Analysis of Locus Accessibility for V(D)J Recombination and its Potential in Generating a Mouse Model for Monitoring RAG Protein Expression in Peripheral B cells

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Table of contents

1. INTRODUCTION 1

1.1 B LYMPHOCYTES 1
1.1.1 ANTIBODIES AND THE B CELL RECEPTOR (BCR) 1
1.1.2 B CELL DEVELOPMENT 3
1.1.3 B CELLS IN AN IMMUNE RESPONSE 4
1.2 GENERATION OF ANTIBODY DIVERSITY 5
1.3 EFFECTORS OF V(D)J RECOMBINATION: THE RAG PROTEINS 6
1.4 CONTROL OF V(D)J RECOMBINATION 8
1.5 ALLELIC EXCLUSION 9
1.6 RAG EXPRESSION IN PERIPHERAL B CELLS 11
1.7 AIM OF THE THESIS 12

2. MATERIALS AND METHODS 14

2.1 MOLECULAR BIOLOGY 14
2.1.1 POLYMERASE CHAIN REACTION (PCR) 14
2.1.2 DNA PREPARATION FROM MOUSE TISSUES AND ES CELLS 16
2.1.3 SOUTHERN BLOT ANALYSIS 16
2.1.4 RNA PREPARATION AND COMPETITIVE RT PCR 17
2.1.5 CLONING OF TARGETING VECTORS 18
2.1.6 TRANSFECTIONS OF 294 CRE AND FLP EXPRESSING BACTERIA 22
2.2 CELL CULTURE AND TRANSFECTIONS 22
2.2.1 ES CELL CULTURE 22
2.2.2 B CELL CULTURE 23
2.2.3 HANDLING OF HELA AND 293 CELLS 24
2.3 IMMUNOSTAININGS 24
2.3.1 FACS ANALYSIS 24
2.3.2 MICROSCOPE SLIDE STAININGS 25
2.3.3 MAGNETIC CELL SORTING 26
2.3.4 ELISA 26
2.4 MICE 26
2.4.1 IMMUNISATION 26
2.4.2 BONE MARROW TRANSFER 27
2.4.3 TYPING PROTOCOLS 27

3. RESULTS 28

3.1 RAG EXPRESSION REPORTER LINE 28

3.1.1 ANALYSIS OF CD21 EXPRESSION 29
3.1.2 GENERATION OF THE CD21 TARGETING CONSTRUCT 33
3.1.3 CRE RECOMBINATION 33
3.1.4 RAG RECOMBINATION 35
3.1.5 TARGETING OF THE CD21 LOCUS 38
3.1.6 ANALYSIS OF CD21 REPORTER MICE 42
3.2 ANALYSIS OF DEPENDENCE OF RAG MEDIATED RECOMBINATION AND TRANSCRIPTION 46
3.3 ANALYSIS OF ALLELIC EXCLUSION IN μMT MICE 49
3.4 CONDITIONAL DISRUPTION OF IGM MEMBRANE EXONS 56

4. DISCUSSION 59

4.1 RECOMBINATION ACTIVATING GENE (RAG) PROTEIN EXPRESSION IN PERIPHERAL B CELLS 59
4.2 V(D)J RECOMBINATION AND TRANSCRIPTION 63
4.3 ESTABLISHMENT OF ALLELIC EXCLUSION IN THE *μ*MT MOUSE 65

5. SUMMARY 70

6. ZUSAMMENFASSUNG 71

7. REFERENCES 73

8. VERSICHERUNG 79

9. ACKNOWLEDGEMENTS 80

10. LEBENSLAUF 81

ABBREVIATIONS

Ab(s)	antibody(ies)
Ag	antigen
BCR	B Cell Receptor
BM	Bone Marrow
bp	Base pair
BSA	Bovine Serum Albumin
CD	cluster of differentiation
cDNA	Complementary DNA
CG	Chicken γ-globin
CyC	CyChrome
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
DNTP	2' deoxyribonucleosidetriphosphate
DTT	dithiothreitol
EDTA	ethylene-diaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EF	embryonic fibroblasts
ES cells	embryonic stem cells
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Flox	*loxP* flanked region
FSC	forward scatter
G418	geneticin
Ig	immunoglobulin
IL	interleukin
i.p.	intaperitoneal
i.v.	inteavenous
kb	kilobase pairs
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
2-ME	2-mercaptoethanol
MACS	magnetic cell sorting
MHC	major histocompatibility complex

mmC	mitomycin-C
mRNA	messenger RNA
neo^r	neomycin resistance
NP	4-hydroxy-3-nitrophenylacetyl
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFU	plaque forming units
RAG	recombination activating gene
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Rosewell-Park-Memorial-Institute cell culture medium
RT	Room temperature
SA	streptavidin
SDS	sodium dodecyl sulfate
TCR	T cell receptor
Tg	transgenic
Tk	thymidine kinase
Tris	Tris(hydroxymethyl)aminoethane
UV	ultraviolet
VSV	Vesicular stomatitis virus
wt	wild type

1. Introduction

The immune system has evolved in vertebrates for the protection of the organism against foreign, potentially pathogenic, microorganisms or parasites. The vertebrate immune system consists of an innate and an adaptive component. The innate immune system functions as a first line of defence against a broad range of pathogens. The adaptive immune system, on the other hand, is characterised by high specificity, ability 'to learn' and memory. Invasion of any foreign agent elicits an immune reaction, which can be divided into two interrelated activities, recognition and response.
Recognition by the adaptive immune system is 'precise'. It has evolved so as to recognise foreign, non-self components with high specificity. Subtle differences in the protein or polysaccharide content of invading microorganisms can be detected and subsequently lead to a different response.
The goal of the elicited immune response is the most efficient clearance of the invading agent. Two distinct, although tightly interdependent, processes can be activated: humoral and cellular responses. The key effectors of the humoral immune response are B lymphocytes, while T lymphocytes are mostly involved in the cellular response pathway.

1.1 B Lymphocytes

1.1.1 Antibodies and the B Cell Receptor (BCR)

B cells are the key effectors of the humoral immune response since they can produce antibodies. Antibodies are tetrameric protein molecules composed of immunoglobulin heavy and light chain heterodimers. Disulfide bonds link the heavy chains, but also exist

between heavy and light chains. Each chain has a constant region (C) and a variable region (V); the variable region is highly divergent and mediates antigen recognition and binding. The constant region is conserved, although there are several IgH constant region variants, termed immunoglobulin isotypes (α,δ,ε,γ) and two different light chains (κ and λ) (Honjo and Alt, 1995).

B cells can secrete antibody but additionally express it on their surface (surface immunoglobulin or sIg), anchored to the membrane. Surface immunglobulin associates with the Igα /Igβ heterodimer which functions as a signal transducing entity. Surface Ig together with Igα /Igβ forms the B Cell Receptor (BCR) (Reth, 1992) (Schamel and Reth, 2000) (Figure 1).

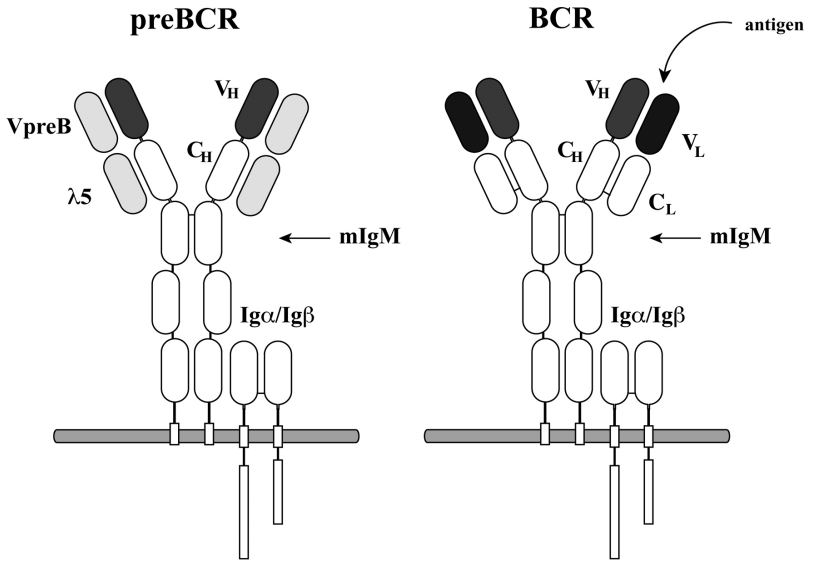


Figure 1: The murine BCR consists of two identical heavy chains (of any isotype: μm, δm, εm, γm and αm; shown here is μm) and two identical light chains (κ or λ) which form the membrane bound immunoglobulin (mIg). The mIg can bind antigen specifically with its variable region (shown in grey) whereas the membrane proximal and transmembrane constant region (shown in white) associates with an Igα/Igβ heterodimer. These molecules contain tyrosine based activation motifs (known as ITAMS) in their cytoplasmic tails thereby coupling the receptor to intracellular signalling molecules.

Depending on the developmental status and the micro environment of the B cell, the strength and quality of BCR transmitted signals control survival, differentiation and proliferation (Healy and Goodnow, 1998; Nemazee, 2000).

1.1.2 B cell development

The governing force in B cell development is the generation of B cells expressing a B cell receptor on their surface and subsequently ensuring that this BCR does not recognise self-antigens. In mice and humans, B cell generation occurs during embryogenesis in liver and spleen. After birth, it is largely restricted to the bone marrow. B cell development in the bone marrow can be divided into distinct steps that are easily identified by the differential expression of surface antigens (Figure2; Hardy et al., 1991; Rajewsky, 1996).

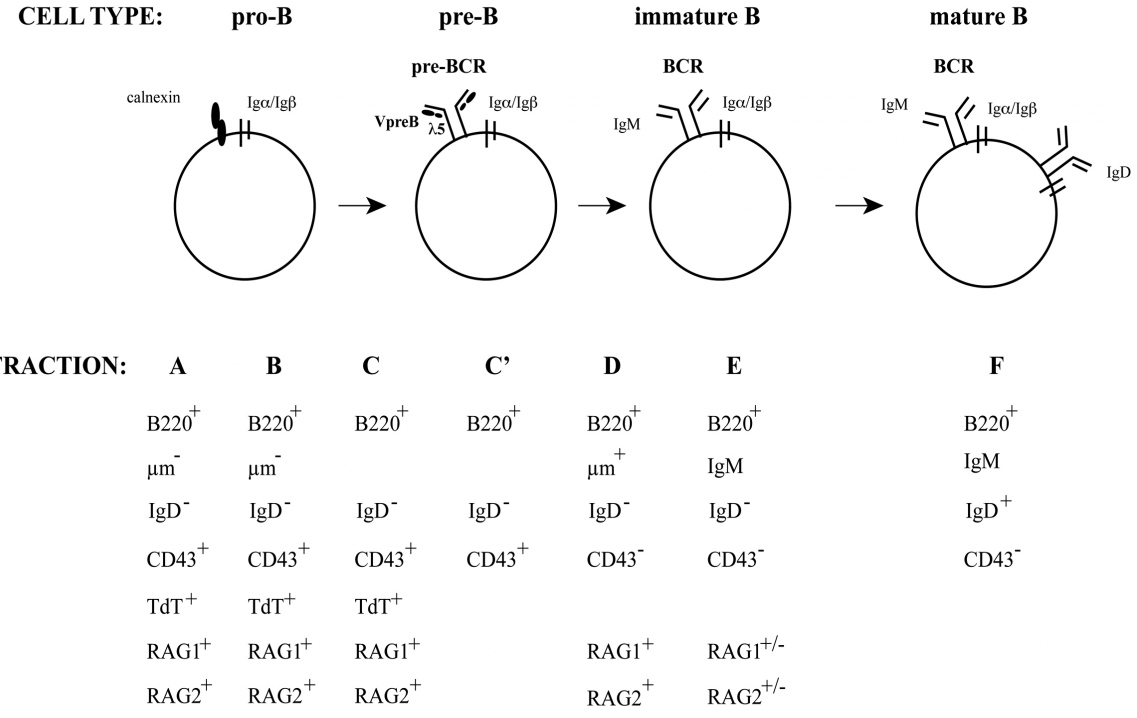


Figure 2: B cell development in the bone marrow. Classification of the various fractions is primarily defined by the rearrangement status of the immunoglobulin heavy and light chains and by the expression pattern of surface markers (adapted from Hardy et al., 1991).

In most cases, B cells synthesize first a heavy chain, which is expressed on the surface of the cells together with surrogate light chains (VpreB and λ5) (Sakaguchi and Melchers, 1986; Takemori et al., 1990) and Igα/Igβ; this complex is known as the pre-B cell receptor and is essential in signalling for cell survival and progression to the precursor (pre-) B cell stage (Kitamura et al., 1991). Light chain is subsequently

produced in pre-B cells, and substitutes the surrogate light chains (Coffman and Weissman, 1983) thus forming the BCR. Immature B cells express a BCR of a single specificity on their surface. Monospecificity is achieved by two processes, termed allelic exclusion and light chain isotype exclusion (Gorman and Alt, 1998) (see Chapter 1.5) which ensure that each lymphocyte expresses Ig protein from only one heavy and light chain locus and thus produces an antigen receptor of a given specificity.
The specificity of the receptor is controlled before cells leave the bone marrow, in order to select against autoreactive B cell receptors. These are eliminated either by modification of the receptor (see Chapter 1.6) or deletion of autoreactive B cells (Nemazee and Buerki, 1989).

1.1.3 B cells in an immune response

Mature Ig expressing B cells (IgM^+, IgD^+) then leave the bone marrow and migrate to peripheral lymphoid organs, where they persist for weeks or months. These B cells can become activated upon encounter with antigen. Multimeric antigens often directly stimulate B cell proliferation and differentiation into antibody producing plasma cells by cross-linking of the BCR, while protein antigens require T cell help for B cell activation (reviewed in Rajewsky, 1996).
In a T cell dependent response an oligoclonal population of antigen activated B cells starts proliferating and differentiating in primary follicles located in peripheral lymphoid organs. This requires the presence of antigen specific helper T cells and follicular dendritic cells (FDC). Rapidly expanding antigen-activated B cells form a structure known as germinal centre, where they undergo isotype switching, affinity maturation and terminal differentiation into plasma and memory cells. Naïve IgM^+IgD^+ cells recruited to the germinal centre can switch to synthesis of other immunoglobulin isotypes (IgG, IgE, IgA) (Esser and Radbruch, 1990). These cells additionally diversify their Ig receptor by directed point mutations of the Ig heavy and light chain genes (Jacob et al., 1991). This later process is called somatic hypermutation and is mediated by a yet elusive enzyme, possibly an error prone DNA polymerase (Goodman and Tippin, 2000). Mutation of Ig heavy and light chains generates a pool of different antibody specificities, which are then selected for their ability to bind the immunogenic antigen with higher affinity, while potentially autoreactive and low affinity mutants are eliminated (Allen et al., 1987; Berek, Jarvis and Milstein, 1987).
The process of affinity maturation contributes to the diversity of the secondary antibody repertoire, but already before, in the naïve B cell compartment a large number of diverse receptor specificities are present. The specificity is conferred to the BCR by the variable region of immunoglobulin heavy and light chains.

1.2 Generation of antibody diversity

The enormous diversity of antigen receptors is primarily achieved by random assembly of the variable region of both heavy and light chain genes by a 'cut-and-join' process known as V(D)J recombination.
Both heavy and light chains are not single gene entities; they are composed of a different number of gene segments, which have to be assembled in order to produce the functional protein. Genes encoding Ig heavy chains (IgH genes) are formed by first rearranging a D_H to a J_H element. Upstream V_H elements are then recombined to the D_HJ_H joint in order to form a $V_HD_HJ_H$ segment, which codes for the variable region of the IgH (Figure 3). Assembly of the light chain is simpler, since only a V_L to J_L joint has to be formed (Figure3).
The murine heavy chain locus contains between 100-1000 V_H elements (Honjo and Alt, 1995), about 15 D_H elements (Chang, Paige and Wu, 1992) and 4 J_H elements. The κ light chain locus consists of 93 Vκ and 5 Jκ gene segments (Thiebe et al., 1999) whereas the λ locus has a different structure with three functional subsets of V, J and C elements. The 'random' rearrangement of these modules in order to generate the variable region of IgH and IgL molecules is mainly responsible for the observed diversity of the antibody repertoire. Another phenomenon, which contributes to the diversity of the antibodies produced, is the random deletion or addition of untemplated nucleotides, called N and P nucleotides, at V(D)J junctions .

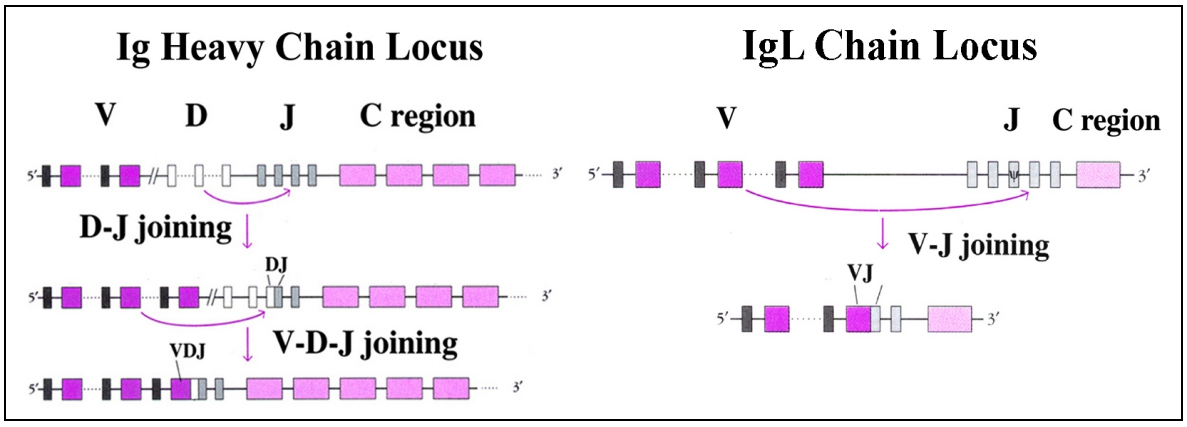


Figure 3: Organisation of the murine immunoglobulin heavy and light chain loci and schematic representation of the steps necessary to form a variable region (adapted from Kuby).

These recombination events take place in the bone marrow: heavy chain rearrangement generally occurs first, in pro-B cells (or using a different nomenclature cells of Fraction A-C'), while light chain rearrangement follows in the pre-B cell compartment (Fraction D) (Hardy et al., 1991; ten Boekel, Melchers and Rolink, 1995). Recombination is not

precise, often leading to out of frame coding units. These recombination events are termed non-productive. If on the other hand, the joints are in a contiguous open reading frame, the rearrangements are referred to as productive. Statistically, less than one third of possible rearrangements leads to a productive joint and therefore to a full-length protein.

1.3 Effectors of V(D)J recombination: the RAG proteins

Many of the enzymes involved in V(D)J recombination have been identified; most of them are ubiquitously expressed double strand break repair enzymes (Ku70, Ku80, DNAPKcs, XCCR4 and DNA ligase IV proteins) (Critchlow and Jackson, 1998) but two are lymphoid lineage specific proteins, named Recombination Activating Genes (RAG1 and RAG2). The RAG proteins were identified about fifteen years ago as the only necessary components for the recombination event (Oettinger et al., 1990; Schatz and Baltimore, 1988). Co-transfection of RAG-1 and RAG-2 expression constructs is sufficient for recombination to occur in many non-lymphoid cell lines. These proteins specifically recognise and bind to conserved sequence motifs flanking the V, D and J segments, named Recombination Signal Sequences (RSS)(Sakano et al., 1979). RSSs are composed of a conserved heptamer, a relatively non-conserved spacer region of 12 or 23 bp and a conserved nonamer (Figure 4a).
The stoichiometry of the reaction is not accurately known, but two RSS sequences, with different spacer regions (12/23 bp rule) (van Gent, Ramsden and Gellert, 1996) are brought together, possibly by two RAG-1/RAG-2 complexes (tetramer consisting of two molecules of each RAG protein) (Bailin, Mo and Sadofsky, 1999). RAG-1 mediates RSS recognition primarily through the nonamer, with a region which is homologous to the DNA-binding domain of bacterial invertases, such as Hin, and to homeodomain proteins (Difilippantonio et al., 1996; Spanopoulou et al., 1996). The DNA-bound RAG-1 recruits RAG-2 and high mobility group proteins (HMG-1, HMG-2) (Aidinis et al., 1999) to the complex. No sequence-specific binding of RAG-2 to DNA has been reported, but the binding of RAG-1 is enhanced by recruitment of RAG-2 and the protein-DNA contacts are then extended into the heptamer region (Swanson and Desiderio, 1998). These contacts seem to distort the DNA around the heptamer-coding region and nicking occurs at the border of the heptamer and the coding sequence; this is followed by a transesterification reaction in which the free 3' hydroxyl group attacks a phosphodiester bond on the opposite strand (van Gent, Ramsden and Gellert, 1996). As a result, two DNA ends are produced: a signal end terminating in a 5'-phosphorylated, double strand break and a coding end terminating in a hairpin. The ligation of the signal end, which occurs at the base of the two heptamers is relatively precise but the coding

joint is deliberately imprecise. The hairpin structure after nucleolytic opening can function as a substrate for deletion of nucleotides or the addition of untemplated (N) nucleotides by terminal deoxynucleotidyl transferase (TdT) (Desiderio et al., 1984; Grawunder and Lieber, 1997). Additionally, short stretches of palindromic (P) nucleotides are generated from asymmetric opening of the hairpin coding ends, producing self-complementary single stranded extensions that can be incorporated into the coding joints (Lewis, 1994).
Studies of artificial recombination substrates have shown that the RSSs are both necessary and sufficient to direct V(D)J recombination. Plasmid substrates containing RSSs can efficiently recombine in cells transfected with RAG expression vectors (Hesse et al., 1987). The recombination signal sequences are asymmetrical and their orientation is important for the recombination event. Depending on the orientation of the RS sequences, recombination can lead either to deletion or inversion of the intervening DNA (Figure 4b).

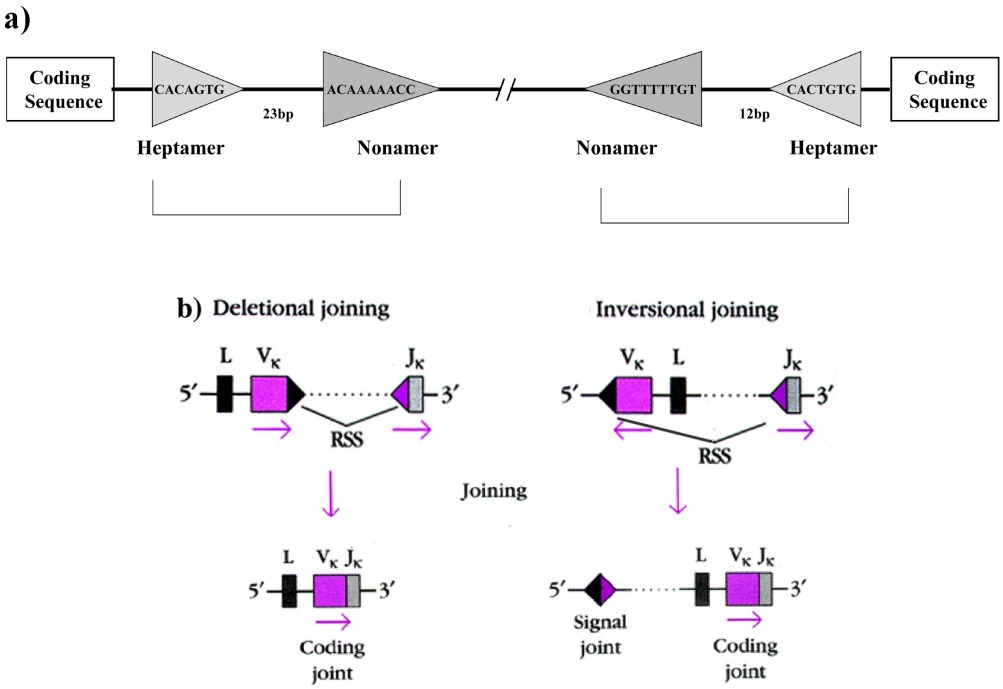


Figure 4: a) The signal sequences consist of a conserved palindromic heptamer and a conserved AT-rich nonamer; these are separated by non conserved spacer regions of either twelve or twenty-three base pairs b) Depending on the orientation of the RSS sequences RAG recombination can lead to deletion or inversion of the intervening substrate.

Both deletional and inversional recombination events occur in vivo. Deletion is more common and takes place during the assembly of the IgH chain and T Cell Receptor loci,

while inversion has been detected in the human and the murine, Igκ locus (Honjo and Alt, 1995; Thiebe et al., 1999).

1.4 Control of V(D)J Recombination

One of the fascinating aspects of VDJ recombination is that there is a strict temporal and tissue specific control of the recombination events. Developing lymphocytes harbour seven complex immune receptor loci (four T Cell Receptor and three Ig) that are rearranged by a common recombinase. RAG proteins are expressed throughout B cell development in the bone marrow, and are also expressed in developing thymocytes (for the rearrangement of TCR loci). Yet, complete rearrangement of Ig loci occurs exclusively in B cells and of TCR loci in T cells (Alt et al., 1992). Furthermore, the rearrangement proceeds in an ordered fashion: in developing B cells IgL loci will start rearranging mainly after productive assembly of the IgH locus (Alt et al., 1981) and a similar phenomenon is observed in T cells. To explain the apparent paradox that recombination does not readily occur, although both the necessary enzymes (RAG1,2) and the substrate for the reaction are present, the 'accessibility hypothesis' was formulated.

This hypothesis postulates that Ig and TCR genes typically reside in a chromatin structure that is refractory to recognition by the recombination machinery (Alt, Blackwell and Yancopoulos, 1987; Yancopoulos and Alt, 1986). In lymphoid cells, developmental signals lead to changes in chromatin that allow the recombinase access to particular gene segments but not to others. To test this hypothesis the overall configuration of rearranging versus non-rearranging loci was analysed. Hence it was reported that active V(D)J recombination correlates with transcriptional activity, DNA demethylation, and increased DNaseI sensitivity of the rearranging loci, all of which can be considered hallmarks of chromatin remodelling.

Although all three of these features have been associated with enhanced V(D)J recombination, none of them perfectly predicts which joining signals will be available for rearrangement. Germline transcription, for example, from unrearranged V_H gene segments can be detected just prior to heavy chain rearrangement (Yancopoulos and Alt, 1986) and seems to correlate with preferential usage of certain V_H elements. Such germline transcripts cannot be detected in B cells that have already rearranged their IgH locus (Mather and Perry, 1981). Also transcription and recombination seem to correlate well in several inducible systems (Schlissel, Corcoran and Baltimore, 1991), but rearrangement in the absence of detectable transcription has been found in other cases (Lauster et al., 1993)(Engler et al., 1991).

Nuclease sensitivity provides another means of monitoring changes in chromatin configuration. Studies with introduced substrates support a relationship between DNaseI

sensitivity and recombination (Ferrier et al., 1989) while DNAseI hypersensitive sites appear in TCR or Ig loci early in lymphoid differentiation (Blasquez et al., 1992).
The methylation patterns of Ig and TCR loci also change during lymphocyte development. Such changes might be important for accessibility of the locus to V(D)J recombination. Introduced Vκ transgenes can recombine in both B and T lineage cells, and -unlike endogenous loci- are hypomethylated in both cell types (Goodhardt et al., 1993). In T cells, there is a correlation between hypomethylation of the TCRβ locus, which is tissue specific and established just prior to the onset of rearrangement, and the subsequent rearrangement of the locus (Burger and Radbruch, 1990). Currently it is believed that methylation per se does not bar access to the recombination machinery but can function as a signal that will switch the chromatin into an inaccessible state after replication (Hsieh and Lieber, 1992).
Recently the accessibility of RS sequences for RAG recombination has been correlated to chromatin remodelling, either via histone acetylation or chromatin remodelling factors, such as the Swi/Snf complex (Hernandez-Munain, McMurry and Krangel, 1999; McBlane and Boyes, 2000; McMurry and Krangel, 2000). At the molecular level, it has been shown that the position of the RSS nonamer and heptamer on the nucleosome affects RAG binding and cleavage (Golding et al., 1999).
The exact way, however, in which chromatin remodelling is orchestrated in order to allow or prohibit V(D)J recombination is not well understood. The emerging picture is that in B and T cell development, during which substantial changes in gene expression occur, Ig and TCR loci are also modified, in order to become accessible substrates for V(D)J recombination.

1.5 Allelic exclusion

Regulating access of the RAG recombination machinery in Ig loci is crucial for yet another phenomenon, called allelic exclusion. As mentioned previously, and as predicted by the clonal selection theory (Burnet, 1976) B cells express an immunoglobulin receptor (and therefore secrete antibody) of a given specificity. This requirement of monospecificity means that of all Ig heavy and light chain loci present in a cell (a total of six: two heavy chain alleles and four light chain alleles) only one heavy and one light chain locus should produce functional protein. Repression of the production of other Ig molecules is known as allelic exclusion, since it is achieved by restricting expression to one of the two alleles from each locus.
Several models have been proposed to explain the phenomenon of allelic exclusion. The stochastic model suggests that allelic exclusion of IgH loci is a secondary effect, due to the low probability of two productive $V_HD_HJ_H$ rearrangements occurring in one cell (Coleclough et al., 1981). In a second model, it was postulated that expression of two different IgH chains on the surface of the cells is toxic (Wabl and Steinberg, 1982), and

thus double producers are eliminated. Heavy chain toxicity however, seems unlikely since B cell generation is not impaired in transgenic mice producing two functional heavy chains (Sonoda et al., 1997). Yet, the increased probability of generating an autoreactive receptor, when two different heavy chains are produced, might further limit the number of double producers.

Allelic exclusion is obviously favoured by both the limited time available for productive rearrangements to occur and the counter-selection of autoreactive cells, but it is actually established as a partial block of V(D)J recombination. In pro-B cells both IgH chain alleles start $D_H \rightarrow J_H$ rearrangements but $V_H \rightarrow D_H J_H$ joining typically occurs only on one allele. If this is productive, it leads to expression of μ heavy chain on the surface of the cells together with surrogate light chains (VpreB and λ5) (Sakaguchi and Melchers, 1986; Takemori et al., 1990) and Igα/β. Expression of this pre-BCR, as it is called, seems to be critical for inhibiting further $V_H \rightarrow D_H J_H$ rearrangements. Analysis of μ chain transgenic mice show that, in fact, $V_H \rightarrow D_H J_H$ rearrangements of endogenous loci are greatly reduced while $D_H J_H$ joints are still formed (Rusconi and Kohler, 1985;Weaver et al., 1985). Gene knockout experiments have also shown that disruption of either the λ5 gene (Loffert et al., 1996) or the membrane exon of the μ chain on one allele (Kitamura and Rajewsky, 1992) leads to impairment of allelic exclusion.

On the other hand, if the $V_H \rightarrow D_H J_H$ joint on one IgH allele is not productive then rearrangement of the other allele is mandatory for cell survival (Kitamura et al., 1991). The pro-B cells that manage to express a pre-B cell receptor, subsequently move on to start rearranging their light chain genes (Reth et al., 1987). Generally, but not always (Novobrantseva et al., 1999; Zou, Takeda and Rajewsky, 1993) light chain rearrangement also proceeds in an ordered fashion: one of the two Igκ alleles starts rearranging first. If this is not productive or is unable to pair with the IgH chain, then there is rearrangement of the other Igκ allele and only if this is equally unsuccessful, recombination will proceed on the Igλ alleles (Hieter et al., 1981) (Muller and Reth, 1988).

This ordered process of rearrangement of the Ig loci yields B cells, which have one BCR specificity. Analysis of the rearrangement "status" of peripheral B cells is a good indication of the progression of cells through the sequential steps of V(D)J recombination. Most cells harbour $D_H J_H$ rearrangements on both alleles, while about 40% of them have a productive and a non productive $V_H D_H J_H$ joint. Only about 0.1% of the cells have productive VDJ rearrangements on both heavy chain alleles (Barreto and Cumano, 2000).

Although allelic exclusion depends on signals coming from the pre-BCR (Papavasiliou et al., 1995), the exact mechanism of inhibiting rearrangement of a previously active locus is not known. Signals from the pre B cell receptor initially lead to down-regulation

of Rag gene expression (Grawunder et al., 1995) but this cannot be sufficient for the establishment of allelic exclusion, since RAG proteins are again present during light chain rearrangement. Somehow, changes in chromatin structure must be induced to render the locus inaccessible to the recombinase.

1.6 RAG expression in peripheral B cells

B cell receptor expression on newly generated B cells marks a second 'checkpoint' in B cell development. The successfully rearranged heavy and light chains now have to be screened for their ability to recognise self-antigens. Cells bearing autoreactive receptors are either eliminated by apoptosis (Nemazee and Burki, 1989) or are forced to modify their BCR specificity by secondary rearrangements of their IgL loci (Gay et al., 1993; Tiegs, Russell and Nemazee, 1993). This process is known as receptor editing and has been well characterised for the Igκ locus (Pelanda et al.,1997) but has also been observed in the IgH locus (Taki et al., 1995) (Kleinfield et al., 1986). Receptor editing involves a new recombination event, which removes the pre-rearranged VκJκ element and leads to the assembly of a different variable region (Tiegs et al, 1993).

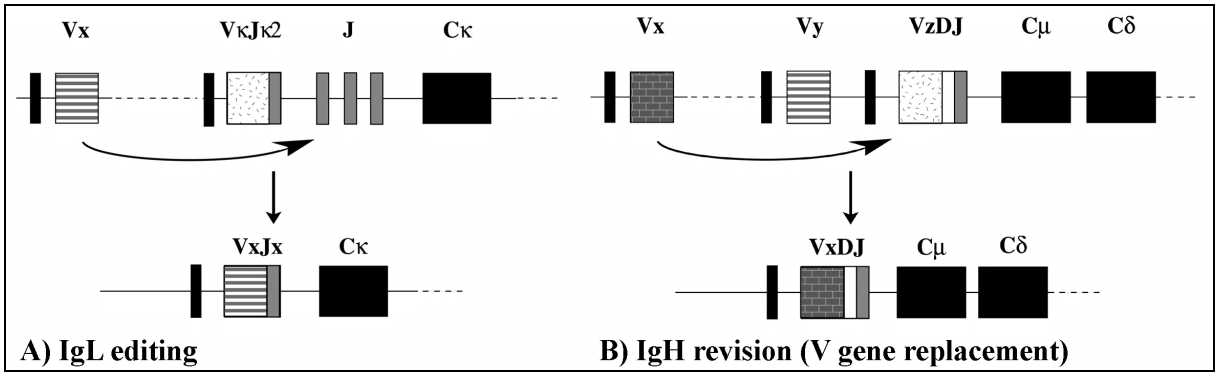


Figure 5:Receptor revision in IgL and IgH loci

More surprising however was the idea that RAG proteins might be involved in antigen receptor diversification also later, in mature B cells, during an immune response. The finding that RAG transcripts and protein can be detected in germinal centre B cells, or upon activation of B cells in culture (Han, 1996; Hikida, 1996; Hikida, 1997), gave rise to the provocative idea that the RAG recombinase could also be involved in affinity maturation through a process called receptor revision (Kouskoff et al.,2000;Papavasiliou

et al., 1997). This can be easily envisaged as happening in the light chain locus, where unrearranged V_L and J_L elements could be brought together, deleting the preformed V_LJ_L joint in a process similar to receptor editing. But even in the heavy chain locus $V_H \rightarrow V_HD_HJ_H$ rearrangements have been reported, mediated by cryptic heptamers present in some V elements (V gene replacement)(Figure5) (Kleinfield et al., 1986; Reth et al., 1986)(Taki, 1995). Receptor revision could be beneficial in deleting rearranged joints which have acquired a crippling mutation during the somatic hypermutation process and which are unlikely to be rescued by a second mutation at the same spot.
The role of RAG proteins, however, in peripheral B cells remains controversial; there is mounting evidence that the observed expression outside the bone marrow is due to residual expression in immature B cells which have not yet downregulated the RAG proteins rather than re-induction upon antibody challenge.

1.7 Aim of the thesis

Germinal centre and activated B cells have been shown to express RAG proteins. Double strand breaks, suggesting ongoing V(D)J recombination, have been detected in the Igκ loci of these cells. In order to assess the extent of V(D)J recombination in peripheral B cells and its contribution in shaping the secondary antibody repertoire we decided to generate a mouse strain in which B cells re-expressing the RAG proteins in the periphery would be labelled. Cells could then be sorted and analysed for secondary recombination.
One way to generate such an indicator mouse strain, is by inserting a reporter gene which will only be activated after RAG mediated recombination in peripheral B cells. There are two essential requirements for such an approach:
-the reporter gene will have to be continuously expressed after recombination has taken place, in order to allow detection of the desired B cell population.
-only B cells expressing the RAG proteins in the periphery should become labelled. RAG mediated inversion should not take place during bone marrow development of B cells, despite expression of RAG proteins.
RAG proteins when expressed can mediate recombination of an artificial reporter substrate; this would provide a stable marker for B cells having expressed the RAG proteins. Additionally, recombination of the substrate would provide evidence that the proteins are fully functional for V(D)J recombination. This recombination event however, should not occur in the bone marrow. Limiting recombination to peripheral B cells could be achieved by taking advantage of differential accessibility of various loci to recombination.
This idea brought us to a second point of interest: correlation of RAG accessibility and transcription. The possibility that a transcriptionally inactive locus would be inaccessible for V(D)J recombination and would become accessible upon induction of transcription

could be analysed in the reporter mice. In parallel, transcription and V(D)J accessibility were analysed in two other systems:
-an inducible system for transcription; efficiency of recombination could be analysed in a stably integrated substrate, before and after induction of transcription
-a mutant mouse strain harbouring a transcriptionally active gene in the IgH locus.

2. Materials and Methods

2.1 Molecular Biology

Common methods in molecular biology were performed according to standard protocols (Sambrook, Fritsch and Maniatis, 1989) unless otherwise stated.
For most cloning purposes heat shock competent XL-1 Blue bacteria were used.
Isolation of plasmid DNA was performed using an alkaline lysis method (Birnboim and Doly, 1979) . For cleaner preparations of plasmid Qiagen columns (Qiagen, Hilden) were used. Targeting vectors were additionally purified on a $CsCl_2$ gradient before transfection into ES cells.
All enzymes used for restriction digests and cloning purposes were from New England Biolabs (NEB, Beverly, USA) unless otherwise stated.
DNA was recovered from agarose gel slices with QIAEXII gel extraction kit (Qiagen, Hilden) according to the manufacturer's instructions.
DNA fragments were sequenced with the TAQ Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and analysed on an ABI377 sequencing machine.
Concentration of nucleic acids was determined by measuring absorption of the DNA solution at 260nm (and 280 nm) in a spectrophotometer (Pharmacia). Concentration of genomic DNA or purified fragments used as probes was determined by agarose gel elecrophoresis and comparison of the intensity of the band with λHindIII ladder.

2.1.1 Polymerase Chain Reaction (PCR)

PCR was used to amplify fragments for cloning, to analyse RAG or Cre recombination events, and to screen mice or ES cells for the presence of targeted alleles. Typically PCR reactions were performed in a volume of 50 µl containing 25 pmol of each primer, 5U

Thermus Aquaticus (Taq) DNA polymerase (Gibco-BRL), 200 µM dNTPs (Pharmacia), 1x PCR Buffer (10 mM Tris-Cl pH=8.3, 50 mM KCl) and $MgCl_2$ (concentration ranging from 1-2 mM). In PCR reactions designed to amplify fragments for cloning purposes a 1:1 mix of Taq (Gibco, BRL) and proof reading PFU (Stratagene) polymerase was used. A list of primers used in PCR reactions is given in Table 1.

#	Name	Sequence
1.	Ex14.5’	GAA TGT CAG TCA TGT ACA GTT GC
2.	Tm2-3’	GGG CTG GCT TAC AAA TCA GAG G
3.	Ex12.5’	GCG TAA ATG ATT CTA AAG GTC ATG G
4.	Ex15.3’	CTG GAT TCC ATT TGT ATC TTC AGG
5.	CD21(ATG)	GGG GAT CCC GGG TGT GCA GCA G
6.	CD21intr.5’	CAA GTT CTT TTC TCT GAT ATA TCA G
7.	CD21intr.3’	TTA CAC TAG TCG ACA GAT TTA TCA CTC ACA ATA TCA CTC
8.	VH81x-5’	GGC CAA GTC GAC TGG AGG TTT TAG TTT GAG CTC ACA GTA ACT TTT GCT CAT TGT GTG TCT TGC AC
9.	VH81x-3’	GGC CGT GCA AGA CAC ACA ATG AGC AAA AGT TAC TGT GAG CTC AAA CTA AAA CCT CCA GTC GAC TT
10.	DFL16.1-5’	AAT TTC GAG GCT TTT TGT GAA GGG ATC TAC TAC TGT GTT TAT TAC TAG
11.	DFL16.1-3’	AAT TCT AGT AAT AAA CAC AGT AGT AGA TCC CTT CAC AAA AAG CCT CGA
12.	PROM 5’	CTA GTC ACC TGC AGT TGT GC
13.	PROM 3’	ACA TCC CTG GTT TGT CCT CTA ACC
14.	LoxP-5’	AAT TTC TAG AGG TAC CAT AAC TTC GTA TAG CAT ACA TTA TACGAA GTT AT
15.	LoxP-3’	AAT TATAAC TTC GTA TAA TGT ATG CTA TAC GAA GTT ATG GTA CCT CTA GA
16.	LoxP-Cµ 5’	AGC TAA TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT AAG CTT CAA TTG
17.	LoxP-Cµ 3’	AGC TCA ATT GAA GCT TAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT ATT
18.	RSS-5’	ATC CTA GGA GGG TTT TTG TAC AGC CAG ACA GTG GAG TAC TAC CAC TGT GCA GGA AGC TTC AGT GTC GAC ACC TG
19.	RSS-3’	GCG GCC GCA ATG GCG CGC CGC TAG CCA GTG TTT TTG TTC CAG TCT GTA GCA CTG TGC AGG TGT CGA CAC TGA AGC TTC CTG
20.	GFP-5’	CAT CGA GCT GAA GGG CAT CGA C
21.	GFP-3’	GTA CAG CTC GTC CAT GCC GAG AG
22.	Cre8	CCC AGA AAT GCC AGA TTA CG
23.	CD19c	AAC CAG TCA ACA CCC TTC C
24.	CD19d	CCA GAC TAG ATA CAG ACC AG
25.	glD42i-5’	TGT CAA GAC TCT CAC TGT AG
26.	glD42i-3’	CCG TCA GCT TCT GCA GCA TG
27.	Mx-CreF	CAT GTG TCT TGG TGG GCT GAG

28.	Mx-CreR	CGC ATA ACC AGT GAA ACA GCA T
29.	JHT1	CAG TGA ATG ACA GAT GGA CCT CC
30.	JHT2	GCA GAA GCC ACA ACC ATA CAT TC
31.	JHT3	CAC AGT AAC TCG TTC TTC TCT GC
32.	BAP31	CTGTAGTCCACTGCAAACTCATC
33.	CD21ex2fr.	TGCTAAACCTTCTATTGTGAGTGA
34.	oligo top	TGCTAAACCTTCTATTGTGAGTGATATTGTGAGTGATAAATCTGTCGACT GGAGGTTTTAGTTTGAGCTCACAGTAACTTTTGCTCATTGTGTCACAGT
35.	oligo bottom	CTGTAGTCCACTGCAAACTCATCCCGAAGGGGTTCGAATTTCGAGGCTT TTTGTGAAGGGATCTACTACTGTGACACAATGAGCAAAAGTTACTGTGA GCTCA

Table 1: List of primers used in PCR reactions and for cloning purposes

2.1.2 DNA preparation from mouse tissues and ES cells

DNA was prepared form mouse tissues and cells by incubation in Laird-Jaenisch (LJ) lysis buffer (100 mM Tris-Cl pH=8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) supplemented with proteinase K (1 mg/ml). Tissues/cells were incubated overnight at 55 °C and undissolved debris was pelleted by centrifugation. DNA was precipitated by addition of equal volume of isopropanol, washed with 70% ethanol, airdried and dissolved in 0.3x TE (Tris-EDTA, pH=8).
Genomic DNA from ES cells was prepared by directly lysing the cells in 96-well plates according to the protocol by Pasparakis et al [Pasparakis, 1995 #5]. 50 µl of lysis buffer (10 mM Tris-HCl pH=8, 10 mM EDTA, 150 mM NaCl, 0.2% SDS and 400 µg/ml Proteinase K) were added to each well and plates were incubated overnight at 55 °C in a humidified chamber. DNA was precipitated with 100 µl of EtOH, and subsequently washed 3x with 70% EtOH. Plates were air dried and restriction digest mix was added directly to each well.

2.1.3 Southern blot analysis

For southern blot analysis 5-15 µg of genomic DNA were digested with appropriate enzymes at 37° C overnight. ES cell DNA was directly digested in the 96-well plate by addition of 35 µl of restriction enzyme mix (20u enzyme, 1x restriction buffer, 100 µgr/ml BSA, 1 mM spermidine, 50 µg/ml RNAse and 1mM DTT) in each well and overnight incubation at 37 ° C
Digested DNA was run on a 0.8% agarose gel, and blotted on a nylon membrane (Hybond N+, Amersham) by dry capillary transfer. Membranes were baked at 80° C for 2 hours, pre-incubated in hybridisation buffer (1% BSA, 1mM EDTA, 0.5M Na-phosphate pH=7.2, 7% SDS) for 1 hour and hybridised overnight at 65 °C. Probes were

radioactively labelled with ^{32}PdGTP (Amersham) using a random primer labelling kit (Gibco). Unincorporated nucleotides were removed by centrifugation on a Sepharose spin column (Pharmacia). After hybridisation membranes were washed four times for 20 minutes in wash buffer (1mM EDTA, 40 mM Na-phosphate pH=7.2,1% SDS) and exposed overnight on autoradiographic films. More stringent washing conditions had to be used for southern blot hybridisation when using the D probes (and specifically the DFL16 probe) because of high unspecific binding. In this case blots were washed once with 1%SDS 1x SSC, twice with1%SDS 0.5x SSC and once with 1% SDS 0.2xSSC at 65 °C. Blots were measured with a Geiger counter and if counts were high they were washed further with 1% SDS 0.2x SSC.

The following probes were used:

-CD21 targeting: the 3' external probe was a SacI-KpnI fragment form pBS-Cr2.5 (M. Alimzhanov), which corresponds to Exon4 of the CD21 gene
-GFP probe (NheI-XhoI fragment from EGFP-C1)
-neo^r probe (XhoI fragment from plasmid neoflox-8)
-Cμ targeting: the 5' external probe was 0.7 kb EcoRI-HindIII fragment from pBR322-Cμ (RH probe)
-DQ52 probe: 1.2 kb XhoI-HindIII fragment from pDQ52
-DFL16 probe: 1.5 kb NsiI-BstEII fragment from plasmid pDFL3.8
All probes were gel purified after restriction digest of the respective plasmids. Concentration of the purified probe was estimated by agarose gel electrophoresis and 50ng were used for the labelling reaction.

2.1.4 RNA preparation and competitive RT-PCR

RNA was prepared from sorted B and T cells using an RNA extraction kit (RNAeasy, Qiagen). RNA was prepared from 10^5 cells and dissolved in 40 μl of DEPC treated water. 10 μl of the RNA preparation were used for the cDNA synthesis reaction using reverse transcriptase (Gibco). For the PCR amplification reaction 1.5 μl of the synthesised cDNA was used. Different combinations of intron spanning primers for the CD21 gene were tested on mRNA from total spleen and two primer pairs were selected for further analysis (primers #1-4). The competitor molecule was generated by PCR amplification of a 600 bp fragment from the CD21 transcript (primers #1, #2), subcloning of this into pBS (using a Topo-TA cloning kit, Invitrogen) and removal of a 150 bp fragment from the middle by restriction digest with BstEII-HincII. The plasmid was religated and removal of the 150 bp fragment was verified by restriction digest. The concentration of the 'competitor molecule' was measured with a spectrophotometer, and the number of molecules included in each reaction (x) was calculated from the measured concentration (x= c[mol/l]*V[l]*N [1/mol], where x number of molecules, c;

concentration [mol/l], V volume of competitor solution included in each reaction and N Avogadro's constant). Known amounts of this plasmid were included in the PCR amplification reaction in order to quantitate the expression of CD21 in different sorted cell populations. Actin primers were used as a control for the presence and the quality of the cDNA template.
Another approach was also used, which involved direct cDNA synthesis from a small number of sorted cells, without prior RNA purification. In this case 40 cells from the different populations were sorted in tubes containing 10 µl of Buffer X (1x Superscript Buffer, 10 mM DTT, 0.5% NP-40, 10 u RNA guard and 5s rRNA). After sorting, 9 µl of 1x first strand buffer (10 pMol oligo-dT, 5 mM dNTPS,) were added to each tube and samples were incubated at 65 °C for 3 minutes. 1 µl of reverse transcriptase (1000 U/µl) was added to each tube after cooling down and the reaction was then done according to standard protocols. 1.5 µl of the synthesised cDNA was used for the PCR amplification reaction.

2.1.5 Cloning of targeting vectors

Generation of targeting vectors and other plasmids is described below. Some cloning intermediates and the final constructs generated are shown in Figure 6.

CD21 targeting construct

To generate the CD21 targeting construct a BamHI-HindIII 3kb fragment, containing the first half of intron I was subcloned from pBSKSCr2HindIII (kind gift from Dr. Molina) into the respective sites of pBSKS+ (Stratagene) (plasmid#1). The upstream XbaI-BamHI fragment was also subcloned from the same plasmid into pBSKS and used as a template for PCR mutagenesis of the first ATG of Exon1 (intoducing a SmaI site in place of the ATG using primer#5 and T7)). The PCR product was blunted by Klenow treatment, digested with NotI-BamHI and subcloned into the NotI-BamHI digested plasmid#1. The resulting plasmid (plasmid#2) was sequenced to verify that no mutations were introduced by the PCR amplification. The second half of intron 1 was PCR amplified (primer#6-#7) from a BAC-Cr2 clone (kind gift of Dr. Molina) in order to introduce a SalI site into Exon 2. The resulting PCR product was blunted by Klenow treatment, digested with HindIII-SalI and cloned into plasmid#2 (plasmid#3).
Plasmid Neoflox-8 (generated by M. Kraus) was modified by partial digest with XhoI followed by Klenow treatment and religation. Plasmids where the downstream XhoI site was destroyed were then selected. This neoflox-8(XhoI) plasmid was cut with NotI and an RSS containing linker (VH81x-oligos #8,9) was inserted destroying the NotI site. Sequence and orientation of the linker was verified by sequencing (plasmid#4).

The GFP cassette was generated by cloning an RSS containing linker (DFL16.1-oligos #10,11) into the EcoRI site of p31HR123-EGFPII (gift of S. Kuppig/ M. Reth) leaving only one EcoRI site 5' of the GFP gene. Sequence and orientation of the linker were verified by sequencing (plasmid #5). In the remaining EcoRI site a *LoxP*-XbaI-KpnI containing linker (oligos #12,13) was cloned. Sequence and orientation of the linker were verified by sequencing (plasmid#6).The GFP-RSS-*LoxP* fragment was cut out from plasmid#6 by XbaI-XhoI digest and cloned into the XbaI-XhoI site of plasmid#4 (plasmid #7).
The final construct (plasmid#8) for the targeting of the CD21 locus was generated by isolating the GFP-neoflox cassete from plasmid #7 by SalI-KpnI digest and inserting it into the SalI-KpnI site of plasmid#3. The short arm of homology (EcoRI-XbaI 1kb fragment from pBSCr2.5) was then inserted in the KpnI site of this plasmid by blunt end cloning.

Conditional Cμm targeting construct

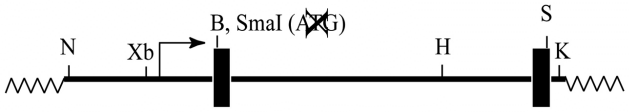
The Cμm targeting construct was generated by subcloning an EcoRV-HindIII fragment from plasmid pBR322-Cμ into the respective site of pBSKS (plasmid#9). Plasmid #9 was digested with HindIII and a *LoxP* containing linker (oligos #10,11) was inserted downstream of Cμ membrane exons1 and 2. The sequence and orientation of the linker was verified by sequencing. A second *LoxP* site was then inserted in the upstream EcoRV site using the same linker which was blunted by Klenow treatment. Sequence and orientation of the plasmid was verified by sequencing (plasmid #10). An FRT flanked neomycin gene (1.4 Kb SalI-XhoI fragment from plasmid pGEM-FRT2neo, generated by Ralf Kühn) was inserted by blunt end cloning in the EcoRI site of plasmid #10 (plasmid #11). The long arm of homology, a 3 kb XbaI-EcoRV fragment from pBR322-Cμ was blunted by Klenow and inserted in the SmaI site of plasmid #11. To generate the targeting construct the short arm of homology was cloned in the HindIII-MfeI sites of plasmid #11 as a HindIII-EcoRI fragment (from pBR322-Cμ) (plasmid #12).

Tet-RSS construct

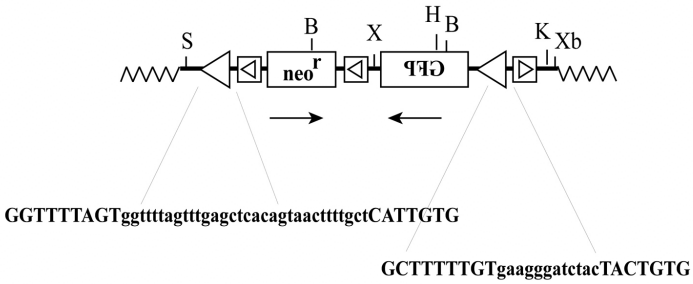
The Tetracycline Responsive Element (TRE) was subcloned from pTRE (Clontech) into the XbaI-XhoI sites of pBSKS (plasmid #13). A double RSS containing linker (oligos #12,13) was inserted in the XbaI site of plasmid #13. Sequence and orientation of the linker were verified by sequencing (plasmid #14). The puromycin acetyltransferase gene was cut out of the pGK-puroCre vector (A. di Nardo) with HindIII-XhoI and cloned into the HindIII-SalI sites of plasmid #14 (plasmid#15). The last step was cloning GFP (NotI-XbaI fragment from EGFP-C1, Clontech) into the AscI-NheI sites of plasmid #15 (plasmid #16).

A) CD21 targeting construct

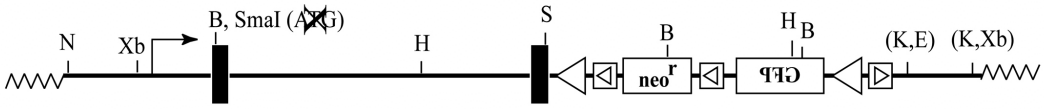
Plasmid#3
(pBS-CD21)



Plasmid #7
pBS-neo-GFP



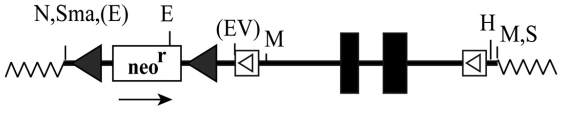
Plasmid #8
CD21 final



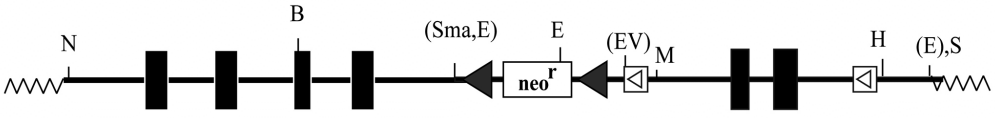
B) Cμm targeting construct

Plasmid #9
pBS-Cμm

Plasmid #11
pBS-Cμm-neo

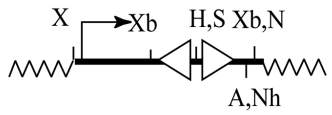


Plasmid #12
pBS-Cμmfinal

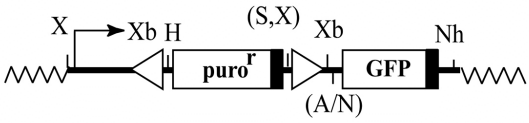


1 kb

C) Tet/RAG reporter construct



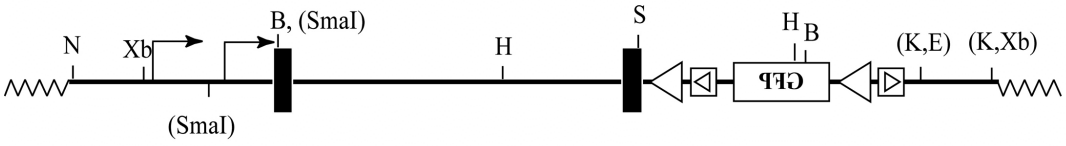
Plasmid #14
pBS-Tet/RSS



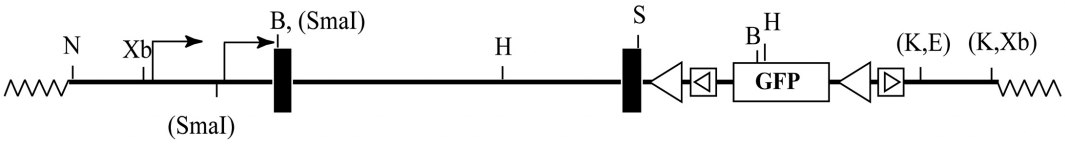
Plasmid #16
pBS-Tet/RSSfinal

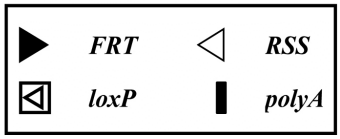
D) CD21 final/modified plasmids

Plasmid #17
CMV(CD21)neodel.



Plasmid #18
CMV(CD21)neodel/GFPinv.





1 kb

Figure 6: Cloning intermediates and targeting constructs generated. All constructs were cloned using pBSKS as vector (indicated by jagged lines). Maps are drawn to scale except for *loxP* and FRT sites and RSS.The sequence of the RSS inserted in the CD21 targeting construct is indicted below the respective plasmids. Transcription is indicated by arrows under the gene and from the respective promoters. Restriction digest sites: B; BamHI, E; EcoRI, EV; EcoRV, H; HindIII, K; KpnI, M; MfeI, Nh; NheI, S; SalI, Sma; SmaI, X; XhoI, Xb; XbaI

2.1.6 Transfections of 294 Cre and Flp expressing bacteria

To test the functionality of the *loxP* and FRT sites used in the targeting constructs plasmids were transfected in 294 bacteria constitutively expressing Cre or FLP recombinase (Buchholz, Angrand and Stewart, 1996). Transfections were typically done by electroporation at 1.8 KV and 25 μF, bacteria were allowed to recover for 1 h at 37 °C and then plated on LB plates. Overnight cultures were grown from single colonies.
In the case of the CD21 targeting construct however, due to the presence of two inverted *loxP* sites a different protocol was used. Growing bacteria at 37 °C resulted in aberrant deletion of the intervening DNA due to high levels of Cre expression. Therefore bacteria were transformed by mild heatshock at 37 °C for 5 minutes and then allowed to recover at 30°C. Thereafter bacteria were grown at room temperature (RT) to ensure low Cre expression.
Transformation of the 294-Cre bacteria was used to modify the CD21 targeting construct (plasmid #). Following the mild transformation protocol single colonies were picked and minipreps grown at 30 °C. All colonies had deleted the *loxP* flanked neomycin gene and had either inverted (plasmid #18) or not inverted (plasmid #17) the GFP cassette. In these plasmids the CMV promoter from pEGFP-N1 (MfeI-NotI fragment) was cloned as a blunt end fragment in the SmaI site of exon1.

2.2 Cell culture and transfections

All tissue culture reagents used were purchased from Gibco-BRL unless otherwise stated.

2.2.1 ES cell culture

Two different embryonic stem (ES) cell lines (Bruce-4 of C57BL/6 origin (Kontgen et al., 1993) and IB10, a subclone of E14.1 ES cell line, of 129 origin (Kuhn, Rajewsky and Muller, 1991) were used for generation of mice. ES cells were handled according to protocols described elsewhere (Pasparakis and Kollias, 1995;Torres and Kuhn, 1997). Cells were grown in DMEM media with 10% heat inactivated FCS (PAN Cat#3302-P9795 Lot#P979549 for Bruce-4 cells and Gibco Cat#16141-079 Lot#34N4764 for IB10), 2mM L-glutamine, 1mM sodium pyruvate, 1x non-essential aminoacids, 0.1 mM β-mercaptoethanol and 10^3 u/ml LIF (leukemia inhibitory factor, Chemicon) on a confluent embryonic fibroblast monolayer. Embryonic fibroblasts (EF) were mitotically inactivated with mitomycin C (mmC, Sigma) (10 μg/ml for 2-3 hours) and rigorously washed to remove traces of mmC before plating ES cells. ES cells were always split before reaching confluence by mild trypsinisation (in the case of Bruce-4 cells trypsin

was supplemented with 2% chicken serum). ES cell transfections were carried out by electroporation using 30 μgr of DNA/10^7 cells in 0.8 ml of transfection buffer (20 mM Hepes, 137 mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 6mM glucose and 0.1mM 2-β-ME) at 240 Volt and 500 μF (Biorad, GenepulserII). DNA was linearised before transfection.
ES cells were selected after electroporation with G418 (160 μg/ml active concentration for Bruce-4 ES cells and 200 μg/ml for IB10 cells) and clones were picked 8 to 9 days after transfection and expanded directly into 96-well plates (Pasparakis and Kollias, 1995). ES cell clones were also frozen directly in the plates: 50 μl of trypsin were added per well and incubated at 37°C for 5 minutes. Subsequently 50 μl of 2x freezing media (FCS with 20% DMSO) were added and wells were overlaid with 100 μl of sterile light mineral oil (Sigma). Plates were there frozen at –80 °C until homologous recombinants were identified.
Specific deletion of the *loxP* flanked neo^r gene was performed by transient transfection of Cre expression plasmid PGK-puro-Cre (A. di Nardo) in ES cells. Transfection conditions are identical as described for the initial transfection. 5 hours after transfection puromycin was added to the ES cell media (final concentration 1.25 mg/ml). Media was changed to non selective media 24-36 hours later and cells were split and replated at a density of 2 x10^3 cells per 10 cm dish on a fresh feeder monolayer. Clones were picked 6-7 days later, and analysed for G418 sensitivity.

2.2.2 B cell culture

B cells were isolated from the spleen and bone marrow of mice. Bone marrow was flushed out from the tibia and femurs using a 24-G needle and spleens were removed, ground to a single cell suspension between glass slides and washed in DMEM, 1% FCS. Erythrocytes were removed by incubating the cells for 3 minutes in Red Blood Lysis buffer (150mM NH_4Cl , $KHCO_3$, 0.1 mM Na_2EDTA pH=7.2) at room temperature. To remove monocytes and granulocytes a Ficoll (Pharmacia, Sweden) gradient centrifugation was performed (2000 rpm, for 20 minutes at RT) and lymphocytes were collected from the interphase. Macrophages were depleted by pre-plating the cells and allowing them to adhere to plastic for 45 minutes; non adherent cells were harvested.
Cells were cultured in DMEM, 5% FCS, 0.1 mM 2 β-mercaptoethanol, 1x penicillin-streptomycin, 2mM L-glutamine. For the LPS activation 20 μgr/ml of LPS (Sigma) was added to the media while 100 U/ml of IL-4 were used. Initially 10^6 cells/ml were plated in a 25 cm^2 flask.
For the culture of bone marrow cells stromal cells (ST2 cells; Ogawa, 1988) were pre-plated in the flasks and treated with mitomycinC for 4 h.

2.2.3 Handling of HeLa and 293 Cells

HeLa Tet-ON cells were purchased from Clontech and used for all HeLa transfections. Cells were cultured according to the company's instructions (DMEM supplemented with 10% FCS, 4 mM L-glutamine, 1x Penicillin-Streptomycin, 100 µgr/ml G418). Transfections were done using Lipofectamine 2000 (Gibco) according to the manufacturer's instructions. For the generation of stable cell lines with the Tet-RSS construct (linearised with NotI) cells were treated with doxycycline containing media (2µg/ml) after transfection. 24 hours later puromycin was added (1 mg/ml) for 48 hours. Selection media was subsequently removed and cells were grown in the presence of doxyxcyclyine until clones were large enough for picking. Cells were then kept in doxycycline containing media supplemented with 500 µg/ml of puromycin.
For the transfection experiment, with the RAG expression vectors, cells were grown in 3 wells of a 6-well plate. In one case doxycycline free media was added, while in the other two wells cells were grown in the presence of doxycycline. Cells were transfected with RAG expression vectors and 48 hours later fresh doxycycline containing media was added to all wells. Cells were trypsinised 24-48 hours later and analysed by flow cytometry. 293 cells (Senapathy and Carter, 1984) were grown in in DMEM supplemented with 10% FCS. Cells were transfected with lipofectamine 2000 (Gibco).

2.3 Immunostainings

2.3.1 FACS analysis

Staining of cells for flow cytometry were performed as previously described. The list of antibodies used for different stainings is given in
Table 2. Antibodies were conjugated to FITC, PE, APC, CyChrome or Biotin. Biotinylated antibodies were visualized with Streptavidin conjugated CyChrome. Stained cells were analysed with a FACSCalibur (Becton Dickinson) and sortings were performed with a FACStar (Becton Dickinson). For the exclusion of dead cells propidium iodide (PI, 02µg/ml, Sigma) was added to the cell suspension just prior to acquisition.
The list of antibodies used is given in Table 2.

Ab Name (Clone)	Specificity	Reference or supplier
RS3.1	IgM[a]	(Schuppel, Wilke and Weiler, 1987)
MB86	IgM[b]	(Nishikawa et al., 1986)
R33-24.12	IgM	(Grutzmann, 1981)

RA3-6B2	B220 (CD45R)	(Coffman and Weissman, 1983)
S7	CD43	(Gulley et al., 1988)
1.3-5	IgD	(Roes, Muller and Rajewsky, 1995)
7G6	CD21/CD35	Pharmingen (Heyman, Wiersma and Kinoshita, 1990;Wiersma, Kinoshita and Heyman, 1991)
8C12	CD21/CD35	Pharmingen (Heyman, Wiersma and Kinoshita, 1990;Wiersma, Kinoshita and Heyman, 1991)
AF6-120.1	MHCII ($I\text{-}A^b$)	Pharmingen (Wall et al., 1983)
6D5	CD19	SBA,Inc
PNA	lectin	(Bramwell et al., 1982)
goat polyclonal	IgG1	Southern Biotechnology associates (SBA)
B1-8µ	NP	(Reth, 1981)
267.7	NP	(Reth, 1981)
1D3	CD19	(Krop et al., 1996)

Table 2: List of antibodies used for immunostainings and Elisa

2.3.2 Microscope slide stainings

In order to stain cells for microscopy, glass coverslips were washed with 70% EtOH, then water and allowed to air dry. Coverslips were then coated with poly-L-lysine (0.1% in water, Sigma) for 1 h and washed with PBS. Cells were resuspended in DMEM and allowed to adhere to the glass slides for 30 minutes at room temperature. Cells were then washed 1x with PBS and fixed with paraformaldehyde (2% in PBS) for 30 minutes at RT. For intracellular staining cells were permeabilized with 0.1% Triton X-100 in paraformaldehyde, washed 3x with PBT-glycine (PBS, 0.05% Tween 20, 50 mM glycine) and blocked for 1 hour at room temperature with PBT-Block (PBS, 0.05% Tween 20, 2% BSA, 0.05% gelatin and 50 mM glycine). Cells were incubated with primary antibody (for FITC coupled antibodies 1:50 dilution was used, while biotinylated antibodies were used at a dilution of 1:200 in PBT-Block) for 45 minutes at RT. Cells were then washed 6x5 minutes with PBT and the secondary reagent was added (strepatividin-TRITC, dilution 1:200 in PBT-Block) for 30 minutes at RT. Coverslips were washed again (6x5 minutes with PBT), rinsed briefly with water and mounted on glass slides with gelvatol.
Slides were stored at 4 °C and analysed with a Leica DM RXA microscope equipped with epifluorescence.

2.3.3 Magnetic cell sorting

Magnetic cell enrichment or depletion was done using MACS magnets and columns according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach). Cells were sorted by positive selection using directly coupled αB220 MACS microbeads and αIgD^{biotin}-streptavidin-microbeads (10 µl of bead suspension in 90 µl PBS/BSA/NaN_3 for 10^7 cells). The purity of the sorted cell populations was tested by FACS analysis using either an αB220 or an αCD19 antibody.

2.3.4 ELISA

Ig serum concentrations were determined by enzyme-linked immunosorbent assays.(ELISA) as described by . Flat bottom microtiter plates (Costar) were coated with 50 µl antibody in PBS overnight at 4 °C. Plates were washed 3 x 5minutes with PBS/0.1% Tween-20 (Sigma). Remaining binding sites were saturated by incubating the plates with PBS/0.5% BSA for 2 hours at 37 °C. Plates were washed 3 x 5minutes with PBS/Tween-20. Serially diluted serum samples were added to the wells (sera were pre-diluted 1:50 in PBS) and incubated for 1 hour at 37 °C. Plates were washed and secondary biotinylated antibody was added and incubated for 1 hour at 37. Detection of the biotinylated antibody was achieved with streptavidin conjugated alkaline phosphatase (AP, Boehringer) and p-nitrophenylphosphate (Boehringer) as a substrate. The OD was measured with an Elisa photometer 30 minutes to 1 hour after addition of the substrate at 405 nm.

2.4 Mice

All animals were kept in conventional mouse facility. Unless otherwise specified all experiments were done with 8 to 12 week old mice.

2.4.1 Immunisation

Mice were immunised by intravenous injection (tail vein) of vesicular stomatitis virus (VSV, $5x10^5$ PFU Plaque Forming Units) or intraperitoneal injection of 4-hydroxy-3-nitrophenylacetyl coupled chicken γ-globulin (NP-CG, 100 µgr/mouse in 200 µl of PBS).

2.4.2 Bone marrow transfer

Bone marrow cells were isolated from femurs and tibia of mice under sterile conditions. Cells were resuspended in sterile PBS (without azide!) at a concentration of 10^7 cells/ml. 200 µl of the cell suspension were injected in the tail vein of sub-lethaly irradiated $RAG1^{-/-}$ mice. Host mice were irradiated with a X ray-source with a total dose of 600 rad Antibiotics were administered in the drinking water of the animals one week before irradiation and for an additional three weeks after reconstitution.

2.4.3 Typing protocols

The genotype of transgenic mice was determined by PCR. The CD21 targeting was confirmed using internal GFP primers (oligos #20,21), the CD19-Cre transgenic line was monitored using oligos #22-24, Mx-Cre with oligos 27-28, JHT with oligos #29-31 and glD42Hi with oligos #25-26.

3. Results

3.1 RAG expression reporter line

In order to assess the extent and significance of RAG re-expression in B cells after bone marrow development a reporter mouse was generated. The idea was to label B cells that express the RAG proteins after the bone marrow rearrangement programme has been completed, and then isolate and analyse them for secondary V(D)J recombination in Ig loci.

The reporter substrate was designed in such a way as to use the ability of the RAG proteins to mediate DNA recombination. It contains a reporter gene, flanked by RSS, which is inserted in the 'wrong' transcriptional orientation. RAG-mediated recombination leads to inversion and expression of the reporter gene. The reporter gene chosen was a variant of green fluorescent protein (GFP). However, it was necessary to establish that RAG mediated recombination of the reporter substrate would not occur in the bone marrow, when the RAG proteins are expressed for primary rearrangement of Ig loci. In several systems (reviewed in Lewis, 1994) accessibility of a substrate for RAG recombination correlates with transcriptional activation. Possibly, one way to exclude recombination of the reporter substrate in developing B cells would be by introducing it into a locus which is not transcribed in progenitor and precursor B cells, but becomes transcriptionally active later.

The above criteria apply to the CD21 gene, which codes for complement receptors 1 and 2. The CD21 protein is expressed only on mature B cells and follicular dendritic cells

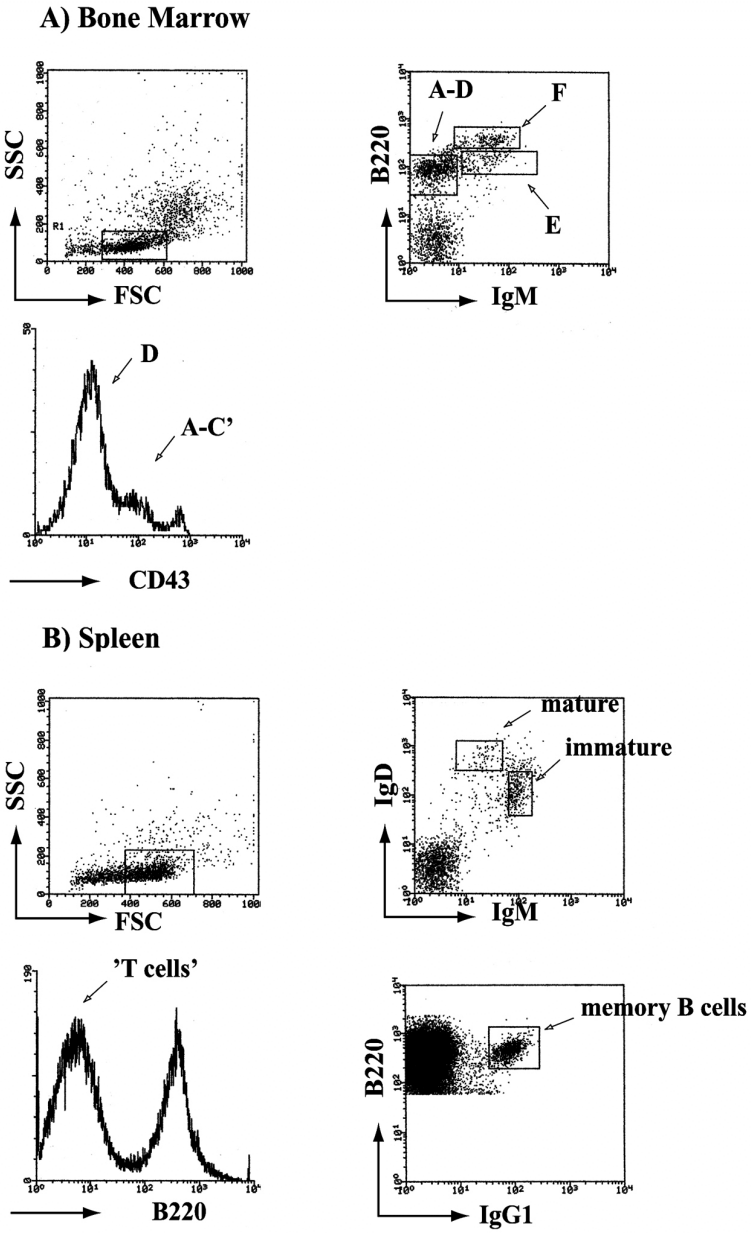
(Kinoshita et al., 1988;Tedder, Clement and Cooper, 1984) which makes the CD21 locus a potentially good candidate for introducing the RAG recombination reporter substrate.

3.1.1 Analysis of CD21 expression

Absence of transcription of the CD21 locus during bone marrow development (when rearrangement of endogenous IgH and IgL loci takes place) is essential for preventing rearrangement of the reporter substrate in progenitor and precursor B cells. Although it has been reported that murine CD21 protein is expressed only on mature B cells, we decided to perform a more extensive analysis of CD21 mRNA levels in different populations of B cells from spleen and bone marrow.
Bone marrow fractions were identified according to cell surface markers (see Figure 2) B220, IgM and CD43. Fractions A-C' (pro-B cells), Fraction D (pre-B cells) and Fractions E and F (immature and mature cells, respectively) from bone marrow were sorted for analysis (Figure 7a). Immature (IgM^{high} IgD^{low}) and mature (IgM^{low} IgD^{high}) B cells and non B cells ($B220^{-}$ cells which by forward-side scatter criteria would belong to the lymphocyte population and are therefore designated as T cells in the subsequent analysis) from spleen were sorted as positive and negative controls, respectively.
Another important consideration is the constitutive expression of the reporter gene in germinal centre and post-germinal centre B cells. Since major differences in gene expression profiles of these B cell subpopulations have been described, we decided to check for CD21 expression levels in isotype switched cells (which are usually post germinal centre B cells). For this reason IgG1 positive cells from immunised animals were also sorted for CD21 mRNA quantitation (Figure 7b).

Figure 7: Sorting of B cells from a)Bone marrow: cells from Fractions A-C', D, E and F were sorted as indicated based on expression of B220, IgM and CD43. Cells from Fraction A-D are $B220^{+}$, IgM^{-} and are further divided into Fractions A-C' and D cells based on CD43 expression. Cells from Fraction E are $B220^{low}$ IgM^{+} while those from Fraction F are $B220^{high}$ IgM^{+}.

b) Spleen: Mature and immature cells were identified based on IgM, IgD expression; immature cells are IgM^{high} IgD^{low} while mature cells are IgM^{low} IgD^{high} In the lower panel B220 negative cells (designated as 'T cells') were sorted as negative controls, and memory B cells were identified as $IgG1^{+}$ cells (the plot is gated on $B220^{+}$ cells).



CD21 mRNA levels were analysed by RT-PCR. Initially RT-PCR was carried out on cDNA prepared from crude lysate of cells sorted into a tube (40 cells/sample)(Figure 8a). Alternatively mRNA was prepared from sorted cells (10^5/sample) and then analysed by competitive RT-PCR. Cells from the IgG1 population (which are representative of the memory B cell pool) were only analysed by the direct RT-PCR protocol, since it was

impossible to obtain enough cells for mRNA extraction and competitive RT-PCR analysis. Two different pairs of intron spanning primers (Ex12.5'-Ex15.3' and Ex14.5'-Tm2.3') were used for amplification. Actin transcripts were also amplified as internal controls for determining sorting efficiency and monitoring the cDNA synthesis reaction (Figure 8a). Additionally, expression of CD21 on germinal centre (GC) B cells was monitored by staining spleen cells from immunised animals for CD21. Peanut agglutinin (PNA) binding (Bramwellet al., 1982) was used to distinguish GC B cells (Figure 8b).

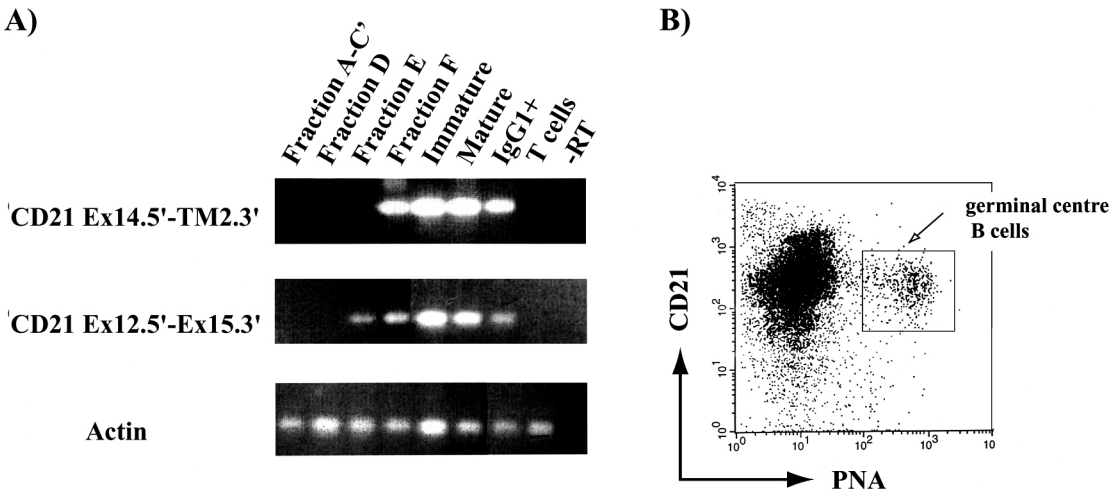


Figure 8: A) Analysis of CD21 expression levels by direct RT-PCR (40 cells/reaction) of different bone marrow fractions, and mature and immature B cells, $B220^-$ cells and $IgG1^+$ cells from spleen. Two different sets of primers, Ex14.5'-Tm2.3' and Ex12.5'-Tm2.3' were used. Actin mRNA was amplified as a control for the cDNA synthesis reaction.

B) Expression of CD21 on germinal centre B cells. Splenocytes from immunised animals (day 13 post immunisation) were stained with αB220, αCD21 and PNA. The majority of PNA^+ cells are $CD21^+$. The plot is gated on $B220^+$ cells.

CD21 transcripts can be detected in immature, mature and $IgG1^+$ cells from spleen but not in T cells. Furthermore, most GC B cells (PNA^+ cells) are CD21 positive. Bone marrow fractions A to D do not have any detectable CD21 transcripts (while actin mRNA can be amplified by RT-PCR from these samples). CD21 expression in Fraction E is ambiguous; mRNA is amplified with one set of primers but not with the other. Probably the two primer pairs have different efficiencies of amplification and if expression is low only one PCR reaction is efficient enough to detect the CD21 mRNA. B cells from Fraction F have detectable levels of CD21 transcripts with both primers pairs.

In order to estimate the level of CD21 expression in the different B cell populations competitive RT-PCR analysis was performed. The procedure relies on the co-

amplification of the sequence of interest with a serially diluted, synthetic DNA fragment of known concentration, using the same set of primers (Becker-Andre and Hahlbrock, 1989;Vu et al., 2000). The competitor molecule should give a distinguishable PCR product from the endogenous gene. In this case, the competitor molecule was a fragment of the CD21 cDNA, which was PCR amplified (using primers Ex14.5' and Tm2.3') from total cDNA from spleen, and subcloned in pBSKS+. An internal 150 bp fragment from this subcloned fragment was removed by restriction digest (HincII-BsteII) and the plasmid was religated. Thus, a piece of DNA which is amplified with the same primer pair but yields a shorter PCR product can compete with the CD21 cDNA for amplification. Different amounts of competitor DNA were initially tested and thereafter, different concentrations were used, so that amplification of the competitor DNA would reflect expression levels of the endogenous gene. For bone marrow Fractions A to E ten-fold serial dilutions (from 10^3 to 1 molecules) were included in each reaction, while for Fraction F and splenic B cells twofold dilutions from $8*10^3$ to 10^3 molecules were analysed. The efficiency of mRNA isolation and cDNA synthesis from each sample was verified by amplification of the actin transcript (data not shown).

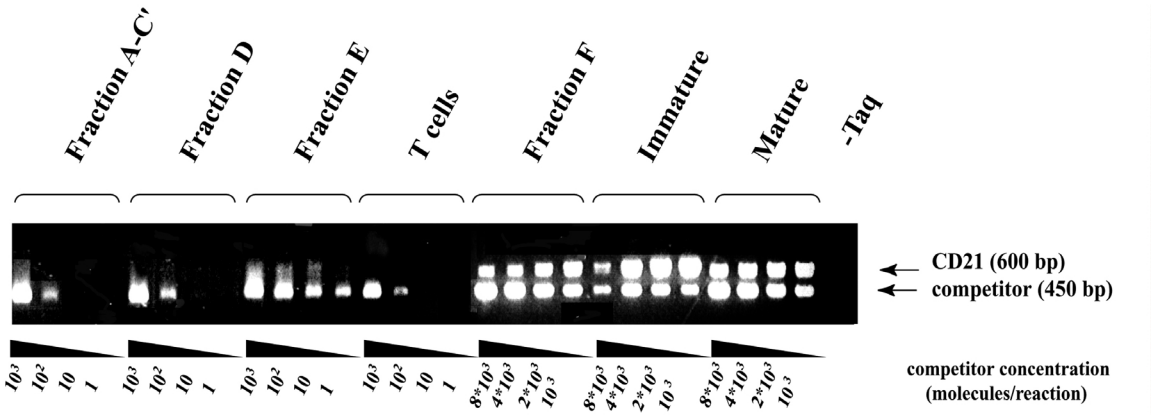


Figure 9: Competitive RT PCR analysis of cDNA from different fractions of bone marrow and splenic B cells and T cells. Primer pair Ex14.5'-Tm2.3' was used. Different amounts of the competitor molecule were included in each reaction as indicated below each lane. The upper band results from amplification of the CD21 cDNA and the lower band from amplification of the competitor molecule. The amount of CD21 mRNA in cells from each B cell fraction can be estimated from the serial dilution of the competitor molecule in which the two bands are of equal intensity.

From the RT-PCR analysis of CD21 transcripts it appears that there is no detectable transcription from the CD21 locus in bone marrow B cells from Fraction A to D (below 100 molecules/10^3 cells). CD21 transcripts appear in Fraction E, which corresponds to immature B cells in the bone marrow and mRNA levels increase subsequently, with highest expression in immature splenic B cells.
This analysis confirms the published reports about CD21 expression profile in B cells. There is no detectable transcription in the early stages of bone marrow development

while CD21 expression is strong in mature B cells, GC and post germinal centre B cells. Therefore the CD21 locus seems suitable for insertion of the recombination substrate.

3.1.2 Generation of the CD21 targeting construct

In order to preserve the desired cell type specific and temporally regulated expression of CD21 we decided to insert the recombination substrate into the CD21 locus by gene targeting. Therefore a targeting vector for the CD21 locus was generated (see Figure 14). This vector contains a neomycin resistance gene (neo^r) for selection in embryonic stem (ES) cells, which is flanked by *loxP* sites and the GFP reporter gene flanked by Recombination Signal Sequences (RSS). A modified version of the EGFP gene was used (gift of S. Kuppig/M. Reth); this is an N-terminal fusion of EGFP (Clontech) with the transmembrane region of BAP-31 (Adachi et al., 1996), which functions as an endoplasmic reticulum (ER) anchoring signal. We chose to use this modified GFP because it could be easier to localise in cryosections from spleen. A third *loxP* site is located downstream of the GFP gene, in the opposite orientation in respect to the other two; this *loxP* site was included in order to allow inversion the GFP gene by Cre mediated recombination.
To avoid disruption of regulatory elements located in the promoter region and the first intron, the neo-GFP cassette was inserted into exon 2 (disrupting the exon but retaining the splice acceptor site) which is located 5 kb downstream of the first exon. The initiation (cc)ATG(g) in the first exon was mutated to (cc)CGG(g) thus introducing an analytical SmaI site for screening. Thus, after RAG mediated inversion (or Cre mediated inversion) the first ATG for translation of the transcribed message will be that of the GFP gene. 3 kb of genomic DNA (including the characterised 1.2 kb promoter region) were included upstream of the ATG mutation as a long arm of homology (LAH) and another 1 kb fragment was cloned downstream of the third *loxP* site as a short arm of homology (SAH). Therefore, insertion of the GFP-neo cassette in the CD21 locus leads to disruption of exon 2 and deletion of exon 3.
Before transfection in ES cells parts of the targeting construct were sequenced (promoter region, coding region of genes and *loxP* and RSS sequences) and the functionality of *loxP* sites and RSS sequences was analysed.

3.1.3 Cre recombination

In order to check the orientation and functionality of the *loxP* sites in the targeting vector an intermediate plasmid generated during the cloning (plasmid #7), which contains the neo-GFP cassette and the three *loxP* sites, was transfected in Cre expressing bacteria (294-Cre; Buchholz et al., 1996) The two *loxP* sites (*loxP*1,2) flanking the neomycin gene are in the same orientation and therefore Cre recombination should lead to deletion

of this DNA segment. The third *loxP* site (*loxP*3), downstream of the GFP gene, is in the opposite orientation and Cre recombination should lead to inversion of the GFP gene. (Figure 10C). Plasmid #7 was transfected in 294-Cre bacteria and overnight cultures were grown from single colonies. DNA was isolated from 10 overnight cultures and digested with HindIII. Surprisingly, this yielded a 3kb fragment, which could only result from complete deletion between *loxP*1 and *loxP*3 (Figure 10A). Sequencing the recovered plasmids showed that in fact deletion had occurred, and only one *loxP* site was left. The sequence of this *loxP* site was an overlap of the two inverted sequences present in the parental plasmid.

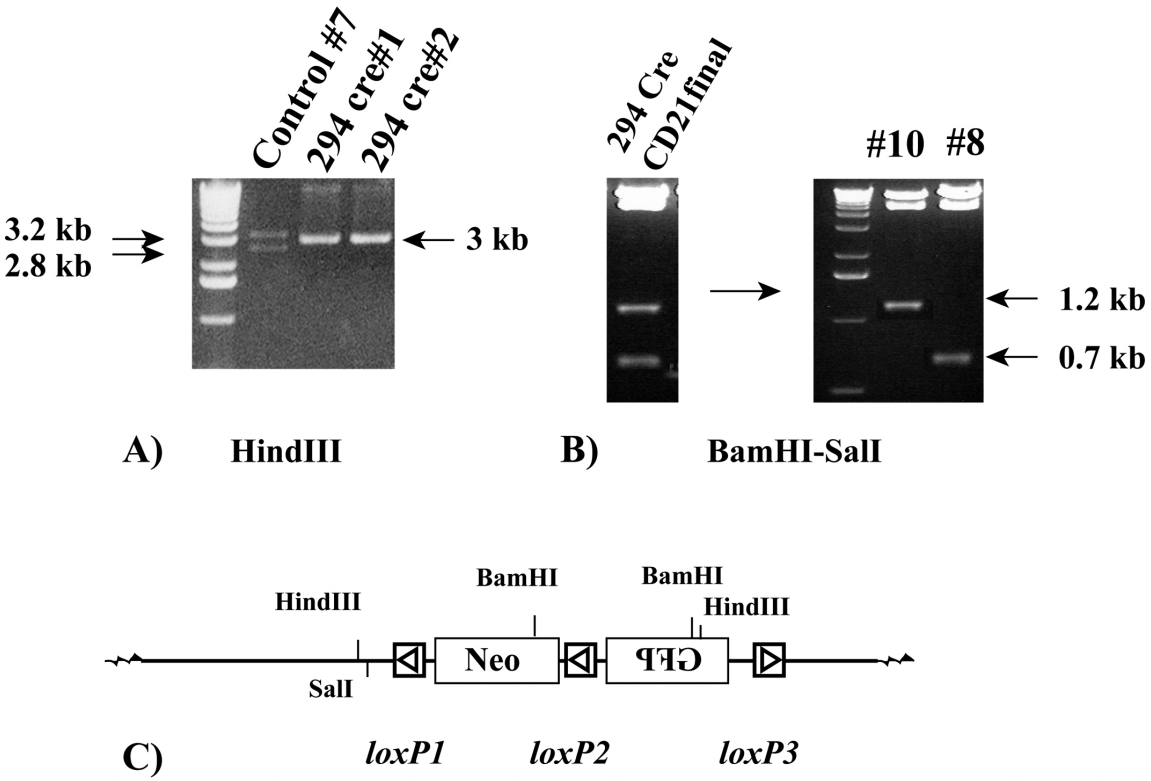


Figure 10: A) Plasmid #7 before and after transformation in 294 Cre bacteria. DNA was isolated from overnight cultures inocculated from single colonies and was digested with HindIII. DNA from two colonies is shown B) Transformation of CD21final plasmid in 294 Cre bacteria. The bacteria were grown at lower temperature; correct recombination events occur, as can be assessed by digesting plasmid DNA with SalI-BamHI. In the minipreps from 294 bacteria both inverted and not-inverted product is there (0.7 and 1.2 kb bands respectively) but subcloning the plasmid gives plasmids which have either inverted (#8) or not inverted (#10) the GFP gene. In all cases the neomycin gene is excised C) Schematic representation of the DNA segment where recombination takes place in both plasmid #7 and CD21 final. Diagnostic restriction digest sites are shown. The length of the resulting fragments is not shown but the map is drawn to scale. Recombination between *loxP*1 and *loxP*2 leads to deletion of the neomycin gene while recombination between *loxP*1 or 2 and *loxP*3 leads to inversion of the GFP gene.

Sequencing again the plasmid showed that the three *loxP* sites were indeed in the opposite orientation, but nevertheless deletion of the intervening DNA sequence occurs. Further analysis of the phenomenon in collaboration with Dr. P. Droege (Aranda et al., 2001) revealed that plasmids deleting one of the two inversely oriented *loxP* sites, which can no longer serve as substrates for the inversion reaction, replicate at a higher rate, diluting out both the parental and the correctly recombining plasmids. In this way, aberrant recombination events, which can occur at as yet undetermined frequencies, are selected. The phenomenon is dependent on high levels of Cre expression, possibly because constant 'flipping' of the substrate hinders its replication and provides a selective advantage for incorrectly recombined plasmids.
It was observed that when the 294-Cre bacteria were not grown at 37 °C, but at room temperature (thereby reducing Cre expression levels) plasmids, which had correctly recombined their *loxP* sites could be isolated. Plasmid yield was improved under these conditions (Figure 10B). Since the inversion can take place in both directions, DNA prepared directly from single colonies of the 294-Cre bacteria contains both possible resulting plasmids: those, which have inverted the GFP gene and those, which have not. These can be distinguished by BamHI-SalI digest, giving either a 0.7 kb or 1.2 kb fragment respectively. Re-transforming the isolated plasmids in E.coli (not expressing Cre) gives plasmids which have either inverted or not inverted the GFP gene (Figure 10B) .
From the above analysis it seems that the *loxP* sites inserted in the CD21 targeting construct are in the right orientation and functional for Cre recombination.

3.1.4 RAG recombination

The RSS sequences introduced in the targeting construct are identical to those found in the IgH locus adjacent to frequently recombining gene segments (VH81x and DFL16.1 specifically) and should therefore be functional in V(D)J recombination. Nevertheless, we wanted to check the functionality of the RSSs in the targeting construct. To do so a modified version had to be created. Modifications were necessary for two reasons; first, expression of the reporter gene in the targeting construct is driven by the B cell specific CD21 promoter, which is not expressed in most tissue culture cell lines and second, it contains the neo^r gene. The neomycin gene has its own promoter and is positioned in the opposite transcriptional orientation from the GFP gene potentially affecting expression of the GFP reporter.
The control plasmid for RAG recombination was generated by transformation of the CD21 targeting vector in 294-Cre expressing bacteria, and selection of clones with a neo^r deletion but not GFP inversion. In this plasmid, the CMV promoter was inserted into the SmaI site (which had been introduced in the ATG of exon 1) just in front of the CD21 promoter (plasmid #17). A second control plasmid, introducing the CMV

promoter in a plasmid, in which the GFP gene had been inverted by Cre, was also generated (plasmid #18).
These two plasmids were transfected in HeLa and 293 cells together with RAG expression vectors (RSV-RAG1 and RSV-RAG2 or CMV-RAG1 ands CMV-RAG2, kind gift of G. Rathbun), or a Cre expression vector (PGK-puro-Cre, A. di Nardo). GFP expression was visualised with a fluorescence microscope after transfection of cells (Figure 11). GFP expression could be detected with both the inverted and the not inverted plasmid.

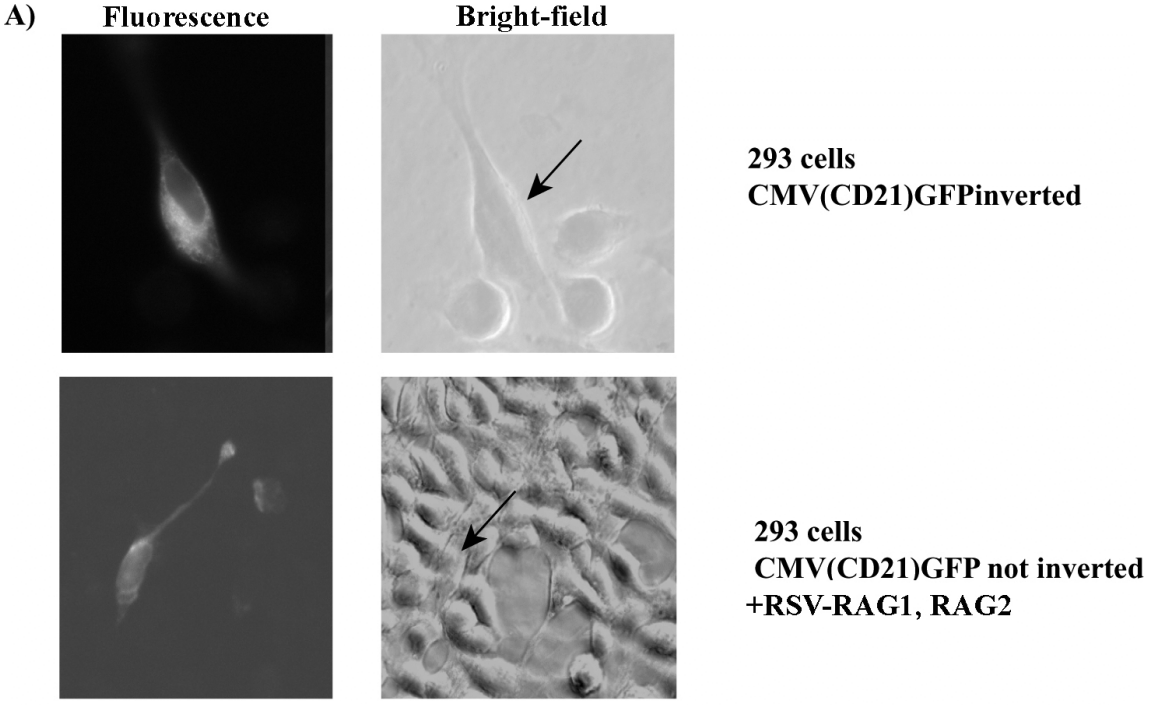


Figure 11: 293 cells transfected with CMV(CD21) GFPinverted or CMV(CD21)GFPnot-inverted plasmids (+RAG expression vectors) were visualised with a fluorescence microscope. GFP expression could be detected in both cell lines

In a separate experiment cells were transfected with the plasmid #17 alone, or together with PGK-Cre expression vector or CMV-RAG expression vectors and analysed by flow cytometry for GFP expression (Figure 12). GFP expressing cells could be detected 72 hours post transfection but no such cells were present when plasmid #10 alone was transfected into the cells. Only 0.4%-1.5% of cells are GFP positive possibly due to low co-transfection efficiency, and the limited time available (in a transient transfection experiment) for synthesis of recombinases, recombination and synthesis of the recombined reporter to occur.

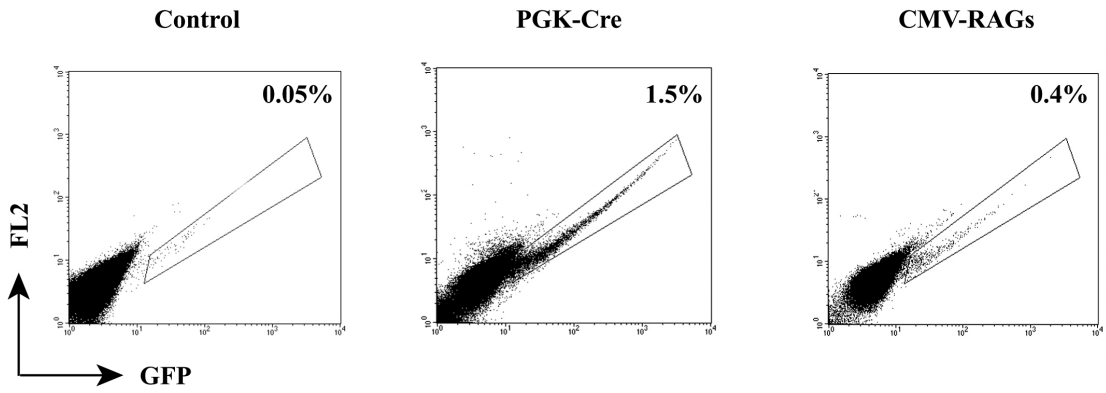


Figure 12: FACS analysis of 293 transfected cells. Control cells were transfected with the plasmid CMV(CD21)GFPnotinverted (plasmid #17). As a positive control plasmid #17 was cotransfected with a Cre expression plasmid. In the last panel plasmid #17 was cotransfected with CMV-RAG expression vectors. Cells were analysed 72 hours after transfection. GFP expressing cells can be detected in both Cre and RAG contransfections but not in cells tranfected with plasmid #17 alone.

In order to verify that the cells observed with the FACS, had really inverted the GFP gene, cells were sorted based on their GFP expression and PCR analysis was performed (using primers BAP31 and CD21Ex2frw). Since there are not many cells expressing GFP in these transient transfection experiments, different samples of 500 cells/tube were sorted. From these cells it was impossible to obtain a clear PCR signal either from the RAG or the Cre transfection experiment (Figure 13B). In fact, in an assay trying to determine the sensitivity of the PCR reaction by titrating a synthetic oligo the detection limit was approximately $5x10^3$ molecules per reaction (Figure 13A).

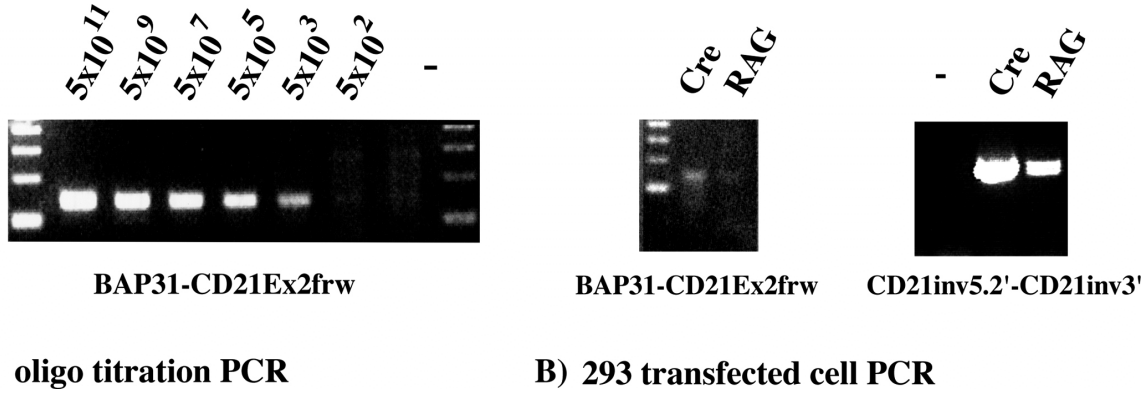


Figure 13: PCR assay using primers BAP31-CD21Ex2frw and CD21inv5.2'-CD21inv3'. A) A synthetic DNA oligo was used as a positive control for the reaction. The sensitivity of the reaction was assessed by serial dilution of the synthetic oligo in cell lysate from 500 sorted 293 cells, starting from $5x10^{11}$ molecules B) DNA from 500 transfected cells (with either plasmid #17+PGK-Cre or

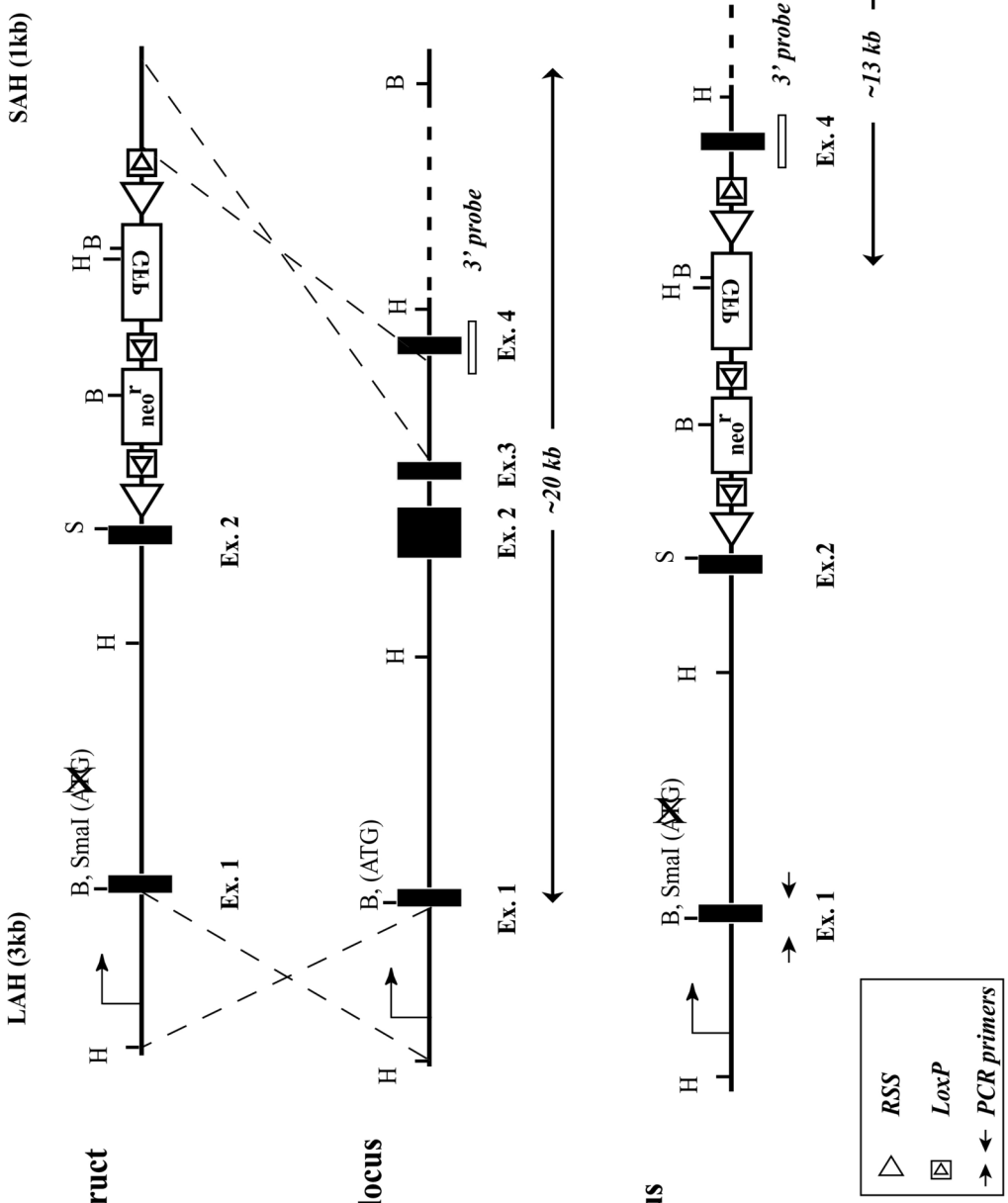
plasmid #17+CMV-RAGs) was PCR amplified with primers BAP31-CD21Ex2frw. A much better PCR product was obtained using primers CD21inv5.2'-CD21inv3' from DNA prepared from 10^4 sorted GFP^+ 293 cells after transfection.

The transfection was therefore repeated and 10^4 cells were sorted from Cre and RAG transfected 293 cells. DNA was isolated and a PCR product was amplified using a different set of primers, CD21inv5.2' and CD21inv3' (Figure 13B).
The RSS can mediate RAG recombination and lead to inversion of the GFP reporter gene in the context of plasmid DNA. The efficiency of the recombination seems lower than that obtained with Cre, judging from the number of GFP^+ cells in the Cre versus the RAG transfection experiment (1.5% and 0.4% respectively). Of course this could also be due to differences in transfection efficiencies, since in the case of RAG recombination two expression plasmids have to be successfully co-transfected together with the reporter plasmid while Cre recombination requires only one additional plasmid.

3.1.5 Targeting of the CD21 locus

After sequencing analysis and functional tests, the targeting vector for the insertion of GFP recombination cassette in the CD21 locus was linearized with NotI and transfected into ES cells (C57BL/6 derived Bruce-4 ES cells). After selection with G418 for 8 days, 400 neomycin resistant clones were picked, expanded and analysed by southern blot hybridisation. The targeting construct, the genomic CD21 locus and the locus after insertion of the substrate by homologous recombination are shown in Figure 14.

Figure 14: Targeting of the CD21 locus. The configurations of the targeting construct, the wildtype locus and the targeted allele are shown. The arms of homology where recombination (indicated by dashed lines) should occur are designated LAH (long arm of homology) and SAH (short arm of homology). The map is shown to scale except for *LoxP* sites and RSS sequences. Restriction sites used for screening are indicated: B, BamHI; S, SalI; H, HindIII . The external probe used to verify the homologous recombination event and the expected sizes of the restriction fragments (in a BamHI digest) are also indicated. PCR primers used forscreening are shown as arrows.


LAH (3kb)
SAH (1kb)
targeting construct
genomic CD21 locus
targeted locus
B, SmaI (ATG)
B, (ATG)
H
S
B
neo^r
CEb
Ex. 1
Ex. 2
Ex.3
Ex. 4
~20 kb
~13 kb
3' probe
RSS
LoxP
PCR primers

Positive ES cell clones were identified by restriction digest either with BamHI or HindIII and hybridisation with an external 3' probe (Figure 15a). The mutation of the ATG of the CD21 gene, introduced in exon 1, could not be verified by southern blot analysis (SmaI digest of genomic DNA was incomplete). Co-integration of the mutation was confirmed by PCR amplification of the first exon (using primers Prom5' and Prom3.2') and restriction digest of the resulting PCR product with SmaI (Figure 15b).

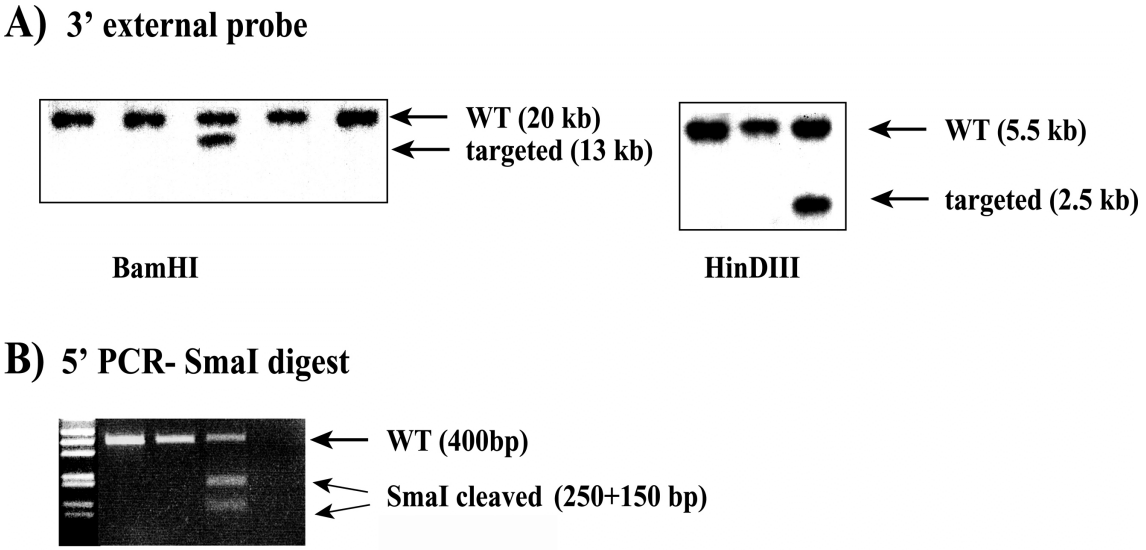


Figure 15: a) Screening of ES cells for homologous recombination in the CD21 locus. Genomic DNA from ES cell clones was digested either with BamHI or HinDIII and hybridised with a 3' external probe (see Figure 14). b) Co-integration of the mutation of the ATG in Exon 1 was verified by PCR and restriction digest of the PCR product.

From the 400 colonies screened, 9 were positive for homologous recombination based on the 3' external probe (and verified by probing with a neomycin or a GFP probe). Only two clones, 3-E9 and 4-B5, showed cointegration of the ATG mutation, as seen by cleavage of the PCR product with SmaI.

The presence of the selection marker (which has its own thymidine kinase promoter) in the CD21 locus might disturb the correct expression of the CD21 gene. Therefore deletion of the neomycin resistance gene (neo^r) in ES cells, before generation of the mice, was necessary. Since the neo^r gene is flanked by two *loxP* sites it can be excised by expression of Cre recombinase. Both homologous recombinant ES cell clones were transiently transfected with a Cre expression plasmid (PGK-puro-Cre) and selected for 36 hours with puromycin (1.25µg/ml). Cells were grown for 8 days and 150 ES cell colonies were picked from each transfection. Clones were analysed for G418 sensitivity and expanded for southern blot analysis. Of the 300 picked clones (from both transfections) 97 were G418 sensitive.

Southern blot analysis was performed using a BamHI digest and part of the sequence of GFP as a probe. Similarly to the analysis of the plasmid substrate for Cre recombination, three recombination events are possible due to the presence of the third *loxP* site 3' of the GFP gene (Figure 14): deletion of the neo^r gene, deletion of the neo^r gene and inversion of GFP, and only inversion of GFP without neo^r deletion (Figure 16).

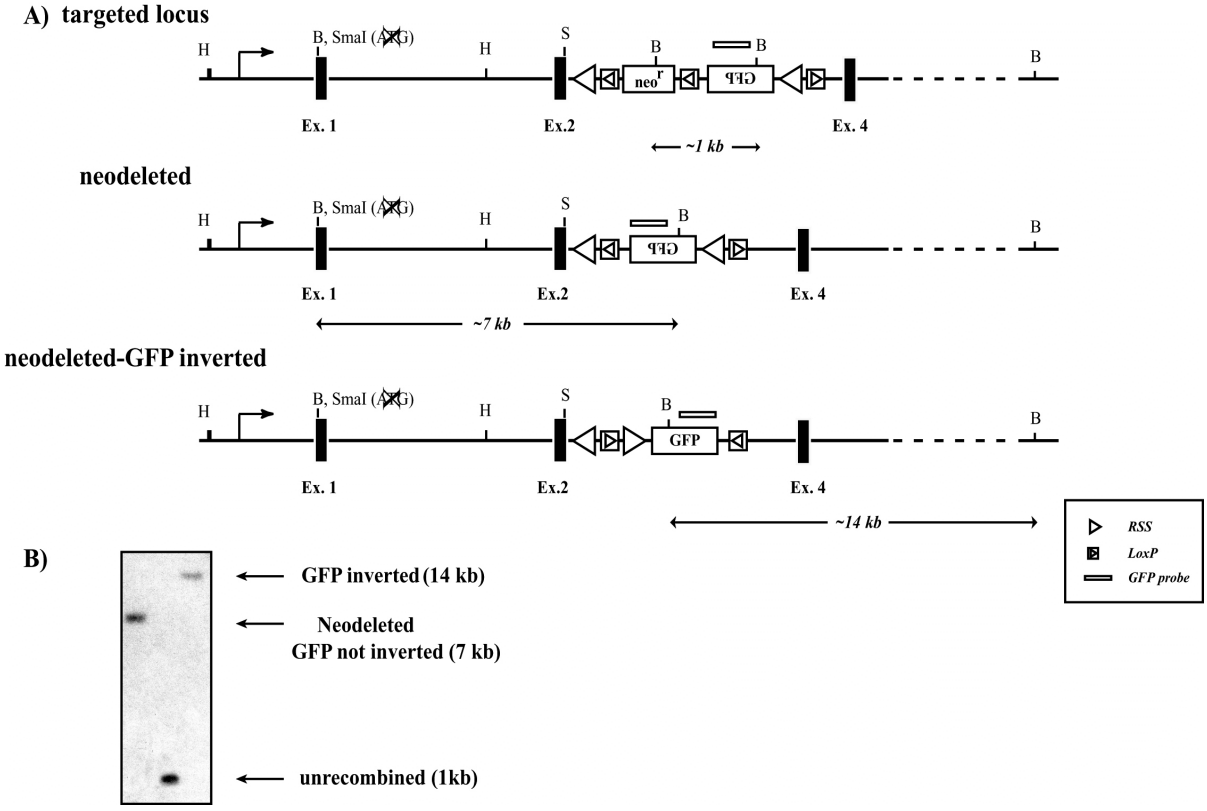


Figure 16: Cre-mediated recombination of the inserted substrate. A) Three possible recombination events can occur (the first two are shown here); neo^r excision only, neo^r excision with concomitant inversion of GFP and only inversion of GFP. B) In the two first cases the recombination event can be monitored by southern blot hybridisation following restriction digest of genomic DNA with BamHI and hybridisation with a GFP probe. Neo^r clones give a 1kb band, neo^r deleted but not inverted 7 kb fragment and neo^r deleted and inverted a 14 kb band

Of the 97 G418 sensitive clones analysed 26 had deleted the neo ^{r} gene without having inverted the GFP gene. In all other cases both deletion and inversion had taken place. Blots were reprobed with a neomycin specific probe to verify excision of the neo^r (data not shown). Two clones, which had deleted the neo gene and had not inverted the GFP gene, 3E9-1B10 and 4B5-3C5 were injected into BALB/c blastocysts for the generation

of chimeric mice. Germline transmission by coat colour was observed for two mice, only one of which had the targeted allele.

3.1.6 Analysis of CD21 reporter mice

Some of the non-transmitting chimeras were used for analysis. In these mice ES cell derived B cells can be discriminated from B cells derived from the blastocyst either by allotypic differences of the IgM molecule (IgM^b and IgM^a respectively or of MHC haplotype). Since RAG reactivation had been reported in germinal centre B cells, the animals were immunised to induce germinal centre formation.
Initially one mouse (chimera 2993) was analysed, 13 days post immunisation with Vesicular Stomatitis Virus. For FACS analysis, cells from spleen and bone marrow were stained with an αIgMb antibody to be able to discriminate ES cell derived B cells ($IgMb^+$) in the chimera. B220 staining was used as a general B cell marker. In this first analysis no FITC coupled antibody was used to allow detection of GFP expressing cells. Splenic cells from immunised C57BL/6 mice were included as negative controls, while as a positive control for GFP expression splenic cells from a GFP expressing transgenic mouse (EBI-2, gift of S. Casola) were analysed.

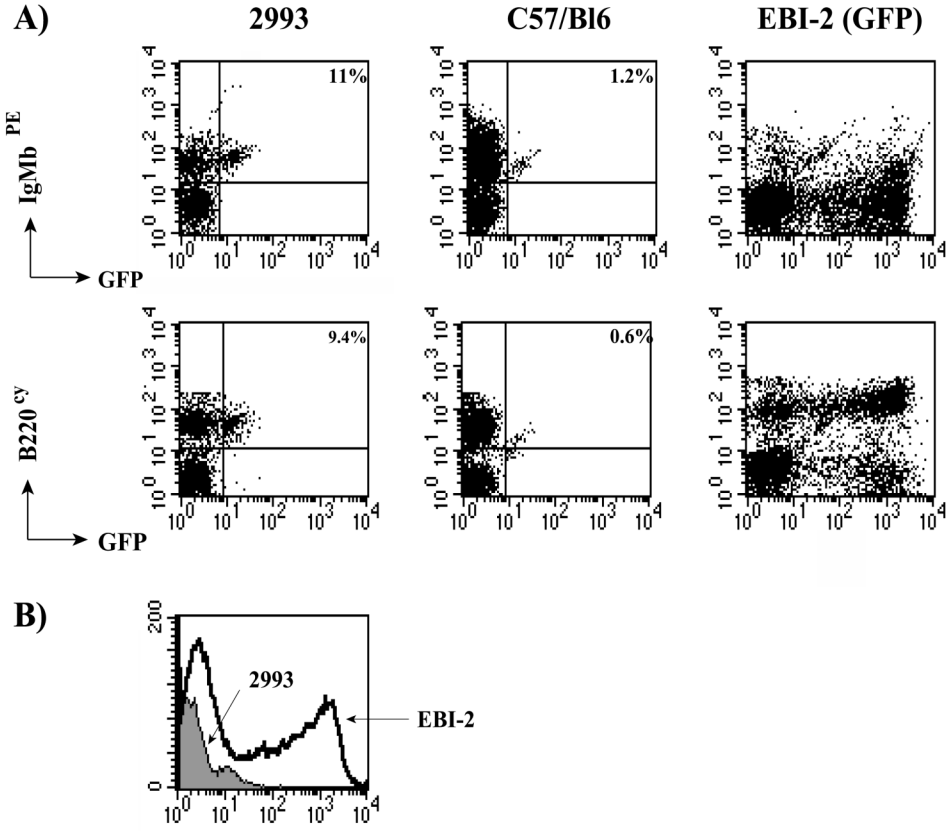


Figure 17: A) FACS analysis of spleen of CD21 chimeras after immunisation. αIgMb staining was used to distinguish ES cell derived B cells from WT BALB/c cells (from the injected blastocysts) and B220 staining to identify B cells. Spleens from C57BL/6 mice were used as negative controls and cells from EBI-2 mice as GFP expressing positive control. B) Histogram overlay of GFP intensity in splenic cells from 2993 chimera and EBI-2 cells.

As can be seen in Figure 17A there is a small population of cells from the chimera which show fluorescence in the GFP channel. These cells also appear in the WT control, but at a much lower frequency (1% compared to 10% of IgMb or B220 positive cells). However, the intensity of the GFP signal is much lower compared to the GFP expressing cells from the EBI-2 mouse (histogram plot overlay in Figure 17B).
In order to confirm that the cells detected in the spleen of CD21-GFP RAG reporter chimeras, represent GFP expressing cells that would result from RAG mediated recombination, DNA from spleen, kidney and thymus of chimeric mice was prepared and analysed by southern blot hybridisation (
Figure 18A). Inversion can be detected on the genomic level by southern blot analysis using a BamHI digest and a GFP probe (as described for Cre recombination in ES cells).The 14 kb band which would result from inversion of the GFP reporter gene cannot be seen by the southern blot analysis. Therefore we designed a more sensitive PCR assay, which would only amplify the inverted substrate (Primers CD21inv5.2' and CD21inv3'). DNA from ES cell clones in which inversion of the GFP reporter gene was obtained by Cre mediated recombination was used as a positive control (Figure 18B).

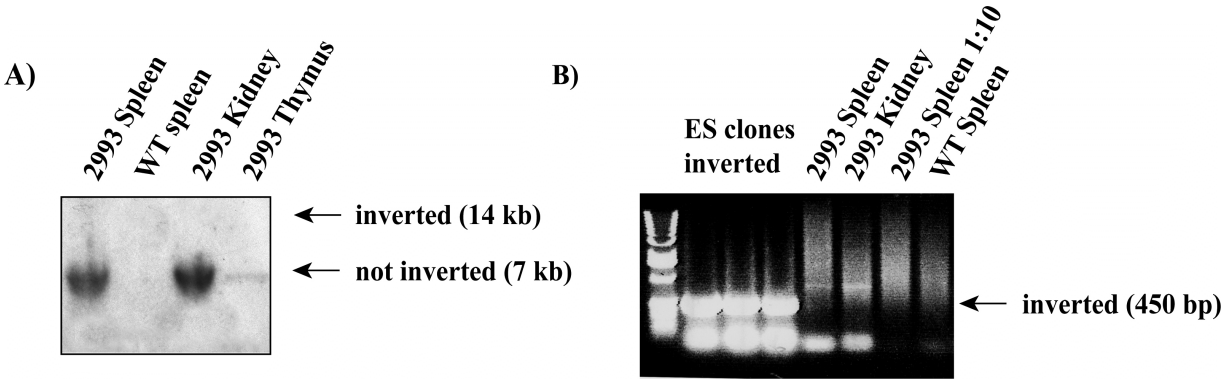


Figure 18: Southern blot (A) and PCR analysis (B) of genomic DNA prepared from total spleen, kindey and thymus of immunised chimera 2993. Southern blot hyibridisation was done using a BamHI digest and a GFP probe, similar to the screening of Cre recombination in ES cells (see Figure 16). RAG mediated inversion would give a 14 kb band similar to Cre mediated inversion. The PCR was performed using primers CD21inv5.2' and CD21inv3' and genomic DNA from Cre inverted ES cells as a positive control.

The PCR analysis of splenic cells from this chimera also failed to detect the inverted substrate. If the percentage of B cells that have undergone RAG mediated inversion is very low, it is possible that the recombination event would be undetectable in DNA prepared from splenic cells, even by PCR.
Therefore more chimeras were immunised (by intraperitoneal injection of NP-CG in alum) and analysed 13 days later. FACS analysis of splenic B cells using αIgMb antibodies as well as an antibody against an MHC class II molecule expressed on C57BL/6 cells (MHC-II I-A^b) was carried out. Using this protocol of immunisation, which was originally used when RAG proteins were detected in GC B cells, a small population of 'green' cells could once more be detected in the chimeras. Also in this case the intensity of the fluorescence was lower compared to the GFP expressing mice used as positive control (data not shown). Bone marrow cells were also analysed by FACS for GFP expression (Figure 19)
The reduced GFP expression in the CD21-GFP-RAG reporter mice could be due to the use of the ER retained BAP31-GFP fusion, since it is known that resting B cells have a restricted ER. LPS stimulation could overcome this limitation since it leads to activation of B cells and formation of B cell blasts with an expanded ER network. Furthemore, LPS in conjunction with IL-4 was described to induce RAG expression in peritoneal cavity and mature B cells (Hikida et al., 1996; Qin et al., 1999). For this reason splenic cells from chimera 54006 were stimulated ex vivo with lipopolysacharide (LPS) and interleukin-4 (IL4). However, no GFP expressing cells could be detected by FACS after stimulation (Figure 19).

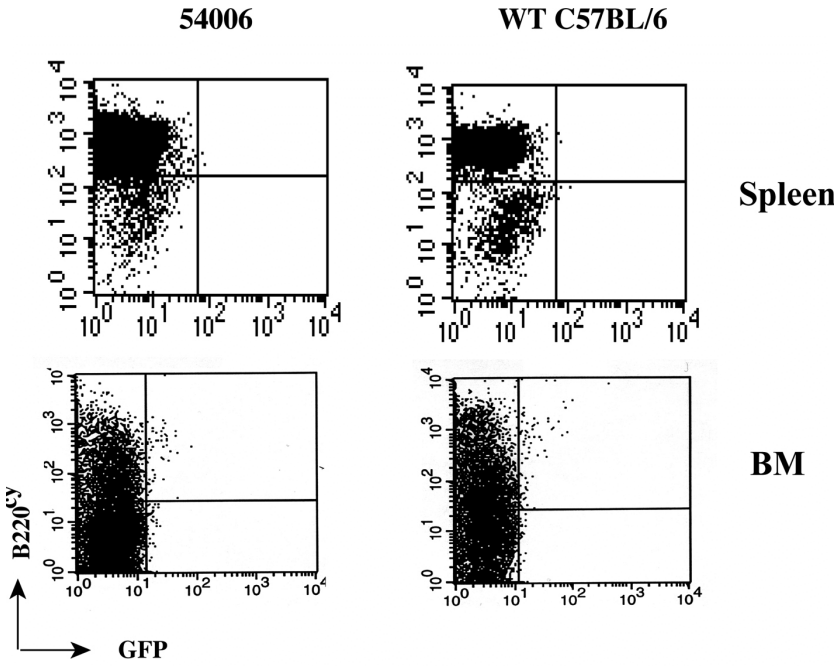


Figure 19: Flow cytometric analysis of LPS stimulated splenic cells from chimera 54006 and a WT C57BL/6 mouse. Cells were kept in culture with LPS+IL-4 for 4 days and stained with αB220. Cells from bone marrow were also analysed by FACS for GFP expression.

We also decided to generate more animals by transfer of bone marrow cells from chimeric mice into sub-lethaly irradiated $RAG^{-/-}$ hosts. All the lymphocytes in the host mice eight weeks after the bone marrow transfer will be derived from the transferred cells (Coligan, 1991). Different populations of cells were sorted from immunised bone marrow chimeras and repeated the genomic analysis. The sorted fractions and number of cells from each fraction are shown in Figure 14. All cells from spleen were sorted based on the lack of MHCII $I\text{-}A^d$ expression, which was used to exclude BALB/c derived cells. Respective populations of splenic cells from immunised C57BL/6 animals were also sorted.

Sample ID	Description	Number of cells
1	BM $B220^{low}IgM^+$	$5x10^4$
2	BM $B220^{high}IgM^+$	$5x10^4$
3	$B220^+$ IgD^+	$2x10^6$
4	$B220^+$ PNA^-	$6x10^5$
5	$B220^+PNA^+$	$2.7x10^5$

Table 3: Sample identification and description of different populations of cells from immunised CD21 chimeras sorted by FACS.

DNA was prepared from the sorted cells and analysed by PCR using the PCR assay developed to detect inversion in 293 cells (see Figure 13). One tenth of the DNA from each sorted fraction was included in the PCR reaction. As mentioned previously by serial dilutions of the synthetic piece of DNA we were able to detect up to $5x10^3$ molecules/reaction. However, mixing the synthetic oligo with genomic DNA from the sorted cells further decreased the sensitivity of the reaction; in this case the PCR assay using $5x10^3$ molecules is not reproducible. Using this PCR assay we were not able to detect RAG recombination in the different populations of sorted cells (Figure 20).

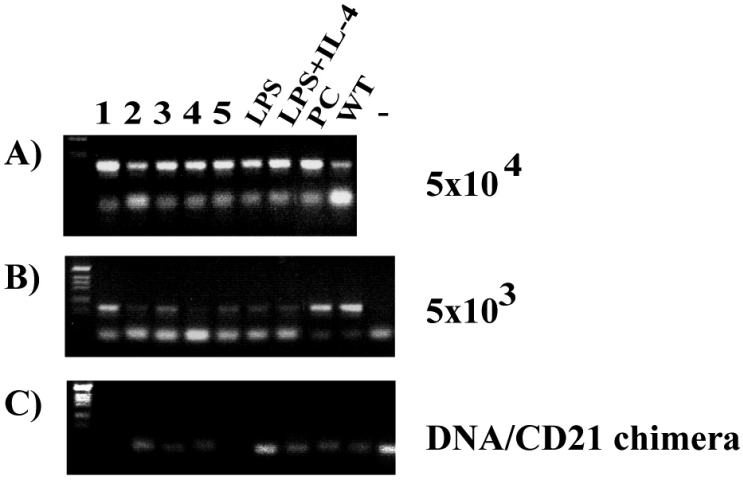


Figure 20: PCR analysis of sorted cells from CD21 bone marrow chimeras. A,B) Synthetic oligo was added in each reaction as a positive control to estimate the sensitivity of the PCR reaction, Two different amounts were added, $5x10^4$ and $5x10^3$ molecules/reaction. The PCR signal in the second case is not very robust. C) PCR analysis of sorted B cell fractions (1-5) and DNA from cells activated in vitro with LPS, LPS+IL-4 and peritoneal cavity (PC) B cells. Wild-type C57BL/6 DNA from spleen was included as a negative control

No RAG mediated inversion in the chimeras analysed could be detected. Unfortunately even by PCR we would be able to detect at best between $5x10^2$ and $5x10^3$ molecules per reaction. Taking into consideration the amount of cells sorted from spleen, at least 5-20% of the cells would have to have inverted the substrate to be detected by this PCR assay and most of the cells from the bone marrow mature and immature fractions. However, total DNA from spleen and thymus shows no detectable inversion.

3.2 Analysis of dependence of RAG mediated recombination and transcription

The CD21-GFP RAG reporter mouse was generated to monitor RAG recombination in mature B cells. In making this reporter mouse we relied on the inability of the RAG proteins to recombine a substrate when it is inserted in a transcriptionally inactive locus. Although there is evidence supporting this hypothesis in Ig and TCR loci (Lewis, 1994) there is no direct system correlating induction of transcription and V(D)J recombination. In order to address this question more directly we decided to use an inducible gene expression system and monitor dependence of recombination on transcription. For this

reason the tetracycline inducible promoter (Tetracycline Responsive Element) was used (Gossen et al., 1995).
The recombination substrate generated is not identical to the one used for the CD21 targeting; in this construct a puromycin resistance gene is flanked by RSS sequences and inserted downstream of the tetracycline responsive element (TRE). Expression of the puromycin^r gene can be induced by tetracyclines and provides a selection marker for generation of stable lines. Additionally it functions as a translational stop cassette (Angrand et al., 1998) preventing expression of the GFP reporter gene, which is located downstream. RAG-mediated recombination leads to deletion of the puromycin^r gene and subsequent GFP expression (Figure 21).

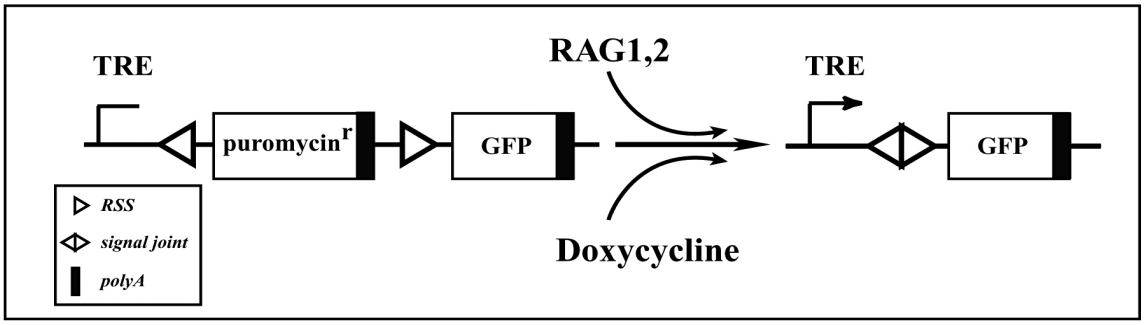


Figure 21: Representation of the RAG recombination substrate generated for transfection in HeLa Tet-On cells. The Tet Responsive Element drives transcription of a puromycin gene in the presence of the inducer (doxycycline). The puromycin gene has a polyadenylation signal, which prevents translation of the downstream GFP and is flanked by Recombination Signal Sequences (RSS). RAG recombination leads to deletion of the puromycin gene and subsequent GFP expression.

The construct was linearised and transfected in HeLa Tet-On cells (Clontech) which express the reverse teracycline-controlled transactivator (rtTA). Upon addition of doxycycline genes driven by the Tet promoter are activated. The activation of expression was assessed using a tet-luciferase construct (data not shown). After transfection cells were selected with puromycin (1 µg/ml) in the presence of doxycycline (2 µg/ml) for 2 days. To reduce stringency of the selction cells were subsequently grown in 500 µg/ml puromycin (and doxycycline) and when clones had reached a reasonable size were picked for analysis. As expected, in the absence of RAG mediated recombination and removal of the puromycin^r gene no GFP expression was detected in these cells.
Only in three, of the 18 clones picked, GFP positive cells could be detected after transfection with the RAG expression vectors. Two of these clones were analysed further. Cells were grown in the presence or absence of doxycycline and transfected either with RSV-RAG1,2 or LTR-RAG1,2 (LTR expression vectors were only used in the presence of doxycycline). 48 hours after transfection all samples were treated with

doxycycline containing media, to induce GFP expression and 36 hours later cells were analysed by FACS (Figure 22).

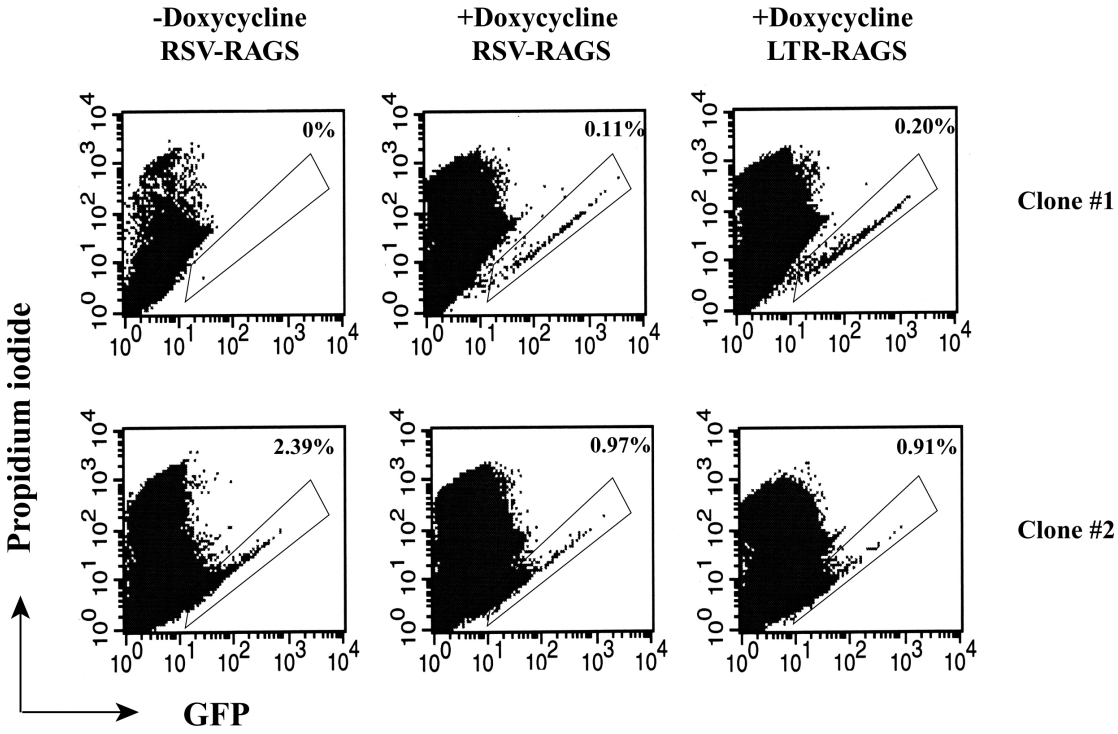


Figure 22: FACS analysis of HeLa cells stably transfected with the recombination substrate and selected for puromycin resistance. Two clones were analysed for GFP expression after transfection with RAG expression vectors in the absence (left panels) or presence (middle and right panels) of doxycycline. The numbers on the upper right corner of each plot indicate percentage of GFP^+ cells.

In clone #1 (top row) no GFP expressing cells were present when transfection with the RAG expression vectors was performed in the absence of the transcriptional inducer. In the presence of doxycycline 0.1% to 0.2% (depending on the RAG expression vector used) of the cells expressed GFP. In contrast clone#2 (bottom row) showed a GFP positive population irrespective of whether the transfection was performed in the presence or absence of doxycycline. Overall the number of GFP positive cells in this clone is much higher than that detected after transfection of Clone#1 (close to 1% with doxycycline and 2.4% in the absence). Therefore transcription could be important for activating RAG recombination but there is a variability in the process, probably due to integration site of the recombination substrate.

3.3 Analysis of allelic exclusion in µMT mice

Another phenomenon where locus accessibility for RAG recombination seems to play a major role is allelic exclusion. Typically immunoglobulin heavy chain (IgH) loci stop rearranging after successful assembly of a heavy chain. There are several mouse mutants, where a breakdown of allelic exclusion has been described (Kitamura and Rajewsky, 1992; Loffert et al., 1996). In most cases this is due to inability of pro-B cells to present a functional pre-B cell receptor on their surface and therefore shut down rearrangement on the other IgH allele. One such mouse model, is the µMT mouse, which contains a neomycin gene disrupting the first membrane exon of IgM (Kitamura et al., 1991). B cells with functional rearrangements on both IgH alleles can be detected in heterozygous µMT mice and these cells are termed 'double producers'. What is surprising however is that the frequency of double producers in the bone marrow, is higher than theoretically predicted (25% versus 12.5%, see Discussion 4.3). The presence of the neomycin^r gene, which has its own promoter, in the IgH locus could be the reason for the observed discrepancy; a transcriptionally active gene could alter accessibility of the locus to RAG recombination and lead to preferential rearrangement of the neomycin^r containing allele. Another idea is that the neomycin^r gene could prohibit establishment of allelic exclusion, and therefore this allele would go on rearranging even after signalling through the pre-B cell receptor has been established.

To test these two possibilities we decided to study the phenomenon in a context, which promotes allelic exclusion. Therefore, the µMT mice were bred to a heavy chain insertion mouse, which is a mouse in which a functional $V_HD_HJ_H$ element has been inserted by gene targeting into the IgH locus (Taki, Meiering and Rajewsky, 1993). In such a mouse most B cells express the pre-rearranged heavy chain, since the inserted $V_HD_HJ_H$ gene in a heterozygous configuration prevents rearrangement of the other heavy chain allele. The heavy chain insertion mouse we chose to use was glD42Hi (Pewzner-Jung et al., 1998), because antibodies produced in this mouse strain can be distinguished from antibodies generated from the µMT allele by allotypic differences (IgM^a and IgM^b respectively). Anti-allotypic staining could be used to distinguish antibodies produced from each allele, and thereby estimate the number of cells harbouring $V_HD_HJ_H$ rearrangements on both chromosomes.

Homozygous µMT mice were crossed to heterozygous glD42Hi. Since the glD42Hi mutation is not bred on a specific genetic background, the µMT mice were also crossed to BALB/c and C57BL/6 mice as controls.

The µMT mutation does not allow the expression of surface IgM (Kitamura et al., 1991), so production of secreted antibody had to be detected. Therefore B cells from spleen and bone marrow of µMT/glD42Hi, µMT/$WT_{C57BL/6}$ and µMT/$WT_{BALB/c}$ and glD42Hi/C57BL6 mice were cultured in the presence of lipopolysaccharide (LPS) to stimulate antibody production. Cells were kept in culture for 4 days and subsequently analysed for IgMa and IgMb production by intracellular staining. Only cells that have

rearranged both IgH alleles (or in the case of the glD42 insertion mouse, the remaining heavy chain allele) can produce antibodies of both allotypes. Splenic cells were analysed either by FACS or allowed to adhere on glass slides, stained and counted under a fluorescent microscope. Cells from bone marrow were only stained on microscope slides since intracellular FACS staining was not reproducible, due to their high autofluorescence. As can be seen from the FACS plots there are no detectable double producers in the spleens of µMT/glD42Hi mice. Both IgM^{b+} cells or $B220^{+}$ are not IgM^{a+}. In contrast, in the µMT/$WT_{C57BL/6}$ splenic cultures, a distinct population of double producers was observed (Figure 23).

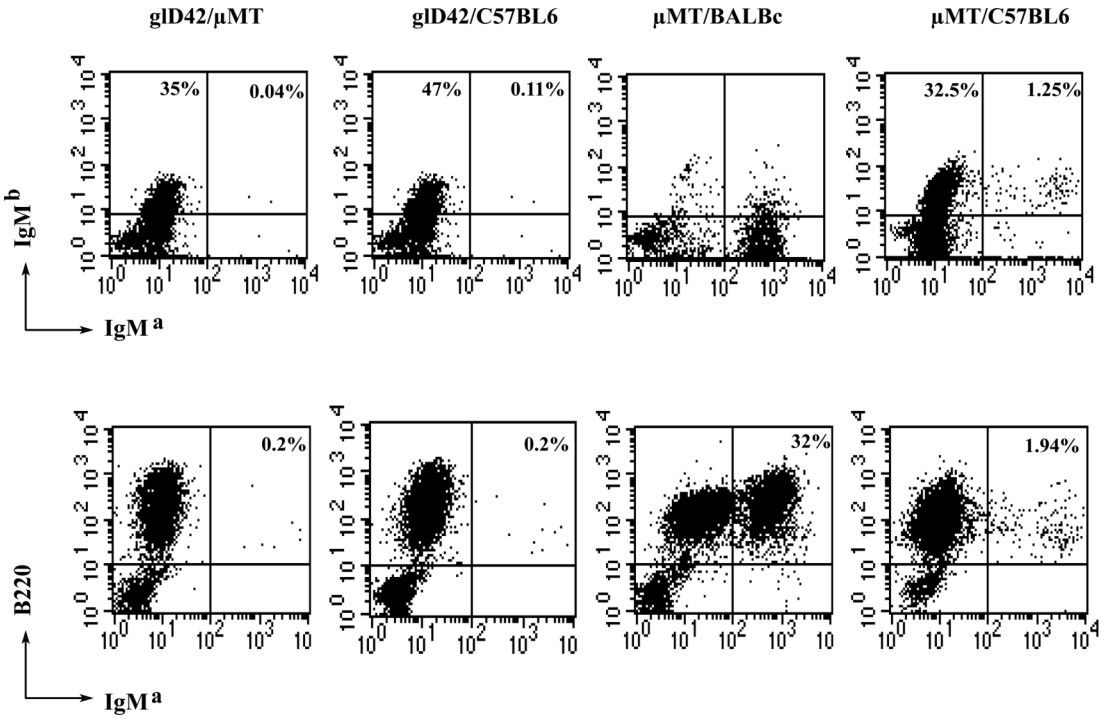


Figure 23: FACS analysis of LPS activated splenic cells from glD42Hi/µMT, glD42Hi/WT, µMT/ $WT_{C57BL/6}$ and µMT/WT_{BalbC}. A) In the upper panel $IgMb^{cychrome}$ versus $IgMa^{FITC}$ staining is shown; B) B220 versus IgMa staining. Most cells are $B220^{+}$ but only 32% of the cells are stained for IgMa even in the positive control (µMT/BALBc).

In the same experiment glD42/C57BL6 mice, which can only produce IgM^{b} antibodies, were included as negative controls, while the µMT/BALBc mice, which can only make IgM^{a} antibodies were used as positive controls. As can be seen from the FACS plots, the glD42/µMT mice have background levels of IgM^{a+} cells (less than 1%). Surprisingly only 32% of $B220^{+}$ cells in the µMT/BALBc mice are IgM^{a+}, probably due to efficiency of the LPS activation and/or intracellular staining. In µMT/C57BL6 heterozygous mice

1.94% of $B220^{+}$ cells are IgM^{a} positive, which assuming the same efficiency of activation and staining as the positive control would correspond to 6.1% of double producers. In the double staining with αIgM^{a} and αIgM^{b} (upper row) approximately 4% of the IgM^{b+} cells are IgM^{a+}.
Cells were also counted on slides after staining with α-IgM^{a} and α-IgM^{b} antibodies.

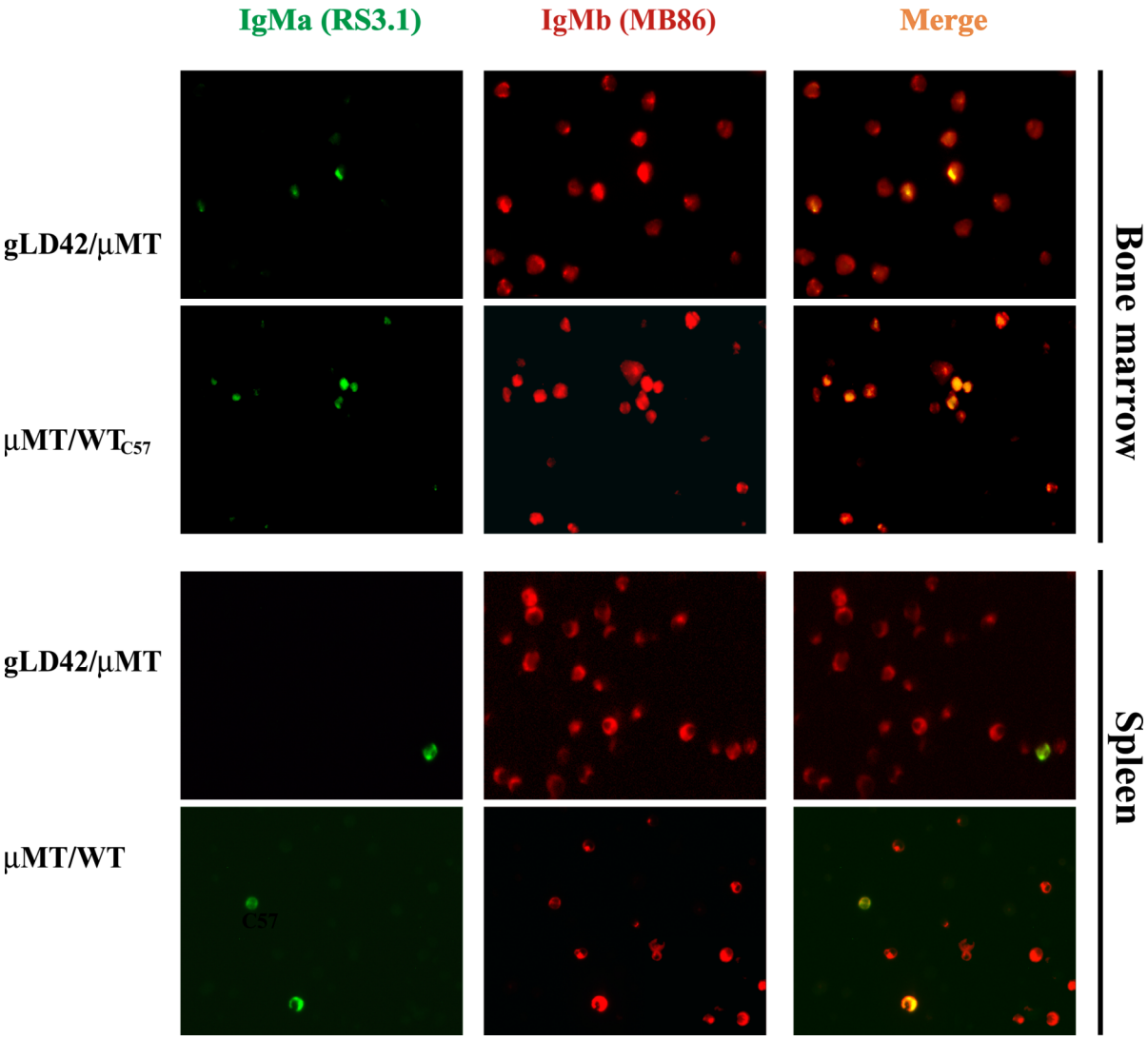


Figure 24: Antiallotypic staining of LPS activated bone marrow cells (upper two rows) and spleen (lower two rows) from glD42Hi/µMT and µMT/ $WT_{C57BL/6}$ mice. IgMa was detected with antibody RS3.1 (green) and IgMb with MB86 (red). Double producers appear yellow in the last panels (merged image).

The number of double producers counted in µMT/C57BL6 mice, in three independent experiments is between 4-7% in the spleen and ~20% in the bone marrow. On the other hand glD42/µMT mice have less than 1% double stained cells in the spleen and ~2% in the bone marrow. The results from FACS analysis and slide stainings are summarised in Table 4.

Strain	FACS stainings		Slide stainings	
	$B220^+$	**IgM^{b+}**	**Spleen**	**Bone marrow**
glD42/µMT	0.5%	0.1%	0.3% (1/476)	2% (2/110)
µMT/C57BL6	6.4%	4.2%	5.8% (39/670)	22.2% (48/216)

Table 4: Percentages of double producers from µMT/C57BL6 and glD42/µMT mice. In the FACS stainings values were calculated either from B220 versus IgM^a staining or IgM^b-IgM^a double staining. The values given for the B220 staining were normalised for efficiency of the IgM^a staining as assessed from the µMT/BALBc positive control. (~30%). Cells on slides were stained for IgM^a and IgM^b; the number of double positive versus single positive (IgM^{b+}) cells counted is given in parenthesis. Values are the means of two independent experiments.

Absence of double producers in the µMT/glD42 cross indicates that the inserted $V_HD_HJ_H$ allele efficiently blocks rearrangement of the neomycin containing allele.
Another way to directly assess the presence of double producing B cells in the mice is by analysis of serum titers of unimmunised animals for IgM^b and IgM^a antibodies by Elisa (Figure 25 and Figure 26 respectively).

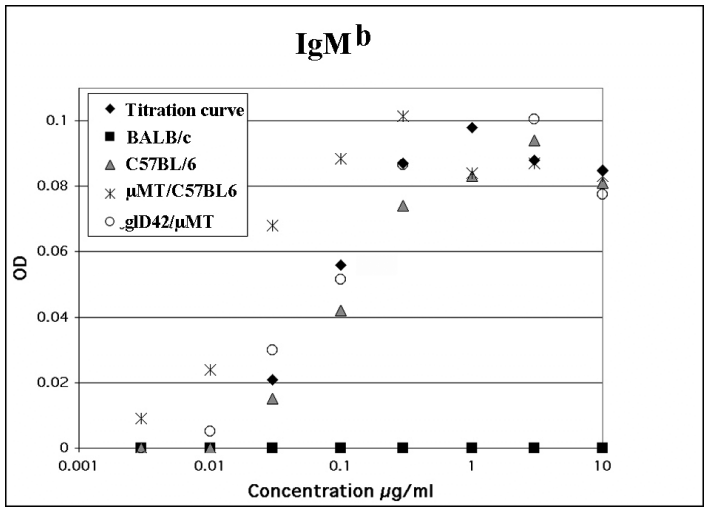


Figure 25: Determination of IgMb titers by an Elisa assay in glD42/µMT and µMT/C57 mice. BALB/c and C57 animals were included as controls. As expected both µMT/C57 and glD42/µMT produce comparable levels of IgMb antibodies.

IgMb titers from the mice were analysed to determine total IgM production (IgM^a+IgM^b). As expected the titers for IgM^b are similar in µMT/C57BL6, glD42/µMT and C57BL/6 animals since all three mice can produce antibodies of the IgM^b allotype, while BALB/c mice show no detectable levels of IgM^b. Measuring IgM^a titers (Figure 26), which can be produced from the µMT allele, would give an indication for the presence or absence of double producers.

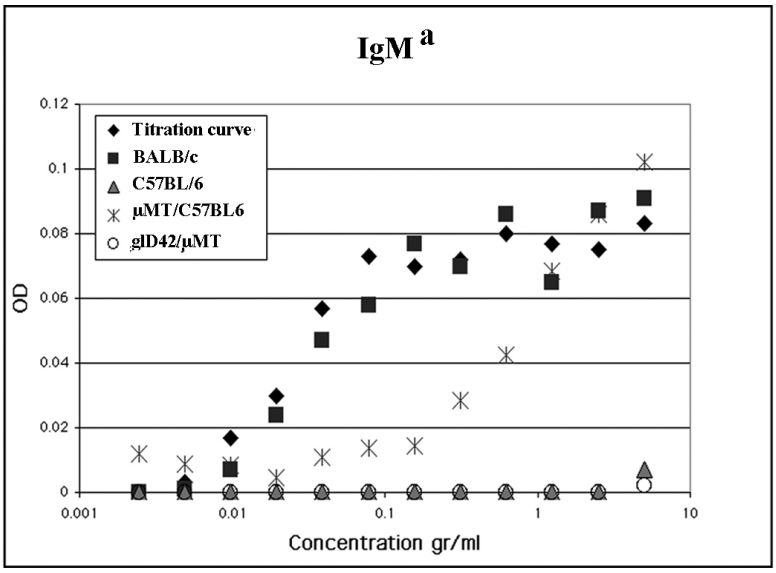


Figure 26: ELISA assay to determine the IgMa serum titers of µMT/C57BL6 and glD42/µMT mice. Sera from BALB/c and C57BL/6 mice were included as controls.

IgM^a titers are very different; BALB/c mice which only express IgM antibodies of the a allotype have approximately 300 µg/ml of IgM^a antibodies in their sera, as estimated from the titration curve, while µMT/C57BL6 mice have 20~10µg/ml. IgM^a antibodies were not detected in the serum of glD42/µMT mice, which supports the observation that no double producers cells are present in the spleen of these mice.

Finally rearrangement of the IgH locus can be directly monitored at the genomic level by southern blot analysis. Two probes DQ52 and DFL16 have been described which bind to regions upstream of DQ52 (which is the last D element of the IgH locus) or upstream of DFL16.1 (which is the 5' most D element) respectively. Most $D_H \rightarrow J_H$

rearrangements will lead to deletion of the region recognised by the DQ52 probe and IgH allele, regardless if this is productive or non productive.
To assess the extent of rearrangement in μMT/C57BL6 compared to glD42/μMT mice and wild-type mice, $B220^+$ and $B220^-$ cells from spleen were isolated by magnetic cell sorting (MACS). The purity of the populations after MACS purification was calculated by immunostaining for CD19 and B220 and analysis by flow cytometry. In all cases the purity of the $B220^+$ cells was more than 90%. The percentage of contaminating $B220^+$ cells in the $B220^-$ population was between 5 and 10% (data not shown).

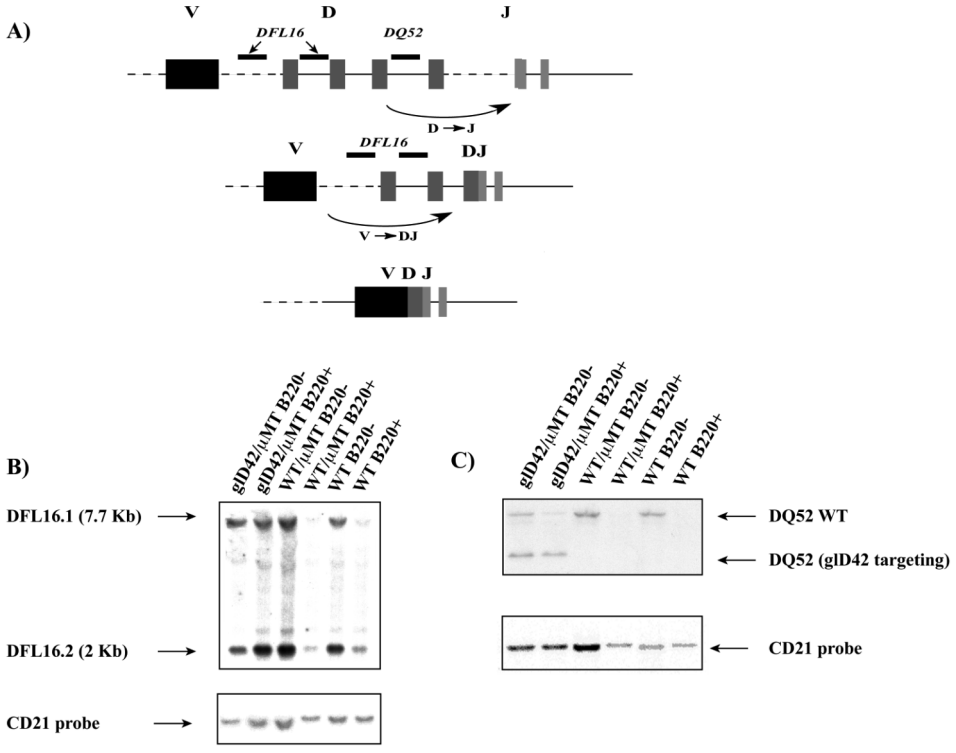


Figure 27: A) Organisation of the 3' region of the IgH locus and binding sites of the two probes used for analysis, DQ52 and DFL16. $D_H \rightarrow J_H$ rearrangement leads to deletion initially of the fragment recognised by the DQ52 probe and $V_H \rightarrow D_H J_H$ rearrangements of that recognised by the DFL16 probe. The DFL16 probe recognises two regions, upstream of DFL16.1 and DFL16.2 respectively B) Southern blots from MACS purified B cells and non B cells ($B220^+$ and $B220^-$ respectively) from spleens of glD42/μMT mice, heterozygous μMT and wildtype mice. To estimate the amount of DNA loaded per lane both blots were reprobed with the CD21 probe (lower panel).

DNA was prepared from $B220^+$ and $B220^-$ cells from wild-type, glD42/μMT and μMT/C57BL6 mice. DNA was digested either with AccI or EcoRI and blots were probed with DFL16 and DQ52 probes respectively. The DFL16 probe binds specifically two DNA fragments, one located upstream of DFL16.1 (7.7 kb) and another upstream of DFL16.2 (2.2 kb). Quantification of the amount of DNA loaded per sample was performed by reprobing the blots with an unrelated genomic probe (either the CD21 probe or in the case of thymus DNA with DFL16 probe the Cμ RH probe was used as a DNA loading control). The intensity of the bands on the southern blots was quantified using Imagequant software and a STORM phosphoimager (Molecular Dynamics) and re-analysed with a quantitation programme written by H. Stoffler. The results are shown in Table 5 as the ratio of the signal of each D probe to the CD21 control probe.

STRAIN	**DQ52**	**DFL16.1**	**DFL16.2**
glD42/μMT $B220^-$	0.61	0.64	1.2
glD42/μMT $B220^+$	0.12	0.71	1.0
glD42/μMT Thymus	0.35	1.9	2.2
μMT/WT $B220^-$	0.57	0.72	1.1
μMT/WT $B220^+$	0	0.08-0.15	0.5
μMT/WT Thymus	n.d	2.2	2.7
WT $B220^-$	0.65	0.9	1.1
WT $B220^+$	0.02	0.4	0.67
WT Thymus	n.d	2	2.2

Table 5: Intensity of the signal of DQ52 and DFL16 probes quantified by phosphoimager and ImageQuant software. The numbers given represent the ratio of the intensity of the bands of either the DQ52 or the DFL16 probe to the CD21 probe. For thymus DNA quantitation the RH probe was used, which due to different binding efficiency compared to the CD21 probe gives a different ratio. However the value is close to 2 in all three mouse strains, suggesting that no rearrangement takes place in thymocytes in these mice.

The loss of the DQ52 probe signal is due to $D_H \rightarrow J_H$ rearrangement which is known to happen in the majority of B cells on both alleles, and can also occur in T cells . In μMT heterozygous mice as well as in WT mice the DQ52 probe signal is almost undetectable in $B220^+$ cells. In $B220^+$ cells from the glD42/μMT mice the DQ52 signal from the wild-type allele is reduced about fivefold (0.12/0.60), suggesting that $D_H \rightarrow J_H$ rearrangement does occur. There is no reduction of the signal from the targeted allele since all J_H elements have been removed by the targeting and no $D_H \rightarrow J_H$ joining can occur. Some loss of DQ52 signal also happens in the thymus of the gld42/μMT mice (0.35/0.6) where $D_H \rightarrow J_H$ rearrangement is known to occur.

Since IgH allelic exclusion is established as a block of $V_H \rightarrow D_H J_H$ rearrangement, analysis of DFL16 probe signal is more informative about the breakdown of allelic exclusion. Southern blot hybridisation shows that there is no significant loss of DFL16 signal in the glD42/μMT mice (comparing $B220^+$ and $B220^-$ cells from spleen) suggesting that no significant $V_H \rightarrow D_H J_H$ rearrangement happens in B cells in these mice. No differences in DFL16 signal intensity are observed in thymus DNA from glD42/μMT, μMT/WT and wild-type mice.
On the other hand μMT heterozygous mice and WT mice show a significant reduction in the signal of the DFL16 probe in DNA from $B220^+$ cells. The reduction is stronger in μMT/WT mice supporting the finding that in these mice there is a higher number of double producers (6-25% compared to 0.1% in wild-type mice (Barreto and Cumano, 2000). Loss of the signal of the DFL16 probe, especially of theDFL16.1 band, makes it hard to quantify both in WT and especially in μMT heterozygous mice. The values in individual experiments and using different quantitation software range from loss of up to 90% (suggesting almost complete rearrangement) to 70% of the DFL16.1 signal.
From the above analysis it appears that allelic exclusion in glD42/μMT mice is not impaired. There are no detectable double producers after LPS stimulation and staining of cells on slides or analysis by flow cytometry, and there are no detectable levels of IgM^a antibodies in the sera of these mice. Furthermore, the genomic locus doesn't seem to undergo $V_H \rightarrow D_H J_H$ rearrangement although some $D_H \rightarrow J_H$ rearrangement does take place. We conclude therefore that the high number of double producers in the μMT heterozygous mice is due to preferential recombination of the targeted, neomycin containing allele during recombination of the IgH locus, rather than rearrangement of this allele during light chain rearrangement.

3.4 Conditional disruption of IgM membrane exons

As previously mentioned, in the μMT mouse (Kitamura et al., 1991) the first membrane exon of the constant region of IgM is disrupted by insertion of a neomycin resistance gene, which blocks the production of membrane bound IgM. The $neomycin^r$ gene has its own promoter (thymidine kinase promoter) and is stably integrated in the genome. Since it is impossible to remove this gene, it is difficult to assess the importance of an actively transcribed gene in the Ig locus and analyse its significance independently of the truncation of the IgM membrane region.
Therefore, we decided to generate a mouse strain (Cμ $membrane^{flox}$) in which the $neomycin^r$ gene is targeted in the constant region of IgM (similar to the situation in the μMT mouse) but can later be removed by a genomic recombination event.
A targeting construct which has a neomycin resistance gene just upstream of the IgM membrane exons was generated. The $neomycin^r$ gene is flanked by Flip Recombinase Target (FRT) sites and can be removed by expression of Flp recombinase. The two

membrane exons, on the other hand, are flanked by *loxP* sites and can be deleted by expression of Cre recombinase.
The targeting construct was generated as described in Materials and Methods and linearized with NotI. Bruce4 ES cells were transfected and selected for G418 resistance. Clones were picked and analysed for homologous recombination by Southern blot hybridization using an EcoRI digest and a 5' EcoRI-HindIII fragment as a probe (RH probe) (Figure 28).

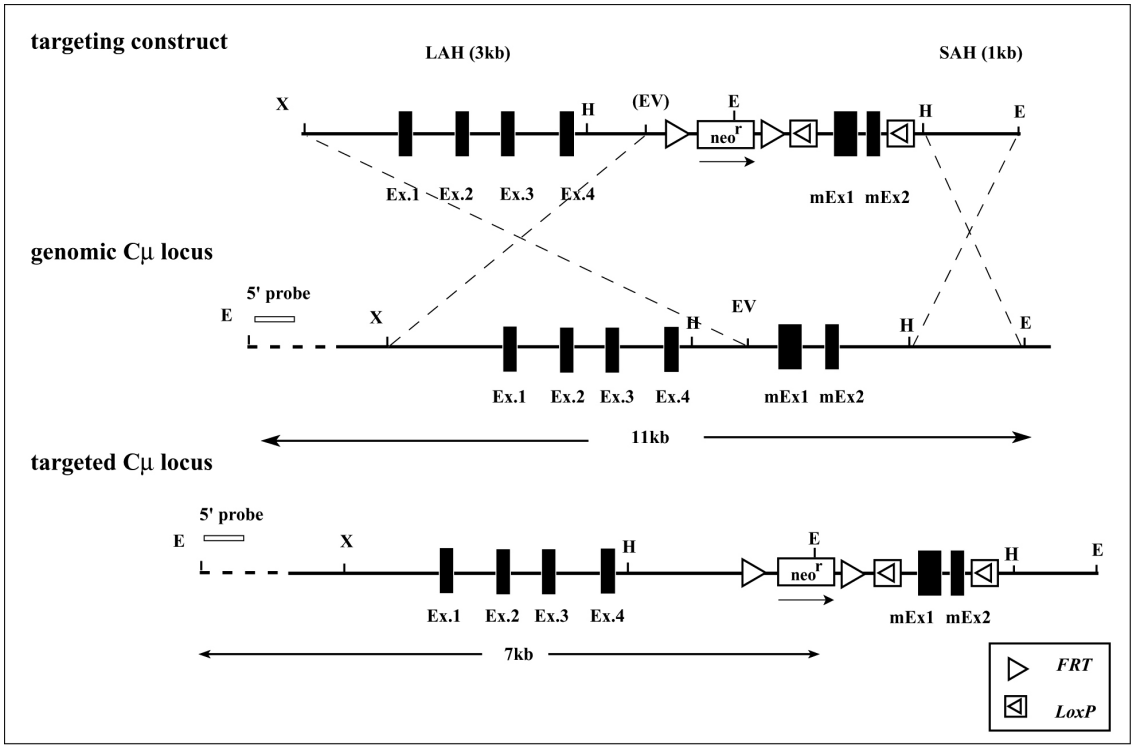


Figure 28: A)Generation of homologous recombinant Cμ membrane^{flox} ES cell clones. The configurations of the targeting vector (top), the wild-type IgM constant region locus (middle) and the targeted locus after homologous recombination (bottom) are shown. The external probe used for screening is shown (open rectangle) as are the diagnostic fragments generated after an EcoRI digest. Arrows underneath the targeting construct indicate the direction of transcription of the selection marker gene. Short arm of homology (SAH) and long arm of homology (LAH) where homologous recombination should take place are indicated by dashed lines. *loxP* sites are indicated as boxed triangles and FRT sites as triangles. Restriction sites displayed: E, EcoRI; H, HindIII; X, XbaI; EV, EcoRV

From 300 clones picked no homologous recombinants were identified. The DNA used for the generation of the targeting construct (long and short arm of homology) is BALB/c derived and polymorphisms in the IgM genomic locus, could reduce the chance of homologous recombination using C57BL/6 ES cells. Therefore the ES cell

transfection was repeated with the same targeting construct but targeting 129 derived ES cells (the subclone of the E14.1 ES cell line, IB10). 600 colonies were picked and analysed by southern hybridization using the same probe and restriction digest as previously described. Three homologous recombinants were identified. Co-integration of the downstream *loxP* site was monitored by PCR. Only one of the three clones (4G6) gave a positive PCR signal (Figure 29).

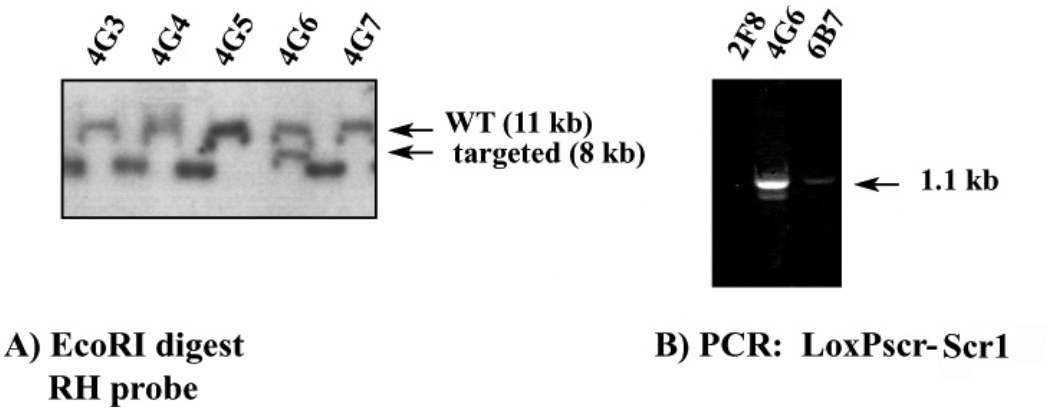


Figure 29: Southern blot analysis of ES cells using the 5' EcoRI-HindII fragment as probe (RH probe). ES cell DNA was digested with EcoRI which gives an 11 kb wild-type band and an 8 kb band in case of homologous recombination. Samples are loaded in two levels which are misaligned (lower band is wildtype band of clones H3,H4, etc. running 0.5 cm below the top lane).

The targeted clone was injected in C57BL/6 and CB20 blastocysts and chimeras were generated. These are breeding for germline transmission with Flp-Deleter (Farley et al., 2000) or CD19-Cre (Rickert, Roes and Rajewsky, 1997) or Mx-Cre (Kuhn et al., 1995) mice, which have been crossed to JHT mice. The progeny will have one JHT allele (which cannot code for immunoglobulin, since the J elements have been deleted) and the targeted allele. Depending on the cross the neomycin gene will be deleted (in the case of the Flp-Deleter cross) or the Cμ membrane exons will be removed by Cre recombination early in B cell development (CD19-Cre) or later after induction of Cre transcription from the Mx promoter. Therefore there is no need to breed homozygous mice,but the effect of the mutation can be directly analysed in the progeny of the chimeras.

4. Discussion

4.1 Recombination Activating Gene (RAG) protein expression in peripheral B cells

Developing B lymphocytes express RAG proteins in order to assemble their Ig receptors. The finding that RAG proteins can also be expressed in peripheral B cells, either in germinal centres and or upon activation with LPS and IL-4, lead to speculation about their possible involvement in the process of affinity maturation (Han et al., 1996;Hikidaet al., 1996). RAG protein expression is not necessarily indicative of ongoing V(D)J recombination, but double-stranded RSS breaks, characteristic of RAG activity were additionally detected in the Igκ loci in splenic B cells *in* and *ex vivo* (Han et al., 1997; Papavasiliou et al., 1997). Therefore a novel mechanism, involving V(D)J recombination, for generation of secondary antibody diversity was postulated.
Initially, such an idea might appear absurd, since the chances of ruining productively rearranged elements, which can already bind antigen with certain affinity are high. The process of affinity maturation however is 'expensive' and massive cell death occurs in the germinal centre reaction (MacLennan, 1994). Receptor revision could be a successful mechanism for the generation of antibody diversity considering that antibodies with similar specificities have identical heavy and different light chains and that V_H and V_L elements can be clustered into families with significant sequence similarity (Kabat and Wu, 1991;Strohal et al., 1989). Therefore, replacement of the light chain variable region, by *de novo* rearrangement, could result in subtle changes of BCR specificity, comparable to those introduced by somatic hypermutation. It should be noted that, recombination intermediates and RAG mRNA are reduced upon antigen receptor engagement in mature B cells (Hertz et al., 1998) (Meffre et al., 1998), which is in contrast to the situation in immature B cells of the bone marrow, where receptor editing is stimulated or maintained by autoreactive receptors (Hertz and Nemazee, 1997). The

objective of receptor revision seems to be the generation of a BCR able to recognise cognate antigen with higher affinity, upon which RAG expression is down-regulated.
Evidence that RAG1 is abundant in apoptotic B cells in the germinal centre (Hikida et al., 1997), suggests that V(D)J recombination could also rescue cells that have acquired a crippling mutation of the rearranged variable region during the process of somatic hypermutation.
After the original observation of RAG expression in peripheral B cells, and in the course of this work, several mouse models for monitoring RAG expression were generated; these are very similar in that they contain a targeted insertion of a reporter gene (GFP) either in the RAG-1 (Kuwata et al., 1999) or RAG-2 locus (Gartner et al., 2000). In another mouse strain GFP is inserted as a BAC transgene using the RAG2 promoter (Yu et al., 1999). B cells coming from the bone marrow, but also T cells in the thymus express GFP, since the respective RAG promoters drive transcription of the reporter gene in these populations. GFP expression decreases subsequently and there is no apparent re-induction after immunisation. Culture of GFP^- and GFP^+ cells with LPS and IL-4 does not lead to GFP expression or maintenance of the signal respectively. Therefore, cells that express RAG proteins in the periphery and can potentially undergo receptor revision seem to belong to a population of immature, transitional B cells (Carsetti, Kohler and Lamers, 1995) whose emigration from the bone marrow is facilitated by immunisation (Nagaoka et al., 2000; Nemazee and Weigert, 2000).
The mouse model we tried to generate differs from the ones previously described in two basic points: a) it assays directly the functionality of the RAG recombinase (for which both RAG1 and RAG2 have to be expressed) and not just activity of the promoter regions and b) if recombination of the substrate occurs the reporter is not transiently expressed but the cells are constitutively labelled.
Since the purpose of the experiment is to label cells that express RAG proteins in the periphery, recombination of the substrate should not occur in the bone marrow. V(D)J recombination does not affect all loci present in a cell, suggesting that there are potential ways to target recombination to specific substrates or regulate the recombination event temporally. Transcription of endogenous Ig and TCR loci seems to correlate well with onset of recombination (Yancopoulos and Alt, 1985)(Goldman et al, 1993) while transcription of V_H elements is not detected in cell lines unable to perform V_H to D_HJ_H recombination (Schlissel, Corcoran and Baltimore, 1991). Therefore, insertion of the reporter substrate into a transcriptionally inactive locus could be used as a potential means to prevent recombination of the substrate during primary Ig rearrangement. Activation of the locus later, in mature B cells, is necessary in order to allow recombination in case of RAG re-expression
The CD21 gene was the only reported example which displayed the desired gene expression profile: no expression (within detection limits) in bone marrow B cells, but constitutive expression in almost all later stages of the B cell life cycle (Hu et al., 1997).

For this reason, and after analysis of CD21 mRNA in sorted B cell populations we decided to target the CD21 gene for insertion of the recombination substrate.
Insertion of the recombination substrate into the CD21 locus could be a problem since the CD21 gene is thus disrupted. Targeted disruption of the CD21 gene is known to impair humoral immune responses and germinal centre formation (Ahearn et al., 1996; Molina et al., 1996). However, only one allele is mutated in the reporter mice and abrogation of one copy of the gene does not affect germinal centre formation. PNA positive B cells, derived from targeted ES cells, were indeed found after immunisation in the chimeras analysed.
Although the general opinion is that transcriptionally silent substrates are refractory to recombination (reviewed in Sleckman, Gorman and Alt, 1996) there have been reports of rearrangement in the absence of detectable transcription (Kallenbach et al., 1993) (Lauster et al., 1993) (reviewed in Lewis, 1994)]. The possibility that recombination of the substrate would occur in the bone marrow, despite the choice of a transcriptionally 'inactive' locus for insertion, could not be excluded. Recombination could also occur in the thymus, where the RAG proteins are expressed for TCR assembly. However, in the chimeras analysed, there was no detectable GFP expression in bone marrow B cells, or inversion at the genomic level in thymus (Figure 14) and bone marrow (Figure 19, 20). Therefore recombination does not occur when the RAG recombination machinery is actively rearranging Ig and TCR loci. This in itself is an interesting observation; recombination signal sequences have been characterised as the DNA elements, which are recognised by the RAG proteins (Difilippantonioet al., 1996; Sakano et al., 1979) and can be cleaved by the RAG1/RAG2 complex *in vitro* (Ramsden, Paull and Gellert, 1997). RSSs are also sufficient in mediating V(D)J recombination of extrachromosomal substrates (Hesse et al., 1987) (Lieber et al., 1988), but it is still not clear whether they alone can direct V(D)J recombination outside Ig and TCR loci, in a locus not usually targeted for V(D)J recombination. Most transgenic substrates analysed for V(D)J recombination are in fact Ig and TCR mini-loci (McMurry et al., 1997) containing not just the RSS sequences but additional enhancer/promoter elements present in these loci . These elements might affect or even be necessary for targeting recombination to specific DNA regions (Engleret al., 1991; Lauster et al., 1993). Only recently there was a report of an episomal, 'chromatinized' substrate containing only RS sequences and a luciferase reporter gene, which was able to undergo V(D)J recombination (Cherry and Baltimore, 1999); still an episomal substrate might not fully mimic the genomic situation. However, in the CD21-GFP-RAG reporter mice generated, the RSS are integrated in the genome and are unable to recombine in developing B and T cells. This suggests, in accord with the accessibility hypothesis, that only the presence of RSS in a locus is not sufficient for V(D)J recombination at this locus to occur. Possibly additional elements present in Ig and TCR loci are necessary for V(D)J recombination, although the elements characterised to date are all related to transcriptional activation of these loci. Therefore,

transcriptional activation of the CD21 gene might render the requirement for Ig or TCR enhancer/promoter elements redundant and allow recombination of the RSS sequences embedded in this locus to occur.
Lack of GFP expressing cells in the spleen or detectable inversion at the genomic level by southern blot analysis or by PCR (Figures 17-20) indicates that RAG recombination does not occur in peripheral B cells either, where the CD21 gene is expressed. Again, this could be an indication of the inability of RSS alone to target V(D)J recombination outside the normally recombining Ig and TCR loci or insufficient RAG expression in peripheral B cells. The latter possibility would be in accordance with observations from the RAG expression reporter mice (Gartner et al., 2000;Kuwata et al., 1999; Nagaoka et al., 2000; Nagaoka, Yu and Nussenzweig, 2000), in which no RAG re-induction was detected in peripheral B cells However, the requirement of additional elements for V(D)J recombination of the chromosomal CD21-reporter substrate cannot be excluded.
It would therefore be interesting to test recombination ability of the CD21 integrated substrate. A plasmid substrate containing identical recombination signal sequences and the same reporter gene, as the ones inserted in the CD21 locus, can recombine in tissue culture cells when co-transfected with RAG expression vectors. But as previously mentioned V(D)J recombination of an extrachromosomal substrate and a genomic substrate could be subject to different limitations. Therefore V(D)J recombination potential of the CD21-reporter substrate should be analysed, either by transfecting mature B cells from the CD21-GFP RAG reporter mice with RAG expression vectors or inducing RAG protein expression by stimulating mature B cells. Transfection of mature B cells however is experimentally demanding (Li, Wickham and Keegan, 2001) and although RAG induction had been described in *ex vivo* isolated cells when treated with LPS and IL4, no GFP expression was detected in B cells from the RAG-GFP reporter mice upon similar treatment (Yu et al., 1999). In fact no recombination was detected in cultured B cells from the CD21-GFP reporter chimeras when treated with LPS and IL-4 (Figure 19). It was not tested whether under these experimental conditions RAG proteins were produced or whether CD21 gene expression was affected in these cells.
In an effort to overcome this problem, a B cell hybridoma line was generated from LPS activated B cells derived from one of the chimeras. Hybridoma clones were screened for CD21 expression; on most clones analysed CD21 expression is not uniform (some cells, but not all express CD21) which could be an indication that hybridoma lines are down-regulating CD21 gene expression. If some clones retain CD21 expression they could be analysed for RAG mediated recombination, after transfection with RAG expression vectors.
The inverse approach is also interesting. Induction of CD21 transcription in RAG expressing (pro- and pre-) B cells could potentially stimulate recombination of the substrate. Recently it has been reported that demethylation by treatment of cells with the methyltransferase inhibitor 5'-aza-2-deoxycytidine induces CD21 transcription. Trichostatin A (TSA), a histone deacetylation inhibitor also induces transcription in progenitor B cells (Schwab and Illges, 2001). If CD21 transcription in bone marrow B

cells was induced then RAG mediated recombination could be 'allowed' to proceed in the CD21 integrated locus.
In brief, analysis of the CD21-GFP RAG reporter strain showed that:
-there is no V(D)J recombination of a reporter substrate inserted into the CD21 locus in bone marrow and thymus.
-V(D)J recombination does not occur in peripheral B cells within the detection limit of our assay (more than 10-20% of cells from different sorted fractions should have inverted the substrate in order to be detectable by PCR, see Figure 20).

4.2 V(D)J recombination and transcription

Ideally we would have liked to assess the relation of transcription and V(D)J recombination in CD21 targeted mice. Another way to investigate relation of transcription and V(D)J accessibility control is by using an inducible expression system. Recombination could then be assayed in this system before and after addition of the transcriptional inducer.
Initially, the interferon inducible Mx promoter (Kuhn et al., 1995) was tested for this purpose but inducibility was poor as analysed either with a luciferase based assay or with a GFP reporter construct. The tetracycline responsive element (TRE) or tetracycline inducible promoter has been well characterised in several cell culture systems (Gossen, Bonin and Bujard, 1993; Gossen and Bujard, 1992; Gossen et al., 1995). A HeLa cell line, which contains the regulatory elements for tetracycline induced transcription (reverse transactivator rtTA) and has been tested for good inducibility and low background expression levels was used.
The V(D)J recombination substrate was stably integrated in the cells by puromycin selection. The puromycin resistance gene functions additionally as a translational stop cassette (Angrand et al., 1998) and being flanked by Recombination Signal Sequences can be removed by RAG recombination. The poly-adenylation signal of the puromycin gene is sufficient to prevent GFP production, since no GFP expressing cells can be detected in isolated clones prior to transfection with RAG expression vectors.
Of the 18 isolated clones only three expressed GFP after transient transfection with RAG expression vectors. There could be several reasons for this; although control transfection of HeLa cells with a CMV-EGFP reporter gene was generally efficient (~50% transfection efficiency) the cotransfection efficiency of the two RAG expression vectors nor the expression levels of RAG1, RAG2 proteins after transfection could be determined. Additionally, the clones were selected on the basis of puromycin resistance but were not analysed for integration of the GFP gene nor for the number of copies

integrated. If more than one copies of the substrate are integrated in a head-to-tail configuration, as is often the case with DNA transgenes, a number of RSSs will be present in this region. In that case recombination between any of the tandemly arranged RSSs can occur and will give unpredictable products, possibly not resulting in GFP expression. Furthermore, tandemly repeated arrays of genes have been implicated in silencing of a locus by condensation into heterochromatin (Dorer and Henikoff, 1997).
Two of the three clones were analysed further for relation of induction of transcription and RAG recombination. In the first clone, GFP expressing cells were detected only when transfection with the RAG expression vectors was performed in the presence of the transcriptional inducer (doxycycline). In the second clone however, GFP expressing cells were present even when transfection was performed in the absence of doxycycline and (theoretically) active transcription of the substrate. The percentage of GFP^+ cells in this clone after RAG transfection was higher than that observed in the first clone. Tet transactivation is sensitive to position effects and basal transcriptional activity in the absence of the inducer can vary depending on the integration site of the substrate (H. Bujard, personal communication). It should be noted, that the cells were selected on the basis of puromycin resistance, and thereby high expression of the puromycin acetyltransferase (PAC) gene by the Tet-Operator. So in a way 'leakiness' is favoured in this system and could affect RAG accessibility to the substrate. It would be therefore informative to use the bi-directional Tet promoter (Baron et al., 1995) and screen clones on one hand for puromycin resistance (and therefore high levels of expression) as before, but with another reporter gene (i.e. luciferase, which is transcribed in the opposite orientation) eliminate clones with high basal transcription.
The clones that have already been generated could also be analysed for basal transcription levels in the absence of doxycycline, either by RT-PCR and amplification of PAC transcripts or by monitoring survival of the cells with increasing concentration of puromycin.
Another surprising observation was that in Clone#2 the percentage of GFP expressing cells is in fact higher in the absence of doxycycline. This could reflect differences either in transfection efficiency (although there have been no reports of doxycycline affecting transfection efficiencies) or expression of the RAG proteins. Indeed, tetracyclines have been reported to down-regulate expression of endogenous genes in certain systems (Amin et al., 1997; Kuzin et al., 2001).
From the preliminary analysis of the Tet inducible lines it was shown:
-that RSS sequences are able to recombine when inserted in a genomic locus. The recombination efficiency however is low (up to 2%). Comparing RAG recombination efficiencies in an extrachromosomal and the stably integrated substrate would be interesting. Efficiency of recombination could be affected by addition of enhancer or

promoter elements from the Ig locus (Engler et al., 1991). although the IgH enhancer does not affect recombination of plasmid substrates (Hesseet al., 1987).
-that V(D)J recombination can be prevented by absence of a transcriptional inducer but this is not always the case.

4.3 Establishment of allelic exclusion in the μMT mouse

Targeted disruption of the IgM membrane exons (μMT mouse) results in an almost complete block in B cell development at the pre-B cell stage (Kitamura et al., 1991). In heterozygous mutant mice normal numbers of cells are produced but loss of heavy chain allelic exclusion is observed (Kitamura and Rajewsky, 1992). If V(D)J rearrangement on the mutant allele is productive, soluble μ chain is expressed. The secreted μ chain however, does not prevent $V_H \rightarrow D_H J_H$ joining on the wild-type IgH allele, and cells expressing two different μ chains (only one of which is membrane bound) can be detected. These cells are termed double producers and are found at a higher than expected frequency (25% instead of 12.2%) in heterozygous μMT mice. Assuming that the frequency of productive $V_H D_H J_H$ joints is 1/3 and that allelic exclusion in these mice was established upon pre-BCR expression from the wild-type allele, the expected frequency of double producers would be 16.7% (10/60). Taking into consideration that 80% of rearrangements when D_H segments are joined to J_H segments in reading frame 3 (RF3) are non-productive (Gu, Kitamura and Rajewsky, 1991; Ichihara et al., 1989), would further reduce the expected frequency of double producers to 12.2%. This situation is schematically demonstrated in Figure A assuming that allelic exclusion is established upon pre-BCR expression (and rearrangements stop at the point indicated by the dashed vertical line). However, the frequency of double producers observed in LPS activated bone marrow cultures was close to 25% (Kitamura and Rajewsky, 1992). Two theories were proposed to explain the observed discrepancy, both of which consider the effect of the actively transcribing neomycin^r gene on the rearranging IgH locus.
Either the neomycin^r containing allele in heterozygous μMT mice is not subject to allelic exclusion and $V_H \rightarrow D_H J_H$ joining on this allele can occur as long as the RAG proteins are expressed (regardless of pre-BCR signalling) (Figure 30A) or V_H to $D_H J_H$ rearrangement on the neomycin^r containing allele is favoured and occurs first in pro-B cells (Figure 30B). Since rearrangement of this allele cannot lead to pre-BCR expression and signalling, rearrangement will always proceed on the other allele.
The two possibilities are depicted in Figure 30. In both cases the observed number of double producers would be identical (approximately 20/60 = 30%). Correcting again for non-productive rearrangements due to stop codons in RF3 joints, the expected frequency of double producers in heterozygous μMT mice would be 24.4% in both cases. This is in accord with the observed frequency of double producers in bone marrow B cells (~25%).

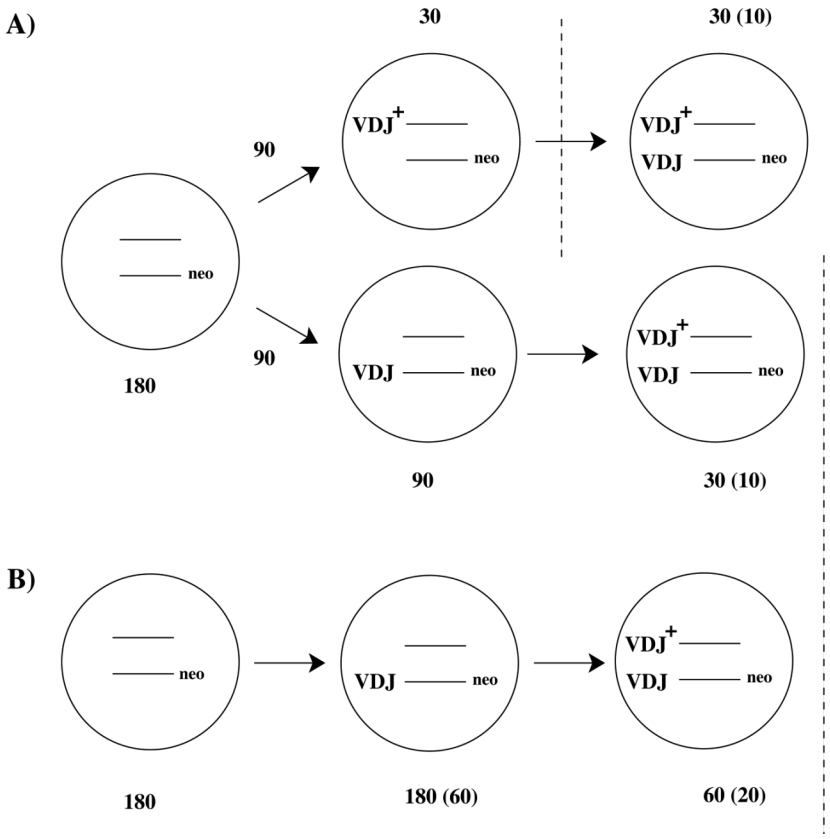


Figure 30: Potential rearrangement of the μMT targeted allele in case of A) breakdown of allelic exclusion or B) preferential rearrangement of the targeted allele before allelic exclusion is established. Starting from an indicative population of 180 cells, numbers indicate cells that survive; all of these need have a productive rearrangement on their WT allele. VDJ^+ indicates productive rearrangements while VDJ both productive and non productive joints. Numbers in brackets refer to cells that have a productive rearrangement on the neo^r containing allele. Dashed lines designate the point at which pre-BCR signalling and allelic exclusion should be established.

In order to discriminate between the two possibilities and assess whether allelic exclusion can indeed be established on the μMT allele we decided to analyse the process in a context which promotes allelic exclusion.

Transgenic mice expressing an Ig heavy chain were found to inhibit rearrangement of endogenous IgH loci (Weaver et al., 1985); similarly heavy chain insertion mice contain a pre-rearranged $V_HD_HJ_H$ element targeted into the IgH locus and can also inhibit rearrangement of the second allele (Taki, Meiering and Rajewsky, 1993) One such heavy chain insertion mouse mutant, the glD42 strain (Pewzner-Junget al., 1998), can produce secreted IgM distinguishable from the one produced from the μMT allele by allotypic differences. The μMT/glD42 cross was therefore generated to analyse the

effect of the neomycin^r gene in a situation where IgH allelic exclusion does not require V(D)J rearrangement and double producers can be detected by a simple anti-allotypic staining. If expression of the glD42 encoded pre-BCR on the surface of the cells is sufficient to prevent rearrangement of the neo^r containing allele, no double producers should be detected in these mice.

Indeed, no double producers were present in *ex vivo* (LPS activated) cultures of cells from spleen and bone marrow from glD42/μMT, as assessed by flow cytometric analysis and on slide stainings. Additionally no antibody production from the μMT allele is detectable in the sera of these mice. The neomycin^r containing allele is therefore subject to allelic exclusion and not rendered constitutively accessible for recombination. The observed number of double producers, in the bone marrow of the μMT heterozygous mice, could be generated due to preferential rearrangement of the neomycin^r containing allele upon the onset of V(D)J recombination in pro-B cells. Once an IgH heavy chain is expressed on the surface of the cells however, rearrangement stops and allelic exclusion is established irrespective of the presence of an actively transcribed gene in the IgH locus.

Analysis of the genomic IgH locus also supports this model. In the glD42/μMT cross there is no significant loss of DFL16 probe signal[*] (Table 5, Figure 27), which would be indicative of $V_H \rightarrow D_HJ_H$ rearrangement. $D_H \rightarrow J_H$ rearrangement does occur in B cells from these mice, but still at a lower level compared to WT or μMT heterozygous mice (as assessed by southern blot analysis using the DQ52 probe). In the latter two cases the DQ52 signal is undetectable, while the glD42/μMT B cells show a five-fold reduction compared to non-B cells. But $D_H \rightarrow J_H$ joining is not subject to allelic exclusion and usually occurs on both IgH alleles (Alt et al., 1984) while D_HJ_H joints have been detected in developing T cells (Born et al., 1988). Loss of DQ52 signal was indeed evident by analysis of DNA from thymus of glD42/μMT mice. The μMT allele is 'open' for $D_H \rightarrow J_H$ recombination but $V_H \rightarrow D_HJ_H$ joining is inhibited by expression of the pre-BCR.

Another point of interest is the extent of V(D)J rearrangement in μMT heterozygous mice. The observed number of double producers (25%) in bone marrow of these mice would indicate that virtually all B cells harbour a $V_HD_HJ_H$ rearrangement on both alleles. Roughly 25% of the rearrangements would be productive in a non-selective situation, and this is indeed detected at the protein level (secreted antibody from LPS activated bone marrow cells, see Figure 24) in μMT/C57BL6 mice. Southern blot analysis however of DNA from sorted B220$^+$ splenic cells from these mice does not show complete loss of the DFL16 probe signal .The signal is strongly reduced (more than in the respective population from wildtype mice), to the extent that precise quantification is difficult; the reduction of the DFL16 probe signal ranges from 5- to 10-fold, suggesting that 20%-40%[**] of B cells have no $V_H \rightarrow D_HJ_H$ rearrangement on the μMT allele. Similar analysis of wild-type B cells shows that ~60% of the cells have no $V_H \rightarrow D_HJ_H$ rearrangements on the second IgH locus. It seems that a higher fraction of B cells in the

μMT heterozygous mice harbour $V_HD_HJ_H$ rearrangements on both alleles compared to wild-type mice, but not all.
It should be considered that the observed number of double producers in the spleen of heterozygous μMT mice is much lower than in the bone marrow (6% compared to 25% respectively (Kitamura and Rajewsky, 1992) (Table4 in Results). Possibly most bone marrow B cells harbour $V_HD_HJ_H$ rearrangements on both alleles (slightly more than 2/3 of which are non-productive on the μMT allele) but during transition to the peripheral B cell pool, cells expressing two different antibodies (even though a BCR of a unique specificity is expressed) are not favoured. Toxicity of free immunoglobulin heavy chain has been proposed as a mechanism for counterselection of double producers (Kohler, 1980) although double-heavy chain insertion mice have normal numbers of B cells and retain expression of both IgH heavy chains (Sonodaet al., 1997). Another possibility is that competition of the two heavy chains for IgL chain pairing, leads to reduced BCR surface density and impaired survival of these cells (Sanchez et al., 2000). Single IgH expressing cells which contain a non-productive rearrangement of the μMT allele or no rearrangement on this allele would then be favoured. Analysis of the serum levels of antibody generated from the μMT allele (IgM^a allotype) also supports the finding that double producers are counterselected. IgM^a antibodies are detectable in μMT heterozygous mice but the amount of IgMa antibodies is only 5% of the total IgM (IgM^a +IgM^b antibody present in the sera). This estimate would be in accordance with the observed frequency of double producers in the spleen.
It would be interesting to analyse bone marrow fractions from μMT heterozygous mice, for genomic rearrangement first to see if the observed 25% of double producers really reflects $V_H\rightarrow D_HJ_H$ rearrangement of both IgH alleles and if so at which stage these double producers are counterselected.
Taken together analysis of the glD42/μMT mice showed that:
-no double producers were present in bone marrow and spleen of these mice by intracellular staining after LPS activation
-no secreted antibody from the μMT allele was detected
in the IgH locus is not sufficient to overcome inhibition of V_H to D_HJ_H joining. On the contrary, it would be interesting to check whether transcription of the $neomycin^r$ gene is affected after pre-BCR signalling and allelic exclusion is established. -no $V_H\rightarrow D_HJ_H$ rearrangement was detected in splenic B cells. Therefore allelic exclusion is established in the glD42/μMT mice, irrespective of the presence of the $neomycin^r$ gene in the IgH locus. The question of the mechanism of establishment of allelic exclusion remains open, but insertion of an exogenous promoter in the Ig locus is not sufficient for maintenance of the accessible conformation of the locus after an IgH heavy chain has been produced. .

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* loss of DFL16 and DQ52 probe signal was determined by comparison of normalised signal from $B220^+$ and $B220^-$ cells according to the formula:

$$\textbf{D probe signal} = \frac{D_{B220+} / C_{B220+}}{D_{B220-} / C_{B220-}}$$

where D probe signal represents the reduction of intensity of DFL16 or DQ52 probe signal; D_{B220+} and D_{B220-} is the intensity of the signal from either of the two D probes and from DNA of $B220^+$ and $B220^-$ cells respectively and C the intensity of the probe used for DNA quantification of each sample (CD21 probe or RH probe).

** Considering that x $B220^-$ cells (which have no rearrangement on either of the two IgH alleles) contain 2x copies of 'D probe recognition sequence' while B cells need to have one productive rearrangement on the IgH locus (and therefore can contain only x 'D probe recognition sequences'). Some B cells have $V_HD_HJ_H$ rearrangements on bothe alleles; if α is the fraction of cells which have $V_HD_HJ_H$ joints on both IgH alleles the above equation can be written as

D probe signal =(1-α)x/2x from which the percentage (2D%) of cells that harbour only one $V_HD_HJ_H$ rearrangement can be calculated

5. Summary

Recombination activating gene proteins (RAG1 and 2) mediate antigen receptor assembly in bone marrow B cells but their expression has also been reported in peripheral B cells. To study the role of this late RAG expression an indicator mouse strain was generated, which would allow labeling of cells that display V(D)J recombination activity after bone marrow rearrangement has been completed. In the mouse line generated (CD21-GFP RAG) a RAG recombination reporter substrate was integrated in the CD21 locus. The idea was that recombination of the reporter substrate in the bone marrow could be prevented by insertion in the CD21 locus, since the CD21 gene is transcriptionally inactive during bone marrow development and is mainly expressed in mature B cells.

Indeed in the CD21-GFP RAG reporter mice no recombination was observed in bone marrow B cells and T cells. Incorporation of RSSs in the CD21 locus is not sufficient for V(D)J recombination to occur even though the RAG proteins are expressed. Recombination was also not detected in mature B cells, although the CD21 locus is transcriptionally active. This could be due to insufficient RAG expression in peripheral B cells to allow V(D)J recombination of the artificial substrate. However, it cannot be excluded that RAG-mediated recombination of genomic substrates requires the presence of additional regulatory elements.

Attempts to analyse dependence of RAG mediated recombination on transcription, using an inducible gene expression system, were also made. Induction of transcription potentiates RAG-mediated recombination of a stably integrated reporter substrate in one case analysed; however absence of the inducer is not limiting for V(D)J recombination in another situation.

Accessibility control of V(D)J recombination is also important for allelic exclusion, which is established in B cells as a partial block of IgH rearrangement. In a mutant mouse containing a neomycin^r gene, inserted in the IgM membrane region (μMT mouse), the frequency of B cells expressing two IgH chains is higher than expected. A rearranged heavy chain however is able to prevent rearrangement of the μMT alelle suggesting that allelic exclusion is properly established despite of the presence of an exogenous promoter in the IgH locus.

6. Zusammenfassung

Die Proteine RAG1 und RAG2 (Recombination Activating Gene Protein) vermitteln die Umlagerung der Antigenrezeptorgene im Knochenmark. Allerdings wurde auch in peripheren B Zellen RAG1 und 2 Proteinexpression gefunden. Um die Funktion dieser späten RAG-Ausprägung zu untersuchen, wurde ein Indikator-Mausstamm (CD21-GFP RAG) generiert. In diesem Mausstamm sollten B Zellen markiert werden, in denen V(D)J Rekombination nach Abschluss der Umlagerungen im Knochenmark stattgefunden hat. Zu diesem Zweck wurde ein RAG-Rekombinationssubstrat durch homologe Rekombination in den CD21 Lokus der Maus eingebracht.
Umlagerung und Ausprägung des Reportergens sollten auf Zellen beschränkt werden, die die RAG Proteine nach vollständiger Immunglobulinumlagerung re-exprimieren. Da es Anzeichen dafür gibt, dass Rekombination von Transkription abhängt, schien ein Weg, dieses Ziel zu erreichen, die Einbringung des Reporter-Konstruktes in den CD21 Lokus. Dieser wird während der Reifung der B Zellen im Knochenmark nicht transkribiert und wird hauptsächlich in reifen B Zellen ausgeprägt.
In den CD21-GFP RAG Mäusen wurden tatsächlich keine Umlagerung des Reportergens in B Zellen des Knochenmarks oder T Zellen detektiert. Dies zeigt, dass trotz vorhandener RAG Protein-Ausprägung in den CD21 Lokus eingebrachte RSS Sequenzen nicht rekombiniert werden. Rekombination des Substrats konnte allerdings auch in reifen B Zellen nicht nachgewiesen werden, obwohl in ihnen das CD21 Gen exprimiert wird. Dies könnte daran liegen, dass die RAG-Expression in der Peripherie nicht ausreicht, um V(D)J Umlagerung eines transgenen Substrats zu vermitteln. Es kann aber nicht ausgeschlossen werden, dass zur RAG-vermittelte Rekombination von genomischen Substraten das Vorhandensein von zusätzlichen regulatorischen Elementen notwendig ist.
Es wurde auch versucht, die Abhängigkeit RAG-vermittelter Umlagerungen von Transkription anhand induzierbarer Genexpression in Zelllinien zu demonstrieren. Leider kann keine eindeutig Aussage getroffen werden, da in einer Zelllinie Rekombination tatsächlich nur nach Gabe von Induktor erfolgte, in einer anderen Zelllinie war V(D)J Rekombination dagegen unabhängig von Induktion der Genexpression.

Die Limitierung von V(D)J Rekombination durch Lokuszugänglichkeit ist auch wichtig für die Allelische Exklusion, die einen partiellen Block von IgH Umlagerungen darstellt. In einer Mausmutante, die eine Neomyzinresistenzkasette in der Transmembranregion eines Allels (μMT Allel) des IgM Gens enthält, wurde eine erhöhte Anzahl von B Zellen die zwei IgH Moleküle ausprägen, gefunden. Eine vollständig umgelagerte schweren Kette auf dem anderen Allel verhindert die Umlagerung des μMT Allels. Dies zeigt, dass Allelische Exklusion trotz Vorhandensein eines transgenen Promoters in dem IgH Lokus etabliert werden kann.

7. References

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Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von (Prof. Dr. K. Rajewsky) betreut worden.

Köln, im October 2001-10-15 Chrysi Kanellopoulou

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1. Aranda, M., Kanellopoulou, C., Christ, N., Peitz, M., Rajewsky, K., and Droge, P. (2001). Altered directionality of the Cre-loxP site-specific Recombinase. *J Mol Biol* **311,** 453-9.
2. Kanellopoulou C., Rajewsky K. (2001). Allelic exclusion is properly established in μMT heterozygous mice in the presence of a functional Ig heavy chain (in preparation).

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10. Lebenslauf

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