Lehrstuhl für Genetik
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"Analysis of humanized antibody diversification in rabbits and mice using transgenic Immunoglobulin heavy chain (IgH) mini-loci"

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To my family
Especially to my son
Basile-Jordan Siewe Jr.

Contents

Abbreviations

Ad	add
ADCC	Antibody dependent cellular cytotoxicity
AID	Activation induced deaminase
AID	Activation induced deaminase
BAC	Bacterial artificial chromosome
BCR	B cell receptor
Bp	basepair
BSA	Bovine serum albumin
C	Constant region
CDR	Complementary determining region
CIP	Calf intestinal phosphatase
D	Diversity region
DNA	Deoxyribonucleic acid
E	enhancer
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetaacetic acid
EtBr	Ethidium bromide
FACs	Flourescence-activated cell sorter
FR	Framework region
HuCμ	Human C mu chain
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IgV	Immunoglobulin V region
J	Joining region
Kb	Kilobase
kDA	Kilo Dalton
KLH	Keyhole limpet hemocyanin
LB	Luria broth
mMRP8	Murine myeloid related protein
Nt	nucleotide
Oligo	oligonucleotide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
RAG	Recombination activating gene
Rpm	Rounds per minute
RSS	Recombination signal sequence
SDS	Sodium dodecyl sulfate
SHM	Somatic hypermutation
TRIS	Trishydroxymethylaminomethan
U	units
V	Variable region

VDJ	Variable-diversity-joining gene segment
WT	Wild tyope
B-globin	Betaglobin house keeping gene

1 Introduction

1.1 The Immune System

The principal function of the immune system is to protect animals from infectious organisms and their toxic products. This system has evolved a powerful range of mechanisms to locate foreign cells, viruses or macromolecules to neutralize these invading molecules and to eliminate them from the body. This surveillance is performed by a system of proteins and cells that circulate throughout the body, the components of which can be divided into two broad categories: the non-adaptive or innate immunity and the adaptive or acquired immunity (Janeway, Jr., 1992; Janeway, Jr., 2001).

1.1.1 The innate or non-adaptive immune system

The innate immune system refers to immunity mediated by cells that respond non-specifically to foreign molecules. These components of the immune system can distinguish between foreign tissues and organisms but are unable to distinguish a particular invader. They carry out their functions by recognizing structures which are shared by most invading micro-organisms, but which are not features of self, for example specific lipopolysaccharides and high-mannose containing oligosaccharides. The components of the innate system can be divided into a cellular and a humoral part. A complex of plasma proteins termed the complement system makes up the main constituent of the humoral arm of the innate system. These complement proteins bind to invaders, a process known as opsonization, thereby marking them for destruction. The cellular arm is composed of macrophages, whose principal function involves destruction of foreign cells by phagocytosis. The lacrimal cells, which secrete lysozymes that destroy the outer surface of many bacteria, are also components of the innate system. Other

elements of the innate immune system include granulocytes and dendritic cells that activate and regulate other immune elements through secretion of cytokines and finally the natural killer cells that destroy foreign cells via cell lysis. Some members of the innate system activate the adaptive system; dendritic cells for example are known to act as antigen presenting cells, thereby creating a link between the innate and the adaptive system. Non-adaptive immunity does not improve with repeated exposure to the same foreign molecule, thus these systems can easily be evaded by mutations. It is presumably the inadequacy of innate recognition that led to the evolution of the adaptive immunity (Janeway, Jr., 1992).

1.1.2   The adaptive or acquired immune system

The adaptive immune system exhibits two major differences to the innate system: first the adaptive immune system is directed against specific molecules, and second, its actions are enhanced by re-exposure, a property termed memory, which enables immunization. Adaptive immunity is mediated by cells called lymphocytes, which synthesize cell-surface receptors or secrete proteins that bind specifically to foreign molecules. These secreted proteins are known as antibodies and the specific molecules they bind to are termed antigens. The immune system contains more than 10^9 lymphocytes distributed throughout the body, enabling them to respond rapidly at any site. Lymphocytes arise continuously from progenitor stem cells- the hematopoietic cells- in the bone marrow. The bone marrow and thymus represent the central or primary lymphoid organs. These are sites of lymphocyte generation and maturation. They then circulate through the blood and lymphatic systems resting and accumulating in specialized structures called secondary lymphoid organs, where actual interaction with antigen occurs. These secondary lymphoid organs include lymph nodes, spleen, tonsils, the Peyer's patches in the small intestines and the appendix. There are two lymphocyte sub-populations: B -and T-cells.

T-cells arise from the common lymphoid progenitor cell in the bone marrow but mature in the thymus. The T-cells serve two major functions, first as coordinators of other acquired immune responses, accomplished by their production of a wide variety of cytokines and surface cell signals, and secondly in that they mediate the primary answer to intra-cellular infections. There are two sub-types of T-cells

- The cytotoxic T-cells or CD8+ cells
- The helper T-cells or CD4+ cells

Each T-cell has a unique surface molecule called a T-cell receptor, which recognizes short peptides (generated from invading pathogens or viruses), displayed on the surface of specialized cells termed antigen presenting cells (APC). The peptide display is usually in conjunction with a membrane glycoprotein molecule called the major histocompatibility complex (MHC). The different T-cell sub-types recognize peptides from different cellular locations, presented in conjunction with different types of MHC molecules.

B-cells are the mediators of humoral immunity. Every B-cell has an Immunoglobulin (Ig) molecule on its surface, termed the B-cell Receptor (BCR). The BCR recognizes a unique three-dimensional moiety on an antigen, termed an epitope. B-cells mature in the bone marrow, and the millions of different B-cells produced daily proceed to circulate throughout the bloodstream. Since each B-cell has a different BCR, each will bind to a different substrate. Recognition and binding of its specific target induces clonal expansion resulting in many B-cells that recognize the same target. Recognition of a specific target leads to the differentiation of B-cells to Memory and Plasma cells.

1.2　　　　Antibody structure and function

1.2.1　　　Antibody structure

Antibodies belong to the immunoglobulin (Ig) protein super-family. The common characteristics shared by the members of this family include the fact that they are made up of a series of domains of related amino acid sequence which possess a common secondary and tertiary structure, as seen in figure 1 below. Antibodies are capable of recognizing and distinguishing between specific molecular patterns (epitopes) of antigens and because antigens are diverse in structure, the repertoire of antibodies must be immense to be able to perform their functions. In mice and humans there are different types of antibody classes known as IgM, IgD, IgG, IgA and IgE, and subtypes of certain classes exist in both humans (G1, G2, G3, G4 and A1, A2) and in mice (G1, G2a, G2b and G3). The structure of the IgG class of antibodies is described below. In humans the IgG class antibody is the most abundant.

The basic structure of an antibody molecule is Y-shaped, composed of two identical 20kDa light (L) chains and two identical 50kDa heavy (H) chains. Disulfide bonds link each heavy chain to a light chain as well as the other heavy chain of the same molecule themselves (Figure 1). In most animals there are only two types of light chains, which are termed lambda (λ) and kappa ($\varkappa$) light chains. In contrast, there are five main heavy-chain classes, often referred to as isotypes, and these determine the functional activity of the antibody molecule.

Extensive sequence analysis of many immunoglobulin chains revealed that the amino-terminal regions are highly variable while the carboxyl-terminal regions are isotype-specific within a given species (Hilschmann and Craig, 1965). The amino-terminal region or variable (V) region of each chain is responsible for antigen recognition and contains sub-regions whose residues have been implicated in actual antigen contact. These sub-regions are referred to as "Complementary Determining Regions" (CDR1, CDR2, CDR3), and are flanked by less variable sub-regions termed "Framework Regions" (FR1, FR2, FR3 and FR4), figure 1.1b. The

carboxyl-terminal region of the antibody molecule is made up of one or more domains which are comparatively constant (C) in sequence and which perform an array of effector functions.
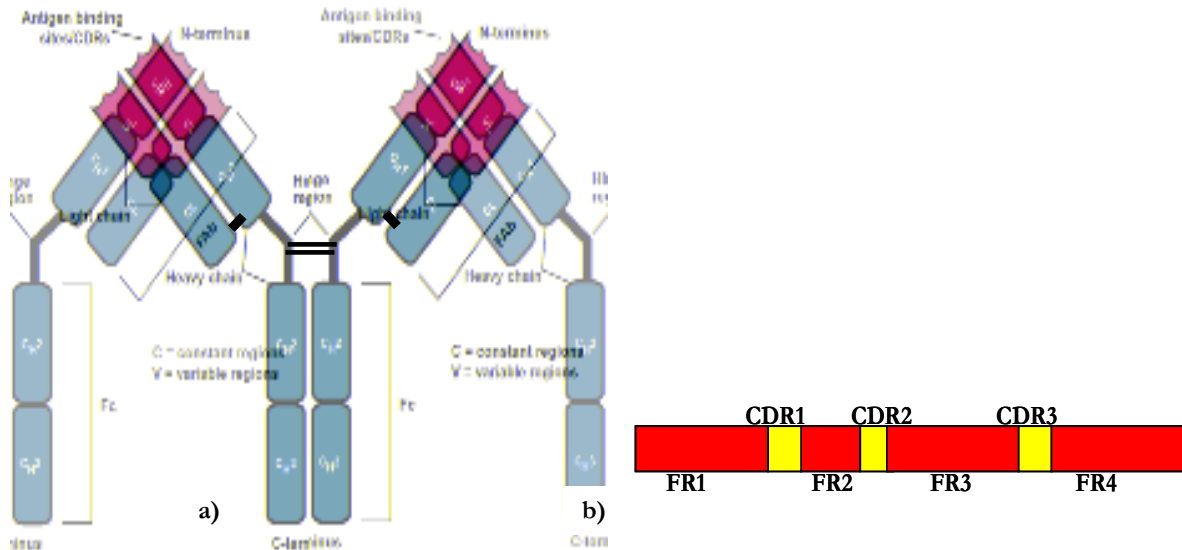
Figure 1.1   Figure 1.1a depicts an antibody molecule (IgG sub-class).Two heavy (H) and two light (L) pairs comprise a single antibody molecule. Within each chain there are two regions described as constant(C) and variable (V) regions. The genes that code for the variable regions in both chains (V_H and V_L genes) generate the highly diverse binding sites capable of binding almost any type of antigen. The constant and hinge regions of the antibody are encoded by C_L, C_H1, C_H2 and C_H3 genes segments. Digestion of an antibody molecule with the protease papain results in three fragments: two identical fragments carrying the antigen-binding activity, called the Fab fragments, for Fragments of antigen binding, the other fragment which carries no antigen-binding activity but crystallizes readily and is termed the Fc fragment (fragment crystallizable) Figure 1.1b depicts the frame work (indicated in red) and the complementary determining regions (indicated in yellow). (figure adapted from Paul, 2003)

1.2.2   *Antibody Function*

As stated above, antibodies are key proteins of the immune system of animals that protect against invading disease. They are distributed evenly throughout the body to react immediately to invading pathogens. Their primary function involves specific binding of antigens; carried out by means of the antigen binding sites, meanwhile the activation of other components of the immune system to eliminate these invading pathogens is performed by the constant region of the antibody. Figure 1.1 depicts a summary of the functions of antibodies.
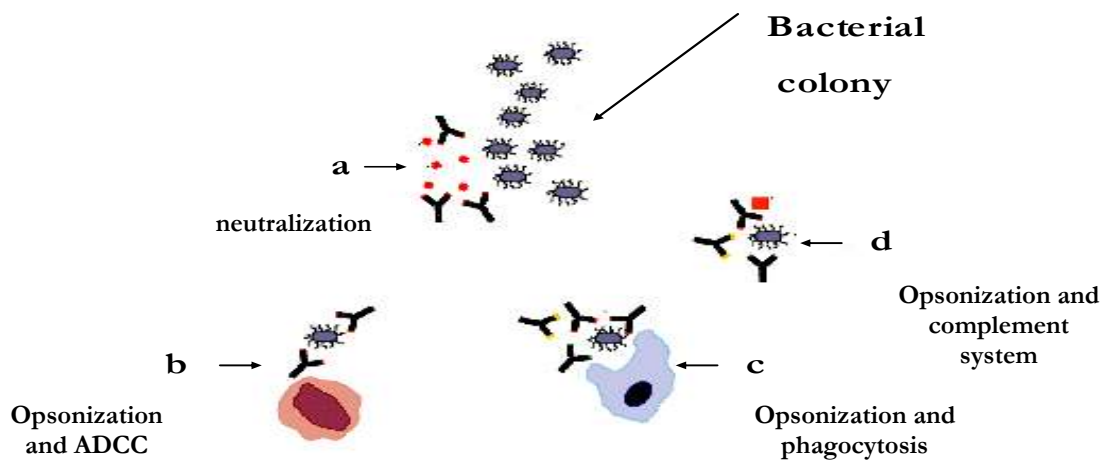
Figure 1.2   The functions of antibodies in the elimination of invading pathogens. Antibodies use a multitude of methods to eradicate invading pathogens. Theses different eradication pathways, a, b, c and d are further elucidated below. (**Red spots** = bacterial toxins, **blue structure** = macrophages, **reddish-brown structure** = natural killer cells, **Y-shaped structures** = antibodies and the **red rectangle** = a component of the complement system of proteins.)

Figure 1.2 shows four pathways employed by antibodies in neutralizing antigens and eliminating pathogens.

    a.   Antibodies can bind to and effectively neutralize invading bacterial toxins

    b. Also, antibodies complexed with antigens by specific binding a process known as opsonization can in turn be bound by other components of the immune system that possess Fc receptors. In this example the natural killer cells are bound to opsonized bacteria, resulting in the lysis of the bound bacteria. This is an example of antibody dependent cellular cytotoxicity (ADCC).

    c. Alternatively, opsonized bacteria can be engulfed by macrophages, scavenger cells of the immune system. Macrophages as well possess Fc receptors and engulf the opsonized pathogen via phagocytosis.

    d. The opsonization of pathogen also leads to recruitment of the complement system. This is a set of plasma proteins that act together in a cascade manner to facilitate removal of coated pathogens by phagocytes or by directly killing them. Here one of the complement components is activated by oposonization of pathogens.

1.3   Creation of antibody diversity

1.3.1   Combinatorial diversity

The mouse was used as a model for the studies involving the creation of antibody diversity and it was thus observed that in the mouse genome, gene segments coding for the variable region are present in multiple copies, scattered along a chromosome (Seidman et al., 1978). This means that during gene rearrangement, different gene segments can be joined together to form a stretch of DNA that codes for an entire variable region. The process of gene rearrangement is restricted to B cells (Dreyer and Bennett, 1965; Hozumi and Tonegawa, 1976; Tonegawa et al., 1977). Furthermore, different heavy chains can pair with different light chains to create a functional antibody molecule. This mechanism termed combinatorial diversity has important consequences:

- It enables a limited number of gene segments to generate an extremely diverse set of proteins;

- Each cell assembles a different set of gene segments in the formation of antibody molecules. This results in the expression of a unique antibody in each cell.
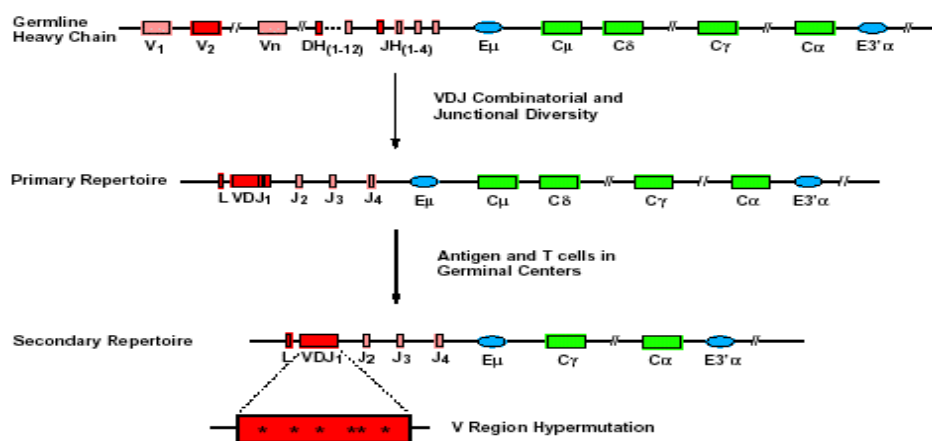
Figure 1.3   Using the example of a mouse heavy chain, the processes leading to the generation of antibody diversity are outlined. Ig gene segments are shown as red and green blocks. The depicted events are discussed in detail in the ensuing sections. (Figure adapted from Paul, 2003)

The rearrangement processes involved in the formation of the V region of an antibody is dependent on the protein products of the recombination activating genes, *RAG-1* and *RAG-2* (Seidman et al., 1978; Alt and Baltimore, 1982). These enzymes belong to a complex of enzymes termed the V (D) J recombinase that play key roles in the rearrangement processes. Germ-line V and D DNA segments are followed by a conserved heptamer sequence CACAGTG and a nonamer sequence ACAAAAACC, separated by an non-conserved spacer sequence of either 12 or 23 base pairs (Lewis and Gellert, 1989; Gearhart and Bogenhagen, 1983). Likewise, all D and J segments are immediately preceded by a consensus nonamer GGTTTTTGT and a consensus heptamer CACAGTG. The two consensus sequences are separated by a short non-conserved spacer sequence of either 12 or 23 base pairs.

This heptamer-spacer-nonamer sequence is called the recombination signal sequence, RSS (figure 1.4). The RSS is recognised by the *RAG-1*: *RAG-2* protein complex (figure 1.4), which cuts one strand of the double-stranded DNA, at the end of the heptamer sequences, thereby initiating the DNA rearrangements.
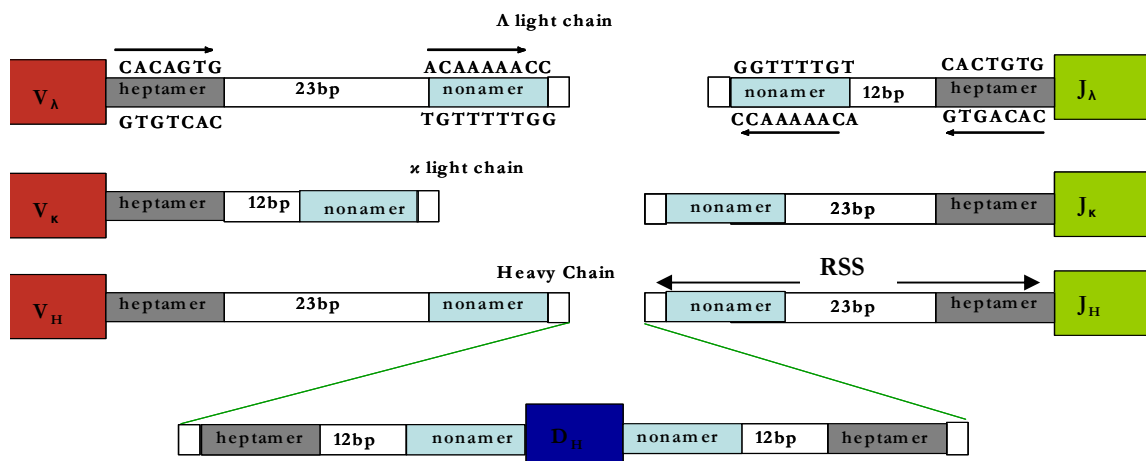
Figure 1.4   The gene segments coding the V regions in both heavy and light chains are flanked by highly conserved nonamer and heptamer sequences. The spacing regions between the conserved sequences are either 12 or 23 bp. This is the basis of the 12/23 bp rule whereby joining invariably involves a 12 bp and a 23 bp recombination signal sequence (RSS). (Figure adapted from Janeway, Jr. et al., 1999)

Joining always involves a 12 bp spacer to 23 bp spacer, a principle known as the "12/23 bp rule". This rule allows joining of the regions in a precise manner, for example in the heavy chain, two D_H regions cannot join with each other nor can a V_H join directly with a J_H. In the case of a light chain joining, a V_L fragment must be joined to a J_L fragment. Antibody diversity is generated in three main ways, two (combinatorial and junctional diversity, figure 1.3) of which result as a consequence of the rearrangement processes by which a functional V region is completed. In humans and mice, these two processes generate a diversified primary antibody repertoire, but in birds, rabbits, cows, pigs, sheep, and horses, there is limited diversity of the primary antibody repertoire, and as described in sections 1.3.5 and 1.3.6, rabbits further diversify their primary repertoire by a process termed gene conversion. The third process is a mutational process which takes place after the gene rearrangements. This mutational process which is most often antigen driven (figure 1.3) is restricted to the rearranged V region (Gearhart and Bogenhagen, 1983) and is elucidated below. The mutational process leads to the generation of the secondary antibody repertoire.

1.3.2   Junctional diversity

Of the three complementary determining regions, the CDR1 and CDR2 sub-regions are encoded within the V gene segment. The third sub-region CDR3 falls at the joint between the V and J gene segments in the light chain. In the heavy chain the CDR3 region is partially coded by the D gene segment (figures 1.5 and 1.6 below). In both heavy and light chain, the diversity of the CDR3 sub-region is highly likely a result of addition and deletion of nucleotides occurring during the formation of the junction (the so-called coding joint, figure 1.5) between the gene segments (Alt and Baltimore, 1982; Lewis and Gellert, 1989). This mechanism is termed junctional diversity, and the added nucleotides are known as P-nucleotides and N-nucleotides (figure 1.5).
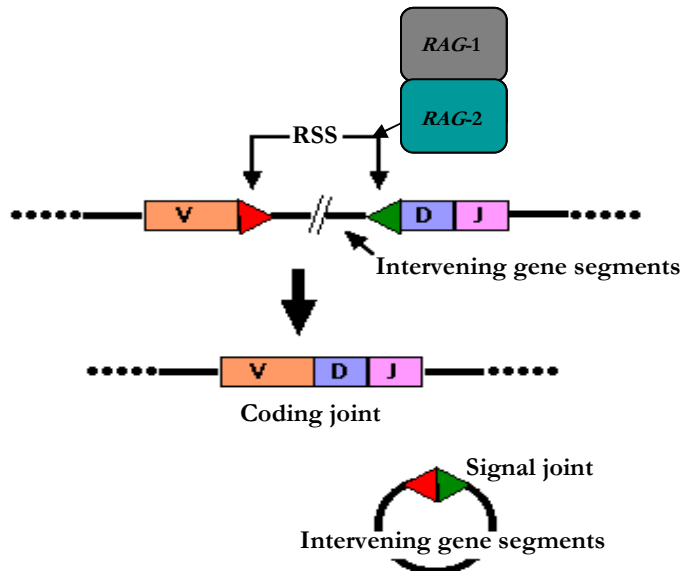
Figure 1.5 Generation of junctional diversity between the V_H and DJ_H genes. The *RAG* protein complex (composed of two proteins *RAG*-1 and *RAG*-2) recognise, bind and cleave the RSS motifs. The segments to be recombined are then brought by the RAG protein complex and the DNA strands the end of gene segments are cleaved by the RAG proteins to form a hairpin, while the ends of the RSS motifs are retained as a double strand break. The rest of the RSS are then joined exactly (signal joint) to form a loop of DNA which plays no further role in the ensuing processes. The double-strand break the end the end of the RSS motifs is the repaired by cell machinery and in this process, the palindromic sequences (P nucleotides) are generated. The resulting joint between the VH and the DJH genes is called the coding joint, and is further diversified by erratic addition of nucleotides (N nucleotides), a process regulated by the enzyme Terminal Deoxynucleotidyl Transferase , TDT (Kunkel et al., 1986; Snow et al., 1987).

1.3.3 *Generation of the light chain*

Detailed analysis of rearranged and germ line immunoglobulin light chain genes revealed that the coding potential of this polypeptide chain lies in three separate DNA portions, the variable V_L, joining J_L and constant C_L gene segments, while the major portion of the signal peptide is encoded by a fourth DNA segment, the leader (L) gene segment (Brack et al., 1978).

Combinatorial diversity in the generation of the light chain would involve the usage of different V_L, J_L and C_L gene segments in different B Cells, resulting in antibodies with different specificities. The V segment encodes the rest of the signal peptide and the first 95 or 96 amino acids of the variable region, while the J segment encodes about 12-14 amino acids of the FR3 region and the C gene segment encodes the whole C region (figure 1.6).
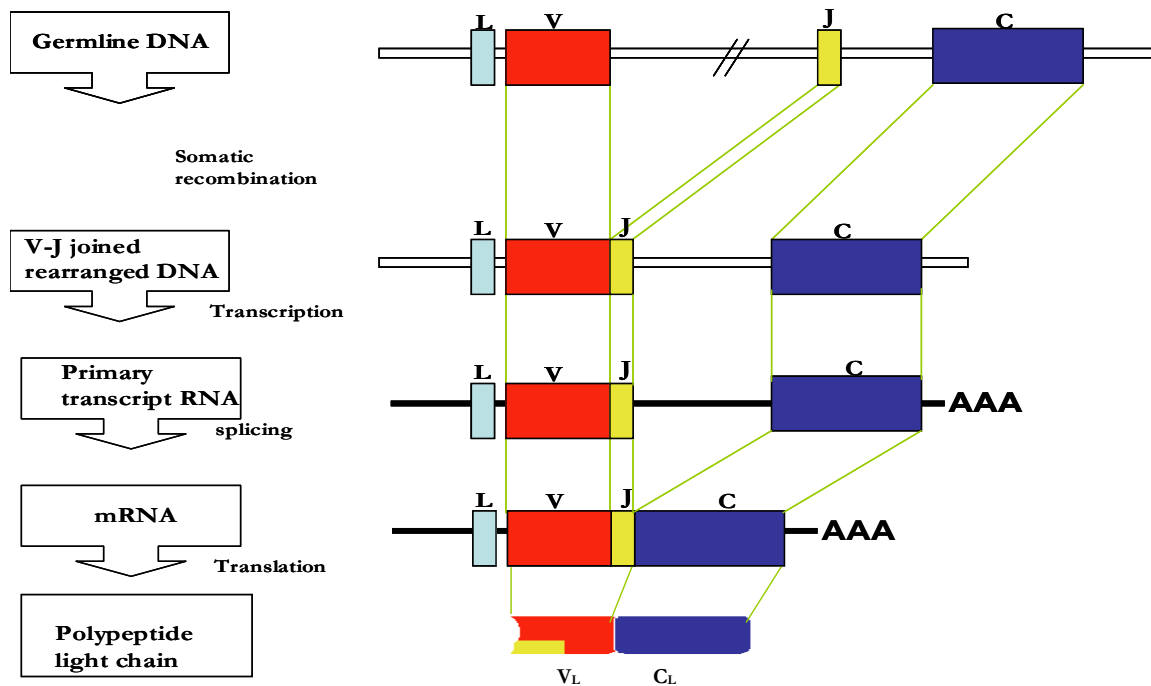
Figure 1.6   Rearrangement events leading to the creation of a light chain. The V segment of the light chain is composed from two segments: a variable (V) and a joining (J) region. These segments which are separated on the genomic DNA level are brought together by somatic recombination to form the functional light chain V region. The C region is encoded in a separate exon, and then is joined to the rearranged VJ segment as a result of splicing, which excises the introns between the leader (L) and the VJ and the VJ and the C segments. The leader directs the nascent protein into the cells secretory pathway. (Figure adapted from Janeway, Jr. et al., 1999)

1.3.4   Generation of the heavy chain

The heavy chain on the other hand is encoded by four separate gene segments. In addition to the V_H, J_H and C_H segments, there is the D_H segment (diversity segment), which encodes the core portion of the CDR3. The process of recombination producing a functional heavy chain occurs in two separate stages: first the D_H segment is joined to the J_H segment, followed by recombination of a V_H segment to the DJ_H to complete the heavy chain variable region. Same as with the light chain, the VDJ segment is joined to the constant-domain coding segment by RNA splicing (figure 1.7). Combinatorial diversity is more pronounced in the heavy chain because different D gene segments can be used in addition to the different V and J segments.
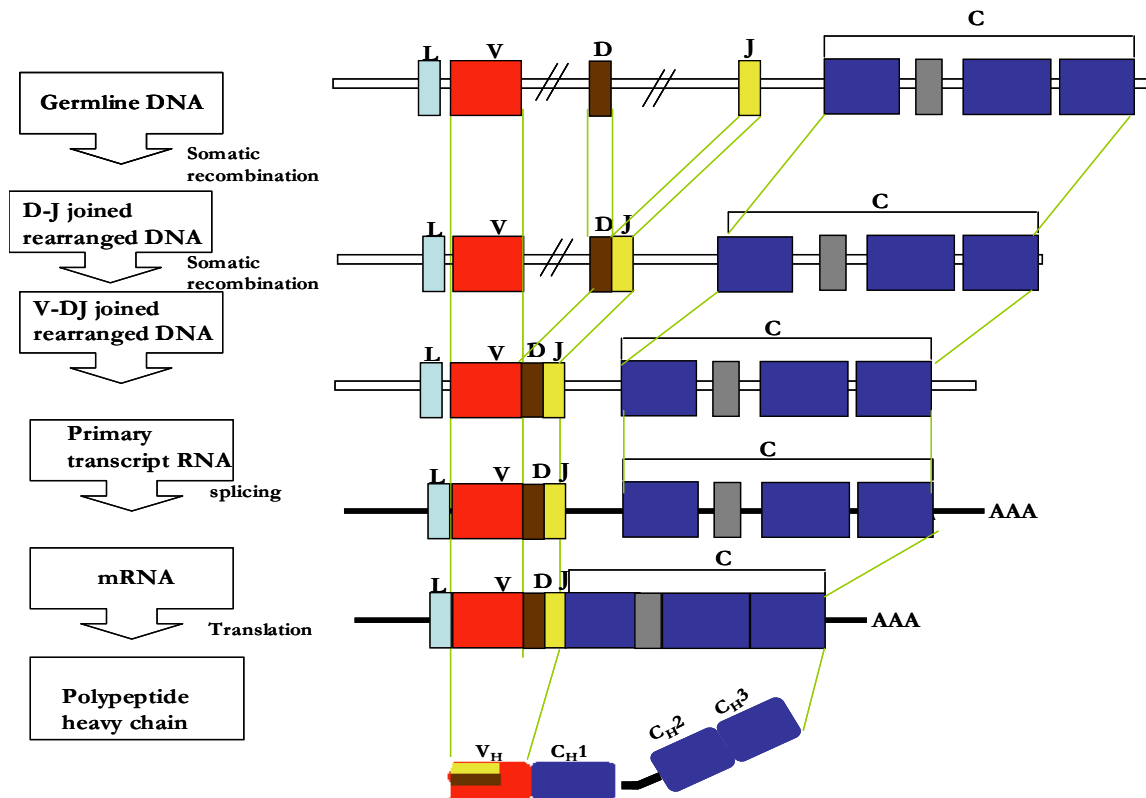
Figure 1.7   Rearrangement events leading to the creation of a heavy chain. Unlike the light chain V region which is composed of just two segments, the heavy chain V region is composed of three segments; in addition to the V and the J segments, there is the diversity (D) segment. Somatic recombination starts with a DJ joining, the V region the rearranges to form a VDJ gene, which forms the complete functional V region. As with the light chain, the c region is encoded on another exon, which is brought to the VDJ gene as a result of splicing. After translation the leader sequence is cleaved. (Figure adapted from Janeway, Jr. et al., 1999)

During the humoral response the first antibodies produced are IgM isotype. As mentioned in section 1.2, the heavy chain isotype determines the effector functions of the antibody. Once the B cells are activated (by antigen binding) they undergo a process called isotype switching or class switching to secrete antibodies of different isotypes, thus acquiring other effector functions against the antigen. Isotype switching does not affect antibody specificity. Isotype switching (Stavnezer, 1996) occurs by site -specific recombination between repetitive sequence stretches called switch sequences. These switch sequences are present upstream of each constant gene region (figure 1.8).
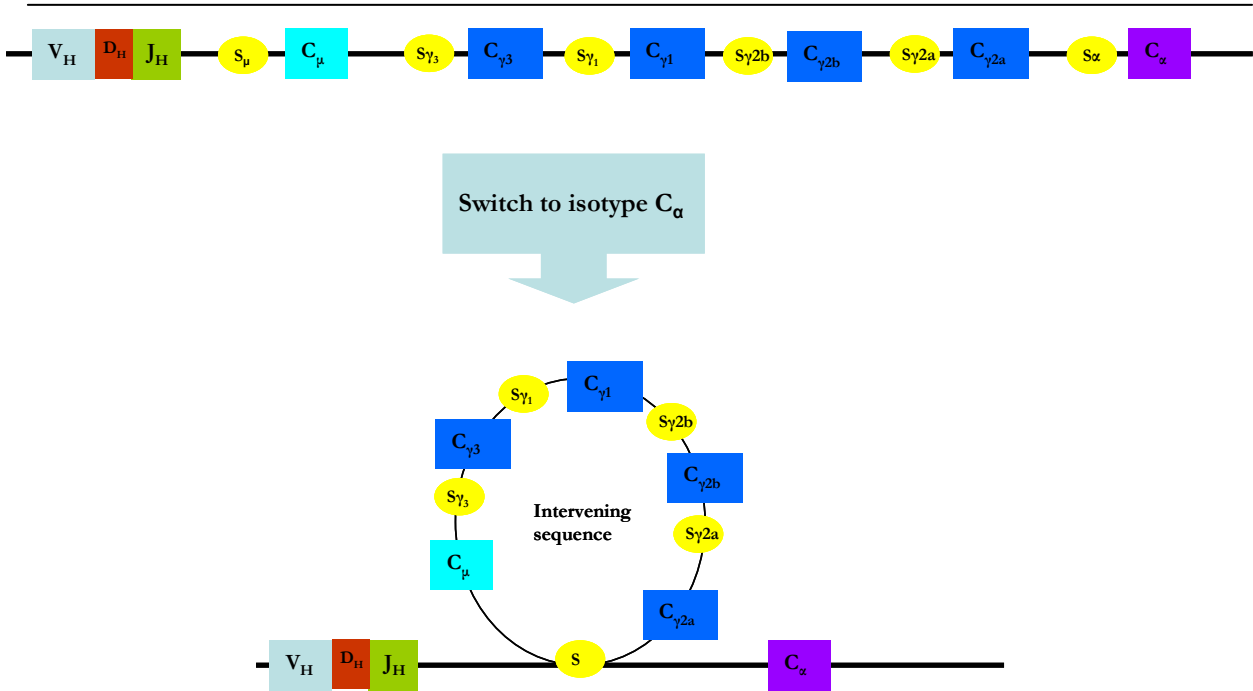
Figure 1.8 Isotype switch to C_α. Shown is an example of class switch using the mouse heavy chain locus. Upstream of the each constant region is the corresponding switch sequence shown as yellow ovals. During the process, site-specific recombination between the corresponding switch sequences results in the deletion of the intervening sequence.

1.3.5 *Somatic mutation*

In most mammals, the above described mechanisms for generating diversity all take place during rearrangement of gene segments in the initial development of B cells in primary lymphoid organs. The antibody repertoire at this stage is labelled the primary repertoire. This antibody repertoire has rather low affinity for antigen; therefore a further mechanism is employed to fine-tune antibodies of the primary repertoire to tightly bind antigens, which greatly increases the versatility and effectiveness of the immune system. This fine-tuning process is termed somatic mutation (the different mechanisms of somatic mutation are discussed in section 1.3.6 below). In humans and mice this process is termed somatic hypermutation (SHM) and its include a high frequency of point mutations- 1 -5 % of base pairs are mutated- and mostly its restriction to rearranged V genes (Gearhart and Bogenhagen, 1983). When immature B cells leave the bone marrow, they circulate via blood to enter the secondary lymphoid organs- the spleen, lymph nodes and the

mucosal-associated lymphoid tissue (MALT). In these secondary lymphoid organs, the immature B cells are restricted in small clusters called primary follicles where they receive survival signals from specialized cells called the follicular dendritic cells. When B cells are activated by antigen binding, they enter the primary follicles and undergo rapid proliferation to form specialized partitions called germinal centres. In these germinal centres, the B cell proliferation is accompanied by an increase in the affinity of antibodies for the particular activating antigen. This process called affinity maturation is a result of somatic hypermutation, (Jacob et al., 1993a; Jacob et al., 1991) with preferential selection for the B cells with higher-affinity immunoglobulins than their germ-line counterparts (Jacob et al., 1993b; Griffiths et al., 1984). Mutations in the CDR regions of the rearranged V gene result in higher affinity for the corresponding antigen.

An outline for a model explaining the events leading to somatic hypermutation (SHM) was proposed by Brenner and Milstein (Brenner and Milstein, 1966). According to this model the process can be divided into three steps: targeting, DNA recognition and cleavage, and repair. These three stages are depicted in figure 1.9.

1.3.5.1 *The targeting phase of SHM*

The process of transcription has been proposed to play a key role in recruiting a B cell specific nuclease to the immunoglobulin locus (a heavy chain locus in this case). A yet unidentified nuclease (green oval) is bound to a protein molecule (light green rectangle) on the Ig enhancer. The interaction between the enhancer and the promoter, places the nuclease next to the transcription initiation complex, shown here as a hatched oval. The enhancer –promoter interaction, also initiates transcription (grey arrow). The deposition of the nuclease on the transcription initiation complex, and the onset of transcription lead to the next stage of the processes resulting in somatic hypermutation.

a)

b)

c)

d)

e)

f)

g)

targeting

DNA recognition
and cleavage

repair
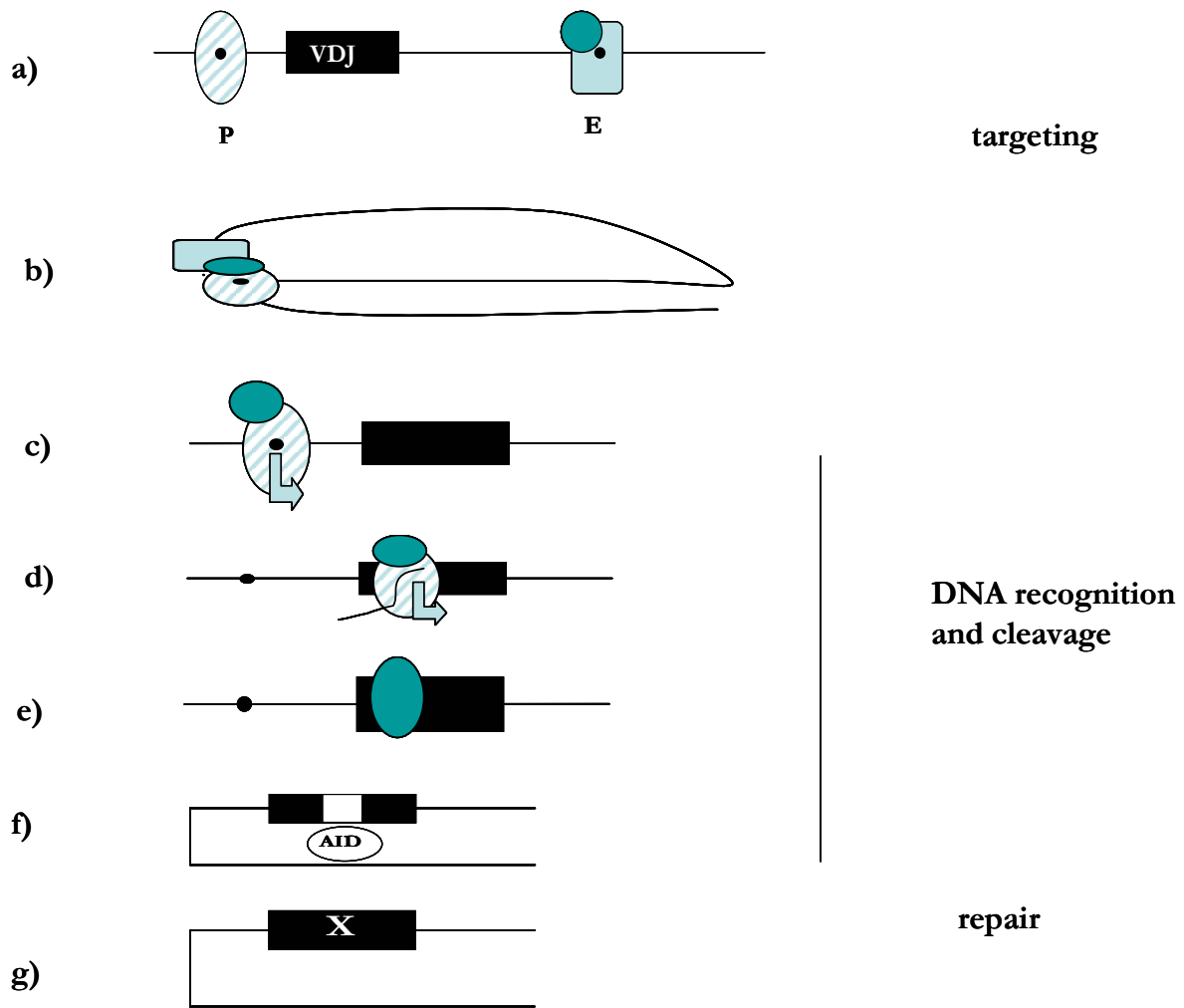
Figure 1.9   The figure depicts the stages leading to the generation of somatic hypermutation, these steps are further explained below. **E** = enhancer, **P** = promoter, **light green hatched oval** = nuclease, **light green oval** =protein molecule on enhancer, **AID** = Activation Induced Deaminase, **X** depicts a somatic mutation, see text for further explanations. (Diagram adapted from Papavasiliou and Schatz, 2002), **a – b** = targeting steps, **c – e** = DNA recognition and cleavage steps, **f - g** = repair steps

1.3.5.2   *The DNA recognition and cleavage phase of SHM*

The structure of the VDJ sequence that is recognized by the nuclease still remains a mystery. Multiple postulations have been brought forth (Papavasiliou and Schatz, 2002), but concrete evidence has not been provided. A tangible hint was provided by mining a vast database of accumulated mutation sequences. After this analysis, mathematical biologists (Rogozin and Kolchanov, 1992); (Rogozin and Diaz, 2004) proposed that the hypermutation mechanism has preferential "hotspots". During transcription, the nuclease moves along the Ig gene with the transcription complex and once the hotspots in the VDJ region are recognised, this nuclease makes a double strand break, (DSB) (Sale and Neuberger, 1998) near the hotspot. This

lesion in the DNA strands creates the template for the repair process, which is the next and final phase of somatic hypermutation.

1.3.5.3 *The repair phase of SHM*

A logical set up for the repair phase would be that the break caused by the B cell specific nuclease is repaired by various DNA repair mechanisms in an error prone manner, thereby introducing a point mutation in the region of the break. Also involved in the repair phase are trans-acting factors. One of these trans-acting factors AID, (Activation Induced Deaminase) has been identified and intensively studied. AID is a protein that was originally identified as an RNA-editing enzyme (Honjo et al., 2002), but the discovery that AID is expressed only in hypermutating B cells (Yang et al., 2005), and is a prerequisite for hypermutation (Gearhart and Wood, 2001; Shen and Storb, 2004; Shen et al., 2005), represented a breakthrough in understanding the mechanism behind the repair phase of SHM. In a model proposed by Neuberger and colleagues (Di Noia and Neuberger, 2002), the role of AID involves the initiation of hypermutation by directly deanimating DNA, thereby causing a lesion in the DNA strand. According to this model, AID induced lesions are consequently repaired by error prone polymerases (reviewed by Gearhart and Wood, 2001), thereby generating point mutations in the VDJ region. Furthermore, the model also suggests a preference for transition mutations (a purine replaced by a purine or a pyrimidine replaced by another pyrimidine) over transversion mutations (a purine replaced by a pyrimidine or otherwise) during somatic hypermutation (Di Noia and Neuberger, 2002) and a supposed link between hypermutation of antibody genes and DNA polymerases (see also Gearhart and Wood, 2001; Xu et al., 2005; Zan et al., 2005).

AID has also been implicated in antibody class switching (Muramatsu et al., 2000; Basu et al., 2005; Schrader et al., 2005), and also proposed to play a role in gene conversion (Sale et al., 2001) the process of gene conversion is described in detail in section 1.3.6 below. Further evidence for the role of AID in gene conversion has been delivered by the cloning, localization and extraction of the protein from rabbit appendix, a site where gene conversion was observed, (Yang et al., 2005).

1.3.6   *Somatic hypermutation versus gene conversion*

Normally, all vertebrates start the creation of the primary antibody repertoire by recombining V, D and J gene segments. In mice and humans, this recombination step leads to considerable diversity since hundreds of VDJ genes are randomly recombined. In these mammals, the secondary repertoire is further diversified by somatic hypermutation discussed above. However, in other vertebrates including rabbits and chicken, the first step of recombination does not lead to significant diversity because only a limited number of variable (V) genes are used (Becker and Knight, 1990; Reynaud et al., 1985). These animals promote diversification of their primary antibody repertoire by making use of somatic gene conversion (Schiaffella et al., 1999; Mage et al., 1999; Sehgal et al., 2000).

The term gene conversion has been used to define the non-reciprocal transfer of information from one chromatid to the other accounting for interallelic recombination. In this scenario, gene conversion is a result of strand exchange between DNA molecules.

In the antibody diversification scenario, gene conversion describes a template-mediated form of somatic mutation whereby the V genes, upstream of the rearranged V gene serve as sequence donors for introducing mutations in the rearranged VDJ. These donor genes are mostly non-functional or pseudogenes because of a multitude of reasons: frameshifts and/ or stop codons and also because they are flanked by suboptimal recombination signal sequences (RSS, mentioned in section 1.3 above). As discussed in section 1.3.5, the protein AID as well as other proteins for example, x-ray repair cross complementing protein 2 and 6 (XRCC2, XRCC6) and RAD51B (Sale et al., 2001), have been shown to play a role in the initiation and regulation of gene conversion. Gene conversion events depend on high sequence homology between donor and recipient V genes. In rabbits, for example, only one V_H gene, V_H1 is preferentially utilized in their VDJ rearrangements (Knight and Becker, 1990) and the upstream V pseudogenes which share very high sequence homology with the rearranged V_H1 and gene serve as

sequence donors for gene conversion (figure 1.10). Diversification of the V_H1 gene in rabbit VDJ rearrangements is characterized by sequence variations which occur primarily as clusters of nucleotide changes and codon insertions or deletions (knight and Becker, 1990; Mage and Knight , 2006). These sequence variations observed in diversified rabbit VDJ rearrangements are typical of gene conversion as they correlate with observations in chicken. Chicken also utilize only a single V_H and V_λ gene in their Ig rearrangements and are known to circumvent these limitations by further diversification using gene conversion (Reynaud et al., 1989). Insertion of nucleotide clusters in the V gene of V(D)J rearrangements and identification of the upstream donor genes are hallmarks of gene conversion and this insertion of nucleotide clusters shows a clear disparity to nucleotide variations resulting from somatic hypermutation for example in mice, where these variations are characterized by scattered single point mutations .
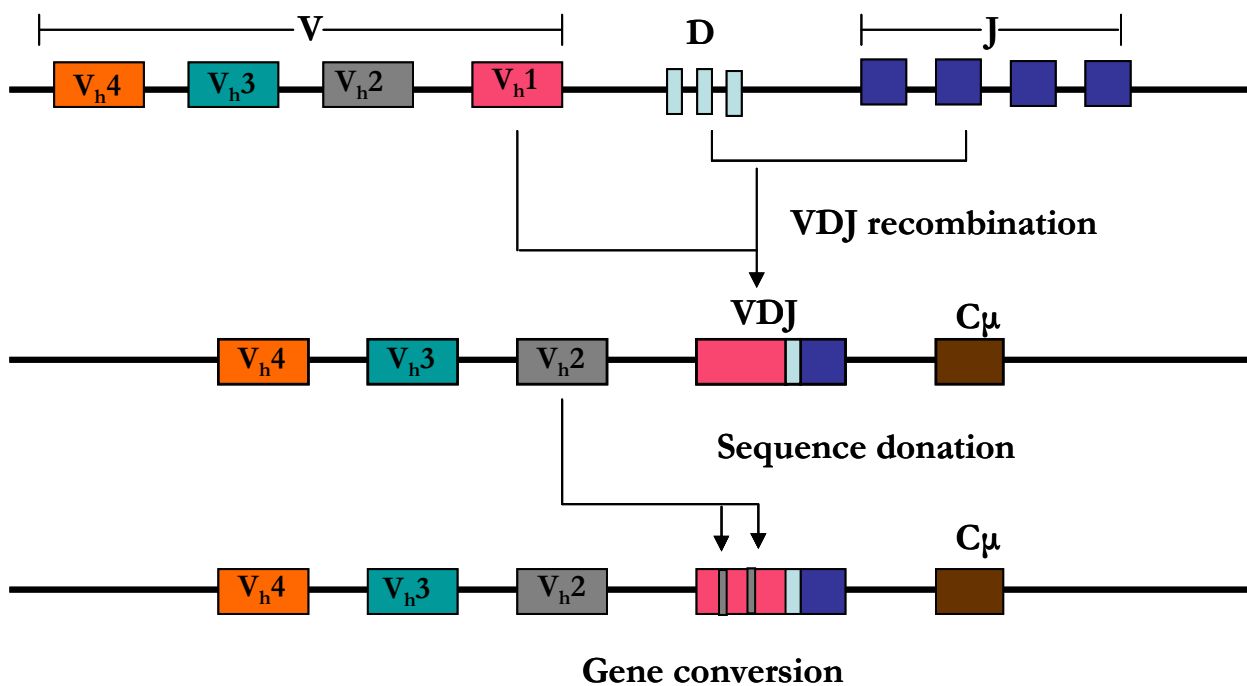
Figure 1.10   Schematic representation of gene conversion. Rabbits preferentially utilize just one of their multiple functional germ-line immunoglobulin genes (V_H1). Thus in rabbits only the V_H1 gene is used in the VDJ recombination which leads to the primary antibody repertoire. Further diversification of the rearranged V_H1 segment in the VDJ gene is by means of gene conversion. In this process, the upstream pseudogenes, (in this figure V_H2, V_H3 and V_H4) which share a high sequence homology to the rearranged V_H1 gene donate nucleotide stretches to the V_H1 gene. In figure 1.9 the donated sequences from V_H2 are shown as grey bands in the V_H1 portion of the rearranged VDJ gene.

In addition to irrevocable evidence of the employment of gene conversion in the diversification of the V gene in V (D) J rearrangements, mutations in the D, J and also in the region immediately 3' of the JH region have been observed. These sequence variations were attributed to somatic hypermutation because the D and J sequences lack potential sequence donors (Crane et al., 1996; Sehgal et al., 2000). The observation of diversification in these regions makes it likely that somatic hypermutation also occurs in the rearranged V_H gene segments, (Winstead et al., 1999; Lanning and Knight, 1997). It can thus be concluded that rabbits employ both gene conversion and hypermutation in the diversification of V(D)J genes.

1.4   Investigating somatic mutation

Intense research is ongoing to elucidate the mechanisms underlying somatic mutation and considerable progress has been made as some of the *cis-* and *trans-*elements regulating Ig rearrangement and somatic mutation have been identified. The *cis-*elements include transcriptional enhancers and promoters while the *trans-*elements include the proteins AID, XRCC2, XRCC6 and RAD51B (reviewed in Maizels, 2005; Jung et al., 2006).

To carry out such investigations, Ig transgenes are extensively used and it has been reported that some of these *cis-*elements function across species boundaries; a chicken light chain was successfully rearranged and expressed in transgenic mice (Bucchini et al., 1987; Lauster et al., 1993; Bulfone-Paus et al., 1995) and human Ig loci have been shown to rearrange and be expressed in transgenic mice (Taylor et al., 1992; Bruggemann et al., 1989; Bruggemann et al., 1991; Popov et al., 1999; Magadan et al., 2002; Jakobovits, 1995; Gallo et al., 2000). Somatic diversification of the transgenic chicken light chain locus was not investigated, but hypermutation of the human Ig loci in the transgenic mice was observed.

As mentioned above in section 1.3.6 somatic hypermutation and gene conversion have been attributed to specific species: mice and humans employ somatic hypermutation meanwhile chicken and rabbits employ gene diversification.

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Nonetheless reports of gene conversion in mice (a *bona fide* hypermutator) have been made (Xu and Selsing, 1994; Tsai et al., 2002), although irrefutable proof is still lacking. Postulations have been made stating that somatic hypermutation and gene conversion constitute alternative pathways of resolving an AID-induced lesion in the V gene of a rearranged V(D)J segment (Maizels, 2005; Neuberger, 2002), but the interactions between the *cis-* and *trans-* elements involved in somatic diversification need further elucidation. An immense contribution to the understanding of Ig diversification in rabbits was made by the construction of a rabbit BAC library, of which three partially overlapping BACs, (Ros et al., 2004) and a fourth BAC were identified and studied. These BACs (figure 1.11) carry sequence information spanning about 0.5MB of the rabbit immunoglobulin heavy chain locus. This sequence information contains coding information for 34 V_H, 11 D, DQ52, six J_H elements and also coding sequences for IgM (with the transcription enhancer, Eμ), IgG and four IgA genes.
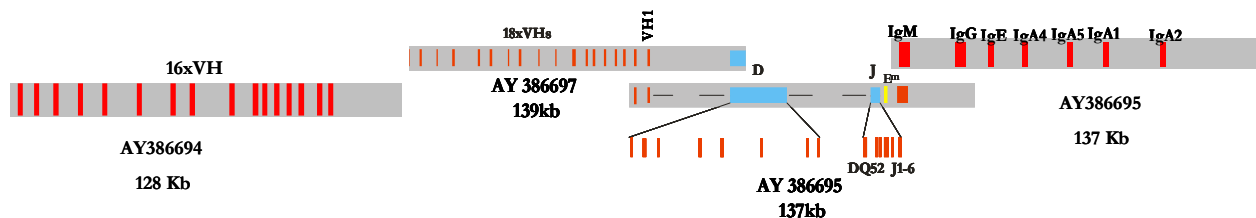
Figure 1.11 Depiction of four BACs (with the above mentioned three overlapping BACs), carrying large parts of the rabbit IgH BAC library. This BAC library was generated in the THP laboratories. The corresponding Genebank Accession numbers are shown with the bars in colour depicting coding sequences of the annotated genes.

As already mentioned, Ig transgenic constructs have been used to investigate the regulators of somatic mutation. To generate such an Ig transgenic construct, with a rabbit backbone, the BAC library was used as a template for a humanization process. This humanization process involved a novel approach whereby only the coding sequences of the Ig gene segments are exchanged for their human

counterparts. In so doing the *cis* elements regulating Ig rearrangements (RSS, promoters) and somatic mutation (transcription enhancers and promoters) are left endogenous. Using this novel approach the J_H and IgM loci of BAC AY386695 (figure 1.11) were substituted with their human counterparts, (Dr. Ros, THP GmbH, personal communication). The humanized BAC AY386695 was further stitched with BACs AY388696 and AY388697 to generate a chimeric IgH locus (Dr. Ros, THP GmbH, personal communication). This 310 Kb heavy chain BAC was termed HC2 (figure 1.11).
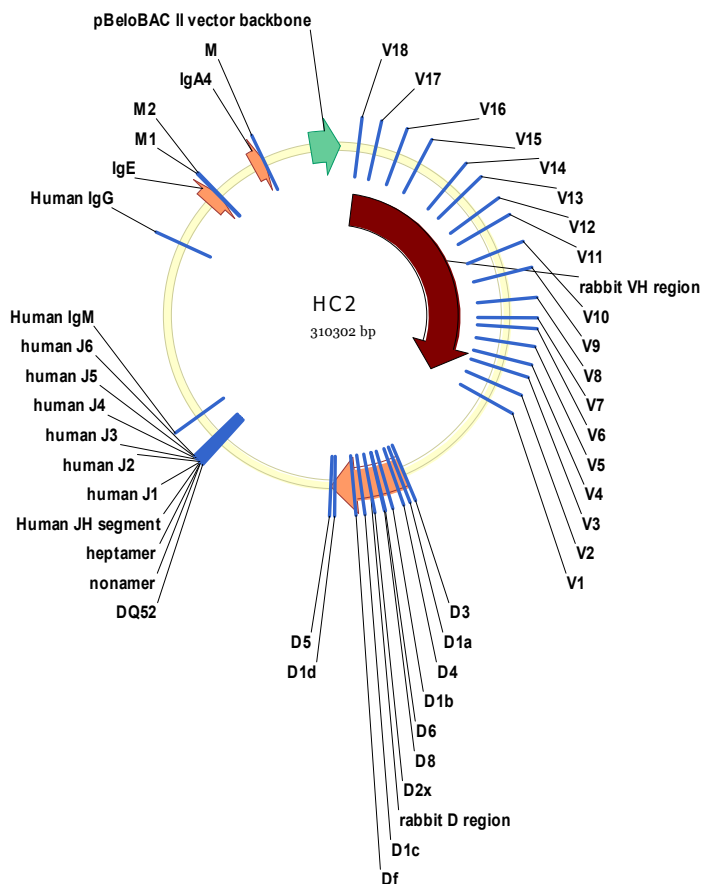
Figure 1.12   A chimeric heavy chain BAC composed of human IgG , IgM and JH (J_H1 to J_H6) portions and the rest of the Ig elements , including the IgA gene segments, 18 VH gene segments (numbered 1 through 18), the D locus (consisting of D3, D1a, D4, D1b, D6, D8, D2x, D1c, Df, D1d and D5) , DQ52 and IgE are rabbit endogenous, nomenclature as published, Ros et al).

Since all the Ig regulatory portions are rabbit endogenous, HC2 transgenic animals would offer an excellent opportunity to study the mechanism of the Ig *cis* elements and their interactions with Ig *trans* elements.

Additionally, more insight to the mechanisms underlying Ig rearrangements and somatic mutation can be obtained by the generation of transgenic animals using a fully human transgenic IgH locus. In a further context, production of functional fully human antibodies by transgenic animals could be of important therapeutic use. It has been reported that three constructs carrying overlapping genomic fragments were able to recombine in a homologous manner to reconstitute a core human IgH locus in transgenic mice (Wagner et al., 1996). In this respect, the generation of a humanized rabbit V_H locus (using BAC AY3886697 as a template) and co-injection with the humanized BAC AY386695 construct would result in the constitution of a core rabbit humanized IgH locus in transgenic animals: since co-injection of two linear constructs have been shown to integrate at the same site.

1.5   Goal of this work

In the present work, the generation of a humanized rabbit V_H locus, using BAC AY3886697 as template is described. This humanized VH locus was co-injected with the humanized AY386695 construct to generate transgenic mice. These transgenic mice analyzed to give an insight to following questions:

➢ Will the rabbit Ig regulatory elements function across species boundaries?

➢ More importantly which method of somatic diversification would result from the interaction between the rabbit Ig *cis* elements (a gene converter) and mouse Ig *trans* elements (a hypermutator)? Gene conversion or hypermutation?

Furthermore, to proffer a more efficient assessment of the different methods of somatic diversification, the HC2 chimeric construct was used to generate both transgenic mice and rabbits and the transgenic animals analysed with following question in mind:

Will the same IgH transgenic construct hypermutate in mice and gene convert in rabbit?

2. Material and Methods

2.1 Bacterial strains

TOP 10 cells (TOPO Kit, Invitrogen, Karlsruhe)	*F- mcrA ⊿(mrr-hsdRMS-mcrBC) Φ80lacZ⊿M15 ⊿lacX74 deoR recA1 endA1 ara⊿139 ⊿(ara,leu)7697 galU galK λ- rpsL (StrR) nupG*
EC 100 (Biozym Scientific ,Oldendorf)	*F⁻ mcrA ⊿(mrr-hsdRMS-mcrBC) φ80dlacZ⊿M15 ⊿lacX74 recA1 endA1 araD139 ⊿(ara, leu)7697 galU galK λ⁻ rpsL nupG*
DH10 B (Invitrogen, Karlsruhe)	*F- mcrA ⊿(mrr-hsdRMS-mcrBC) Φ80lacZ⊿M15 ⊿lacX74 deoR recA1 endA1 ara⊿139 ⊿(ara,leu)7697 galU galK λ- rpsL (StrR) nupG tonA*
XL2 Blue (Stratagene, Heidelberg)	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr) Amy Camr].‡*

2.2 Commercially acquired Vectors and plasmids

Vector/plasmid	Source
pBeloBAC II	New England Biolabs, Frankfurt
pGEM	Promega , Mannheim
PCR II	Invitrogen , Karlsruhe
pGPS1.1	New England Biolabs, Frankfurt am Main
pSC101αβγ-tetra	Gene Bridges, Dresden
pRep-Genta	Gene Bridges, Dresden

2.3 Oligonucleotides (synthesised at Metabion, Munich)

2.3.1 *PCR amplification of human V_H genes from genomic DNA*

VH Gene (gene bank Accession #)	Primer sequence	Primer designation
VH3-9 (M99651)	forward: 5'-atagagagattgagtgtg-3'	BS1
	reverse: 5'-tcctgtcttcctgcag-3'	BS2
VH3-11 (M99652)	forward: 5'-agagacattgagtggac-3'	BS3
	reverse: 5'-agggaggtttgtgtc-3'	BS4
VH3-13 (M99653)	forward: 5'-actagagagatattgagtgtg-3'	BS5
	reverse: 5'-aggcattctgcaggg-3'	BS5
VH3-20 (M99657)	forward: 5'-tcatggatcaatagagagatg-3'	BS6
	reverse: 5'-tgcagggacgtttgtg-3'	BS8
VH3-30 (M99663)	forward: 5'-agagagactgagtgtg-3'	BS9
	reverse: 5'-actagagagatattgagtgtg-3'	BS10
VH3-15 (M99654)	forward: 5'-actagagagattaagtgtg-3'	BS11
	reverse: 5'-tcacactgacctccc-3'	BS12
VH3-43 (M99672)	forward: 5'-tgagtgtgagtgaacatg-3'	BS13
	reverse: 5'-accagctcttaaccttc-3'	BS14
VH3-64 (M99658)	forward: 5'-tgagtgtgagtggac-3'	BS15
	reverse: 5'-tgacgctgatcagtg-3'	BS16
VH3-66 (X92218)	forward: 5'-tctgaccaatgtctcctg-3'	BS17
	reverse: 5'-aggtttgtgtctgggc-3'	BS18
VH3-D (Z18898)	forward: 5'-tgtgtggcagtttctg-3'	BS19
	reverse: 5'-actgactctgatcagtg-3'	BS20
VH3-73 (Z27508)	forward: 5'-agtggacgtgtgtgg-3'	BS21
	reverse: 5'-tcgtggtctcagatg-3'	BS22

2.3.2 *PCR amplification of human VH genes sub-cloned in TOPO vectors (incorporated restriction sites are shown in colour, yellow shading= AscI site, red shading = MluI site)*

VH gene	Primer sequence	designation
VH3-9 (M99651)	forward:5'- tt ggcgcgcc tgtcgtctgtgtttgcag gtgtcc-3'	BS23
	reverse: 5'-ttgc acgcgt gcagggaggtttgtgtctgggctcagcctgagggcccctcactgtgtc atcttttgcac-3'	BS24
VH3-11 (M99652)	forward:5'- tt ggcgcgcc tgtcgtctgtgtttgcag gtgtcc-3'	BS25
	reverse: 5'-ttgc acgcgt gcagggaggtttgtgtctgggctcagcctgagggcccctcactgtgtc tctcg-3'	BS26
VH3-13 (M99653)	forward:5'- tt ggcgcgcc tgtcgtctgtgtttgcag gtgtcc-3'	BS27
	reverse: 5'-TTGC ACGCGT gcagggaggtttgtgtctgggctcagcctgagggcccctcactgtgtc Tcttg-3'	BS28
VH3-20 (M99657)	Forward: 5'- TT GGCGCGCC tgtcgtctgtgtttgcaggtgtc-3'	BS29
	reverse: 5'-TTGC ACGCGT gcagggaggtttgtgtctgggctcagcctgagggcccctcactgtgtc TCTC-3'	BS30
VH3-30 (M99663)	forward: 5'- TT GGCGCGCC tgtcgtctgtgtttgcag GTGTCCAGTGTC-3'	BS31
	reverse: 5'-TTGC ACGCGT gcagggaggtttgtgtctgggctcagcctgagggcccctcactgtgtc TTTCG -3'	BS32
VH3-15 (M99654)	forward: 5'- TT GGCGCGCC tgtcgtctgtgtttgcag gtgtcc-3'	BS33
	reverse: 5'-TTGC ACGCGT gcagggaggtttgtgtctgggctcagcctgagggcccctcactgtgtcTgtgg-3'	BS34
VH3-43 (M99672)	forward: 5'- TT GGCGCGCC tgtcgtctgtgtttgcag gtgtcc-3'	BS35
	reverse: 5'-TTGC ACGCGT gcagggaggtttgtgtctgggctcagcctgagggcccctcactgtgtc Tcttttgcac-3'	BS36
VH3-64 (M99658)	forward: 5'- TT GGCGCGCC tgtcgtctgtgtttgcag gtgtcc-3'	BS37

	reverse: 5'-TTGC ACGCGT gcagggaggtttgtgtctgggctcagcctgagggcccctcactgtgtc tctcgcac-3'	**BS38**
VH3-66 **(X92218)**	forward: 5'- TT GGCGCGCC tctgaccaatgtctcctg-3'	**BS39**
	reverse: 5'- TTGC ACGCGT gcagggaggtttgtgtctgggctcagcctgagggcccctcactgtgtc aggtttgtgtctgggc-3'	**BS40**
VH3-D **(Z18898)**	forward: 5'- TT GGCGCGCC tgtgtggcagtttctg-3'	**BS41**
	reverse: 5'- TTGC ACGCGT gcagggaggtttgtgtctgggctcagcctgagggcccctcactgtgtc actgactctgatcagtg-3'	**BS42**
VH3-73 **(Z27508)**	forward: 5'- TT GGCGCGCC agtggacgtgtgtgg-3'	**BS43**
	reverse: 5'- TTGC ACGCGT gcagggaggtttgtgtctgggctcagcctgagggcccctcactgtgtc tcgtggtctcagatg-3'	**BS44**

2.3.3 PCR amplification of rabbit spacer sequences from BAC template (incorporated restriction sites are shown in colour, yellow shading= AscI site, red shading = MluI site)

Rabbit spacer	Primer sequence	Primer designation
Spacer 1-2	forward: 5´ tt ggcgcgcc aggggagtgcggctccac 3´	**BS45**
	reverse: 5´ ttgc acgcgt tggtcaggacactgtcac 3´	**BS46**
Spacer 2-3	forward: 5´ tt ggcgcgcc aggggcgcgcggctccac 3´	**BS47**
	reverse : 5´ ttgc acgcgt tgatcacgaaactgtcac 3´	**BS48**
Spacer 3-4	forward: 5´ tt ggcgcgcc aggggcgcgcggctccac 3´	**BS49**
	reverse : 5´ ttgc acgcgt tatctgttggtctcttcttc 3´	**BS50**
Spacer 4-5	forward: 5´ tt ggcgcgcc aggggagtgcggctccac 3´	**BS51**
	reverse : 5´ ttgc acgcgt tggtcaagacactgtcac 3	**BS52**
Spacer 5-6	forward: 5´ tt ggcgcgcc agggacgcacggctccac 3´	**BS53**
	reverse: 5´ ttgc acgcgt tggtcaggaagctgtcac 3´	**BS54**
Spacer 6-7	forward: 5´ tt ggcgcgcc agggatgcgcggctccag 3´	**BS55**
	reverse : 5´ ttgc acgcgt tggtcaggacactgtcac 3´	**BS56**
Spacer 7-8	forward: 5´ tt ggcgcgcc aggggagtgcggctccac 3´	**BS57**
	reverse: 5´ ttgc acgcgt tggtcaggacactgtcac 3´	**BS58**
Spacer 21-22	forward: 5´ tt ggcgcgcc ggggcccgcggctccac 3´	**BS59**
	reverse : 5´ ttgc acgcgt tggtcaggaagctgtcac 3´	**BS60**
Spacer 22-23	forward: 5´ tt ggcgcgcc agggacgtgaggctctac 3´	**BS61**
	reverse : 5´ ttgc acgcgt tggtcagggcactgtcac 3´	**BS62**

2.3.4 Oligonucleotides for the modification of the pGEM linker

```
            Fse I              Asc I              MluI
5' GGCCGC GGCCGGCC ATCGAT GGCGCGCC TTCGAA ACGCGT A 3'       BS63
3'      CG CCGGCCGG TACGAT CCGCGCGG AAGCTT TGCGCA TTCGA 5'  BS64
```

2.3.5 Oligonucleotides for the modification of the pBeloBAC II linker

```
            Fse I              Asc I              MluI
5' GATC GGCCGGCC ATCGAT GGCGCGCC TTCGAA ACGCGT TA GGGATAACAGGGTAATA3'   BS65
3'    G CCGGCCGG TACGAT CCGCGCGG AAGCTT TGCGCA AT TTCGA 5'               BS66
```

2.3.6 Oligonucloetides for the PCR amplification of the gentamycin selection marker *(incorporated restriction sites are shown in colour, yellow shading= AscI site, green shading = FseI site).*

| Forward | 5' CCA GGCCGGCC TGGAGTTGTAGATCCTCTACG 3' | BS67 |
| Reverse | 5' CCA GGCGCGCC AAGATGCGTGATCTGATCC 3' | BS68 |

2.3.7 Oligonucloetides for the PCR amplification of the kanamycin selection marker *(incorporated restriction sites are shown in colour, yellow shading= AscI site, green shading = FseI site).*

| Forward | 5' CCA GGCCGGCC ATTACACCAGTGTCAGTAAGCG 3' | BS69 |
| Reverse | 5' CCA GGCGCGCC TCAGAAGAACTCGTCAAGAAGGCG 3' | BS70 |

2.3.8 Oligonuclotides for the modification of BAC AY386695, via ET-cloning *(incorporated restriction sites are shown in colour, yellow shading= AscI site, green shading = FseI site).*

| up | 5' AAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGA GGCCGGCC TCAGAAGAACTCGTCAAGAAGGCG 3' | BS71 |
| Down | 5' ATCGTGCGTCTTGAGCGCCCCCTGGTAGAGCCGCGCGACCCTGGCGCGCC ATAACACCAGTGTCAGTAAGCG 3' | BS72 |

2.3.9 Oligonuclotides for genotyping

Priming region	sequence	designation
Human Cµ. forward1	5'-aagggcccatcggtcttc-3'	BS73
Human Cµ. reverse1	5'-acggtgggcatgtgtgag-3'	BS74
Human Cµ. forward2	5'-gccccactcttgcccctcttcctg-3'	BS73A
Human Cµ. reverse2	5'-ggtctcccccgtgttccattcctc-3'	BS74B
VH3-23. forward	5'-acatactacgcagactccgtg-3'	BS75
VH3-23. reverse	5'-tcactgtgtctttcgcacag-3'	BS76
Beta-globin. forward	5'-acccatggacccagaggttc-3'	BS77
Beta-globin. reverse	5'-tctcaggatccacgtgcagc-3'	BS78

2.3.10 PCR oligos for VDJ amplification

VDJ gene	Primer sequences	designation
HC2 rabbit .forward	5'-aacgcgggacactgagtc-3'	BS79
HC2 rabbit .reverse	5'-gttccctggccccagtagt-3'	BS80
HC2 mice.forward	5'-Tgtagatgacactacaggcaaagg-3'	BS81
HC2 mice.reverse	5'-ctacctggcctccctccca-3'	BS82
3HuVs.I5-6 mice.forward	5' -ttctcctggtcgctgtgctcaaag -3'	BS83
3HuVs.I5-6 mice. reverse	5'-tctcctcaggtaggaacggcctc-3'	BS84

2.5 Protein Reagents

2.5.1 Restriction enzymes

All restriction enzymes employed were purchased from New England Biolabs, Frankfurt otherwise stated.

2.5.2 DNA modifying Proteins

Calf Intestinal Phosphatase (CIP)	New England Biolabs, Frankfurt am Main
pfu ultra-DNA-polymerase	Stratagene, Heidelberg
T4-DNA-Ligase	New England Biolabs, Frankfurt am Main
Herculase Hotstart polymerase	Roche, Mannheim
Proteinase K	Invitrogen, Karlsruhe

2.5.3 Antibodies

Bitoin conjugated rabbit-anti-human IgM(609-4631)	BIOMOL , Hamburg
rabbit-anti-human IgM(609-4131)	BIOMOL , Hamburg
Fluorescein Isothiocyanate (FITC) conjugated rabbit -anti-KLH	BIOMOL , Hamburg
R-Phycoerythrin conjugated rabbit-antimouse	BD Biosciences Pharmingen, San Diego
CD45R/B220 (RA3-6B2)	
Lectin from Arachis hypogaea, lyophilised powder biotin conjugated (L6135-1MG)	BIOMOL , Hamburg

2.5.4 Other Protein reagents

Albumin bovine serum	Sigma, Taufkirchen
Human IgM purified Immunoglobulin (ELISA standard)	Sigma, Taufkirchen
Key hole lymphocyanin	Sigma, Taufkirchen
Freunds' Complete Adjuvant (FCA)	Sigma, Taufkirchen
Freunds' Incomplete Adjuvant	Sigma, Taufkirchen

2.6   *Other reagents*

Aceton	VWR International
Agarose NA	Amersham Pharmacia Biotech, Freiburg
Agarose, Ultra pure	Invitrogen, Karlsruhe
Ammonium sulphate	Carl Roth, Karlsruhe
Ammoniumcatate	Carl Roth, Karlsruhe
Bacto Agar	Becton Dickinson , Heidelberg
Bacto Peptone	Becton Dickinson , Heidelberg
BioTrap Starterkit	Schleicher & Schuell, Dassel
Carbenicillin	Carl Roth, Karlsruhe
Chloramphenicol	Sigma , Taufkirchen
Deoxynucleotide mix	Stratagene, Heidelberg
DL-Dithiothreitol (DTT)	Sigma , Taufkirchen
EDTA	Carl Roth, Karlsruhe
Ethanol	Carl Roth, Karlsruhe
Ethidium Bromide 1% w/v in water	Carl Roth, Karlsruhe
Glycerin	Carl Roth, Karlsruhe
Human genomic DNA	Becton Dickinson , Heidelberg
Isopropanol	Carl Roth, Karlsruhe
Kanamycin sulphate	Sigma, Taufkirchen
L (+) Arabinose	Sigma, Taufkirchen
Polyoethylenesorbitan (Tween 20)	Sigma, Taufkirchen
Sodium acetate	Carl Roth, Karlsruhe
Sodium chloride	Carl Roth, Karlsruhe
Sodium dihydrogen phosphate	Carl Roth, Karlsruhe
Sodium dodecyl sulphate (SDS)	Carl Roth, Karlsruhe
Sodium hydroxide	Carl Roth, Karlsruhe
Spermidine	Sigma , Taufkirchen
Gentamycin	Carl Roth, Karlsruhe
Tris	Carl Roth, Karlsruhe
TrisEDTA (TE buffer)	Sigma, Taufkirchen

2.7   Standards and kits

2.7.1   *DNA size standards*

100 bp DNA ladder	Fermentas , St. Leon-Rot
1 kb DNA ladder	Fermentas , St. Leon-Rot
Lambda DNA PFGE marker	Amersham Pharmacia Biotech, Freiburg

2.7.2   *Kits for DNA isolation and purification*

Fast plasmid minikit	Eppendorf, Hamburg, Hamburg
GENECLEAN turbo kit	Qbiogene, Mannheim
Macherey & Nagel Plasmig Kit Midi	Macherey & Nagel, Düren

2.8   *General material*

Agarose Gel Trap	Bio-Rad, Munich
Autoclave	H+P AG, Oberschleißheim
Centrifuge 5417R	Eppendorf, Hamburg
Centrifuge Avanti J-25	Beckman Coulter, USA
Electroporator, Gene Pulser II	Bio-Rad, Munich
ELISA reader	Bio-Rad, Munich
ELISA reader	Bio-Rad, Munich
Freezer, Minus 20	Liebherr, Ochsenhausen
FACScan Apparatus	Becton Dickinson , Heidelberg
Freezer, Minus 80	VWR International
Gel documentation equipment	Bio-Rad, Munich
Ice generator	VWR International
MaxiSorp microtiter plate	NUNC , wiesbaden
PH meter, MP 225	Mettller Toledo, Switzlerland
Pulse Field Electrophoresis Equipment (Chef Mapper)	Bio-Rad, Munich
Robocycler PCR maschines	Stratagene , Heidelberg
Safety hood	BDK GmbH, Sonnenbühl
Spectrophotometer, SmartSpec 3000	Bio-Rad, Munich
Thermomixer	Eppendorf, Hamburg
Vortex, MS2	IKA Works, Wilmington
Waterbath shaker, SW-22	Schubert & Weiss, Munich

2.9   Solutions and Media

2.9.1   *LB-Medium*

10g	Tryptone
5g	Yeast extract
5g	NaCl
To 1L	Ad H_2O

Autoclave

2.9.2   Agar plates

10g	Tryptone
5g	Yeast extract
5g	NaCl
15g	Select Agar
To 1L	Ad H_2O

Autoclave

2.9.3   ELISA   Reagents

Coating buffer	4,29g Na_2CO_3*$10H_2O$; 2,93g $NaHCO_3$ ad 1l H_2O (pH 9.6 with in HCL)
PBS 10x	80g NaCl;2,4g KH_2PO_4; 14,4g Na_2HPO_4 ad 1L H_2O (pH set to 7.4 using HCL or NaOH)
ELISA Buffer	0,05% (v/v) Tween 20 in PBS
Blocking Buffer	0,1% (w/v) BSA in PBS

2.9.4   Cutting Buffer (digestion of biopsie samples)

2,5 ml 1M Tris, pH set to 7.5 using HCL
5.0 ml 0,5M EDTA pH 8,0
1,0 ml 5M NaCl
250 µl 1 M DTT
127µl Spermidine (500mg/ml)
Ad to 50 ml steril water

2.9.5   Antibiotics solutions

Antibiotic	Stock solution	Dilution
Ampicillin	50mg/ml in water	1:500
Carbenicillin	50mg/ml in water	1:500
Chloramphenicol	25mg/ml in Ethanol	1:1000
Gentamycin	50mg/ml in water	1:10000
Kanamycin	50mg/ml in water	1:2500
Tetracycline	5mg/ml in Ethnol	1:1000

All working solutions were sterile filtered and stored at -20°C

2.9.6 *TAE Buffer*

Tris	40mM
Acetic acid	20mM
EDTA	1mM pH was usually measured to be 8.5

2.10 Molecular biology methods

2.10.1 *Transformation of E.Coli by electroporation*

Electroporation involves the application of high-density electric fields of short duration to reversibly permeabilize biomembranes. This is believed to create pores in the cell membrane through which the DNA molecules can easily pass from the medium into the cytoplasm.

Normally, 50 µl thawed electrocompetent cells (TransfroMAx EC100 cells, Epicentre, Madison, Wisconsin, USA, genotype *-F⁻ mcrA ⊿(mrr-hsdRMS-mcrBC) φ80dlacZ⊿M15 ⊿lacX74 recA1 endA1 araD139 ⊿(ara, leu)7697 galU galK λ⁻ rpsL nupG*)were mixed with the desired amounts of DNA on ice, then transferred to pre-chilled 1mm electroporation cuvettes. The cuvette was then placed into an electroporator (Gene Pulser II, Bio-Rad, Munich) and pulsed, standard settings used unless otherwise stated are; 25 µF , 2,5 KV, and 200 Ω. Thereafter, 1ml of LB medium at room temperature was added, the cells were shaken gently at 37°C, 175 rpm for one hour. Different amounts of the electroporated cells were then platted on LB plates, containing the appropriate antibiotics and finally the inverted plates were incubated overnight at 37°C.

2.10.2 *Chemical Transformation of E.Coli*

The E.c*oli* strain XL2 Blue was transformed with the use of $CaCl_2$/Heatshock-method (Cohen et al., 1972), briefly, the cells were thawed on ice 5 µl of DNA added and incubated for further 30 minutes on ice. After incubation the

cells/DNA mixture was pulsed for 30 seconds at 42°C placed briefly on ice and supplemented with 250 µl of LB medium. The bacteria were then incubated at 37°C and 175 rpm for one hour and plated on LB plates containing the appropriate selective antibiotics and finally the inverted plates were incubated overnight at 37°C.

2.10.3   *Cloning PCR Products*

PCR products were cloned using a rapid and selective method. This was achieved by means of commercially purchased kits. Invitrogen, The Netherlands, offers different kits for cloning varying PCR products. The TOPO Zero Blunt Kit was used to clone blunt end PCR products, obtained by amplification with proof reading poymerase meanwhile TOPO TA kit was used to create PCR products with A overhang, finally to clone large PCR products, >3kb, the TOPO XL kit was used.

2.10.4   *Restriction endonuclease digestion of DNA*

Restriction endonucleases were used to digest double-stranded DNA for analytical or preparative purposes. These restriction enzymes were purchased from New England Biolabs, unless otherwise stated, and used according to the manufacturers' instructions. A typical reaction consisted of a 20 µl volume containing about 1µg of DNA, 1x reaction buffer (from supplier), and if required 100 µg/ml BSA. The reaction was incubated at the recommended temperature (usually 37°C) for times from 30 minutes to overnight. Complete digestion was confirmed by agarose gel electrophoresis.

2.10.5   Ethanol precipitation of DNA

DNA was precipitated from aqueous solutions using salt and alcohol. A 1/10 volume of 3.3 M sodium acetate, pH 5.2 and a 2.5 volume of ice-cold absolute ethanol were added to the DNA containing aqueous solution. After overnight incubation at -20°C, the solution was centrifuged at 14000 rpm, (Eppendorf 5417R) 4°C for 30 minutes. The resulting pellet was washed with ice-cold 70% ethanol, dried and dissolved in water.

2.10.6   Dephosphorylation of linearized plasmid DNA by CIP

To prevent the re-ligation of linearized vector and to promote the insertion of the desired DNA fragment (insert), calf intestinal phosphatase (CIP) was used to dephosphorylate the 5' ends of the linearized vector. Dephosphorylation was carried out directly following plasmid linearization. 1Unit CIP (New England Biolabs) was added to the digestion mixture. After one hour incubation at 37°C, the mixture was loaded to agarose gel in which the CIP and restriction enzymes were inactivated. The dephosphorylated plasmid was gel-extracted and precipitated with ethanol.

2.10.7   Ligation of DNA fragments

DNA fragments bearing either sticky ends, meaning both vector and insert have compatible overhangs or blunt ends, meaning the ends of the vector and insert have no overhangs can be ligated *in vitro* with T4 DNA ligase.
Linearized, dephosphorylated vector was mixed with insert DNA, usually in vector: insert molar ratios of 1:5 or 1:10. Total DNA was at 200 ng in a total volume of 20 µl, containing 1x ligase buffer and 1U T4 DNA ligase. The ligation mixture was incubated at 16°C overnight and was subsequently used to transform bacteria.

2.10.8   *Mini-prep: small-scale preparation of plasmid DNA*

Following amplification of plasmid DNA in E.*coli*, DNA was extracted using a rapid method ('mini-prep') to screen for clones with the correct insert. Single colonies were picked from agar plates, and used to inoculate 3 ml of LB medium containing the suitable selective antibiotic and cultured overnight at 37°C, 225 rpm. For medium and high copy plasmids up to a size of about 7 kb, mini-prep DNA extraction was done using the Eppendorf, (Hamburg) "fast-plasmid kit". For larger plasmid and single copy BAC vector plasmids, the Qiagen (Qiagen, Hilden, Germany) "plasmid purification kit". In both cases, the extraction was performed following the manufacturers' instructions and reagents supplied in the kit. The extracted DNA was analyzed by restriction digestion.

2.10.9   *Midi-prep: large-scale preparation of plasmid DNA*

For preparations of large amounts of plasmid DNA, the Macherey & Nagel Nucleobond BAC 100 purification kit was used. About 20 μl of a bacterial culture from a clone known to contain the desired insert was used to inoculate 500 ml LB medium containing the appropriate antibiotic and grown overnight. In the case of medium and high copy plasmids, 100 ml of LB was used. Extraction was carried following the manufacturers instructions with the reagents supplied in the kit.

2.10.10   *Genomic DNA preparation*

Genomic DNA was obtained from mouse tail-tip or rabbit tear-tip biopsies. . Tissue samples were incubated overnight in a lysis buffer consisting of 375 μl cutting buffer, 20 μl 20%SDS and 5 μl Proteinase K. The debris was gotten rid off by centrifuging at 14000 rpm for 2 minutes in a table centrifuge (Ependorf 5417R). The supernatant was transferred in fresh tubes, the DNA was precipitated by

adding 400µl Isopropanol and again centrifuged for two minutes at 14000 rpm (Ependorf 5417R).The pellet was washed with 400 µl 70% Ethanol, centrifuged again for two minutes at 14000 rpm (Ependorf 5417R) and the pellet washed again with 400 µl 100% Ethanol, dried and re-dissolved in 200 µl 0.1 TE buffer. 2 µl were used for PCR reactions.

2.10.11 Quantification of DNA solutions

The concentration of DNA was determined by spectrophotometry. The ultraviolet (UV) absorption was measured at a wavelength of 260 nm (OD_{260}) using a quartz cuvette. For double-stranded DNA one OD_{260} corresponds to approximately 50 µg/ml DNA. In addition, the OD_{260} was measured to estimate the purity of the nucleic acid sample. A ratio A_{260}/A_{280} significantly less than 1.8-2.0 would indicate phenol or protein contamination.

2.10.12 Agarose gel electrophoresis

Conventional gel electrophoresis is commonly used to separate and analyze nuleic acids. Separation of DNA molecules from 100 bp to 20 kb was usually performed on agarose gels (Ultra pure Agarose, Invitrogen) in 1x TAE buffer; the agarose percentage was used according to the DNA sizes to be separated. Gel run was performed with variable time and volt conditions according to separation range and agarose percentage using an electrophoresis power supply (Bio-Rad, Munich). For size comparison, a DNA molecular ladder was loaded on the gel next to the samples. After gel run, DNA was stained with the intercalating fluorescent reagent ethidium bromide (EtBr), which was added either, to the gel before solidification or in TAE buffer for after-run staining at the concentration of 0,5 µg/ml. Stained DNA was visualized on a UV-transilluminator at a wavelength of 254 nm and photographed with a gel documentation apparatus (Bio-Rad, Munich).

2.10.13   Pulse field gel electrophoresis (PFGE)

Conventional gel electrophoresis has an upper limit to the sizes that can be resolved. Pulse field gel electrophoresis overcomes the problems of resolution of large DNA fragments by using two sets of electrodes that are fixed at angles to each other. The current is alternated between these sets of electrodes at defined intervals. Separation is based upon the potential of smaller DNA molecules to reorient faster than larger DNA molecules. PFGE was used for the resolution of high weight molecular DNA (20-200 kb), derived from the restriction digestion of BAC DNA. Pulse Field Agarose was used at a concentration of 1% in 0.5% TBE. Run was carried out in 0.5% TBE using the Chef-Mapper apparatus (Bio-Rad, Munich). λ mix DNA standard was used for size comparison. The temperature was maintained at 14°C, by means of a cooling system. The run time and settings were generated automatically according to input of separation range. This was done by software delivered with the apparatus. Gel was stained with EtBr after the run and visualized as described above.

2.10.14   Extraction of DNA fragments from agarose gels

After gel electrophoresis, the desired DNA bands were excised with a scalpel under irradiation with low-energy long wavelength UV light (320 nm) to minimize damaging the DNA. The DNA fragments were eluted from the gel pieces by means of the GENECLEAN kit (Qbiogene) according to the provided protocol.
In case of preparation of fragments to inject into fertilized oocytes for the generation of transgenic mice, as well as after PCR amplification of Human V_H genes and antibiotic selection cassettes, contact with EtBr and UV irradiation was absolutely avoided. After EtBr-free gel run, the gel was stained with 0.5%

Methyleneblue , de-stained by washing with water and the desired bands were the excised after visualization on a transilluminator.

In case of high molecular weight fragments, the DNA was extracted by means of electroelution, using the BioTrap apparatus according to the provided protocol. The DNA was thereafter precipitated with EtOH.

2.10.15   *Polymerase chain reaction (PCR)*

This method of DNA amplification had different applications, for example production of DNA fragments to sub-clone and genotyping of transgenic animals. According to the specific purpose, PCR amplifications were realized from various templates, genomic DNA, cloned DNA like plasmids and BACs. Furthermore different types of DNA polymerases (sources stated below) were chosen and the reaction settings were according to the manufacturers' instructions.

In general, otherwise stated, PCR reaction mixes were set up with 1x PCR buffer, 4mM of a deoxyribonucleotide triphosphate mixture (dNTP), 4 picomoles of each pimers, 1Unit of DNA polymerase and the amount of DNA template was specifically determined in each case.PCR reactions were performed in Strategene Robocylcler PCR machines.

2.10.16   *Sequencing*

Sequencing was performed by the company AGOWA GmbH in Berlin. For all fragments sub-cloned in the TOPO vectors, the sequencing primer was the T7 primer, present on the commercially acquired vector.

2.10.17 BAC modification via homologous recombination in E.coli

BAC modification using homologous recombination allows amendments of large DNA constructs like BACs, PACs and Cosmids, where the normal cloning techniques based on the use of restriction enzymes and ligation cannot be applied. Using this targeted homologous recombination in *E.coli* insertions, deletions and point mutations can be introduced in such large DNA constructs. Normally used *E.coli* strains are deficient in the recombination machinery; an exogenous recombination has to be imported. For this purpose several approaches have been established(Yang et al., 1997; Muyrers et al., 1999; Yu et al., 2000; Swaminathan et al., 2001). In this work the method proposed by Stewart and co-workers (Zhang et al., 1998) was used for BAC modification.

Briefly, the exogenous plasmid expressing the proteins which mediate homologous recombination is transformed into competent cells carrying the target BAC. In this case the plasmid used was the pSC101αβγ-tetra plasmid. The plasmid expresses the phage recombinase proteins Redα, Redβ and Redγ under the control of an arabinose inducible promoter, and a temperature sensitive origin; propagation is permissible at 30°C.

To make competent cells for the transformation, overnight cultures of the cells carrying the target BAC are generated by using glycerol stocks to inoculate 1.0 ml LB medium containing the selective antibiotics. This is carried out in a 2 ml Eppendorf tube with a punctured lid and shaken overnight at 37°C, 1050 rpm in a heating block (Eppendorf, Hamburg).

The next day 20 µl of the overnight culture is used to inoculate three 2 ml tubes, each with 1,4 ml LB medium and the selective antibiotic. The cultures are grown for two to three hours till OD_{600}=0.2-0.25, shaking at 37°C, 1050 rpm. One of the three inoculated tubes is used for the measurement. Once the desired OD is attained the two remaining tubes are spun for 30 seconds at 11,000 rpm in a pre-cooled (between -5°C and +2°C, Ependorf 5417R) centrifuge. The supernatant is quickly discarded and the pellet washed by re-suspending in 1 ml ice-cold 10%

glycerol, the wash step is repeated twice. After the final wash step the supernatant is discarded leaving 20 to 30 µl cell suspension in the tube. Keeping the tube on ice 1µl (0.2 to 0.5 µg) of the pSC101αβγ-tetra plasmid is added to the one tube of the freshly made competent cells and as a negative control 1µl water is added to the second tube. The mixture is then transferred to an ice-cold 1mm electroporation cuvette and electroporation carried out at 1350 V, 10 µF, 600 Ω using the Bio-Rad Gene Pulser II. After electroporation the cells were re-suspended in 1ml sterile LB medium and incubated at 30°C, 1050rpm for 70 minutes. Varying amounts from 10µl-100µl are platted on LB plates with the selective antibiotics. The pSC101αβγ-tetra plasmid has a tetracycline selection cassette. The day after, about six colonies from the plates were used to inoculate 3ml LB medium with the selective antibiotics and grown at 30°C, the selective temperature for the propagation of the pSC101αβγ-tetra plasmid. After DNA isolation as described above, the presence of the transformed plasmid was confirmed by restriction digestion.

About 20 µl bacterial cultures of positive clones were used to inoculate 3 ml LB medium again with the selective antibiotics, this time the cultures were incubated at 37°C. After DNA isolation the intactness of the target BAC was confirmed by restriction digestion. Now that the homologous recombination mediating plasmid has been successfully transformed into cells carrying the target BAC, the next step is to create a targeting construct carrying the desired modification to be introduced in the BAC. This targeting construct is created by PCR amplifying a selection cassette, with oligonucleotides carrying a 50 bp homology to the target BAC and preceding these 50 bp homologies are the desired mutations to be introduced (figure 2.1). The PCR product is gel analysed under EtBr free conditions; the gel is stained with Methylenblue as described above and purified using the GENECLEAN kit (QBIOGENE).

Next the bacterial cells carrying both the target BAC and the pSC101αβγ-tetra plasmid are made competent for the transformation of the targeting construct.

Six Eppendorf (Hamburg) tubes are set each with 1.4ml LB medium containing the selective antibiotic –in this case neo-, and inoculated with 15-20µl of fresh

overnight culture of the cells carrying the target BAC and the pSC101αβγ-tetra plasmid. The cells are propagated at 30°C, 1050 rpm till OD_{600}= 0.15-0.18 is reached. One or two of the six tubes are used for OD measurements. As soon as the optimal OD_{600} is attained 20 % L (+) Arabinose is added to a final concentration of 0.1-0.2% thereby inducing expression of the recombinase proteins, one tube is left un-induced as a negative control.
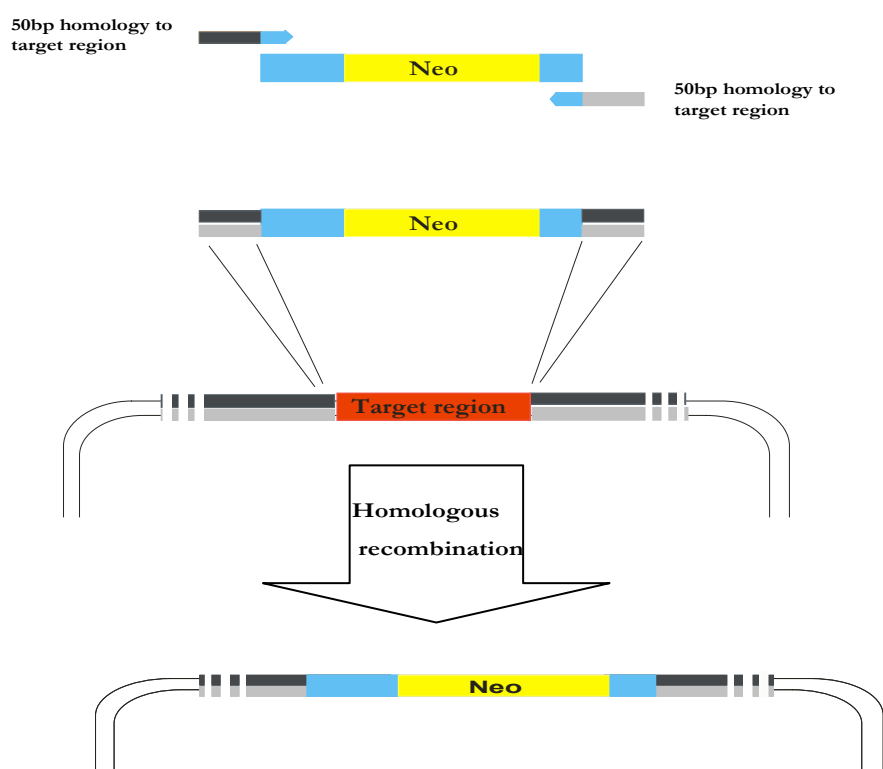
A selection cassette is PCR amplified with primers carrying desired sequences as well as 50 bp homolgies (shown here in grey and black) to the target region

The purified PCR product is transformed into freshly made competent bacteria carrying, the target BAC as well as the pSC101αβγ-tetra plasmid, which mediates homologous recombination.

The pSC101αβγ-tetra plasmid is induced using L-Arabinose to produce the proteins which regulate and direct homologous recombination between the 50 bp arms of the PCR product and tht corresponding region in the target BAC. Transformants are selected because of the presence of the neo cassette and the intactness of the insert is confirmed by colony analysis

Figure 2.1 A schematic view of BAC modification by homologous recombination as described by (Zhang et al., 1998).

The tubes are then incubated at 37°C, 1050 rpm for 50 minutes, until an OD_{600}=0.35-0.4 is attained. Three tubes two induced and the un-induced control are then spun down at 11000 rpm, for 30 seconds in a pre-cooled Eppendorf (Hamburg) centrifuge, and the pellet re-suspended in 1ml ice-cold 10% glycerol. The wash step is repeated twice and after the third time the supernatant is discarded leaving 20 to 30 µl cell suspension is left in the tubes. Still keeping the tubes on ice, 1-2 µl about 0.3-0.5µg of the targeting construct is added to one of the induced tubes and to the non-induced negative control.

The same volume of sterile water is added to the second induced tube as a second negative control. After mixing briefly by pipetting very gently up and down, the mixture is transferred to an ice-old 1mm electroporation cuvette and electroporated at 1350 V, 10 µF, and 600 Ωs. The cells are then re-suspended in 1ml LB medium and incubated for 70 minutes at 37°C shaking at 1050rpm. 3x 330 µl of the sample probe and 1x 330 µl of the negative controls are plated on LB plates with selective antibiotics and incubated overnight at 37°C. Colony analysis is then carried out and positives confirmed by restriction digestion and sequencing.

2.11 Transgenic constructs

The first step in the generation of the transgenic constructs was the customisation of two commercially acquired plasmid vectors, (pGEM and pbeloBAC II) to accommodate the employed cloning strategies.

2.11.1 Modifying the pGEM vector

The initial step involved the modification of the linker region to provide desired restriction sites: *Fse*I, *Asc*I and *Mlu*I. To modify the pGEM linker, the synthesized lyophilized oligonucleotides, BS63 and BS64, were dissolved in distilled H_2O to a final concentration of 10 ng/µl. Annealing reactions were set up using varying concentrations of the two oligonucleotides. Three different set ups with each 2.6 ng, 7 ng and 13 ng of each oligonucleotide in a total volume of 20 µl were supplemented with 2 µl 10x Roche T4-ligase buffer. The reactions were heated in a water bath at 97°C for 2 minutes and cooled to room temperature. The pGEM vector was opened by double restriction digestion with *Not*I and *Hind*III, and the annealed linker was then ligated to the opened vector (figure 2.2a).

Varying ratios of opened vector: annealed linker from 1:2, 1:5 and 1:10 were set up in a total volume of 10 µl, supplemented with 1 µl 10X T4-ligase Buffer (Roche, Mannheim) and 1µl T4-Ligase (Roche) and incubated overnight at 16°C. A

negative control reaction with no vector DNA was also set up. The following day, XL2-Blue cells were chemically transformed with 5 µl of the ligation reactions, and the insertion of the desired sites was confirmed by restriction digestion.
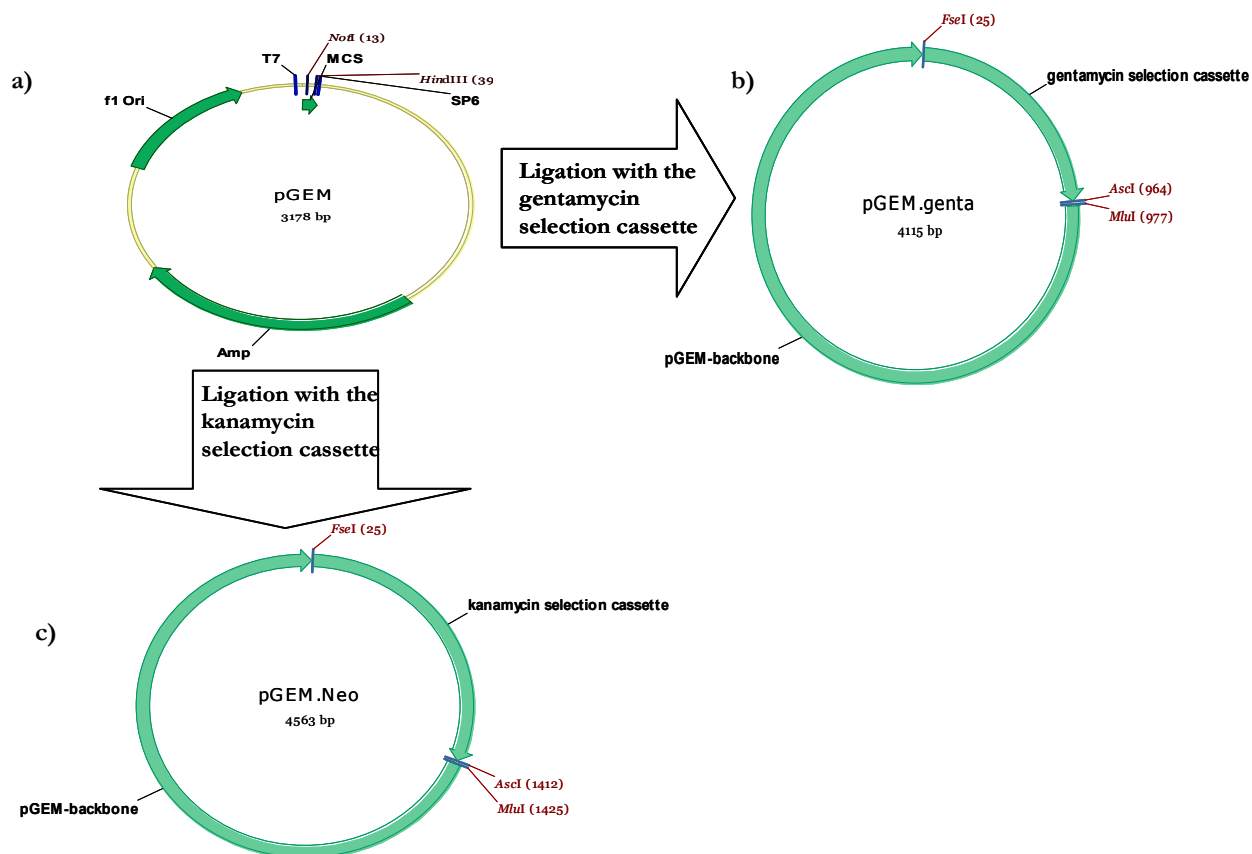
Figure 2.2   Generation of customized pGEM.genta and pGEM.neo vectors. After the linker modification of a commercially acquired pGEM vector (inserting the sites FseI-AscI-MluI, in that order) two different selection cassettes, gentamycin (figure 2.2 b) and kanamycin (figure 2.2c) were ligated into the *Fse*I and *Asc*I sites.

After this confirmation, using the pRep-Genta (Gene Bridges, Dresden) plasmid as Template, the gentamycin selection cassette was amplified by primers BS67 and BS68. The PCR product was then double digested with *Fse*I and *Asc*I (intergrated in the primers) and the digested gel-purified product was ligated with the modified pGEM vector also opened with *Fse*I and *Asc*I. The resulting vector was tagged pGEM.Genta (figure 2.2b).

A second pGEM vector tagged pGEM.neo (figure 2.2c), was also generated following the same scheme but or this vector a kanamycin selection cassette was PCR amplified with oligos BS69 and BS70, double digested with *Fse*I and *Asc*I sites and ligated with the modified pGEM opened with the same restriction enzymes.

2.11.2   *Modifying the pBeloBAC II (pBBII) vector*

A commercially acquired pBBII vector was modified and used as the backbone for building the final transgenic construct. The pBBII vector is the "state of the art" vector used in building large constructs; inserts of up to 500 kb can be readily accommodated by the vector (Woo et al., 1994). This BAC vector system is based on the *E.coli* F factor, and in bacteria such plasmids carrying the F factor are maintained in low copy number (Shizuya et al., 1992). This low copy maintenance in bacteria effectively reduces the potential for recombination between DNA fragments (inserts) carried by the plasmid.
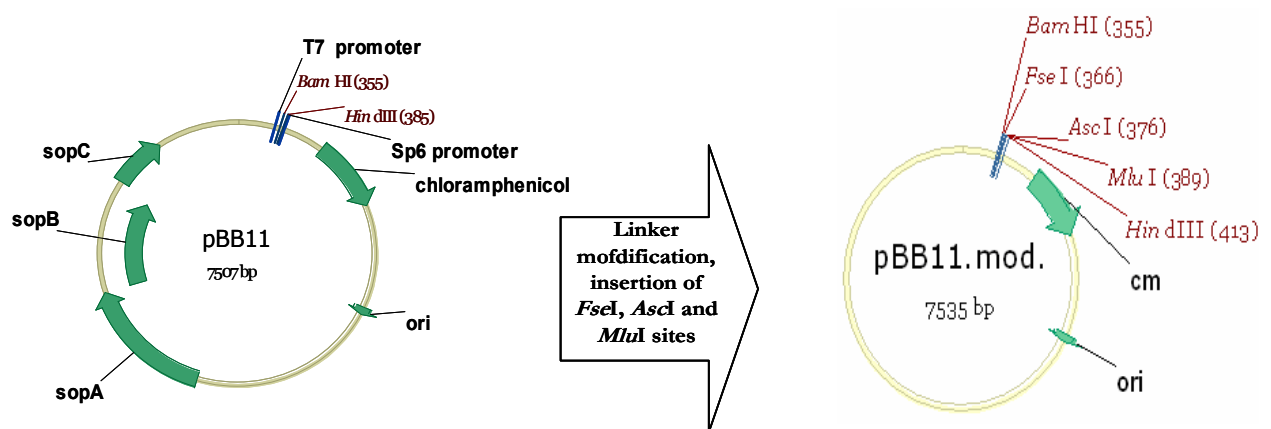
Figure 2.3   The pBBII vector was opened with *Bam*HI and *Hind*III and the linker modified using oligos BS65 and BS66 to carry restriction sites for *Fse*I, *Asc*I and *Mlu*I. The reactions set up were same as described for the pGEM vector in section 2.11.1. The insertion of these sites was confirmed by restriction digestion (data not shown).

This property is of paramount importance for the humanization procedure of the rabbit VH locus, because this locus is composed of highly homologous segments (Ros et al., 2004). Other advantages offered by the pBBII vector includes: easy transfection of such large plasmids into *E.coli* by electroporation and also the genetic stability of the cloned DNA (Shizuya et al., 1992). The commercially acquired pBBII vector was double digested with *Bam*HI and *Hind*III and the linker modified as described in section 2.4 using oligonucleotides BS65 and BS66. The modified linker had additional restriction sites for *Fse*I, *Asc*I and *Mlu*I. Theis modified pBBII vector was named pBBII.mod (figure 2.3).

2.11.3 Generation of rabbit V_H spacer and human V_H plasmid libraries

As mentioned in section 1.4 the humanization process in this work employs a novel approach whereby only the coding sequences of the rabbit V_H locus are exchanged for their human counterparts. In this process the regulatory elements are left rabbit endogenous. BAC AY3886697 was used as the template for this humanization process. The rabbit IgH locus fulfils two prerequisites vital for gene conversion:

- A functional V_H C most proximal gene (since rabbits preferentially use this gene in Ig rearrangements, section 1.3.6)

- The upstream genes share great sequence homology to this V_H C most proximal gene, enabling them to serve as sequence donors during gene conversion.

In the humanization process these properties had to be maintained to generate a functional transgenic rabbit V_H mini-locus. Consequently the human VH genes used for the humanization process all belonged to the same family, the VH3 family of genes (Brezinschek et al., 1995), thus ensuring sequence homology and the V_H C most proximal gene was the human VH3-23 gene which is the most abundantly used human V_H gene (Brezinschek et al., 1995).

The starting point of the humanization process was the creation of two small plasmid libraries:

- One composed of rabbit V_H spacer sequences carrying the regulatory elements

- And the second composed of the coding sequences of the human VH3 gene family.

To generate the rabbit spacer library, BAC AY386697 was digested with *Bstb*I (data not shown) and the digested DNA separated using PFGE, and the DNA extracted by electro-elution. This was to generate small templates to be used in PCR amplification of the rabbit V_H spacer sequences. The desired amplicon is shown in figure 2.4
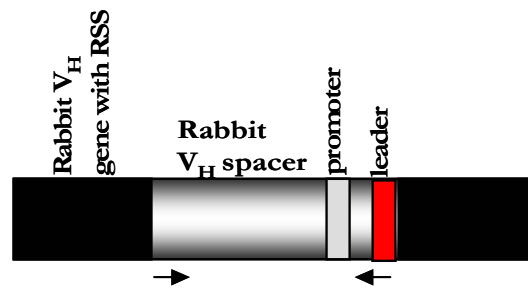
Figure 2.4   The rabbit spacer sequences were PCR amplified with primers BS45-BS62, shown here as arrows. The forward primer carries an *Asc*I site while the revers primer carries a *Mlu*I site. The obtained amplicon is a rabbit spacer sequence, with the promoter and leader sequences flanked by *Asc*I and *Mlu*I restriction sites

Following this scheme, spacer sequences I1-2, I2-3, I3-4, I4-5 and I5-6 were successfully amplified (nomenclature by Ros et al., 2004). Subsequently it was attempted to sub-clone the different PCR products in TOPO vectors, but only spacer I5-6 could successfully be sub-cloned. It was thereafter excised by digestion with *Asc*I and *Mlu*I and sub-cloned into the customized pGEM.genta vector.

VH gene	Genebank number	VH gene	Genebank number
VH3-23	**M99660**	VH3-48	M99675
VH3-66	**X92218**	VH3-21	M99658
VH3-11	**M99652**	VH3-64	M99682
VH3-30	**M99663**	VH3-D	Z18898
VH3-9	**M99651**	VH3-74	Z17392
VH3-13	**M99653**	VH3-33	M99665
VH3-72	**Z27508**	VH3-7	M99649
VH3-73	**Z27508**	VH3-43	M99672
VH3-15	**M99654**	VH3-49	M99676

Table 2.1   the 18 members of the human VH3 gene family. The members in bold could be successfully amplified and sub-cloned in the TOPO vectors.

To generate the human VH gene plasmid library, human genomic DNA was used as template in PCR reactions with primers BS1-BS22. The PCR products were then sub-cloned in TOPO vectors and the inserts sequenced. Table 2.1 shows the human VH3 gene family members.

To furnish the humanized V_H locus with the functionality of a rabbit endogenous locus capable of undergoing Ig rearrangements, it was of paramount importance to supplement these sub-cloned human V_H coding sequences with rabbit regulatory portions consisting of the splicing signal and the recombination signal sequence. To achieve this goal, the sub-cloned human V_H genes were again PCR amplified, with

> **Up: 5´- AscI-(rabbit-splice site)-(huV-specific)-3´**
>
> **Down: 5´-MluI-(rabbit-RSS)-(huV-specific)-3´**

Figure 2.5   Scheme for   the primers used to supplement the human VH genes with rabbit regulatory sequences. **Up** = forward PCR primer, **down** = reverse PCR primer, **huV** = human V_H gene segment.

the primers (figure 2.5) carrying the desired rabbit regulatory sequences followed by restriction sites. The human VH genes now with the rabbit regulatory sequences were again sub-cloned in TOPO vectors and the inserts sequenced. Finally, the different inserts were excised with *Asc*I and *Mlu*I and sub-cloned in different pGEM.neo vectors, thus generating a plasmid library of human V_H genes.

2.11.4   Generation of a chimeric (rabbit V_H spacer I5-6 and human V_H) plasmid library

After creating the plasmid libraries in section 2.11.3, the next step involved linking the rabbit V_H spacers to the human V_H genes resulting in a humanized rabbit VH mini locus.
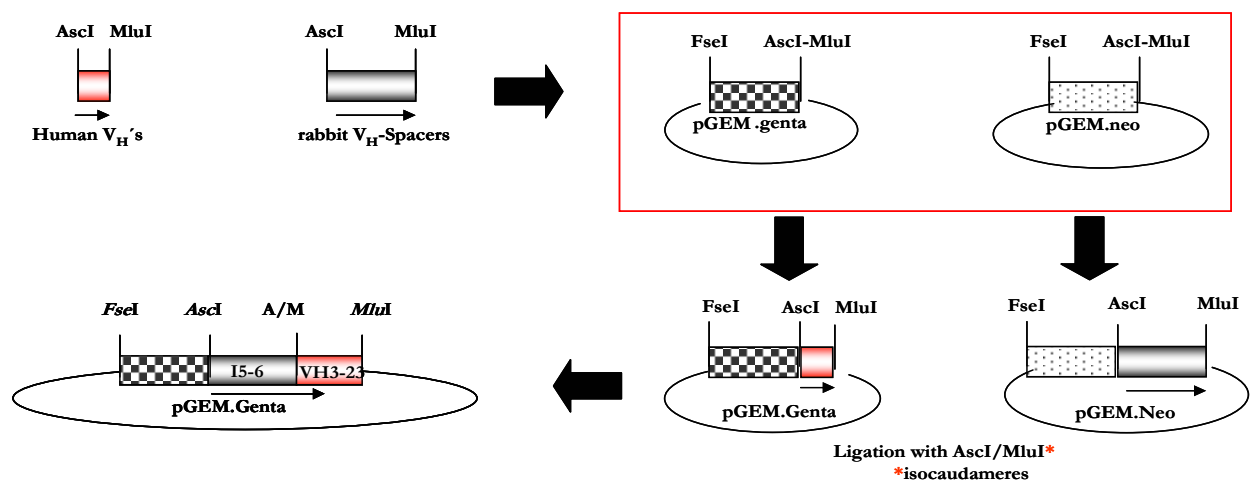
Figure 2.6   Generation of the chimeric plasmid library. As described in section 2.11.4, two separate libraries were first generated, one containing ten members of the human VH3 (huV) gene family, sub-cloned in the modified pGEM.neo vector the other library with just the rabbit V_H spacer I5-6 in the modified pGEM.genta vector. To generate the chimeric plasmid library with units of I5-6 followed by a huV gene segment, the usage of the two different selection cassettes (genta and neo) as well as the isocaudemeric property of the restriction enzymes *Asc*I and MluI were exploited, see text below for further explanation. A/M = the resulting sequence after ligation between *Asc*I and *Mlu*I compatible ends.

To achieve this goal, another plasmid library was generated, with units composed of a human V_H gene linked and rabbit spacer I5-6. A scheme is shown in figure 2.6. To link the rabbit I5-6 spacer to huV gene segments, two factors were exploited:

- The fact that the plasmid backbones of the two libraries (section 2.11.3) carried different selection cassettes
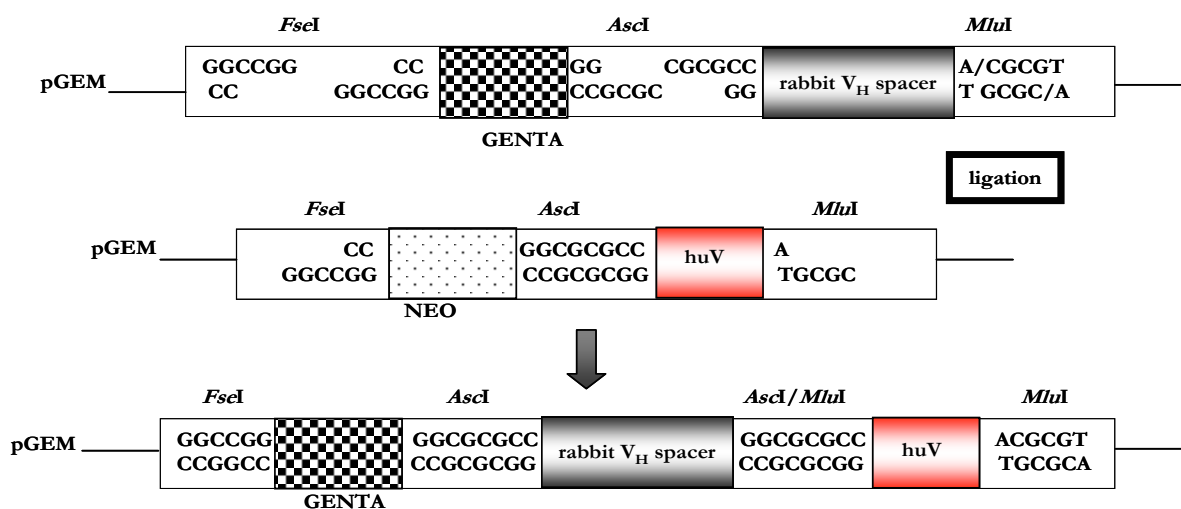
Figure 2.7   The ligation strategy (showing the isocaudemeric property of *Asc*I and *Mlu*I) employed in generating the chimeric plasmid units in pGEM backbone. After restriction digestion with *Asc*I and *Mlu*I compatible protruding termini ends are generated. Upon ligation a new sequence is created neither cleavable by *Asc*I nor by *Mlu*I.

- The Isocaudemeric property of the restriction enzymes *Asc*I and *Mlu*I (figure 2.7)

To generate the first chimeric unit, the pGEM.neo carrying the human VH3-23 gene, (pGN.VH3-23), was opened with *Fse*I and *Asc*I, thereby excising the kanamycin selection cassette. Simultaneously, the pGEM.genta vector carrying the rabbit spacer I5-6 (pGG.I5-6) was also double digested with *Fse*I and *Mlu*I. The segment composed of the gentamycin cassette, followed by I5-6, was gel purified and ligated to the opened pGN.VH3-23 vector, exploiting the Isocaudemeric property of *Asc*I and *Mlu*I. After ligation, positive clones were selected on gentamycin plates so only clones with the gentamycin cassette hence the complete insert were able to grow. Colonies were further screened by restriction digestion. Three of such chimeric units were constructed: pGG.I5-6.Vh3-23, PGN.I5-6.VH3-64 and pGG.I5-6.VH3-73.

After generating the last plasmid library with the chimeric units, the final step involved re-building the humanized mini VH locus in the pBBII.mod vector.

2.11.5   Re-building the humanized VH locus in the pBBII.mod vector

As mentioned in section 1.4, the co-injection of overlapping fragments leads to homologous recombination and ultimately the reconstitution of a core locus. Following this principle, the construction of the humanized V_H locus with an overlapping region to the humanized BAC AY386695, would lead to the reconstitution of a core humanize rabbit IgH locus upon co-injection of the two fragments.

To create such an overlapping region, the pBBII.mod vector was first complemented with a 17kb region homologous to construct AY386695 (figure 2.8).
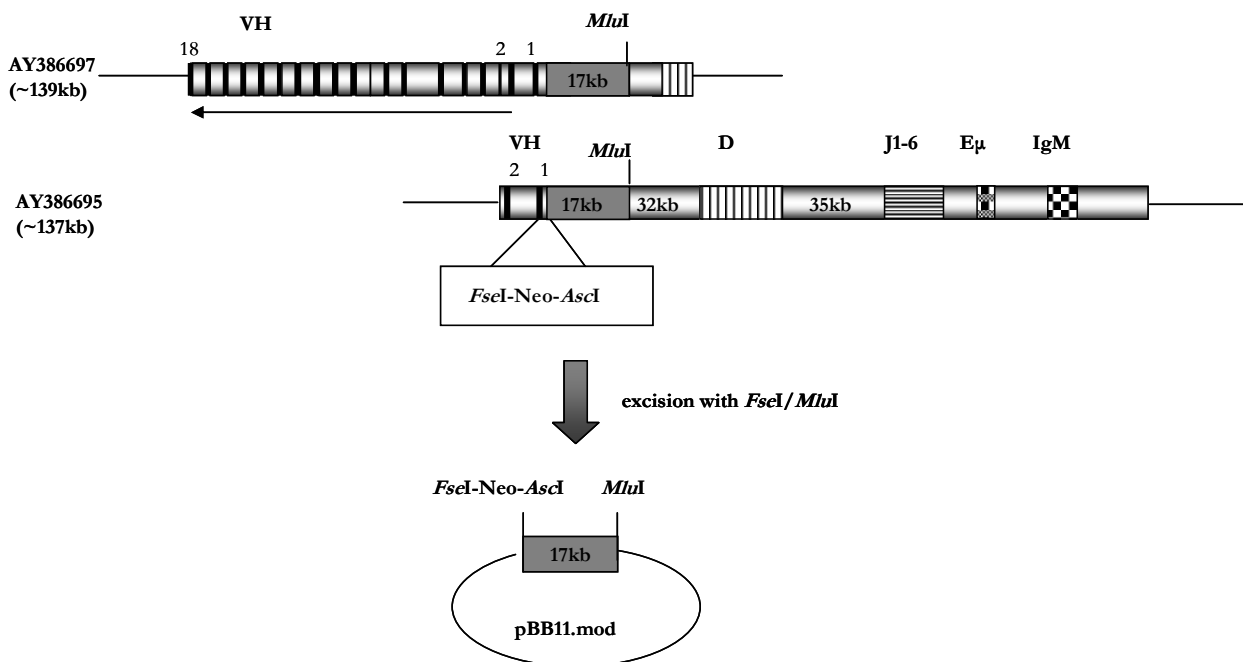
Figure 2.8   Sub-cloning of a 17kb homologous region in the pBBII.mod vector. BACs AY386697 and AY386695 share a large homologous region, a 17kb region, shown here in grey, was sub-cloned in the pBBII.mod vector. After modification of BAC AY386695, a double digestion with *Fse*I and *Mlu*I ensued and the resulting 17kb flanked by *Fse*I and *Mlu*I restriction sites. pBBII.mod was opened with *Fse*I and *Asc*I and the 17kb fragment sub-cloned. The pBBII.mod vector was now named pBBII.mod.17kb

48

The first step involved the modification of the BAC AY386695 via ET cloning (section 2.10.17). This modification involved the insertion of a kanamycin cassette flanked by *Fse*I and *Asc*I restriction sites. This fragment was inserted immediately after the V_H1 gene segment (figure 2.8). Positive clones were selected on kanamycin, and colonies were analyzed by restriction digestion .After this insertion, a double digestion of the modified AY386695 with *Fse*I and *Mlu*I (figure 2.8) was performed. The 17 kb fragment flanked by *Fse*I and *Mlu*I sites was excised, the DNA purified by electro-elution and finally ligated with pBBII.mod opened with *Fse*I and *Asc*I, another ligation exploiting the isocaudemeric properties of *Asc*I and *Mlu*I. Clones were selected on kanamycin and chloramphenicol and colonies analyzed by restriction digestion with *Apa*I (data not shown), the vector was now tagged pBBII.mod.17kb. After optimizing pBBII.mod for is role as the acceptor BAC of the humanized VH locus, the next steps involved re-building the three chimeric units in this pBBII.mod vector.
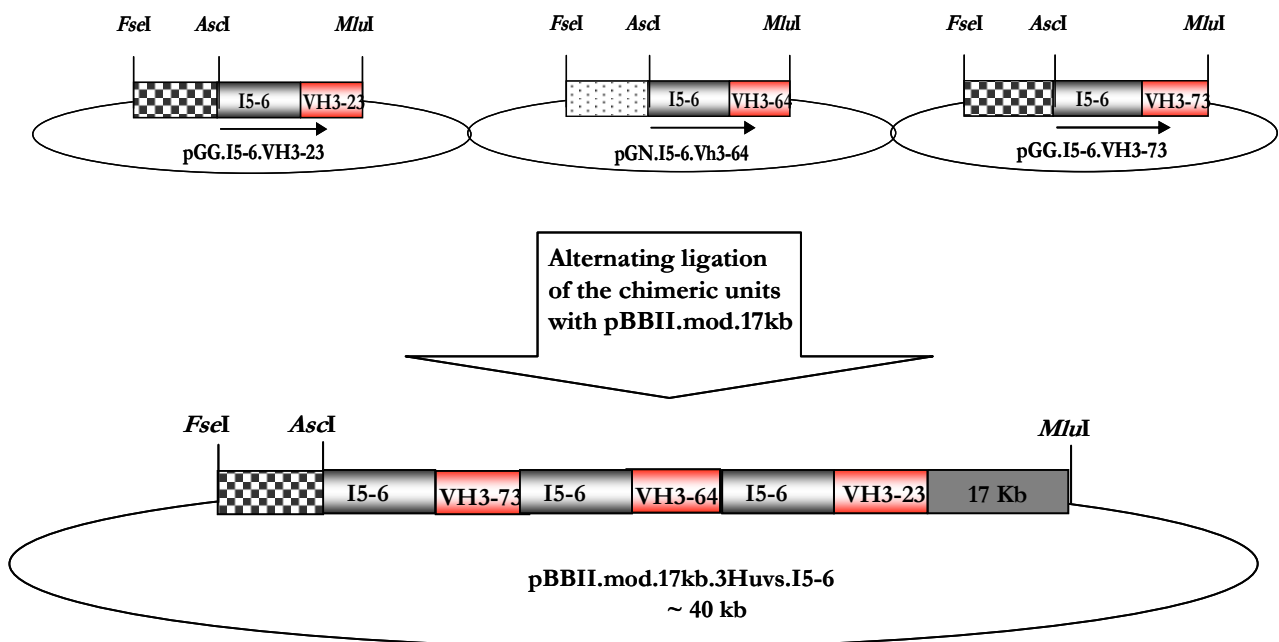
Figure 2.9   The three units of the chimeric plasmid library are integrated in the pBBII.mod vector equipped with the 17kb overlap to the humanized AY386695. To perform this task, ligation cycles were performed, with alternating selection of positive clones on gentamycin or kanamycin. The ligation steps exploit the Isocaudemeric compatibility of *Asc*I and *Mlu*I. As mentioned above the first V_H gene was the human VH3-23 gene. The final construct (about 40 Kb) was a humanized rabbit VH mini-locus capable of

The pBB11.mod BAC vector was opened with *Fse*I and *Asc*I, thereby excising the neomycin selection cassette. The first chimeric unit, pGG.I5-6.VH3-23 (figure 2.9) was also digested with *Fse*I and *Mlu*I excising the piece composed of the gentamycin selection cassette, spacer I5-6 and VH3-23. This insert was then ligated with the opened pBB11.mod BAC vector and colonies selected on gentamycin.

The selection on gentamycin ensures that only clones with the desired insert can survive. This construct was subsequently prepared for the ligation of the second chimeric unit pGN.I5-6.VH3-64 by excising the neomycin cassette with *Fse*I and *Asc*I. Again, the second unit was also digested with *Fse*I and *Mlu*I but this time the insert carried a neomycin selection cassette. After ligation the colonies were selected on neomycin, again only clones with the desired insert can survive the selection. This ligation cycle was repeated with the last chimeric unit pGG.I5-6.VH3-73 and the final construct is a humanized V_H locus (figure 2.9), with human V_H elements VH3-23, Vh3-64 and VH3-73 interspersed by the rabbit spacer I5-6 and other rabbit regulatory sequences required for successful rearrangement of the V_H locus *in vivo*. An in addition it also carries the 17kb overlap to the humanized construct AY 388695, thus enabling homologous recombination after co-injection of both BAC constructs.

2.12 Generation of transgenic animals

The dominating technique leading to random integration of a transgenic construct in the mice genome involves the micro-injection of DNA into the pro-nucleus of a developing zygote. Transgenesis was performed by pro-nuclear injection as described by Gordon *et al* (Gordon et al., 1980). After fertilization of a mouse egg, the male and female pro-nuclei stay separated for a few hours before they fuse to create the zygotic nucleus allowing a 3-4 hour window ideal for DNA micro-injection (Auerbach, 2004). During this time span, these independent pro-nuclei can be visualized under a microscope and DNA can be injected into the larger male pro-nucleus. The nuclear membranes then break down and the pro-nuclei fuse to

give the zygotic nucleus. The eggs which survived the injections are then transferred to the oviducts of pseudopregnant mice (Auerbach, 2004). In this work micro-injection experiments were performed by Dr. Imgard Thorey in the facilities of the Munich Gene Centre under supervision of Dr.Vet. Ingrid Renner-Mueller. Generation of transgenic rabbits was out-sourced to the company BioProtein Technologies, Paris. The pubs were then bred in the facilities of THP GmbH.

2.13 Animal breeding

FVB/N strain mice were bred in the facilities of the Gene Centre Munich under the supervision of Dr. Ingrid Renner-Müller. A2 allotype rabbits (Mage et al., 1984; Esteves et al., 2004) were bred in the THP facilities after pro-nuclei injections were carried out at BioProtein Technologies, Paris.

2.14 Genotyping transgenic animals

Transgenic mice and rabbit founders were genotyped by using isolated genomic DNA, in PCR reactions for the transgenic constructs. Genomic DNA was obtained from mice tail tip and from rabbit ear tip biopsies. The PCR settings included an initial denaturation step was at 94°C for 8 mins, followed by a denaturation for 45 secs at 45°C, the annealing temperature was primer dependent while the elongation time was amplicon-size dependent. The 35 cycles were followed by a final extension step at 72°C for 5 mins. Summarized below (Table 2.2) are the different conditions for the respective PCR reactions. The *herculase hotstart* polymerase (ROCHE, Mannheim) was employed in all reactions.

Genotyping PCR reactions				
Amplicon	Step	Temp.	time	Cycle #
	Initial denaturation	94°C	8 mins	1
	Denaturation	94°C	45 secs	
Murine myeloid related protein (mMRP)	Annealing	62°C	45 secs	
8; used for murine genomic DNA quality	Elongation	72°C	45 secs	
control (326 nt PCR product)				

				35
Rabbit β-globin gene, used for rabbit genomic DNA quality control (200 nt PCR product)	Annealing Elongation	58°C 72°C	45 secs 30 secs	
Human Cμ fragment, used to detect transgenic rabbits (672 nt PCR product using primers 73 and 74, and 450 nt using 73A and 73B)	Annealing Elongation	63°C 72°C	50 secs 50 secs	
Human Vh3-23 gene fragment, used to detect transgenic mice (185 nt PCR product)	Annealing Elongation	60°C 72°C	40 secs 20 secs	
	Final extension	72°C	5 mins	1

Table 2.2 The PCR settings used in genotyping the transgenic rabbits and mice

After each PCR reaction the obtained product was analysed by gel electrophoresis. Founders were again crossed with wild type animals to generate F_1 progeny. The F_1 progeny were again genotyped using the PCR reactions and settings in Table 2.2.

2.15 Immunization of transgenic animals

To investigate the employment of the translocus in the production of antigen specific antibodies, transgenic animals were immunized with keyhole limpet hemocyanin (KLH). Hemocyanins are copper-containing oxygen-transporting proteins found in many arthropods and mollusc species. KLH with a molecular weight of about 400 kDa, is isolated from the Californian giant keyhole limpet *megathura crenulata*, a marine gastropod. KLH has been recognized as a potent immunogenic antigen, a property accepted to be related to its sugar constituents. Based on this immunostimulatory property, KLH is being used not only as an immunoactivator but also in medical research and clinical studies (Kurokawa et al., 2002). In this study immunizations were done via the subcutaneous route with injections at four different sites. Before immunizations the animals were bled (pre bleed, an average of about 150 ul blood was obtained).

To immunize rabbits, 500 μl of keyhole limpet hemocyanin (KLH) solution in PBS was mixed with 500 μl of Freunds' Complete Adjuvants and used pro rabbit.

Mice were immunized with 50 µl of same mixture. After four weeks the rabbits were boosted with a mixture of 500 µl of KLH solution in PBS mixed with 500 µl of Freunds' Incomplete Adjuvants, while mice were boosted after three weeks with 50 µl of same mixture pro mouse.

2.16   Immunohistochemistry methods

2.16.1   Flow cytometry and cell sorting

Flow cytometry is a powerful tool employed in the characterization and enumeration of cells. In multicellular organisms, all the cells have identical DNA content but their proteins vary tremendously. Therefore, it would be very useful if cells could be separated from each other based on their differential expression of cell-surface proteins moreover, it is important to know the amount of cells expressing a particular cell-surface protein. A mixed population of cells are labelled with specific monoclonal antibodies coupled to fluorescent dyes. This suspension is then forced through the nozzle of the FACS apparatus, with a larger volume of saline, creating a fine stream of liquid containing cells spaced singly at intervals. As the singular cells pass through the laser beam, each cell scatters the laser light thereby exciting the dye molecules bound to the cells. The fluorescence emissions give information on the binding of the monoclonal antibodies and hence on the expression of the cell-surface proteins by each cell. In the apparatus shown below, these signals are conveyed to the computer which decides their fate by passing an electric charge via the nozzle, through the liquid stream of cells simultaneously with the passage of a single cell in the form of a droplet.

Droplets containing a charge can then be deflected from the main stream of droplets as they pass between the plates of opposite charge, rather like an electron cathode-ray tube. In this way specific subpopulations of labelled cells can be purified from a heterogeneous population of cells, and collected for downstream purposes.
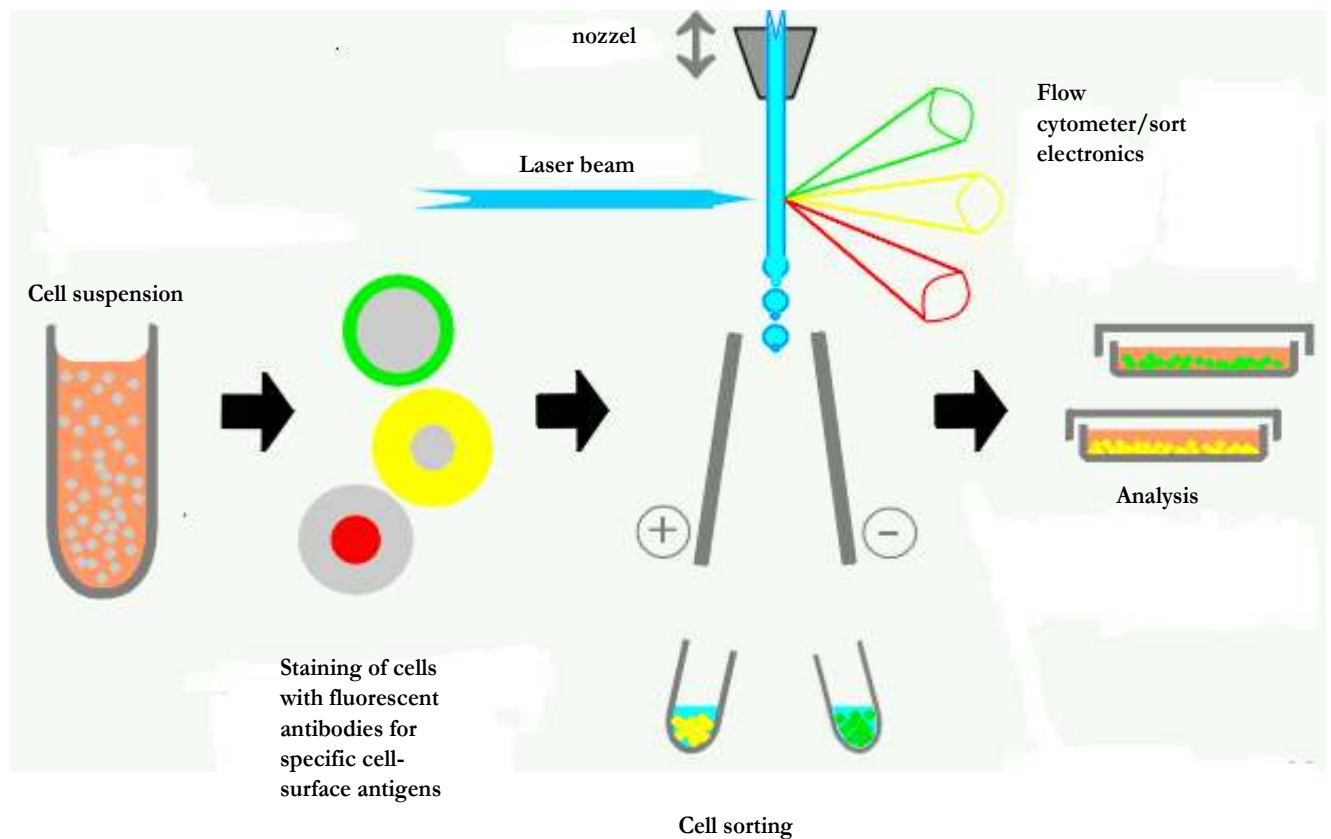
Figure 2.10   A depiction of the Fluorescence-Activated Cell Sorter (FACS) principle. The process begins by forcing the stained cells through the nozzle thereby generating a thin liquid film of cells with the cells passing through the laser beam one cell at a time. These cells are then scanned by the laser. The laser beam is scattered by the passing cell, depending on the size and granularity of the cell. During the scattering process, the laser light excites the fluorescent dye tagged to the antibody which in turn emits a colour of light detected by the photomultiplier tube. Cells stained with a particular dye can be sorted into a tube for further analysis.
(Picture taken from www.zellsorter.de)

Data from the flow cytometer can be displayed in different forms using the software from the supplier, for example as a histogram or in the form of a two-dimensional scatter diagram. Furthermore by examining large numbers of cells, flow cytometry can deliver quantitative data on the percentage of cells bearing different molecules.

To stain B cells from peripheral blood, 100 µl of blood was placed in a 15 ml falcon tube. The red blood cells are lysed by addition of FACS lysing solution (BD Biosciences Pharmingen, Mannheim) and incubating 10 minutes on ice. The cells are then centrifuged for 5 minutes at 250x g, 4°C (Eppendorf centrifuge 5417R).The cell pellet is resuspended in 500µl CellWash solution (BD Biosciences Pharmingen, Mannheim) and the primary antibodies are added in the appropriate working solutions. Following incubation on ice for 15 minutes, the cells are again

pelleted by centrifugation at 250x g, 4°C (Eppendorf centrifuge 5417R) and resuspended in 2 ml cell wash. The detection antibodies were then added, the cells incubated for 15 minutes on ice and analysed using the flow cytometer (FACScan, BD Biosciences Pharmingen, Mannheim). When preparing cells for sorting, the steps with cell wash were done with PBS supplemented with 2% FCS. Before sorting the suspension was pressed through a sieve. Sorting was performed in the laboratory of Dr. Ellwart, GSF institute of molecular immunology. The sorted cells were collected in PBS supplemented with 20% FCS and genomic DNA was extracted as described above.

2.16.2   *Enzyme-Linked Immunosorbent Assay (ELISA)*

ELISA is an immunohistochemical technique used to detect proteins subsequent to their reaction with specific antibodies. In a standard ELISA a specific antigen is immobilized on the polystyrol-surface of a 96-well-microtier plastic plate. After addition of an antibody preparation containing antibodies against the antigen the interaction can be detected and evaluated photometrically. Alternatively, an antibody (the "capture" antibody) can be coated on the microtiter plate and a protein preparation can be tested for the presence of reacting antigens, in this case a second labelled antibody (the "detecting" antibody) is used for detection. This alternative form is termed sandwich ELISA (figure 2.11).

To detect the production of KLH specific antibodies after immunization of transgenic animals with KLH, the standard ELISA was used. 100μl of the KLH protein (10μg/ml) was pipetted in the wells of the MaxiSorp microtiter plate (NUNC). 100μl BSA (1μg/ml) was also pipetted in the uncoated blank wells and the plate was incubated overnight at 4°C.
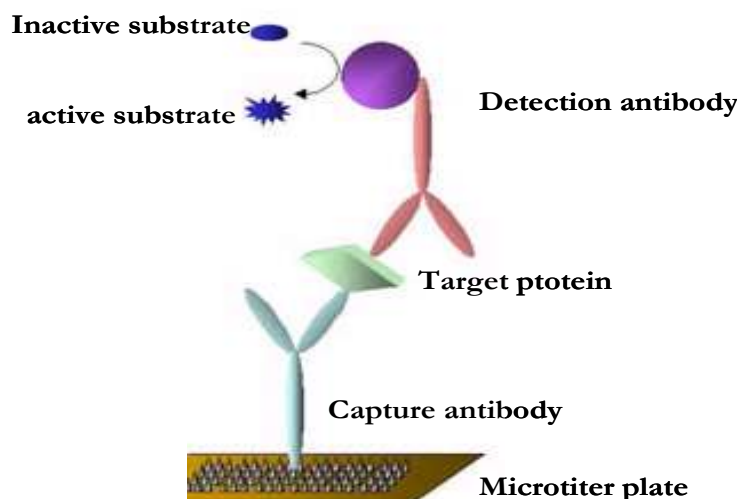
Figure 2.11   Principle of the sandwich ELISA (further described in text), (adapted from www.uni-hamburg.de)

Unbound protein was discarded by washing the plate 2x (wash steps were performed using the Wellwash machine, Thermo Labsytems) Then 200 µl of blocking buffer was pipetted in the wells and to block residual sticky sites on the plate. After incubating for 1h at room temperature, the plate was again washed 2x with 200 µl washing buffer and 100 µl of the diluted serum preparation (varying dilutions 1:10,1:20,1:40, in blocking buffer) were then pipetted in the wells and the plate incubated for 3h at room temperature.

After incubation, the plate was again washed 6x and 100µl of 1:8 (diluted in blocking buffer) anti-human IgM (609-4631, Biomol, Hamburg) antibody was pipetted into the wells. 100 µl of blocking buffer was pipetted in the blank control wells. The plate was then incubated for 1h at room temperature and the wash step repeated after incubation. 100 µl of a 1:8000 dilution of peroxidase-streptavidin-conjugate (Sigma, Taufkirchen) was pipetted into the wells and incubated for 30 minutes at room temperature. After the incubation, the plate was again washed and 100 µl of a substrate solution (3,3'5,5'-tetramethylbenzidine base TMB, Sigma, Taufkirchen) was pippetted into the wells. The plate was further incubated until the substrate, a colourless substrate (Chromogen, figure 2.3) is converted blue by the peroxidase. The reaction was stopped by addition of 100 µl 1N H_2SO_4 whereby the solution turned yellow.

The absorption was then measured at λ= 450nm in an ELISA reader (Bio-Rad, Munich). The obtained absorption values were used to calculate the different antigen concentration, in comparison to the blank control. The calculated data were then represented as a histogram.

To quantify the amount of serum antibodies containing human μ chains used in the transgenic animals after KLH immunization, the sandwich ELISA method was used. Plates were coated with 100μl of a 1:2500 dilution of the anti-human IgM antibody (609-4131, BIOZOL, Hamburg), the "capture" primary antibody in this case and tested with serum from the transgenic animals. The secondary "detection" antibody was the biotin conjugated anti-human IgM (609-4631, Biomol, Hamburg). A 1:200 dilution was used.

Finally, detection was done using the peroxidase-streptavidin-conjugate, as described above.

After absorption readings (ELISA reader, BioRad München), the different levels of antibodies containing chains were calculated by interpolation on calibration curves generated using a human reference serum (I-8260, human IgM reagent grade, Sigma, Taufkirchen).

3. Results

3.1 3HuVs.I5-6 transgenic mice
3.1.1 Purification of transgenic constructs for micro-injection

To generate 3HuVs.I5-6 transgenic mice, the constructs built in section 2.11 were first analyzed by restriction digestion before purifying them for micro-injection. As described in section 2.11 three plasmid libraries were generated:

1. The first library consisted of nine human VH genes in the modified pGEM vectorbackbone

2. The second library was composed of the rabbit spacer I5-6 in the modified pGEM.genta vector.

3. The final library consisted of three chimeric contructs, composed of the rabbit I5-6 spacer and different human VH genes: pGG.I5-6.VH3-23, pGN.I5-6.VH3-64 and pGG.I5-6.VH3-73.

These chimeric constructs were then further stitched together exploiting the isocaudemeric properties of the restriction enzymes *Asc*I and *Mlu*I and also the alternating selection cassetes in the different vectorbackbones. The final humanized rabbit mini VH locus, pBBII.mod.17kb.3HuVs.I5-6 as well as the intermediate constructs pBBII.mod.17Kb and pBBII.mod.17kb.VH3-23.I5-6 3.9 were double- digested with *Xma*I and *Hind*III, and separated by gel electrophoresis.

a)

b)

c)

d)
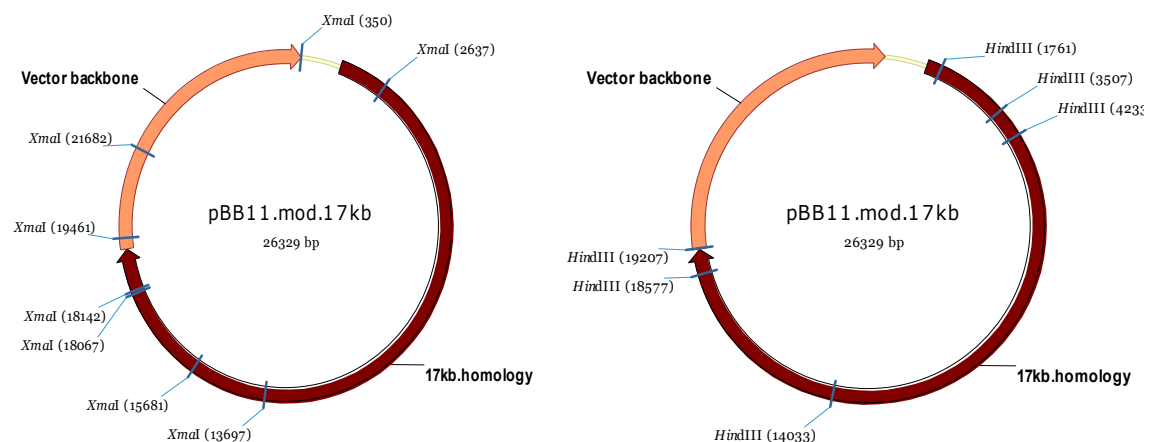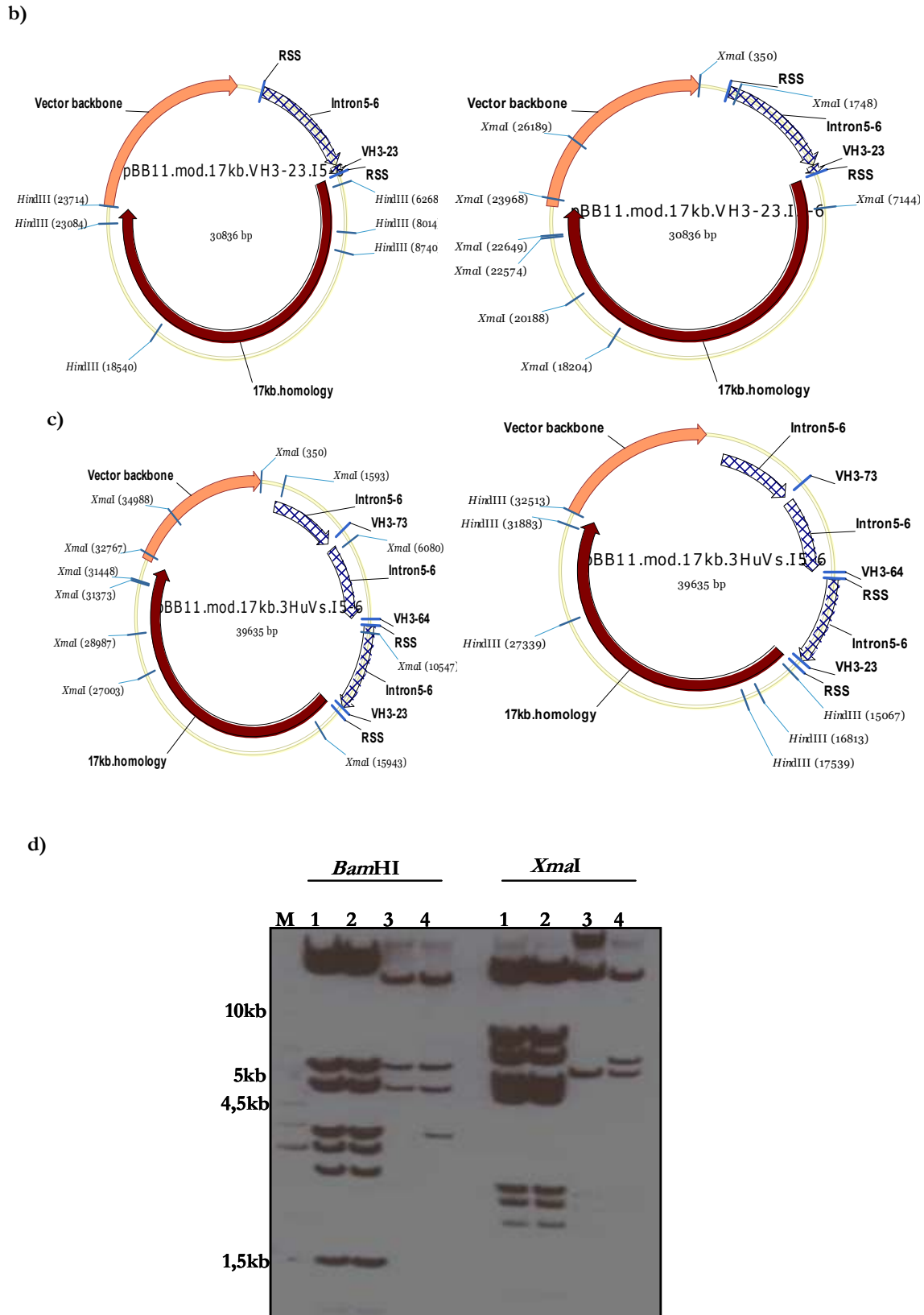
Figure 3.1   Restriction analyses of intermediate constructs as well as the final humanized rabbit mini V_H locus. **a)** map of pBBII.mod.17Kb, **b)** pBBII.mod.17kb.VH3-23.I5-6 **c),** map of pBBII.mod.17kb.3HuVs.I5-6, maps showing the restriction sites for *Xma* I and *Bam*HIThe constructs were digested with *Bam*HI and *Xma*I, and separated by agaros gel electrophoresis, **d)** M = 1 kb marker, 1 = pBBII.mod.17kb.3HuVs.I5-6 clone # 2, 2 = pBBII.mod.17kb.3HuVs.I5-6 clone #5, 3 = pBBII.mod.17kb, 4 = pBBII.mod.17kb.VH3-23

Two different clones of the final construct were analyzed, and after confirmation that the constructs were intact it was then proceeded to purify the constructs for micro-injection.

As described in section 2.11, the humanized mini VH locus was to be co-injected with the humanized AY386695, with an overlapping region to ensure homologous recombination of the two fragments. The two constructs (pBBII.mod.17kb.3HuVs.I5-6 and AY386695) were therefore digested for purification as such that a 2kb overlapping region (figure 3.2a) resulted. pBBII.mod.17kb.3HuVs.I5-6 was double digested with *Asc*I and *Mlu*I, while the humanized AY3986695 construct was digested with *BsiW*I (figure 3.2a).
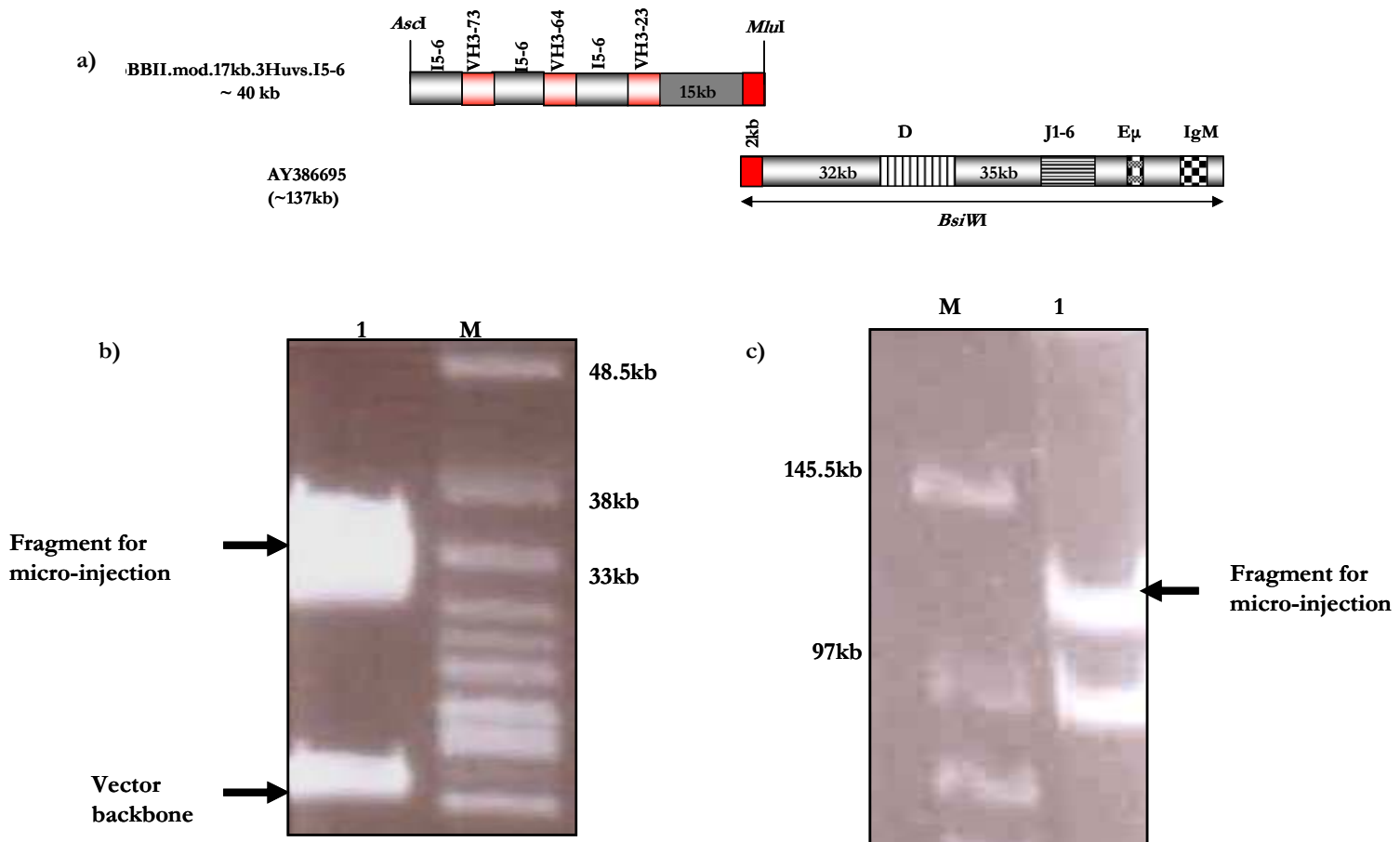
Figure 3.2   Purification of the constructs for co-injection. Figure 3.2a is a schematic depiction of the constructs after restriction digestion. The 2 kb homology is shown in red. Figure 3.2b is a PFGE picture of the separation of construct pBBII.mod.17kb.3HuVs.I5-6 after restriction digestion with *Asc*I and *Mlu*I. M = lambda mix ladder, 1 = digested DNA). The construct without the vector backbone (~ 33kb) was excised and the DNA isolated by electro-elution. Figure 3.2c shows a second PFGE picture of the separation of the humanized AY 386695 after digestion with *BsiW*I. The fragment with the humanized JH and Eμ and IgM portions (~100kb), was gel excised and the DNA isolated by Electro-elution. Figures 3.2b and 3.2c show the portions of the gel stained with Etbr for visualization purposes, the rest of the gel with the separated DNA to be purified for micro-injection was stained with methylenblue.

The restriction digestions guaranteed that the vector sequences were removed before micro-injection as recommended (Chada et al., 1985; Krumlauf et al., 1985) . After restriction digestion, the fragments were separated by PFGE (figures 3.2b and 3.2c) and the DNA isolated from the respective excised fragments by electro-elution.

3.1.2　　Genotyping 3HuVs.I5-6 transgenic founder mice

A litter comprising ten pups was obtained from micro-injected zygotes. These pups were numbered 733-1 to 733-10. As described in section 2.13, to genotype F_0 generation animals, genomic DNA was extracted from tail-tips and a quality control PCR reaction was performed by PCR amplification of the mouse MRP8 gene (figure 3.16a), subsequently a second PCR reaction with primers specific for the human VH3-23 gene was performed (figure 3.16b).
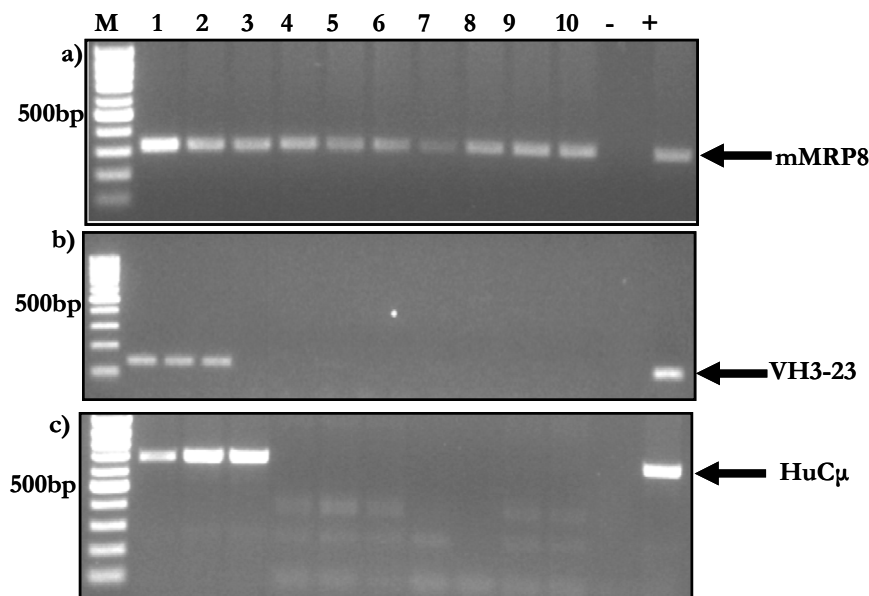
Figure 3.3　　DNA was extracted from the tail-tips from a litter of 10 pups a) quality control of the isolated DNA was done by PCR amplifying the murine myeloid Related Protein (mMRP)8, a calcium binding protein (Marenholz et al., 2004) b) Primers specific for of the human VH3-23 gene (~ 185 bp) revealed three mice (733-1, 733-2 and 733-3) to be transgenic for the humanized VH locus. C) These same mice were also transgenic for the humanized constant region as revealed by PCR amplification with primers specific for the human Cμ (~675bp). Positive controls (+) of for the PCR reactions were plasmid DNA. **M** = 1Kb marker, **numbers 1 – 10** = mice 733-1 to 733-10, **-** = water for the *mMRP8* PCR, and wild type mouse genomic DNA for the VH3-23 and HuCμ PCR reaction.

Mice positive for the human VH3-23 gene consequently have the humanized V_H mini-locus. Finally, since the humanized V_H locus was co-injected with the humanized constant region (figure 3.2), a second PCR for the humanized constant region was done by using primers specific for the Cµ region (figure 3.3c). Three mice were double-positive for humanized portions, 733-1, 733-2 and 733-3. This result hints that the co-injected fragments could possibly have recombined homologously to generate an intact locus: an integral humanized rabbit locus carrying all the Ig elements obligatory for effective VDJ rearrangements. The next steps involved generating F_1 generation mice and further investigate if the transgenic locus was functionally employed in the transgenic mice.

3.1.3   Generation and genotyping F1 transgenic mice

The transgenic founder mouse 733-2 was backcrossed with wild type FVB mice to generate F_1 generation animals. The F_1 generation animals (a litter of 13 pups) were genotyped using the PCR reactions described in 3.3 and three transgenic mice proved to be transgenic.
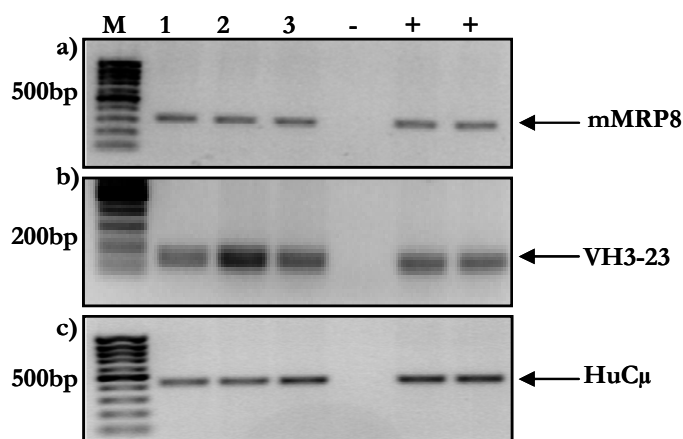
Figure 3.4   After extraction of genomic DNA, the quality control was carried out by PCR amplification of the mMRP8 gene, figure 3.4a. Subsequently, the intergration of the humanized V_H locus was confirmed by PCR amplification of the VH3-23 gene figure 3.4b. Three mice 733-2-4, 733-2-6 and 733-2-12 proved to be transgenic for the humanized V_H locus. These same mice were also transgenic for the human IgM portion on the co-injected AY386695 figure 3.4c. **M** = 1 kb ladder, **1** = 733-2-4, **2** = 733-2-6, **3** = 733-2-12, **-** = negative control, (mMRP8 PCR reaction, water, VH3-23 and HuCµ PCR reactions, a wildtype mouse DNA), **+** = positive controls, DNA from transgenic mouse 733-2.

Figure 3.4 shows three transgenic F1 generation mice (733-2-4, 733-2-6, 733-2-12) from the founder 733-2. Mouse 733-2-4, carrying both the humanized VH locus (figure 3.4b) as well as the human Cμ, (figure 3.4c) was employed in further analyses to characterize the transgenic locus.

3.1.4 Expression of antibodies carrying human Cμ chains in serum of transgenic mouse 733-2-4

To further characterize the transgenic animals it was investigated if the humanized locus was functionally employed in these animals. This was carried out first by performing an ELISA assay (figure 3.5) to investigate for secretion of antibodies carrying the human Cμ chain in serum of the transgenic animals.

Anti-human IgM ELISA
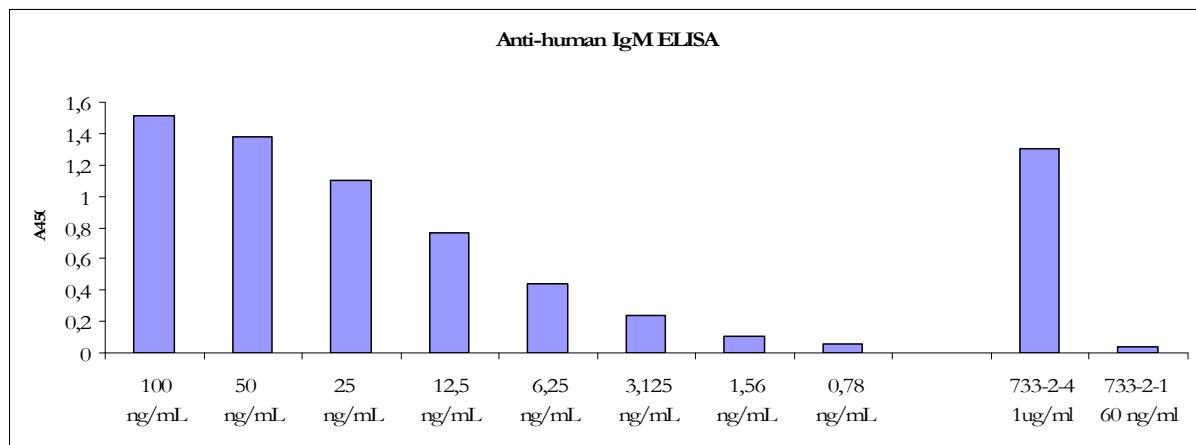
Figure 3.5 A sandwich-ELISA for Human Cμ antibodies. Plates were coated with a rabbit unlabeled Anti-Human Cμ antibody (Biomol, Hamburg) and tested with serum from mouse 733-2-4 (F1 generation transgenic mouse from founder 733-2), a 733-2-1 (non-transgenic littermate from 733-2-4), and a human IgM purified Ig standard (Sigma, Taufkirchen) was used for calibration. Detection was done with a biotinylated rabbit anti-human Cμ antibody (Biomol, Hamburg), and developed with horseradish peroxidase (sigma, Taufkirchen). The calculated expressions of serum antibodies containing human IgM chains are indicated. This experiment was repeated with three different serum dilutions, with similar results.

As can be seen from figure 3.5, there is expression of serum antibodies containing human Cμ chains, proving that the translocus is functionally employed in the transgenic mouse. The observed titre (1 ug/ml) is relatively low, but not uncommon in other plasmid based-based Ig transgenic mice (Wagner et al., 1994).

3.1.5   Expression of antibodies containing human Cμ chains on transgenic B cell surface

To further examine the observed low titre of serum antibodies containing human Cμ it was next investigated which percentage of B cells expressing these human μ chains on their surface. Flow cytometry technique (FACS, figure 3.18) weas used to perform this quantification. Blood was obtained from the transgenic mouse 733-2-4 as well as a non-transgenic littermate, 733-2-1.
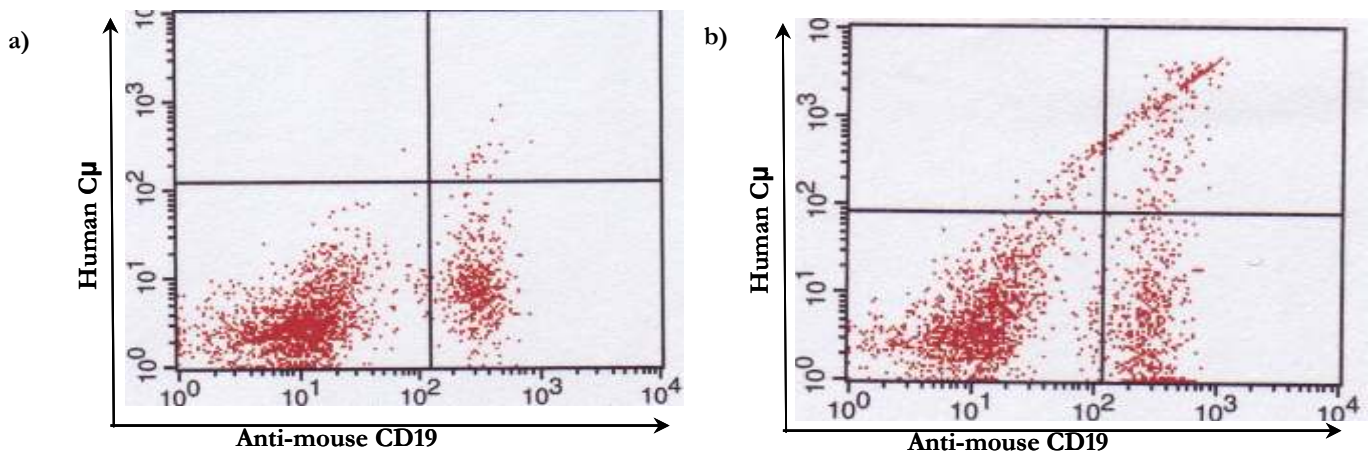
Figure 3.5   Expression of human μ chains on the surface of transgenic B cells. Blood cells from mouse 733-2-4 and a 733-2-1 were double-stained with Biotin-conjugated anti-human Cμ and phycoerythrin (PE)-conjugated anti- mouse CD19 antibodies and detected with streptavidin-PE-CY5. Lymphocytes were gated and stained cells quantified a) the dot-plot diagram of the mouse 733-2-1 shows 0.93% of the gated cells double positive b) 10.07% of the transgenic mouse gated cells are double-positive (figure 3.5b,upper right quadrant).

After staining and analysis by flow cytometry (FACS), only 10.07% (figure 3.5b, upper right) of all B cells express antibodies containing human Cμ chains. This could be an explanation for the low serum titre measured by ELISA in figure 3.4.

3.1.6   Transgenic antibody diversity and response to antigen challenge

After detecting antibodies containing human Cμ chains in the serum of the transgenic mouse 733-2-4, it was next investigated if the antibodies from translocus underwent effective rearrangement event and diversity in response to antigen challenge. Effective rearrangements are to be expected because there are previous

reports of effective rearrangement of a chicken light chain locus (also a gene converting animal) in transgenic mice (Bucchini et al., 1989). Such rearrangements would hint that rabbit Ig *cis-* elements function in mice, correlating with reports of the functionality of Ig *cis-* elements across species boundaries (Bucchini et al., 1989; (Mendez et al., 1997; Gallo et al., 2000). Moreover, in this study not only was effective rearrangement to be investigated but also the interactions between the Ig *cis-* and *trans-* elements required for somatic mutation, and more important which method of somatic diversification is employed to diversify the V_H gene of the humanized rabbit IgH mini-locus in the transgenic mice.

Antigen challenge of the transgenic mice would provide an insight to both issues at hand: the functionality of the transgenic locus would be defined by generation of antigen specific serum antibodies and these antigen specific antibodies would be expected to be have undergone diversification to augment specific binding.

Mice 733-2-4 (transgenic) and 733-2-1 (non-transgenic littermate) were primed with Keyhole limpet hemocyanin (KLH) coupled with Freunds' Complete Adjuvant (section 2.14). The mice were boosted after three weeks and euthanized three days later. Serum for an ELISA assay was extracted before (pre-immune) and (post-immune) three weeks after immunization. The extracted sera were titred by "sandwich" ELISA methods (figure 3.6) on microtitre plates. As can be seen from figure 3.6, transgenic mouse 733-2-4 secretes serum KLH specific human Cμ antibodies. This is evident from the increase of the level of these antibodies after KLH immunization, in comparison to the non-transgenic control, 733-2-1.

This proves the humanized transgenic Ig mini-locus is not only successfully rearranged but is also employed in the production of antigen specific antibodies.

After detecting transgenic antigen specific antibodies, the next step was to investigate if these transgenic antibodies underwent somatic diversification and more important which method of somatic mutation is employed.
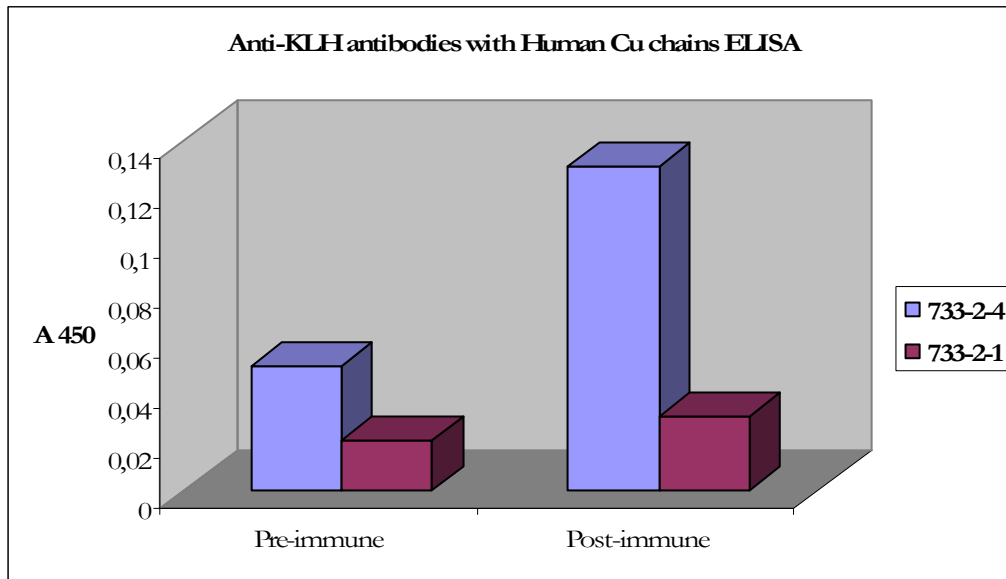
Figure 3.6   Plates were coated with KLH protein and KLH specific antibodies carrying human Cμ antibodies in the sera were detected with a biotinylated rabbit anti-human IgM antibody (BIOMOL, Hamburg) and developed with horseradish peroxidase (Sigma, Taufkirchen). Data are presented as mean +SD. Error bars indicate standard deviation. Two different assays were performed with three different serum dilutions, and both Assays with similar results.

Classically, somatic diversification is investigated by cloning the expressed V genes from the germinal centers in lymphoid organs and comparing the obtained V sequences to their germline counterparts (Klix et al., 1998). To clone these expressed V regions the appropriate lymphoid organ is of significance, since the B cells from the organ of choice must be in germinal centers, where B cell maturation occurs. It has been shown in sheep (Reynaud et al., 1991) and mice (Gonzalez-Fernandez and Milstein, 1993), that the peyers patches are the optimal organs for acquiring diversified V(D)J sequences. In this work, the peyers patches were also the organ of choice for the PCR amplification of transgenic VDJ sequences. To PCR amplify the expressed VDJ gene from the transgenic IgH mini-locus, specific PCR primers were designed (figure 3.7a). The forward primer was designed such that it had a hybridizing region common to all three human V_H genes of the transgenic IgH mini-locus. To achieve this, a nucleotide alignment the leader sequences of the three human genes VH3-23, VH3-64 and VH3-73 was done (figure 3.7b). A sequence stretch consensus to all three leaders (figure 3.7b, highlighted in red) was used as the forward PCR primer (BS83). Similarly, since the human JH sequence used in the expressed VDJ gene is unknown, the reverse PCR

primer (BS84) was designed to carry a sequence common to all the human J_H sequences (figure 3.7 c, highlighted in red) on the transgenic construct and also a sequence hybridizing to the JH-C intron (the intron between the JH and C loci).
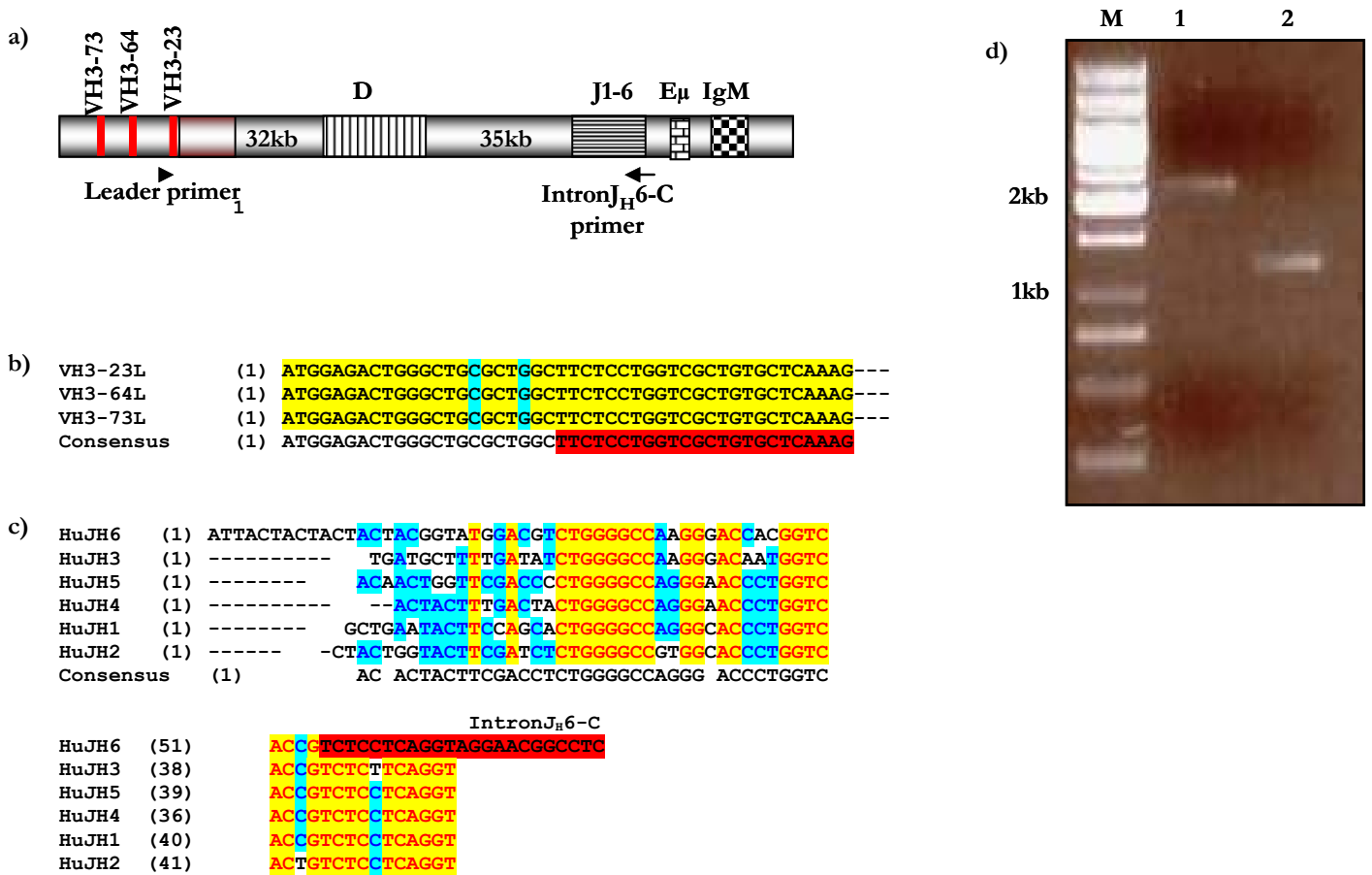
Figure 3.7   PCR amplification of VDJ genes, a) primers were designed such that the forward primer (highlighted in red), hybridizes to the leader sequence common to all three V_H genes in the transgenic construct and b) the reverse primer (also highlighted in red) hybridizes partly to the JH6 and to the Intron JH6-Cμ sequences c) Using these primers two PCR bands were obtained, shown is 1 μl of the purified products. The different sizes are due to different JH sequences used in the VDJ gene. **M** = 1 kb marker, **1** = V_H1DJ_H4 PCR product, **2** = V_H1DJ_H6 PCR product.

Using these primers, VDJ genes of two different sizes were obtained (figure 3.7d). The obtained PCR products of different sizes can be explained by the J_H usage in the expressed VDJ genes. The J_H4 and J_H6 genes are preferentially used in humans and in transgenic mice with a human Ig locus, the same preferential usage was observed (Gallo et al., 2000). The obtained PCR sequences were then sub-cloned in TOPO vectors and sent for sequencing. A total of 20 sequences were analyzed but for convenience purposes, a nucleotide alignment of just four representative clones is shown, alignment 5a, (appendix). As is evident from the nucleotide alignment

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(dots represent identical nucleotides to the germline sequence) the humanized rabbit IgH mini-locus undergoes effective re-arrangements in the transgenic mice. Not only can V, D and J genes can be attributed also junctional diversity (N-D-N) is apparent. All the four clones shown in the nucleotide alignment use the human VH3-23 and JH6 genes (CDR and FR regions are indicated, annotations according to the International Immunogenetics Information Systems (IMGT), Montpellier, France) and even portions of the D2x sequence are identified. The usage of the human VH3-23 and JH6 genes, coincides with the observation of preferential usage of human Ig genes in transgenic mice (Gallo et al., 2000). As described above, diversification is analyzed by comparing the obtained VDJ sequence to the germline counterpart, in this case the germline counterpart being human VH3-23 gene. In the nucleotide alignment, dots represent identical bases and as is evident no somatic mutation is observed.

Alignment 5b shows an amino acid alignment of the four representative clones, of these four only clones BS-2-44 and BS-2-45 have sequences in frame and code for functional proteins. This shows that the obtained sequences code for functional proteins albeit no somatic mutation is evident.

The above results give a strong hint that the transgenic locus undergoes effective re-arrangements and gives rise to specific antibodies in response to antigen challenge. Again this demonstrates the observation that Ig *cis* elements are conserved amongst species, in this case rabbit Ig *cis* sequences are functional in mice. Furthermore these results also give the first hint that interaction between the Ig *cis* and *trans* elements determine the method of somatic diversification employed. As seen in the alignment from alignment 5a, the Ig locus of a gene converting animal (rabbit) does not undergo somatic diversification in mice, a hypermutator.

To further examine this hypothesis a more effective comparison is required; a comparison of the somatic diversification methods of both rabbits and mice transgenic for the same Ig locus. This comparison was performed with rabbits and mice transgenic for the HC2 construct (figure 1.11). If the hypothesis is to be confirmed, it would be expected that this IgH mini-locus undergoes gene

conversion in the transgenic rabbits and as above successful rearrangements but no somatic diversification in the transgenic mice.

3.2 Comparing somatic diversification of antibodies in mice and rabbits using a transgenic humanized mini-locus

3.2.1 Genotyping HC2 transgenic mice

The HC2 construct described in section 1.4 was generated in the THP GmbH laboratories. To generate transgenic mice, the HC2 construct was rendered vector-less by double-digestion with *AsiS*I and the isolated DNA used for micro-injection (data not shown).

A litter of 11 pups was obtained from the micro-injected zygotes, and the pups numbered 670-1 to 670-11. Of these 11 pups, three proved to be transgenic for the chimeric HC2 construct. Genotyping was done using PCR technique Figure 3.8.
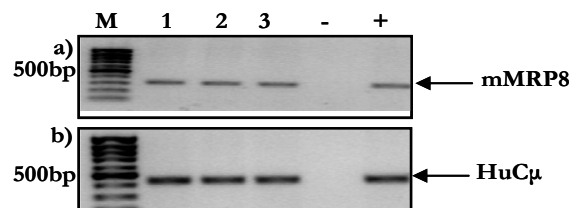
Figure 3.8   Genotyping HC2 F_0 transgenic mice. After isolation of genomic DNA, the DNA quality was controlled by PCR amplifying the mMRP8 protein figure 3.8a; subsequently the PCR amplification for the human Cμ portion was carried out (using oligos BS 73A and BS74A), figure 3.8b. **M** = 1 kb ladder, **1** = mouse 670-1, **2** = 670-6, **3** = 670-8, **+** = DNA from mouse 733-2 (positive control), **-** = negative control, water for the mMRP8 PCR reaction and a Wild type mouse genomic DNA for the HuCμ PCR reaction.

After positively identifying three transgenic mice, mouse 670-8 was backcrossed with wild type FVB mouse to generate F1 generation animals. Two litters of 13 pups were obtained, with six transgenic animals, Figure 3.9.
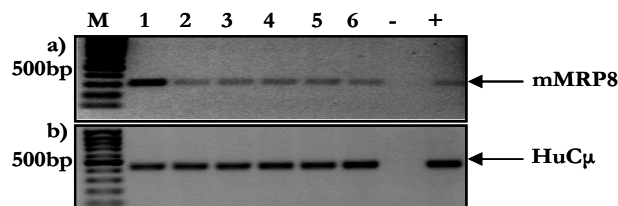
Figure 3.9   Using PCR technique, six F_1 transgenic mice were identified. Quality control of the genomic DNA was assured by the mMRP8 PCR amplification, figure 3.9a and subsequently PCR amplification of the human Cμ gene, figure 3.9b. M = 1 kb marker, **1** = mouse 670-8-1, **2** = 670-8-2, **3** =670-8-5, **4** = 670-8-6, **5** = 670-8-10, **6** = 670-8-14, **-** = negative control, water for the PCR reaction and wild type genomic DNA for the HuCμ PCR reaction.

3.2.2   Employment of the translocus in antibody production

To investigate serum antibodies containing human Cμ, serum was obtained from transgenic mouse 670-8-1 and a non-transgenic littermate 670-8-3 and an ELISA for serum antibodies containing human Cμ chains was performed, figure 3.10.

Anti-human IgM ELISA

A450

| Value bars (ng/mL): 100, 50, 25, 12,5, 6,25, 3,125, 1,56, 0,78; 670-8-1 1,36ug/ml; 670-8-3 70ng/ml |
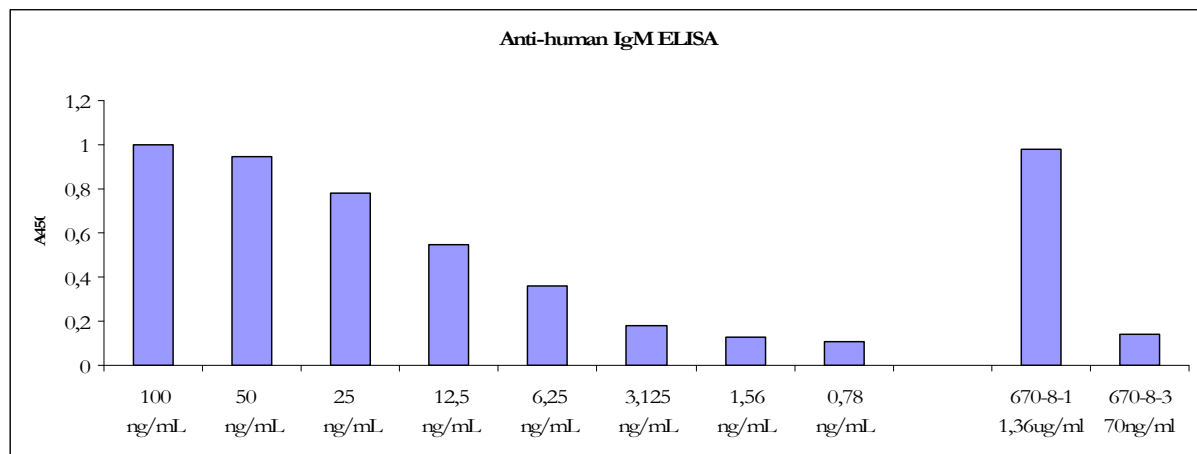
Figure 3.10   To detect serum antibodies containing human Cμ chains, sera was obtained from mice 670-8-1 and 670-8-3 and used in a "sandwich" ELISA. Microtiter plates were coated with an unconjugated rabbit anti-human Cμ antibody (Biomol, Hamburg), and tested with the obtained sera, and a human IgM purified standard (Sigma, Taufkirchen) was used for calibration. Detection was done using a biotinylated rabbit anti-human Cμ antibody and developed with horseradish peroxidase (Sigma, Taufkirchen). The calculated expressions of serum antibodies containing HuCμ chains are indicated. The assay was performed twice with similar results.

It is evident from figure 3.10 that the transgenic mouse 670-8-1 produces serum antibodies containing human Cμ chains, proving that the translocus was employed in the transgenic mouse, and as observed for mouse 733-2-4, the measured titre was low (1,36 ug/ml).

Subsequently, it was investigated if the translocus was employed in the production of antigen specific antibodies. As in section 3.1.6, this was carried out by investigation for transgenic antibodies in response to KLH immunization. Mice 670-8-1 and 670-8-3 were immunized with KLH and serum extracted before (pre-immune serum) and four weeks after immunization (post-immunization) figure 3.11.
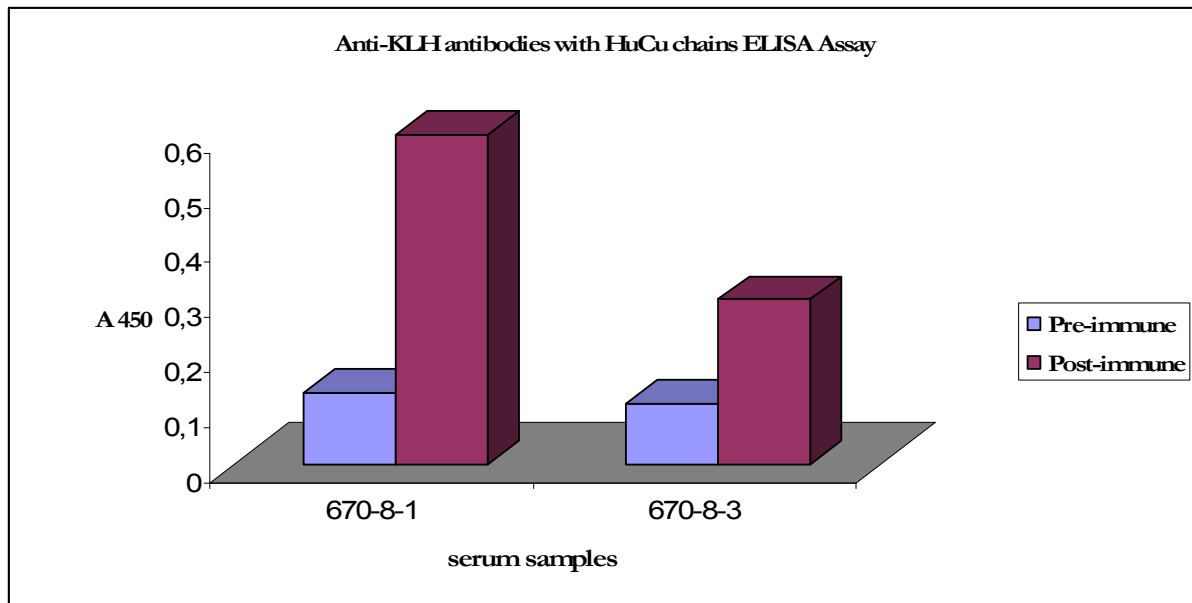
Figure 3.11   Using pre-immune and post-immune sera from KLH immunized mice 670-8-1 (transgenic mouse) and 670-8-3 (non-transgenic littermate) to investigate KLH specific antibodies. Microtiter plates were coated with KLH protein and KLH specific antibodies were detected with a biotinylated rabbit anti-human antibody (BIOMOL, Hamburg), and developed with horseradish peroxidase. Two different assays were performed with three different serum dilutions, and both Assays with similar results.

From figure 3.11 it is evident that the translocus is employed in the production of KLH specific antibodies. This is concrete evidence that the translocus is functionally rearranged and hence employed in transgenic mouse. Following this observation it was then investigated if these antibodies had undergone somatic mutation.

3.2.3   Antibody diversification

After showing that HC2 locus is functionally employed in the transgenic mouse, the next step was to determine the method of somatic mutation used by the transgenic antibodies.

Since the goal is an effective comparison of the methods of somatic diversification used by HC2 transgenic rabbits and mice and as mentioned before, rabbit utilize the CH region most proximal VH gene in 80% of their VDJ rearrangements, it would be expected that the HC2 rabbits in similar fashion preferentially use the VH1 gene present on the transgenic construct. Therefore, to offer an effective

assessment of the methods somatic diversification between HC2 transgenic rabbits and mice, the compared VDJ genes must carry the same VH gene. Consequently, the primers used for the PCR amplification of the rearranged VDJ gene in the HC2 mice was designed as such that the forward primer (BS81) hybidizes in the spacer 1-2 (nomenclature by Ros et al., 2004) and the reverse primer (BS82) in the intron JH-C, between the humanized JH and HuCH loci. Using these primers, it is guaranteed that the amplified rearranged VDJ gene carries the first VH gene in the HC2 construct; the VH1 gene.

  As described in section 3.16, the organs of choice for VDJ PCR amplifications are the Peyers' patches. The immunized mouse 670-8-1 was boosted four weeks after immunization, killed three days later and the Peyers' patches extracted. Using the isolated DNA as template, re-arranged VDJ genes were PCR amplified figure 3.25, the isolated DNA sub-cloned in TOPO vectors and positive clones sent for sequencing.
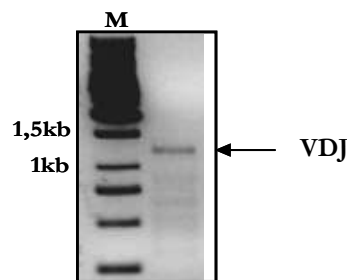
Figure 3.12   Using the DNA extracted from the PP as PCR template and the PCR primers, the VDJ was amplified, the ~ 1,2kb band was excised, gel-purified, sub-cloned in TOPO vectors and sent for sequencing.

In the nucleotide alignment c, the sequences of seven representative VDJ clones are shown (a total of 20 clones were analyzed); V_H1 and J_H6 gene segments could be identified as well as portions of D2x as shown by the comparison to germline sequences, whereby dots represent identical residues. The amino acid alignment d shows that the clones BS-2-220, BS2-2-221 and BS-2-222 contain stop codons, while the other sequences are "in-frame", coding for functional proteins. Rearrangement of these VDJ clones is evident and also junctional diversity, but again as observed in section 3.1.6 somatic mutation is not evident, not even in the clones with "in-frame" sequences. This observation further solidifies the previously

mentioned hypothesis that the Ig *cis* elements of a gene converting animal, cannot interact with the Ig *trans* elements of a somatic hypermutating animal to result in somatic mutation.

It has been reported that, though unfractionated populations of B lymphocytes from PP transgenic mice have already undergone hypermutation, B cell populations in the germinal centres show the most extensive somatic mutation (Gonzalez-Fernandez and Milstein, 1993).To exploit this possibility, the FACS technique was used to sort for PP B cells in germinal centres Figure 3.13, and DNA isolated from the enriched cells used as PCR template for a second VDJ PCR amplification.
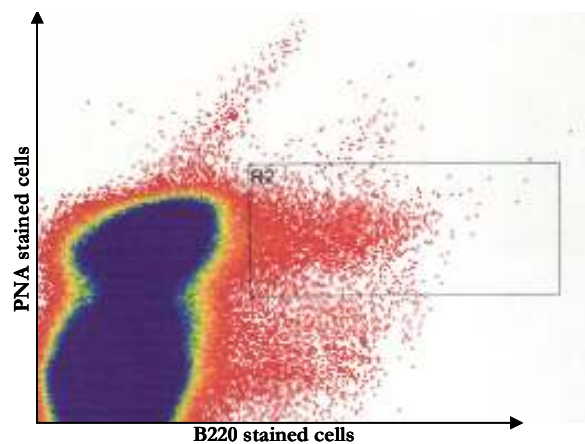
Figure 3.13   Cells from the Peyers patches were double-stained with Biotin-conjugated anti-peanut agglutinin, PNA (this is a plant lectin which is used as a marker for germinal center B cells (Butcher et al., 2005; Butcher et al., 1982) and a PE-conjugated B220 (a murine B cell marker). The double-stained cells PNA$^+$B220$^+$ were sorted (R2, gated cells) and genomic DNA extracted and a VDJ PCR reaction repeated.

The PCR product (data not shown) was sub-cloned in TOPO vectors and clones sent for sequencing. A nucleotide alignment of (alignment e) shows the sequence of four representative VDJ clones (a total of 15 clones were analyzed). Again, effective rearrangement is evident, as well as junctional diversity (N-D-N). Usage of the VH1, JH6 as well as portions of the D1v gene segments is evident. In the alignment, dots represent identical nucleotides to the germline sequences. Though these clones are obtained from PNA$^+$B220$^+$ B cells from the PP of a immunized animal, no somatic hypermutation is manifested.

One can assume that in the HC2 transgenic mice, the complete machinery of Ig *cis* and *trans* elements, and especially the necessary interactions necessary for somatic mutation is lacking.

The results from the HC2 transgenic mice consolidate the theory postulated in section 3.1, saying that a rabbit IgH locus can effectively rearrange in mice but the Ig trans regulatory elements required for somatic mutation are not present, hence no somatic mutation is occurs. The concluding corroboration for this hypothesis would be gotten by investigation of somatic mutation in HC2 transgenic rabbits.

3.2.4   Genotyping HC2 transgenic rabbits

A litter of 8 pups was obtained from the micro-injected zygotes, and the pups numbered 2001-2003, genomic DNA was obtained from ear-tip biopsies and transgenic integration investigated by PCR technique, figure 3.14.
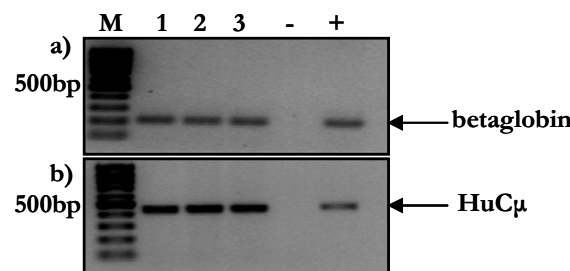
Figure 3.14   After isolation of genomic DNA from ear-tip biopsies, the quality of the DNA was controlled by PCR amplification of the rabbit ubiquitous betaglobin gene, figure 3.14a. Subsequently using oligos BS73A and BS74A, the 475bp portion of the human Cμ gene was also PCR amplified, (figure 3.14b) confirming integration of the HC2 construct. **M** = 1 kb ladder, **1** = rabbit 2002, **2** = 2003, **3** = 2004, **-** = negative control (water for the betaglobin PCR, and for the HuCμ PCR, WT DNA).

Figure 3.26 shows the three F_0 generation HC2 rabbits. Rabbit 2003 was backcrossed with wild type rabbits to generate F_1 generation animals. From a litter 5 pups, one transgenic F_1 generation rabbit was identified, figure 3.15.
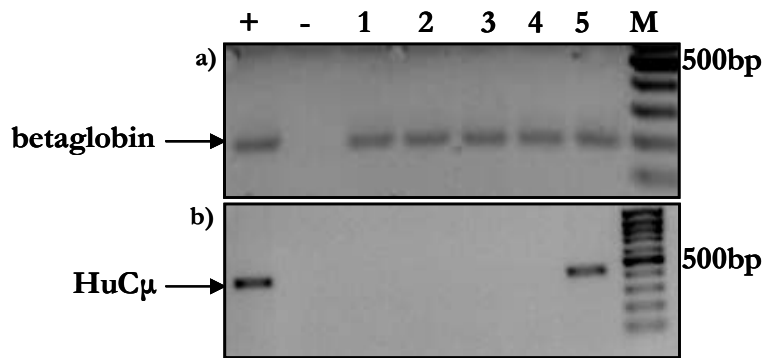
Figure 3.15   Genomic DNA was obtained as usual from ear tip biopsies and the quality control betaglobin PCR reaction performed, figure 3.15a. Subsequently, the PCR amplification of the HuCμ was performed (figure 3.15b) with one F_1 rabbit transgenic for the HC2 locus. **M** = 1kb marker, **1** = 2014, **2** = 2015, **3** = 2016, **4** = 2015, **5** = 2017, **-** = negative control (water for the betaglobin PCR, and for the HuCμ PCR, WT DNA).

HC2 rabbit 2017 was then further analysed for expression and employment of the translocus.

3.2.5   Employment of the translocus in antibody production

An ELISA assay was performed to detect serum antibodies with HuCμ chains, figure 3.16.
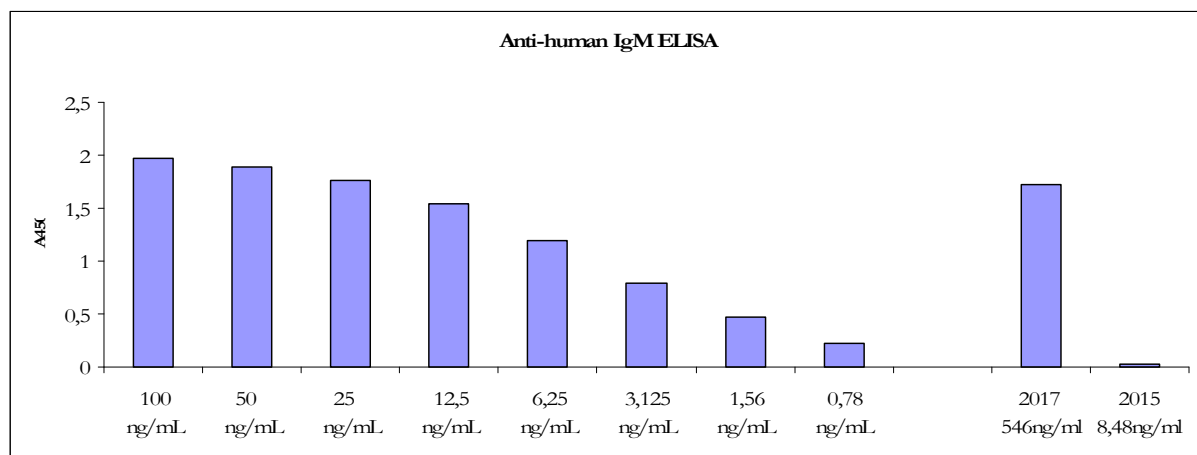
Figure 3.16   Microtiter plates were coated with an unconjugated rabbit anti-human Cμ antibody (Biomol, Hamburg), tested with the obtained sera from rabbits 2017 and non-transgenic littermate 2015, and a human IgM purified standard (Sigma, Taufkirchen) was used for calibration. Detection was done using a biotinylated rabbit anti-human Cμ antibody and developed with horseradish peroxidase (Sigma, Taufkirchen). The calculated expressions of serum antibodies containing HuCμ chains are indicated. The assay was performed twice with similar results.

Following detection of serum antibodies containing HuCμ chains, it was next investigated if these antibodies also participated in the production of antigen specific antibodies. As above for the transgenic mice, rabbits 2017 and 2012 were

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primed with KLH and boosted four weeks later. Pre – and Post - immune sera from both animals were extracted and an ELISA assay (figure 3.17) for KLH specific antibodies with HuCµ chains carried out.
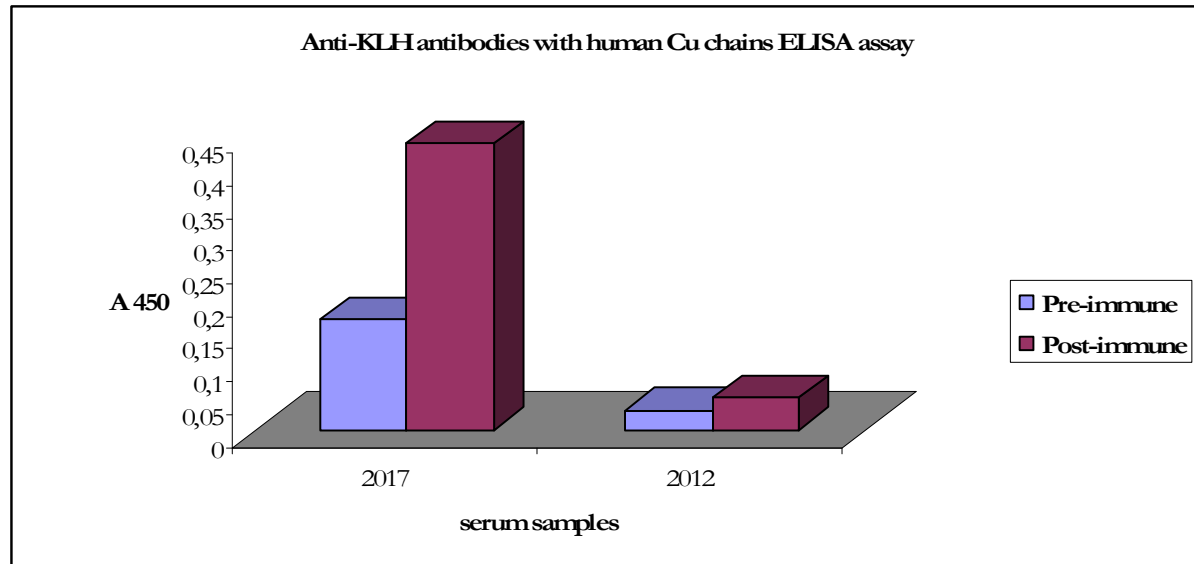
Figure 3.17   Using pre-immune and post-immune sera from KLH immunized rabbits 2017 (transgenic) and (non-transgenic littermate) 2012 to detect for KLH specific antibodies. Microtiter plates were coated with KLH protein and KLH specific antibodies were detected with a biotinylated rabbit anti-human antibody (BIOMOL, Hamburg), and developed with horseradish peroxidase. Two different assays were performed with three different serum dilutions, and both Assays with similar results.

As is evident from figure 3.17, rabbit 2017 does have serum antibodies with HuCµ chains specific for KLH.

The next step was therefore to investigate if these antigen specific antibodies had undergone somatic mutation.

3.2.6   *Antibody diversification*

Four day after the boost, rabbit 2017 was euthanized and the Peyers' patches extracted. Genomic DNA was isolated from the PP and used as a template for the PCR amplification of VDJ genes (figure 3.18). As mentioned above, the primers (BS79 and BS80) were designed to exclusively amplify VDJ rearrangements containing the VH1 gene of the HC2 construct.
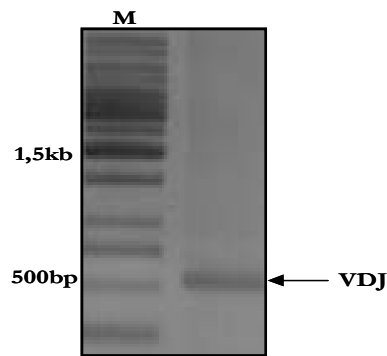
Figure 3.18   Using a forward primer specific for the VH1 gene and the reverse primer specific for JH4, the VDJ gene (~500bp PCR product) was amplified, sub-cloned in TOPO vectors and sent for sequencing.

Alignment 5g (Appendix) depicts the nucleotide sequences of four representative clones (a total of 22 clones were analyzed). This alignment shows usage of the V_H1 and human J_H4 gene segments, with some portions of the D1v identified. The dots represent identities to germline sequences. As is evident from the alignment these clones have undergone somatic mutation in the form of gene conversion and also some hypermutation. The VH genes on the HC2 construct which have served as sequence donors are highlighted in red beneath the corresponding clones and the donated sequence is highlighted in grey. For example, the V_H4 gene served as donor to clone BS-2-275 and the donated sequence starts from nucleotide 109 to 115 (shaded grey).

The amino acid alignment h shows that all the sequences except for BS-2-278 code for functional proteins. This means that the sequenced clones represent KLH specific transgenic antibody genes which have undergone affinity maturation contrary to the sequences obtained from the transgenic mice above.

This proves that the rabbit Ig *cis* elements require species specific *trans* elements to regulate somatic mutation.

# 4.	Discussion

Following successful gene rearrangement, immunoglobulin V genes are further diversified to amplify their function of binding a multitude of foreign antigen, a process called affinity maturation. In humans and mice affinity maturation is attained by somatic hypermutation (Tonegawa, 1983), a non-templated mutational process during which single nucleotide substitutions are introduced in and around the rearranged V region. On the other hand, chicken and rabbits employ a templated mutational process, gene conversion (Reynaud et al., 1987; Becker and Knight, 1990); although they have been reports of gene conversion in mice (Xu and Selsing, 1994; Tsai et al., 2002).

Using Ig transgenic animals, the mechanisms underlying the gene rearrangement events and the subsequent somatic mutation are still under investigation. Consequently, some Ig *cis* and *trans*- acting regulators have been identified (Jung et al., 2006), and it has also been reported that some of these Ig *cis* regulators function across species boundaries (Mendez et al., 1997; Bucchini et al., 1987; Bruggemann et al., 1991).

In this study, the generation of transgenic animals carrying a rabbit humanized IgH mini-locus is described, with groundbreaking work in the generation of Ig transgenic rabbits. The humanization process described involves a novel approach where only the coding sequences of the rabbit IgH locus are substituted for their human counterparts. The resulting humanized IgH mini-locus contained the required regulators of V gene rearrangement: RSS and splice site, as well as Ig *cis* acting elements including the VH promoter and enhancer regions; all these regulatory elements remaining rabbit endogenous.

It was shown that the rabbit humanized IgH mini-loci successfully rearranged and were employed in transgenic mice and rabbits, but no gene conversion was observed in the transgenic mice, as opposed to transgenic rabbits, where gene conversion of the translocus was observed.

4.1   3HuVs.I5-6 transgenic mice

4.1.1   *Transgene integration*

Initial genotyping of transgenic founders and F1 generation mice was done with PCR technique using genomic DNA from tail tip biopsies. Three different PCR reactions were performed: PCR amplification of the mouse ubiquitous MRP8 gene, the human VH3-23 gene on the 3HuVs.I5-6 construct and finally amplification of a portion of the human Cμ portion of the humanized AY386695. Transgenic mice were positive for both the VH3-23 and the HuCμ portions hinting that the co-injection of the two fragments with a 2 kb overlapping region could have resulted in homologous recombination and the formation of an intact humanized IgH locus.

Since the study was focused on the functionality of the translocus, confirmation of transgene integration using PCR methods sufficed; though by using this rather simple technique, neither the number of integrated sites nor the exact number of integrated copies can be determined.

4.1.2   *Transgene expression*

Using the F1 transgenic mouse 733-2-4, serum antibodies containing human Cμ chains could be identified by "sandwich "ELISA method. Though the measured titre was rather low, it was not uncommon for other plasmid-based generated transgenic mice (Wagner et al., 1996). Furthermore, by flow cytometry it could be confirmed that only about 10% of the B cells express the transgenic human Cμ chains on their cell surface. This low percentage is a feasible explanation for the measured titre of serum antibodies containing human Cμ chains. These observations give the first concrete hints the translocus was functionally employed in the transgenic mouse. To consolidate this preliminary observation, the functionality of the translocus was further investigated by detecting antigen specific serum antibodies.

This was done by immunizing mice with KLH, a potent immunogenic antigen. After immunization, antigen specific antibodies were detected using a "sandwich ELISA". This assay confirmed the presence of serum KLH specific antibodies carrying human Cμ chains.

These experiments reveal that the humanized rabbit IgH min-locus successfully rearranged and was functional in the transgenic mice. The functionality of the mini-locus was confirmed by the detection of serum KLH specific antibodies carrying human Cμ chains. Furthermore, these results confirm the functionality of rabbit IgH *cis* regulators in mice, correlating with the observation of functional rearrangement of a rabbit IgL locus in transgenic mice (Bucchini et al., 1987).

4.1.3   *Somatic diversification of humanized antibodies*

After showing that the humanized mini-locus rearranged and was functionally employed in the transgenic mice, it was next investigated if the antibodies carrying human Cμ chains underwent somatic diversification.

This was done by PCR amplification of rearranged VDJ genes. The organ of choice for the amplification is of paramount importance, since only B cells in germinal centres have undergone affinity maturation; therefore the organ of choice for the VDJ amplification must contain B cells in germinal centres. The mice Peyers' patches (PP) have been reported as optimal organs for PCR amplification of diversified Ig genes (Gonzalez-Fernandez and Milstein, 1993). In this study the PPs of the transgenic mice were therefore also used as the organ of choice for the PCR reactions. Using PCR primers specific for the translocus, two different VDJ PCR products were obtained. These two different VDJ PCR products are explained by prefential J_H usage in the transgenic mice.   In humans the J_H6 and the J_H4 genes are preferentially used in rearranged VDJ genes, (Brezinschek et al., 1995). This preferential usage has also been reported in mice transgenic for the human IgH locus (Gallo et al., 2000). The VDJ_H6 PCR product was sub-cloned and sequenced.

For simplicity reasons, the sequences of just four representative of 20 clones are shown. Upon sequence analysis, done by comparing the obtained sequences to their germ-line counterparts, the usage of the human VH3-23 and JH6 gene segments could be identified. It was difficult to identify D gene segment usage, because most often only portions of the D gene segment are incorporated in the rearranged VDJ gene (Gallo et al., 2000), furthermore, the D segment is the core of the CDR3 region which contribute highly to junctional diversity, whereby nucleotides are erratically added and deleted. The observed junctional diversity of the four representative clones proved that the obtained sequences constitute diverse repertoire and not a mono- or oligo expansion of a limited set of rearrangements. Of the four sequences two have an open reading frame; with the clone BS-2-44 containing a CDR3 region of about 10 amino acids. A length similar to the average CDR3 length reported in adult human sequences (Sanz, 1991).

Although combinatorial (different J_H usage) and junctional diversity were observed, the obtained VDJ clones showed no point mutations; the hallmarks of somatic hypermutation.

The observed junctional diversity is a viable explanation for the detection of serum KLH specific antibodies in the transgenic mice. This would correlate with other reports where antigen binding in transgenic mice was mostly dictated by CDR3 diversity (Suarez et al., 2005). This means that the obtained sequences coding for the humanized antibodies are comparable to human counterparts.

It can be concluded that the rabbit Ig *cis* regulatory elements do permit successful rearrangements of V gene in the transgenic mice, but the Ig *trans* regulatory elements required for the interactions with the *cis* elements to promote somatic mutation are missing.

To ratify this hypothesis, it was necessary to perform a more comprehensive comparison of the methods of somatic mutation between rabbits and mice. This comparison would be more significant by employing rabbits and mice transgenic for the same Ig locus. Thus, transgenic rabbits and mice were generated using the HC2 construct; a humanized rabbit IgH mini-locus with 18 VH, 11D, DQ52, IgE

and IgA4 rabbit gene segments and six JH, IgM and IgG human gene segments. As with the 3HuVs.I5-6 construct all the described Ig *cis* regulatory elements (Maizels, 2005; Jung et al., 2006; Perlot et al., 2005) remained rabbit endogenous.

4.2 HC2 mice

4.2.1 Transgene intergration

Founder animals where genotyped by using genomic DNA obtained from ear-tip biopsies as PCR template for the PCR amplification of a portion of the human $C\mu$ gene segment present on the HC2 translocus. Transgenic founder, 670-8 was then back-crossed with WT mice to generate F_1 generation transgenic animals. F_1 animals were again genotyped by PCR technique and for simplicity reasons only mouse 670-8-1 was used in further experiments.

4.2.2 Transgene employment and expression

Using the F1 transgenic mouse 670-8-1, serum antibodies containing human $C\mu$ chains could be identified by "sandwich "ELISA method. Again low serum titres were observed. To further characterize the employment of the translocus, it was investigated antigen specific antibodies containing $HuC\mu$ chains were produced upon antigen challenge.

After KLH immunization, sera from mouse 670-8-1 and a non-transgenic littermate 670-8-3 were investigated for KLH specific antibodies carrying $HuC\mu$ chains. Using the ELISA technique the production of KLH specific antibodies with $HuC\mu$ chains was confirmed in transgenic mouse 670-8-1 in contrary to the non-transgenic mouse 670-8-3 where the production of KLH specific antibodies was not observed.

These results correlate with the observations for the 3HuVs.I5-6 above, proving that the HC2 transgenic locus was successfully rearranged and functional in the transgenic mouse.

4.2.3   *Somatic diversification of the humanized antibodies*

Following detection of antigen specific antibodies with HuCμ chains, indicating successful rearrangement and functionality of the HC2 translocus in the transgenic mouse, it was next investigated if these antibodies underwent somatic diversification.

Using genomic DNA isolated from the PPs of transgenic mouse 670-8-1, VDJ sequences were PCR amplified, sub-cloned in TOPO vectors and sequenced. For simplicity reasons, only the sequences from six representative of 20 clones are shown. Sequence analysis done by comparing obtained sequences to the germ-line counterparts confirmed usage of the rabbit V_H1, D2x (portions), as well as human J_H6 gene segments was confirmed. Furthermore, extensive junctional diversity is evident, proving the obtained sequences constitute a diverse repertoire of rearranged VDJ genes.

As is evident from the amino acid alignment of the obtained sequences, sequences from clones BS-2-226,227,228 and 231 code for functional humanized antibodies, whereas sequences from clones BS-2-220 and BS-2-221 contain stop codons.

Since these antibodies have been shown to be KLH specific, it would be expected that the obtained sequences show high somatic mutation. In contrary no somatic mutation is evident; the point mutations in clone BS-2-220 (position 168, A to T mutation, position 176, C to G mutation, and position 177, G to A) most probably represent *Taq* polymerase error (Keohavong and Thilly, 1989), in addition this clone contains a stop codon.

Normally, a very high percentage of the VDJ sequences obtained from the PPs of transgenic mice have undergone somatic mutation (Gonzalez-Fernandez and Milstein, 1993; Delbos et al., 2005). Furthermore VDJ sequences from B cells in

germinal centres show the highest mutation rates (Gonzalez-Fernandez and Milstein, 1993). In this study, $B220^+PNA^+$ B cells were sorted from PPs of transgenic mouse 670-8-1 and VDJ sequences PCR amplified. Successful rearrangement is evident (15 clones were analayzed) with the usage of rabbit V_H1, D1v (portions) as well as human J_H4 gene segments. Junctional diversity is also evident but again no somatic mutation events in the rearranged V region are apparent.

The results from the HC2 mice go a long way to buttress the already proposed hypothesis of the lack of necessary Ig *trans* elements to interact with the rabbit Ig *cis* elements to regulate somatic mutation.

Investigation of the HC2 rabbits gave the final substantiation to the hypothesis.

4.3 HC2 transgenic rabbits

4.3.1 *Transgene integration*

Founder rabbits were genotyped by PCR amplification for the HuCμ gene of the HC2 construct. Following this confirmation, F_1 generation rabbits were generated by back crossing transgenic founder TPO3 with WT animals. The F_1 transgenic rabbits were again genotyped by PCR amplification for the HuCμ gene. The F_1 HC2 transgenic rabbit 2017 was thereafter used in the investigations. The generation of rabbits transgenic for a humanized IgH mini-locus represent groundbreaking work in rabbit transgenesis.

4.3.2 *Transgene employment and expression*

Using the ELISA technique, transgenic rabbit 2017 was shown to have serum titres of antibodies containing HuCμ chains Upon immunization with KLH, these antibodies containing HuCμ chains were shown using ELISA technique to be employed in the production of KLH specific antibodies.

4.4.3   *Somatic diversification of humanized antibodies*

A chicken IgL locus, (a gene converting animal) has been shown to successfully rearrange in transgenic mice (Bucchini et al., 1987; Lauster et al., 1993). Though rearrangement was observed, neither expression nor somatic mutation was investigated. In the present study, expression of the humanized rabbit IgH locus is shown and to investigate re-arrangement and somatic diversification, genomic DNA from the PPs of the KLH immunized rabbit 2017 was isolated and used in the PCR amplification of VDJ genes. Primers were specific for VDJ genes carrying V_H1 and human J_H4. The pre-selection for VDJ genes with the V_H1 was to ease comparison with the obtained VDJ sequences from the transgenic HC2 mice with rearranged V_H1 gene. And furthermore, rabbits preferentially utilize the V_H1 gene in their VDJ genes (Becker et al., 1990; Becker and Knight, 1990).

The nucleotide sequence alignment obtained from four representative of 22 clones show usage of the rabbit V_H1, portions of D1v and human J_H4 gene segments. Successful re-arrangement is also evident as well as high junctional diversity. Somatic mutation is also obvious. The hallmarks of gene conversion are apparent: stretches of nucleotides in the re-arranged VDJ clone "donated" by other upstream VH genes (Becker and Knight, 1990). In the nucleotide alignment g, the donor VH genes (shaded in red) are aligned under the rearranged VDJ clones with the donated sequences (grey shadings). All the identified donor sequences are present on the HC2 construct and furthermore, the gene conversion events are also concentrated in the CDR regions, leading to amino acid substitutions as is evident in the amino acid alignment h. All the obtained sequences from the clones, code for functional proteins except, clone BS-2-278, which contains a stop codon. This proves that the sequences code for functional antibodies which have undergone affinity maturation to bind KLH.

In addition to gene conversion, rabbits use hypermutation to diversify the rearranged VDJ genes (Schiaffella et al., 1999). In the obtained sequences, point mutations typical of point mutations are also evident. These point mutations are

summarized in the table below. Since the nucleotide analysis is based on PCR amplifications, Taq polymerase error must be taken into account, but as mentioned above, these PCR errors are composed mainly of A*T to G*C transitions. As is evident from the table below, there is a bias for transitions over transversions (ratio 6:4) a ratio comparable to other published data (Lanning and Knight, 1997).

Table 4.1 Dissection of the somatic hypermutation events from HC2 rabbit

Mutation	Number	Clone	Position	Region
A → N	1	BS-2-275	58	CDR1
G → N	3	BS-2-277	17, 99,169,	FR1, FR1, FR2,
C → N	4	BS-2-278	69, 185	FR1, CDR2,
		BS-2-276	179, 181	CDR2, CDR2
T → N	2	BS-2-278	9, 248	FR1, FR3
Total Mutations	**10**			
Transitions				
G → A	1			
T → C	2			
C → T	3			
Total Transitions	**6**			
Transversions				
A → T	1			
G → C	2			
C → G	1			
Total Transversions	**4**			

From these data it is apparent that, the humanized antibodies from the HC2 mini-locus undergo somatic gene conversion and hypermutation; the mechanisms of affinity maturation described for rabbit Ig genes. Finally, the hypothesis that rabbit Ig *cis* elements require, species specific Ig *trans* elements to regulate somatic mutation is thus consolidated.

In the investigations of the mechanisms underlying somatic mutation, it has previously been postulated that Ig *trans* elements, play a decisive role in the method of somatic mutation employed in the diversification of rearranged IgV genes, (Maizels, 2005). The postulations describe somatic hypermutation and gene conversion to be two methods which could be used to repair a lesion caused by the protein AID in the rearranged IgV gene, figure 4.1. Using chicken bursa lines capable of undergoing gene conversion constitutively it has been shown that in the deficiency of species specific Ig *trans* factors, for example the genes implicated in

homologous recombination, there is a shift from gene conversion to hypermutation (Neuberger, 2002; Sale et al., 2001).

In this study, using a novel transgenic approach a genetic system was used to established that these Ig *trans* acting factors function in a species specific, since the humanized rabbit IgH mini-loci successfully rearranged in transgenic mice but no somatic mutation was evident in contrary, the HC2 locus rearranged and underwent somatic mutation in transgenic rabbits.
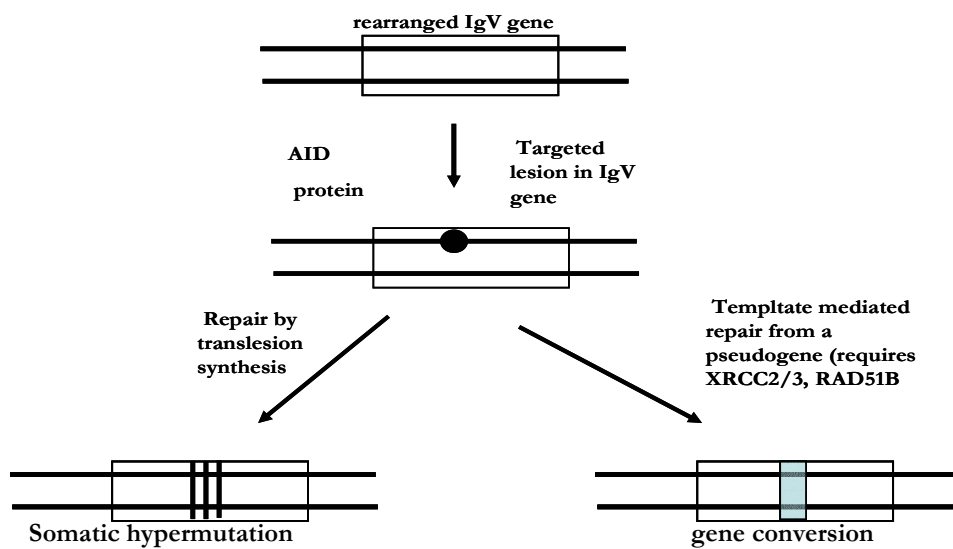
Figure 4.1   Hypermutation and gene conversion represent alternative methods used to repair an AID induced lesion in the rearranged IgV gene. In the absence of *trans* acting factors for example the genes implicated in homologous recombination (XRCC2, XRCC3 and RAD51B), there is a shift in the method of repair from gene conversion to hypermutation, (figure adapted from Neuberger, 2002).

.

5   Sequence Alignments

a)

```
                10        20      FR1     40        50        60        70        80
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Vh3-23-D2x-JH6  tgtttgcaggtgtccagtgtgaggtgcagctgttggagtctggggggaggcttggtacagcctggggggtccctgagactc
BS-2-42         ................................................................................
BS-2-43         ................................................................................
BS-2-44         ................................................................................
BS-2-45         ................................................................................

                 90       100     110     CDR1    130       140    FR2    0        160
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Vh3-23-D2x-JH6  tcctgtgcagcctctggattcacctttagcagctatgccatgagctgggtccgccaggctccagggaaggggctggagtg
BS-2-42         ................................................................................
BS-2-43         ................................................................................
BS-2-44         ................................................................................
BS-2-45         ................................................................................

                         CDR2        190       200       210       220       230       240
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Vh3-23-D2x-JH6  ggtctcagctattagtggtagtggtggtagcacatactacgcagactccgtgaagggccggttcaccatctccagagaca
BS-2-42         .........A......................................................................
BS-2-43         ...........................................|....................................
BS-2-44         ................................................................................
BS-2-45         ................................................................................

                250       260       270       280    FR3   )         300       310       320
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Vh3-23-D2x-JH6  attccaagaacacgctgtatctgcaaatgaacagcctgagagccgaggacacggccgtatattactgtgcgaaa~~~~~~~
BS-2-42         ............................................................................G.~~~~~
BS-2-43         ............................................................................G.~~~~~
BS-2-44         ............................................................................G.~~~~~
BS-2-45         ............................................................................G.~~~AT

                          330       34(   CDR3    50        360       3 0       380       390       400
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Vh3-23-D2x-JH6  ~~~~~~~~gttactatagttatggttatgcttatgctacc~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~a
BS-2-42         ~~~~~~~~GTCCC........................T       FR4             ~~~~~~~~~~~~~~~~~~~
BS-2-43         ~~~GTATCC.GGT......A.CT.G.GCTTA...TACT~~~~                   ~~~~~~~~~~~~~~~~~~~
BS-2-44         ~~~~~~~GT.GGT......AC....A...GC                          T  ~~~~~~~~~~~~~~~~~~~
BS-2-45         TTCCTGGTTT......CTAC..CTAC~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                410       420       430       440       450       460
        ....|....|....|....|....|....|....|....|....|....|....|....|....|
Vh3-23-D2x-JH6  ttactactactactacggtatggacgtctgggggccaagggaccacggtcaccgtctcctcaggt~
BS-2-42         ~~~~~.................................................~
BS-2-43         ~~~~~.................................................~
BS-2-44         ~~~~~..................................................~
BS-2-45         ~~~~~~~.................................................
```

Right-side annotations for part a): **VH3-23**, **N-D-N**, **JH6**

b)

```
                10      FR1   )         CDR1  40   FR2    CDR2       70    FR3   )
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
VH3-23-D2x-JH6  VQCEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKN 80
BS-2-42         ...................................................                             51
BS-2-43         .............................................................................. 80
BS-2-44         .............................................................................. 80
BS-2-45         .............................................................................. 80

                     9(   CDR3  00        110     FR4    :0        130
        ....|....|....|....|....|....|....|....|....|....|....|....|..
VH3-23-D2x-JH6  TLYLQMNSLRAEDTAVYYCAKVTIVMVMLMLPLLLLLRYGRLGPRDHGHRLLR          133
BS-2-42                                                                       51
BS-2-43         ....................R.SWL                                     105
BS-2-44         ....................R.GYSTGNGYYYYGMDVWGQGTTVTVSSG             129
BS-2-45         ....................RDRVYPGYSTRSTTTTTVWTSGAKGPRSPSPQ          132
```

c)

```
                 10        20      FR1      .0        50        60        70        80
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D2x-Jh6   gtgttccagtgtcagtcggtgaaggagtccgagggaggtctcttcaagccaacggataccctgacactcacctgcacagtc
BS-2-220      ................................................................................
BS-2-221      ................................................................................
BS-2-222      ................................................................................
BS-2-226      ................................................................................
BS-2-227      ................................................................................
BS-2-228      ................................................................................
BS-2-231      ................................................................................

                   90       10   CDR1       120      FR2       40        150      160
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D2x-Jh6   tctggattctccctcagtagcaatgcaataagctgggtccgccaggctccagggaacgggctggaatggatcggagccat
BS-2-220      ................................................................................
BS-2-221      ................................................................................
BS-2-222      ................................................................................
BS-2-226      ................................................................................
BS-2-227      ................................................................................
BS-2-228      ................................................................................
BS-2-231      ................................................................................

                 CDR2    180       190     2.. 210       220       230       240
              ....|.       ..|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D2x-Jh6   tggtagtagtggtagcgcatactacgcgagctgggcgaaaagccgatccaccatcaccagaaacaccaacctgaacacgg
BS-2-220      .......T.......GA.............................................................
BS-2-221      ................................................................................
BS-2-222      ................................................................................
BS-2-226      ................................................................................
BS-2-227      ................................................................................
BS-2-228      ................................................................................
BS-2-231      ................................................................................

                 FR3   260       270       280       290       300       310       320
              ....       |....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D2x-Jh6   tgactctgaaaatgaccagtctgacagccgcggacacggccacctatttctgtgcgaga~
BS-2-220      ....................................................G.~
BS-2-221      ....................................................G.~
BS-2-222      ....................................................G.~
BS-2-226      ....................................................~
BS-2-227      ....................................................~
BS-2-228      ....................................................~
BS-2-231      ....................................................~

                 CDR3  340       350       360       370       380       390       400
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D2x-Jh6   ~~~gttactatagttatggttatgcttatgctacc~
BS-2-220      ~~~~............................CG
BS-2-221      ~~~~~TA.CC.......AG.AC..G.T
BS-2-222      ~~~~~TA.CC.......AG.AC..G.T.T
BS-2-226      ~~~~C...........................GG
BS-2-227      ~~~~GA.......................C..
BS-2-228      ~~~~GA..GG.CCC......AG.AC..GCC
BS-2-231      ~~~~G.........................G.

                      410       420     430    FR4    450       460
              ....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D2x-Jh6   ~~attactactactacggtatggacgtctggggccaagggaccacggtcaccgtctcctcaggt~
BS-2-220      ~~~~~...........................................................~
BS-2-221      ~~~~~...........................................................~
BS-2-222      ~~~~~...........................................................~
BS-2-226      ~~~~~...........................................................~
BS-2-227      ~~~~~...........................................................~
BS-2-228      ~~~~~...........................................................~
BS-2-231      ~~~~~...........................................................~
```

VH1

N-D-N

JH6

d)

```
                 10      FR1       30  CDR1    FR2   CDR2      70        80
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D2x-JH4   VQCQSVKESEGGLFKPTDTLTLTCTVSGFSLSSNAISWVRQAPGNGLEWIGAIGSSGSAYYASWAKSRSTITRNTNLNTV
BS-2-220      .............................................................C.RT..............
BS-2-221      ...............................................................................
BS-2-222      ...............................................................................
BS-2-226      ...............................................................................
BS-2-227      ...............................................................................
BS-2-228      ...............................................................................
BS-2-231      ...............................................................................

                   FR3       100      CDR3     120       130
              ....      |....|....|....|....|....|....|....|
VH1-D2x-JH4   TLKMTSLTAADTATYFCARVTIVMVMLMLPLLLLLLRYGRLGPRDHGHRLLR
BS-2-220      .................G
BS-2-221      .................VSWL
BS-2-222      .................VSWL
BS-2-226      .................HYSYGYAYATATVWTSGAKGPRSPSPQ
BS-2-227      .................DIVMVMLMLPTTTTVWTS.AK...SPSPQ
BS-2-228      .................DRALVIV.A.L...RYGRLGPRDHG.RLLR
BS-2-231      .................DYSYGYAYGTTTTTVWTS.AK...SPSPQ
```

e)

```
                   10        2(   FR1  30        40        50        60        70        80
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D1v-HJH4    gtccagtgtcagtcggtgaaggagtccgaggggaggtctcttcaagccaacggataccctgacactcacctgcacagtctc  80
424             ...........................................................................  80
427             ...........................................................................  80
429             ...........................................................................  80
432             ...........................................................................  80


                            90        100  CDR1         120       130   FR2            150      160
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D1v-HJH4    tggattctccctcagtagcaatgcaataagctgggtccgccaggctccagggaacgggctggaatggatcggagccattg  160
424             ...........................................................................  160
427             ...........................................................................  160
429             ...........................................................................  160
432             ...........................................................................  160


                        17    CDR2     0         190       20        210       220       230      240
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D1v-HJH4    gtagtagtggtagcgcatactacgcgagctggggcgaaaagccgatccaccatcaccagaaacaccaacctgaacacggtg  240
424             ...........................................................................  240
427             ...........................................................................  240
429             ...........................................................................  240
432             .........G......C..........................................................  240


                      250       270       280       290       300    CDR3          320
                ....|....|....|....|....|....|....|....|....|....|....|....|....|...|
VH1-D1v-HJH4    actctgaaaatgaccagtctgacagcgcgggacacggccacctatttctgtgcgaga~~~~~agctacgatgactatgg  314
424             ...........................................~~~~~G.T.CT.AG.T.TGC...  315
427             ...........................................~~~~~GT.GGGTT..AGTAC...  315
429             ...........................................~~~~~G.T.CT.AG.T.TGC...  315
432             .....C.....................................~~AAG.TGC.T..A.T.....  317


                      330       340       350       360       370       380   FR4        400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|...|....|
VH1-D1v-HJH4    tgattac~~~~~~~~~~~~~~~~~~~~actactttgactactggggccagggaaccctggtcaccgtctcctc  367
424             .AG.AGA~~~~~~~~~~~~~~~~~~~~......................~~..  343
427             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~........................~~..  338
429             .AG.AGA~~~~~~~~~~~~~~~~~~~~......................~~..  343
432             .CC.GCTTATGCTAGCA~~~~~~~~~~~~......................~~..  356
```

VH1

N-D-N

JH4

f)

```
                  10       FR1        30  CDR1       FR2       CDR2      0        80
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D1v-HJH4    VQCQSVKESEGGLFKPTDTLTLTCTVSGFSLSSNAISWVRQAPGNGLEWIGAIGSSGSAYYASWAKSRSTITRNTNLNTV  80
BS-2-424        ...........................................................................  80
BS-2-427        ...........................................................................  80
BS-2-429        ...........................................................................  80
BS-2-432        ...........................................................................  80


                 FR3                    100        CDR3       120
                .       ....|....|....|....|..        ..|....|
VH1-D1v-HJH4    TLKMTSLTAADTATYFCARSYDDYGDYTTLTTGAREPWSPSPQ  123
BS-2-424        ...................DPQLCW  105
BS-2-427        ...................VGL  102
BS-2-429        ...................DPQLCW  105
BS-2-432        ..................VVVVLVLLLLRYGRL.PRDHGHRLLR  125
```

g)

```
                10      FR1   30      40      50      60      70      80
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D1v-HJH4 ~tgtccagtgtcagtcgg~~~tgaaggagtccgagggaggtctcttcaagccaacggataccctgacactcacctgcaca  76
BS-2-275     G................................................C............................  77
VH4          G..........~~~...G.......AG......C..GA........................................  77
BS-2-276     G..........~~~..............................................................  77
VH11         G........GA.CAGC..........G......C..GG...C...TGGA.GA.T....T...................  80
BS-2-277     G.......A...~~~..............................................................  77
VH9          ~.......A.~~~................................................................  76
BS-2-278     G......C...~~~..........................................T....................  77
VH6          ~........GA.CAGC........GAA....C..GG..C...TGG..GAT..........................  79

                90      100    CDR1   120     130    FR2    150     160
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D1v-HJH4 gtctctggattctccctcagtagcaatgcaataagctgggtccgccaggctccagggaacgggctggaatggatcggagc  156
BS-2-275     .....................GT...G.G.G.T..................................A.  157
VH4          ...................T...G.G.G.T...........................AT.....A.  157
BS-2-276     .C................A...A....T.CAG...GG.............................  157
VH11         .C................A.......T.CAG...GG...................G..........AC......TA  160
BS-2-277     ..................A...T.......................................  157
VH9          ...................T.......................................AT  156
BS-2-278     .C...C........A..A........G.T..............................  157
VH6          .C...C.....A..A........G.T............G...........AC......TT  159

                170     180    CDR2   200     210     220     230     240
             .|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D1v-HJH4 cattggtagtagtggtagcgcatactacgcgagctgggcgaaaagccgatccaccatcaccagaaacaccaacctgaaca  236
BS-2-275     ............................................................  237
VH4          ............................................................  237
BS-2-276     ......................T.T...................................  237
VH11         ....A..TACG...........AA....T....G.....T....T...~~~~.A~.C.TCA.G.  234
BS-2-277     .....C.....................................................  237
VH9          ....AA....TA....A.....................................GA.....  236
BS-2-278     ..................G........................................  237
VH6          ....AA..C.G.......A.TA..........TG.....T......T....CG...A.GC..A.....  239

                250     FR3   70      280     290     300     310     320
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D1v-HJH4 cggtgactctgaaaatgaccagtctgacagccgcggacacggccacctatttctgtgcgaga~~~~~~~  298
BS-2-275     ....................................................~~~~~TCTAA  304
VH4          ...........................................GA~........301
BS-2-276     ...............................................~~~~~TCTAAT  305
VH11         ....GA.....................T......A.........C...GA~  298
BS-2-277     ..........................................................  299
VH9          ..........................................................  298
BS-2-278     ......C...........................................~~TTC  302
VH6          ....T.....C......A...............CTATTT..G.GC.A.A  295

                330     CDR3   350     360     370     380     390     400
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D1v-HJH4 ~~~~agctacgatgactatggtgattac~actactttgactactggggccagggaaccctggtcaccgtctcctcaggt  372
BS-2-275     AACG.C~CTACGATGACTAT.ACGGG~~~~.......................  355
VH4                                                             301
BS-2-276     ACGAG.~~CTACGATGACTAT.ACGGG~~~~......................  355
VH11                                                            298
BS-2-277     ~~~G.T.CTG.T.ATAGTATTG.TACGGG..........................  348
VH9                                                             298
BS-2-278     GATGCT.CTG.T.ATAGTATTG.TA.GGG~~~~....................  350
```

VH1

N-D-N-JH4

h)

```
                10      FR1        CDR1   FR2       CDR2       70      80
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
VH1-D1v-HJH4 VQCQSVKESEGGLFKPTDTLTLTCTVSGFSLSSNAISWVRQAPGNGLEWIGAIGSSGSAYYASWAKSRSTITRNTNLNTV  80
BS-2-275     ....................A.........RYGVI............T..................  80
BS-2-276     ....................A....IN.YRMG............V...................  80
BS-2-277     .....................N.Y.................T...................  80
BS-2-278     ..R...............F..A..TI....MI.......................  59

                FR3        100    CDR3       120
             ....|....|....|....|....|....|....|....|
VH1-D1v-HJH4 TLKMTSLTAADTATYFCARSYDDYGDYTTLTTGAREPWSPSPQ  123
BS-2-275     ...................KTT.D..DGYFDYWGQG  117
BS-2-276     ...................NTR.D..DGYFDYWGQG  117
BS-2-277     ...................DPGYSIGTGLT.GARE  115
BS-2-278                                         59
```
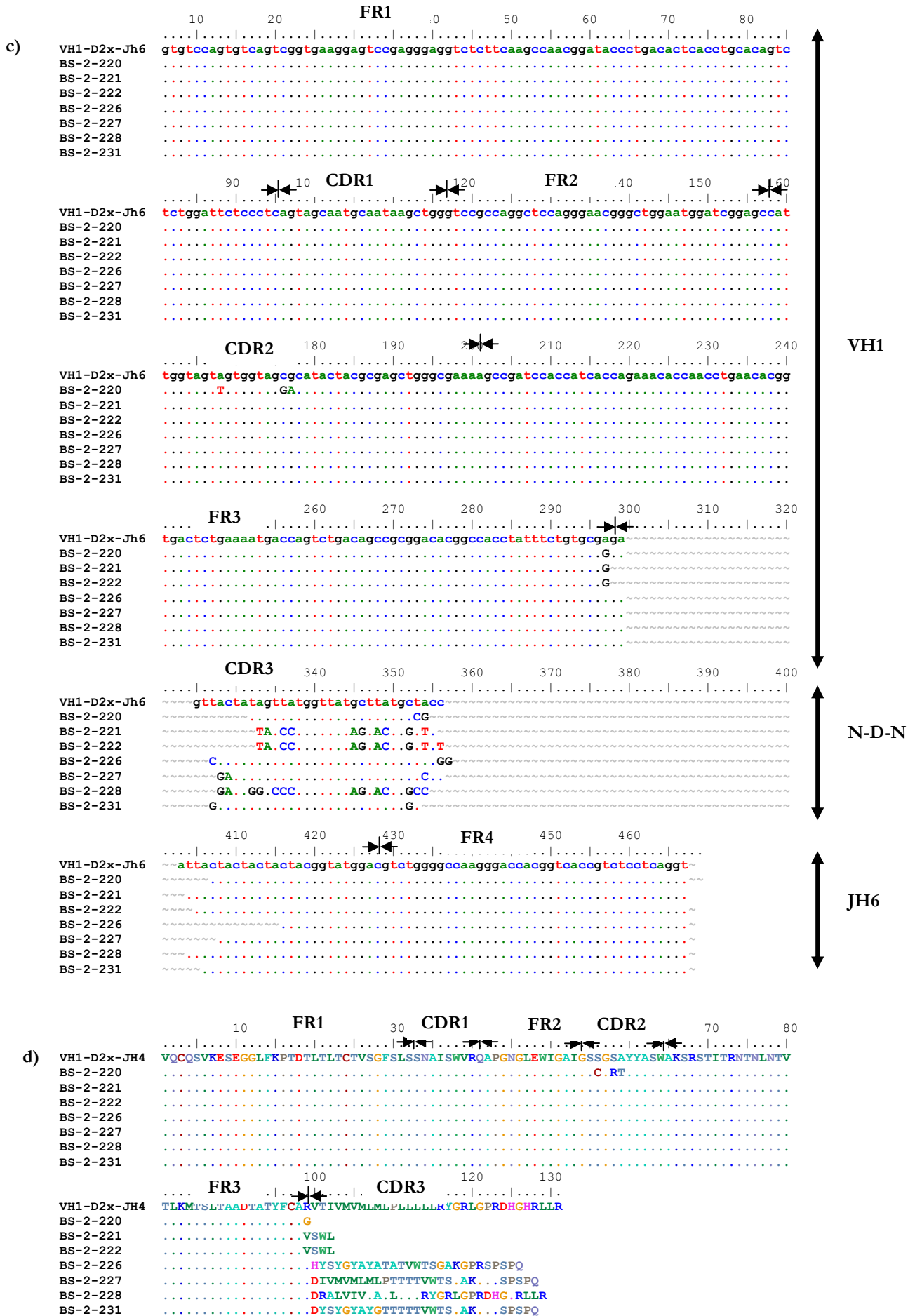
6. Summary

Somatic mutation of rearranged immunoglobulin genes (IgH and IgL) is a key process because it leads to the diversification of the antibody chains, a vital progression in the affinity maturation process whereby antibody-antigen binding is enhanced.

Two different mechanisms of somatic mutation have been described and attributed to different species: somatic hypermutaion (in man and mouse) occurs by fixation of individual non-templated nucleotide substitutions, whereas gene conversion (in rabbits, sheep, birds and cattle) occurs by templated substitutions with sequences donated by upstream pseudogenes to the rearranged IgV gene segment. These two processes have been described to be alternative methods employed in revamping a lesion caused by the protein Activation Induced Deanimase (AID) in the rearranged IgV gene segment. The decision which method is thereby used depends on interplay of *cis* (Ig promoter and enhancer) and *trans* (genes involved in homologous recombination) elements.

In this work a transgenic vector is created using a novel approach, whereby the coding sequences of a rabbit IgH locus are substituted with their human counterparts.

In the first part of the study, this transgenic vector is used to generated transgenic mice, and somatic diversification of the humanized antibodies investigated; the question posed being what would be the result of the interaction between rabbit (a gene converting animal) *cis* regulatory elements (on the transgenic vector) and the trans elements of the mouse host (a hypermutating animal)? The results advocate a species specific activity of the Ig *cis* and *trans* elements as no somatic hypermutation was observed, albeit successful rearrangement and employment of the translocus.

In the second part of the work, a second humanized rabbit transgenic vector was used to generate transgenic rabbits and mice and the somatic diversification of the humanized antibodies investigated. Mice and rabbits transgenic for the same locus proffer a much comprehensible comparison of somatic hypermutation in these animals. The question to be answered in this second part was would the humanized antibodies undergo somatic hypermutation in the transgenic mice and gene conversion in the transgenic rabbits? The results give credit to the hypothesis from the first part: the humanized antibodies from the transgenic mice showed no somatic hypermutation, while those from the transgenic rabbits did undergo effective gene conversion.

In total these results argue for a species specific interaction of Ig *cis* and *trans* regulatory elements in determining the method of somatic mutation employed.

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