THE STRUCTURE AND TRANSCRIPTION OF A RAT RT1 Bα CLASS II GENE

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ABSTRACT

The major histocompatibility complex of the rat (RT1 complex) encodes two sets of class II molecules referred to as RT1 B and RT1 D. The RT1 B_α gene was isolated from a Sprague-Dawley ($RT1^b$) rat genomic library using a rat RT1 B_α chain cDNA as a hybridization probe. The coding and the majority of the intron DNA sequence was determined. The structure of the RT1 B_α gene is equivalent to that of H-2 and HLA α chain genes. Comparison of the nucleotide and predicted amino acid sequences of the RT1 B_α gene to those of the H-2 and HLA genes revealed a high degree of overall sequence conservation. However, two regions of the first external domain (α1), residues 19-23 and 45-78, exhibit marked sequence variation. Two blocks of conserved nucleotide sequence were identified in the 5' promoter region of the RT1 B_α gene that have been described in all MHC class II genes sequenced to date. These conserved sequences may be involved in the co-ordinate regulation of expression of class II genes. The cloned RT1 B_α gene was efficiently transcribed when transfected into mouse L cells.

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LIST OF ABBREVIATIONS

ATP	2'-Adenosine 5'-triphosphate
BIS	N,N', methylene-bis-acrylamide
BSA	Bovine Serum Albumin
cDNA	Copy Deoxyribonucleic acid
CTL	Cytotoxic T-lymphocyte
CTP	2'-Cytosine 5'-triphosphate
DEP	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DNase I	Deoxyribonuclease I
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
F_1	First filial generation
FACS	Fluorescence Activated Cell Sorter
FITC	Fluorescine Isothiocyanate
GLφ	polymer of L-glutamic acid, L-lysine, and L-phenylalanine
GTP	2'-Guanosine 5'-triphosphate
H-2	Histocompatibility 2 Complex of the mouse
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethylsulfonic Acid
HLA	Human Leukocyte Associated
I	Immune response region
Ia	Immune Associated
IPTG	Isopropylthiogalactoside
Ir	Immune Response

L	Litre
LB	Luria-Bertani
LMP	Low Melting Point
Ltk +/-	L-cell + or - the Thymidine Kinase Gene
MHC	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Response
mRNA	Messenger Ribonucleic Acid
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
RNA	Ribonucleic Acid
RNase	Ribonuclease
SDS	Sodium Dodecylsulphate
SSC	Standard Saline Citrate
TAE	Tris-HCl Acetate Buffer
TBE	Tris-HCl Borate Buffer
TE	Tris-HCl EDTA Buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TK	Thymidine Kinase Gene
Tris-HCl	Tris (hydroxymethyl) aminomethane
tRNA	Transfer Ribonucleic Acid
TTP	2'-Thymidine 5'-triphosphate
UV	Ultraviolet light
V	Volts
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside
xgpt	E.coli xanthine guanosine phosphoribosyl transferase

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CHAPTER 1

INTRODUCTION

The Major Histocompatibility Complex

The Major Histocompatibility Complex (MHC) is a set of linked genes specifying at least three families of gene products known as the Class I, II, and III molecules. The Class I and II molecules are cell surface glycoproteins whereas the Class III molecules are serum proteins. The MHC encoded molecules are involved in a number of biological phenomena mostly immunological in nature (Kaufman et al.,1984). All vertebrate species studied possess an MHC (Gotze,1977) and some invertebrates exhibit functions characteristic of the MHC (Hildeman et al.,1979; Scofield et al.,1982).

A. Detection and History of the MHC

Although all mammalian species are now known to contain MHC regions in their genomes, the MHC's of mouse and human are by far the most well characterized. In fact these two species have been used almost exclusively in the historical definition of this genetic system.

1. The Class I Molecules

a) Graft Rejection

In 1903 Jensen began experiments on the transplantation of live tumours from one mouse to another. He observed that the more closely related the two strains of mouse were, the greater the success of the transplant. A number of studies over the next 10 years suggested that the success or failure of tumour grafts was under the control of several dominant genes (Little and Tyzzer,1916). In order to simplify analysis of these transplantation genes inbred strains of mice were developed.

Experimental transplantation of tumours between inbred strains of mice clearly showed that the donor and recipient mice must be of the same inbred strain in order for the graft to be accepted (Gorer, 1936). Crosses between donor and recipient mouse strains demonstrated that several independently segregating genes were responsible for graft rejection (Gorer, 1937), and Snell (1948) called these genes histocompatibility genes. The development of congenic strains of mice differing only at the genetic loci controlling graft rejection allowed further genetic mapping of the histocompatibility genes. These genes have been assigned to linkage group IX (Gorer et al.,1948) and later to chromosome 17 (Allen et al.,1955). Further comparison of the susceptibility to a given tumour amongst many congenic strains of mice has identified cross-over events which separate the histocompatibility genes into two loci (Snell,1953).

In humans the practice of skin grafting to treat superficial injury dates from the mid 1800's (Barnstable et al.,1979). By 1927 Bauer established that the acceptance or rejection of a skin graft was also under genetic control by observing that of all grafts, only autografts and homografts between identical twins survived permanently. Gibson and Medawar (1943) noted that rejection was an immunological phenomenon by correctly associating the rapid rejection of a graft in a patient sensitized by the previous rejection of a homograft from the same donor with a secondary type immune response. Further work in rabbits demonstrated that the sensitizing antigens were also carried on leukocytes (Medawar,1946).

b) Genetics and Serology

The linkage of the histocompatibility genes to serologically detectable cell surface antigens resulted from a series of experiments by Peter Gorer in 1936. He immunized rabbits with cells from strain A mice raising an antiserum which could detect cell surface proteins (antigens) on strain A mouse cells but not on C57BL strain or CBA strain mouse cells (Gorer,1936). Anti-strain A serum was also raised in C57BL strain mice and found to have identical specificity to the serum raised in rabbit. When tumours derived in strain A mice were transplanted into A, C57BL, CBA, and various hybrids bred amongst these three strains, the ability to accept the graft segregated absolutely with the ability to react with anti-A serum. These data were interpreted to show that tumour graft rejection was under the control of as few as two genes, one of which encoded the strain A antigen (Gorer, 1937) which was called antigen II. In concurrent studies Snell observed that the histocompatibility gene associated with antigen II was remarkable in its ability to elicit graft rejection, and led to the designation of the mouse MHC as the H-2 complex (Counce et al.,1956).

Further genetic and serological analyses identified recombination events which separated the H-2 region into two loci designated H-2K and H-2D (Gorer,1956,1959; Amos,1955; Allen,1955; and Snell,1953,1971). Each of these loci was shown to have serologically defined alleles and encode cell surface glycoproteins now called Class I antigens.

A similar genetic and serological approach has identified a homologous genetic region in rats, now called the RT1 complex (Bogden,1960).

The human MHC (HLA Complex) has been defined almost entirely through serological analysis. The first antigens identified were those thought to be involved in graft rejection, and are the products of the HLA-A, HLA-B, and HLA-C loci. They were first detected using antisera derived from individuals who had undergone multiple blood transfusions (Dausset, 1958), and antisera from women who had been sensitized to human lymphocyte antigens by fetal maternal stimulation (Van Rood et al., 1958; Payne and Rolf, 1958). The separation of the HLA complex into loci depended on population genetics and statistical analysis of many antisera assayed on several different target cells. Each locus has been shown to be extremely polymorphic with up to 50 alleles identified at a single locus. Alleles originally assigned by statistical methods have now been confirmed through family studies (reviewed in McMaster, 1981; Barnstable et al.,1979). The antigens encoded by the HLA-A, HLA-B, and HLA-C loci are also called Class I molecules.

2. The Class II Antigens

a) Immune Response Genes

Although a genetic factor regulating immune responses was described as early as 1938 it was not until well defined synthetic antigens became available in the early 1960's that detailed analyses were attempted (Barnstable et al,1979). Levine and colleagues challenged two strains of guinea pig with a simple polypeptide antigen, poly-L-lysine, one strain could respond by producing antibody whereas the other could not (Levine et al.,1963). Further studies in guinea pig (Levine et al.,1963) and in mouse (McDevitt and Sela,1965) involving genetic crosses between responder and non-responder animals showed that the ability to respond to the antigen challenge was under the control of a single dominant

gene. This gene and later genes were called the immune response genes. Inbred strains of rat (Amerding et al.,1974) and mouse (McDevitt and Chinitz,1969) were used in linkage experiments to assign the immune response genes to a region (called the I or immune response gene region) within the previously defined MHC.

b) Immune Associated Antigens

In an attempt to serologically identify the products of the immune response genes congenic mice and guinea pigs were developed. These animal strains differ only in the immune response (or I) region of the MHC, and were used to raise anti-I region sera of exquisite specificity. Studies in mouse and in guinea pig showed that anti-I region sera reacted with highly polymorphic cell surface glycoproteins expressed only on B-lymphocytes and macrophages (Shreffler and David,1975; Schwartz et al.,1976; and Sachs et al., 1977). The antigens described in this manner were termed immune associated or Ia, and are now called Class II antigens.

The availability of anti-I region sera allowed the further subdivision of this region through the detection of cross-over events between loci. Specific sera have identified the A, E, and/or C subregions or loci in mice (Shreffler et al.,1976).

c) Mixed Lymphocyte Responses

The mixed lymphocyte response (MLR) involves the mixing of lymphocytes from different individuals in an <u>in vitro</u> culture. If the cells are cultured in the presence of ^{3}H-thymidine, cell proliferation can be measured by the incorporation of radioactivity (Bain et al.,1964). In an early study 0% of monozygotic twins and 50% of dizygotic twins showed stimulation of lymphocyte proliferation in this

type of assay, clearly suggesting a genetic basis (Bain et al.,1964). The MLR response was linked to the MHC by demonstrating that lymphocytes from siblings who were identical at HLA Class I loci did not react with each other whereas lymphocytes from unrelated but HLA Class I identical individuals did react in 90% of cases (Bach and Amos,1967). These results suggested that the genes which regulate mixed lymphocyte responses were linked to but separate from the HLA Class I genes (Yunis and Amos,1971).

The development of standardized assays for typing cells allowed a similar type of analysis as that used to study the genetics of the HLA Class I antigens, to be applied to the genes that induce a mixed lymphocyte response. Using the analytical techniques of population genetics and statistics a large number of mixed lymphocyte response types have been defined. Family studies have shown that MLR types behave as alleles at a single locus, now called the D or Class II locus (Barnstable et al.,1979).

During analysis of the immune associated or Class II antigens in mice, it was found that cells bearing Class II antigens could cause the alloreactive proliferation of unprimed T-lymphocytes in a response identical to the mixed lymphocyte response observed in humans (Freilinger et al.,1974). Furthermore, anti-Class II antiserum could block the mixed lymphocyte response stimulated by Class II bearing cells (Freilinger et al.,1974). These findings clearly associated the Class II antigens to the genes controlling the mixed lymphocyte response.

Cepellini and colleagues (1969) observed that HLA typing sera could also inhibit mixed lymphocyte responses using human cells. These sera were not reactive with the Class I antigens encoded by the HLA-A, HLA-B,

or HLA-C loci, and yet reactivity segregated with the HLA complex in families (van Leeuwen,1973).

d) Class II Antigens are encoded by Class II Genes

The evidence that the Class II antigens are the products of the Class II genes is threefold. First the Class II antigens and the Class II genes have been mapped to the same sub-region of the MHC. Secondly anti-Class II antisera block mixed lymphocyte responses which are known to be under Class II gene control. Finally a number of studies directly link Class II antigen structure to an immune response.

When two non-responder mice were crossed the F_1 progeny were unexpectedly found to respond to the same antigen. This suggested that the response to this antigen L-glutamic acid, L-lysine, L-phenylalanine (GLφ) was under the control of a recessive gene (Dorf and Benacerraf,1975). Later it was shown that two genes were involved, one of which was mapped to the I-A subregion of the H-2 complex (Dorf and Benacerraf,1975), and the other to the I-E subregion (Jones et al.,1978). Cook et al. (1979) later showed that the E_α chain was I-E encoded whereas the E_β chain was I-A encoded. Furthermore, these two chains must associate in order to form a functional Class II molecule. Antisera raised against GLφ responder mouse cells were also found to block the immune response to GLφ. The ability of anti-Ia sera and monoclonal antibodies to block immune responses established the close functional relationship between Immune response genes and Immune associated antigens (Schwartz et al.,1976; Baxevanis et al.,1980). Further confirmation came with the advent of cDNA cloning and gene transfer technologies. Mice of the H-2^b haplotype are non-responders to GLφ due to a failure of cells of this haplotype to express Class II E_α

chain molecules. The creation of transgenic mice in which functional H-2 E molecules were reconstituted by the introduction of a cloned E^a_α gene into mice which produce no endogenous E_α molecules proved conclusively that the Class II antigens are the products and effectors of the Class II genes (LeMeur et al.,1985; Yamamura et al.,1985).

B. The Structure of MHC Products

The use of antisera developed against the products of the genes encoded by the MHC has led to the identification of three families or classes of molecules designated I,II, and III. The genetic loci encoding each class of MHC product has been identified in mouse and human whereas only loci encoding the Class I and II molecules have been identified in the rat.

1. The Class I Molecules

The Class I molecules are highly polymorphic transmembrane glycoproteins normally expressed on all nucleated cells. These proteins are approximately 350 amino acids in length, have a molecular weight of 45,000 , and can be sub-divided into three extramembrane domains (Klein et al.,1981). Class I molecules are normally found associated with the non-MHC encoded glycoprotein β2-microglobulin (Figure 1) and function in regulating the recognition of antigen by cytotoxic T-lymphocytes (CTL) (Klein et al.,1983; Kaufman et al.,1984; Sporer et al.,1979).

a) Structure of the Rat Class I Molecules

The rat Class I molecules have not been characterized to the same degree as the murine Class I molecules. Most of the information available on the rat concerns the identification and preliminary characterization of the products of the Class I loci.

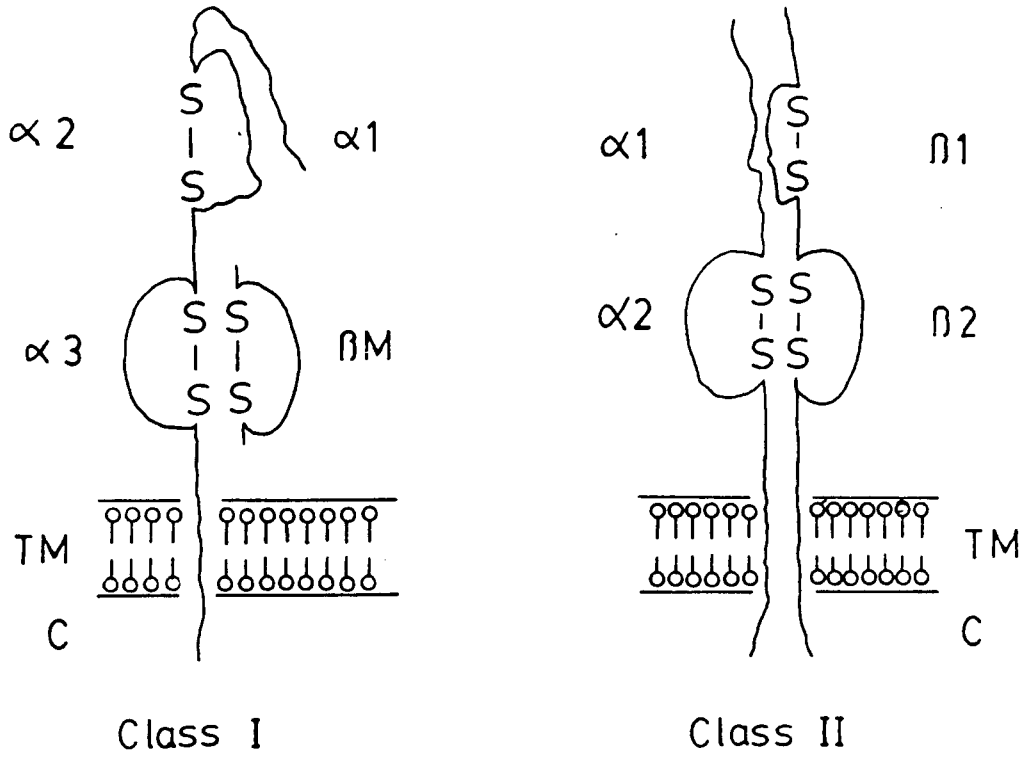

Figure 1: The structure of the MHC encoded Class I and Class II proteins. The domain structure of the proteins are illustrated. α1, α2, α3, β1, and β2 denote the extracellular domains, C denotes the cytoplasmic tail, and the transmembrane domain, designated TM, is shown spanning the plasma membrane. βM is beta-2-microglobulin, and S-S denotes intra-domain disulphide bonds.

The predominant species of transplantation antigen immunoprecipitated from rat cell membranes was shown to have a molecular weight of 45,000 (Sporer et al.,1979). These molecules were found to be associated with a 12,000 molecular weight protein, presumed to be β2-microglobulin, on the membranes of all nucleated cells. Through sequential immunoprecipitation studies it was demonstrated that there were more than one species of rat class I molecule (Sporer et al.,1979; Natori et al.,1979). N-terminal amino acid sequence data confirmed a second Class I species of molecular weight 42,000 (Blankenhorn et al.,1978) which was later genetically mapped to the RT1.C locus (Kohoutova, 1980). This 42,000 molecular weight Class I protein showed a restricted tissue distribution compared to the RT1.A encoded proteins in that it could not be demonstrated on erythrocyte or hepatocyte membranes (Haustein,1982). The lower molecular weight and the restricted tissue distribution led Haustein (1982) to propose that the RT1.C locus was the rat homolog of the murine Qa/Tla complex.

Class I proteins highly similar in size and distribution to those from the RT1.A locus have now been identified and mapped to the RT1.E (Kunz et al.,1982) and to the RT1.F (Misra et al.,1982) loci. Hybridization studies using rat Class I probes have identified at least 20 different DNA bands which hybridize to the probes. This finding when compared to similar studies in mouse suggest the rat genome contains about 20 Class I genes (Gunther et al.,1985).

b) Comparison to the Mouse

Extensive studies on the Class I proteins from the mouse have revealed detailed information on the structure of these molecules. The proteins have a molecular weight of 45,000, are transmembrane proteins,

and are found associated with β2-microglobulin on the surface of all nucleated cells (Goding,1981; Michaelson,1981; and Robinson et al.,1981). Amino acid sequence data derived from several different mouse haplotypes showed that the protein was composed of 5 domains; 3 extramembrane, a transmembrane region, and a cytoplasmic domain (Coligan et al.,1981) (see Figure 1). Each of the three extramembrane domains was about 90 residues in length. Characteristic di-sulphide bridges were found in the α2 and α3 domains as well as up to three potential glycosylation sites in each of these domains (Maloy et al.,1982). Furthermore the β2-microglobulin protein was found to associate with the α3 domain (Yokoyama and Nathenson,1983).

2. The Class II Molecules

The Class II molecules are composed of two non-covalently associated transmembrane glycoproteins called the α and β chains with molecular weights of 29,000 to 34,000 and 24,000 to 28,000 respectively (Figure 1). Class II molecules show a restricted tissue distribution and are found on B-lymphocytes, antigen presenting cells such as macrophages and dendritic cells, and some activated T-lymphocytes. Class II molecules are also highly polymorphic and function in regulation of antigen recognition by T-lymphocytes (Kaufman et al.,1984: Klein et al.,1983: McMaster and Williams,1979).

a) The Structure of the Rat Class II Molecules

The structure of the rat Class II gene products were first examined by radiochemical labelling and immunoprecipitation (Sachs et al.,1977) of proteins from thymic epithelial reticular cells. Similar procedures were used to isolate proteins from mouse thymic epithelial reticular cells (Cullen et al.,1976) and human B-lymphocytes (Springer et

et al.,1977). These antigens were shown to be composed of two non-covalently bonded glycoprotein chains of molecular weight 25,000 to 30,000 and 32,000 to 36,000 respectively.

Further characterization of rat Class II antigens was achieved using monoclonal antibodies. Rat Class II protein molecules were isolated from detergent solubilized rat thymocyte membranes by lentil lectin affinity chromatography, size fractionated, and used to produce mouse monoclonal antibodies specific for rat Class II proteins. One monoclonal MRC OX3 was shown to detect Class II determinants on rat B-lymphocytes, some thymocytes, spleen cells from various strains of rat, and several human lymphoblastoid cell lines as determined by binding assay or flow cytometry. Furthermore, binding studies on inbred strains of mouse with known recombinational events within the Class II region of the MHC showed that MRC OX3 identified proteins which mapped to the mouse Class II A region. This monoclonal antibody was used in antibody affinity chromatography of solubilized rat spleen membranes to purify an RT1.B molecule. RT1.D molecules were purified in a similar fashion using the monoclonal antibody MRC OX17. Rat Class II molecules isolated in this manner were shown to consist of two non-covalently linked glycoprotein chains of apparent molecular weight 29,000 and 24,000 designated the α and β chains respectively (reviewed in McMaster and Williams,1979).

Further studies have shown that Class II molecules are expressed on B-lymphocytes, spleen cells, some thymocytes, a small percentage of bone marrow cells, and kidney tissue (McMaster and Williams,1979; Mason and Gallico,1978). Class II molecules in human have been described as being expressed on bone marrow derived cells such as B-lymphocytes,

macrophages, and dendritic cells; non-bone marrow derived cells such as thymus, intestinal, and kidney epithelium; and some tissues after stimulation such as mammary glands during lactation, or contact sensitized epidermal cells (Daar et al,1984).

Serological studies have shown the rat Class II molecules to be highly polymorphic. More recently two dimensional denaturing polyacrylamide gel electrophoresis (PAGE) (Sawicki and Wettstein,1984) has also been used to identify polymorphism in Class II molecules. Studies on the isolation of Class II membrane proteins as well as hybridization studies searching for Class II genes have been unable to demonstrate more than one α chain and two β chains at each of the B and D loci (Diamond et al,1985; Gunther,1985).

b) Comparison to Mouse and Human

The murine and human Class II molecules have provided most of the structural information known about these molecules. Limited proteolysis of intact dimeric molecules and separated chains, as well as structure deduced from cloned Class II genes or cDNAs has given rise to the structure shown in Figure 1 (Kaufman et al.,1984; Hood et al.,1983; Mengle-Gaw and McDevitt,1985). As described above Class II molecules are composed of two non-covalently linked polypeptide chains, designated α and β. The observed difference in the molecular weights of the α and β chains is due to an additional N-linked carbohydrate side chain on the α molecule (Germain and Malissen,1986). Both chains are transmembrane glycoproteins consisting of two large external domains, a hydrophobic transmembrane region, and a short hydrophilic cytoplasmic tail (Mengle-Gaw and McDevitt,1985). The external domains of both chains are about 90 amino acids in length, and the β2, and α2 domains contain disulphide

bridges highly characteristic of immunoglobulin constant region domains, MHC Class I membrane proximal (α3) domains, and β2-microglobulin (Larhammar et al.,1982). The β1 domain also contains a disulphide bridge whereas the α1 domain does not (Shackelford and Strominger,1983). There are two potential glycosylation sites in the α chain; one in the α1 domain at residue 78, and a second in the α2 domain at residue 118 (Shackelford and Strominger,1983; Kaufman and Strominger,1982; Kaufman et al.,1984; and Mengle-Gaw and McDevitt,1985). The α2 and β2 domains are believed to form characteristic tertiary structure of the immunoglobulin fold. Biochemical data and DNA sequence identity to immunoglobulin constant region domains suggests that the α2 and β2 domains associate to form a β-pleated structure creating an immunoglobulin-like three dimensional configuration (Kaufman and Strominger,1982: Larhammar et al.,1982). It has been suggested that the α1 and β1 domains also form interacting β-pleated sheets (Mengle-Gaw et al,1984), and that the transmembrane regions form α helices (Travers et al.,1984).

Data derived from the nucleotide sequence of RT1 B_α (Wallis and McMaster,1984), RT1 D_β (Robertson and McMaster,1985), and RT1 D_α (Holowachuk,1985) Class II cDNAs as well as the RT1 B_β gene (Eccles and McMaster,1985) have shown that rat Class II molecules are highly homologous to those of human and mouse.

3. The Class III Molecules

The Class III molecules are components of the complement system, C2, C4, 21-hydroxylase and factor B (Klein et al.,1981). While these molecules are encoded by the MHC and have immunological function they bear no structural or functional resemblance to the Class I or II

molecules. In fact the failure to find any evidence of functional or evolutionary ties between the Class III molecules and the rest of the MHC has led Klein (1983) to argue for their exclusion from discussions of the MHC. The Class III molecules will not be discussed further.

C. Genetic Organization of the MHC

The use of serological reagents in the analysis of recombinational events between animals of different haplotypes has been used to construct genetic maps for the rat (RT1), mouse (H-2), and human (HLA) major histocompatibility complexes. A comparison of the organization of the MHC's of rat, mouse, and human is presented in Figure 2.

1. Structure of the Rat RT1 Complex

a) Historical Perspective

The MHC of the rat is known as the RT1 complex. Historically the rat MHC has been referred to as the Ag-B or H-1 complex (Howard,1983), although these designations are no longer in use. The RT1 complex has not been assigned to a particular chromosome, nor has the orientation of the RT1 complex been determined with reference to the centromere (Howard,1983).

The loci of the RT1 complex were defined by analysis of genetic crosses between rats of different serological haplotypes. Recombination events occurring between regions encoding defined MHC phenotypes allowed the division of the RT1 region into specific loci. The first two loci defined in this manner were those encoding the Class I and Class II MHC functions and were called the A and B loci respectively (Stark et al.,1977: Butcher and Howard,1977: Gunther et al.,1978). Further studies identified and mapped other Class I loci which were called the RT1.C (Kohoutova et al.,1978), RT1.E (Kunz et al.,1982), and another proposed

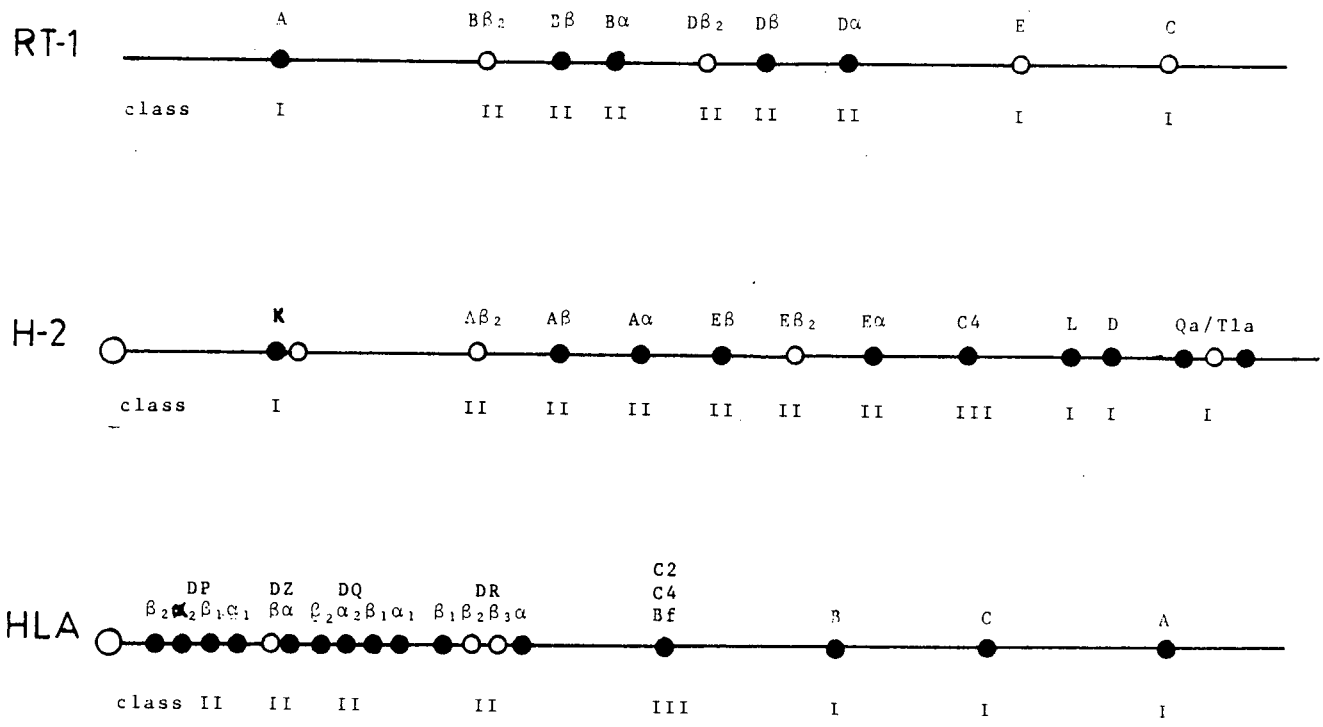

Figure 2: Organization of the MHC in rat, mouse, and man. Class I and Class II loci are shown. The MHC has been assigned to chromosome 17 in the mouse and chromosome 6 in human. No chromosomal assignment has been made for the rat RT1 complex. The large open circles indicate the location of the centromere with respect to the MHC where known. Shaded circles denote genes which are known to be expressed, whereas open circles denote genes which are not expressed or for which expressibility has not been determined.

locus RT1.F (Misra et al.,1985). In addition a second Class II locus was identified and called the RT1.D locus (Lobel and Cramer,1981). There have been no Class III loci identified in the rat to date.

b) Structure of the RT1 Complex

The orientation of the known loci of the RT1 complex is shown in Figure 2. It has been proposed that the RT1.A locus encodes the classical Class I transplantation antigens, which are expressed on all nucleated cells. Data from studies using monoclonal antibodies (Misra et al.,1985) and restriction fragment length polymorphisms (RFLP) (Gunther et al,1985) have shown these Class I loci to be highly polymorphic, identifying up to 19 different alleles.

The RT1.C and RT1.E loci have been shown to contain Class I like genes by hybridization with rat Class I gene probes (Gunther et al.,1985), however the products of these loci do not exhibit typical Class I histocompatibility behaviour (Stark and Gunther,1982). The molecules encoded by the RT1.C and RT1.E show a more restricted tissue distribution and lower levels of expression when compared to RT1.A encoded molecules (Diamond et al.,1985). Furthermore, the location of the RT1.C locus in relation to the other RT1 loci (Figure 2) resembles that of the H-2 Qa/Tla loci, which coupled with the non-classical Class I behaviour has led several researchers to call the RT1.C locus the homolog of the Qa/Tla region of the mouse (Misra et al.,1985: Gunther et al.,1985). More than 80% of the rat Class I gene clones which have been mapped are localized in the RT1.C region. In the mouse the majority of Class I genes have been localized to the Qa\Tla region.

The RT1.B and RT1.D regions have been shown to encode Class II molecules highly homologous to the A and E loci of the mouse H-2 complex

(Fukomoto et al.,1982). Each locus contains one α chain gene and probably two β chain genes ordered as shown in Figure 2 (Blankenhorn and Cramer,1985; Diamond et al.,1985; and Blankenhorn et al.,1983).

2. The Structure of the Mouse H-2 Complex

The availability of many inbred, congenic, and recombinant congenic strains of mice and highly specific alloantisera has allowed the H-2 complex to be characterized in great detail (Hood et al,1983). Figure 2 shows the structure of the H-2 complex and its orientation on chromosome 17. Comparison of the overall structure of the H-2 complex to that of the rat RT1 complex shows striking similarities in the number and orientation of loci, reflecting their closeness in evolutionary time.

The H-2 complex encodes about 30 Class I genes in 4 regions as follows: 2 K genes, 2 D/L genes, at least 10 Qa 2,3 genes, and at least 13 Tla genes (Weiss et al., 1984). The K and D/L loci are extremely polymorphic with at least 50 different alleles having been identified in both wild and inbred populations of mice (Hood et al.,1983; Weiss et al.,1984). The Qa/Tla loci appear to be less polymorphic, but as described above encode a greater number of Class I genes (Klein et al.,1983).

The Class II region (or I region) contains 7 genes encoding an A_{α}, $A_{\beta 3}$, $A_{\beta 2}$ pseudogene, $A_{\beta 1}$, E_{α}, $E_{\beta 1}$, and $E_{\beta 2}$ genes oriented as shown in Figure 2 (Steinmetz,1982; 1985). With the exception of the E_{α} gene (Mathis et al., 1983) each of these genes shows considerable polymorphism (Steinmetz,1985).

3. The Structure of the Human HLA Complex

Initially serological reagents were used to define the number and organization of HLA encoded genes in humans. More recent studies using

cosmid mapping and gene counting experiments have supported much of the early data. A comparison of the HLA complex to rat and mouse is shown in Figure 2. The HLA-A, B, and C loci are the human Class I regions, and are homologous to the Class I regions of mouse and rat. The human Class II genes are encoded in the D region of the HLA complex. Comparison of the orientation of the MHC complex between these three species reveals a striking difference. In rat and mouse the Class II region is surrounded by two Class I encoding regions whereas in humans the Class I and Class II regions are adjacent. Gill and colleagues (1982) have suggested that during evolution the ancestral Class I and Class II genes duplicated to form multiple loci adjacent to each other. Following the divergence of man and rodents an inversion event occurred in rodents placing the Class II region between two Class I regions. Further duplication events would give rise to the genetic map seen today.

The human Class I molecules have been shown to be highly polymorphic with 10 to 50 alleles identified serologically at each of the HLA-A ,B, and C loci (Bodmer, 1984). Serological and biochemical studies have shown evidence for human homologs to the murine Qa/Tla region, however no genes have been identified or mapped to the HLA region (Steinmetz and Hood,1983).

The human Class II region has been shown to contain at least three loci called HLA-DR, HLA-DQ, and HLA-DP. The HLA-DQ and HLA-DR loci are homologous to the H-2 A, H-2 E, and RT1 B, RT1 D loci respectively, however there is no rodent homolog for the HLA-DP locus. In addition the human HLA-DP locus is the only known Class II locus encoding two α chain genes (Auffray et al.,1984; Servenius et al.,1984). The HLA-D loci also

exhibit marked polymorphism with 16 HLA-DR, 3 HLA-DQ, and 6 HLA-DP alleles identified to date (Klein, 1986; Bodmer,1984).

D. The Structure of the Class I and Class II Genes

1. Class I

The DNA encoding the 340-350 amino acids of Class I proteins is separated into eight exons which correspond precisely to the domain structure of the protein (Hood et al.,1983). Exon 1 encodes the leader peptide, and exons 2, 3, and 4 encode the three external domains of the protein ($\alpha 1$, $\alpha 2$, and $\alpha 3$ respectively). The transmembrane region is encoded in exon 5. The cytoplasmic domain is encoded in exons 6, 7, and 8. In addition exon 8 also encodes the 3' untranslated region. The Class I genes of mouse and rat (Hood et al.,1983) are highly similar in structure, however the human Class I genes show a variation in that the cytoplasmic domain is encoded in two rather than three exons (Malissen et al.,1982).

In general the Class I genes possess the characteristics common to all eukaryotic genes. Intron/exon boundaries conform to the GT\AG rule (Breathnach and Chambon,1981), the 5' flanking sequences contain the CAAT and TATA promoter elements upstream from the initiation codon, and a polyadenylation signal can be found 400 nucleotides 3' to the termination codon (Hood et al.,1983).

2. Class II

The structure of the Class II genes corresponds closely to the domain structure of the protein in rat, mouse, and human (Figure 3). Class II β genes of mouse (Larhammar et al.,1983) and rat (Eccles and McMaster,1985) are encoded in six exons whereas the human HLA-DC_β gene has only 5 exons (Schenning et al., 1984). Exon 1 codes for the leader

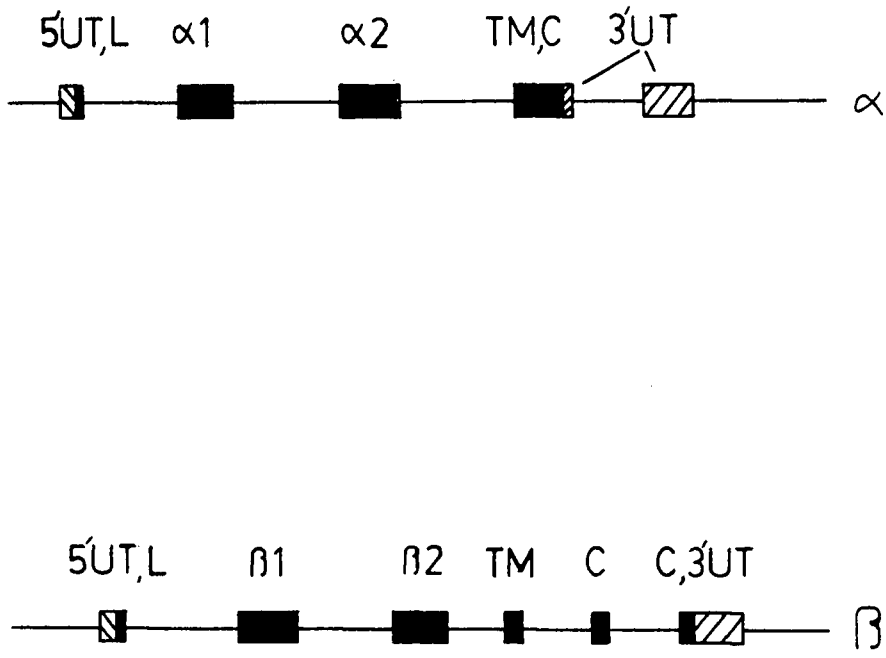

Figure 3: Structure of Class II genes. The H-2 E_α chain gene (Mathis et al., 1983) and RT1 B_β chain gene (Eccles and McMaster, 1985) are shown as examples of Class II α and β chain gene structures. Shaded boxes denote coding sequence and cross-hatching denotes untranslated sequences. L denotes the leader peptide, α1, α2, β1, and β2 denote the exons encoding the external domains of the proteins; TM denotes the transmembrane region: C denotes the cytoplasmic region of the protein; and 3'UT denotes the untranslated region found in the 3' flanking region.

peptide and the first four amino acid residues of the first external domain. Exons 2 and 3 each encode the first and second external domains respectively. Exon 4 contains the sequence for the transmembrane region, and exon 5 the cytoplasmic region. Interestingly human Class II_β genes do not have a separate exon for the cytoplasmic region but instead encode the 3' untranslated sequence in a single exon (Schenning et al., 1984). The sixth exon in rodents contains 8 residues of the cytoplasmic domain and the 3' untranslated sequence.

The Class II α genes of mouse (Benoist et al.,1983) and human (Mathis et al.,1983) are encoded in five exons. Exon 1 encodes the leader peptide and 3 to 4 residues of the α1 domain. Exons 2 and 3 encode the α1 and α2 domains respectively. In contrast to one exon per domain, the transmembrane region, cytoplasmic tail, and several residues of 3' untranslated region are all encoded in exon 4. Exon 5 contains the remainder of the 3' untranslated sequence, which is unusual in that the 3' untranslated sequences are normally not split between exons.

E. Functions of the MHC

1. Functions Assigned to the Class I Antigens

a) Graft Rejection

The primordial function of the MHC may have been in self non-self recognition. Although tissue transplantation is only encountered surgically or in animal experiments today, there are examples of MHC regulation of natural transplantation in the invertebrates. The tunicate Botryllus primigenus lives in colonies sharing a common vascular system and encased in a common gelatinous tunic. Parental organisms sexually reproduce to form tadpole like larva which undergo metamorphosis to form oozooids which in turn form colonies by budding. Parental individuals

are reabsorbed by fusion with newly formed buds. The fusion process appears to be under the control of genes encoded by an ancestral MHC like gene complex (Scofield et al.,1982).

Although tissue transplantation cannot be the function for which the Class I genes have evolved, they nonetheless present a formidable obstacle to the surgical transplantation of tissue in medicine. The strong allogeneic response elicited in the recipient by donor Class I and Class II antigens ultimately causes the rejection of the graft. The careful matching of donor and recipient for shared Class I and other histocompatibility antigens has improved the success of organ transplants, however with the exception of identical twins - matches are difficult to achieve without the aid of immunosuppressive drugs.

b) Restriction of Cytotoxic T-lymphocyte (CTL) Activity

The Class I molecules have been shown to play an important role in the recognition of virus infected cells by syngeneic Cytotoxic T-lymphocytes (CTL). Zinkernagel and Doherty (1974) showed that T-lymphocytes isolated from an H-2 type K^kD^k animal infected with Lymphocytic choriomeningitis virus (LCMV) would kill Lymphocytic choriomeningitis virus infected target cells of H-2 type K^kD^k, but neither Lymphocytic choriomeningitis virus infected cells of H-2 type K^dD^d, nor uninfected cells of H-2 type K^kD^k were killed. Similar studies in mouse (reviewed by Ploegh et al.,1981) and human (McMichael et al.,1977) have confirmed this phenomenon known as MHC restriction.

Cytotoxic T-lymphocytes function in the surveillance of the immune system, constantly searching for and destroying cells which display altered self Class I antigens on their cell surfaces (Hood et al.,1983). Some highly oncogenic viruses such as human adenovirus appear to escape

the immune system by altering the expression of Class I molecules on the surface of cells they infect (Schrier et al.,1983). Reintroduction of functional Class I genes into Adenovirus 12 transformed cells results in loss of oncogenicity by the virus (Tanaka et al.,1985). Furthermore, introduction of Class I genes and de novo synthesis of product in fibrosarcoma cells reduced their tumourigenicity and metastatic potential (Wallach et al.,1985). This suggests that lack of expression of Class I molecules can result in evasion of the immune system by tumour cells. However, induction of Class I antigens in other tumours increases tumour growth (Brickwell et al.,1985). Nonetheless Class I proteins are instrumental in determining the targets of cytotoxic immune activity.

c) Allelic Polymorphism and Function

One of the most outstanding features of the Class I antigens is the extreme polymorphism in the primary structure of these proteins. Serological studies using alloantisera and monoclonal antibodies developed in MHC congenic mice have defined over 50 different allelic determinants at each Class I locus (Klein et al.,1981). Similarly, many Class I alleles have been demonstrated in wild rats (Wagener,1979).

The conservation of Class I antigen polymorphism through the evolution of mammals suggests that it plays a role in the function of these molecules. Studies directed towards the understanding of structure-function relationships have employed the spontaneous Class I mutants naturally occurring in mice (Nathenson et al.,1986).

Tryptic peptide mapping, amino acid sequencing, and most recently DNA sequencing (Nairn et al.,1980; Pease et al.,1983; and Weiss et al.,1983) have been used in the detailed structural analysis of the H-2

K^b family of spontaneous *in vivo* mutant Class I molecules and their parent molecules. Comparison of structural data to functional studies using anti-K^b antisera and alloreactive Cytotoxic T-lymphocytes (CTL) allowed the identification of regions of functional importance within the Class I molecule.

These studies have identified one region in each of the α1 (residue 70 to 90) and the α2 (residue 150 to 180) domains to which anti-Class I antisera were directed. Furthermore, disruptions in these sequences, even of a small nature, grossly altered the reactivity of these Class I molecules with specific T-cells, apparently by altering the conformational determinants formed by the interaction of the α1 and α2 domains (reviewed in Nathenson et al.,1986). Clearly primary sequence polymorphism is involved in the regulation of the functional interaction of Class I bearing cells and lymphocytes.

A study of the Cheetah *Acinonyx jubatus* showed that this species exhibited considerably less genetic variation than other mammals. The MHC, normally the most polymorphic genetic region, shows little or no genetic variation as demonstrated by skin grafting experiments. In 14 out of 14 cases skin grafts were accepted without complications between unrelated animals. The apparent monomorphism of the cheetah has been attributed to a population bottleneck. Perhaps the worst consequence of this monomorphism is the susceptibility of the cheetah to the Coronavirus and the fatal disease Feline Infectious Peritonitis it causes. The Cheetah population apparently lacks any individuals carrying the appropriate MHC allele to mount a effective immune response to the Coronavirus, and as a result entire populations have been decimated

(O'Brien et al.,1985). This example may show the importance of MHC polymorphism in the protection of populations from infectious diseases.

2. Functions Assigned to the Class II Antigens

a) Activation of T-lymphocytes

Activation of T-lymphocytes in an immune response requires the recognition of Class II molecules and antigen by the T-cell's antigen receptor. This phenomenon was first called the mixed lymphocyte response in which the activation of T-lymphocytes is by Class II antigens presented by allogeneic lymphocytes. Physiologically, presentation of antigen to T-cells is the function of macrophages and dendritic cells, the so called "antigen presenting cells". Lipsky and Rosenthal (1973) demonstrated that antigen must be processed by the antigen presenting cell, and presented to the T-cell associated with Class II molecules in order to be immunogenic. Later work showed that isolated membranes containing Class II molecules could also activate T-lymphocytes provided that the antigen had been "pre-processed" by proteolytic digestion (Watts et al.,1984). B-cell lymphomas (Ben-Nun et al.,1984), and L-cells (Norcross et al.,1984) have been transfected with cloned Class II genes and both express functional transfected Class II molecules on their cell surfaces. B-cell lymphomas are known to be capable of antigen processing (Shimonkevitz et al., 1982), however the finding that the L-cell fibroblastoid cell line also processes antigen was unexpected (Shastri et al.,1985). Furthermore, it suggests that the physiological pathways involved in antigen processing are not confined to the lymphoreticular system (Germain and Malissen, 1986). It is clear that recognition of processed antigen in the context of Class II proteins by T-lymphocytes activates these cells for participation in immune responses to antigen.

b) Restriction of Helper T-lymphocyte Activity

The Class II molecules are also involved in the regulation of antibody responses by B-lymphocytes. B-cells respond to antigen by producing antibody only when activated by helper T-lymphocytes. T-cell activation of B-cells requires the recognition of Class II molecules on the cell surface of the B-lymphocyte. Several investigators have demonstrated that the B-cell must share the same MHC haplotype as the activating T-cell in order to respond and synthesize antibody (Katz,1973; Kappler and Marrack,1978). More recently Lanzavechia (1985) has proposed a mechanism for B-cell activation by T-cells. Antigen molecules can be bound by immunoglobulin receptors on the B-lymphocyte cell surface and absorbed by endocytosis. The antigen can then be processed by the B-cell and presented along with Class II molecules on its cell surface. Recognition of processed antigen and Class II molecules by helper T-cells results in the induction of antibody production by the B-cell.

Class II molecules are also involved in the regulation of cell mediated immunity. Von Boehmer (1978) immunized female mice with cells from male mice of the same strain, where male antigens are recognized as foreign by the female recipient in a Class I mediated cytotoxic T-lymphocyte response. He noted that reconstitution of chimeric mice which were high responders to male antigen with cells from two different mice which were low responders to male antigen gave different results. In one case the chimeric mice remained high responders while the other became low responders. The lack of immune response in the one case was found to be due to histoincompatibility between the helper T-cell and the responding cytotoxic T-lymphocyte at the Class II locus.

c) Allelic Polymorphism

The majority of the nucleotide and protein sequence variation between Class II alleles is clustered in several regions of the N-terminal domains of both α and β chains (Benoist et al.,1983). Furthermore, comparison of Class II protein sequences between different species such as rat, mouse, and man (Eccles and McMaster,1985; Robertson and McMaster,1985; and Wallis and McMaster,1984) shows that interspecies variation in Class II proteins is also clustered in regions of the α1 and β1 domains. The non random nature of the polymorphism in the α1 and β1 domains suggests that these regions function as the site of recognition for antigen and /or the T-cell receptor (Mengle-Gaw and McDevitt,1985).

Several investigators have examined the relationship between structural variation in Class II molecules and their function. Wakeland and colleagues (1985) studied two mouse alleles H-2 A^{kv1} , and H-2 A^{kv2} which can be differentiated serologically using monoclonal antibodies and functionally using H-2 A^{k} restricted alloreactive T-lymphocytes. Tryptic digest and amino acid sequence mapping localized the regions responsible for the observed differences to residues 43-71 of the α chain, 26-29 of the β chain, and 95-106 of the β chain. Site directed mutagenesis has been used to introduce amino acid changes in specific regions of the β1 domain of murine H-2 A^{b} molecules (Cohn et al.,1986). Analysis of the serological and functional changes caused by the H-2 A_{β} chain mutations identified two sites (residues 9-13, and 65-67) responsible for the restriction of Class II activity. It was clear that changes in the primary structure of Class II molecules in the so called regions of allelic hypervariability resulted in changes in the

functional recognition of these molecules by antibodies and allorestricted T-lymphocytes.

A recent study (Landais et al.,1986) examined the relationship between the regions of allelic variability of the H-2 A_α molecule and the immunogenicity of the molecule. Chimeric A_α molecules were constructed from the cDNA clones encoding the α chain of H-2 A^k and H-2 A^b mice. The chimeric A_α chains were introduced into mouse L-cells by DNA mediated gene transfer techniques along with a cDNA encoding a complete H-2 A_β molecule. The Class II molecules expressed on the cell surface were then characterized using monoclonal antibodies specific for determinants on the A_α chain, and which distinguish the A^k and A^b alleles. The residues which defined k allele and b allele were localized to positions 69-76 and 53-59 of the α1 domain respectively. The authors noted that despite the extensive polymorphism in α1, at residues 11-15, 53-59, and 69-76 in both the A^k and A^b molecules, the monoclonal antibodies tested recognized only one hypervariable region in differentiating the two alleles. This apparent limited spectrum of reactivity exhibited by the anti-A_α reagents could be caused by the inaccessibility of polymorphic regions due to protein conformation. Alternatively the allelic amino acid substitutions may not alter the protein structure enough to be immunogenic. Evidence suggests that amino acid substitutions between the A^k and A^b α chains such as arginine to threonine at position 56 sufficiently changes the local charge to significantly alter the local structure (Landais et al.,1985). Similarly hydrophilicity plots predict that all three of the hypervariable regions are exposed on the surface of the A_α molecule (Landais et al.,1986). It appears that mice simply cannot respond, by

producing antibody, to all the available polymorphism in the Class II molecules. This may explain the observation that serologically the H-2 A_α chains appear less polymorphic (Klein and Figueroa,1981) than would be predicted from the comparison of allelic nucleotide sequences (Benoist et al.,1983). Determination of the three dimensional crystal structure of Class II molecules may allow the fine detail of Class II antibody interaction to be examined.

Despite the extensive work done on the effects of structural variation in Class II molecules on recognition by antibody it is not known if the T-cell receptor recognizes Class II molecules in a similar manner. Preliminary results from a number of laboratories suggest that Class II molecules may be recognized in a different manner than antibody. Two murine cell lines JE50 and JE67 express mutant A^k_α chains which are indistinguishable by anti-A_α monoclonal antibodies, but are distinguishable using T-cell hybridomas specific for hen egg ovalbumin and restricted by H-2 A^k (Allen et al.,1985). Allogeneic T-cells have been found which clearly respond to α1 domain determinants localized to residues 53-59 and 69-76 of H-2 A^k_α chains whereas monoclonal antibodies were all directed against residues 69-76 (Landais et al.,1986). These data are consistent with reports that A_α A_β chain association and the resulting tertiary structure are responsible for the restriction properties of Class II molecule T-cell receptor interaction (Cohn et al.,1986; Lechler et al.,1986).

The molecular role of polymorphism in the interaction of Class II molecules, antigen, and T-cell receptors is unclear. Helper T-lymphocytes recognize antigen displayed on the surface of antigen

presenting cells through a single T-cell receptor molecule (Hood et al.,1985). In addition the T-cell receptor must also recognize self Class II molecules before the T-cell can respond to the antigen. The molecular mechanism of co-recognition of antigen and Class II molecule through a single T-cell receptor molecule is a highly controversial subject (reviewed, Schwartz,1985). One model suggests that Class II molecules interact physically with antigen either before or during binding of the T-cell receptor. The T-cell receptor could recognize the Class II-antigen complex, or some "altered self" epitope resulting from a conformational change in the Class II protein mediated by antigen binding. Alternatively, the T-cell receptor may bind antigen and Class II protein molecules independently accounting for the observed dual specificity without requiring Class II-antigen interaction.

Watts, Gaub, and McConnell (1986) created artificial planar membranes containing Class II proteins, which were capable of stimulating antigen specific Class II restricted T-lymphocytes to proliferate in the presence of antigen. Fluorescent dyes were coupled to both the Class II molecules and to the antigen. Using the property that excited fluorochromes can transfer energy to other fluorescent dye molecules in close proximity (less than 40 °A) these authors demonstrated that during T-cell recognition of antigen, Class II molecules, T-cell receptor molecules, and antigen are closely associated in a complex. Similarly, Ashwell and Schwartz (1986) examined the dose response of a single specific T-cell clone to antigen presented in the context of two different Class II molecules. Although the T-cell can recognize the antigen in the context of both Class II molecules ($E_\beta{}^k E_\alpha{}^k$ or $E_\beta{}^b E_\alpha{}^k$) the response was significantly reduced using the $E_\beta{}^k E_\alpha{}^k$ Class

II molecule. These authors attribute the difference in dose response to a difference in the affinity of the antigen to the two MHC molecules, suggesting a physical interaction between antigen and Class II molecule.

The molecular role of the polymorphic regions of Class II molecules has not been resolved. Analysis of a number of T-cell receptor β chain sequences has identified as many as seven significantly hypervariable regions (Patten et al.,1984). Three hypervariable segments are located at positions similar to those found in immunoglobulin molecules, and are believed to interact with the hypervariable segments in T-cell receptor α chains to form an antigen binding site (Davis, 1985; Patten et al.,1984). Four hypervariable regions appear to be located on the outside of the T-cell receptor molecule and may be involved in interactions with polymorphic determinants on Class II molecules (Davis, 1985). The available data do not exclude the "altered self" hypothesis where the T-cell receptor recognizes a novel epitope which results from an antigen induced conformational change in the Class II molecule (Schwartz, 1985; Ashwell and Schwartz, 1986).

d) T-cell Differentiation

The development of a self MHC restricted antigen specific repertoire of T-cell receptor molecules which is not spontaneously autoreactive has been an unresolved issue in immunology for some time (Kronenberg et al.,1986). Studies using chimeric mice in which the reconstituting cells are of a different MHC haplotype have supported the model that developing T-cells are "educated" in the thymus. According to this model only those T-cells with receptors capable of interacting with self MHC and antigen are allowed to emigrate from the thymus and populate the peripheral lymphoid organs. T-cells which recognize antigen

alone or non-self MHC molecules do not leave the thymus and are believed to die in the thymus (Zinkernagel et al.,1978; Schwartz, 1984). An alternative hypothesis suggests that only self restricted T-cells are activated in peripheral circulation. Recent evidence has shown that the thymus is the first site where the T-cell receptor is expressed, and therefore "education" or induction of tolerance is unlikely to occur before the entry of the T-cells into the thymus. Furthermore, the large number of non-productive rearrangements observed in T-cell receptor genes may account for some of the cell death believed to occur in the thymus (Kronenberg at al.,1986; Cohn and Epstein,1978). These data favour the thymic education theory, however they are by no means conclusive. Nonetheless MHC antigens are key in the development of T-cell antigen receptor repertoire.

e) Non-Response

Attempts to explain the phenomenon of immunological non-responsiveness have led to much controversy amongst immunologists. Non-response to antigen has been examined by a number of investigators and five models have been proposed. 1) Non-response is due to the absence of functional MHC molecules. Jones et al. (1981) showed that mice of MHC haplotypes b,s,or f, which have deletions in the promoter of the E_α gene, are incapable of expressing E Class II molecules on their cell surface. As a result these animals are non responders to any antigen which associates with E Class II molecules. 2) the repertoire of variable regions of the T-cell receptor genes is incomplete, resulting in no appropriate receptor for some antigens. 3) Schwartz (1978) in describing a theory for development of self tolerance suggested that some T-cell clones are selected against, effectively deleting them from

the repertoire. Deletion of T-cells which cross react with self determinants would make these animals non-responders to antigens requiring the deleted T-cell to mount a response. 4) Rosenthal (1978) proposed a "determinant selection" hypothesis in which antigen, MHC molecule, and T-cell receptor all interact. If some of these interactions were of low affinity they may not be strong enough to elicit an immune response, resulting in the non-responder phenotype. 5) Non-response is due to regulatory cells such as T-suppressor cells which block the immune response.

f) Evolutionary Function

The role of Class II proteins in the presentation of antigen to the immune system has led many investigators to propose that polymorphism across a species ensures that at least one individual will have a Class II allele appropriate for mounting an immune response against any new pathogen (reviewed Klein and Figueroa,1981). It would therefore be an advantage for an individual to express as many different MHC molecules as possible, as this would provide the maximum protection against infectious diseases. In agreement with this idea is the finding that Class II loci (RT1.B and RT1.D) are expressed co-dominantly (Klein and Figueroa,1981), providing two different sets of Class II molecules for potential antigen interaction. Heterozygous individuals should also be at a selective advantage, due to hybrid Class II molecules formed by the independent assortment of allelic α and β chain molecules. Consistent with this is the finding that 85% of all wild mice in a random bred population, and 90% of humans are heterozygous at their Class II loci (Klein and Figueroa,1981). Heterozygote advantage has also been demonstrated by Palm (1969,1970) in crosses between homozygous inbred

rats which are non-responders to a given antigen. He found that the heterozygous offspring could respond to the antigen, and therefore had a selective advantage over their parents.

3. MHC Functions Not Assigned to the Class I or Class II Molecules

Meruelo and Edidan (1980) have postulated that MHC encoded molecules function as general receptors binding antigen, ligands, or other receptors. The polymorphism of MHC molecules as well as the multiplicity of loci would enable the MHC to be involved in the modulation of a wide variety of processes. The MHC has been associated with functions as diverse as variation in the response of a tissue to androgen in animals of different MHC haplotype, to the expression of different MHC loci at different stages of development.

It has been noted that individuals carrying certain alleles at some MHC loci have a higher incidence of specific diseases. The association of MHC alleles and disease may be due to a direct physical relationship in which the MHC locus contributes to the disease phenotype, or the MHC marker may simply show linkage disequilibrium with an undefined disease locus. The most striking disease associations are found in humans where individuals carrying the HLA-B27 allele have an 85 times higher chance of developing the autoimmune disease Ankylosing Spondylitis (Bodmer and Bodmer,1978). A similar relationship is seen in rats where Insulin Dependent Diabetes Mellitus is strongly associated with the $RT1^{u}$ haplotype of BB strain rats (Colle et al,1981; Jackson et al,1984). To date very little is known about the mechanism of association between an MHC allele and a disease.

F. Objectives and Rationale for Studying the RT1 Class II Genes

Recent advances in molecular genetics have greatly increased our knowledge of the structure of individual MHC genes and their products. Many of the human and murine Class II genes have now been well characterized, however the structure of the rat RT1 Class II genes remains largely unknown. The study of the rat RT1 Class II genes is important in that it allows the structure of the rat genes to be compared to the equivalent genes in mouse and human. Comparisons of closely related species such as rat and mouse which diverged about 8 million years ago, and more distantly related species such as rodent and human which diverged about 80 million years ago will lead to a better understanding of the relationship between the structure of the rat Class II genes and their evolution, polymorphism, and expression.

This thesis describes the isolation and characterization of a rat RT1 B_α Class II gene. The structure of this gene will be compared to the equivalent human HLA-DQ_α gene (Okada et al.,1985) and to the mouse H-2 A_α cDNA sequence (Mathis et al.,1983). This approach will identify genetic regions which have been conserved throughout evolution and are therefore likely to be of functional importance. Furthermore, the evolutionary relationships between the three gene sequences will be identified and discussed. The RT1 B_α gene is the first rodent "A" Class II gene for which the complete sequence and gene structure has been described.

The MHC shows the highest degree of polymorphism of any known mammalian genetic system. This polymorphism has been selected for during evolution and is related to the function of MHC molecules. Comparison of the gene encoding the RT1 B_α molecule to the equivalent gene sequences

from mouse and human, as well as an allelic rat RT1 B_α cDNA (Wallis and McMaster,1984) may reveal mechanisms for the generation and maintenance of polymorphism.

Finally, structural comparisons between the RT1 B_α and other Class II genes may identify conserved structural sequence elements important in the regulation of the transcription of these genes, and therefore their expression.

Chapter 2

MATERIALS AND METHODS

A. Materials

1. Enzymes

Restriction endonucleases were from Pharmacia Molecular Biology Division, Montreal, Quebec; New England Biolabs, Beverly, Maryland,USA; or Bethesda Research Laboratories (BRL), Bethesda, Maryland, USA.

T4 DNA Ligase, DNA Polymerase I, DNA Polymerase (Klenow Fragment), and Mung Bean Nuclease were from Pharmacia, New England Biolabs, or BRL. Deoxyribonuclease I (DNase I) was from Pharmacia or Boehringer Mannheim GmbH, and Ribonuclease A was from Sigma Chemical Co., St. Louis, Missouri, USA. Proteinase K was from Bethesda Research Labs.

2. Electrophoresis Chemicals

Agarose was supplied by BRL and Sigma Chemical Co., Acrylamide by Bio-Rad Laboratories, Richmond, California, USA, and British Drug House (BDH), Toronto, Ontario.

Ammonium Persulphate, N,N'-Methylenebis-acrylamide, and N,N,N',N'-Tetramethyl-ethylenediamine (TEMED) were supplied by Bio-Rad Laboratories, BDH, and Sigma Chemicals. All other chemicals were supplied by Sigma Chemicals.

3. Bacterial Culture Media

All Bacto products (Tryptone, Yeast Extract, and Agar) were from Difco Laboratories, Detroit, Michigan, USA. Pre-mixed Luria-Bertani (LB) broth mix was supplied by Gibco Laboratories, Maddison, Wisconsin, USA. Antibiotics were from Sigma Chemicals.

4. Tissue Culture Products

Dulbecco's Modified Eagle Medium (DMEM) and Tissue culture dishes were from Gibco Laboratories, while antibiotics were from Sigma Chemicals.

5. General Chemicals and Supplies

General laboratory chemicals and reagents were purchased from Sigma Chemicals, British Drug Houses, Toronto, Ontario, and J.T. Baker Co., Phillipsburg, New Jersey, USA. Laboratory supplies were from Canlab, and Western Scientific, both of Vancouver, British Columbia.

B.Bacterial Strains, Vectors, and Media

1. Vectors

The rat liver genomic library was supplied in the lambda vector Charon 4A (Jagodzinski, unpublished). Charon 4A ($A_{am}32$, B_{am1}, lac 5, bio 256, KH 54, BW 2, nin 5, and QSR 80) (Blattner et. al.,1977) phage particles contained rat DNA fragments 15,000 to 20,000 nucleotides in length generated by partial digestion with the restriction endonuclease Hae III and cloned into the Eco R1 site.

The plasmid pUC9 (Vieira and Messing, 1982) was used for the subcloning and maintenance of DNA fragments isolated from the Charon 4A library. Small DNA fragments for sequencing were sub-cloned into the M13 phage vectors mp9 (Messing and Vieira, 1982), mp18, and mp19 (Yanisch-Perron et al.,1985).

2. Bacterial Strains

The host cell used for growth of the Charon 4A phage was Escherichia coli strain LE392 [F^-, hsd R514(r_k^-,m_k^-), supE44, supF58, (lac IZY)6, gal K2, gal T22, met B1, trp R55%, and lambda$^-$] (Leder et al., 1977; Murray et al., 1977).

Plasmid pUC9 was maintained in E. coli K-12 strain JM83 cells (ara, lac-pro, strA, thi, φ80dlacZM15) (Messing, 1979). The M13 phage were grown in E. coli K-12 strain JM101 cells [supE, thi, (lac-proAB),(F-traD36, proAB, lacIqZ,M15)] (Messing et al., 1981).

3. Media

Luria-Bertani (LB) media: 10g Bacto-tryptone, 5g Bacto Yeast Extract, and 10g NaCl per liter of dH_2O. (for plates add 1.5% Bacto-agar)

2xYT media: 16g Bacto-tryptone, 10g Bacto Yeast Extract, and 5g NaCl per liter of dH_2O.(for plates add 1.5% Bacto-agar)

M9 Salt media: 6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, and 1g NH_4Cl per liter of dH_2O. Sterilize, and when cool add : 2.0ml 1M $MgSO_4$, 10ml 20% glucose, and 0.1ml 1M $CaCl_2$ per liter.

Top (T) agar: 10g Bacto-tryptone, 5g NaCl, and7.5g Bacto-agar per liter.

C.Basic Techniques of Molecular Biology

1. Restriction Endonuclease Digestion of DNA

Each restriction enzyme has its own optimal reaction conditions, which are supplied by the manufacturer. Typically 2 to 10μg of DNA was suspended in 20μl of a solution of 10mM Tris-HCl pH 7.5, 1mM DTT, and 0 to 100mM NaCl depending on the enzyme. Two to ten units of the appropriate endonuclease were then added and digestion allowed to proceed for 1 to 2 hours at the recommended temperature.

2. Agarose Gel Electrophoresis

a) Qualitative Agarose Gel Electrophoresis

Agarose gel electrophoresis of DNA samples was carried out using 0.7% to 1.0% agarose, containing 1μg/ml EtBr, and Tris Borate buffer

(TBE) (90mM Tris-HCl, pH 8.3, 90mM Boric acid, and 25mM EDTA) or Tris Acetate buffer (TAE) (40mM Tris-HCl, pH 8.0, 20mM NaOAc, and 2mM EDTA) (Maniatis et al.,1982). Glycerol-dye solution (20% glycerol, 0.1% bromphenol blue) was added to samples in a 1 to 1 (dye:DNA sample) ratio prior to loading the gel. DNA fragments were subjected to electrophoresis at 12 to 20 Volts/cm for 15 to 30 minutes. DNA bands were visualized under short wave ultraviolet (UV) light (254nm).

b) Preparative Agarose Gel Electrophoresis

In order to recover specific DNA fragments from agarose gels DNA was electrophoresed and bands visualized as described above. The desired DNA band(s) were located and the segment of the gel containing the fragment excised from the gel with a razor blade. The DNA was then isolated from the gel slice by one of two methods.

i) Electroelution in Dialysis Bags

The gel slice was placed in a dialysis bag containing buffer at one half the concentration of the running buffer. the bag was then sealed and placed partially submerged in a gel box such that the gel fragment is perpendicular to the current. Current was passed through the bag (100V for 30 to 60 minutes) until all the DNA has migrated out of the gel slice and onto the wall of the dialysis bag. The polarity of the current was reversed for 90 seconds to release the DNA from the wall of the dialysis bag. The DNA solution was then removed from the bag and the DNA recovered by precipitation with ethanol (McDonell et al.,1977).

ii) Low Melting Point (LMP) Agarose Gels

A number of grades of agarose are available which melt at 65°C, which is well below the melting point of DNA, and remain molten at 37°C. Gel slices cut from LMP agarose gels were melted at 65°C and diluted in

65°C TE buffer containing 0.2M NaCl. The solution was loaded on a Bethesda Research Laboratories (BRL) NACS PREPAC^{TM} column equilibrated in the same buffer. The DNA fragments were selectively bound to the resin in the column while the agarose was not. The column was washed with 5ml TE buffer containing 0.2M NaCl to remove all of the agarose. The DNA fragments were then selectively eluted using a small volume (600µl) of TE buffer containing 1.0M NaCl. The eluted DNA was recovered by ethanol precipitation.

Alternatively LMP agarose gel slices were melted at 65°C, diluted with TE buffer, and the agarose removed by repeated extraction with phenol. Again the DNA was recovered from solution by ethanol precipitation (Weislander,1979).

3. Polyacrylamide Gel Electrophoresis

Polyacrylamide gels (Maniatis et al.,1982) containing 6% to 8% acrylamide, and 7.5M Urea were routinely used for DNA sequencing. Glass plates (36x20cm) were scrubbed well with detergent and treated with 2% dimethyl-dichlorosilane to siliconize the plates. The glass plates were then taped together using 0.35mm plastic spacers. Forty milliliters of gel solution (8%) was prepared as follows: 25g Urea, 10ml 40% acrylamide solution [91.2g acrylamide, 4.8g BIS (N,N'-methylenebisacrylamide), and distilled water to 240ml; deionized with Amberlite^{TM} MB-3 resin], 2.5ml 10x TBE (0.9M Tris-HCl pH 8.3, 0.9M Boric acid, and 0.25M EDTA), and 20ml dH_2O. The gel solution was warmed to dissolve the urea and de-aerated under vacuum. The gel was polymerized by the addition of 330µl 10% Ammonium Persulphate, and 20µl TEMED (N,N,N',N'-tetramethylethylenediamine), quickly poured between the glass plates,

and allowed to set. Sequencing gels were electrophoresed in 1x TBE buffer at 35 watts per gel.

D. Isolation and Purification of Nucleic Acids

1) Large Scale Preparation of Plasmid DNA

The procedure used was that of Birnboim and Doly (1979) as modified by Maniatis (1982). A culture of the bacterial strain containing the plasmid was prepared by inoculating 40ml of Luria-Bertani (LB) media containing antibiotic (50μg/ml for ampicillin; 10μg/ml for tetracycline) with a single bacterial colony, and incubating the culture overnight at 37°C, with shaking.

Four liters (L) of M9 media containing antibiotics were inoculated with 10ml of the overnight culture prepared above. The 4L of culture was shaken vigorously at 37°C until the optical density of the culture reached an $O.D._{600}$ of 0.7. Chloramphenicol was added to a final concentration of 250μg/ml and incubation continued at 37°C overnight.

The bacterial cells were collected by centrifugation at 4000 x g for 10 minutes at 4°C, and resuspended in 80ml of a Solution 1 [25% sucrose; 50mM Tris(hydroxymethyl)aminomethane-HCl(Tris-HCl), pH 8.0; 1mM Disodiumethylenediaminetetracetic acid (EDTA); and lysozyme 4mg/ml (added fresh)]. The suspension was incubated at 22°C for 5 minutes, followed by addition of 80ml of ice cold Solution 2 [0.2N NaOH; 1.0% Sodium Dodecylsulphate (SDS)], gentle mixing and incubation on ice for 10 minutes. The cell lysate was cleared by addition of 120ml of cold Solution 3 (60ml 5M Potassium Acetate; 11.5ml glacial acetic acid; and 28.5ml dH_2O), incubation on ice for 10 minutes, and centrifugation at 16,000 x g for 30 minutes at 4°C.

The supernatant was transferred to clean tubes and the DNA precipitated by the addition of 0.6 volumes of cold isopropanol. After 1 to 2 hours of incubation on ice the precipitated DNA was collected by centrifugation for 30 minutes at 4°C, and 16,000 x g. The supernatant was discarded and the pellets dried under vacuum for 5 minutes. The pellets were resuspended in TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA)

The DNA solution was extracted three times with an equal volume of phenol/chloroform (1:1,v:v) to remove proteins. The DNA was then precipitated from solution by the addition of 3M NaOAC (1/10th volume) and 95% Ethanol (2 volumes) and stored at -20°C for 2 to 18 hours. The precipitated DNA was collected by centrifugation at 27,000 x g for 20 minutes at 4°C. DNA pellets were dried and resuspended in TE buffer.

Further purification involved the use of Cesium Chloride (CsCl) equilibrium gradients to separate bacterial chromosomal DNA from plasmid DNA. Essentially (for 4L of initial culture) 80g of CsCl was dissolved in 70ml of TE buffer containing the DNA. Two ml of Ethidium Bromide (EtBr) (10mg/ml) was added and the final CsCl concentration adjusted to 35% brix using an Atago refractometer. The gradients were divide into 8 Beckman "quick seal" tubes (12ml) and sealed. Ultracentrifugation was at 60,000rpm for 20 hours at 15°C in a Beckman Ti 70.1 rotor (331,000 x g). The DNA bands were visualized under UV light (254nm) to locate the plasmid DNA band. The gradient was fractionated by puncturing the tube with an 18 gauge needle and aspirating the band with a syringe. The CsCl-DNA solution was diluted with TE buffer, butanol extracted to remove the EtBr and the DNA precipitated with ethanol as described above.

2. Large Scale Isolation of Phage (Charon 4A) DNA

The procedure used was a scaled up version of the plate lysate protocol described by Maniatis et al.,(1982). Briefly (for 20 plates) 6ml of an overnight culture of Escherichia coli (E. coli) strain LE392 (Leder et al.,1977) was infected with $2x10^9$ plaque forming units (pfu) of bacteriophage by incubation together at 37°C for 20 minutes. Following infection 80 to 100ml of Top (T) agar supplemented with thymidine (50µg/ml) and magnesium (10mM) was added and the mixture plated onto 20 LB agar plates (also supplemented with magnesium and thymidine). The plates were incubated at 37°C overnight to allow growth.

After overnight growth the plaques on each plate should have reached confluence. Each plate was then overlaid with 5ml of SM buffer (100mM NaCl, 10mM Tris-HCl pH 7.5, and 10mM $MgSO_4$) and allowed to stand at 4°C for 2 hours. The buffer containing the phage particles was collected and centrifuged at 27,000 x g for 10 minutes to remove debris. The phage particles were precipitated by the addition of 0.15 volumes 5M NaCl and 0.3 volumes of a 50% solution of Polyethylene Glycol (PEG 6000) and incubation on ice for 120 minutes. The phage particles were collected by centrifugation at 27,000 x g for 5 minutes at 4°C. The phage pellet was resuspended in 10ml Deoxyribonuclease Activation buffer (10mM Tris-HCl pH 7.5, 5mM $MgCl_2$, and 100µg/ml Bovine Serum Albumin). In order to remove bacterial nucleic acids 100µg of Deoxyribonuclease I (DNase I) and 1.0mg Ribonuclease a (RNase) were added and digestion allowed to proceed at 37°C for 30 minutes. Debris was removed by centrifugation at 1500 x g for 10 minutes.

The supernatant was transferred to clean tubes and SDS and EDTA were added to concentrations of 0.05% and 25mM respectively. Proteinase

K (1mg) was added and allowed to digest the phage coat for 60 minutes at 68°C. Digested protein was removed by repeated phenol and chloroform extractions as described above. The phage DNA was ethanol precipitated and used directly or was further purified by CsCl equilibrium gradient centrifugation as described above.

3. Isolation of High Molecular Weight Eukaryotic DNA

Eukaryotic cells (10^6 - 10^7) may be collected from tissue culture or from whole tissue. Cells were washed twice with cold Phosphate Buffered Saline (PBS) and collected by centrifugation at 500 x g for 10 minutes at 4°C. The PBS was carefully removed at the cells resuspended in a solution of 150mM NaCl, 50mM EDTA, and 50mM Tris-HCl, pH 8.0. Sarcosine was added to a final concentration of 2.5mg/ml. Proteinase K (20µg) was added to the solution and digestion allowed to proceed at 50°C for 3 to 8 hours. Following digestion protein was removed by repeated phenol and chloroform extractions. DNA was precipitated using ethanol, and collected by centrifugation. The final pellet was resuspended in 200ul TE buffer, and typical yields were 700ug.

E. Isolation of High Molecular Weight RNA from Eukaryotic Cells

Cells harvested from tissue culture or homogenized tissue ($5x10^7$ cells) were washed twice in 10 ml of a solution of 150mM NaCl and 0.1% Diethylpyrocarbonate (DEP). Cells were collected by centrifugation at 500 x g for 10 minutes at 0°C. The cells were lysed by resuspension of the pellet in 3ml of 7.5M Guanidine Hydrochloride (GuHCl), 0.5% sarcosine, and 10mM Dithiothreitol (DTT). The viscosity of the solution was reduced by trituration of the solution through an 18 gauge needle.

The lysate was carefully layered on a 3ml cushion of 5.7M CsCl and 25mM sodium citrate in a 5ml Beckman Cellulose Nitrate centrifuge tube.

The gradient was ultracentrifuged for 21 hours at 20°C and 36,000rpm in a Beckman SW50.1 rotor. The supernatant was carefully removed leaving the transparent, gelatinous RNA pellet in the tube. The pellet was resuspended in 200µl of TE buffer containing 0.1% SDS and extracted twice with phenol/chloroform (1:1,v:v). The RNA was ethanol precipitated as described above for DNA (Glisin et al.,1974: Ullrich et al.,1977).

F. Labelling of Probe DNA by Nick Translation

DNA fragments to be used as probes in hybridization reactions were radioactively labelled by nick translation (Rigby et al.,1977). A typical nick translation reaction contained 200 to 500ng of probe DNA, 5ul 10x nick translation buffer (500µM Tris-HCl, pH 7.5, 50mM $MgCl_2$), 1.0µl 50mM DTT, 2µl Bovine Serum Albumin (BSA)(2mg/ml), 2ul each of dGTP and dTTP (500µM), 1.5ul each of dATP and dCTP (35µM), 1ul $CaCl_2$(10mM), 2.5µl DNase I (200pg/ul; freshly diluted), 17.5µl dH_2O, 2.5ul each of [α^{32}P]dATP and [α^{32}P]dCTP (10µCi/µl), and 2 units DNA Polymerase I. The reactions were incubated at 16°C for 60 minutes. Reactions were stopped by heating to 68°C for 10 minutes. Unincorporated nucleotides were removed by gel filtration chromatography using Sephadex G-50 gel matrix (Pharmacia) in a 25 x 1cm column. Elution was with TE buffer, and 400µl fractions were collected. The labelled probe DNA eluted in the void volume (fractions 7-9) and was concentrated by ethanol precipitation. Typical nick translation reactions yielded $3.0x10^7$ counts per minute (cpm) at $5x10^7$ cpm/µg of DNA. Probe DNA was denatured by incubation at 100°C for 10 minutes followed by quick cooling in an ice water bath immediately prior to use in hybridization reactions.

G. Screening the Rat Genomic Library

A HaeIII rat liver library was provided by Drs. L. Jagodzinski and J. Bonner (unpublished). The library was constructed by the partial digestion of Sprague-Dawley ($RT1^b$) rat liver DNA to give fragments 15,000 to 20,000 nucleotides in length. These fragments were cloned into the EcoR1 site of the lambda vector Charon 4A using EcoR1 linkers.

The library was screened essentially as described by Woo (1979) using the radioactively labelled cDNA insert from the plasmid pRIa.2 (Wallis and McMaster,1984) as the probe.

1. Plating the Library

The library stock (titered at $2.0x10^9$ pfu/ml) was diluted with SM buffer to give a plating density of $2.0x10^4$ plaques per plate. To this was added 300ul per plate of a fresh overnight culture of E. coli LE392, and the suspension incubated at 37°C for 10 minutes to allow infection. Following infection 4ml per plate of Top agar (10g Bacto-tryptone, 5g NaCl, $MgSO_4$ to 10mM, Thymidine to 0.05mg/ml, 7.5g Bacto-agar, and 1L dH_2O) was added and the mixture plated on LB agar plates supplemented with $MgSO_4$ (10mM) and Thymidine (0.05mg/ml). Once the Top agar had set the plates were inverted and incubated at 37°C overnight.

In order to locate a single copy gene in a rat DNA library (genome complexity $3.0x10^9$ nucleotides) $3.0x10^5$ recombinant phage each containing $2.0x10^4$ nucleotides of rat DNA must be screened. Therefore to screen the rat library for the $RT1B_\alpha$ gene $2.0x10^6$ plaques were examined (ie. 100 plates at $2.0x10^4$ plaques per plate).

2. Amplification

Nitrocellulose filters (sterile Millipore™ HA) were soaked in an overnight culture of E. coli LE392 cells and allowed to air dry for 60

minutes. The filters were then placed carefully on the agar surface of the 100 plates containing the phage plaques. The plates were allowed to stand 10 minutes. After marking the plates and the filters to allow orientation at a later time, the filters were carefully peeled off the plates and placed on fresh LB agar plates supplemented with $MgSO_4$ and Thymidine. The plates were then incubated at 37°C overnight.

3. Plaque Hybridization

The nitrocellulose filters were lifted off the agar plates and placed on Whatman 3MM filters soaked in a solution of 0.5N NaOH, and 1.5M NaCl for 15 minutes. This procedure denatures the phage particles and fixes the DNA to the nitrocellulose filter. The filters were neutralized by transferring the filters to Whatman 3MM paper soaked in 0.5M Tris-HCl, pH 7.4, and 1.5M NaCl for 15 minutes. The filters were then air dried, baked at 68°C for 2 hours to fix the DNA, and pre-hybridized at 68°C overnight in 6xSSC (1xSSC 0.15M NaCl, 0.015M Sodium Citrate) and 1x Denhardt's (0.04% polyvinylpyrrolidone, 0.04% ficoll, and 0.04% BSA).

The filters were hybridized in a solution of 6xSSC, 0.5% SDS, and 1x Denhardt's containing denatured ^{32}P labelled DNA probe. Hybridization was carried out at 68°C overnight with at least $1x10^6$ cpm per filter. the filters were washed three times for 60 minutes each in 1xSSC, 0.5% SDS at 60°C. After air drying the filters were autoradiographed at -20°C using Kodak XAR-2 x-ray film and intensifying screens.

The original plaque containing plates, filters, and films were aligned and plaques with positive hybridization signals identified. Positive plaques were excised from the plate using the tip of a sterile pasteur pipet, and suspended in 2ml of SM buffer. Following addition of

a single drop of chloroform these phage stocks were stable at 4°C indefinitely.

4. Second and Third Screens

In order to insure that plaques hybridizing with the probe were indeed positive a second and third screen were performed. Essentially phage stocks from plaques selected in the first screen were titered to determine the number of phage present. Each stock was then plated at a density of 500 plaques per plate as described above. A dry sterile nitrocellulose filter was placed on each plate for 10 minutes and then denatured directly, omitting the amplification step. Plaque hybridization was carried out exactly as described. Plaques identified as positive in the second screen were isolated, plated, and screened a third time.

Three independent recombinant phage clones positive for the RT1 B_{α} gene after 3 screens were chosen for further analysis. They were designated Lambda 7, 8, and 16. DNA stocks from each of these phage were prepared as described in Section A.

H. Analysis of Recombinant Clones

1. Restriction Mapping

Ten microgram (μg) amounts of DNA were digested in 20μl volumes with a number of different restriction enzymes. Enzymes were used singly or in pairs. Digested DNA samples were electrophoresed on qualitative agarose gels with DNA standards of known molecular size. The DNA fragments were visualized under UV light (254nm) and photographed to create a permanent record. From the sizes of the DNA fragments the relative locations of the restriction enzyme cleavage sites were deduced.

2. Southern Blot Analysis

DNA fragments which had been separated electrophoretically in agarose were denatured by soaking the gel in 0.5M NaOH, 1.5M NaCl for 30 minutes. Following neutralization of the gel by soaking for 30 minutes in a solution of 0.5M Tris-HCl, pH 7.6, 1.5M NaCl the DNA was transferred unidirectionally to GeneScreen^{TM} Transfer membrane (New England Nuclear) according to the method of Southern (1975). The DNA was then immobilized on the membrane by baking at 80-100°C for 2-4 hours.

Prehybridization conditions were routinely 2.5x Denhardt's (0.1% polyvinyl-pyrrolidone, 0.1% ficoll, and 0.1% BSA), and sheared Herring Testis DNA (100µg/ml) as carrier. Membranes were sealed in plastic bags and incubated at 60°C for 4 to 20 hours. Hybridizations were routinely carried out in the following solution: 0.3M NaCl, 0.06M Tris-HCl(pH 8.0), 0.002M EDTA, 0.5x Denhardt's (0.02% polyvinyl-pyrrolidone, 0.02% Ficoll, and 0.02% BSA), 1.0% SDS, and carrier DNA (≥100µg/ml). Denatured probe DNA ($5.0x10^6$cpm at ≥ $5.0x10^7$cpm/µg) was added and the entire mixture sealed in a plastic bag with the membrane. Hybridizations were carried out at 60°C for 12-24 hours.

Membranes were washed twice in 100ml of 0.3M NaCl, 0.06M Tris-HCl(pH 8.0), and 0.002M EDTA at room temperature for 5 minutes. The stringency was then increased and the membranes washed for 30 minutes at 60°C in 100ml the above solution with SDS added to 1.0%. This wash was repeated a second time, the membrane air dried, and exposed to Kodak XRP-1 film at -20°C with intensifying screens. Comparison of the films and the original photograph of the gel allowed identification of DNA bands containing sequences homologous to the probe DNA.

I. Sub-cloning of DNA Fragments Into the pUC Plasmids

1. Isolation of specific DNA fragments

Lambda 7 was shown to contain the RT1 B_α gene on two EcoR1 fragments 7,800 and 8,100 nucleotides in length by restriction mapping and Southern analysis. Phage DNA (20µg) was digested with EcoR1 and subjected to electrophoresis on a preparative agarose gel. The desired bands were excised from the gel, and the DNA fragments isolated as described in Section C. DNA fragments were resuspended in dH_2O to a final concentration of 50-100ng/µl.

2. Ligation

The pUC family of plasmid vectors (Vieira and Messing, 1982) have been used to maintain subcloned fragments of DNA. The plasmid vector pUC9 was digested with EcoR1 to completion, phenol/chloroform extracted, and ethanol precipitated. Vector DNA was resuspended in dH_2O at a concentration of 10 to 20 ng/µl. Ligation conditions were as follows: 10-20ng vector DNA (EcoR1 cut pUC9), 20-100ng of the DNA fragment to be subcloned (isolated above), 2µl 10x ligation buffer (0.5M Tris-HCl, pH 7.8, and 0.1M $MgCl_2$), 2µl 10mM ATP, 2µl 50mM DTT, 1-2 units T4 DNA ligase, and dH_2O to 20µl. The reaction was incubated at 16°C overnight.

3. Transformation of DNA into Bacteria

Introduction of recombinant pUC plasmids into the bacterial host E. coli strain JM83 (Vieira and Messing, 1982) allows propagation of the plasmid.

a) Preparation of Competent Cells

In order to be able to take up the recombinant plasmid DNA bacterial cells must be made "competent" by treatment with $CaCl_2$ (Morrison, 1979; Maniatis et al.,1982; Messing et al.,1981). JM83 cells

were grown in 50ml of 2xYT medium at 37°C with vigorous shaking until the $O.D._{600nm}$ reached 0.6. The cells were collected by centrifugation at 1500 x g for 10 minutes at 4°C. The cell pellet was gently resuspended using a Pasteur pipet in 16ml cold 100mM $CaCl_2$, and allowed to stand on ice for 30 minutes. The cells were again collected by centrifugation at 1500 x g for 10 minutes at 4°C. The pellets were gently resuspended in 5ml of a 100mM $CaCl_2$ solution, and transformed immediately.

b) Transformation

A small portion of the ligation reaction (3-5µl) was added to 45µl of TEN 7.5 buffer (10mM Tris-HCL, pH 7.5, 1mM EDTA, and 100mM NaCl), and 200µl of freshly made competent JM83 cells. The suspension was incubated on ice for 30-40 minutes, followed by heat shock at 42°C for 2 minutes. LB broth (1ml) was added and the mixture incubated at 37°C for 60 minutes. A small volume (100µl) was then used to inoculate LB agar plates containing ampicillin (50µg/ml), Isopropylthiogalactoside (IPTG) (160 µg/ml), and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) (40µg/ml) utilizing the spread plate technique. The plates were incubated at 37°C overnight. Colonies containing recombinant plasmid (identified by their white colour) were picked and cultured in 5ml of LB broth containing 50µg/ml ampicillin at 37°C overnight. Sterile glycerol was then added to a final concentration of 15% and the culture frozen at -70°C. Frozen stock cultures of pRT1B.4 and pRT1B.5 were stored at -70°C, and were thawed as required for isolation of plasmid DNA.

J. DNA Sequence Analysis

1. Preparation of DNA for Shotgun Cloning into M13 Vectors

One approach to sequencing a gene was to generate random DNA fragments 200 to 500 nucleotides in length, and sub-clone them into M13 vectors. Random fragments were generated by two methods:

a) Digestion with Restriction Enzymes

Restriction enzymes which have four nucleotide recognition sites will cut DNA on average once in every 256 nucleotides. Plasmid DNA (pRT1B.4 and pRT1B.5) were digested with the enzymes AluI, RsaI, and HaeIII which result in blunt ended fragments, or Sau3A which results in cohesive ends. Fragments were phenol/chloroform extracted and precipitated with ethanol prior to ligation.

b) Sonication

Random DNA fragments were also generated using the sonication procedure of Deininger (1983). Whole plasmid DNA (20µg) was resuspended in a solution of 0.5M NaCl, 0.1M Tris-HCl (pH 7.4), and 0.01M EDTA. The DNA solution was cooled on ice and subjected to five 5 second bursts on a Biosonik IIA Sonicator set at 60, with mixing between each burst. Sonicated DNA fragments were collected by ethanol precipitation, and fractionated on a preparative LMP agarose gel. The region of the gel containing DNA 200 to 500 nucleotides in length was excised, and the DNA recovered using a NACS PREPAC^{TM} column as described above.

The ends of sonicated DNA fragments were repaired for blunt end ligation by treatment with the enzyme Mung Bean Nuclease (Kowalski et al.,1976). The reaction was carried out as follows: 2µl 10x S1 buffer (0.3M NaOAc pH 4.5, 0.5M NaCl, and 0.01M $ZnSO_4.7H_2O$), and 10units Mung Bean Nuclease were added to 10µg of DNA in 90µl of TE buffer, and

incubated at 37°C for 10 minutes. The reaction was stopped by extraction with phenol/chloroform, and the DNA recovered by precipitation with ethanol.

2.Subcloning DNA into M13 Vectors

a) Ligation

Random DNA fragments with blunt ends were ligated into M13 mp9 or mp18 (Messing and Vieira, 1982; Yanisch-Perron et al., 1985) Replicative Form (RF) cut with SmaI. Fragments with cohesive ends were ligated into M13 mp9, mp18, or mp19 RF cut with an enzyme generating a compatible cohesive end. Ligations were carried out under the following general conditions: 2µl vector DNA (10-20ng/µl), 2µl 10x ligation buffer (0.5M Tris-HCl pH 7.8, and 0.1M $MgCl_2$), 2µl 10mM ATP, 2µl 50mM DTT, 1-2units T4 DNA Ligase, 20-100ng of DNA to be cloned, and dH_2O to 20µl. Reactions were incubated at 16°C overnight.

b) Transformation of M13 RF into Bacteria

The bacterial host for the M13 phage vectors was JM101 (Vieira and Messing,1982). JM101 cells were made competent prior to transformation as described for JM83 in Section G. The transformation reaction incubated 3µl of the ligation mix with 45µl TEN 7.5 buffer, and 200µl fresh competent JM101 cells together on ice for 30-40 minutes. The cells were then heat shocked at 42°C for 2 minutes followed by the immediate addition of 3.0ml 2xYT media with 0.75% agar (autoclaved and cooled to 42°C), 20µl 100mM IPTG, and 40µl 2% X-gal. The mixture was then poured onto LB agar plates and allowed to set. Plates were incubated at 37°C overnight. Recombinant plaques appeared as clear colourless plaques.

3. Isolation and Growth of Recombinant M13 Phage

Clear plaques were isolated from agar plates using the tip of a sterile Pasteur pipet. The piece of agar containing the plaque was physically excised from the plate and placed in 2ml sterile 2xYT medium. Plaques were stored for up to several weeks at 4°C in this form.

Recombinant M13 phage were grown by adding 20µl of an overnight culture of JM101 cells to the agar plug in 2xYT medium, and incubating overnight at 37°C with aeration.

4. Preparation of Template DNA

The overnight M13 phage cultures were transferred to sterile (1.5ml) microfuge tubes (Eppendorf) and centrifuged for 5 minutes (15,000 xg) to pellet the cells. One ml of supernatant was transferred to a clean microfuge tube, and 250µl of a solution of 20% PEG, 2.5M NaCl added. The tubes were mixed and allowed to stand at room temperature for 30 minutes. The phage particles were collected by centrifugation at 15,000 x g in a microfuge. The PEG supernatant was removed with a pipet, and the pellet resuspended in 100µl TE buffer. The phage coats were removed by three phenol/chloroform extractions, and the DNA recovered by ethanol precipitation. Phage DNA pellets were routinely resuspended in 20µl dH_2O.

5. Screening Recombinant M13 Phage DNA

Recombinant phage containing DNA encoding the RT1 B_α gene were identified by hybridization with the cDNAs pRIa.2 (Wallis and McMaster, 1984) and pACD3 (Benoist et al.,1983). Template DNA (2µl) was spotted on nitrocellulose membranes (Millipore HA) and allowed to air dry. The DNA was denatured by soaking the filter in a solution of 0.5N NaOH and 1.5M NaCl for 15 minutes, followed by neutralization in a solution of 0.5M

Tris-HCl pH 7.6 and 1.5M NaCl for 15 minutes. Template DNA was immobilized on the membrane by baking the filter at 68°C for 4 hours.

Membranes were prehybridized in 5ml of a solution of 5x Denhardt's (0.2% PVP, 0.2% Ficoll, and 0.2% BSA), 5x SSC (0.75M NaCl, and 0.075M Sodium Citrate), 50% formamide (deionized), 50mM phosphate buffer pH 6.8, 0.1% SDS, and 250µg/ml sheared Herring testis DNA. Membranes were sealed in plastic bags and incubated at 42°C for 2 to 18 hours.

The prehybridization buffer was replaced with hybridization buffer, the bag re-sealed, and incubated at 42°C overnight. Hybridization buffer contained 1x Denhardt's (0.15% PVP, 0.15% Ficoll, and 0.15% BSA), 5x SSC, 50% formamide, 20mM phosphate pH 6.8, 0.1% SDS, 100µg/ml carrier DNA, and denatured radioactive probe ($2x10^6$ cpm per filter; $5x10^7$ cpm/µg).

The membranes were washed three times in 2x SSCP (0.3M NaCl, 0.03M NaOCit, 1.0mM EDTA, 0.02M phosphate, and 0.1% SDS) at 20°C for 15 minutes per wash. Four additional washes were carried out in 0.2x SSCP at 50°C and 15 minutes per wash. Membranes were air dried and autoradiographed using Kodak XRP-1 film at -20°C with intensifying screens (Dupont Lightning Plus). Templates which hybridized to the probe were sequenced.

6.Di-deoxy DNA Sequencing

M13 DNA templates were sequenced using the di-deoxynucleotide chain termination protocol of Sanger (Sanger et al.,1977).

a) Reactions

In a microfuge tube (eppendorf) mix 2µl 10x Hinf buffer (100mM Tris-HCl pH 8.0, 70mM $MgCl_2$), 1µl(0.2 pmoles) M13 primer (17 base universal sequencing primer), and 5µl template DNA (1.0µg). The tube was

heated to 75°C and allowed to slowly cool to room temperature over 45 minutes. Next 1µl 15µM dATP, and 1.5µl [α^{32}P]dATP (10µCi/µl) were added, and the tubes mixed. The reaction mixes were dispensed into 4 microfuge tubes at 2µl to the A and T tubes and 2.5µl to the G and C tubes. To the tubes 1.5µl of the appropriate termination mix (see Appendix 1 for the preparation of termination mixes) was added. 0.6units of Klenow DNA Polymerase was added to each tube and incubated at room temperature for exactly 15 minutes. One microliter of 0.5mM ATP was added and incubation at room temperature continued for a further 15 minutes. The reactions were stopped by addition of 5µl dye mix (98% formamide, 10mM EDTA, 0.2% bromphenol blue, and 0.2% xylene cyanol) and heating to 100°C for 5 minutes.

b) Gels

Reactions were loaded directly on acrylamide gels prepared as described above, and electrophoresed for approximately 1.5 to 3 hours. Gels were dried and exposed to Kodak XRP-1 film overnight at -20°C. DNA sequence data was analyzed by the computer programs of Staden (1980), and Delaney (1982).

K. Transfection of Mouse L Cells with RT1 Class II Genes

1. Growth and Maintenance of L cells in Tissue Culture

The thymidine kinase-deficient mouse L-cell line Ltk^-A^- (provided by C. Stammers) was maintained in monolayer cultures in Dulbecco's modified Eagle medium (DMEM) supplemented with heated 10% fetal calf serum (FCS), 100 IU/ml Penicillin G, 100µg/ml Streptomycin Sulphate, and 50µg/ml Amphotericin B (Fungizone). Cells were grown in 100x20mm tissue culture dishes at 37°C, in an atmosphere containing 5% CO_2.

2. Transfection by Calcium Phosphate Co-precipitation

Transfections were carried out essentially as described by Graham and Van der Erb (1973).

a) Preparation of the Precipitate

Approximately 5-10µg of cosmid or phage DNA and 100ng of plasmid pOPF (containing the Tk gene) were mixed together and diluted to a final volume of 450µl with dH_2O. To this was added 50µl of 2.5M $CaCl_2$ and the solution mixed thoroughly. This $CaCl_2$ DNA mixture was added one drop at a time to 500µl of 2x HEBES buffer pH 7.1 (200mM NaCl, 50mM Hepes pH 7.1, and 1.5mM Na_2HPO_4) in a sterile 5ml plastic tube with mixing between drops. The precipitate was allowed to form at room temperature for 30 minutes prior to addition to the cells.

b) Transfection

L-cells were plated at a density of $8x10^5$ cells per 20x100mm tissue culture dish and grown at 37°C overnight as described above. The following morning 5ml of fresh DME medium was added to each culture and cells incubated at 37°C for 1-2 hours. The DNA precipitate was added such that the DNA was spread over the entire surface of the plate and the plates incubated at 37°C for 4 to 6 hours. The media was removed from the dish and the cells were washed with 5ml PBS buffer. The cells were glycerol shocked by addition of 5ml PBS buffer containing 15% glycerol. After 1 minute the PBS glycerol solution was removed and the cells washed with 5ml of PBS buffer. Cells were then grown in 10ml DMEM, containing 10% FCS, for 48 hours to allow expression of the Tk gene. Tk^+ cells were selected by growing the transfected cells in HAT medium for 2-3 weeks. Transformed Tk^+ cell colonies were harvested as mixed

populations of cells (pools) or individually as clonal populations and maintained in monolayer culture as described above.

3. Analysis of Transformed Cells

a) Cell Surface Expression of RT1 Class II Molecules

Transformed Tk^+ L-cells transfected with rat Class II B genes were analyzed for cell surface expression of RT1 B molecules using the mouse monoclonal antibody MRC OX6 (McMaster and Williams, 1979) which detects a non-polymorphic determinant found on all rat Class II molecules. Briefly, cells ($1x10^6$) growing in monolayer culture were washed with PBS buffer (containing no Mg^{2+} or Ca^{2+} ions) and loosened from the bottom of the tissue culture dish by incubation of the culture in 1-2ml 0.6mM EDTA in PBS buffer for 5 minutes. The cells were collected by washing the plate with several milliliters of PBS buffer containing 10% fetal calf serum.

Cells were collected by centrifugation at 500 x g for 5 minutes at 4°C. The cell pellet was resuspended in 50µl of a solution of MRC OX6 antibody (20µg/ml), and incubated on ice for 45 minutes to allow binding. Unbound antibody was removed by washing the cells twice with 1ml PBS buffer containing 0.1% bovine serum albumin (BSA). Again cells were collected by centrifugation as described above. The amount of MRC OX6 bound to the cell surface was quantified using a second antibody (rabbit anti-mouse IgG) labelled with ^{125}Iodine or Fluorescein Isothiocyanate (FITC) followed by analysis using a Gamma counter or by Flow Cytometry (FACS IV) respectively. Again the cells were incubated for 45 minutes on ice in 1ml of the second antibody. Unbound antibody was removed by washing the cells twice in 5ml PBS buffer containing 0.1% BSA. The cells were collected and suspended in 1ml PBS (+ 0.1% BSA)

buffer and analyzed on the FACS IV or Gamma counter. Cells which show high levels of bound fluorescence or radioactivity were expressing RT1 B molecules on their cell surfaces.

b) Hybridization Analysis

In order to investigate the intracellular expression of rat Class II genes high molecular weight DNA and RNA were isolated from Tk^+ L-cells (transfected with RT1 B genes), and hybridized with the RT1 B_α probe pRIa.2.

i) Southern Blot Analysis

Genomic DNA (2µg) was digested with the appropriate restriction endonuclease to completion and run on a 0.5% agarose gel (7x10 cm) in 1x TBE buffer. The gel was denatured by soaking in a solution of 0.5N NaOH, 1.5M NaCl for 60 minutes, followed by neutralization in 0.5M Tris-HCl pH 7.4, 1.5M NaCl for 60 minutes. DNA was transferred to nitrocellulose using the capillary blot procedure of Southern (1975), and 20x SSC as the transfer buffer. Following overnight transfer the membrane was baked at 80°C for 2 hours. The membrane was prehybridized in a solution of 50% formamide, 3x SSC, 1mM EDTA, 0.1% SDS, 10mM Tris-HCl pH 7.5, 10x Denhardt's solution, 0.05% Na pyrrophosphate, 100 µg/µl sheared Herring Testes DNA, and 50µg/µl tRNA at 37°C for 18 hours. The prehybridization solution was removed and replaced with hybridization solution containing ^{32}P-labelled RT1 B_α probe (pRIa.2), and incubated at 37°C for 18-24 hours (hybridization solution and prehybridization solution are the same). Following hybridization the membrane was washed for 60 minutes at 22°C in 2x SSC; twice for 90 minutes at 50°C in 0.1x SSC, 0.1% SDS; and rinsed four times in 0.1x SSC. Membranes were air dried and exposed for 2 to 5 days.

ii) Northern Blot Analysis

RNA suspended in H_2O was mixed with 3 volumes glyoxal premix (67µl DMSO, 23µl 6M deionized glyoxal, 10µl 0.25M phosphate buffer pH 6.6) and incubated at 50°C for 60 minutes. The sample was then cooled to room temperature and sample buffer added (50% glycerol, 10mM phosphate buffer, and 0.4% bromphenol blue). The sample was run on a 1.5% agarose gel using 10mM phosphate buffer pH 6.5 at 3-4 volts/cm for 2 hours. The RNA was transferred to GeneScreen by capillary blot procedure with 25mM phosphate buffer. Following overnight transfer the membrane was baked for 2 hours at 80°C. Hybridizations were carried out as described in Section H above.

Chapter 3

ISOLATION AND CHARACTERIZATION OF RT1 Bα GENOMIC CLONES

A. RESULTS

1. The Plasmid pRIa.2

Wallis and McMaster (1984) have isolated and determined the nucleotide sequence of an RT1 B_α^u cDNA contained in the recombinant plasmid pRIa.2. The cDNA encodes the carboxy-terminal 129 amino acids of the RT1 B_α^u chain and 239 nucleotides of 3' untranslated sequence. The cDNA has been used extensively as a DNA probe in the studies on the structure and transcription of the RT1 B_α gene described in this thesis.

Figure 4 shows a restriction map of the cDNA insert of pRIa.2. The 218 nucleotide Pst1/HpaII fragment was used as a probe for the 5' portion of the RT1 B_α gene whereas the remaining HpaII/Pst1 fragment (nucleotide 218-733) was used as a probe for the 3' end of the RT1 B_α gene.

2. Isolation of RT1 B_α Genomic Clones

A Sprague-Dawley ($RT1^b$) rat liver DNA genomic library constructed in the Lambda vector Charon 4A was screened with radioactively labelled cDNA pRIa.2 (Wallis and McMaster,1984) and from a screen of $1x10^6$ recombinant phage, two strongly hybridizing clones were identified. Southern blot analysis of these recombinant phage, designated Lambda 7 and Lambda 8, is shown in Figure 5. Lambda 7 and 8 overlap and together span 30,000 nucleotides of rat DNA. Two EcoR1 fragments of approximately 8,000 nucleotides in length hybridized to the RT1 B_α cDNA probe in Lambda 7 whereas a single 9,000 nucleotide fragment in Lambda 8 hybridized to the probe. A 4,000 nucleotide BamH1 fragment hybridized to the RT1 B_α cDNA probe in Lambda 7 whereas a 16,000 nucleotide fragment

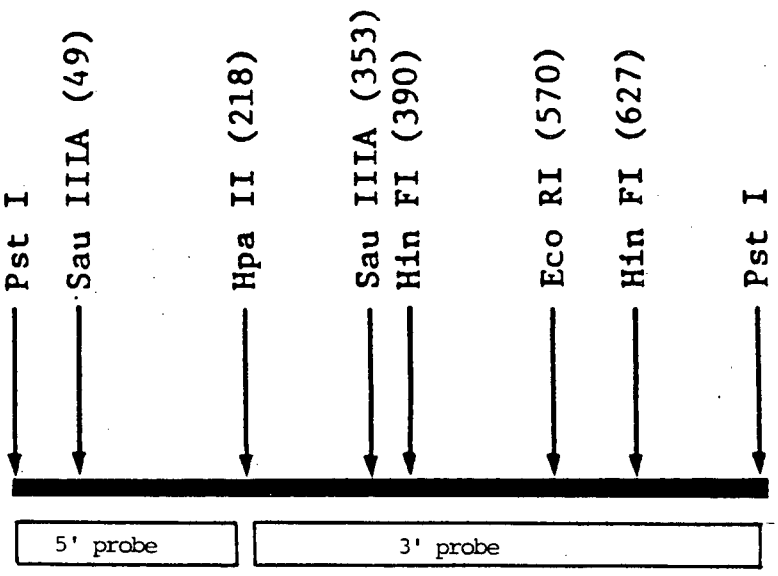

Figure 4: Restriction map of the RT1 B_α cDNA insert (haplotype $RT1^u$) of plasmid pRIa.2 (from Wallis and McMaster,1984). This cDNA was used extensively as an RT1 B_α probe. The 218 nucleotide PstI/ HpaII fragment was used as a probe for the 5' portion of the RT1 B_α gene, while the remainder of the cDNA was used as a probe for the 3' portion of the RT1 B_α gene.

hybridized in Lambda 8. Furthermore hybridizations of EcoR1/BamH1 double digested Lambda 7 and 8 DNA detected bands of 1,900 and 1,100 nucleotides in length for Lambda 7 and 1,900 nucleotides in length for Lambda 8. The restriction map illustrated in Figure 6 shows the orientation of the RT1 B_α gene encoded by Lambda 7 and 8. From these data it appears that the EcoR1 site of Lambda 7 which separates the two fragments detected by the pRIa.2 cDNA probe coincides with the EcoR1 site at position 570 of the pRIa.2 cDNA (Figure 4). One clone, Lambda 7, therefore should contain the entire RT1 B_α gene (as the fragments which hybridized to the RT1 B_α cDNA were flanked by 7,000 nucleotides of DNA in each direction) and was chosen for further study. Lambda 8 was not studied further.

The two EcoR1 fragments of Lambda 7 with homology to the RT1 B_α cDNA were isolated from agarose gels and subcloned into the EcoR1 site of the plasmid vector pUC9. The recombinant plasmids were designated pRT1B.4 and pRT1B.5 (Figure 8A). Southern blot analysis of isolated plasmid DNA hybridized with cDNA probes for the 5' and 3' regions of the gene (see section 1) is shown in Figures 7A and 7B. Figure 7A shows a blot of pRT1B.4 and pRT1B.5 DNA hybridized with the 218 nucleotide Pst1/HpaII fragment of the pRIa.2 cDNA. It is clear that the 5' region of the RT1 B_α gene maps to plasmid pRT1B.4 as the probe for the 5' end of the gene does not hybridize to pRT1B.5. The 530 nucleotide HpaII/Pst1 fragment of the pRIa.2 cDNA hybridizes to both plasmids (Figure 7B). Comparison of the hybridization patterns of pRT1B.4 DNA with the 5' and 3' probes shows that the fragments detected are identical. This suggests that the carboxy-terminal 129 amino acids of the RT1 B_α chain are encoded in a 2,000 nucleotide BamH1/Sma1 fragment located near the 3'

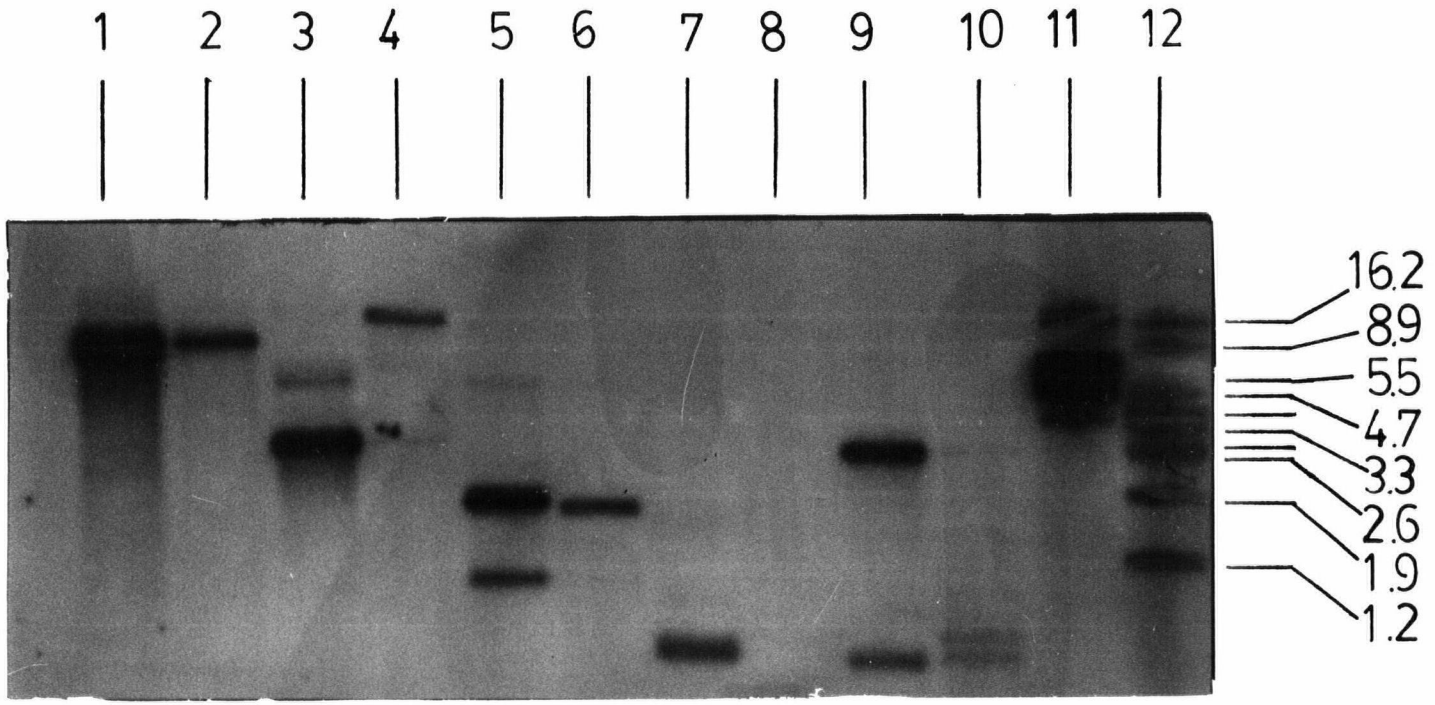

Figure 5: Southern Blot of Lambda 7 and Lambda 8 DNA. Phage DNA was hybridized with the cDNA insert of pRIa.2. Lane 1: EcoR1 digested Lambda 7 DNA; Lane 2: EcoR1 digested Lambda 8 DNA; Lane 3: BamH1 digested Lambda 7 DNA; Lane 4: BamH1 digested Lambda 8 DNA; Lane 5: EcoR1/BamH1 digested Lambda 7 DNA; Lane 6: EcoR1/BamH1 digested Lambda 8 DNA; Lane 7: Alu1 digested Lambda 7 DNA; Lane 8: Alu1 digested Lambda 8 DNA; Lane 9: HpaII digested Lambda 7 DNA; Lane 10: HpaII digested Lambda 8 DNA; Lane 11: EcoR1 digested Lambda DNA standards; and Lane 12: EcoR1/BamH1 digested Lambda DNA standards. The RT1 B_{α} gene encoded in Lambda 7 appears to be contained in two EcoR1 fragments of approximately 8,000 nucleotides in length, whereas the gene is encoded in a single 10,000 nucleotide EcoR1 fragment in Lambda 8. Each lane contains approximately 2µg of digested DNA.

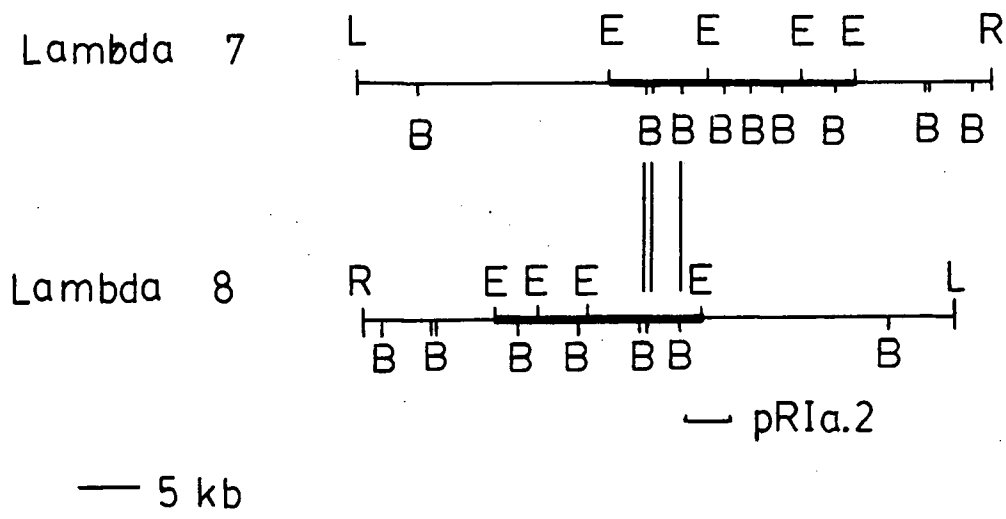

Figure 6: Restriction map of the RT1 B_α gene containing phages Lambda 7 and Lambda 8. The two recombinant phages are aligned to show the RT1 B_α gene in 5' to 3' orientation. The heavy lines indicate DNA fragments inserted into the EcoR1 site of Charon 4A during the construction of the library. EcoR1 sites are indicated with an "E" and BamH1 sites are indicated with a "B". The bar labelled "pRIa.2" denotes the fragments which hybridize to the pRIa.2 cDNA probe.

end of the insert of pRT1B.4. The data from these blots as well as DNA sequence data (presented in Chapter 4) were used to generate the restriction maps shown in Figures 8B and 8C.

3. Genomic Southern Blot Hybridizations

In order to determine if Lambda 7 contained a structurally intact RT1 B_α gene the hybridization patterns of rat genomic DNA and Lambda 7 DNA using the RT1 B_α cDNA probe were compared. When predicting the expected size of genomic fragments from recombinant phage the method of construction of the library from which the phage was isolated must be considered. The library which Lambda 7 and 8 were isolated was constructed from partially HaeIII digested rat DNA cloned into the EcoR1 site of Charon 4A using EcoR1 linkers. Therefore the terminal EcoR1 sites on fragments cloned into Charon 4A (heavy lines in Figure 6) may not be present in genomic DNA. Comparison of the restriction maps of Lambda 7 and Lambda 8 predicts genomic DNA fragment sizes of 9,000 and 7,800 nucleotides for EcoR1 digested DNA; and 3,000 nucleotides for BamH1 digested DNA. Figure 9 shows a Southern blot of high molecular weight Wistar strain rat liver DNA, digested with a number of restriction enzymes, and hybridized with the cDNA insert of pRIa.2. Figure 9 shows that EcoR1 fragments of 9,000 and 7,800 nucleotides in length and a BamH1 fragment of 3,000 nucleotides in length were detected exactly as predicted. Furthermore, the fragment sizes detected in Figure 9 with the enzymes BglII and SmaI are as predicted from the nucleotide sequence of the RT1 B_α gene contained in Lambda 7 and discussed in Chapter 4.

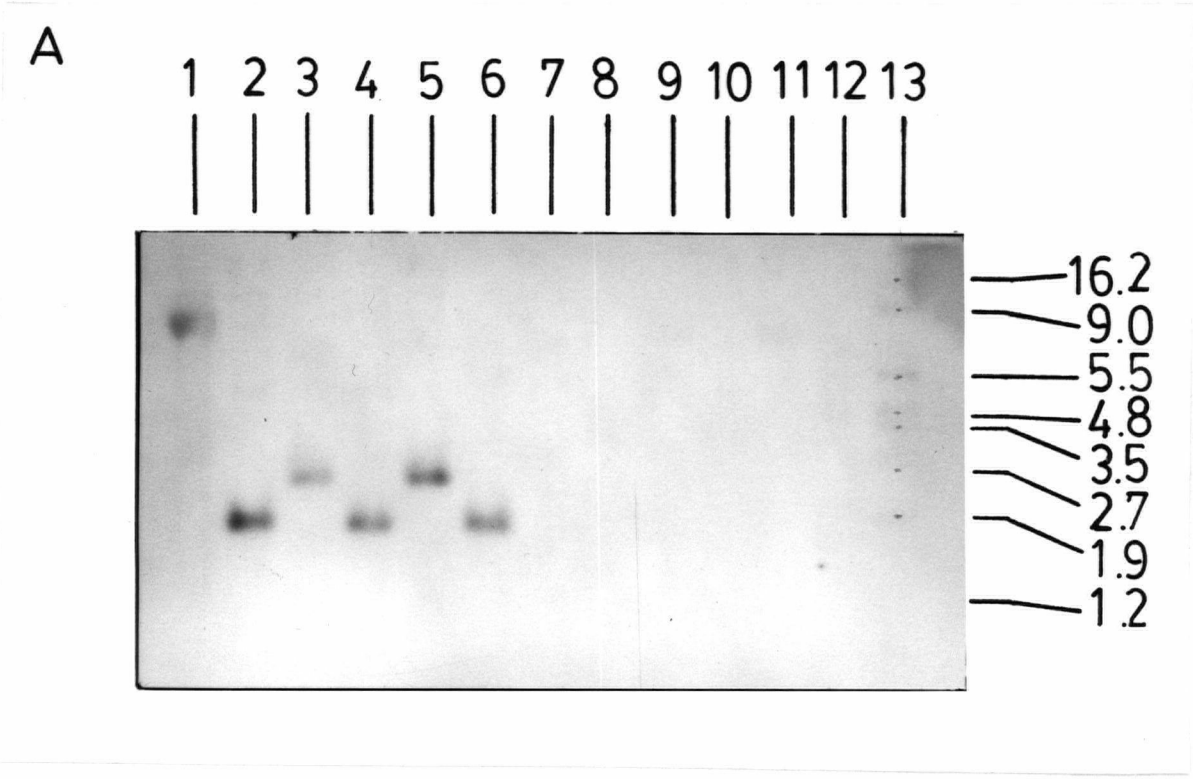

Figure 7A: Southern blot of plasmid pRT1B.4 and pRT1B.5 DNA. Plasmids were digested with various enzymes and hybridized with fragments of pRIa.2. Lanes 1 to 6 contain pRT1B.4 DNA and Lanes 7 to 12 contain pRT1B.5 DNA; Lane 13 contains EcoR1/BamH1 digested Lambda DNA standards. The restriction enzymes used are Lanes 1 and 7: EcoR1; Lanes 2 and 8: BamH1; Lanes 3 and 9: Sma1; Lanes 4 and 10: EcoR1/BamH1; Lanes 5 and 11: EcoR1/Sma1; and Lanes 6 and 12: BamH1/Sma1. Each lane contains approximately 2µg of digested DNA. Blot (A) was hybridized with the 218 nucleotide Pst1/HpaII fragment (1-218) of the cDNA insert of plasmid pRIa.2 (see Figure 4). No hybridization was seen to pRT1B.5 DNA using this probe.

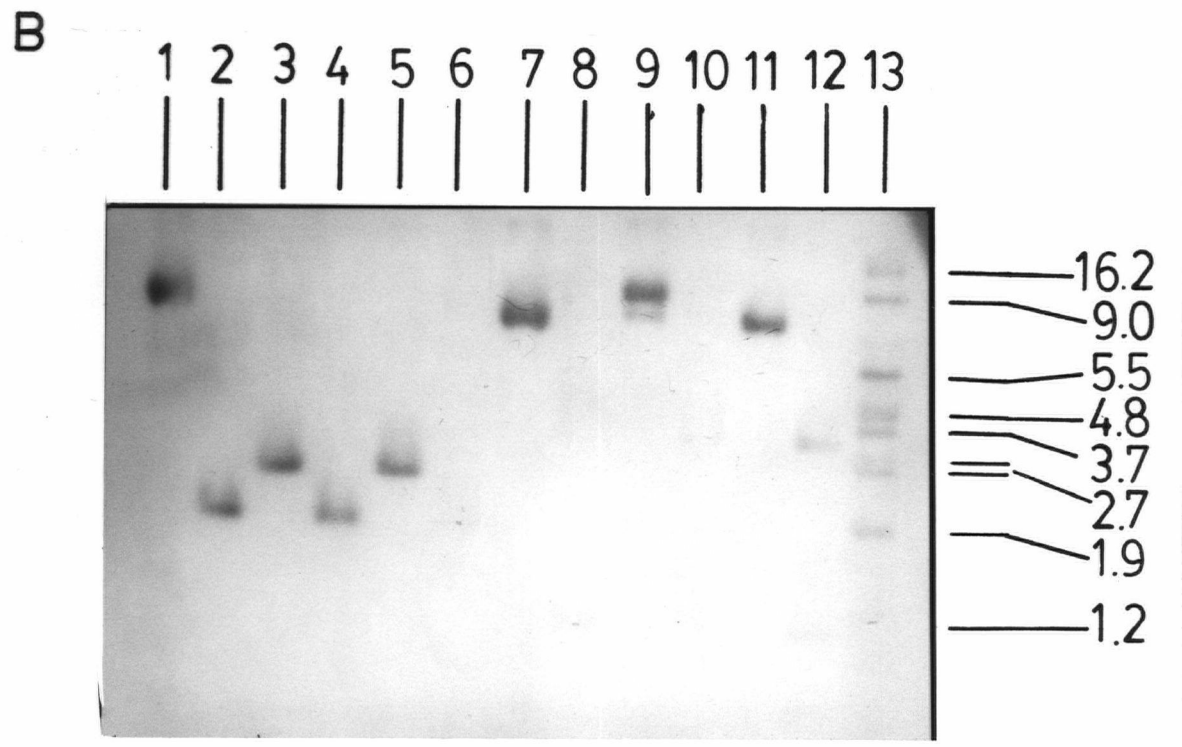

Figure 7B: Southern blot of plasmid pRT1B.4 and pRT1B.5 DNA. Plasmids were digested with various enzymes and hybridized with fragments of pRIa.2. Lanes 1 to 6 contain pRT1B.4 DNA and Lanes 7 to 12 contain pRT1B.5 DNA; Lane 13 contains EcoR1/BamH1 digested Lambda DNA standards. The restriction enzymes used are Lanes 1 and 7: EcoR1; Lanes 2 and 8: BamH1; Lanes 3 and 9: Sma1; Lanes 4 and 10: EcoR1/BamH1; Lanes 5 and 11: EcoR1/Sma1; and Lanes 6 and 12: BamH1/Sma1. Each lane contains approximately 2µg of digested DNA. Blot (B) was hybridized with the 561 nucleotide HpaII/Pst1 fragment (218-733) of the cDNA insert of plasmid pRIa.2 (see Figure 4).

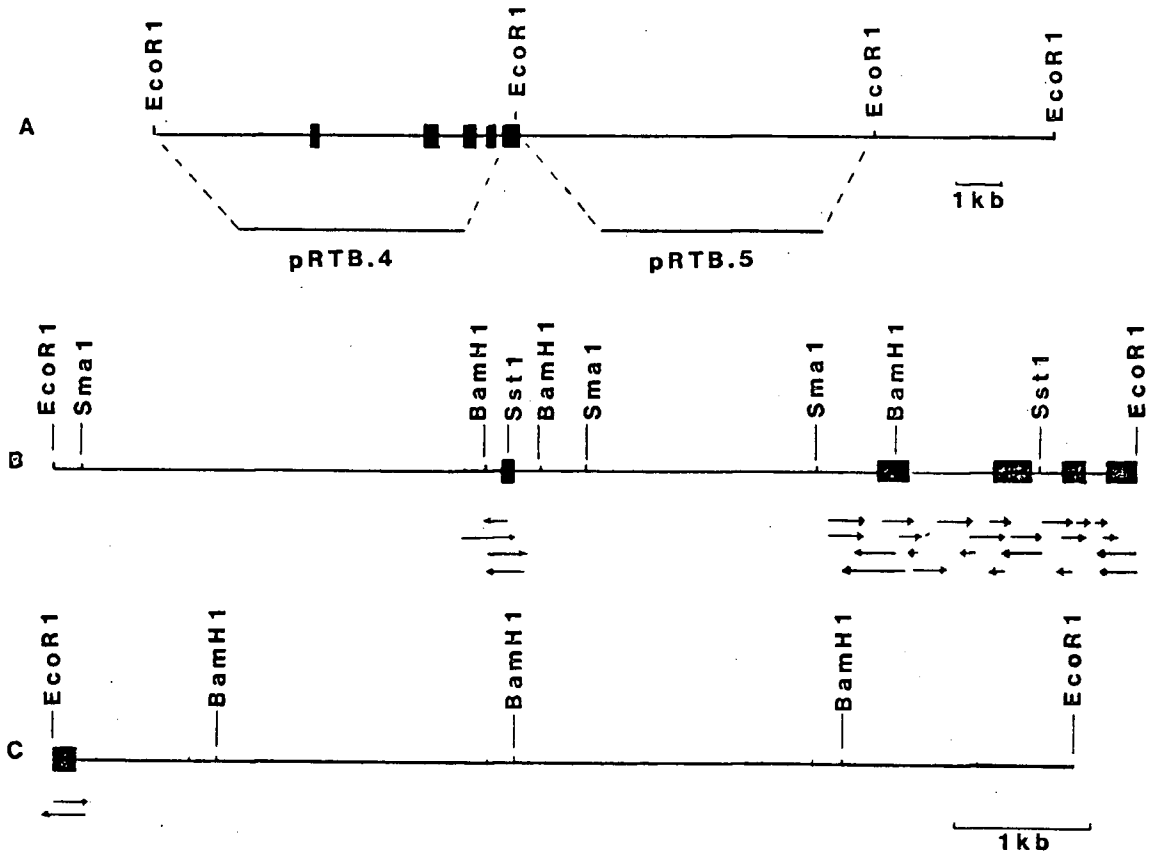

Figure 8: (A) Restriction map of lambda 7. Shaded boxes indicate the exons of the RT1 Bα gene. (B) Restriction map of pRT1B.4. Arrows below the map indicate the fragments used to sequence the RT1 B_α coding regions contained in this plasmid. (C) Restriction map and the fragments used to sequence the RT1 B_α coding regions contained in the plasmid pRT1B.5.

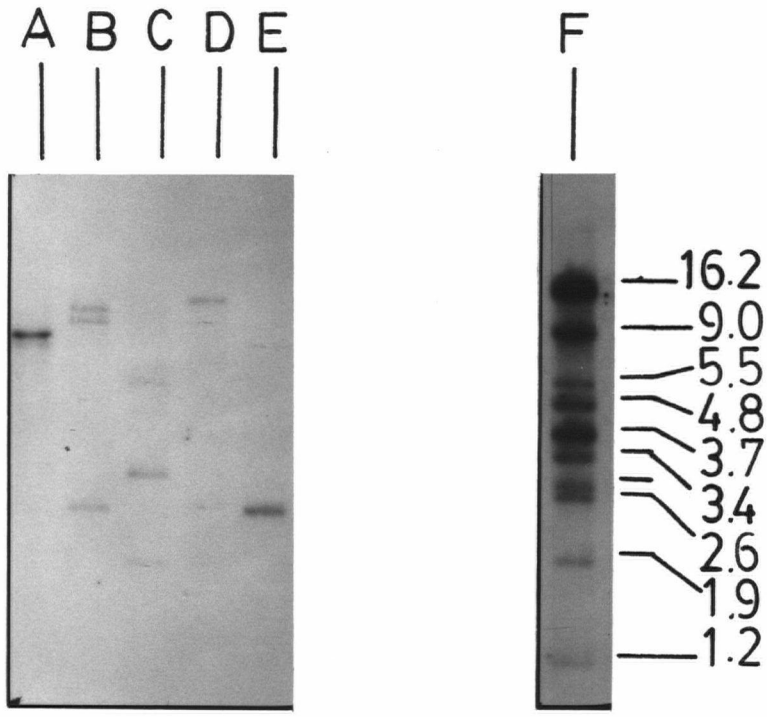

Figure 9: Genomic Southern Blot of Wistar Rat Liver DNA. Approximately 2µg of high molecular weight Wistar rat DNA was digested with the enzymes EcoR1 (Lane A), BamH1 (Lane B), and Sma1 (Lane C) as well as combinations of these enzymes (EcoR1/BamH1 Lane D; EcoR1/Sma1 Lane E). DNA transferred to a Genescreen membrane were hybridized with the cDNA insert of plasmid pRIa.2. Lane F contains EcoR1/BamH1 digested Lambda DNA as molecular weight standards.

B. DISCUSSION

The RT1 B_α cDNA insert of plasmid pRIa.2 was used to screen a Sprague-Dawley ($RT1^b$) rat genomic library. From $1x10^6$ recombinant phage two overlapping clones designated Lambda 7 and Lambda 8 were isolated (Figure 6). Hybridization studies showed that the entire RT1 B_α was potentially encoded in two Lambda 7 EcoR1 fragments of approximately 8,000 nucleotides in length. In order to facilitate the characterization and subsequent sequence analysis of the RT1 B_α gene the two EcoR1 fragments of Lambda 7 were isolated and subcloned into the pUC9 plasmid vector. The RT1 B_α recombinant plasmids were called pRT1B.4 and RT1B.5 (Figure 8A, 8B, and 8C). Restriction mapping and hybridization studies localized the RT1 B_α gene to several fragments of these two plasmids.

Comparison of the restriction fragments detected by hybridization with the RT1 B_α gene probe (pRIa.2 cDNA) in Lambda 7 DNA and in rat genomic DNA has been used to determine if the gene encoded by Lambda 7 is intact. Figure 9 shows that EcoR1 fragments of 9,000 and 7,800 nucleotides in length and a 3,000 nucleotide BamH1 fragment were detected in Wistar genomic DNA and that these fragments corresponded exactly to the fragment sizes predicted from the restriction map of Lambda 7. The RT1 B_α gene encoded by Lambda 7 appears to be intact and identical to that detected by the RT1 B_α gene probe.

Chapter 4

ANALYSIS OF THE DNA AND PREDICTED AMINO ACID SEQUENCE OF THE RT1 Bα GENE

A. RESULTS

1. The Nucleotide Sequence

The approach used to sequence the RT1 B_α gene was to cleave the entire gene into random fragments, identify fragments containing coding sequence, by hybridization with an RT1 B_α cDNA, and determine the sequence of those clones only. This strategy was chosen as it is very efficient in that the structure and organization of a gene can be determined by sequencing only a small portion of the entire DNA contained in a gene (ie coding sequence including intron/exon boundaries as well as 5' and 3' flanking sequence).

The plasmids pRT1B.4 and pRT1B.5 were cleaved into random fragments of 200 to 400 nucleotides in length by two methods. Whole plasmid was sonicated to generate random fragments of varying size. These fragments were size fractionated and isolated using preparative agarose gels. Fragments were rendered blunt ended using Mung Bean nuclease (Kowalski and Kroeker,1976) and blunt end ligated into SmaI cut M13 mp9 or mp18 (Messing,1983). Random fragments were also generated by digestion of whole plasmid with the restriction endonucleases AluI, RsaI, and HaeIII which generate blunt ended fragments. These endonucleases have tetranucleotide recognition sequences and therefore generate fragments which are on average 300 nucleotides in length. Fragments generated by restriction endonuclease were also ligated into SmaI cut M13 mp9 or mp18. Clones containing coding sequence were identified by hybridization with the plasmid pRIa.2 (RT1 B_α cDNA; Wallis and McMaster,1984). As

plasmid pRIa.2 does not contain the 5' end of the RT1 B_α gene the cDNA pACD3 which contains the coding region of the entire mouse H-2 A_α gene was used as a hybridization probe for the 5' end of the RT1 B_α gene (Benoist et al.,1983). Positive clones were sequenced using the di-deoxy nucleotide chain termination method of Sanger et al. (1977). Sequences obtained from random fragments were overlapped by cloning specific fragments into the M13 vectors mp9, mp18, and mp19 (Messing,1983; Norrander et al.,1983). Figures 8B and 8C illustrate the fragments used to determine the coding sequence of the RT1 B_α gene. The complete nucleotide sequence of the coding regions, most of the intron sequences as well as the predicted amino acid sequence are shown in Figure 10. The intron and exon junctions of the RT1 B_α gene were mapped by comparison of the genomic DNA sequence to the cDNA sequence of pRIa.2 and pACD3 and by searching the genomic DNA sequence for the consensus sequence for nuclear mRNA splice junctions (Breathnach and Chambon, 1981). Furthermore, the 3' end of each predicted intron in the RT1 B_α sequence was preceded by variations of the consensus sequence 5'-CTGAC-3' that is found 20 to 50 nucleotides upstream from the 3' splice junction of most rat introns (Keller and Noon, 1984). The organization of the RT1 B_α gene closely resembles the structures of the H-2 and HLA class II α genes in that the gene is divided into 5 exons (Figure 11) which corresponds to the domain structure of the protein (Mathis et al. 1983; Okada et al. 1985; Das et al. 1983). Exon 1 encodes the leader peptide of 23 amino acids as well as the first 5 amino acids of the first external domain (α1). Exon 2 encodes the remaining 83 amino acids of the α1 domain and exon 3 encodes the complete α2 domain consisting of 94 amino acids. In contrast to one exon per domain, exon 4 encodes the

Figure 10: The nucleotide and predicted amino acid sequence of the RT1 Bα gene. The numbers correspond to the amino acid positions in the mature protein. Sequences underlined in the 5' flanking sequences are (5' to 3'): the putative CAAT box, two highly conserved putative regulatory sequence elements, the TATA box, and the site of transcript initiation. The translation termination codon is indicated by ***. The underlined sequences 3' to the coding region are the 3' untranslated region of the gene. ....indicates sequences conserved in all Class II genes (Okada et al. 1985).

CTCCAGGGAGTTCTCCTATCCTCTTCCAGGCCTCTCAATACAAAGTCTGCAGTTAGCAACTGTGACGTCATCACAGGGAAATTTTCTGAT

TGGTCTGTCCGGTTTGGTTTGAGTGTAAGATCTCCTGGGCTGGATCCTCACAATCTCTTGAAGACTCAGGAGAGCAGCTACAGAGACCAC

-23 -1 1

MetProLeuSerArgAlaLeuIleLeuGlyValLeuAlaLeuThrThrMetLeuSerProCysGlyGlyGlnAspAs

CTAGAGAACAGAGATGCCGCTCAGCAGAGCTCTGATTTTGGGGGTCCTCGCCCTGACCACCATGCTCAGCCCCTGTGGAGGTCAAGACGA

5

pIleGluA

CATTGAGGGTGAGTTGTGCAGCTGAGGGATGCCTGGAGCCGGGAAATGGAAAATCTACAGAAGAGGGAGATACAAATTGGCTAAGATATA

AATCCTCAATATTAGACAATCTAACAACTGTGTGAGCCCTTCTTCGGGAGTCTCTGGTTGGCGACCACAG...Intron.1..2.0.KB..

GATCTATTAGACACAAAACCTAACCAGAGAAGGAAATATGCGGACCTGAACCTTTGCAGACAAAGCTTCCTCCCTCTGCTCAGTTGTAAG

CTACTTGGTCTGAGCCGTCTGTGTACCAAAGAGTGGACAAGGCAGTCAGGATAGCAGAAATCAAACACATGGCATCCCACCATTGTGCGG

CGCGTGTCACAGAGGGAAAGACAGGAGTGTAACCCACACTGAGAGTGGTAGGGAGACGCGGGTGGTGGAAATGCATCAAAAACAATGATG

TTCACGGTACTCGTCAGAGCATTTTCTACCCGTTTGTTTTAAAACATTCTTTTCCTCTTCCTGTTTTCCACTTTCCCACCATCCACCTAA

7

laAspHisValGlySerTyrGlyIleThrValTyrGlnTyrHisGluSerLysGlyGlnTyrThrHisGluPheAspGlyA

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spGluArgPheTyrValAspLeuAspLysLysGluThrIleTrpArgIleProGluPheGlyGlnLeuIleSerPheAspProGlnGlyA

ACGAGAGATTCTATGTGGACTTGGATAAGAAGGAGACCATCTGGAGGATCCCCGAGTTTGGACAACTGATAAGCTTTGACCCCCAAGGTG

88

laLeuArgAsnIleAlaIleIleLysHisAsnLeuGluIleLeuMetLysArgSerAsnSerThrProAlaValAsnG

CACTTCGAAATATAGCTATAATAAAACACAATTTGGAAATCTTGATGAAGAGGTCAAATTCAACCCCAGCTGTCAACGGTACGTGCTCAC

CACCCTGCTCCCCCCTCCCTTCCCTGGGGGGTTAGGGGATGTGATCAGGACATTAATGGTCCAATAAAATCCTCTCCTTTCCAAGGAGAC

CCCGGATCTCTTCATCACTGGAGACTCATCCCCTCTCTTCTTGTTTTTAAAGATTTATTTATTTATTATATCTAAGTACACGGTAGCTGT

CTTCGGACACACCAGAGAGAGGGCATCAGATCCCATTACAGATGGTTGTGAGCCACCATGTGGTTGCTGGGAATTGAACTCAGGACCTCT

GGAAGAGCAGTCAGTGCTCTTAACCACTGAGCCATCTCTCCGGCCCATCCCTTCTCTTCTTAAAAATCACACATTTCATGTAATATACAG

CTCACTATTCCACAATGCTCTCCTCGAATCTTTTGGAAAAGCCCCGACAGACATCCTACCTGAACTCCCTAGACGAGAGGGGCACAGGGA

TCAAGTGGAGGGCACATTAGCTCCAAGCAGAGGAATCGGGCTCTTTGACTTTCATATGTGTAAGACTTGTCAAGAAGGCTCTCTCACTAG

90

luValProGluAlaThrValPheS

ACAGTGCAGGGCTTCAGGGAGAGAAGCTCCCATCAGCCTCTGACATCCAGACCATTTCCTTTGCAGAGGTTCCTGAGGCGACCGTGTTTT

erLysSerProValLeuLeuGlyGlnProAsnThrLeuIleCysPheValAspAsnIlePheProProValIleAsnIleThrTrpLeuA

CCAAGTCCCCTGTCCTGCTGGGTCAGCCCAACACCCTCATCTGCTTTGTAGACAACATCTTTCCTCCTGTGATCAATATCACATGGTTGA

rgAsnSerLysProLeuThrGluGlyValTyrGluThrSerPheLeuIleAsnSerAspTyrSerPheHisLysMetAlaTyrLeuThrP

GAAACAGCAAGCCACTCACAGAAGGCGTTTATGAGACCAGCTTCCTTATCAACAGTGACTATTCCTTCCACAAGATGGCTTACCTCACCT

182

heIleProSerAsnAspAspIleTyrAspCysLysValGluHisTrpSerLeuAspGluProValLeuArgHisTrpG

TCATCCCTTCCAACGACGACATTTATGACTGCAAGGTGGAGCACTGGAGCCTGGACGAGCCGGTTCTAAGACACTGGGGTGTGTATGAGC

TCTGCCACTTCTGGCACTTTCTCGTTCACTGTCACTTCTGGAACAGCATGGCTTCTGGCTTCAAGTGACCAAAACTCACTTTCCACTCTT

AAAGTTTCTAAGGCTAGAGTTCGTGTCATCCCATTAGCCCAGCCTCAGAGACCAGTAGGTTAACTCCTCCTCACCTGGTCCTGTCTTACA

184

luProGluIleProAlaProMetSerGluLeuThrGluThrValValCy

CATACATGAGTCCCTTCTGACTCAAAGCTTCTCTCTCTCAGAACCTGAGATTCCAGCCCCCATGTCAGAGCTGACAGAGACTGTGGTCTG

sAlaLeuGlyLeuSerValGlyLeuValGlyIleValValGlyThrIlePheIleIleGlnGlyLeuArgSerValAlaProSerArgHi

TGCCCTGGGGTTGTCTGTGGGTCTCGTGGGCATCGTGGTGGGCACCATCTTCATCATTCAAGGCCTGCGATCAGTGGCTCCCTCCAGACA

233

sProGlyProLeu***

CCCAGGGCCGTTGTGAGTCACACCCTGGGAAAGAAGGTAAGGGTTTGTGTTTGTGGGAAACAAAGGCACGCATGGGGAGGGGAAGGAAGG

GGAGACATGAAAGTTTGTTGTTGGAACTTTGCTGGAGCCTCTGAACCCATCTTTCCTGTGTGTTTTTGTTGTAGGTGCGTGGCCCTCTACA

GGGAAGATGTAGTGTGTGGGGGTGACCTGGCACAGTGTGTTTTCTGGCCCAATTCATCGTGTTCTCTCTCTTCTCCTGGTGTCTCCCATC

TTGCTCTTCCCTTGGCCCCCAGGCTGTCCACCTCATGGCTCTCACGCCCTTGGAATTCTCCCCTGACCTGAGTTTCATTTTTGGCATCTT

CCAAGTCGAATCTACTATAGATTCCGAGACCCTGATTAATGCTCCACCAAACCAATAAACCTCTTATAAGTTGTCTTCTTTGTTTTTCTG

TCTCCCATTTTGGAGCTTACTGGAGCCAGGCCCATGTAATCCTGTGTCCAGTGTCACAGGAATAACCAATGCAGCCCAGAATGGTTTGGT

ATCTTAGCCAAGGTTGACTTTCAAATTTTTTTGTTCAGCTGACATCTCACG

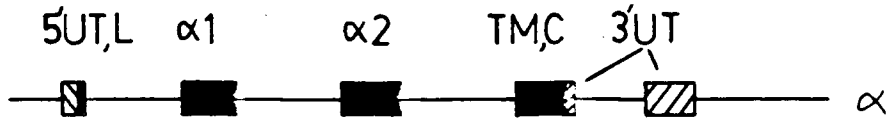


Figure 11: Structure of RT1-B_α Class II gene. The gene is divided into 5 exons: Shaded boxes denote coding sequence and cross-hatching denotes untranslated sequences. L denotes the leader peptide, α1, and α2, denote the exons encoding the external domains of the proteins; TM denotes the transmembrane region: C denotes the cytoplasmic region of the protein; and 3'UT denotes the untranslated region found in the 3' flanking region.

connecting peptide (or membrane proximal region) of 13 amino acids, the transmembrane region of 23 amino acids, the cytoplasmic region of 15 amino acids and 23 nucleotides of the 3' untranslated region. The remainder of the 3' untranslated region is encoded separately in exon 5. An intron in the 3' untranslated region is unusual and is distinctive to MHC class II α genes. With most genes, including the MHC class II β and class I genes, the cytoplasmic domain of the protein and 3' untranslated region are encoded by a single exon (Eccles and McMaster, 1985; Larhammar et al. 1983; and Larhammar et al. 1982).

The organization of the HLA DQ_α gene (Auffray et al., 1984) is similar to that of the RT1 B_α gene except that exon 1 encodes a leader peptide of 23 amino acids and only 4 amino acids of the α1 domain (which is one amino acid shorter). Thus the RT1 B_α and HLA DQ_α genes are structurally more related to each other than to the H-2 E_α and HLA DR_α genes.

2. Analysis of the Predicted Protein Sequence

Comparison of the predicted amino acid sequence of the RT1 B_α gene to that of the H-2 A_α and the HLA DQ_α genes (Table 1) shows that the amino acid sequence of these three molecules is highly conserved. The addition of the predicted amino acid sequence from the RT1 B_α gene provides values for the level of sequence conservation in the leader peptide and α1 domains. Within the α1 domain of the RT1 B, H-2 A and HLA DQ molecules there are conserved amino acids at 39 of 88 positions (44%). Even higher levels of conservation are found in the remaining α chain domains (Wallis and McMaster 1984). This level of conservation is similar to that found in the comparison of RT1 D_β ,H-2 E_β and HLA DR_β chain sequences (Robertson and McMaster 1985). A comparison of 26 MHC

Table 1: Protein Sequence Identity between Class II α Chains of Rat, Mouse, and Human

Domain	Percent Sequence Identity	
	RT1 B_α: H-2 A_α	RT1 B_α: HLA-DQ_α
leader peptide	91	74
α1	80	59
α2	84	79
connecting peptide	92	84
transmembrane region	100	91
cytoplasmic tail	86	73
overall	85	73

class II α chain sequences has recently been reported which shows in detail the distribution of species specific and allele specific differences found in MHC class II genes of rat, mouse, rabbit and human (Figueroa and Klein, 1986).

Conserved amino acid residues are observed at residue 82 and 122 where the sequence Asn-Ser-Thr, a potential site of N-linked glycosylation, is observed. Other residues conserved across species are Cysteine residues at position 111 and 167, Phenylalanine at position 117, Tryptophan at positions 125 and 182, and Aspartate at position 146 all of which are characteristic of immunoglobulin constant region domains (Travers et al.,1984). Class II molecules are a member of the immunoglobulin gene superfamily (Hood et al. 1985). In addition the RT1 B_α, H-2 A_α, and HLA-DQ_α molecules all have the following conserved residues: Phenylalanine 149, Proline 159, and Tryptophan 182 which are characteristic of MHC antigen membrane proximal domains (Travers et al.,1984).

The structure of the coding sequence of Class II genes can be compared between species. The RT1 B_α, and H-2 A_α genes contain 23 amino acids of the leader peptide and 5 residues of the α1 domain in exon 1, 83 residues of the α1 domain in exon 2, 94 residues of the α2 domain in exon 3, and the remaining 51 residues of the protein plus 23 nucleotides of 3' untranslated sequence in exon 4 (see Figure 4). The HLA DQ_α gene is highly similar in structure except that exon 1 is shorter and encodes 23 residues of leader peptide and only 4 residues of the α1 domain (which is one amino acid shorter in human than in rodent). In contrast the H-2 E_α and HLA DR_α genes contain 25 residues of leader peptide and 2 residues of the α1 domain in exon 1, 82 residues of the α1 domain in

exon 2, 94 residues of the α2 domain in exon 3, and the remaining 51 residues of the protein plus 11 nucleotides of 3' untranslated sequence in exon 4. The H-2 A_α, RT1 B_α, and HLA DQ_α molecules are structurally more related to each other than to the E_α homologs H-2 E_α or HLA DR_α, and similarly for the H-2 E_α and HLA DR_α molecules. These findings suggest that the two Class II loci represented by the H-2 A_α and E_α molecules diverged by gene duplication prior to the speciation of mammals.

3. The Promoter

The 5' untranslated region of the RT1 B_α gene contains several blocks of conserved nucleotide sequence that are thought to be involved in the regulation of class II gene expression. The cap site, which has been proposed to represent the site of initiation of RNA transcription and conforms to the consensus sequence 5'-PyNNNPyAPyPyPyPyPy-3'; where Py represents Pyrimidine nucleotides and N can be any nucleotide (Breathnach and Chambon, 1981), is located at -50 with respect to the initiator methionine codon at position +1 (underlined in figures 10 and 12). Similarly, the Hogness or TATA box plays an important role in determining the site of initiation of transcription and is usually found 25 nucleotides upstream from the initiation site (Breathnach and Chambon, 1981). An A-T rich region 25 nucleotides upstream from the putative cap site (underlined in Figures 10 and 12) may serve as a TATA box in the RT1 B_α gene.

Analysis of the 5' flanking sequence of the RT1 B_α gene reveals two segments of highly conserved DNA sequence found in all MHC class II sequences published to date (Saito et al. 1983, Okada et al. 1985). Figure 12 shows a consensus sequence of the conserved 5' region of MHC

```
          -100        -90         -80         -70         -60         -50         -40         -30         -20         -10         -1
RT1 Ea      AAGTCTGCAGTTAGCAACTGTGACGTCATCACAGGGAAATTTTCTGATTGGTCTGTCCGGTTTGGTTTGAGTGTAAGATCTCCTGGGCTGGATCCTCACAATCTCT

HLA DQa                 GCTAGTAACTGAGATGTCACCATGGGGGATTTTTCTAATTGGCCAAAA

H-2 Ea                  CCTAGCAACAGATGTGTCAGTCTGAAACATTTTTCTGATTGGTTAAAAGTTGAGTGCTTTGGATTTTAATCCCTTTTAGTTCTTGTTAATTCTGCCT

HLA DRa         CCTTCCCCTAGCAACAGATGCGTCATCTCAAAATATTTTTCTGATTGGCCAAAGAGTAATTGATTTGCATTTTAATGGTCAGACTCTATTACACCCCACATTC


a CONSENSUS             NYTAGYAACNGNNRYGTCANYNYRRRRNANTTTTCTRATTGGYYNRNR


H-2 AB          ATGTCTACCCAGAGACAGATGACAGACTTCAGCTCCAA TGCTGATTGGTTCCTCACTTGGGACCAACCCTGACACTCTGGGATTTCAGATCACTCTAGGCTACA
HLA DOB         ATGTCTGCCTAGAGACAGATTAGGTCCTTCAGCTCCAG TGCTGATTGGTTCCTTTCCAAAGGACCATCCAATCCTGCCACGCAGGGAAACATCCACAGGTTTTT
H-2 EB          ATCTCTAACTAGCAACTGATGATGCTGGACTCCTTTGA TGCTGATTGGCTCCCAGCACTGGCCTTACCCAATCCAGTGGCAAAGCAGTGAATGTCCTGTCTCTT
HLA DRB         ATCTCTGACCAGCAACTGATGATGCTATTGAACTCAGA CGCTGATTCATTCTCCAACACTAGATTACCCAATCCAGGAGCAAGGAAATCAGTAACTTCCTCCCT

B CONSENSUS             NCYAGNRACNGATGANRNNNNNNNNNCTYNRR YGCTGATTNNYTCYYY
```

Figure 12: Alignment of the promoter regions of Class II α and β genes. [HLA DQ_α (Okada et al. 1985), H-2 E_α (Mathis et al. 1983), HLA DR_α (Das et al. 1983), H-2 A_β (Larhammar et al., 1983), HLA DQ_β (Schenning et al. 1984), H-2 E_β (Saito et al.1983), and HLA DR_β (Larhammar et al. 1985)] Underlined sequences identify the promoter element (-90 to -50), the TATA box (-28), and the cap site (-1).

class II α and β genes incorporating the sequence data from the RT1 B_α gene. This alignment shows that the overall structure of these two short highly conserved regions are separated by 20 nucleotides in α genes and 19 nucleotides in β genes and have been maintained throughout the evolution of class II genes. The α and β consensus sequences do, however, differ in the regions flanking the 3' underlined sequence. It is likely, therefore, that these regions may be important in the regulation of expression of class II genes.

Many eukaryotic promoter regions have a conserved sequence 5'-GGPyCAATC-3' 70 to 80 nucleotides upstream from the cap site of unknown function called the CAAT box (Breathnach and Chambon, 1981). Class II β genes that have been sequenced have CAAT boxes located between the conserved regulatory region and the TATA box (Larhammar et al. 1985; Gillies et al. 1984), whereas many α genes do not appear to have CAAT boxes (Das et al. 1983; Larhammar et al. 1983). Analysis of the RT1 B_α 5' flanking region reveals an atypical CAAT box at position -103. This location is unusual in that in those Class II genes which have CAAT boxes the CAAT box is found between the TATA box and the conserved regulatory element not 5'to it.

4. Allelic and Interspecies Variation in RT1 Bα Genes

Figure 13 compares the nucleotide and predicted amino acid sequence of the RT1 B_α gene (haplotype $RT1^b$) and the RT1 B_α cDNA, pRIa.2, (haplotype $RT1^u$) (Wallis and McMaster, 1984). The cDNA insert of pRIa.2 encodes the carboxy terminal 129 amino acids of the mature RT1 B_α protein which includes the α2 domain, connecting peptide, transmembrane region and cytoplasmic domains. Figure 13 shows that

```
                                                                                                                  124
Bb : Gln Pro Asn Thr Leu Ile Cys Phe Val Asp Asn Ile Phe Pro Pro Val Ile Asn Ile Thr
Bu :
     CAG CCC AAC ACC CTC ATC TGC TTT GTA GAC AAC ATC TTT CCT CCT GTG ATC AAT ATC ACA

                                                                                                                  144
Bb : Trp Leu Arg Asn Ser Lys Pro Leu Thr Glu Gly Val Tyr Glu Thr Ser Phe Leu Ile Asn
Bu :
     TGG TTG AGA AAC AGC AAG CCA CTC ACA GAA GGC GTT TAT GAG ACC AGC TTC CTT ATC AAC
                                 G                                                TC
                                 Val                                              Ser
                                                                                                                  164
Bb : Ser Asp Tyr Ser Phe His Lys Met Ala Tyr Leu Thr Phe Ile Pro Ser Asn Asp Asp Ile
Bu :
     AGT GAC TAT TCC TTC CAC AAG ATG GCT TAC CTC ACC TTC ATC CCT TCC AAC GAC GAC ATT
     CC      C
     Pro     His
                                                                                                                  184
Bb : Tyr Asp Cys Lys Val Glu His Trp Ser Leu Asp Glu Pro Val Leu Arg His Trp Glu Pro
Bu :
     TAT GAC TGC AAG GTG GAG CAC TGG AGC CTG GAC GAG CCG GTT CTA AGA CAC TGG GAA CCT
                                    G                                 A
                                    Gly                               Lys
                                                                                                                  204
Bb : Glu Ile Pro Ala Pro Met Ser Glu Leu Thr Glu Thr Val Val Cys Ala Leu Gly Leu Ser
Bu :
     GAG ATT CCA GCC CCC ATG TCA GAG CTG ACA GAG ACT GTG GTC TGT GCC CTG GGG TTG TCT
         G
         Val
                                                                                                                  224
Bb : Val Gly Leu Val Gly Ile Val Val Gly Thr Ile Phe Ile Ile Gln Gly Leu Arg Ser Val
Bu :
     GTG GGT CTC GTG GGC ATC GTG GTG GGC ACC ATC TTC ATC ATT CAA GGC CTG CGA TCA GTG
           C                                                                      AT
                                                                                  Asp
                                    233
Bb : Ala Pro Ser Arg His Pro Gly Pro Leu
Bu :
     GCT CCC TCC AGA CAC CCA GGG CCG TTG TGAGTCACACCCTGGGAAAGAAGGTGCGTGGCCCTCTACAGGG
      GC                            C T                                          C

Bb :
Bu : AAGATGTAGTGTGTGGGGGTGACCTGGCACAGTGTGTTTTCTGGCCCAATTCATCGTGTTCTCTCTCTTCTCCTGGTGT
                A                                C                    T

Bb :
Bu : CTCCCATCTTGCTCTTCCCTTGGCCCCCAGGCTGTCCACCTCATGGCTCTCACGCCCTTGGAATTCTCCCCTGACCTGA

Bb :
Bu : GTTTCATTTTTGGCATCTTCCAAGTCGAATCTACTATAGATTCCGAGACCCTGATTAATGCTCCACCAAACCAATAAA
                                                                G
```

Figure 13: Alignment of the RT1 B_α gene ($RT1^b$) with the allelic RT1 B_α ($RT1^u$) cDNA pRIa.2 (Wallis and McMaster, 1984). Nucleotide and amino acid substitutions are shown.

between the RT1 B_{α}^{b} and $RT1_{\alpha}^{u}$ alleles there are 16 nucleotide changes resulting in 8 amino acid substitutions of which 50% are conservative.

Comparison of the 3' untranslated sequences between RT1 B_{α} alleles shows that these sequences are 98% conserved. In the mouse comparison of the 3' untranslated sequences between the six H-2 A_{α} alleles (Benoist et al.,1983) showed that the average conservation of nucleotide sequence in this region was 97%. The same comparison between RT1 and H-2 genes shows 95% conservation of nucleotide sequence, however the 3' untranslated sequence is only 80% conserved between the HLA-DR_{α} and H-2 E_{α} genes (Mathis et al.,1983) The high level of conservation between the RT1 B_{α} and H-2 A_{α} genes and alleles in the 3' untranslated region suggests that these sequences may play a role in the functional expression of class II molecules.

Comparison of the nucleotide and protein sequence of the coding sequence of the RT1 B_{α} gene to that of H-2 A_{α} alleles (Benoist, 1983, Figueroa and Klein, 1986) (Figure 14) reveals several striking features. Over the entire α chain protein, 11 of the 20 inter-species amino acid differences occur at positions identified as allelically variable in the H-2 molecule. The inter-species nucleotide and amino acid changes are clustered primarily in two regions of the RT1 B α1 domain at amino acid positions 19 to 23 and 45 to 78. The regions which have been designated as allelically variable in H-2 A_{α} occur at amino acid positions 8 to 16 and 45 to 78. The close association of regions of inter-species and allelic variability suggests that allelic variability may occur at the same locations in the RT1 B α1 domain.

```
      1
RT1 Bα:Gln Asp Asp Ile Glu Ala Asp His Val Gly Ser Tyr Gly Ile Thr Val Tyr Gln Tyr His 20
          *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
H-2 Aα:Glu Asp Asp Ile Glu Ala Asp His Val Gly Ser Tyr Gly Ile Thr Val Tyr Gln Ser Pro

RT1 Bα:Glu Ser Lys Gly Gln Tyr Thr His Glu Phe Asp Gly Asp Glu Arg Phe Tyr Val Asp Leu 40
              *   *   *   *       *   *   *   *   *   *       *   *   *   *   *
H-2 Aα:Gly Asp Ile Gly Gln Tyr Thr Phe Glu Phe Asp Gly Asp Glu Leu Phe Tyr Val Asp Leu

RT1 Bα:Asp Lys Lys Glu Thr Ile Trp Arg Ile Pro Glu Phe Gly Gln Leu Ile Ser Phe Asp Pro 60
        *   *   *   *   *       *           *   *   *       *   *           *       *
H-2 Aα:Asp Lys Lys Glu Thr Val Trp Met Leu Pro Glu Phe Ala Gln Leu Arg Arg Phe Glu Pro

RT1 Bα:Gln Gly Ala Leu Arg Asn Ile Ala Ile Ile Lys His Asn Leu Glu Ile Leu Met Lys Arg 80
        *   *       *   *   *           *   *   *   *   *   *       *   *
H-2 Aα:Gln Gly Gly Leu Gln Asn Ile Ala Thr Gly Lys His Asn Leu Glu Ile Leu Thr Lys Arg

RT1 Bα:Ser Asn Ser Thr Pro Ala Val Asn Glu Val Pro Glu Ala Thr Val Phe Ser Lys Ser Pro 100
        *   *   *   *   *       *   *       *       *   *   *   *       *   *   *
H-2 Aα:Ser Asn Ser Thr Pro Ala Thr Asn Glu Ala Pro Gln Ala Thr Val Phe Pro Lys Ser Pro

RT1 Bα:Val Leu Leu Gly Gln Pro Asn Thr Leu Ile Cys Phe Val Asp Asn Ile Phe Pro Pro Val 120
        *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
H-2 Aα:Val Leu Leu Gly Gln Pro Asn Thr Leu Ile Cys Phe Val Asp Asn Ile Phe Pro Pro Val

RT1 Bα:Ile Asn Ile Thr Trp Leu Arg Asn Ser Lys Pro Leu Thr Glu Gly Val Tyr Glu Thr Ser 140
        *   *   *   *   *   *   *   *   *   *           *       *   *   *   *   *   *
H-2 Aα:Ile Asn Ile Thr Trp Leu Arg Asn Ser Lys Ser Val Thr Asp Gly Val Tyr Glu Thr Ser

RT1 Bα:Phe Leu Ile Asn Ser Asp Tyr Ser Phe His Lys Met Ala Tyr Leu Thr Phe Ile Pro Ser 160
        *           *       *   *   *   *   *   *           *   *   *   *   *   *   *
H-2 Aα:Phe Phe Val Asn Arg Asp Tyr Ser Phe His Lys Leu Ser Tyr Leu Thr Phe Ile Pro Ser

RT1 Bα:Asn Asp Asp Ile Tyr Asp Cys Lys Val Glu His Trp Ser Leu Asp Glu Pro Val Leu Arg 180
          *   *   *   *   *   *   *   *   *   *       *       *   *   *   *
H-2 Aα:Asp Asp Asp Ile Tyr Asp Cys Lys Val Glu His Trp Gly Leu Glu Glu Pro Val Leu Lys

RT1 Bα:His Trp Glu Pro Glu Ile Pro Ala Pro Met Ser Glu Leu Thr Glu Thr Val Val Cys Ala 200
        *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
H-2 Aα:His Trp Glu Pro Glu Ile Pro Ala Pro Met Ser Glu Leu Thr Glu Thr Val Val Cys Ala

RT1 Bα:Leu Gly Leu Ser Val Gly Leu Val Gly Ile Val Val Gly Thr Ile Phe Ile Ile Gln Gly 220
        *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
H-2 Aα:Leu Gly Leu Ser Val Gly Leu Val Gly Ile Val Val Gly Thr Ile Phe Ile Ile Gln Gly

RT1 Bα:Leu Arg Ser Val Ala Pro Ser Arg His Pro Gly Pro Leu 233
        *   *   *           *   *   *   *   *   *   *
H-2 Aα:Leu Arg Ser Gly Gly Thr Ser Arg His Pro Gly Pro Leu
```

Figure 14: Comparison of the predicted amino acid sequences of the RT1 B_α and H-2 A_α molecules. Amino acids highlighted in boldface are positions showing allelic variation in the mouse (Benoist et al.,1983). Underlined sequences indicate regions of nucleotide sequence hypervariability in the mouse (Benoist et al., 1983; Landais et al. 1985; and Landais et al. 1986). Asterisks denote amino acid sequence identity between rat and mouse.

5. Molecular Evolution

The rates of nucleotide divergence among DNA encoding RT1, H-2, and HLA Class II α genes was calculated using the method of Perler (Perler et al. 1980). Comparison of the coding sequences codon by codon allows the number of silent (those substitutions which do not result in an amino acid change) and replacement substitutions (nucleotide substitutions which result in an amino acid change) to be evaluated (Perler et al. 1980). The number of potential silent and replacement substitutions are also determined. From these numbers the rates of divergence are calculated (Perler et al.,1980) and these rates are expressed as the percentage of the number of observed substitutions versus the number of potential substitutions and were corrected for multiple mutation events (Perler et al. 1980). Comparison of the percentage silent and replacement substitutions between loci such as the RT1 B and D, or H-2 A ans E loci (Table 2) shows a large accumulation of mutations of both types. Furthermore, there is very little difference between species in the values reflecting inter-locus mutation. These data support the view that the ancestral Class II gene duplicated into two loci (ie RT1 B and D homologs) prior to the speciation of mammals.

Comparison of the percentage silent substitutions for each domain (Table 3) among species is approximately 45 with two exceptions. The values for exon 2 (27.7%) and exon 4 (19.6%) in the comparison of RT1 to H-2 probably reflects the low number of observed silent substitutions. The value for exon 2 (119.1%) in the RT1 to HLA comparison is likely due to multiple mutations at the same nucleotide position which would have accumulated over the 80 million years since rodent and human diverged. When the RT1 B_α or H-2 A_α chain sequence is compared to that of the HLA

Table 2: Sequence Divergence of Class II α Chain Genes

	Percentage Corrected Divergence*	
	Silent Sites	Replacement Sites
RT1 Bα: H-2 Aα	29.6	9.8
RT1 Bα: HLA-DQα	59.4	16.2
H-2 Aα: HLA-DQα	49.8	17.4
RT1 Bα: H-2 Eα	123.4	44.4
RT1 Bα: HLA-DRα	109.8	39.1
H-2 Eα: HLA-DRα	70.1	15.7
RT1 Bα: RT1 Dα	107.9	34.9
H-2 Aα: H-2 Eα	121.3	45.4
HLA-DQα: HLA-DRα	101.0	38.0

* The percentage corrected divergence of each pair of sequences was calculated as described by Perler et. al. (1980) except that all three categories of substitutions were used for each weighted average.

Table 3: Sequence Divergence of Class II α Chain Genes by Exon

	Silent Substitutions			Replacement Substitutions		
	Observed	Potential	Percent*	Observed	Potential	Percent*
RT1Bα:H-2Aα						
exon 2 (α1)	9	54	27.7	25	209	14.3
exon 3 (α2)	10	61	46.4	15	220	7.8
exon 4 (TM)	9	43	19.8	3	110	3.2
overall	26	159	29.6	43	539	9.0
RT1Bα:HLADQα						
exon 2 (α1)	22	53	119.1	39	209	29.6
exon 3 (α2)	16	61	44.1	28	220	14.6
exon 4 (TM)	10	41	48.2	5	110	5.3
overall	47	157	59.4	72	539	17.4

Percent* Percent corrected divergence for each pair of sequences was calculated as described (Perler et al. 1980).

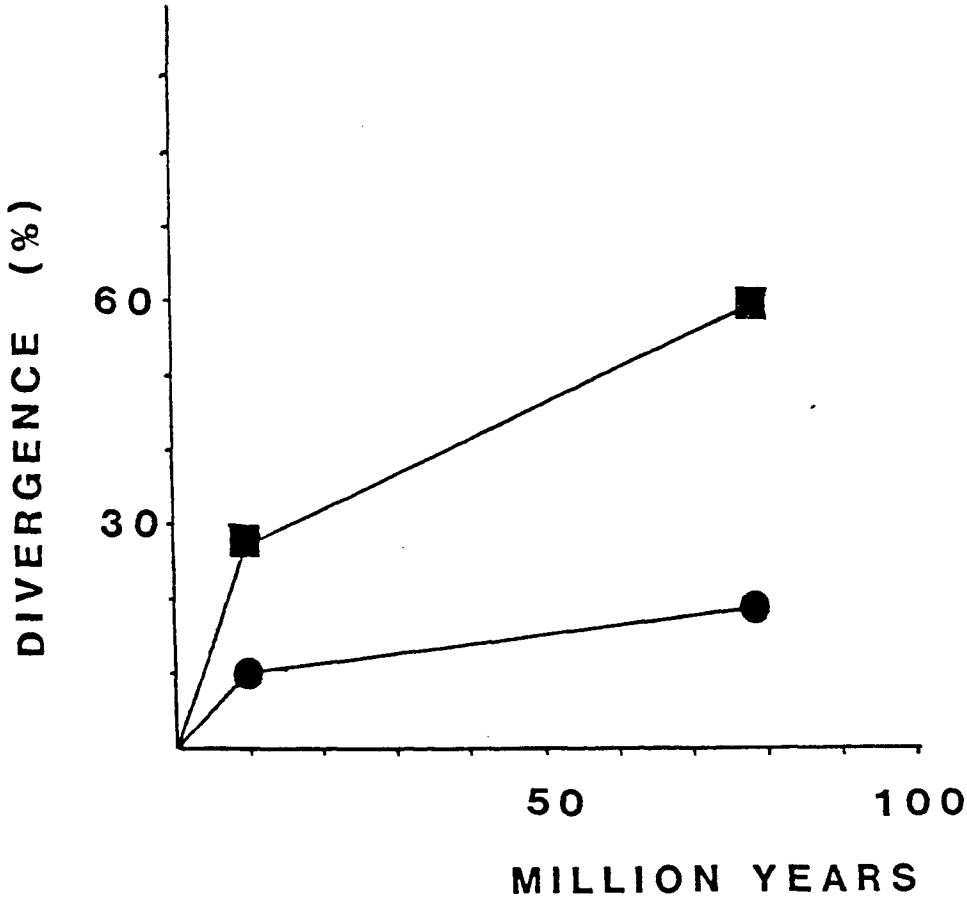


Figure 15: Plot of the percentage corrected divergence of MHC Class II α molecules versus evolutionary time. Values are taken from Table 1. Silent substitutions (■), replacement substitutions (●).

DQ_α it is apparent that the accumulation of both replacement and silent substitutions is not linear with evolutionary time and reaches a plateau (Figure 15). For example, after 8 million years of evolutionary time, represented by a comparison of RT1 and H-2 sequences, there is an overall rate of 9% for replacement substitutions and 29.6% for silent substitutions. After 80 million years of evolutionary time, represented by a comparison of RT1 and HLA sequences, replacement substitutions have increased to only 17 percent and silent substitutions have increased to 59.4%. This strongly suggests that replacement substitutions can only be tolerated to a certain level before they are selected against. Alternatively, it has been proposed that rodents may be evolving more rapidly than humans perhaps due to increased opportunity for selection to act as a result of the shorter generation times of rodents (Wu and Li, 1985). A similar observation has been made in the analysis of the DNA sequence of the β chains encoded by the RT1 D, H-2 E, and HLA DR genes (Robertson and McMaster, 1985).

B. DISCUSSION

1. Structure of the RT1 B_α Gene

The organization of the RT1 B_α gene was determined by comparison of the genomic sequence to the cDNA sequence of pRIa.2 and pACD3. The gene is organized into 5 exons separated by 4 introns and encompassing about 5,000 nucleotides of DNA. The intron/exon structure of the RT1 B_α gene closely resembles that of all Class II α genes and is virtually identical to the H-2 E_α and HLA-DQ_α genes. Structurally the Class II α genes vary primarily in the length and composition of each exon. All variations fall into one of two groups corresponding to the two Class II loci (RT1.B and RT1.D in rat). For example the RT1 B_α and HLA-DQ_α genes

encode 23 residues of leader peptide and 5 amino acids of the α1 domain in exon 1, whereas the H-2 E_α and HLA-DR_α genes encode a leader peptide of 25 amino acids and 2 residues of the α1 domain (Mathis et al.,1983). Thus the RT1 B_α and HLA-DQ_α genes are structurally more related to each other than to the H-2 E_α or HLA-DR_α genes, suggesting that the two Class II loci represented by the RT1 B_α and RT1 D_α molecules diverged by gene duplication prior to the speciation of mammals.

Further evidence for this duplication and its timing come from comparisons of the nucleotide sequences of the coding regions of the RT1, H-2, and HLA Class II α genes. Nucleotide substitutions, both silent and replacement, can be expressed as a rate of sequence divergence (observed substitutions/ potential substitutions) and reflect the mutation rate between the two sequences (Perler, 1980). Table 2 summarizes the rates of divergence of DNA encoding Class II α genes. The largest number of both silent and replacement substitutions occurs between sequences encoded by different loci irrespective of species. For example, the rates of divergence for silent substitutions between H-2 A_α:H-2 E_α, HLA-DQ_α:HLA-DR_α, and RT1 B_α:RT1 D_α are 121.3%, 101.0%, and 107.9% respectively. The lowest rates are observed in comparisons of closely related species at the same locus (for example RT1 B_α:H-2 A_α). This data supports the view that the duplication event which created the two main Class II loci occurred before the speciation of mammals.

Comparison of the predicted amino acid sequence of the RT1 B_α gene to that of the H-2 A_α and HLA-DQ_α genes (Table 1) shows a high degree of overall sequence conservation. The RT1 B_α molecule shows protein sequence identity with the H-2 A_α molecule at 85% of its residues, and with the HLA-DQ_α molecule at 73% of its residues. These values are

similar to those reported for comparisons of the Class II A_β and E_β proteins (Eccles and McMaster,1985; Robertson and McMaster,1985) and reflect the evolutionary distance between rat, mouse, and man.

There are differences in the levels of conservation of protein sequence when individual domains of Class II α molecules are compared. The amino acid sequence of the α1 domain (residues 1-88) is the least conserved when the RT1 B_α molecule is compared to mouse and human (59%-80%). The concentration of the protein sequence variation in the α1 domain suggests that this domain may be involved in antigen recognition by T-lymphocytes (see Class II Polymorphism below).

The α2 domain (residues 89-182) shows a much higher level of sequence conservation (79%-84%) than the α1 domain (Table 1). This domain is as conserved between rodent and human (79% RT1 B_α:HLA-DQ_α) as between rat and mouse (84%) suggesting a functional constraint has prevented the divergence of this domain during the evolution of Class II α genes. The RT1 B_α chain has a number of highly conserved residues such as cysteines at positions 111 and 167 (which form a characteristic disulphide loop), phenylalanine 117, tryptophan 125, aspartate 146, and tryptophan 182 which are all characteristic of immunoglobulin constant region domains (Travers et al.,1984). In addition there are a number of residues such as phenylalanine 149 and proline 159 which are conserved in MHC membrane proximal domains but are not found in immunoglobulin constant region domains (Lee et al.,1982; Travers et al.,1984). These highly conserved residues are likely to be involved in maintaining the structure of the RT1 B_α molecule, and the immunoglobulin-like structure of the RT1 B_α Class II molecule is consistent with the view that immunoglobulin and MHC proteins evolved from a common ancestral gene.

The transmembrane region of the RT1 B_α protein is the most conserved of all domains. Comparison of transmembrane sequences between rat and mouse shows 100% conservation of protein sequence. The transmembrane domain is also highly conserved between rat and human (90%). Similar levels of sequence conservation have been seen in comparisons of rodent and human Class II A_β (Eccles and McMaster,1985) and E_β (Robertson and McMaster,1985) molecules. The high degree of sequence conservation observed between RT1 B_α, H-2 A_α, and HLA-DQ_α transmembrane regions is unusual, and not seen in other membrane glycoproteins. Murine Class I H-2 K^b and H-2 D^b share identity at only 75% of transmembrane residues (Reyes et al.,1982) while H-2 K^b and HLA-B7 have only 30% shared amino acid sequence in the transmembrane region (Coligan et al.,1981). The transmembrane region is composed of a series of hydrophobic residues believed to form an α-helical configuration which spans the lipid membrane (Travers et al.,1984). The conserved nature of Class II α and Class II β chain transmembrane regions suggests that this region may mediate interactions between α and β chains within the membrane insuring that interactions such as RT1 B_α and D_β do not occur. The lack of protein sequence conservation between RT1 B_α and D_α molecules (69%) (Holowachuck,1985) and RT1 B_α and D_β (17%) supports this view.

The cytoplasmic regions of the RT1 B_α molecule are also highly conserved when compared to the H-2 A_α and HLA-DQ_α molecules, showing 86% and 73% sequence identity respectively. The conservation of sequence in this region suggests functional importance for this domain, most likely in interactions with cytoplasmic proteins.

Comparison of nucleotide sequences encoding evolutionarily related proteins can be used to calculate the rate of divergence between the sequences. Table 3 summarizes the rate of divergence of DNA encoding Class II α chains from rat, mouse, and human, expressed as the percentage of silent and replacement substitutions. The low number of replacement substitutions observed in all domains except the α1 reflects the high degree of sequence conservation discussed above. When the number of replacement substitutions is plotted against evolutionary time (figure 15) it is apparent that replacement substitutions do not accumulate linearly. This strongly suggests that replacement substitutions have been selected against during evolution, and can only be tolerated to a certain level before they affect the function of the protein. In contrast there are 2 to 3 times more replacement substitutions in the α1 domain as in the rest of the molecule in both the rat/mouse and rat/human comparisons. Selection appears to have favoured replacement substitutions in the α1 domain.

2. The Promoter Region

Examination of the 5' flanking sequence of the RT1 B_{α} gene (Figures 10 and 12) shows that this gene contains a number of conserved nucleotide sequences characteristic of all eukaryotic promoters. Conserved sequences identifying the TATA box, and "cap" site of initiation of mRNA synthesis have been found in all Class II genes examined (Mathis et al.,1983; Saito et al.,1983; Larhammar et al.,1983; Okada et al.,1985; Das et al.,1983; and Larhammar et al.,1985) and are known to be involved in the regulation of the initiation of transcription (Breathnach and Chambon,1981).

Upstream from the TATA box are two blocks of sequence found to be conserved in the RT1 B_{α} gene and in all Class II genes examined (Saito et al.,1983; Okada et al.,1985). It is likely that these regions may be important in the regulation of transcription of Class II genes. The marked similarity between these conserved regions in both α and β chain genes suggests that these sequences may be involved in the coordinate regulation of Class II gene expression (Collins et al.,1984). The differences in the putative regulatory region between α and β genes appear to flank the 3' underlined element in Figure 12, and may account for the differential levels of α and β transcript production that have been reported (Germain et al.,1985). The nucleotide spacing between the two elements of the putative regulatory region (underlined in Figure 12) of 19 or 20 nucleotides is reminiscent of the recombination signals of immunoglobulin genes (Early et al.,1980). The spacing between the two elements is such that they would be separated by two turns of the DNA helix (10.4 nucleotides per turn) and therefore would be located on the same side of the DNA molecule available to interact with regulatory factors. In recent studies it has been demonstrated that the expression of MHC Class II α and β genes involves a trans-acting factor or factors (de Preval et al.,1985; Salter et al.,1985; and Guardiola et al.,1986) which may possibly interact with the two sequence elements of the putative regulatory region.

The putative CAAT box found at position -103 is atypical in both structure and location. In Class II β genes which have been sequenced the CAAT box is located between the TATA box and the conserved regulatory region (Larhammar et al.,1985; Gillies et al.,1984) whereas

many α genes do not have CAAT boxes (Das et al.,1983; Larhammar et al.,1983). Furthermore there is marked variability in the distance between the cap site and the conserved regulatory region (-78 to -48 in the RT1 B_α gene) amongst α and β genes. A deletion may have occurred during the evolution of Class II α and β genes resulting in the deletion of the CAAT box in some α genes. Alternatively the CAAT box at -103 is not necessary for Class II α gene expression.

3. Polymorphism

The study of polymorphism is important in understanding how it has been generated and maintained throughout evolution, as well as its role in the control of protective immunological responses. The association of disease with various MHC alleles makes the genetic polymorphism of the MHC useful in the diagnosis of disease susceptibility, and may lead to a better understanding of the mechanisms of pathogenesis.

Comparison of allelic Class II sequences localizes the polymorphic regions of the protein. Figure 13 shows a comparison of the RT1 B_α gene (haplotype $RT1^b$) and the RT1 B_α cDNA pRIa.2 (haplotype $RT1^u$). The α1 domains could not be compared as the cDNA pRIa.2 is incomplete (Wallis and McMaster,1984). The amino acid changes are few, and randomly distributed over the α2, transmembrane, and cytoplasmic domains of the protein. It is interesting that only one of the positions of allelic variability in Figure 13 occurs at a position which shows allelic variability in the mouse H-2 A_α molecule (Benoist et al.,1983). Class II α chain polymorphism has been localized to the α1 domain in mouse (Benoist et al.,1983; Landais et al.,1986) and human (Gorski and Mach,1986). The close relationship between the RT1 B_α, H-2 A_α, and HLA-

DQ_α molecules suggests that allelic polymorphism in the RT1 B_α molecules would also be localized in the α1 domain.

The 3' untranslated sequences of the RT1 B^b_α and RT1 B^u_α alleles are also highly conserved, showing identity at 98% of nucleotides. A similar value of 96% sequence identity is seen when the 3' untranslated sequences are compared between mouse H-2 A_α alleles (Benoist et al.,1983). Comparison of 3' untranslated sequences between species shows that the RT1 B_α and H-2 A_α genes are 95% homologous whereas the RT1 B_α and HLA-DQ_α genes are quite divergent (30% homology). The high level of 3' untranslated sequence conservation in rodents reflects the close evolutionary distance between rat and mouse. Conservation of non-coding DNA in Class I and Class II genes has been implicated in gene conversion and other non-reciprocal recombination events (Weiss et al.,1983: Kourilsky et al.,1983).

The comparison of the nucleotide and predicted amino acid sequences of the RT1 B_α gene and H-2 A_α alleles (Benoist et al.,1983) in Figure 14 shows that over 50% of the interspecies amino acid differences occur at residues which show allelic variability in the mouse H-2 A_α molecule. Furthermore, the interspecies changes are clustered in two regions, residues 19-23 and 45-78. The regions of allelic variability in the mouse A_α chain are residues 11-15 and 45-78 (Benoist et al.,1983). Although Landais et al. (1986) designate residues 11-15, 53-59, and 69-78 as allelic variables, examination of the highlighted residues in Figure 14 suggests that most of the allelic variation is spread over residues 45-78. The close association of regions of interspecies variation between the RT1 B_α and H-2 A_α antigens and regions of allelic variation in the mouse H-2 A_α antigen suggests that allelic polymorphism

in the RT1 B_α molecule would also be localized to residues 45 to 78 of the α1 domain.

Recent studies have shown that one region of the class II protein which allows phenotypic differentiation of alleles by antibody comprises residues 43 to 71 (Wakeland et al. 1985). Furthermore, this is the same region which is functionally recognized by T-lymphocytes (Germain et al. 1985). It is clear that the region at positions 45 to 78 is of prime importance to the function of class II molecules.

Many investigators have tried to elucidate the molecular mechanisms which generate Class II polymorphisms. It has become apparent that no single mechanism is responsible, and in fact several mechanisms may act concomitantly.

Comparison of the nucleotide sequences reported for a number of allelic Class II genes from human and mouse to the RT1 B_α gene, described in this study, allowed the number of silent and replacement substitutions (substitutions which result in amino acid changes) to be evaluated. The results showed that despite the observed clustering of amino acid changes in the α1 and β1 domains of the protein, there was no evidence for a higher mutation rate in these parts of the molecules. Replacement substitutions appeared to be subject to negative selection pressures eliminating these substitutions from the second external domain, the transmembrane region, and the cytoplasmic region. In contrast the first external domain behaves as if replacement substitutions were selectively neutral or perhaps even undergoing positive selection. The authors conclude that Class II polymorphism could arise from random point mutations. Selective pressures would eliminate mutations causing replacement substitutions over the majority

of the molecule while allowing replacements to accumulate in the α1 and β1 domains (Gustafsson et al.,1984).

Gene conversion has been demonstrated to generate polymorphism in MHC Class I and Class II molecules. Several authors have shown that the H-2 K^{bm1} allele has been derived from a conversion event involving the Qa 2.3 gene locus as the donor sequence, and the H-2 K^{b} gene as the recipient (Mellor et al.,1983; Weiss et al.,1983). Similarly one allelic murine Class II β chain gene may have been derived by a gene conversion event. Comparison of the nucleotide sequences of the H-2 E_{β}^{b} and H-2 A_{β}^{b} revealed that the allelic variant H-2 A_{β}^{bm12} resulted from a gene conversion in which the E_{β}^{b} gene was the donor and the A_{β}^{b} gene the recipient (Widera and Flavell,1984; Mengle-Gaw et al.,1984). This recombination event transferred 14 nucleotides from exon 2 of the E_{β} gene to exon 2 of the A_{β} gene (Widera and Flavell,1984; Mengle-Gaw et al.,1984). Recently gene conversion between two HLA-DR_{β} loci has been shown to have resulted in a new HLA-DR specificity (Gorski and Mach,1986). These authors demonstrated that the HLA-DR3/Dw3 haplotype was the result of a conversion event between a segment of the βIII locus and the βI locus, as donor and recipient respectively, of the HLA-DRw6/Dw18 haplotype. The region of the protein involved in both loci was the β1 domain.

It has been postulated that gene conversion can cause both the increase in sequence diversity and also the decrease in sequence diversity in affected genes (Klein and Petes,1981). Gene conversion can generate polymorphism between genes whose spontaneous mutation rate is higher than the conversion rate, whereas when the conversion rate is higher than that of spontaneous mutations, between the two genes,

sequence homogenization occurs (Klein and Petes,1981; Ernst et al.,1981). For example the β1 domains of murine Class II β chain genes show a high degree of sequence variation indicating that selection is maintaining a high mutation rate. Therefore in this case gene conversion would be predicted to lead to the generation of further polymorphism. As described above gene conversion has clearly been shown to be responsible for Class II β chain polymorphism. Conversely, several authors have suggested that gene conversion is responsible for the lack of polymorphism seen in globin (Liebhaber et al.,1981) and immunoglobulin (Schrier et al.,1981) genes.

Interestingly there is no evidence for gene conversion as a source of polymorphism in Class II α genes. Gene conversion events between loci such as the RT1 B_α and D_α, or two allelic RT1 B_α genes in a heterozygous wild animal could potentially have made a limited contribution to polymorphism, however no such conversions have been detected (Benoist et al.,1983). The low level of polymorphism amongst H-2 E_α or RT1 D_α alleles when compared to H-2 A_α or RT1 B_α alleles (Mathis et al.,1983; Palmer,1985) led to the proposal that an intrinsic structural difference exists between these duplicated loci. This difference would allow site specific or region specific mutagenic events to occur. For example changes in chromatin structure may alter the accessibility of repair enzymes resulting in a higher apparent mutation rate in that region. Furthermore, selection could eliminate undesirable mutations or fix others (Benoist et al.,1983).

Allen and colleagues have raised a series of monoclonal antibodies and T-lymphocyte clones reactive with one antigen associated with one Class II molecule. They were able to demonstrate that this protocol

generated a family of T-cell clones and monoclonal antibodies each reactive with a different determinant on the same molecular structure. It is clear that Class II molecules and antigen interact to produce a number of different determinants thereby increasing diversity (Allen et al.,1985).

Recombinational hot spots have also been identified in the I-A region of the murine MHC of wild mice. The unusually high number of crossover events occurring in the region between the A_α and E_α loci suggests that this may be a mechanism of shuffling of sequences bearing different haplotypes in heterozygous animals (Steinmetz et al.,1986).

It is clear that there is no single mechanism for the generation of polymorphism in Class II genes. Random mutation is likely the main source of sequence diversity. Phenotypic selection will conserve structurally important residues, while allowing others to mutate freely. Gene conversion in some Class II genes, such as H-2 A_β (Mengle-Gaw et al., 1984), leads to further diversity by dispersing mutations through interallelic transfer of sequences.

It is clear from the above discussion that the majority of the inter-species variation between the Class II α molecules of rat, mouse, and human resides in the α1 domain. The high degree of overall conservation of Class II genes structure and sequence and the demonstrated sequence variability in certain regions of the α1 domain is consistent with the view that conservative selection is acting on structurally important residues while functionally important regions are undergoing positive selection for replacements generating polymorphism.

Chapter 5

STUDIES ON THE TRANSCRIPTION OF THE RT1 Bα GENE

A. RESULTS

1. Transcription of the Cloned RT1 Bα Gene

DNA mediated gene transfer has been used to study the structure, function, and regulation of expression of cloned Class II genes (Eccles et al.,1986;Germain and Malissen,1986). In order to eliminate problems with the serological differentiation of introduced and endogenous Class II molecules, as well as technical difficulties, cell lines that do not express Class II molecules, such as the mouse fibroblastoid L-cell line, have been used as recipients for cloned Class II genes. L-cells have been successfully transfected with a number of mouse (Malissen et al.,1984) and rat (Diamond et al.,1985; Eccles et al.,1986) Class II genes, and expression of Class II molecules detected on the cell surface.

DNA mediated gene transfer experiments were carried out in order to determine whether the cloned RT1 B_α gene contained in Lambda 7 could be transcribed. Lambda 7 DNA was transfected into thymidine kinase (TK) deficient mouse L-cells by co-transfection with cosmid 21.3 DNA containing an RT1 B^a_β gene (Diamond et al.,1985) and plasmid DNA containing the Herpes Simplex Virus thymidine kinase gene using the Calcium phosphate procedure as described by Graham and Van der Erb (1973). L-cells were also transfected with cosmid 13.1 DNA which contains both the RT1 B^a_α and B^a_β genes (Diamond et al.,1985; Eccles et al.,1986). Stable transfectants were selected for thymidine kinase expression in HAT medium, and Ltk^+ colonies pooled and maintained in

culture. For each experiment a fraction of the Ltk^+ pool was plated at a density such that well separated colonies resulted. Individual colonies were then trypsinized and transferred to microtitre tissue culture dishes. Several colonies from each experiment were isolated in this way clonal populations of each were grown and maintained in culture.

Cell surface expression of transfected RT1 B genes was analyzed using the monoclonal antibody MRC OX6 directed against non-polymorphic determinants on all rat RT1 Class II B_β molecules (McMaster and Williams,1979). The level of rat Class II cell surface expression was evaluated by indirect binding assay. L-cell pools or clones were incubated with saturating levels of MRC OX6, washed, and incubated with saturating levels of ^{125}I or FITC (Fluorescein iso-thiocyanate) labelled second antibody reagent. The amount of antibody bound was then determined by Gamma counting or fluorescence activated cell analysis.

Table 4 shows the results of a typical transfection experiment. Pooled and clonal populations of cells were labelled with MRC OX6 and iodinated second antibody. The results show that the pooled populations of L-cells transfected with cosmid 13.1 DNA express cell surface RT1 B_α molecules on at least some of the cells in the population. Furthermore, these cells express RT1 B_α molecules at high levels. In L-cells transfected with Lambda 7 and cosmid 21.3 DNA no cell surface expression of RT1 B_α molecules could be detected.

Figure 16 shows the analysis of another transfection experiment using a Fluorescence Activated Cell Sorter (FACS IV). Pooled populations of transfectants selected for thymidine kinase expression were labelled with antibody as described above and analyzed on the FACS IV cell

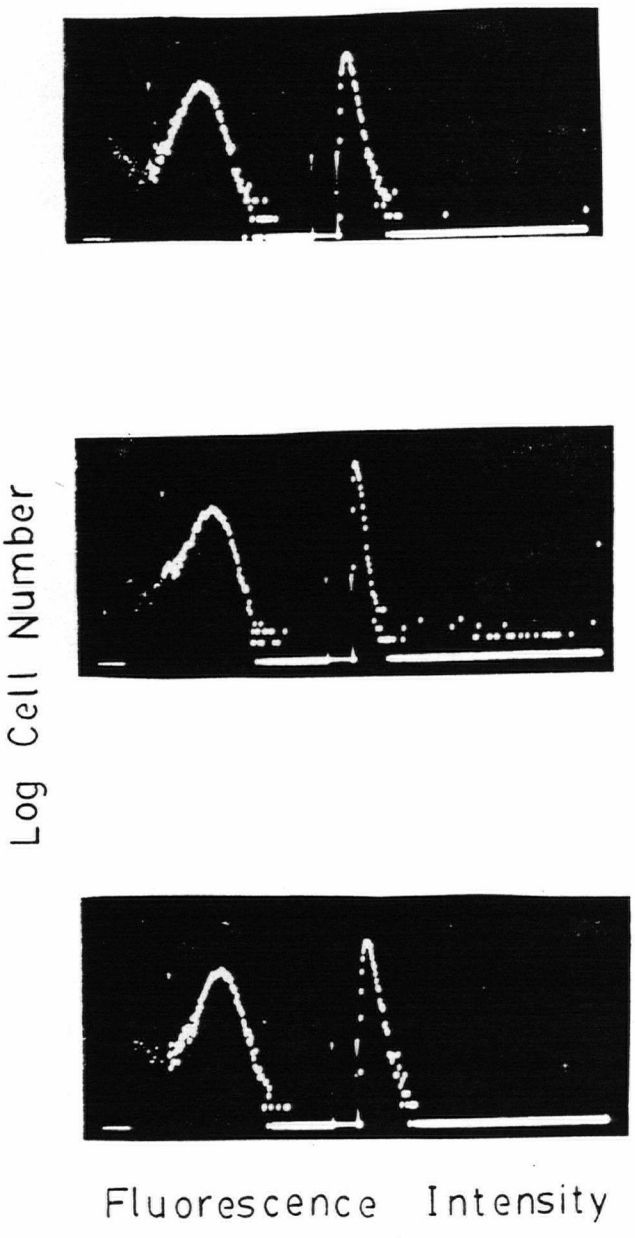


Figure 16: FACS profiles of L-cells transfected with RT1.B genes. A: Untransfected L-cells labelled with the rat specific monoclonal antibody W3/13 (McMaster and Williams, 1979) as a negative control. B: L-cells transfected with cosmid 13.1 DNA (RT1 B^a_α B^a_β) labelled with the rat class II specific monoclonal antibody MRC OX6. A significant number of these cells show increased fluorescence indicating cell surface Class II gene expression. C: L-cells transfected with lambda 7 DNA (RT1 B^b_α) and cosmid 21.3 DNA (RT1 B^a_β) labelled with MRC OX6. No significant increase in fluorescence is seen indicating the absence of class II molecules on the surface of these cells.

Table 4: Expression of Class II Genes on Transfected Ltk^+ Cells

Cells	Antibody	α Gene	β Gene	Counts(cpm)
Ltk^-	none	-	-	3,016
Ltk^-	$\alpha H\text{-}2^k$	-	-	30,824
pool A	MRC OX6	cos 13.1	cos 13.1	10,112
clone A-1	MRC OX6	cos 13.1	cos 13.1	36,172
clone A-2	MRC OX6	cos 13.1	cos 13.1	22,374
clone A-3	MRC OX6	cos 13.1	cos 13.1	3,314
pool B	MRC OX6	Lambda 7	cos 21.3	7,960
clone B-1	MRC OX6	Lambda 7	cos 21.3	1,114
clone B-2	MRC OX6	Lambda 7	cos 21.3	5,488
clone B-3	MRC OX6	Lambda 7	cos 21.3	2,530
clone A-1	W3/13	cos 13.1	cos 13.1	7,760
clone B-1	W3/13	Lambda 7	cos 21.3	3,314

First antibodies used: $\alpha H\text{-}2^k$, mouse anti-mouse MHC Class I antibody which detects molecules expressed on the surface of all mouse cells (used as a positive control); MRC OX6, detects rat Class II genes expressed on the cell surface; and W3/13 (Williams et al.,1979), which detects non-Class II molecules found on rat T-lymphocytes but not on rat B-lymphocytes (used as a negative control).

The second antibody was Rabbit anti-mouse IgG antibody labelled with radioactive iodine (^{125}I).

sorter. Populations of cells transfected with cosmid 13.1 DNA contain cells which express high levels of RT1 B_α molecules, and these cells account for about 50% of the total TK+ population. In populations of cells transfected with Lambda7 and cosmid 21.3 DNA no sub-population of cells expressing RT1 B molecules could be detected.

Figure 17 shows a Southern blot of high molecular weight DNA isolated from transfected L-cells, and hybridized with radioactively labelled RT1 B_α probe (pRIa.2 cDNA). The probe detects the single endogenous copy of the homologous H-2 A_α gene in the untransfected L-cell genome (the 8,000 nucleotide band in Lane A). DNA isolated from Clone 1 cells transfected with cosmid 13.1 DNA shows strong hybridization with the RT1 B_α probe (lane C). Comparison of the intensity of these bands to those in Lanes D, E, and F (digests of isolated cosmid 13.1 DNA) suggests that Clone 1 cells have many copies of the RT1 B_α gene integrated into its genome. In contrast L-cells transfected with Lambda 7 and cosmid 21.3 DNA show two bands of approximately 8,000 nucleotides corresponding to the Lambda 7 encoded RT1 B_α gene. Furthermore, the intensity of these bands is similar to that of the endogenous RT1 B_α gene of L-cells, suggesting that cells transfected with Lambda 7 DNA have one or two copies only integrated into their genomes.

Northern blot analysis (Figure 18) using a RT1 B_α probe (pRIa.2 cDNA) of RNA isolated from the two clones of L-cells transfected with Lambda 7 and cosmid 21.3 DNA showed a single band of 1,100 nucleotides. The α chain transcript of the Lambda 7 RT1 B^b_α gene from these two

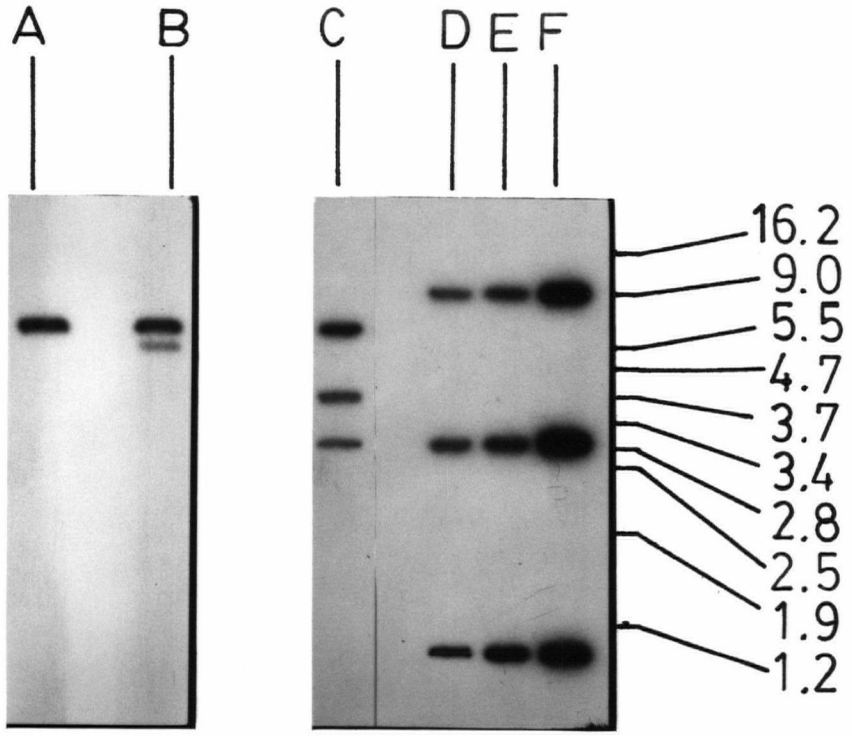


Figure 17: Southern blot analysis of high molecular weight DNA isolated from transfected L-cell clones. Approximately 2.0μg of DNA was digested with the restriction enzyme EcoR1, electrophoresed on a 1% agarose gel, transferred to Genescreen and hybridized with an RT1 B_{α} gene probe (pRIa.2 cDNA). Lane A: Untransfected L-cell DNA, Lane B: DNA from L-cells transfected with Lambda 7 and cosmid 21.3 DNA, Lane C: Clone 1 DNA (L-cells transfected with cosmid 13.1 DNA), Lane D: Cosmid 13.1 DNA digested with EcoR1 (5μg), Lane E: Cosmid 13.1 DNA digested with EcoR1 (10μg), and Lane F: Cosmid 13.1 DNA digested with EcoR1 (15μg). Lanes A and B are 5 day exposures, whereas Lanes C,D,E, and F are 8 hour exposures.

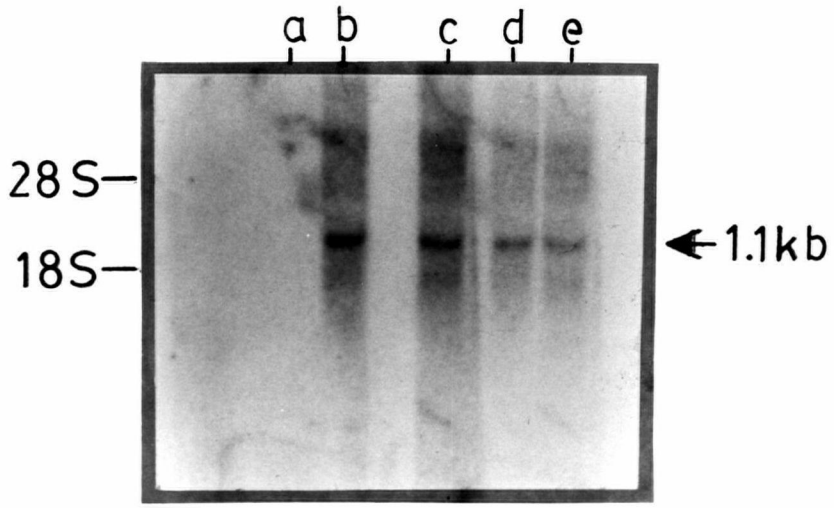


Figure 18: Northern blot analysis of RT1 Bα chain RNA. Total RNA (20 μg) was hybridized with the RT1 B^{u}_{α} cDNA insert of pRIa.2. RNA from: (a) L cells; (b) rat spleen; (c) L cells transfected with cosmid 13.1 (RT1 $B^{a}_{\alpha}B^{a}_{\beta}$); (d) L cells transfected with lambda 7 (RT1 B^{b}_{α}) and cosmid 21.3 (RT1 B^{a}_{β}) clone a ; (e) same as d except clone b.

clones (Lanes D and E) was indistinguishable from that of the RT1 B^a_α gene of cosmid 13.1 (Lane C) and of rat spleen total RNA (Lane B). Thus the RT1 B^b_α gene of Lambda 7 is efficiently transcribed when transferred to L-cells.

B. DISCUSSION

DNA mediated gene transfer is a powerful tool in the study of the regulation of gene transcription. Cloned DNA can be introduced into a variety of different cell lines to examine the effects of cell type and stage of differentiation on transcription. Manipulations of the cloned DNA, such as deletion or mutation of specific residues, allows the analysis of promoters, enhancers, and transcript processing when the altered genes are introduced into a cell line which does not endogenously transcribe the gene.

Class II genes from rat (Eccles et al.,1986; Diamond et al.,1985) and mouse (Malissen et al.,1984) have been introduced into the mouse L-cell fibroblastoid cell line which does not transcribe endogenous Class II genes. Expression of Class II antigens on the cell surface has been shown to be directly proportional to the amount of Class II transcript within the cell, suggesting that the primary level of control of Class II gene expression is transcription (Germain and Malissen,1986). Thus cell surface expression of Class II antigens can be used to evaluate transcriptional activity in addition to Northern blot analysis.

DNA mediated gene transfer was used to introduce Lambda 7 DNA encoding an RT1 B^b_α gene into mouse L-cells. A number of studies have demonstrated a requirement for α/β dimers in the cell surface expression of Class II molecules (Malissen et al.,1983; Murphy et al.,1980). Therefore Lambda 7 DNA was co-transfected with cosmid 21.3 DNA which

encodes an RT1 B^a_β molecule (Diamond et al.,1985). As a control, L-cells were also transfected with cosmid 13.1 DNA which encodes RT1 B^a_α B^a_β molecules, and has been successfully introduced into L-cells previously (Eccles et al.,1986).

As described above, one approach to evaluating the transcriptional activity of Class II genes introduced into Class II^- cell lines is to analyze cell surface expression. L-cells transfected with RT1 B genes were analyzed using a monoclonal antibody directed against determinants on the RT1 B_β chain. Table 4 and Figure 16 show the results of typical transfection experiments. L-cells transfected with Lambda 7 and cosmid 21.3 DNA have no detectable RT1 B_α antigen on their cell surface, whereas L-cells transfected with cosmid 13.1 DNA clearly have detectable RT1 B_α molecules expressed on their cell surface.

L-cells transfected with RT1 B_α genes were analyzed by Southern Blot hybridization with an RT1 B_α gene probe. Cells transfected with cosmid 13.1 or Lambda 7 DNA were shown to contain copies of the RT1 B_α gene, however cells transfected with cosmid 13.1 DNA appear to have many copies of the RT1 B_α gene integrated into their genome whereas cells transfected with Lambda 7 DNA contain only one or two copies.

Northern blot analysis of L-cells transfected with RT1 B_α genes is shown in Figure 18. The RT1 B_α genes contained in Lambda 7 and cosmid 13.1 are clearly transcribed when introduced into L-cells, and the transcript is indistinguishable from the RT1 B_α gene of rat spleen cells.

The primary form of regulation at the protein level appears to be the requirement for the correct association of α and β chains in the cytoplasm before they can be expressed on the cell surface. Studies

involving the introduction of cloned Class II genes into cells which are Class II^+ or Class II^- clearly show the requirement for both chains in order to get cell surface expression. Cloned H-2 A^k_β genes were transfected into a murine lymphoblastoid cell line which expresses H-2^d Class II molecules. Immunoprecipitation of cell surface proteins using an anti-H-2^k monoclonal antibody showed that A^k_β molecules were only expressed in association with A^d_α molecules (Germain et al.,1983; Ben-Nun et al.,1984). Transfection of cloned murine (Malissen et al.,1984; Norcross et al.,1984) and human (Rabourdin-Combe and Mach,1983) into mouse L-cells, which express no endogenous Class II molecules, showed that cell surface expression only occurred when suitable α and β chains were available. That is to say in no case were α or β chain molecules detected alone on the membranes of transfected cells.

The failure of L-cells transfected with the RT1 B_α gene encoded by Lambda 7 to express RT1 B_α molecules on their cell surface despite the efficient transcription of the gene could be due to a number of reasons. The most obvious reason for the absence of cell surface RT1 B molecules is the absence of a functional β chain. Although Lambda 7 was always co-transfected with the cosmid 21.3 which contains an RT1 B^a_β gene (Diamond et al.,1985) the cosmid may not have been integrated into the L-cell genome, or a mutation may have occurred preventing efficient transcription. As this study was primarily concerned with the transcriptional activity of the RT1 B^b_α gene cosmid 21.3 was not investigated further.

Immunoprecipitation of intra-cellular α or β chains using monoclonal antibodies co-precipitates a third glycoprotein termed the invariant chain (Ii)(Jones et al.,1979). This non-polymorphic, non-MHC

encoded protein has not been detected on the cell surface (Sung and Jones,1981) and has been shown to be induced by interferon (Koch et al.,1984). These data suggested that the invariant chain plays a role in the assembly of Class II molecules. Recent studies using functional murine (Germain and Malissen,1986) and rat (Eccles et al.,1986) Class II α and β genes and cell lines which express no endogenous invariant chain genes showed that transfected cell lines were capable of expressing Class II genes in the absence of Ii molecules. The invariant chain does not appear to be required for Class II expression, however it may aid in the cytoplasmic assembly of Class II molecules. In this study L-cells transfected with cosmid 13.1 DNA have multiple copies of exogenous RT1 B_α genes integrated into their genome. It is possible that excess copies of Class II genes can overcome the normal regulatory mechanisms which function on single copy genes. The absence of invariant chains cannot be ruled out as a mechanism for the lack of expression of RT1 B_α molecules on the cell surface of Lambda 7 transfected L-cells.

The intracellular levels of α and β chain molecules, as reflected by transcript levels, also play a role in expression of Class II molecules. Germain and colleagues (1985) have transfected murine Class II α and β genes into L-cells and examined cell surface expression. Haplotype matched genes pairs ($A^k_\alpha A^k_\beta$) resulted in high levels of expression in primary transfectants whereas haplotype mismatched gene pairs ($A^d_\alpha A^k_\beta$) did not. Further analysis showed that higher levels of transcript (particularly A_α transcript) were required to achieve expression of haplotype mismatched gene pairs. Recombinant Class II molecules containing the Aβ1 domain of one allele and the remainder of the molecule from a second allelic β chain were used in similar studies

to map the region of the Class II molecule controlling inter-chain assembly to the β1 domain.

The transfection experiment involving Lambda 7 (RT1 B^b_α) and cosmid 21.3 (RT1 B^a_β) is clearly a haplotype mismatched situation. Figure 18, however shows that L-cells transfected with Lambda 7 DNA have RT1 B^a_α transcript levels equivalent or slightly lower than those found in L-cells transfected with cosmid 13.1 DNA, which is a haplotype matched situation. Thus RT1 B^b_α transcript levels may be insufficient for cell surface expression of RT1 B^b_α molecules in this haplotype mismatched situation.

Germain and Quill (1986) have also examined the control of isotype selection in the assembly of Class II molecules. Cloned murine Class II genes were transfected into mouse L-cells in mixed isotype gene pairs (eg. $A_\alpha E_\beta$) and cell surface expression examined. Unexpectedly these authors found that $A_\beta{}^k$ could not pair with $E^{a/k}_\alpha$ molecules whereas A^d_β and $E^{a/k}_\alpha$ could pair. Since the N-terminal domain of the A_β molecule contains the bulk of the inter-allelic variation the function of isotype pairing was assigned to this region.

Clearly the polymorphic α1 and β1 domains of Class II molecules regulate the association of α and β chains thereby also regulating the cell surface expression of Class II molecules.

Chapter 6

GENERAL DISCUSSION

Serological studies have assigned Class II molecules an essential role in immune function, and there is a considerable amount of information concerning the molecular mechanisms by which Class II molecules control immune responses. Recombinant DNA technology has made it possible to examine the genetic structure and organization of Class II genes and its relationship to the evolution, polymorphism, and expression of these genes.

Determination of the structure of the Class II genes and the glycoproteins they encode has revealed a number of highly conserved amino acid residues. All Class II genes contain conserved regions centering on two cysteine residues (residues 111 and 167 in the RT1 B^b_α molecule) in the membrane proximal domain which are thought to form an intrachain disulphide loop characteristic of immunoglobulin constant region domains (Travers et al.,1984). MHC Class I molecules, Thy-1 antigen molecules, and β2-microglobulin molecules also contain these characteristic conserved residues (Travers et al.,1984) suggesting that these molecules evolved from a common ancestral gene (Hood et al.,1983). Kaufman and colleagues (Kaufman et al.,1984) have proposed that the members of the so called immunoglobulin supergene family evolved from an ancestral gene which closely resembles the contemporary Class II β chain genes.

Figueroa and Klein have proposed an evolutionary scheme for the Class II genes of mouse and human (Figueroa and Klein,1986). The determination of the nucleotide sequence of the RT1 B_α, RT1 B_β (Eccles and McMaster,1985), RT1 D_β (Robertson and McMaster,1985) and RT1 D_α

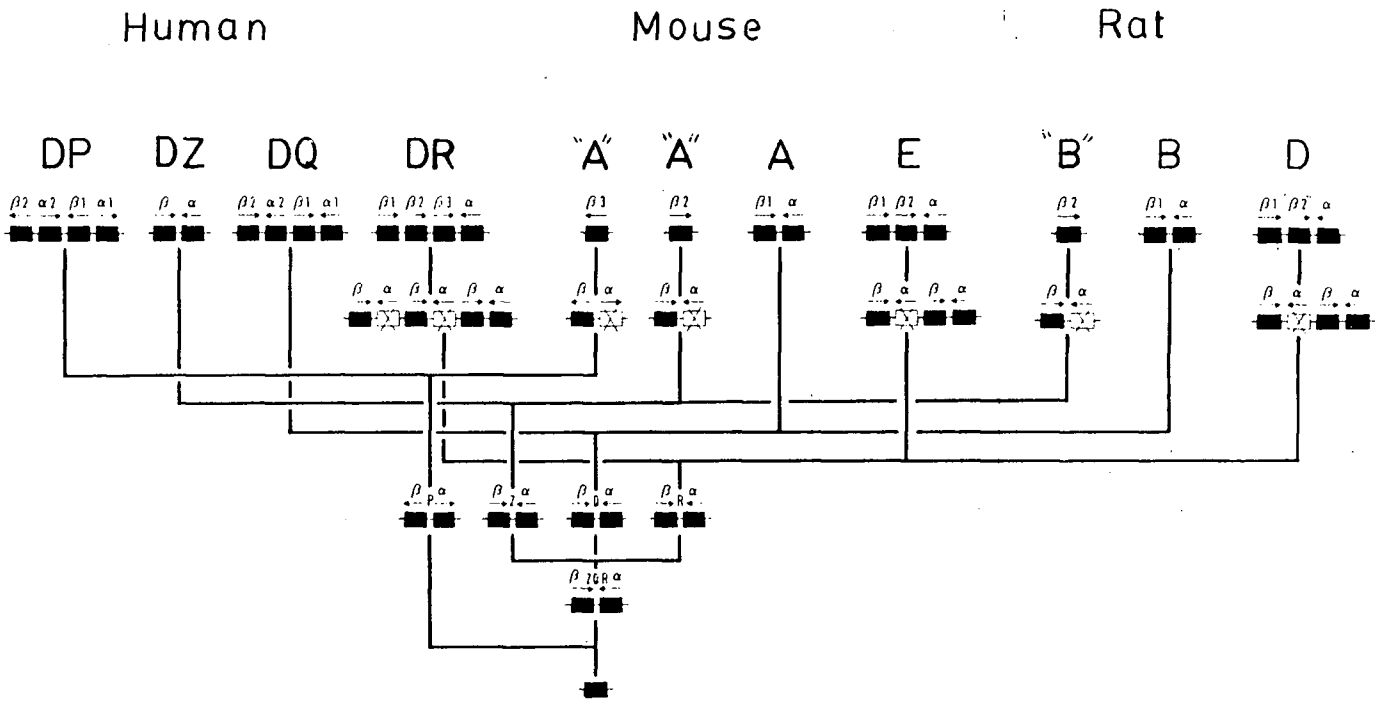


Figure 19: Postulated evolution of Class II MHC genes. Each rectangle represents one gene. Crossed rectangles indicate deleted genes. Arrows show the direction of transcription 5' to 3'. (adapted from Figueroa and Klein,1986).

(Holowachuck,1985) genes has allowed the rat to be added to the evolutionary scheme as shown in Figure 19. The ancestral gene duplicated early in evolution to form the elements from which the α and β genes have evolved. The duplication event may have occurred twice, once giving rise to the ancestors of the HLA-DP locus through an inversion duplication, and once giving rise to the ancestral α and β genes of the main evolutionary lineage through a tandem duplication. The ancestors of the three contemporary Class II loci were formed by further duplications of the β-α unit prior to the speciation of mammals. Class II evolution continued through further duplication of the β-α unit followed by mutation and deletion of some genes. Although multiple α and β genes have been demonstrated for each Class II locus only the α1 and β1 genes at each locus are known to be functional in rat, mouse, and human (Figueroa and Klein,1986). Clearly the evolutionary relationships between the RT1 B_α gene and its murine and human homologs determined in this study contribute significantly to the overall understanding of Class II gene evolution.

The extreme serological and functional polymorphism of Class II antigens depends on variability in the primary structure of the protein (Kaufman et al.,1984). Comparison of nucleotide and predicted amino acid sequences of a number of H-2 A_α chain molecules showed that structural polymorphism is confined to two subregions (residues 11-15 and 45-78) of the α1 domain (Benoist et al.,1983; and Landais et al.,1986). A similar comparison of the allelic rat RT1 B^b_α gene and the RT1 B^u_α cDNA pRIa.2 (which encodes the α2, transmembrane, cytoplasmic, and 3' untranslated domains; Wallis and McMaster,1984) showed that the protein sequences of

these alleles are highly conserved (Figure 13). This suggests that any significant polymorphism must reside in the α1 domain.

When the structure of the RT1 B_α and H-2 A_α genes are compared (Figure 14) it was apparent that these molecules were highly homologous. The RT1 B_α and H-2 A_α molecules share sequence identity at 85% of residues, whereas H-2 A_α alleles share identity at 89% of residues (Benoist et al.,1983). There are two regions of interspecies sequence variability (residues 19-23 and 45-78) one of which corresponds exactly to a region of allelic variability (residues 45-78) in the mouse H-2 A_α molecule. The close association of regions of interspecies and allelic variability at residues 45 to 78 suggests a role for polymorphism in this region in the function of Class II α molecules. Furthermore amino acid changes introduced into this region profoundly alter the serological properties of the molecule identifying residues 45 to 78 as functionally important (Wakeland et al.,1985).

Despite the extreme polymorphism of Class II α chain molecules, structural comparisons across species reveal that these molecules are highly conserved. A plot of amino acid changes (replacement substitutions) against evolutionary time (the sequence divergence between rat and mouse represents 8 million years of evolution whereas the divergence between rodent and man represents 80 million years) shows that replacement substitutions do not increase linearly with evolutionary time but reach a plateau (Figure 15). This strongly suggests that conservative selection is acting to maintain the structure of Class II α chains. In contrast Table 3 shows that the amino acid sequence of the α1 domain is not nearly as conserved as in the rest of the molecule. This data suggest that conservative selection acts on

residues (Gustafsson et al.,1984) and positive selection for replacement substitutions acts on others. In this way polymorphism can be maintained throughout evolution while conserving the overall structure of the molecule.

Class II gene expression has been shown to be directly proportional to the Class II mRNA content of the cell, suggesting that transcription is the primary level of control for Class II genes (Germain and Malissen,1986). Dissection of the structure of Class II molecules also provides insight into the control of expression of the genes encoding these molecules. Class II genes, like most eukaryotic genes, contain a number of highly conserved sequences important for the correct initiation of transcription. The conserved sequences identifying the CAAT box, TATA box, and "cap" site have been found in the RT1 B_{α} gene and several other Class II α and β genes from rat (Eccles and McMaster,1985), mouse (Mathis et al.,1983; Saito et al.,1983; Larhammar et al.,1983), and human (Okada et al.,1985; Das et al.,1983; Larhammar et al.,1985). Furthermore, these sequences are known to be involved in the regulation of initiation of transcription (Breathnach and Chambon, 1981).

Comparison of the rat RT1 B_{α} gene to a number of Class II genes revealed a highly conserved element found in the 5' flanking sequences of all α and β chain genes (Saito et al.,1983). This element is located 70 to 140 nucleotides 5' to the site of transcript initiation, and consists of 10 and 14 nucleotide sequences separated by 19 or 20 nucleotides. Features of these elements allow those from α and β chain genes to be differentiated, although the element is highly homologous in both genes (Okada et al.,1985). The duplication of the primordial Class

II gene into α and β genes is thought to be the first step in the evolution of Class II genes (Klein, 1986). Therefore the conservation of this structure between the evolutionarily distant α and β genes has been suggested to be involved in the coordinate regulation of α and β gene transcription (Saito et al.,1983).

Mice of the H-2 E_{α}^{b} or E_{α}^{s} haplotypes do not express Class II E molecules on their cell surfaces. Northern blot analysis determined that non-expression was due to the absence of E_{α} gene transcript. Further characterization revealed a 600 nucleotide deletion encompassing most of the first exon and at least 200 nucleotides of the promoter region. The correlation of lack of transcription with the absence of the promoter element, CAAT box , and TATA box clearly suggests these sequences are important in the regulation of transcription (Mathis et al.,1983).

Studies involving the construction of recombinant plasmids containing 2.7kb of 5' flanking sequence from the H-2 E_{β}^{d} mouse gene linked to a selectable marker (xgpt; expression of this gene allows growth in the presence of mycophenolic acid) have allowed the identification of tissue specific enhancer elements in Class II genes. These constructs were transfected into B-cell lymphoma cells, myeloma cells, or L-cells and the drug resistant colonies counted. More colonies, reflecting a higher level of xgpt gene transcription, were seen in the Class II+ B lymphoma cells, suggesting the presence of a tissue specific enhancer element or elements in the 5' sequences of Class II genes. Further analysis has localized the element or elements to the -600 to -2,000 region of the E_{β}^{d} gene (Gillies et al.,1984).

Introduction of the H-2 E_{α}^{a} gene complete with 2,000 nucleotides of 5', and 1,400 nucleotides of 3' flanking sequence into

(C57B1/6xSJL)F1 mice has also demonstrated enhancer elements in E_α genes. These F1 mice do not transcribe their endogenous E_α genes due to deletions in the E_α promoter regions. Introduction of an E^a_α gene by DNA mediated gene transfer restored the cell surface expression of the H-2 E molecules to levels comparable to the H-2 A molecules (Lemeur et al.,1985; Yamamura et al.,1985).

The P388D1 mouse macrophage cell line expresses no detectable Class II molecules on its cell surface. Addition of γ interferon to the growth media induces the expression of H-2 A^d and E^d Class II molecules after several days. Furthermore, introduction of a cloned H-2 E^b_β gene by gene transfer techniques, followed by γ interferon treatment led to the expression of $A^d_\alpha A^d_\beta$, $E^d_\alpha E^d_\beta$, and $E^d_\alpha E^b_\beta$ molecules. Clearly interferon stimulation results in the coordinate regulation of transcription of Class II genes (Folsom et al.,1984).

Other compounds such as prostaglandins (Snyder et al.,1982), glucocorticosteroids (Aberer et al.,1984), and lipopolysaccharide bacterial toxins (Steeg et al.,1982) have been shown to suppress Class II gene transcription, and therefore expression on macrophages.

Tissue specific trans-acting factors have also been implicated in the regulation of Class II gene expression. Early studies on the human B-lymphoid cell line (B-LCL) showed that these cells had untranscribed but structurally intact allelic HLA-DR1 and HLA-DR3 Class II genes. Fusion of these Class II defective cells with another B-lymphoid cell line which expresses HLA-DR2 antigens on its cell surface restored the expression of the HLA-DR1 and DR3 molecules on the surface of the fusion products. The authors concluded that the defect in the B-LCL cell line must be in some trans-acting transcription factor contained in the

second cell line (Gladstone and Pious, 1980). One form of congenital severe combined immunodeficiency (SCID) is also characterized by the absence of Class II gene products expressed on the surface of patient lymphocytes. Southern and Northern hybridization studies showed that the HLA-DR, DQ, DP, α, and β genes were all structurally intact but none were transcribed in these patients. Interferon γ, a known inducer of Class II gene expression, did not alleviate the global block in Class II transcription. Family studies showed that this condition was inherited as an autosomal recessive trait, however the trait did not segregate with the HLA. In one family with two children with identical MHC haplotypes one child has SCID while the other does not. In a second family two affected siblings have different MHC haplotypes (de Preval et al., 1985). The authors conclude that these patients are defective in a trans-acting factor which controls the expression of all Class II genes. This factor is unlinked to the MHC, and the factor controls a function or product essential for the action of γ interferon on Class II genes.

Recent studies have provided more direct evidence for the role of trans-acting factors in the control of Class II gene expression. Guardiola and colleagues (1986) have studied the Raji human B-lymphoma cell line which expresses high levels of Class II gene products. Mutagenesis and immunoselection resulted in a variant cell line which did not express HLA-DR, HLA-DQ, or HLA-DP molecules due to a block at the level of transcription. Fusion of these cells with Class II positive mouse B-lymphoma cells restored the expression of the human Class II genes and their products. Analysis of the fusion products by hybridization with probes for various mouse chromosomal markers suggested that the gene encoding the trans-acting factor was located on

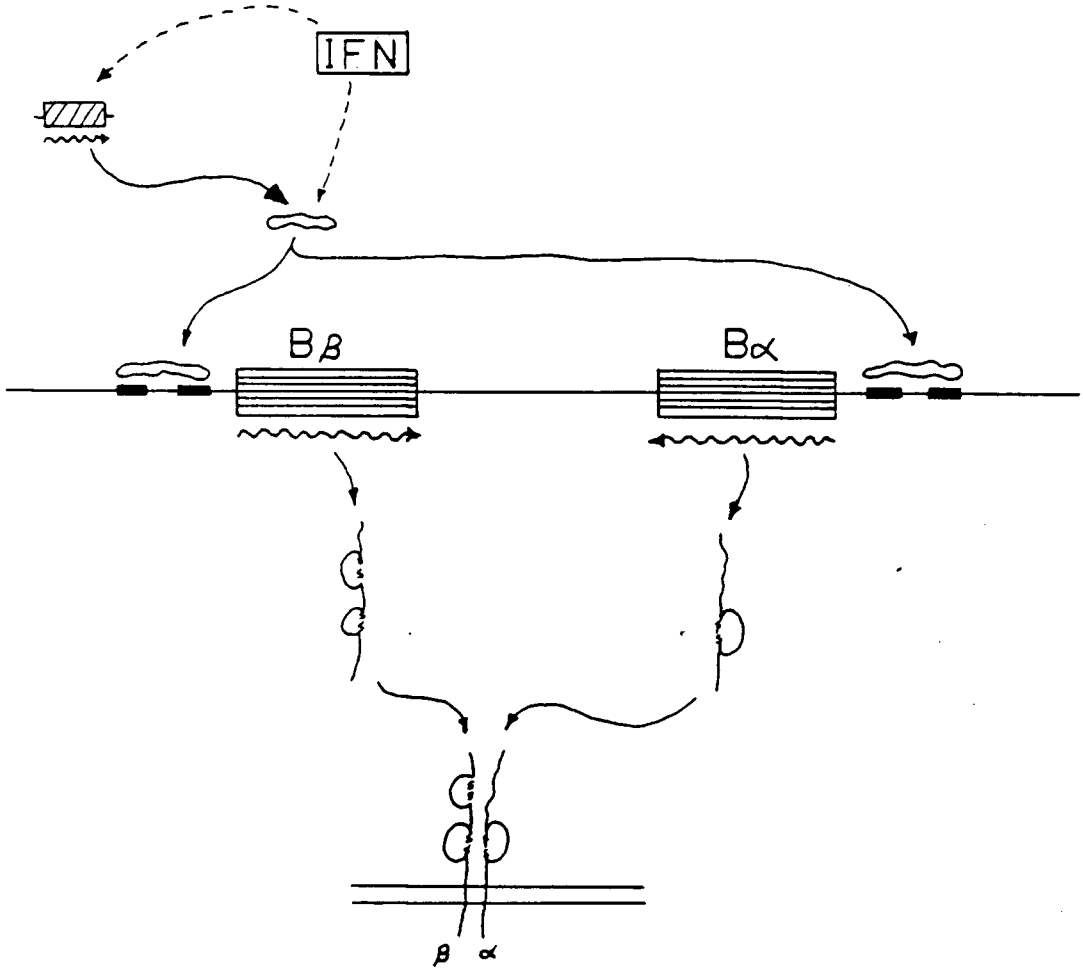
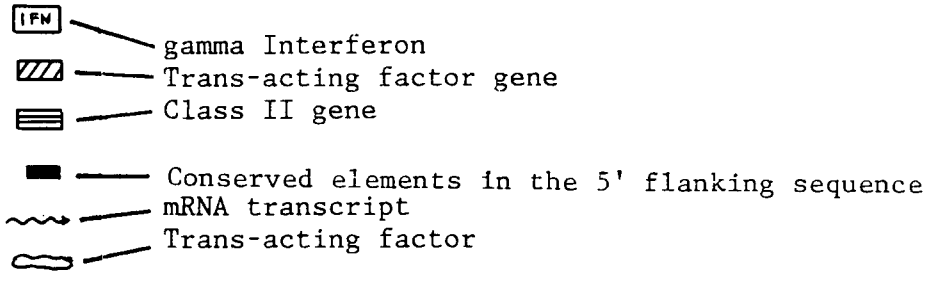


Figure 20: Model for the regulation of Class II gene expression. Non-MHC encoded genes produce trans-acting factor(s) which coordinately regulates the transcription of both Class II α and β genes by binding to the conserved elements in the 5' flanking sequences of both genes. Class II transcripts are translated and inserted into the cell membrane to form functional class II molecules expressed on the surface of the cell. Factors such as prostaglandins and ɣ interferon may act on the trans-acting factor(s) or the gene(s) encoding them to mediate Class II gene expression.



mouse chromosome 16. In Class II$^-$ Raji cells have been transfected with genomic DNA isolated from Ia$^+$ murine B-lymphoma cells. Transfectants with restored human Class II gene expression have been shown to contain stably integrated mouse DNA. These data strongly suggest that trans-acting factors are required for Class II gene expression, and that the specific gene or genes will soon be identified.

Figure 20 illustrates a model for the control of Class II gene expression. The highly conserved elements in the 5' flanking sequence identified in the RT1 B_α gene and in all Class II genes are likely targets for trans-acting factor(s). The control of transcriptional activity by a trans-acting factor(s) regulates the expression of Class II molecules on the cell surface. Although some regulation of Class II expression can occur at the protein level (discussed in Chapter 5) the primary level of control appears to be at the level of transcription. Other factors such as γ interferon and prostaglandins which are known to induce Class II gene transcription may act in a synergistic manner with a trans-acting factor(s) or possibly in the regulation of trans-acting factor gene expression.

In summary, the addition of the RT1 B_α nucleotide sequence to the list of known Class II gene sequences has provided evidence supporting Figueroa and Klein's model for the evolution of Class II genes. In addition comparison of the structure and sequence of the RT1 B_α gene to other Class II α genes has localized interspecies variation to the α1 domain. Furthermore these regions coincide with the known functionally polymorphic residues identified in allelic mouse sequences, and positive selection appears to favour replacement substitutions in this region. In

contrast structurally important residues have been highly conserved during the evolution of Class II α genes.

Finally, sequence analysis has identified a promoter element in the 5' flanking sequence of the RT1 B_{α} and all Class II genes which may be the target for factors involved in the regulation of Class II gene expression.

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APPENDIX 1

Preparation of Termination Mixes for M13 Dideoxynucleotide Sequencing

(from G. Winter, unpublished)

1. Dilute Deoxyribonucleotide Triphosphate (dNTP) stock solutions to a final concentration of 0.5mM with distilled water (dH_2O).

2. Prepare dNTP mixes as follows:

stock	dTTP	dCTP	dGTP	dATP
0.5mM dTTP	4µl	80µl	80µl	80µl
0.5mM dCTP	80µl	4µl	80µl	80µl
0.5mM dGTP	80µl	80µl	4µl	80µl
dH_2O	20µl	20µl	20µl	20µl
	184µl	184µl	184µl	260µl

3. Prepare Dideoxynucleotide Triphosphate (ddNTP) mixes from 10mM stocks to give the following final concentrations:

ddATP	0.2mM
ddCTP	0.2mM
ddTTP	2.0mM
ddGTP	1.6mM

4. Titrate the ddNTP mixes to prepare the termination mixes.

For example to prepare the "A" termination mix dATP mix and ddATP are mixed in several ratios;

5µl dATP mix	5µl dATP mix	5µl dATP mix
5µl 0.2mM ddATP	5µl 0.1mM ddATP	5µl 0.05mM ddATP
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10µl	10µl	10µl

Repeat this procedure for the other three nucleotides, and use the 3 sets of termination mixes to sequence a wild type M13 phage template.

5. Determine the optimum ratio for each termination mix from the sequencing resµlts, and make 2-5ml of each. Aliquot in 100µl amounts and store at -20°C.