.

Comparison of NH1 with class I HLA genomic sequences shows about 82% sequence similarity. The NH1 amino acid sequence most closely resembles that of the HLA-B locus, with 4 amino acid substitutions, whereas a greater number of substitutions can be seen between NH1 and HLA A or B locus sequences. Residues at position 187, 199 and 211 are conserved between all the BoLA sequences.

Likewise these residues are conserved between the HLA class I molecules, but with different residues from those present in the BoLA molecules. Therefore residues 187, 199 and 211 are species specific and differentiate between the bovine and human class I MHC molecules (Figure 1b).

Two weeks following transfection 5.4% of cells were showed binding to IL-A88 and were thus expressing a BoLA antigen. The expressing cells were enriched by three rounds of FACS sorting yielding a 100% BoLA class I expressing population (figure 2). These cells have now been kept in continuous culture for over a year with no appreciable loss of expression of the BoLA antigen. The identity of the expressed antigen was investigated in a standard lymphocytotoxicity assay used for MHC typing bovine PBM. Three allo-antisera were used to define the w11 specificity Ed 73, Ed 76 and Ed 110. Two of these three routinely killed the transfected L-cells in the assay, and failed to kill the control L-cells. Serum ED 76 which failed to react is a low titre serum used neat when typing with bovine PBM. Control L-cells were negative for all the w11 sera while both the control and the transfected L-cells were negative with non-w11 sera.

Isoelectric focusing results for BoLA class I antigens from PBM of the donor animal are presented in figure 3 Between 8 and 10 bands are seen following precipitation with the mAb w6/32. Bands that have previously been assigned to w11 and w10 haplotypes (Joosten et al 1988) are indicated. A greater number of bands ($\sim$50) are seen following precipitation with mAb IL-A88, some corresponding to those seen for w6/32 can be assigned to haplotypes, the additional bands have not been assigned to BoLA haplotypes at present. Six bands are seen by IEF following precipitation of MHC antigens from PBM using allo-antiserum ED102, which is specific for the BoLA w11 haplotype. The pattern of bands for mouse class I

molecules precipitated from the control L-cells is identical using the anti-H2 mAb (MCA17) and the anti-HLA mAb (w6/32). The anti-BoLA mAb (IL-A88) does not recognise the mouse class I antigens while the anti-murine mAb does not cross-react with the bovine products.

Using the IEF technique to investigate the transfected cells, the same bands are seen arising from the murine class I antigens, for w6/32 and alloantisera, but in addition a number of bands are revealed corresponding to those assigned to the w11 haplotype on PBM. For w6/32 one major band and a minor band are seen, corresponding to the major bands for w11 from PBM. Additional faint bands are also present. For the alloantiserum (ED 102) one prominant band can be assigned to the BoLA product, which coincides with the major band seen when w6/32 is used with the transfected cells. This band is seen following IL-A88 precipitaion from PBM, however not as a major band, while it is not revealed when IL-A88 is used with the transfected cells. Instead two strong bands are seen with lower PI and a faint band at much higher PI, each of these bands correspond to bands seen when using IL-A88 with PBM, but are faint or absent with w6/32 or the alloantisera.

The CTL line specific for the w11 antigen only killed Tah infected target cells expressing the BoLA w11 class I serological phenotype in the cytotoxicity assay. Targets expressing other BoLA class I antigens were not killed. The anti-w11 specific CTL recognised w11 on the L-cells transfected with 19.1 and on cells from the donor animal equally well. The level of killing of these cells in the cytotoxicity assay with the anti-w11 CTL line at various effector:target ratios is shown in figure 4. Untransfected Ltk^- cells were not killed by this CTL line. Neither control nor transfected L cells were recognised by another effector cytotoxic T cell line specific for the BoLA w10 class I phenotype. The results of cytotoxicity assay were

considerably better when adherent cells were harvested 16 hr before the assay and incubated in non-adherent petri dishes than when freshly harvested cells were used. Using freshly harvested cells results were sometimes indistinguishable from spontaneous chromium release.

DISCUSSION

In these experiments only the bovine class I heavy chain is introduced into the recipient murine cells, however, it has been shown that heterologous association between mouse $ß_2$ microglobulin and HLA class I heavy chains suffices for expression at the cell surface (Fellous et al 1977, Arce-Gomez et al 1978, Brodsky and Parham 1982, Barbosa et al 1982, Lemonnier et al 1982). It has also been established that free $ß_2$M will exchange with that associated with the heavy chain at the cell surface (Hyafil and Strominger 1979, Schmidt et al 1981, Kefford et al 1984, Bernabeu et al 1984), in this study the cells were cultured with foetal calf serum which contains large quantities of bovine $ß_2$M. Thus, it is likely that the expressed bovine heavy chain combines with the bovine light chain at the cell surface giving rise to a molecule resembling an authentic bovine molecule.

From the 49 clones obtained from the library, 15 appeared to be reasonably complete, in that both 3' and 5' coding sequence was present, however significant levels of expression was only detected for one of the clones, 19.1. We do not know whether this is because the other 14 clones are either incomplete or represent unexpressed pseudogenes or if other of the clones were expressed and we failed to detect the expression. The latter is quite likely in that few anti-BoLA class I mAb are available and their precise specificities are not known. The IL-A88 mAb used for selection of the transfected cells shows the broadest specificity of those available on

the basis of the IEF analysis of precipitated antigens, nevertheless we do not know if it recognises the products of all the BoLA class I loci.

In the microlymphocytotoxicity test the 19.1 transfected L-cells were recognised by two out of three sera recognising the w11 antigen, the third serum that did not elicit killing was at low titre and has to be used neat in tests with bovine cell, it is possible that the L-cell are more robust than PBM and hence it is below the concentration limit for lysis of the transfected L-cells. None of the BoLA allo-antisera reacted with murine cells in the microlymphocytotoxicity test, showing an absence of xenogenic reaction. In contrast HLA alloantisera show xenogenic reactivity that obscured specific lysis of transfected cells, and required absorption prior to their use with murine cells (Lemonnier et al. 1983, Jordan et al, 1983, Sodoyer et al 1986).

For IEF analysis, BoLA antigens were precipitated with mAbs directed either against HLA (w6/32) or BoLA (IL-A88). For PBM the bands revealed with w6/32 have been assigned to different serological specificities for some haplotypes, including, w10 and W11 (Joosten et al, 1988). In addition to the bands that correspond to those precipitated with w6/32, IL-A88 precipitates a large number of additional bands that have not been assigned to haplotypes. Considerably fewer bands are seen for the transfected cells, assigned to the BoLA product, compared with PBM. With different bands being revealed by the different antibodies, possibly due to variation in the specificities. IL-A88 recognises an epitope on the heavy chain of the class I molecules and is not dependent on conformation (S.Morzaria, ILRAD; personal communication), while w6/32 is dependent on the molecule being in its' native conformation in association with $ß_2$m (Parham et al, 1979). It is possible that the more basic bands precipitated from PBM with IL-A88 arise from immature class

I molecules which are not associated with $ß_2$m; the use of a mAb that is directed against $ß_2$microglobulin (B1G6), produces a banding pattern similar to that of w6/32 (S.W.A. unpublished observations). The reason for the different patterns, seen with the three antibodies, from the transfected cells is not known. From the restriction mapping there is only one BoLA gene within the DNA that was transfected, it is therefore most likely that the bands seen for IL-A88 are processing intermediate while the major band seen by w6/32 and the alloantisera represent the mature molecule.

The same patterns of bands are seen for animals of the same serological types in the population, this holds true even across breeds from different countries (R. Oliver personal communication). If the multiple bands seen on IEF arise from the products of different loci it is likely that recombination between the loci would result in different banding patterns being seen for the different haplotypes in the population, unless there is strong linkage disequilibrium between the BoLA class I loci. Another explanation of the multiple bands is that they are processing intermediates of a single gene product. It is therefore interesting to note that the transfected cells give rise to fewer bands than are assigned to w11 from PBM. Either L-cells do not have the same processing intermediates for the BoLA antigens as the bovine cells, but nevertheless give rise to a fully identifiable BoLA w11 antigen. Alternatively the multiple bands seen following precipitation with the mAbs are the products of different genes. The nature of the multiple class I bands seen by IEF is the subject of further study (Al-Murrani et al , manuscript in prep)

We have therefore succeeded in transfecting a BoLA class I gene into murine L-cells, which is expressed from its own promoter. Although the transfection and expression of other BoLA class I genes has been described (Toye et al 1990), these have been by shotgun transfection of genomic DNA and therefore the genes involved

are not available for characterisation. These transfected cells were only characterised on the basis of interaction with alloantisera. Significantly in this paper we have used a cloned gene the product of which is recognised as a BoLA w11 antigen by reaction with alloantisera, by isoelectric focusing following immunoprecipitation and by w11 specific alloreactive cytotoxic T cells. Thus by three different criteria the expressed antigen appears to be an authentic BoLA w11 antigen. We are now carrying out experiments to determine whether it is functionally active and if the transfected cells will serve as a bovine antigen presenting cells, restricted by w11.

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FIGURE 1a. *COMPARISON OF THE AMINO ACID TRANSLATION OF THE FOURTH EXON OF NH1 WITH THE SAME REGION OF THE 5 BoLA cDNA CLONES*

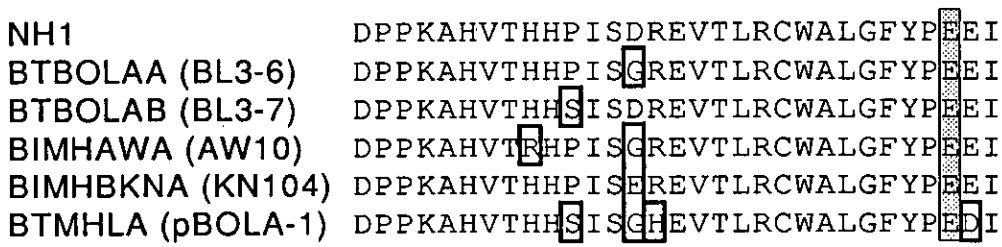
Potential bovine-specific conserved residues are shaded. Residues which differ from the NH1 sequence are boxed.

FIGURE 1b. *COMPARISON OF AMINO ACID TRANSLATION OF THE FOURTH EXON OF NH1 WITH THE SAME REGION OF THE 4 GENOMIC HLA CLONES.*

The potential bovine-specific glutamate residue is shaded and differences from NH1 are boxed.

a.

Third Domain (Exon 4)



b.

Third Domain (Exon 4)

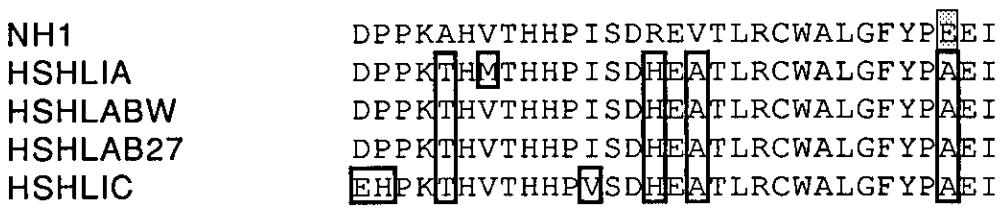


FIGURE 2. HISTOGRAMS OF FACS ANALYSIS OF THE STAGES OF SORTING THE CLONE 19.1 TRANSFECTED POPULATION

The figures show relative fluorescence of transfected cell populations stained with the following antibodies.

9a. Control Ltk^+ cells stained with mAb ILA-88 and FITC conjugated RAMIg.

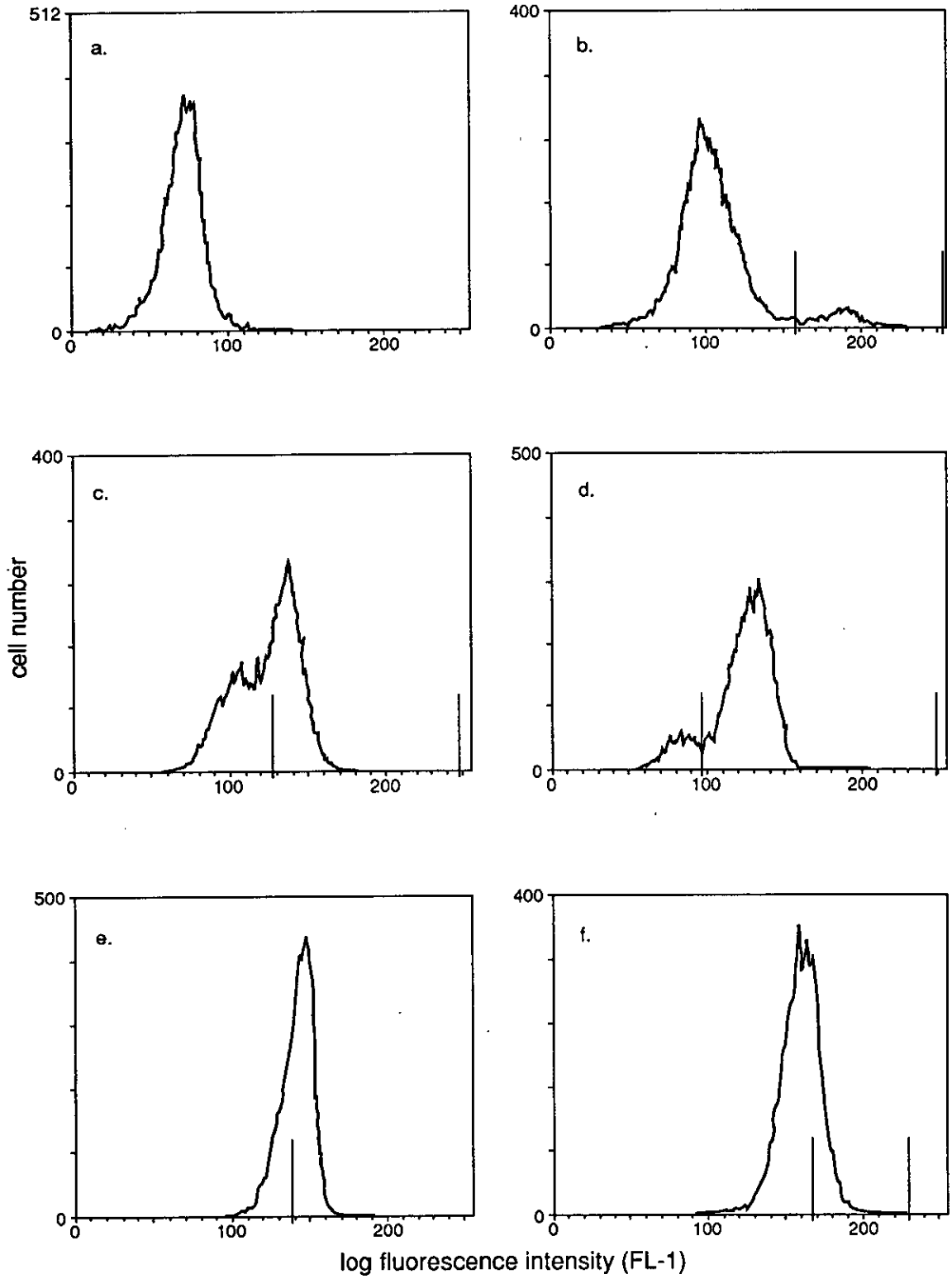
9b. Transfected Ltk^- cells similarly stained. 5.4% of the cells fell within the gates indicated and were sorted and cultured to subconfluence.

9c. Transfected cells after one round of sorting. 51.9% of the cells fell within the gates indicated and were similarly sorted and cultured.

9d. Transfected cells after two rounds of sorting. 80% were positively stained.

9e. Transfected cells after the final (third) round of sorting. 100% were positively stained.

Figure 9f shows the distribution of positive staining on brightest cells sorted from Figure 9e. The brightest of these cells were further sorted and the cells maintained as a stock of transfectants.


a.
b.
c.
d.
e.
f.
512
400
500
0
100
200
cell number
log fluorescence intensity (FL-1)

230

FIGURE 3. 1D-IEF ANALYSIS OF IMMUNOPRECIPITATE FROM PBM OF 10769, Ltk⁻ AND Ltk 19.1

Cells were labelled with ^{35}S methionine and samples were prepared for electrophoresis as described in method. IEF gels were prepared and run as described in methods mAbs used for precipitation were w6/32, MCA 172 and ILA-A88 while the alloantiserum used was Ed102. Tracks 1, 4, 7 and 10 are PBM from 10769; tracks 2, 5, 8 and 11 are Ltk⁻ and tracks 3, 6, 9 and 12 are Ltk 19.1. As expected no bands were precipitated in tracks 4 and 8.

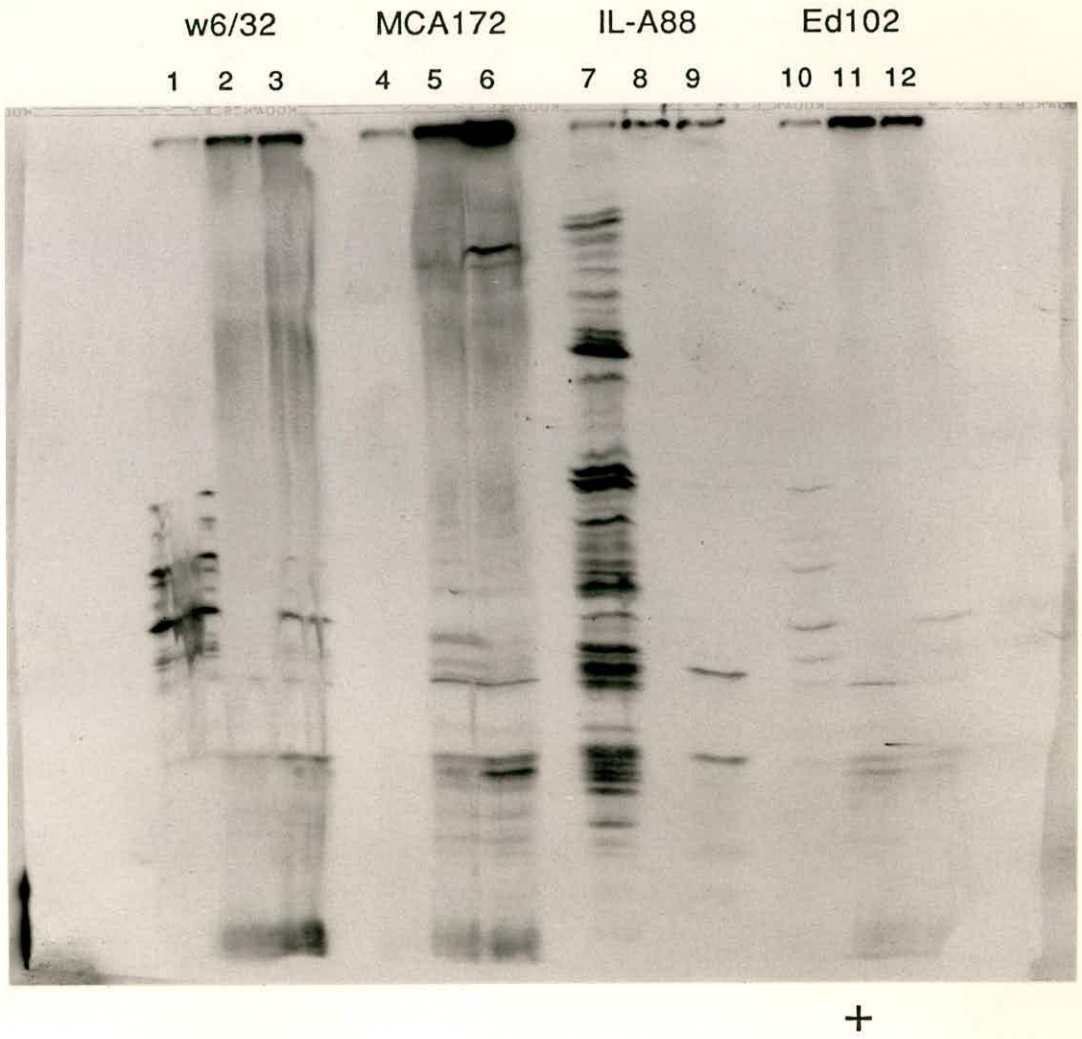

w6/32
MCA172
IL-A88
Ed102
1 2 3
4 5 6
7 8 9
10 11 12
+

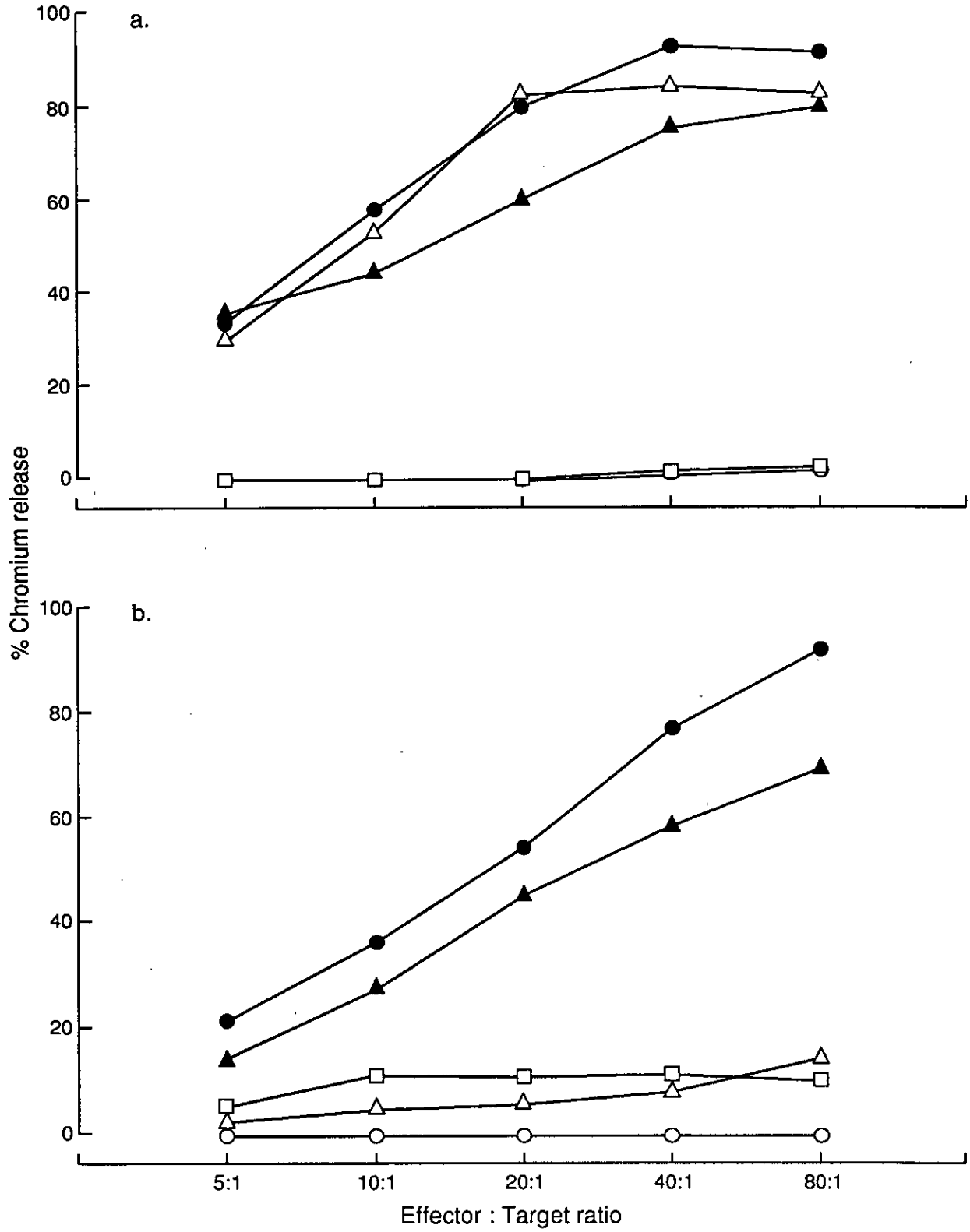
FIGURE 4. *CYTOTOXICITY ASSAY*

Cytotoxicity assays using:

a) anti-w11 CTL and

b) anti-w10 CTL

of a panel of cells as follows: 10779 TaH with BoLA type w10/w11 (●); 10557 TaH with BoLA type w11/- (▲); 2836 TaH with BoLA type w6/Ed99 (○); Ltk⁻ (□) and Ltk 19.1 (△). The assays were performed as described in methods.


a.
b.
100
80
60
40
20
0
% Chromium release
5:1
10:1
20:1
40:1
80:1
Effector : Target ratio