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Transcription, Editing, and Switching of Antibody Genes

A thesis submitted to the faculty of the Rockefeller University in partial
fulfillment of the requirements for the degree of Doctor of Philosophy

by

Rafael Casellas

April 1, 2002

The Rockefeller University

New York, New York 10021

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To my parents, wife, and children

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I thank Dr. Martin Weigert for supplying me with useful reagents, insights, and time, without which the editing section of this thesis would have been impossible.

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Abstract

B lymphocytes are the antibody producing cells of the immune system. During B cell lymphopoiesis in the bone marrow, immunoglobulin molecules are assembled from V, D, and J gene segments through a process known as V(D)J recombination. Because of its stochastic nature, V(D)J recombination is expected to create self-reactive specificities. In targeted animals carrying autoantibodies, these specificities were shown to be silenced by deletion, anergy, and receptor editing. While anergy and deletion physically eliminate the autoreactive clone, receptor editing salvages it by replacing its immunoglobulin receptor through ongoing V(D)J recombination. Once a functional non-self reactive receptor is generated, B lymphocytes migrate to the periphery. Upon antigen encounter, B cells undergo another recombination process, class switching, by replacing their heavy chain μ constant region with downstream isotypes, such as γ, α, or ε. The isotype choice is dictated by a variety of mitogens and cytokines produced during the immune response. In the first part of this thesis, I describe experiments that elucidate the role of DNA double-stranded break repair in class switch recombination. In the second part, I determine how frequently B cell receptors are successfully replaced *in vivo* by editing. Finally, I provide evidence that secondary gene recombination, as well as allelic exclusion of light chain genes is under transcriptional regulation.

Chapter 1: Introduction

The Antibody Molecule

Antitoxins and the clonal selection theory

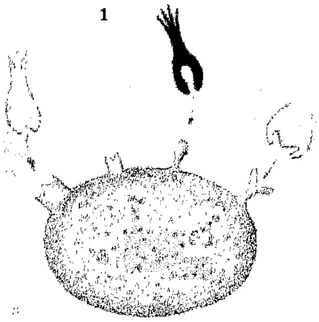
The first experiments proving the physical existence of antibodies were performed in 1890 by von Behring and Kitasato (Behring and Kitasato, 1890), who showed that injections of serum obtained from rabbits immunized with tetanus or diphtheria toxins could prevent disease in mice infected with such pathogens. The unknown substance present in serum which provided protection upon transfer was salt-precipitated a year later by Tizzoni and Cattani and named antitoxin.

These initial observations on passive immunity were soon generalized by the work of Paul Erhlich with plant toxins and by Jules Bordet, who demonstrated that a protective response could even be generated against whole cells (erythrocytes). The more inclusive term antibody, thereby replaced antitoxin.

In an attempt to explain the formation of antibodies, Paul Erhlich advanced a comprehensive theory (Ehrlich, 1900) (Figure 1-1), which postulated that antigens induced cell damage by binding to pre-existing chemical "side-chains" or receptors (antibodies) at the surface of host cells. This binding would result in a selective cell surface depletion of those side chains specific for the antigen. To compensate for their loss, the cell would produce an excess of receptors, which will then appear in the serum as free antibodies. Although highly speculative, Ehrlich's theory was surprisingly insightful for two reasons: first it implied that the antigen-antibody interaction would signal the cell nucleus to manufacture large amounts of antibody. Secondly, the theory stressed the antibody's chemical nature and suggested that its specificity for antigen would be dictated by the stereochemistry of its binding site. These ideas fascinated chemists such as Svante Arrhenius (Arrhenius, 1907) and others, whose theoretical and experimental contributions established the basis of immunochemistry. This field, which dominated the first half of the twentieth century, was more concerned with the chemical nature of the antigen-antibody interaction than with its biological consequences.

Figure 1-1 Paul Erhlich's side-chain theory. 1-2 Antigens bind to the surface of somatic cells by interacting with unknown receptors. 3-4 As the cell surface receptors are blocked, the cell tries to compensate for their functional absence by generating an excess of receptors, which eventually are secreted in large amounts in the serum (adapted from (Ehrlich, 1900)).

1



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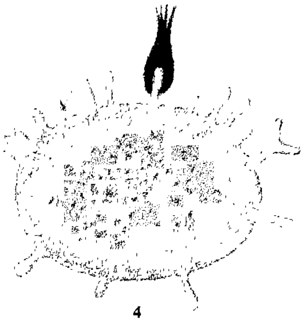

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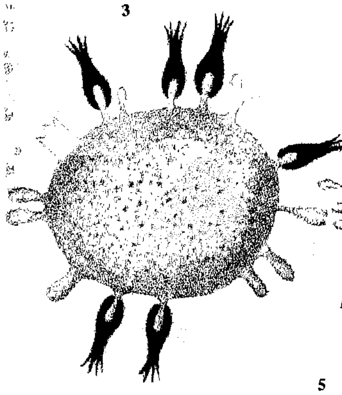

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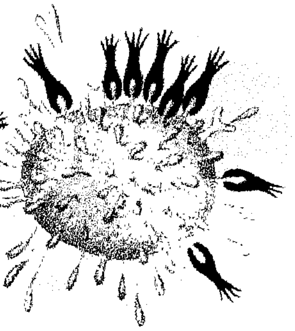

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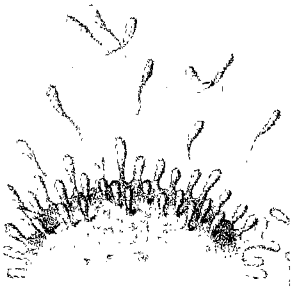
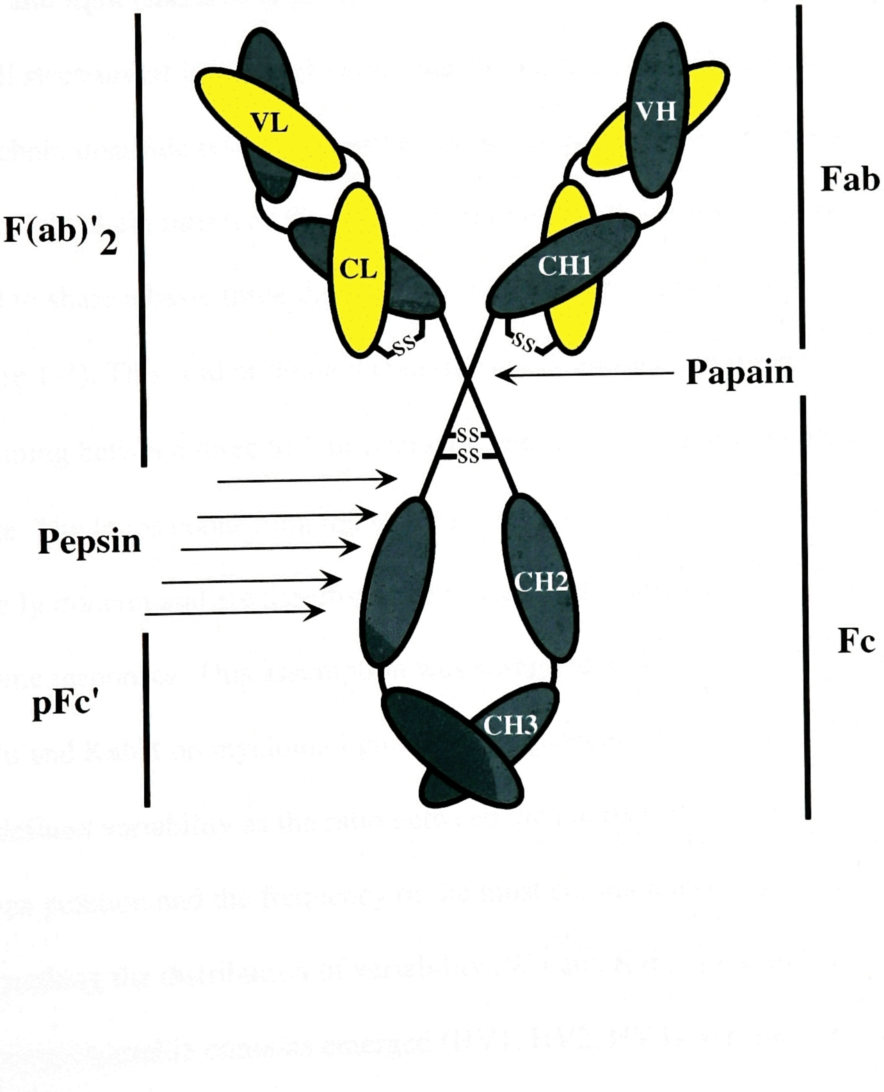


At the time Paul Erhlich proposed his comprehensive theory, scientists had only immunized experimental animals with a limited number of antigens, namely toxins. However, the work of Obermeyer, Pick, and Landsteiner in the second decade of the twentieth century clearly demonstrated that antibodies could be elicited against a myriad of antigens, including substances that would never be found in nature (such as haptens coupled to protein carriers). These observations established the basis of a new biological puzzle: how could an animal generate antibodies against synthetic antigens? As Felix Haurowitz and Linus Pauling surmised, antigens could convey the information necessary for the synthesis of the antibody molecule (Pauling, 1940). The instructive or template theory, as it became known, proposed that antigens served as templates to nascent antibody molecules, which would coil around them creating the complementarity. The idea of instruction was widely accepted by chemists and biochemists working in the antibody problem. The theory, however, failed to explain several of the more important biological aspects of immunity such as the presence of long term memory responses, the increase in antigen specificity upon repeated immunizations, and perhaps more importantly, it did not explain how immunological tolerance was acquired. These objections were the basis for a new theory conceived by the ideas of Jerne, Talmage, Burnet, and Lederberg, the clonal selection theory (Burnet, 1959). This theory revisited Ehrlich's concept that antibody recognition patterns pre-existed before the introduction of antigen into the animal, and proposed that diversity was generated by somatic mechanisms which randomly generated the antibody specificity in individual cells. The antibody-antigen interaction at the cell surface would result in the clonal expansion of that particular cell and the secretion of immunoglobulins. Tolerance to self would in turn be achieved by deletion of self-reactive clones early in development. An important corollary of the clonal selection theory was that lymphocytes were monospecific, i.e. "one cell/one antibody". This notion was tested and corroborated by Nossal and Lederberg on single cell cultures and Edelman's work establishing Bence Jones proteins, isolated from multiple myeloma patients, as immunoglobulins carrying a single specificity. The clonal selection theory was, as recognized by Burnet himself, a Darwinian answer to the more Lamarckian instructionalistic view of the antibody problem.

The first successful attempt to identify antibody molecules was accomplished in1939 by Tiselius and Kabat (Tiselius and Kabat, 1939) who demonstrated that hyperimmunization of rabbits increased the concentration of gamma globulins in serum and that this fraction contained antibody activity. Since gamma globulins are large molecular weight proteins, it was inferred that further characterization of antibodies, now termed immunoglobulins, necessitated splitting them into smaller, easily handled fragments. In 1959, Rodney R. Porter succeeded digesting rabbit IgG with limiting concentrations of the proteolytic enzyme papain. This generated two discrete fragments: a monovalent fragment with antigen binding activity (Fab), and a second fragment which retained the antibody's effector functions and crystallized readily into a lattice, termed Fc (Porter, 1959) (Figure 1-2). It should be noted that the inability of the antigen specific Fab fragments to crystallize correlated with chemical heterogeneity, i.e. differences in amino acid sequence, a prelude to the characterization of variable domains. Edelman and Poulik, on the other hand, assumed that antibodies, like many other proteins, would be composed of a number of independent polypeptides held together by disulfide bounds.

When methods that usually disrupt such bonds were tested, myeloma globulins were separated into two distinct chains, which based on their sizes on starch gels were subsequently termed heavy (H) and light (L) chains (Edelman and Poulik, 1961). Similar studies using the protease pepsin were successful in digesting antibodies into an $F(ab')_2$ fragment with bivalent antigen binding activity, and several small fragments derived from the Fc portion of the molecule, the largest termed pFc' (Nissonoff et al., 1960). These pioneer studies were fundamental in deciphering the structure of immunoglobulins by establishing the existence of heavy and light chains. In addition, they predicted that antigen binding and antibody effector functions were located in distinct protein domains.

Figure 1-2 Schematic diagram of an IgG molecule. Immunoglobulin domains are represented as dark ovals for heavy chains and as clear ones for light chains. SS indicates the inter- and intrachain disufide bridges. Treatment of the molecule with the proteolytic enzyme papain (or tripsin) generates three fragments: a crystalizable Fc portion, and two identical Fab fragments. Pepsin digestion, on the other hand, cleaves the IgG molecule below the heavy chain interchain disulfide bonds, yielding the entire F(ab)'2 fragment and numerous smaller polypeptides from the Fc domain, the largest one being pFc'. VL and CL: variable and constant region of the light chain; VH: variable region of the heavy chain; CH1 to CH3: constant regions 1 to 3 of the heavy chain.


VL
VH
Fab
F(ab)'$_2$
CL
CH1
SS
SS
Papain
SS
SS
Pepsin
CH2
Fc
pFc'
CH3

Sequencing studies in the 1960s subdivided light chains, and later heavy chains, into an amino terminal V (variable) region, which varied substantially in amino-acid composition between different antibodies, and a C (constant) domain, whose sequence was conserved between antibodies of the same isotype (Hilschmann and Craig, 1965). In addition, X-ray crystallography confirmed the proteolytic experiments of Porter and Edelman by showing that all immunoglobulins were indeed composed of two pairs of heavy and light chains of approximately 25 and 55 kD (Cummingham et al., 1968). The overall structure of immunoglobulins was found to be stabilized by a series of inter and intra-chain disulfide bounds as well as strong hydrophobic interactions created at heavy/light chain interface (Figure 1-2). Perhaps most interestingly, both chains were found to share a basic three-dimensional structure, the immunoglobulin (Ig) fold (Figure 1-3). This fold or domain consists of two antiparallel β-pleated sheets, each containing between three to four β strands joined at their hydrophobic core by a disulfide bridge. The loops connecting the strands are not critical for generating the proper folding of the Ig domain and are therefore free to vary and contribute to the diversity required for immune responses. This assumption was sustained at the time by the comparative studies of Wu and Kabat on myeloma light chains sequences (Wu and Kabat, 1970). Kabat and Wu defined variability as the ratio between the number of different amino acids found at a given position and the frequency of the most common amino acid seen at that position. By graphing the distribution of variability (Wu and Kabat plot) in heavy chain V regions three hypervariable domains emerged (HV1, HV2, HV3), separated by four relatively invariant or framework regions (FR1, FR2, FR3, FR4) (Figure 1-4). The conserved framework regions correspond to the β strands in the immunoglobulin fold domain, while the hypervariable domains constitute the loops connecting the strands. All HV loops from heavy and light chain V regions are brought together at the apex of the Fab fragment, generating the antigen-binding site. Since this site forms a surface complementary to the antigen, the hypervariable regions are also known as complementarity determining regions or CDRs.

Figure 1-3 Schematic representation of the V and C domains of an immunoglobulin light chain. The β strands composing the Ig domain are represented as arrows. The shading of the arrows distinguishes between the two antiparallel β-pleated sheets, each containing three (shaded) and four (unshaded) β strands. The disulfide bridge connecting the two β sheets are depicted as black bars. Adapted from (Edmundson A et al., 1975).

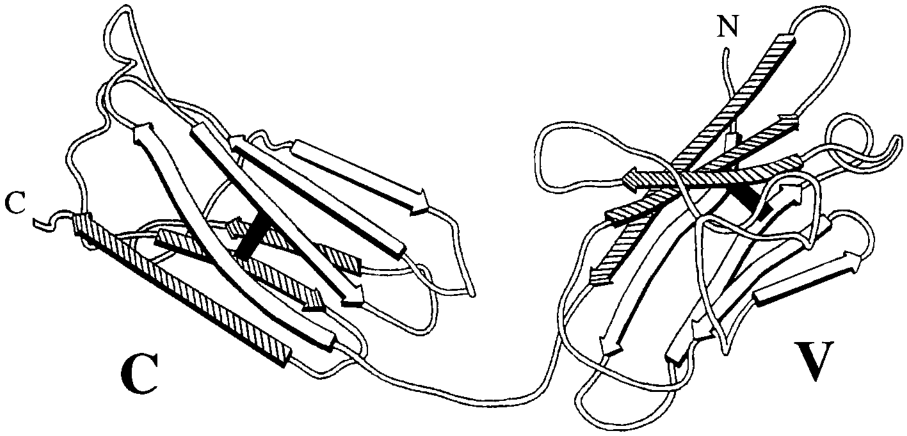
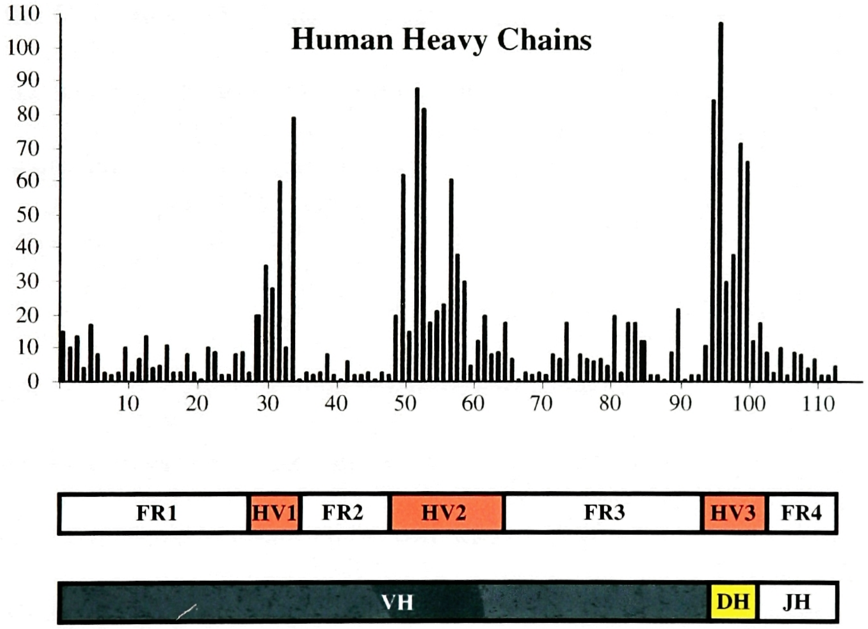

N
C
C
V

Figure 1-4 Variability plot generated by comparing human heavy chain sequences. Y axis: degree of variability, X axis amino acid position for the variable region of the heavy chain. From these comparisons the framework (FR) and hypervariable (HV) domains were defined as shown below. For comparative purposes the approximate distribution of the variable (VH, dark box), diversity (DH, gray box), and joining (JH, white box) heavy chain domains is aligned below. Variability plot based on (Kabat et al., 1991).


Human Heavy Chains
110
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
110
FR1
HV1
FR2
HV2
FR3
HV3
FR4
VH
DH
JH

Generation of Diversity

The first studies of light chain amino-acid composition clearly indicated the existence of a V domain, whose amino-acid sequence varied from antibody to antibody, and a conserved C domain. This raised the possibility that immunoglobulin chains were the result of a fusion of two genes, as advanced by Dreyer and Bennet (Dreyer and Bennett, 1965). This hypothesis, which challenged Garrod's "one gene one polypeptide" idea (Garrot, 1923), was validated by the work of Tonegawa and coworkers (Tonegawa et al., 1977). Their study showed that a light chain mRNA probe containing both the V and the C regions hybridized to different fragments of genomic DNA pre-digested with restriction enzymes and separated by electrophoresis. Shortly after, RNA-DNA R-loop and sequencing experiments not only confirmed that V and C regions were coded by different genes but also defined a new set of genes, the J segments(Brack and Tonegawa, 1977). These data shed light for the first time into the mechanism of diversity by suggesting that a given V gene segment was joined to a J segment through a recombination process, later termed V(D)J recombination. A meticulous comparison between the kappa J region DNA with known antibody protein sequences revealed more variability at the junction point between the V and J segments. This new diversity, called junctional, predicted that in addition to successful rearrangements, "forbidden" or out of frame recombinations would also occur.

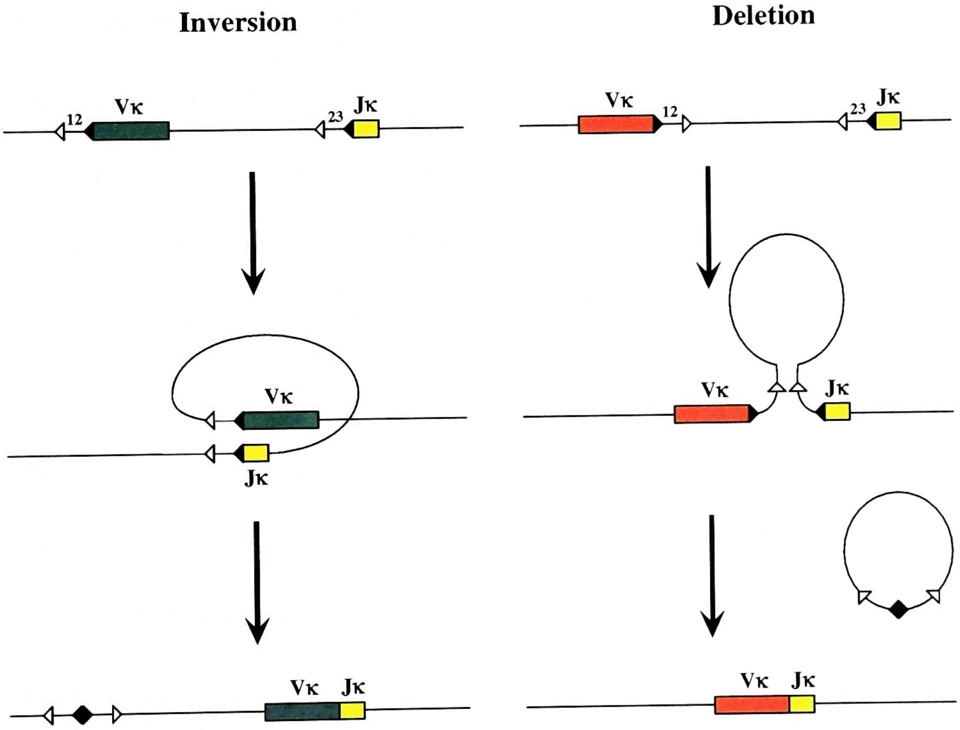
Heavy chain assembly turned out to be more complicated since three independent gene segments were involved: VH, D, and JH of approximately 98, 3-7, and 12-17 amino acids respectively. The VH encodes a leader peptide sequence and both the CDR1 and CDR2 regions. The CDR3 is encoded by the D segment and the VH-D and D-JH junctions. The remainder of the J segment encodes the last framework region. Conversely, the light chain is encoded by two genes: VL, which comprises all light chain CDRs and the JL segment. Despite variations in their number and organization, nearly all species studied contain immunoglobulin gene segments arranged into clusters along the same chromosome.

V(D)J Recombination

At the heavy chain locus, V(D)J recombination initiates random D to JH joining, followed by VH to DJH rearrangements (Early et al., 1980). The V(D)J recombinase complex recognizes recombination signal sequences (RSSs) flanking the gene segments involved. RSSs are composed of a palindromic conserved heptamer: CACAGTG, followed by the nonamer ACAAAAACC. These elements are separated by a non-conserved spacer of either 12 ± 1 or 22 ± 1 base pairs which constitute one and two complete turns of the DNA helix presumably allowing the DNA groove of both RSSs to be aligned during the recombination process (Hesse et al., 1989). In fact, recombination will only rarely occur between gene segments flanked by like spacers, a phenomenon known as the '12-23' rule (Early and Hood, 1981).

Depending on the alignment of the gene segments on the chromosome, V(D)J recombination can occur by two mechanisms: inversions and deletions (Figure 1-5). When the recognition sequences are facing each other, recombination proceeds by deletion of the intervening DNA as circles containing the fused heptamers. Inversions occur when the RSSs are found in the same orientation in the chromosome. In these events, heptamers and nonamers are retained in the chromosome and no DNA is lost. It has been estimated that approximately 55% of all Vκ genes are in opposite orientation with respect to the Jκ segments (Thiebe et al., 1999).

Figure 1-5 Inversional and deletional recombination of the kappa locus. Inversions occur when the RSSs of the Vκ and Jκ genes are in the same orientation in the chromosome (open triangles: nonamers, closed triangles: heptamers, 12 and 23: DNA spacers). When the recognition sequences are facing each other (approximately 40% of the time), the recombination proceeds by deletion of the DNA intervening sequences as circles.


Inversion
Deletion
12
Vκ
23
Jκ
12
Vκ
23
Jκ
Vκ
Jκ
Vκ
Jκ
Vκ
Jκ
Vκ
Jκ

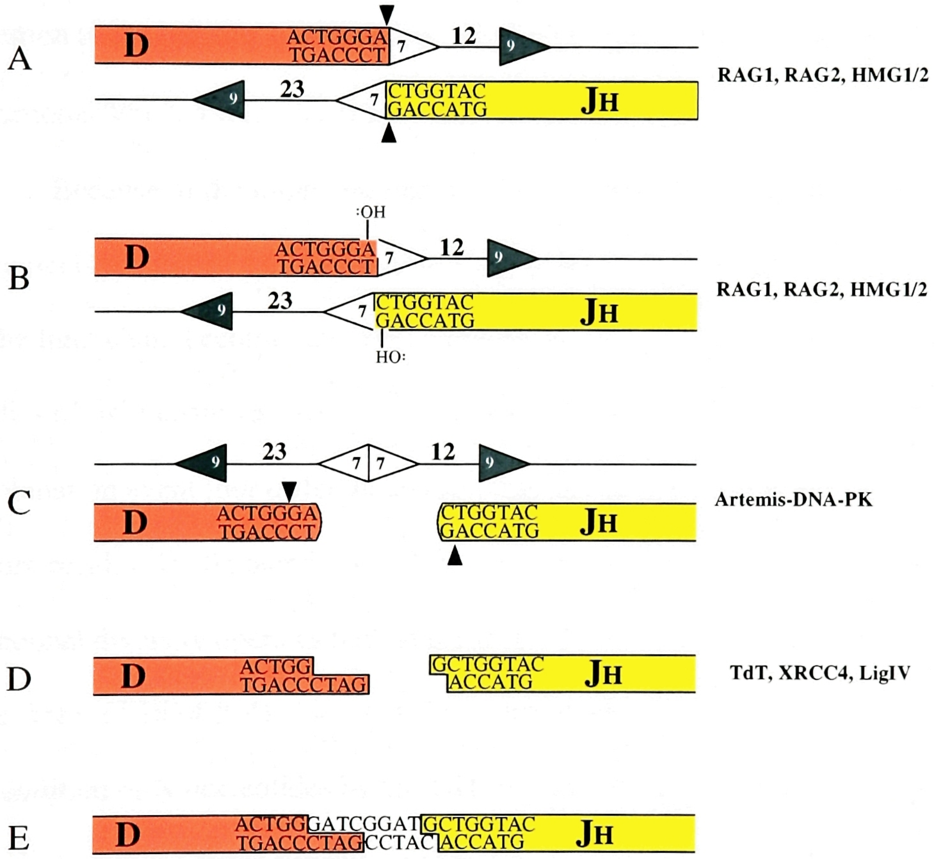
To isolate the genes involved in immunoglobulin recombination, Schatz and Baltimore relied on the fact that only immature lymphoid cell lines carried out V(D)J gene assembly, however, upon transfection of human or mouse genomic DNA, 3T3 fibroblasts displayed V(D)J recombinase activity (Schatz and Baltimore, 1988). The cloning and sequencing of the locus responsible for such an activity revealed the presence of two genes with no apparent sequence similarity: RAG-1 and RAG-2 (Oettinger et al., 1990; Schatz et al., 1989). These genes are only separated in the mouse genome by 8kb and are aligned in opposite transcriptional orientation with respect to each other.

Much of what is known with regard to the molecular details of immunoglobulin gene recombination has been inferred from cell-free in vitro assays. The current model of V(D)J recombination derived from such studies describes first the recognition and cleavage of the heptamers signal sequences mediated by RAG1 and RAG2 proteins (McBlane et al., 1995) (Figure 1-6). The stereochemistry of this reaction shows that RAG proteins generate first a nick at the 5' end of the heptamer. The 3' hydroxyl group created by the nick then attacks the phosphodiester bond at the end of the 7-mer of the bottom strand (transesterification). This reaction creates a DNA hairpin at the coding end and a blunt end at the signal end. Although RAG proteins can cleave DNA alone in vitro, other factors such as the high mobility group (HMG) proteins have been shown to enhance the reaction (Sawchuk et al., 1997).

Hairpin intermediates were first characterized in SCID mice, which accumulate such unprocessed ends due to a deficiency in the catalytic subunit of the DNA dependant protein kinase (DNA-PKcs) (Roth et al., 1992). This kinase, in association with two other factors, Ku70 and Ku80, form a protein complex not only required for V(D)J recombination, but also for repair of IR-induced DNA damage (Nussenzweig et al., 1996). Ku70 and Ku80 bind as a heterodimer with high affinity to DNA ends. This Ku-DNA interaction is also believed to recruit the catalytic subunit DNA-PKcs to the DNA ends and enhance its kinase activity (Gottlieb and Jackson, 1993). From these observations it can be concluded that the accumulation of coding ends in *scid* mice would result from failure of DNA-PKcs to phosphorylate and activate the hairpin endonuclease. Hairpin opening was postulated to be carried out by the RAG1 and RAG2 proteins (Besmer et al., 1998), and the nonhomologous end-joining factors Mre11, RAD50, and Nbs1 (Paull and Gellert, 1999). Nevertheless, this activity is most likely carried out by the novel identified gene Artemis, whose mutation in humans causes X-ray sensitivity and SCID (Moshous, 2001). In vitro studies clearly demonstrate that Artemis forms a physical complex with DNA-PK and acts in hairpin opening and overhang processing (Ma et al., 2002).

The opening of hairpins at asymmetric positions is believed to generate palindromic repeats at some coding joints (P nucleotides) (Figure 1-6C). Another enzyme, terminal deoxynucleotide transferase (TdT), catalyses the addition of non-template dependant nucleotides (N nucleotides) to the open DNA ends. N-nucleotide additions are commonly found in heavy chain but rarely seen in light chain genes, perhaps as a result of transcriptional downregulation of the TdT gene by the μ-surrogate light chain complex on the surface of preB cells (Wasserman et al., 1997). The addition of P and N nucleotides, as well as the deletions created by the imprecise joining reaction generate junctional diversity. The final step of V(D)J recombination involves the joining of the processed DNA ends. This step was shown to be mediated by two genes, the gene mutated in the x-ray complementation group 4 (XRCC4) and Ligase IV (Grawunder et al., 1997).

Figure 1-6 Proposed model for V(D)J recombination. A- Nicking. The RAG1 and RAG2 proteins recognize and bind to the recombination signal sequences (7: heptamer; 9: nonamer; 12 and 23: DNA spacers). RAG1 and 2 then cleave the RSSs at the 5' end of the heptamer. B- Transesterification. The 3' nucleophilic OH group created by the nick (bases A and G) attacks the phosphodiester bond at the end of the 7-mer of the bottom strand (bases T and C) generating hairpins at the coding ends and blunt ends at the signal ends. C- Signal and Coding End processing. DNA-PKcs, Ku70, and Ku80 form a protein complex around the DNA ends generated by the transesterification reaction. Signal ends are joined bluntly, while the hairpins formed at the coding ends are opened by either RAG1 and RAG2 or the Mre11/RAD50/Nbs1 complex. D and E- N and P nucleotide additions. The terminal deoxynucleotide transferase (TdT) catalyses the addition of non-template dependant nucleotides (N nucleotides) at the opened hairpin ends. XRCC4, Ligase IV, and probably a polymerase seal the DNA gaps.


A
D
ACTGGGA
TGACCCT
7
12
9
23
9
7
CTGGTAC
GACCATG
JH
RAG1, RAG2, HMG1/2
B
:OH
D
ACTGGGA
TGACCCT
7
12
9
23
9
7
CTGGTAC
GACCATG
JH
HO:
RAG1, RAG2, HMG1/2
C
23
9
7
7
12
9
D
ACTGGGA
TGACCCT
CTGGTAC
GACCATG
JH
Artemis-DNA-PK
D
D
ACTGG
TGACCCTAG
GCTGGTAC
ACCATG
JH
TdT, XRCC4, LigIV
E
D
ACTGGGATCGGATGCTGGTAC
TGACCCTAGCCTACACCATG
JH

Shortly after V and J gene segments were discovered, Weigert and coworkers performed a detailed analysis of Vκ21 protein sequences and proposed that antibody variability could be in part explained by combinatorial diversity, i.e. the random associations of V, D, and J DNA segments (Weigert et al., 1978). Assuming no bias in the combination process, the maximal collection of antibody specificities possible would be equal to the product of the number of V, D, and J genes present in each of the Ig loci. Translating this assertion to the recently characterized mouse Vκ locus (Thiebe et al., 1999) results in 100 (functional Vs) X 4 (functional Js) = 400 potential Vκ genes generated.

Because of the imprecise nature of V(D)J recombination, the point at which V and J segments are joined can vary over a range of several nucleotides. As a result, codon 96 of the light chain becomes the most variable position of the molecule, as comparative studies of light chain sequences reveal (Wu and Kabat, 1970). Theoretically, in every V-J combination event four different amino acids can be placed at position 96, which would in turn result in1600 potentially different Vκ genes (400 X 4). For the heavy chain locus junctional diversity operates both at the level of V-D and D-J joints increasing diversity by a factor of 16 (4 X 4). As noted above, heavy chain junctions are also diversified by the addition of N nucleotides by the TdT enzyme. Equally important, the random opening of hairpin intermediates generates palindromic (P) nucleotides in the process. Because of the extent of junctional diversity, antibodies show the greatest diversity at the CDR3, the portion of the Ig molecule which contributes the most to the antigen binding pocket.

Somatic Hypermutation

Burnet conceived the clonal selection theory to a great extent by extrapolating the Darwinian concepts he had acquired while working on bacterial genetics. He envisioned that, much as antibiotics select resistant bacterial cells, antigens could also select "improved" lymphocyte clones that could arise as a result of somatic mutations "in that region of the genome concerned with immunologically significant pattern" (Burnet, 1959). The first direct evidence for somatic diversification of antibody variable regions came from a comparison of λ1 protein sequences to the number of Vλ1 genes (Weigert et al., 1970). The study showed that of 19 λ1protein sequences analyzed, 12 were identical and 7 differed by two or three amino acids. The interpretation was that there existed only one λ1 gene in the genome and that the substitutions had arisen, as predicted by Burnet, by somatic mutational events.

The mechanism of somatic hypermutation, which is confined to germinal center B cells, randomly introduces mutations downstream of the VH and VL promoters modifying the affinity of the immunoglobulin antigen binding site. Those B cells expressing higher affinity for the antigen are selected for further expansion and differentiation. B cells that loose the affinity for the antigen in question, or those that react to self-antigens (change in specificity) are eliminated through apoptosis.

Class Switch Recombination

The existence of different isotypes was first hinted by the work of Bauer and Stavitsky, who discovered that after immunization, antigen specific antibodies appeared first with a sedimentation coefficient of 19S, followed within several days by the additional synthesis of a 7S form (Bauer and Stavisky, 1961). The results demonstrated that distinct molecular weight antibodies were generated during an immune response. The favored interpretation at the time was that different plasma cells synthesized the 19S or the 7S immunoglobulins. The idea that a single cell could "switch" from one isotype to another was first proposed by Nossal in 1964 (Nossal et al., 1964). Nossal isolated single antibody producing cells at different time points after immunization and characterizated the antibodies they produced. In the first 5 days after antigen challenge, all plasma cells isolated produced the heavy 19S antibody form (IgM). At day 7 and later, only 7S (IgG) containing cells could be found. However, at days 6 and 7 single cells were isolated producing both IgM and IgG. That this switching was the result of another recombination event was later suggested by the demonstration that IgG1 cells shared the same variable domain with their IgM predecessors (Gearhart et al., 1975). Honjo's finding that specific CH genes were deleted in mouse myeloma cells, and that deletion accompanied class switching unequivocally validated the recombination theory (Honjo and Kataoka, 1978).

Heavy chain constant regions exist in five classes: mu (μ), delta (δ), gamma (γ), epsilon (ε), and alpha (α). Accordingly, individual immunoglobulins are named based on their constant region isotype: IgM, IgD, IgG, IgE, and IgA. In the mouse, CH genes span a region of approximately 200Kb in chromosome 12 and are organized from 5' to 3' in the order μ, δ, γ3, γ1, γ2b, γ2a, ε, and α. The human CH locus resides in chromosome 14q32 and is composed of two copies of the γ–γ–ε–α unit. Contrarily to the light chain κ and λ constant regions, the CH proteins are functionally divergent as to their ability to dimerize, interact with different Fc receptors, activate the complement cascade, or their capacity to cross the placenta.

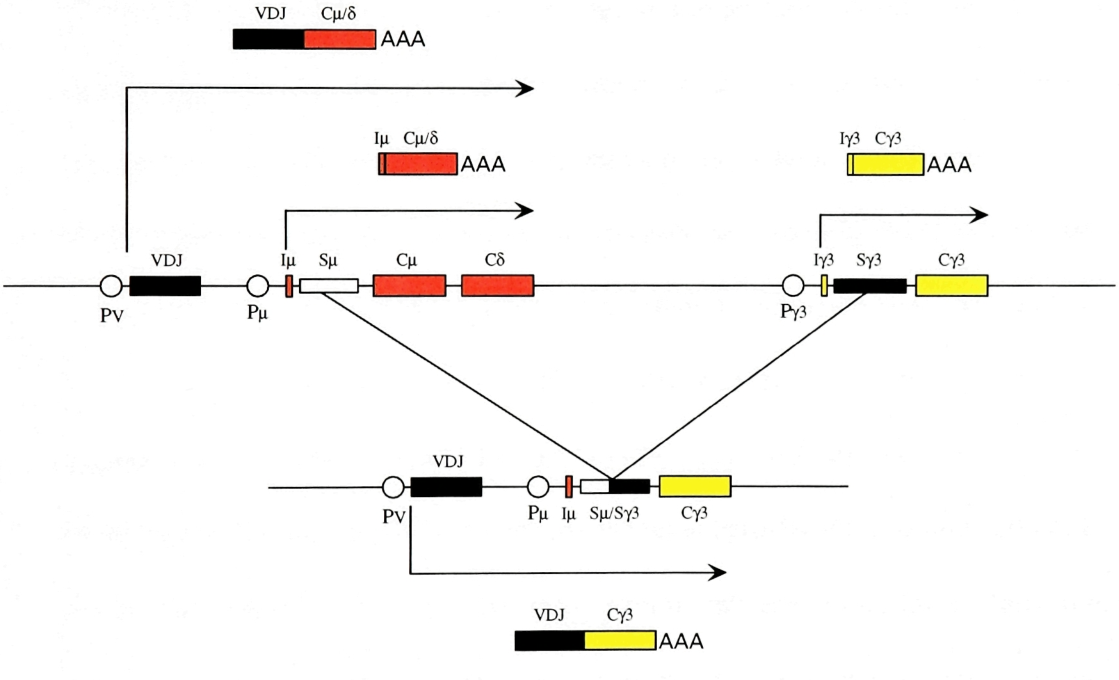
Isotype switching can be induced both in vivo or in cell culture upon activation with a variety of mitogens and cytokines. For example, culturing mouse B cells in the presence of lipopolysacharide (LPS) and interleukin 4 (IL4), induces switching from IgM to IgG1 and IgE. In the absence of IL4, however, LPS activates recombination to IgG3 and IgG2b. In vivo studies indicate that switching is not a random process. In the mouse, immunization with soluble antigens generates IgG1 responses, while carbohydrate antigens stimulate switching to IgG3. Viruses, on the other hand, induce IgG2a responses in mice and IgG3 and IgG1 secretion in humans. In summary, the environmental stimuli elicited by an immune response appear to target switching to particular isotypes. Switch factors include IL4, IL5, IL10, INF-γ, TGF-β, LPS, and CD40 ligand.

Class switch recombination occurs between highly repetitive DNA sequences, known as switch (S) regions, located 5' of each constant region gene, with the sole exception of Cδ, which is co-transcribed with Cμ, and requires mRNA differential splicing for its expression. Although all S regions are primarily composed of the nucleotide repeats GAGCT and TGGGG, the exact sequence composition and length varies among CH genes. Unlike V(D)J recombination, isotype switching occurs anywhere within or near these S regions, with no apparent regard to the sequence specificity at the site of recombination(Davis et al., 1980).

Switching results in deletion of chromosomal DNA located between the two S regions engaged in the recombination process (Figure 1-7). As shown for V(D)J recombination, switching is restricted to transcriptionally active loci (Stavnezer-Nordgren and Sirlin, 1986). This observation led to the hypothesis that transcription may render the chromatin accessible to the DNA binding proteins involved in the recombination process. This hypothesis, known as the accessibility model, propose that cytokines target switch recombination by inducing transcription of specific heavy chain constant regions. Interestingly, recent work suggests that proper splicing of switch transcripts and not transcription *per se* is required for recombination to occur (Lorenz et al., 1995).

Although the identity of the alleged class switch recombinase remains unknown, recent reports indicate that a B cell specific cytidine deaminase (AID) is required for class switching and somatic hypermutation both in humans and mice (Muramatsu et al., 2000; Revy et al., 2000). In addition to this novel factor, as will be discussed in Chapter 3, some of the components known to participate in both V(D)J recombination and DNA double-stranded break repair (DNA DSB), such as DNA-PKcs, Ku70, Ku80, Nbs1, and the phosphorylation of histone H2AX are also directly involved in isotype switching (Casellas et al., 1998; Manis et al., 1998; Petersen, 2001; Rolink et al., 1996).

Figure 1-7 Class Switching to γ3. Prior to switch recombination, Cytokines and B cell activators specifically target the genes involved in the reaction (μ and γ3) by initiating germline transcription from promoters (Pμ and Pγ3) upstream of the respective CH genes. The I exons are spliced to the C exons deleting the switch regions (S) from the germline transcripts. Upon switching, the intervening DNA is deleted from the chromosome bringing the Cγ3 into proximity to the VDJ gene so that the mature transcript initiated from the V promoter (PV) now contains the Cγ3 domain.


VDJ
Cμ/δ
AAA
Iμ
Cμ/δ
AAA
Iγ3
Cγ3
AAA
VDJ
Iμ
Sμ
Cμ
Cδ
Iγ3
Sγ3
Cγ3
PV
Pμ
Pγ3
VDJ
PV
Pμ
Iμ
Sμ/Sγ3
Cγ3
VDJ
Cγ3
AAA

B cell development

As previously explained, the clonal selection theory proposed that lymphocytes were monospecific and that clones of lymphocytes were selected and expanded upon interaction with antigen. Antibodies, however, have been found to regulate the antigen independent phase of B cell development. Therefore, in addition to cellular selection, the B cell repertoire is shaped by receptor selection.

The earliest committed B cell precursors are pre-pro-B cells (Hardy et al., 1991). These cells have their immunoglobulin (Ig) loci in germline configuration but can be distinguished from other bone marrow cells by a series of cell surface markers and because they only differentiate into B lymphocytes (Allman et al., 1999). Pre-pro-B cells do not express components of the B cell receptor, as a consequence, commitment to the B cell pathway precedes antibody gene recombination and is receptor independent.

Development of pre-pro-B cells into Pro-B cells is distinguished by the acquisition of new cell surface markers and the expression of a primordial form of the BCR receptor composed of Igα, Igβ and calnexin (pro-BCR). Igα and Igβ are BCR signaling components that are associated with membrane bound Igμ (mIgμ) in more mature B cells. These Ig superfamily members activate cellular signaling pathways through cytoplasmic immune receptor tyrosine activating motifs (ITAMs) that recruit src and syk family kinases (reviewed by (Meffre et al., 2000)).

The first immunoglobulin gene recombination events occur between diversity (D) to junction (JH) segments in pro-B cells, followed by VH to DJH recombination (Alt et al., 1984; Tonegawa, 1983). The switch from DJH to VDJH is likely to be regulated at the level of VH gene accessibility and appears to require Pax-5 and IL-7 (Corcoran et al., 1998; Nutt et al., 1997). Thus, as previously proposed for class switching, transcription *per se* may render VH gene segments accessible to the recombination machinery (Yancopoulos and Alt, 1985). Alternatively, that cis-regulatory elements present in Ig promoters and enhancers might recruit factors that remodel chromatin domains independently of transcription (Alt et al., 1984).

Expression of mIgμ results in assembly of the pre-BCR and marks the transition to the pre-B cell stage. The pre-BCR is composed of mIgμ, ψL, Igα and Igβ. This receptor is a key checkpoint regulator in B cell development and its primary functions are to trigger B cell differentiation, clonal expansion, and heavy chain allelic exclusion.

Two models were advanced to explain heavy chain allelic exclusion: the "stochastic" model suggested that random joining of Ig genes would rarely produce two in frame Ig heavy chains (Coleclough et al., 1981), whereas the "regulated" model proposed feedback control of recombination by a productively rearranged antibody (Alt et al., 1984). The regulated model was validated by experiments showing that a mIgμ transgene inhibited further V(D)J recombination (Nussenzweig et al., 1987).

Less is known about the nuclear events that govern allelic exclusion and pre-B cell development, but two mechanisms are likely to contribute. First mIgμ signaling in pre-B cells down-regulates the expression of RAG1 and RAG2 (Grawunder et al., 1995). Second, mIgμ expression appears to make VH genes less accessible to the recombinase (Constantinescu and Schlissel, 1997; Stanhope-Baker et al., 1996).

In conclusion, pre-BCR signaling regulates heavy chain allelic exclusion and induce pre-B cell development. Lymphocytes that fail to assemble a pre-BCR fail to progress in development and are deleted. Therefore, antibody mediated selection of lymphocytes begins in the antigen independent phase of the B cell pathway, earlier than originally envisioned by the clonal selection theory.

Following clonal expansion of mIgμ producers, pre-B cells arrest in G1, express RAG proteins, Igκ germline transcripts and undergo light chain gene recombination. Successful light chain gene rearrangements lead to BCR assembly and replacement of the ψL in the pre-BCR by Igκ or Igλ.

To maintain the one cell one antibody rule, allelic exclusion would have to be imposed on light chains as well as heavy chains. Evidence for light chains allelic exclusion was first observed in rabbits and rats by means of anti-allotypic antibodies (Pernis et al., 1965; Tsukamoto et al., 1984). However, light chain allelic exclusion has never been measured precisely, and in contrast to heavy chains, many of the experiments with transgenic mice carrying pre-rearranged light chain genes showed only partial exclusion (Ritchie et al., 1984; Rusconi and Kohler, 1985). In addition, normal human B cells and myelomas synthesizing two light chains have been described (Bernard et al., 1981; Giachino et al., 1995; Kwan et al., 1981). Finally, single cell sequencing experiments show two or more in frame light chain genes in approximately 10 % of all mature B cells (Yamagami et al., 1999a). Despite this, in chapter 4 we show that light chain allelic exclusion is a highly efficient process under physiological conditions.

One of the mechanisms likely to contribute to light chain allelic exclusion is asymmetric demethylation of the Igκ alleles (Mostoslavsky et al., 1998). Demethylation is thought to render one of the two Igκ alleles preferentially accessible for VJκ rearrangement (Mostoslavsky et al., 1998). The idea that undermethylation is required for recombination, is further supported by experiments with V(D)J recombination substrates in cell lines (Cherry et al., 2000; Hsieh and Lieber, 1992). Another potential mechanism thought to regulate light chain allelic exclusion involves feedback control of B cell development by the BCR. In this model a non-self reactive BCR would signal the transition from the pre-B cell stage to the immature B cell stage where RAG expression and V(D)J recombination are turned-off (Grawunder et al., 1995; Monroe et al., 1999; Yu et al., 1999). High affinity cross-linking of self-reactive BCRs, or lack of signaling due to failure to assemble a functional BCR, would be expected to trap developing B cells in a compartment where they continue to undergo secondary recombination (Gay et al., 1993; Tiegs et al., 1993; Yamagami et al., 1999a; Yamagami et al., 1999b).

The idea that BCR signaling regulates the checkpoint between the pre-B cell and immature B cell stage is supported by gene targeting experiments on the cytoplasmic domain of Igα, as in its absence, immature B cell development is inefficient (Torres et al., 1996).

B cell tolerance

Receptor Editing

Early studies with B cell lines showed that Ig gene rearrangements could continue even after assembly of functional BCRs (Feddersen and Van Ness, 1985). Furthermore, DNA episomes excised from the genome by V(D)J recombination showed occasional in frame VJκ genes that had been displaced by nested recombination between upstream Vκs and downstream Jκs (Harada and Yamagishi, 1991). These secondary rearrangements can occur at both heavy and light chain genes, but the mechanism of replacement is different at the two antibody loci. Cryptic recombination signal sequences

(RSSs) embedded in many heavy chain genes allow upstream V_H genes to recombine with pre-existing V(D)Js to produce hybrid V_H genes (Kleinfield et al., 1986). In contrast, secondary Vκ rearrangement entirely replaces pre-existing VJκ genes by nested recombination events to downstream Jκs (Harada and Yamagishi, 1991).

The potential physiologic significance of secondary recombination was first appreciated from studies on transgenic mice that carried anti-double stranded DNA or anti-MHC antibodies (Gay et al., 1993; Tiegs et al., 1993). In these models, self-reactive antibodies were replaced by secondary light chain gene recombinations (Radic et al., 1993). In contrast to anergy and deletion (see below), this new mechanism of tolerance termed "receptor editing" spares auto-reactive B lymphocytes by replacing their receptors and is therefore an example of molecular selection.

Whether receptor editing is due to specific RAG re-induction or is simply the result of random recombination and selection was not determined at the time. Support for specific RAG re-induction came from experiments with bone marrow derived immature transgenic B cells where BCR cross-linking *in vitro* resulted in increased RAG expression and secondary light chain rearrangements (Melamed and Nemazee, 1997; Tiegs et al., 1993). However, antigens that mediate clonal deletion of transgenic receptors *in vivo* appear to arrest B cell development in a recombination competent B cell stage (Chen et al., 1995b; Hartley et al., 1993). Studies with gene targeted mice identified the pre-B cell compartment as the site for gene replacement suggesting that editing is the result of developmental arrest followed by random rearrangements and selection (Pelanda R et al., 1997).

Although receptor editing is likely to be important in shaping the antibody repertoire, there have been few measurements of the extent of editing in normal B cells. Igλ expressing mouse hybridomas were used to examine Igκ genes that suffered recombining sequence (RS) mediated Cκ deletion (Retter and Nemazee, 1998). In these cells 50% of the Igκ genes were found to harbor potentially productive VJκ rearrangements suggesting that these light chain genes might have been silenced by editing. However, only 5% of B cells in mice express Igλ and only some of these have undergone Cκ deletion by RS recombination. By using light chain knock-in animals in chapter 4 we estimate that one out of four receptors in the B cell repertoire are the result of light chain editing.

Deletion

Immature B cells are the first B lineage cells to express surface BCRs, they display surface IgM, but little or no IgD. B cells remain in the immature compartment for an average of 3.5 days (Osmond, 1993), and it is in this compartment that self-reactive B cells failing to edit their receptors are deleted or anergized (Sandel and Monroe, 1999).

Immature B cells differ from mature B cells in that they are particularly susceptible to BCR induced apoptosis (Sandel and Monroe, 1999). Thus, these B cells conform to Lederberg's idea that tolerance is established by deletion of self-reactive B cells during early development (Lederberg, 1959). That tolerance is an active process and signaled through the BCR was initially suggested by experiments in which mice treated with anti-immunoglobulins from birth were found to be B cell depleted (Lawton et al., 1972). The assumption linking these experiments to tolerance was that anti-IgM antibodies would mimic auto-antigens by crosslinking the BCR. This assumption was subsequently corroborated in transgenic mice (Chen et al., 1995b; Hartley et al., 1991; Nemazee and Burki, 1989; Okamoto et al., 1992). B cells bearing anti-self-reactive antibodies in the appropriate background are efficiently eliminated in large numbers (Nemazee and Burki, 1989), and the degree of B cell elimination is dependent on the strength of receptor crosslinking (Hartley et al., 1991; Okamoto et al., 1992).

Anergy

Anergy is the second mechanism by which self-tolerance is induced in immature B cells. This phenomenon was originally observed in cultures of developing B cells exposed to different concentrations of anti-μ antibodies (Pike et al., 1982). B cells cultured in the presence of high concentrations of anti-μ did not develop and underwent deletion. In contrast, lower concentrations of anti-μ allowed B cell development but abrogated normal B cell function as determined by decreased proliferation and antibody production upon mitogen exposure (Pike et al., 1982). This hypo-responsive or anergic state has been studied extensively in mice carrying anti-lysozyme (Goodnow et al., 1988) or anti-single stranded DNA antibodies (Erikson et al., 1991; Tsao et al., 1993). Anergic B cells are short lived, and have difficulty in developing from the immature into the long-lived B cell compartment in the spleen (Cyster et al., 1994; Fulcher and Basten, 1994). Chronic exposure to antigen in anergic cells is also associated with decreased expression of the BCR, possibly due to a selective block in BCR transport from the endoplasmic reticulum (Bell and Goodnow, 1994).

BCR signaling pathways that induce anergy have been characterized in mice carrying loss of function mutations. For example, absence of the protein phosphatase SHP in anti-HEL transgenic B cells exposed to soluble HEL leads to an exaggerated calcium response and apoptosis (Cyster and Goodnow, 1995). Thus SHP is a negative regulator of BCR signaling and modulates the threshold for anergy (Bolland and Ravetch, 1999). In contrast anti-HEL specific B cells rendered deficient in CD45 are under-responsive to BCR crosslinking by soluble HEL and are more difficult to anergize (Cyster et al., 1996; Healy et al., 1997). These and similar studies performed in CD19 deficient animals indicate that BCR signaling is down-regulated in anergic B cells (Inaoki et al., 1997). Whether this feature is a consequence of diminished levels of cell surface IgM or the activation of inhibitory molecules has not been determined.

The BCR is an essential regulator of immature B cell development. Mutations in the cytoplasmic domains of Igα and Igβ have dramatic effects on immature B cell differentiation (Gong, 1996; Reichlin, 2001; Torres et al., 1996). In the absence of the cytoplasmic domain of Igα, BCR with a single functional Igβ cytoplasmic domain produce few pre-B and immature B cells, most of which fail to progress to the mature B cell stage (Torres et al., 1996). Igα and Igβ are likely to regulate immature B cell development in part through Btk and Lyn in conjunction with BCR modulating co-receptors such as CD19, CD22 or CD45, since disruption of these genes interferes with the establishment of anergy and immature B cell development.

Transcription of Ig genes

Transcription of immunoglobulin genes is one of the most well studied systems of tissue-specific gene expression. Most Ig genes contain three regulatory elements which are necessary for proper tissue specific transcription: the V promoter, an intronic enhancer (EH or Eκ), and a 3' enhancer downstream of the C gene. Associated with these, a variety of lymphoid restricted and general trans-acting factors regulate transcription throughout B cell ontogeny.

The V Promoter

All V promoters, whether belonging to heavy or light chain genes, share two cis-regulatory elements: the octamer binding site, which consists in an eight base pair consensus motif (ATTTGCAT), and approximately 40 base pairs downstream the TATA box. In vitro and transgenic studies clearly show that these two elements are necessary and sufficient to impart lymphoid-restricted expression. Insertion of an octamer sequence in proximity of a basal TATA promoter, for instance, recapitulates B cell specific transcription (Wirth et al., 1987). Mutations in the consensus motif of the octamer element, on the other hand, severely reduces Ig gene transcription (Grosschedl and Baltimore, 1985). Paradoxically, this same octamer motif seems to be a transcriptional control element in promoters which are active in all tissues such as histone H2B (LaBella et al., 1988), snRNAs (reviewed by (Hernandez, 2001)), and other more restricted genes such as Igα (Hashimoto et al., 1994), and Igβ[Malone, 2000 #623].

In addition to the octamer and TATA boxes, light chain promoters are furnished with a variety of additional sequence elements, which are conserved only in a given Vκ subgroup or family (Bemark et al., 1998a). In chapter 5 we show that these elements, although unable to provide specificity by themselves, play an important role in the regulation of recombination, editing, and allelic exclusion of Igκ genes.

Octamer Binding Factors

Ig gene transcriptional specificity is given by two members of a family of transcription factors, the POU family of homeobox proteins Oct-1 and Oct-2, which recognize the same octamer binding DNA motif.

The POU family is defined by the homeodomain, which consist of an N-terminal POU-specific and a C-terminal POU-homeo subdomains, both of which form a clamp around the octamer binding site (Fig. 1-8 and (Phillips and Luisi, 2000)). Members of the POU family of transcription factors display distinct tissue distribution. B lymphocytes express the ubiquitous Oct-1 protein, in addition to Oct-2, whose expression is almost entirely confined to B cells (Staudt et al., 1986). By virtue of its restricted expression, Oct-2 was believed to be responsible for lymphoid specific transcription(Staudt and Lenardo, 1991). Nevertheless, genetic ablation of Oct-2 did not appear to affect Ig gene expression, perhaps evidence for a functional overlap between Oct-1 and Oct-2(Corcoran et al., 1993).

OcaB

Comparative *in vitro* transcriptional assays with purified Oct-1 or Oct-2, supplemented with either B cell or non-B cell nuclear extracts suggested that an additional B cell specific activity was responsible for higher expression of Ig genes.

Crude purification of this factor indicated that only Ig genes but not H2B promoters were transcriptionally enhanced. Further purification led to the characterization of a novel lymphoid factor: OcaB (Gstaiger et al., 1995; Luo et al., 1992; Luo and Roeder, 1995; Strubin et al., 1995).

OcaB is capable of interacting with Oct-1 or Oct-2 heterodimers when bond to the octamer of Ig gene promoters. OcaB/POU domain/octamer ternary complex is highly selective and occurs only in a subset of such sequences, namely those containing an adenine at position 5 of the octamer (Gstaiger et al., 1996). Crystal structure studies of the ternary complex clearly show that OcaB makes a pair of hydrogen bonds with adenine 5 (Chasman et al., 1999). In the ternary complex, OcaB N-terminus interacts with the POU homeodomain, while its C-terminus interacts with the POU-specific domain of Oct-1 or Oct-2 (Fig. 1-8A and (Chasman et al., 1999)).

These *in vitro* and cell culture studies predicted that *in vivo* ablation of OcaB would lead to an arrest in B cell development. Paradoxically, mice deficient for OcaB showed almost wild type numbers of bone marrow B cells and no obvious defect in Ig gene transcription (Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996). In the periphery, humoral immune responses against T cell dependant antigens were impaired and germinal centers did not form upon immunization (Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996).

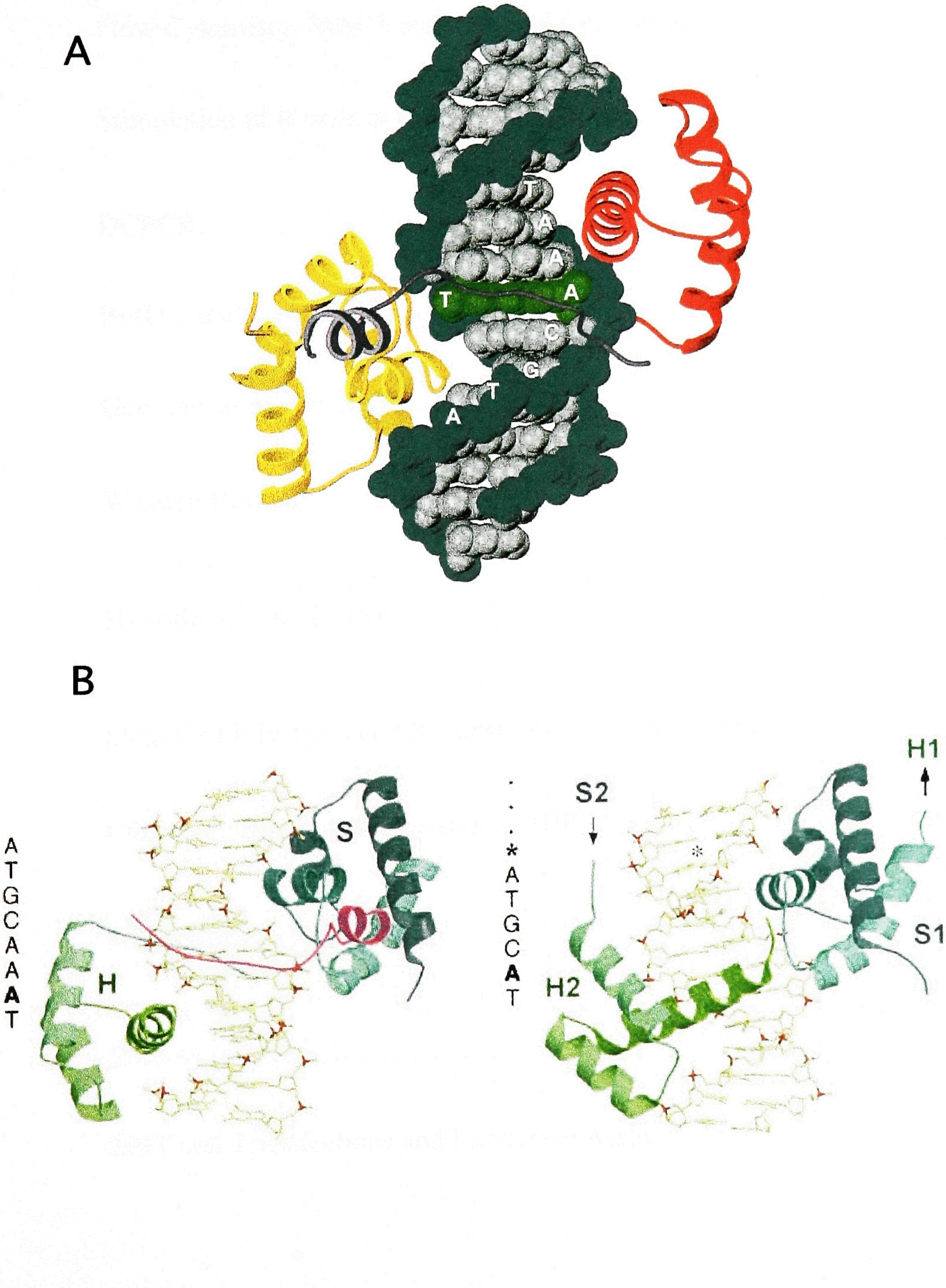
Recent structural studies of the Oct-1/OcaB/Octamer ternary complex may provide an explanation for the normal Ig gene transcription seen in OcaB$^{-/-}$ mice (Remenyi et al., 2001; Tomilin et al., 2000). These studies show that dimerization of Oct-1 on heavy chain promoters appears to preclude recruitment of OcaB to the octamer site

(Figure 1-8B, and (Remenyi et al., 2001; Tomilin et al., 2000)). Thus, OcaB may not be able to interact with heavy chain promoters *in vivo*. In chapter 5, we demonstrate that OcaB does interact with kappa gene promoters and that in the absence of OcaB there is no expression, or editing of a subset of pre-recombined light chains.

Figure 1-8

(A) Structure of the ternary complex composed of OcaB, Oct-1 POU element, and octamer binding site. The OcaB peptide (purple) interacts with the octamer sequence of Ig gene promoters. The critical adenine at position 5 is shown in green. At its C-terminus, OcaB interacts extensively with the Oct-1 POU specific domain (in yellow) via a short α-helix. Both the Oct-1 POU specific domain and the Oct-1 homeodomain form a clamp like structure around the octamer sequence. Reprinted from (Chasman et al., 1999).

(B) Oct-1 (in green) binding to a PORE (left) and a MORE (right) motif. The OcaB molecule (in purple) is capable of interacting with the Oct-1-PORE element but is excluded from the closed Oct-1MORE dimer complex. Reprinted from (Tomilin et al., 2000).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H1
S1
H2

Chapter 2: Material and Methods

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Antibodies

Annexin V [Biotin]	PharMingen/BD Bioscience
BrdU [Biotin]	CALTAG Laboratories
BrdU [FITC]	PharMingen/BD Bioscience
CD19 [Biotin]	PharMingen/BD Bioscience
CD19 [PE]	PharMingen/BD Bioscience
CD22.2 [FITC]	PharMingen/BD Bioscience
CD24/HSA [Biotin]	PharMingen/BD Bioscience
CD24/HSA [PE]	PharMingen/BD Bioscience
CD24a/HSA [FITC]	PharMingen/BD Bioscience
CD25 [PE]	PharMingen/BD Bioscience
CD43 [Biotin]	PharMingen/BD Bioscience
CD43 [FITC]	PharMingen/BD Bioscience
CD45/B220 [APC]	PharMingen/BD Bioscience
CD45/B220 [Biotin]	PharMingen/BD Bioscience
CD45/B220 [Cychrome]	PharMingen/BD Bioscience
CD45/B220 [FITC]	PharMingen/BD Bioscience
CD45/B220 [PE]	PharMingen/BD Bioscience
CD45/B220 [PerCP]	PharMingen/BD Bioscience
CR1/CR2 (CD35/CD21) [FITC]	PharMingen/BD Bioscience
CR1/CR2 (CD35/CD21) [purified] biotinylated	PharMingen/BD Bioscience
FAS [Biotin]	PharMingen/BD Bioscience
FAS [PE]	PharMingen/BD Bioscience
GL7 [FITC]	PharMingen/BD Bioscience
GL7/Ly-77 [purified] – biotinylated	PharMingen/BD Bioscience
hCκ (rat anti-human kappa) [FITC]	Biosource International
IgD [Biotin]	Southern Biotechnology Ass.
IgD [PE]	PharMingen/BD Bioscience
IgD [purified] – conjugated with AMCA	PharMingen/BD Bioscience
IgM [Biotin]	PharMingen/BD Bioscience
IgM [PE]	PharMingen/BD Bioscience
IgM F(ab) [Cy3]	Jackson ImmunoResearch
IgM F(ab) [Cy5]	Jackson ImmunoResearch
IgM F(ab) [FITC]	Jackson ImmunoResearch
Lak27 (anti 3-83) [Biotin]	Casellas et al., Science 2001
Lambda ($\text{mC}\lambda_{1+2}$) [FITC]	PharMingen/BD Bioscience
mCκ [FITC]	PharMingen/BD Bioscience
mCκ [PE]	Southern Biotechnology Ass.
mCλ [PE]	Southern Biotechnology Ass.
Streptavidin-APC (SA-APC)	PharMingen/BD Bioscience
Streptavidin-Red 613 (SA-Red 613)	GibcoBRL
Streptavidin-Red 670 (SA-Red 670)	GibcoBRL
IgG1 [biotin]	PharMingen/BD Bioscience
IgG3 [biotin]	PharMingen/BD Bioscience

IgE [biotin]	PharMingen/BD Bioscience

Flow Cytometry, MACS isolation and Cell Sorting

Immunofluorescent stainings were performed on either cultured cells, or primary cells from bone marrow, spleen, lymph nodes, or peritoneal cavity. Red blood cells were depleted by ACK treatment (0.15M NH4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA, pH to 7.2-7.4). Between 0.5 x 10^6 to 1 x 10^6 cells were stained for 15' at RT. Stained samples were gated according to standard forward- and side-scatter values and analyzed on a Becton and Dickinson FACSscan fluorescence-activated cell sorter with CELLQuest software. B cells requiring purification were incubated with anti-CD43, anti-CD19, or anti-B220 microbeads (Myltenyi Biotech) and run through a MACS magnet. To specifically purify mCκ or hCκ$^+$ populations, cells were first stained with anti-mCκ or anti-hCκ antibodies followed by an anti-rat IgG microbead incubation and positive selection. When cells required >90% purity a FACSVantage instrument was used to cell sort the samples. Staining of intracellular antigens was performed with the Intracellular Staining Kit (Immunotech).

Stimulation of B cells in Culture

Spleenic B cells from 6-8 week-old mice were cultured for 3-4 days in complete RPMI medium (RPMI 1640 (Gibco BRL) with 10% Fetal Calf Serum (Sigma), 1% Antibiotic-Antimycotic (Gibco BRL), 1% L-Glutamine (Gibco BRL), 2% MEM Amino Acids Solution, 1% Sodium Pyruvate Solution (Cellgro), 10mM HEPES buffer (Gibco

BRL), and 53mM 2b-mercaptoethanol (Fisher Scientific)) with the addition of 25mg/ml LPS (E.Coli 0111:B4; Sigma), or LPS and IL4 (50U/ml; Gibco BRL).

DCPCR

To increase the sensitivity of the DC-PCR assay (described by (Chu et al., 1992)) we added a nested amplification step to the basic protocol. Following ligation, 5ng of DNA were denatured at 94°C for 5 minutes followed by 30 cycles of amplification with the first primer set at (94°C for 15'', 66°C 1.5', 72°C 1') and a final amplification at 72°C for 10 minutes. 2 ml from each first cycle reaction was further amplified for 30 cycles with a second set of primers in the presence of $a^{32}P$ dCTP (4mCi). The cycle conditions were 94°C for 15'', 68°C 1.5', 72°C 1'. PCR products were analyzed by 8% PAGE and the products visualized and quantitated with a phosphorimager. PCR primers were: Sm-Sg1 first set: S1: 5' GAGCAGCTACCAAGGATCAGGGA 3' and S2: 5' CTTCACGCCACTGACTGACTGAG 3'; Sm-Sg1 second set of primers: S3: 5' GGAGACCAATAATCAGAGGGAAG 3' and S4: 5' GAGAGCAGGGTCTCCTGGGTAGG 3'. For the nicotinic acetyl choline receptor (nAChR) primers used were: First set: A1: 5' GCAAACAGGGCTGGATGAGGCTG 3' and A2: 5' GTCCCATACTTAGAACCCCAGCG 3'. For the second set:_A3: 5' GGACTGCTGTGGGTTTCACCCAG 3' and A4: 5'GCCTTGCTTGCTTAAGACCCTGG 3'.

BrdU Labeling and Kinetics

BrdU labeling was performed using the Pharmingen BrdU Kit following the manufacturers instructions. Data from Igκ$^{h/m}$ and Igκ$^{\alpha HEL/h}$ BrdU injected mice was subject to linear regression analysis and the entry point for each of the populations was calculated based on the regression formula y=Bx-A. The equations obtained were as followed: Igκ$^{h/m}$ hCκ population: y=1.44x-6.33, entry point [4.4, 0]; mCκ population: y=1.40x-6.35, entry point [4.5, 0]. Igκ$^{\alpha HEL/h}$ hCκ population: y=2.34x-10.8, entry point [4.61, 0]; mCκ population: y=5.29x-13.6, entry point [2.57, 0]. Three mice were sacrificed for each time point.

Genomic and RTPCR

Switch Recombination Project:

Total RNA extracted from cultured cells was reverse transcribed with Superscript II (Life Technologies). 5-100ng cDNA was then amplified in the presence of ^{32}P dCTP (4uCi; DuPont) in a 25 ml PCR reaction with 10 pmoles of each primer. To detect germline sterile transcripts the following primers were paired: 1. Iγ3 : γ3-5' CAAGTGGATCTGAACACA and : γ3-3' GGCTCCATAGTTCCATT 3' (expected product 350 bp); 2. Iγ2b : γ2b-5' CCTGACACCCAAGGTCACG : γ2b-3' CGACCAGGCAAGTGAGACTG (expected product 345 bp); Iγ1: γ1-5' CAGCCTGGTGTCAACTAG and : γ1-3' GCAAGGGATCCAGAGTTCCAG (expected product 341 bp); Igβ : Igβ-5' GATGACGGCAAGGCTGGGATGGAGGAA and Igβ-3' CTCATTCCTGGCCTGGATGC (expected product 142 bp). Mature γ3 and γ2b transcripts were amplified by combining a V$_H$B1-8 specific primer V$_H$B1-8

CAAGGGCAAGGCCACACTG with either Cγ3 CCACTGCTGCCTGAGCCATCTC (expected product 313 bp) or Cγ2b CAGGTGACGGTCTGACTTGG (expected product 414 bp). Mature γ1 transcripts were amplified by combining a second V_HB1-8 specific primer V_HB1-8' CCAGCTACTGGATGCACTG 3' with Cγ1 GGACAGCTGGGAAGGTGTG 3' (expected product 440 bp). All reactions were performed for 30 cycles. Amplified samples were analyzed by 8% PAGE and visualized by phosphorimaging.

Editing Project:

Vκ-Jκs from Igk$^{m/h}$, IgH$^{3H9/+}$Igk$^{Vk4/h}$, Igk$^{aHEL/h}$, and Igk$^{Vk4/h}$ B cells were amplified by RT-PCR with the following primers: 3' primer mCk:ACGCCATTTTGTCGTTCACTGCCA (for) or hCk: GAGTTACCCGATTGGAGGGCGTTA (Igk$^{m/h}$). 5' primer was as in (Schlissel and Baltimore, 1989).

V(D)J recombination of Vκ8, 3-83 was determined in DNA extracted from sorted immature Ig$\kappa^{h/h}$OcaB$^{-/-}$ and OcaB$^{+/+}$ B cells in a nested PCR approach using the following set of primers: Vκ8 set 1: 5'CAGTGGAAATCAAAAGAACTACTTGA, Jκ2-3'/1 TTTTCCCTCCTTAACACCTGATCTG; Vκ8 set 2: same 5' as before, Jκ2-3'/2 GGTTAGACTTAGTGAACAAGAGTTGAGAA; 3-83 set 1: 5' CATGTCTGCATCTCTAGGGGAACG, Jκ2-3'/1 as above, followed by a second PCR using Jκ2-3'/2 (30 cycles each step, Tm= 62°C).

αHEL, 3-83, and Vκ4 germline transcripts were amplified from cDNA generated from cell sorted Ig$\kappa^{h/h}$OcaB$^{-/-}$ and OcaB$^{+/+}$ pre-B cells by nested PCR as following:

αHEL set 1: 5’ATGGAGTTTCAGACCCAGGTACTC, 3’ GTAGGAGGCTGAAGCACTGTG, αHEL set 2: 5’ as before, 3’CGATCAGGGACCCCAATGTATC; 3-83 set 1: 5’ ATGGATTTTCAGGTGCAGATTTTCAGC, 3’TAGCTAGACCCTAAGGAGCTAG; 3-83 set 2: 5’as before, 3’ATGGTGACCCGTTCCCCTA; Vκ4 set 1: 5’ ATGGATTTTCAGGTGCAGATTTTCAGC, 3’GTTAGAGGGTTTTGTTCCAGC, Vκ4 set 2: 5’ as before, 3’CCTATAAATCCAGAATTTGGTAGAAGTG (30 cycles each step, Tm=61°C); Actin 5’ TACCACTGGCATCGTGATGGACT, Actin3’ TCCTTCTGCATCCTGTCGGCAAT (30cycles, Tm=60°C).

αHEL and 3-83 mature transcripts were amplified from $\mathrm{IgM}^{+}\mathrm{B220}^{\mathrm{low}}$ cell sorted $\mathrm{Ig}\kappa^{\mathrm{h/h}}\mathrm{OcaB}^{-/-}$ and $\mathrm{OcaB}^{+/+}$ B cells with the following primers: αHEL 5’ GGGGCATCCAACCGATACAT, hCκ 3’ GAGTTACCCGATTGGAGGGCGTTA; 3-83 5’ same as for genomic V(D)J PCR plus hCκ 3’ (35 cycles, Tm=62°C).

To assess the presence of OcaB and Lyn in spleens before and after hybridoma fusion, genomic DNA was amplified with the following primers: OcaB 5’ CTGTGGCTATGAAGGGGAATGTCACT, 3’ CTGCTGGAATGCTATAGAGTGGTTC (40 cycles, Tm=64°C); Lyn 5’ CATAGCCTGAGTTAGTTCCCTAGC, 3’ TCACATATGAACATGTGTGTACATGTC (35 cycles, Tm=60°C).

Western Blotting

2ml of mouse serum was diluted in 100ml PBS and incubated with 20ml of protein-A sepharose beads (Pierce) for 1 hour. The beads were washed three times with PBS and the bound proteins separated by 8% reducing PAGE before blotting and visualization with alkaline phosphatase conjugated goat anti-mouse IgG (Pierce).

Hybridomas and ELISA

Hybridomas and genomic PCR assays used to characterize the light chain targeted allele from OcaB$^{-/-}$ IgH$^{3H9/+}$Igκ$^{V\kappa4/h}$spleenic B cells were performed as described (Prak and Weigert, 1995). mCκ-hCκ double producing hybridomas were detected by coating 96-well plates with 50μl of rat anti-hCκ (Biosource), or rat anti-mCκ (Southern Biotechnology). Following addition of serum unknowns, 50μl of biotinylated rat anti-mCκ or rat anti-hCκ antibody, respectively, was added. ELISAs were finally developed with SA-horseradish peroxidase.

Hybridoma cell lines were cultured in RPMI-1640 media supplemented with 15% FCS, 2% Hybridoma Cloning Factor, 1% HT, 1% Penicillin/Streptomycin, 1% L-Glutamine, 1% Sodium Pyruvate, 1% non-essential amino acids, 2-β-ME to a final concentration of 50μM, and 1% HEPES buffer.

Stem Cell Infections and Reconstitution of RAG1$^{-/-}$ mice

OcaB$^{-/-}$Igκ$^{V\kappa4/h}$ bone marrow donors were injected with 5mg of 5FU (Sigma) in PBS 5 days before infection and maintained in a pathogen free environment. Wild type and L32P human OcaB were cloned into the retroviral expression vector, pMIG (Hawley et al., 1994). Retroviruses were generated by transfecting Bosc 23 cells with 10 μg of plasmid DNA. 48hs after transfection viral supernatant was span with bone marrow isolated OcaB$^{-/-}$Igκ$^{Vκ4/h}$ stem cells. The efficiency of infection (presence of GFP) was assessed 72hs post-infection by microscopy and cells were transferred by tail vein injections into RAG1$^{-/-}$ recipients that have been previously irradiated with 300rad (Cs-137 source). Recipient mice were maintained in a pathogen free facility with the addition of 25mg/ml neomycin and 13mg/ml of polymyxin in the drinking water. 8 weeks after transfer, the animals were sacrificed and spleenocytes isolated and stained with anti-mCκ (PE), anti-hCκ (Biotin/SA-APC), and PcP conjugated B220.

Chromatin Immunoprecipitation (CHIP) Assay

Spleenic B cells from OcaB$^{-/-}$Igκ$^{Vκ4/h}$ and OcaB$^{+/+}$Igκ$^{Vκ4/h}$ control animals were stained with anti-mCκ PE antibodies (Southern Biotechnology) and then positively selected by using anti-rat IgG microbeads (Myltenyi Biotech). Cells were aliquoted into samples of 2 x 10^6 cells and protein was cross-linked to DNA by adding formaldehyde (up to 1%) directly to the cell suspension and incubating at 37°C for 10'. Samples were washed twice with ice cold PBS containing 1mM PMSF, 1μg/ml aprotinin, and 1μg/ml pepstatin A, and finally resuspended in 200μl of SDS Lysis Buffer (Upstate Biotechnology) containing protease inhibitors as before. DNA was sheared to lengths between 200 and 1000 bps with 4 sonicating pulses of 10" at 60% output power with a micro ultrasonic cell disrupter (Kontes); at this time samples were processed using the

Upstate Biotechnology H3-H4 Chromatin Immunoprecipitation kit. To precipitate protein-DNA complexes, 4μl of both anti-acetyl histone H3 and anti-acetyl histone H4 antibodies (Upstate Biotechnology), or 4μl of anti-Oct-1 antibody (Santa Cruz Biotechnology) were added to each sample. DNA was recovered by phenol-chloroform/ethanol precipitation and resuspended in 25μl TE.

DNA was diluted in a 2 fold series. Vκ4 (knock-in gene and promoter), Eμ, and MyoD were amplified in the presence of (^{32}P)dATP. Products were run on a 8% PAGE. Primers used: Vκ4 promoter 5': AACTGTCTAGGCTATATCTTGTTATTTG; 3': TGTTGTATTTGAATTCAGGTAGCTAG, Vκ4 knock-in gene 5': TCTCTCTACGTTGCTTTTTTCCAG, 3': CAATTTTGTCCCCGTGCCGAACGTGAATG, MyoD 5': CGCCCTACTACACTCCTATTG, 3': AAGGTTCTGTGGGTTGGAATG; Eμ 5': GTAAGAATGGCCTCTCCAGG, 3': ACAATCTAGTGTGGAACATTCCTC (30 cycles, Tm=60°C)

Methylation (SNuPE) Assay

With the exception of thymic cells, Spleenic mCk$^+$ B cells from OcaB$^{-/-}$Igκ$^{Vκ4/h}$ and controls were positively selected as described for the CHIP assay. Genomic DNA was extracted by standard methods from 2 x 10^6 cells. Vκ4 samples were then restricted with PstI and Cμ controls with HindIII. After a cleaning step, restricted DNA was resuspended to around 0.25ng/μl and bisulphite conversion was carried out as previously described (Clark et al., 1994). Vκ4 and Cμ were then nested PCR amplified with the following primers: Vκ4 set 1: 5' AGTGGTAGTGGGTTTGGGA, 3' CTCTTTCTTCTACATTCCCCTTAC; Vκ4 set 2: 5' primer as in set 1, 3' CTTTCTTCTACATTCCCCTTACAAAACT; Cμ set 1 5' TTGAGGATAGGGGGTAAGTA, 3' TTCCACAAACTTCCCATCCTTTA; Cμ set 2: 5' primer as in set 1, 3' CCACAAACTTCCCATCCTTTAACCA (35 cycles, Tm=60°C). Products were purified and sequenced. In a 25μl PCR reaction 10-50ng of product were incubated for 1 cycle (95°C 1', 50°C 2', 72°C 1') with 1μCi of either (^{32}P)dCTP or (^{32}P)TTP and 25pmols of SnuPE primer. Vκ4 SNuPE primer: GAGTTAGTGTGAAAATTGAG, Cμ SNuPE primer: AATTTTAATGTAAATGTGTT. The reaction was stopped with 10μl of denaturing loading dye and run in a 15% denaturing polyacrylamide gel. Percentage of TTP or dCTP incorporation was quantified by phosphoimager analysis (Molecular Dynamics). % of CpG methylation was calculated as C/(C + T) X 100.

EMSA

Oligonucleotides probes containing the octamer from each promoter were annealed and filled in with (^{32}P)dATP and Klenow at 5:1mol/mol (α^{32}P)dATP:oligonucleotide ratio (3pmols of annealed primers/ Klenow reaction). Probes were then purified with ProbeQuant G-50 Micro Columns (Pharmacia). EMSA was carried out as previously described(Luo et al., 1992). Oligonucleotide probes were: Vκ8 upper: CTGATGATATTTTCTGTCAGCTTTGCATGGGTTCCTCCAGCCCA, Vκ8 lower: TCAGTGGGCTGGAGGAACCCATGCAAAGCTGACAGAAAATATCA, Vκ4 upper: CTGATAGGCTATATCTTGTTATTTGCATATCTCATTTTCAGTAA, Vκ4 lower: TCAGTTACTGAAAATGAGATATGCAAATAACAAGATATAGCCTA, 3-83 upper: CTGATAGACTGTATCTTGCTATTTGCATATTTCATTTTCAGTAA, 3-83 lower: TCAGTTACTGAAAATGAAATATGCAAATAGCAAGATACAGTCTA, αHEL upper: CTGATTGTATTTGCTGACTGCTTTGCATAAGTCTGTCCAGTCAA, αHEL lower: TCAGTTGACTGGACAGACTTATGCAAAGCAGTCAGCAAATACAA, PORE upper: CTGAAAGTTAAAATCTCATTTGAAATGCAAATGGAAAAGCAAG, PORE lower: TCAGCTTGCTTTTCCATTTGCATTTCAAATGAGATTTTAACTT, MORE upper: CTGAAAGTTAAAATCTCATGCATATGCATGGAAAAGCAAG, MORE lower: TCAGCTTGCTTTTCCATGCATATGCATGAGATTTTAACTT.

293T cell Transfections and Luciferase Assay

293T cells transient transfections were performed in 48 well plates using 3μl of FuGene transfection reagent (Roche). Cells were maintained in complete DMEM media supplemented with 10% fetal calf serum, 1% glutamine, 1% sodium pyruvate, 1X nonessential aminoacids, and 1X antibiotic-antimycotic. Light chain promoters were cloned into the –37tk-luc enhancerless vector as described (Tomilin et al., 2000). As an internal transfection control 5ng of renilla luciferase reporter vector (Promega) was cotransfected per well. The total amount of transfected DNA was equalized to 300ng with a carrier plasmid (pBluescript). 36hs post-transfection, luciferase activity was measured by using the Dual-Luciferase Reporter System (Promega) in a single-sample luminometer. The pCG-Oct1 and pEV-OBF1 vectors were previously described (Tomilin et al., 2000).

Chapter 3: The role of DNA Repair in Class Switch Recombination

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Introduction

During an immune response, B lymphocytes maintain their antigen binding specificity but can change the antibody constant region subclass they produce by a DNA recombination process known as class switching (reviewed by (Stavnezer, 1996)). Switching occurs between highly repetitive DNA sequences, known as switch regions, which are located 5' of the μ, γ, α, and ε constant region (CH) genes (Shimizu et al., 1982). Switch recombination is preceded by DNA demethylation, increased deoxyribonuclease I-hypersensitivity, and germline transcription of the implicated CH genes (Lorenz and Radbruch, 1996).

Like V(D)J recombination, switching involves DNA deletion (Honjo and Kataoka, 1978) by a mechanism whereby intervening sequences are excised in the form of circular DNA (Iwasato et al., 1990; Matsuoka et al., 1990; von Schwedler et al., 1990). In addition, switching resembles V(D)J recombination in that a DNA double-stranded break (DSB) intermediate appears to be part of the switch reaction (Wuerffel et al., 1997)

Resolution of the broken DNA ends in V(D)J recombination requires several non-lymphoid specific proteins that were first defined by transfection and genetic studies in mutant cell lines and mice. These include the catalytic subunit of the DNA dependent protein kinase (DNA-PK_{CS}), Ku, and XRCC4 (reviewed by (Fugmann et al., 2000)). In addition, the Nijmegen breakage syndrome protein (Nbs1) and the phosphorylated form of histone H2AX create visible foci at the V(D)J recombining sites *in vivo*, thus implicating these DNA repair factors in the resolution of V(D)J derived DNA lesions (Chen et al., 2000).

In contrast to V(D)J recombination, the role of DNA DSB repair in switch recombination in mature B cells has not been evaluated in detail.

Here we report on switch recombination in the absence of Ku80, and H2AX. We find that B cells that are deficient in Ku80 are unable to complete switch recombination, while switching is only impaired in the absence of H2AX. Switching, as V(D)J recombination, is an end-joining mechanism that occurs at the G1 stage of the cell cycle. Additionally, the novel B cell specific cytidine deaminase (AID) is required for the formation of Nbs1 and γH2AX foci at switch recombination sites.

Results

B cell Reconstitution of Ku80 deficient mice

Ku80$^{-/-}$ mice are unable to repair DNA DSBs and show a phenotype that includes proportional dwarfism, and a profound disruption in both T and B lymphocyte development (Nussenzweig et al., 1996; Zhu et al., 1996). In the absence of Ku80, developing lymphocytes cannot repair the DNA breaks produced by RAG1 and RAG2 during V(D)J recombination. The result of the DSB repair deficiency is that both T and B cell development is arrested at early precursor stages in Ku80$^{-/-}$ mice (Nussenzweig et al., 1996; Zhu et al., 1996).

To determine whether the absence of B cells in Ku80$^{-/-}$ mice was solely due to impaired V(D)J recombination, we introduced pre-rearranged targeted Igμ (Sonoda et al., 1997) and Igκ (Pelanda et al., 1996) genes into the Ku80$^{-/-}$ background. Fig.3-1 displays the light and heavy chain locus before and after targeting insertion of the rearranged vectors. The mice resulting from these crosses are referred to as Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ mice. Like Ku80$^{-/-}$ controls, Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ mice were proportional dwarfs and had no mature T cells. However, bone marrow B cell development in Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ mice differed from Ku80$^{-/-}$ mice in that B cells progressed beyond the pro-B cell stage (Fig. 3-2). FACS analysis of Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ bone marrow showed that in the presence of pre-rearranged μ and κ Ig genes, Ku80 deficient B cells progressed to the B220^{+}CD43^{-} pre-B cell stage and developed into immature and mature B220^{+}IgM^{+} B cells (Fig. 3-2). Mature B cells expressing surface IgM were also found in peripheral lymphoid organs such as spleen, but in 6-8 week old mice the total number of B cells in

Ku80$^{-/-}$IgH$^{B1\text{-}8/+}$Ig$\kappa^{3\text{-}83/+}$ was only 15-25% (n=10) of that found in wild type and Ku80$^{+/-}$ IgH$^{B1\text{-}8/+}$Ig$\kappa^{3\text{-}83/+}$ littermate controls. This low number of mature B cells in the periphery of the reconstituted mice may result from absence of T cells and T cell dependent B cell clonal expansion. Alternatively the relative B lymphopenia could be due to the documented inability of Ku80$^{-/-}$ cells to repair DNA double-stranded breaks incurred during normal proliferative responses (Nussenzweig et al., 1996).

We conclude that pro-B cell arrest in Ku80$^{-/-}$ mice is a function of impaired resolution of DNA breaks resulting from V(D)J recombination and that Ku80 is not essential for other aspects of antigen independent B cell development.

Figure 3-1 Igκ and IgH wild type locus and their predicted structure after insertion of the rearranged constructs. The 3' κ and H enhancers (E) are depicted as open circles, and the kappa constant region (C) as black squares.These prearranged loci were introduced in the Ku80$^{-/-}$ background. These mice are referred throughout the text as Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ mice.

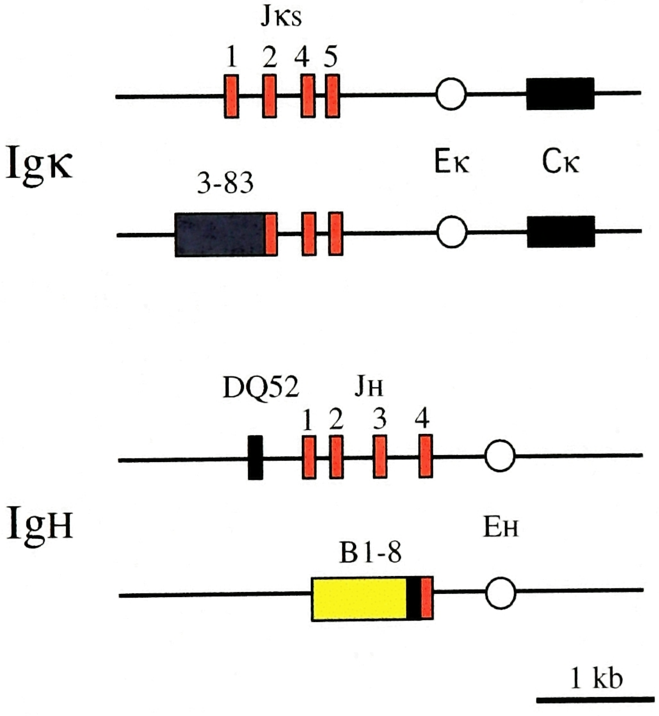
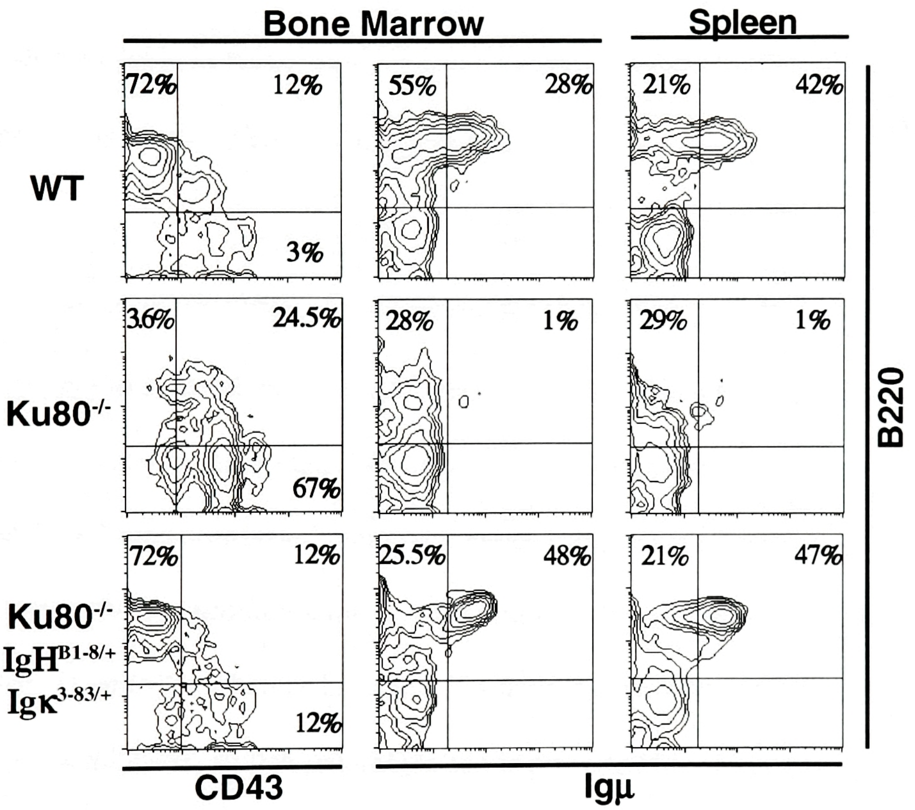

Jκs
1 2 4 5
Igκ
3-83
Eκ
Cκ
DQ52
JH
1 2 3 4
IgH
B1-8
EH
1 kb

Figure 3-2 Reconstitution of B cell development in Ku80$^{-/-}$ mice. Bone marrow and spleen samples from 6-10 week-old wild type, Ku80$^{+/-}$ and Ku80$^{-/-}$IgH$^{B1\text{-}8/+}$Ig$\kappa^{3\text{-}83/+}$ mice analyzed for B cell maturation. Cell percentages were calculated from total gated populations. Bone marrow cells were stained with anti-B220 and anti-CD43 and splenocytes with anti-B220 and anti-IgM antibodies.


Bone Marrow
Spleen
WT
72%
12%
3%
55%
28%
21%
42%
Ku80-/-
3.6%
24.5%
67%
28%
1%
29%
1%
Ku80-/-
IgHB1-8/+
Igκ3-83/+
72%
12%
12%
25.5%
48%
21%
47%
CD43
Igμ
B220

Normal Induction of Class Switching in Ku80$^{-/-}$ mice

Consistent with the lower than normal number of peripheral B cells in Ku80$^{-/-}$ IgH$^{B1-8/+}$Igκ$^{3-83/+}$ mice, the level of circulating IgM was 30% of that found in wild type mice as measured by an IgM specific ELISA (not shown). In contrast to IgM, secondary Ig isotypes, normally found in the serum of un-immunized animals in the mg ml^{-1} range, were not detectable in Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ mice. Western blotting with specific goat anti-mouse IgG showed no IgG heavy chains in the serum of the reconstituted mice (Fig. 3-3).

Absence of secondary Ig isotypes in Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ mice could be due to a cell autonomous deficiency in switch recombination or might be a consequence of the absence of T cells in these mice. To discriminate between these two alternatives, we isolated B cells and stimulated them with either LPS, or the combination of LPS plus IL-4 to activate switch recombination in cell culture. LPS induces mouse B cells to switch from μ to γ3 and γ2b, whereas LPS plus IL4 activates switching to γ1 and, to a lower extent, ε. In all cases, switch recombination is preceded by sterile transcription of the switch targeted CH genes (Fig. 3-4A). To determine whether Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ B cells could respond to either LPS or LPS plus IL-4 by activating switch transcription, we measured germline sterile transcripts by a semi-quantitative RT-PCR assay using Iγ3, Iγ1 (Snapper et al., 1988), and Iγ2b specific primers. Igβ mRNA was used as a B cell specific loading control. Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ resembled control B cells in that Iγ2b, Iγ3, and Iγ1 germline transcripts were specifically induced by LPS and LPS plus IL-4 respectively, although the levels of sterile transcripts found in reconstituted mice was 10-30% lower than those found in wild-type mice, as assayed by phosphorimaging (Fig. 3-4B). We conclude that Ku80$^{-/-}$IgH$^{B1-8/+}$Ig$\kappa^{3-83/+}$ B cells are competent to respond to signals that induce switch recombination *in vitro.*

Figure 3-3 IgG heavy chain (500 kDa) expression in Ku80$^{-/-}$IgH$^{B1\text{-}8/+}$Ig$\kappa^{3\text{-}83/+}$ mice and controls. 2ml of serum from wild type (+/+), and Ku80$^{-/-}$IgH$^{B1\text{-}8/+}$Ig$\kappa^{3\text{-}83/+}$ (-/-) mice was analyzed by PAGE, and blotting with goat anti-mouse IgG visualized with alkaline phosphatase.

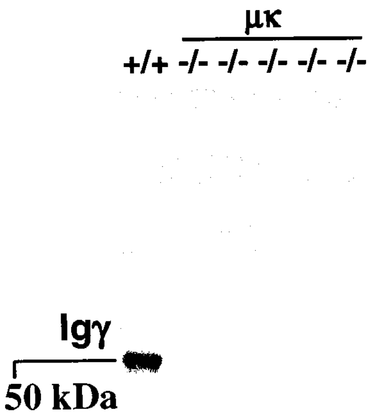
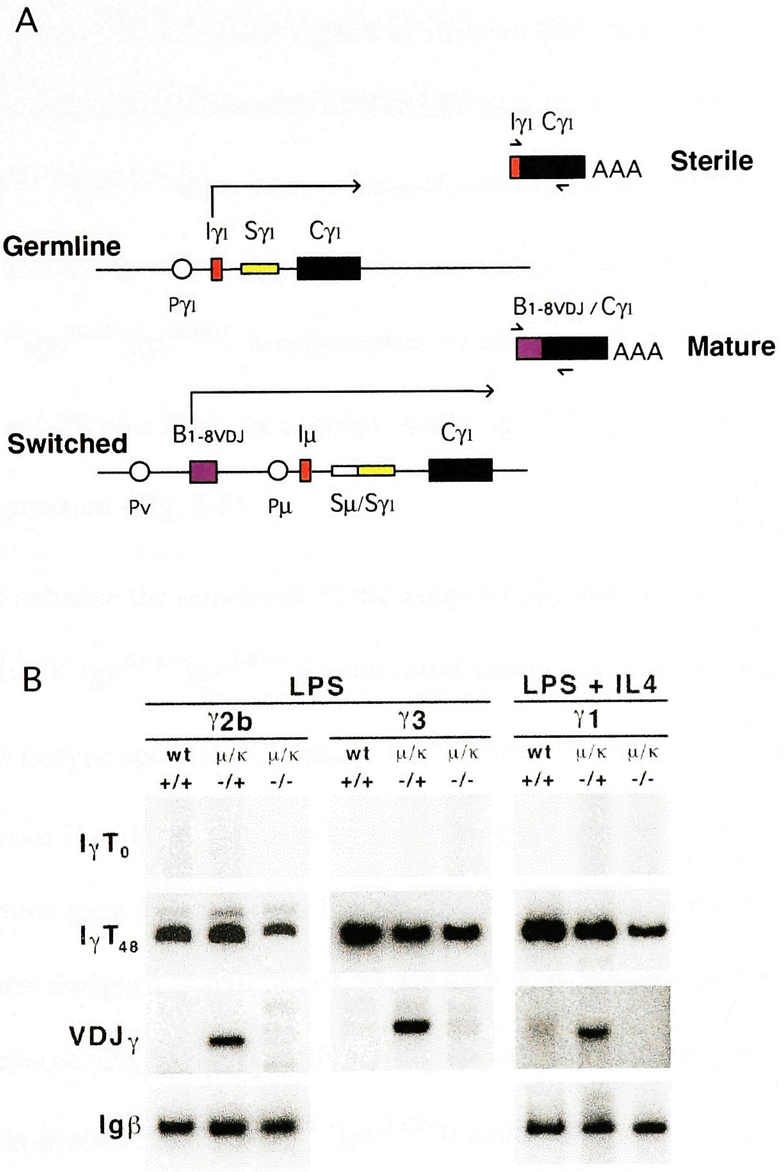

μκ
+/+ -/- -/- -/- -/- -/-
Igγ
50 kDa

Figure 3-4 Ig germline and mature switch transcription

(A) IgG1 locus before and after switching as it is brought in proximity to the targeted B1-8 heavy chain. The I exon (I), switch (S), and constant (C) regions for the μ and γ1 are indicated. Their respected promoters are shown as open circles. Switch recombination is preceded by the generation of sterile transcripts which initiate at the I exons. By using I and C specific primers, as depicted, the induction of sterile transcripts is assayed by RT-PCR. To determine the presence of mature transcripts after class switching, primers specific for the targeted variable B1-8 and C region of interest were used as depicted.

(B) cDNA was prepared from wild type (+/+), $Ku80^{+/-}IgH^{B1\text{-}8/+}Ig\kappa^{3\text{-}83/+}$ (-/+ μ/κ), and $Ku80^{-/-}IgH^{B1\text{-}8/+}Ig\kappa^{3\text{-}83/+}$ (-/- μ/κ) unstimulated B cells (T_0) or B cells that were cultured with LPS or LPS plus IL-4 for 48 hours (T_{48}). Germline (Iγ) and mature (VDJγ) transcripts for γ2b, γ3, and γ1 were detected by PCR, while mature transcripts were absent in reconstituted Ku80 deficient mice. B cell specific Igβ mRNA was used to normalize each RT-PCR reaction.

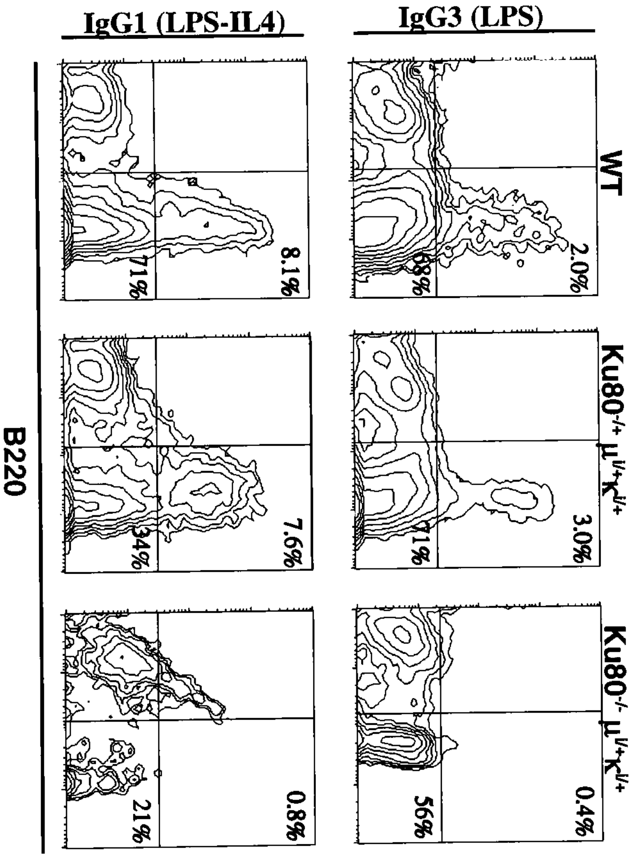

A
Iγ1 Cγ1
AAA
Sterile
Germline
Iγ1
Sγ1
Cγ1
Pγ1
B1-8VDJ / Cγ1
AAA
Mature
Switched
B1-8VDJ
Iμ
Cγ1
Pv
Pμ
Sμ/Sγ1
B
LPS
LPS + IL4
γ2b
γ3
γ1
wt
μ/κ
μ/κ
+/+
-/+
-/-
IγT0
IγT48
VDJγ
Igβ

Lack of switch recombination in the absence of Ku80

To determine whether Ku80$^{-/-}$IgH$^{B1\text{-}8/+}$Igκ$^{3\text{-}83/+}$ B cells can produce secondary antibodies in response to switch signals in vitro we first measured cell surface expression of γ3 and γ1 after stimulation with LPS or LPS plus IL-4 respectively. B cells from Ku80$^{-/-}$IgH$^{B1\text{-}8/+}$Igκ$^{3\text{-}83/+}$ mice were compared with wild type and Ku80$^{+/-}$IgH$^{B1\text{-}8/+}$Igκ$^{3\text{-}83/+}$ B cells after staining with isotype specific antibodies (Fig. 3-5). As expected, wild type and Ku80$^{+/-}$IgH$^{B1\text{-}8/+}$Igκ$^{3\text{-}83/+}$ B cells expressed cell surface γ3, and γ1 following culture with LPS or LPS plus IL-4. In contrast, Ku80$^{-/-}$IgH$^{B1\text{-}8/+}$Igκ$^{3\text{-}83/+}$ showed no secondary isotype expression (Fig. 3-5).

To enhance the sensitivity of the assay for secondary isotypes and to determine whether Ku80$^{-/-}$IgH$^{B1\text{-}8/+}$Igκ$^{3\text{-}83/+}$ B cells could produce mature secondary Ig mRNAs we devised an isotype specific PCR assay. IgH$^{B1\text{-}8/+}$Igκ$^{3\text{-}83/+}$ mice show a highly homogeneous B cell compartment in which the great majority of the cells express the B1-8 replacement gene (Papavasiliou F et al., 1997). Primers specific for B1-8 combined with primers designed to hybridize Cγ3, Cγ2b, or Cγ1 regions can therefore be used to measure mature γ2b, γ3, or γ1 mRNAs (Fig. 3-4A). As expected, mature γ mRNAs were abundant in control Ku80$^{+/-}$IgH$^{B1\text{-}8/+}$Igκ$^{3\text{-}83/+}$B cells stimulated with LPS or LPS and IL-4 (Fig. 3-4B). In contrast, B1-8/Cγ2b, or B1-8/Cγ3 mRNAs were detected at very low levels, and B1-8/Cγ1 were not detected in Ku80$^{-/-}$IgH$^{B1\text{-}8/+}$Igκ$^{3\text{-}83/+}$B cells. Dilution analysis and quantitation by phosphorimaging showed that the levels of B1-8/Cγ2b, or B1-8/Cγ3 were at least ten fold lower than those found in Ku80$^{+/-}$IgH$^{B1\text{-}8/+}$Igκ$^{3\text{-}83/+}$ B cells.

Figure 3-5 Cell surface expression of secondary Ig isotypes. Flow cytometry analysis of splenocytes from wild type, Ku80$^{+/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ and Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ after LPS or LPS plus IL-4 stimulation. Cells were cultured for 3 days and stained with anti-B220 and anti-IgG3 (LPS) or anti-IgG1 (LPS-IL4). Percentages from total gated populations are shown.


IgG1 (LPS-IL4)
IgG3 (LPS)
B220
WT
71%
8.1%
68%
2.0%
$Ku80^{-/+}\ \mu^{I/+}\kappa^{I/+}$
34%
7.6%
71%
3.0%
$Ku80^{-/-}\ \mu^{I/+}\kappa^{I/+}$
21%
0.8%
56%
0.4%

Presence of DNA ds-break switch intermediates in Ku80$^{-/-}$ B cells

When B cells are culture with appropriate mitogens and cytokines, DNA double-stranded breaks are induced in the targeted switch regions (Wuerffel et al., 1997). To determine whether these DNA breaks were present in Ku80$^{-/-}$IgH$^{B1-8/+}$Ig$\kappa^{3-83/+}$B cells, we used a $\gamma3$ switch region specific double stranded DNA break assay (Wuerffel et al., 1997, Fig. 3-6A). We find that DNA double-stranded breaks are generated in $\gamma3$ switch regions from Ku80$^{-/-}$ B cells as well as the Ku80$^{+/+}$ controls upon 4-hour activation with LPS. These $\gamma3$ switch specific breaks are undetected in T or B cells stimulated with mitogen alone (PHA and anti-delta-dextran respectively), but are induced when B cells are cultured in the presence of mitogen plus cytokines (anti-delta-dextran and IL5, Fig. 3-6B). These data indicates that the formation of DNA breaks is unaffected by the absence of Ku80.

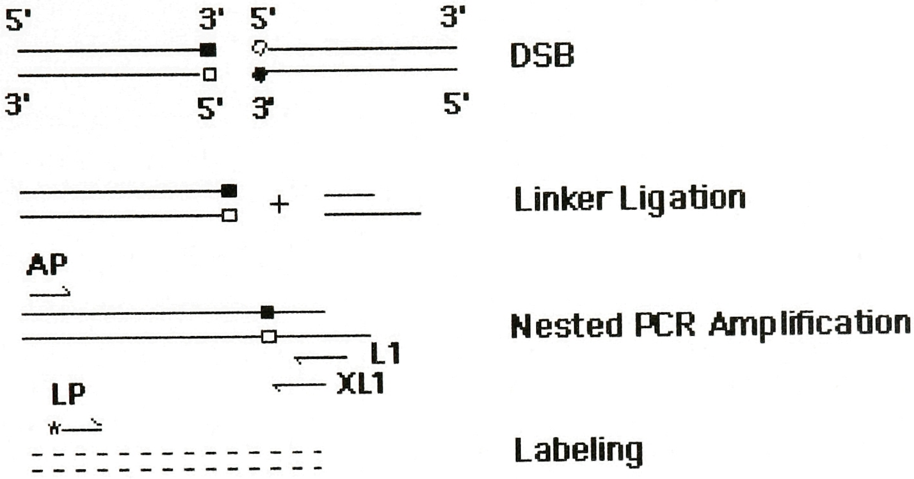
To determine whether the deficiency in switch transcription and protein production was due to a failure to complete switch recombination at the DNA level, we assayed for μ–$\gamma1$ switched DNA directly using a previously described $\gamma1$ digestion-circularization PCR (DC-PCR) assay which detects recombination between μ and $\gamma1$ switch regions ((Chu et al., 1992) Fig. 3-7A). We used the non-rearranging acetylcholine receptor (Ach) gene as a positive control for our digestion and ligation reactions (Chu et al., 1992). All of the DNA samples were positive in the Ach DC-PCR reaction. In addition, μ–$\gamma1$ rearrangement was present in DNA from both wild type and Ku80$^{+/-}$IgH$^{B1-8/+}$Ig$\kappa^{3-83/+}$ control B cells stimulated with LPS and IL-4. In contrast, Ku80$^{-/-}$IgH$^{B1-8/+}$Ig$\kappa^{3-83/+}$ showed a complete absence of μ–$\gamma1$ DNA recombination (Fig. 3-7B).

Figure 3-6 γ3 switch breaks in stimulated B cells.

(A) Schematic diagram of the DSB assay. A linker which will ligate only to double-stranded blunt ends containing a 5'-phosphate is ligated directly to purified genomic DNA. Broken DNA fragments, tagged with the linker, are PCR amplified for 15 cycles using the linker primer (L1) and a locus specific primer (AP). An aliquot of this reaction is used in a second 15 cycle PCR amplification using AP and a nested linker primer (XL1), that overlaps the ligation junction. The amplified products are then radioactively labeled with a nested locus specific labeling primer (LP) and the precise positions of the DSBs are determined by denaturing PAGE.

(B) Ku80$^{+/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ ($\mu/\kappa^{+/-}$) and control Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ ($\mu/\kappa^{+/-}$) splenocytes were stimulated with LPS for the indicated times and assayed for DNA breaks (Wuerffel et al., 1997). Switch breaks are 107nt long and are found in live B cells undergoing switch recombination (Wuerffel et al., 1997). As control, wild type (wt$^{+/+}$) B cells were stimulated for 41hs with anti-delta-dextran (D) antibodies, which induce proliferation but not switching, and anti-delta-dextran plus IL5 (D+IL5), which stimulate B cells to switch from IgM to IgG3. In addition, T cells (T) stimulated for 4hs in the presence of PHA do not show switch specific breaks. L = DNA ladder.

A


5'
3'
5'
3'
DSB
3'
5'
3'
5'
Linker Ligation
AP
Nested PCR Amplification
L1
XL1
LP
Labeling

B

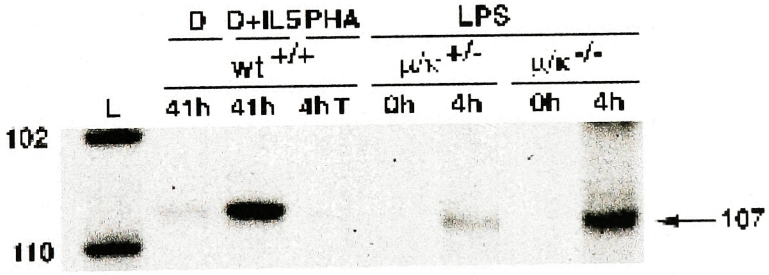
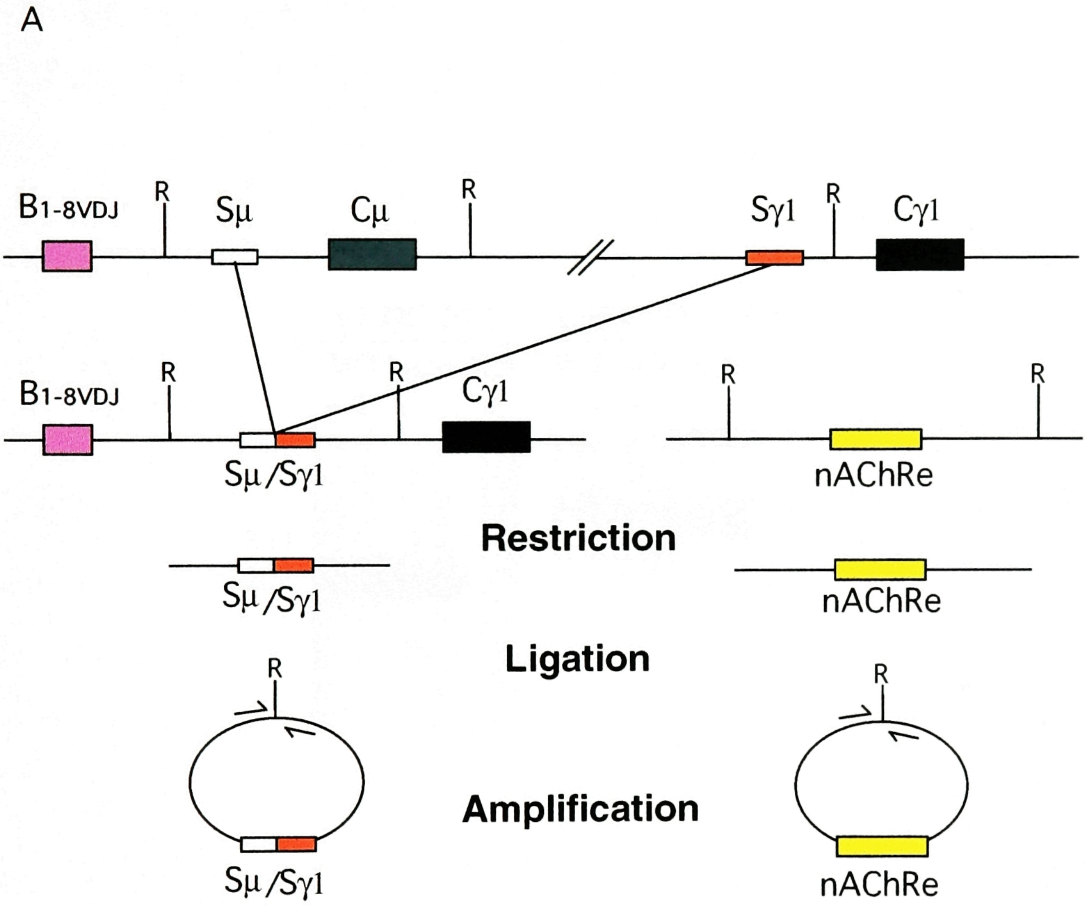
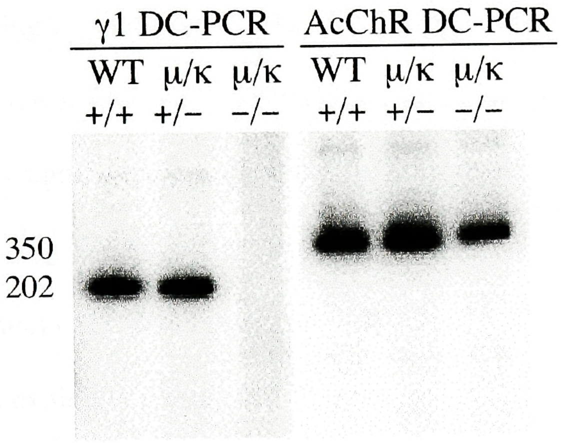

D
D+IL5
PHA
LPS
wt +/+
μ/κ +/-
μ/κ -/-
L
41h
41h
4hT
0h
4h
0h
4h
102
110
107

Figure 3-7 Digestion circularization mediated PCR assay for switch recombination.

(A) Strategy for the digestion circularization polymerase chain reaction (DCPCR) assay of the murine Sμ-Sγ1 class switching (adapted from (Chu et al., 1992)). The upper graph represents schematically the nonrearranged B1-8 locus depicting the μ and γ1 switching (S) and constant (C) regions. Upon EcoRI digestion, ligation, and PCR amplification using specific primers, a product is only obtained if Sμ-Sγ1 recombinations are present on the genomic DNA sample. Using this strategy, another circular DNA fragment containing a portion of the acetylcholine receptor (nAChRe) is created and amplified. This latter PCR product is present regardless of chromosomal rearrangement status thus serving as an internal quantitative control. R represents EcoRI sites.

(B) Semiquantitative nested DC-PCR was performed on DNA extracted from wild type, Ku80$^{+/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$, and Ku80$^{-/-}$IgH$^{B1-8/+}$Igκ$^{3-83/+}$ splenocytes that had been stimulated with LPS and IL4 for 3 days. Mouse nicotinic acetylcholine receptor gene was used as an internal control (Chu et al., 1992). The absence of switch DNA rearrangement in Ku80 deficient B cells was confirmed by overexposure or by using higher concentrations of genomic DNA (up to 100 ng per reaction, data not shown). The size of the amplified products is indicated.


A
B1-8VDJ
R
Sμ
Cμ
R
Sγ1
R
Cγ1
B1-8VDJ
R
R
Cγ1
R
R
Sμ/Sγ1
nAChRe
Restriction
Sμ/Sγ1
nAChRe
Ligation
R
R
Amplification
Sμ/Sγ1
nAChRe


γ1 DC-PCR
AcChR DC-PCR
WT +/+
μ/κ +/−
μ/κ −/−
WT +/+
μ/κ +/−
μ/κ −/−
350
202

Formation of Nbs1 and γ-H2AX foci at switch recombination sites

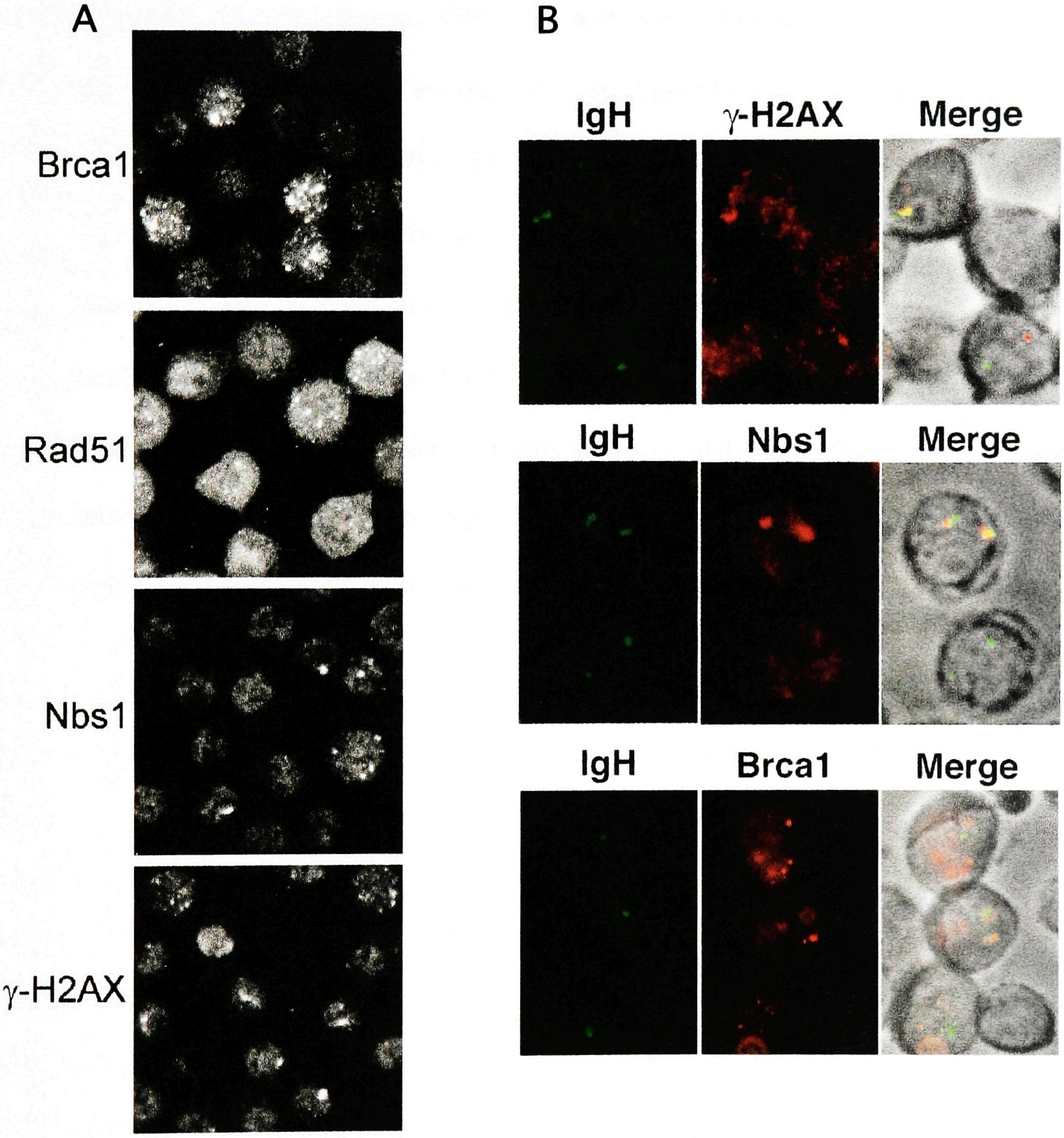
To determine whether DNA repair factors associate with DSBs at the switch regions, we next examined the intracellular localization of γ-H2AX, Nbs1, Rad51, and Brca1 in activated B cells by immunofluorescence. Brca1 and Rad51 are required for homologous recombination (HR); the Mre11/Rad50/Nbs1 complex has been implicated in both HR and non-homologous end-joining (NHEJ); γ-H2AX is critical for recruiting these repair factors to DSBs (Paull et al., 2000) and facilitates NHEJ in *Saccharomyces cerevisiae* (Downs et al., 2000). All four proteins showed diffuse nuclear staining in the majority of resting B cells from C57BL/6 wild-type mice. High local concentrations of these factors (nuclear foci) were detected in a very small percentage of cells (<5%), which increased significantly when the cells were stimulated to undergo CSR *in vitro* with LPS+IL4 (Fig. 3-8A). After three days of stimulation, 37% of the B cells contained discrete Brca1 foci (12 ± 6 per cell) and 43% contained Rad51 foci (7 ± 3 per cell); the remaining cells exhibited a weak diffuse nuclear staining pattern (Fig. 3-8A). Many of the stimulated B cells also formed Nbs1 foci (32% contained, on average, 3± 2 per cell) and γ-H2AX foci (40% contained, on average, 4.5± 3 per cell).

To examine whether Nbs1/γ-H2AX/Brca1 foci are associated with sites of CSR in B cells, we performed immunocytochemistry staining followed by fluorescence in situ hybridization (ICC-FISH) to simultaneously visualize DNA (IgH loci) and protein (Nbs1, γ-H2AX, Brca1 or Rad51) in LPS+IL4 stimulated lymphocytes (Fig. 3-8B). Approximately 15% of cells in a given optical section contained at least 1 Nbs1 or γ-H2AX focus. Coincidence of either signal with one or both IgH alleles was detected in 69% of the cells with Nbs1 foci and 76% of the cells with γ-H2AX foci (Fig. 3-8B). This co-localization was specific since only 3-5% of the stimulated B cells showed co-localization of Nbs1 or γ-H2AX with TCRα or Igκ. In contrast, neither Rad51 nor Brca1 foci co-localized with IgH, although the average number of these foci per cell was greater than the number of Nbs1 or γ-H2AX foci (Fig. 3-8 and not shown).

Figure 3-8 Nbs1, γH2AX foci formation at sites of class switch recombination

(A) Distribution of Brca1, Rad51, Nbs1 and γ-H2AX, in activated wild-type B cells. Confocal images were optically sectioned at 0.5 mm intervals and merged into a maximum projection.

(B) Colocalization of DNA repair foci with the IgH locus. B cells were stained with anti-γ-H2AX, anti-Nbs1, or anti-Brca1 antibodies [ICC (red)] followed by DNA FISH (green) detection of the IgH region. Cells were visualized by phase contrast microscopy and the images were merged to determine co-localization (yellow). Fluorescence images in (B) and (C) represent a single optical section.


A
Brca1
Rad51
Nbs1
γ-H2AX
B
IgH
γ-H2AX
Merge
IgH
Nbs1
Merge
IgH
Brca1
Merge

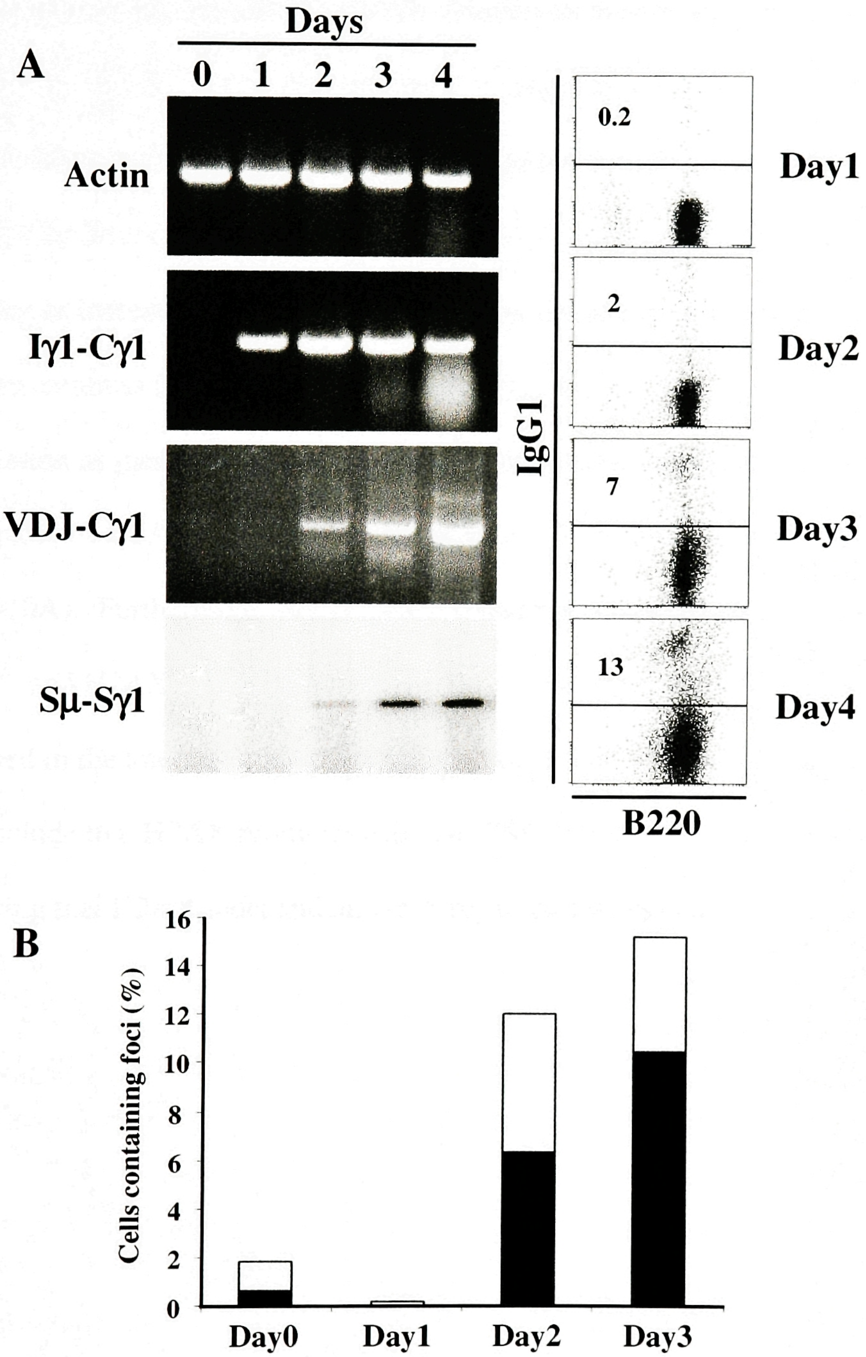
Kinetics of DNA repair foci formation in switching cells

DNA lesions associated with CSR would be expected to form after initiation of germline transcription and prior to expression of mature switch transcripts (Stavnezer et al., 1988; Yancopoulos et al., 1986). To determine when IgH-associated Nbs1/γ-H2AX foci appear relative to these events, we assayed germline Iγ1 transcription (Iγ1-Cγ1), mature IgG$_1$ transcripts (VDJ-Cγ1), Sμ-Sγ1 DNA rearrangements, and surface IgG$_1$ expression (Figs. 3-9A). Germline Iγ1 transcription was present 24 hours after stimulation. Sμ-Sγ1 DNA, mature IgG$_1$ transcripts, and surface IgG$_1$ expression were barely detectable at 48 hours, but were clearly present after 72 hours. IgH-associated Nbs1 foci began accumulating in B cells 48 hours poststimulation (Fig. 3-9B), after the detection of Ig germline transcripts but before high levels of completed recombination, arguing that focus formation is coincident with initiation of CSR.

Figure 3-9 Kinetics of Nbs1 foci formation at switch regions

(A) Actin control, germline sterile Iγ1-Cγ1 transcripts, mature switch VDJ-Cγ1 transcripts assayed by RT-PCR; and Sμ-Sγ1 DNA rearrangements assayed by digestion-circularization PCR (DC-PCR) at days 0-4 of LPS+IL4 culture. Cell surface expression of IgG_1 detected by flow cytometry. Percentages from total lymphocyte gated populations are indicated.

(B) Analysis by ICC-FISH for colocalization of Nbs1 foci with IgH.


A
Days
0
1
2
3
4
Actin
Iγ1-Cγ1
VDJ-Cγ1
Sμ-Sγ1
IgG1
0.2
Day1
2
Day2
7
Day3
13
Day4
B220
B
Cells containing foci (%)
0
2
4
6
8
10
12
14
16
Day0
Day1
Day2
Day3

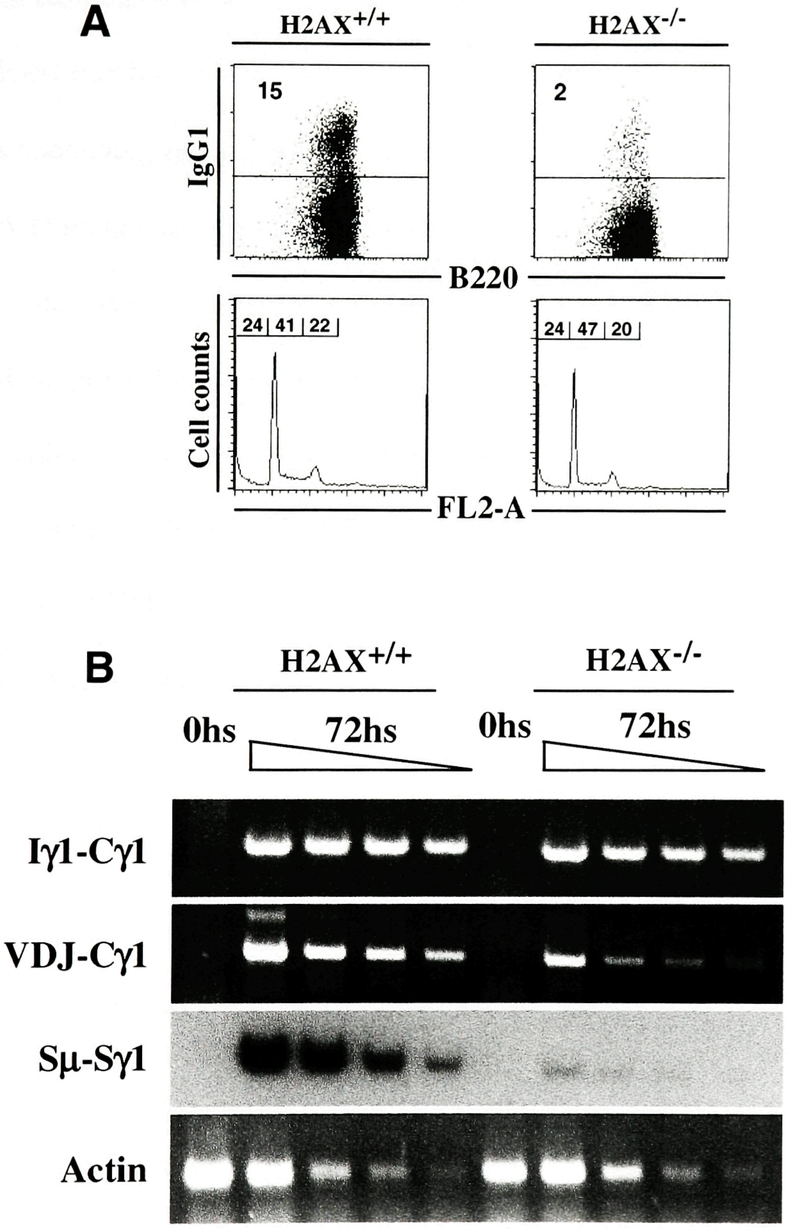
Switch Recombination is impaired in $H2AX^{-/-}$ mice

To determine the functional significance of γ-H2AX focus formation at sites of CSR, we examined the effects of H2AX ablation on switch recombination. B cells isolated from $H2AX^{-/-}$ mice (Nussenzweig, A. unpublished) were stimulated with LPS and IL4 under conditions identical to those used for ICC-FISH analysis and CSR was measured by flow cytometry. B cells from $H2AX^{-/-}$ mice (n=8) exhibited impaired switching as indicated by a 50%-86% reduction in surface IgG1 levels relative to littermate controls (Fig. 3-10A). This deficiency was not due to a difference in proliferation as measured by cell number, ^{3}H-thymidine incorporation ($H2AX^{+/+}$: 2120 ± 244 c.p.m. vs. $H2AX^{-/-}$:1910 ± 102 c.p.m. at day 3 of culture), or cell cycle distribution (Fig. 3-10A). Furthermore, sterile switch transcripts were induced at similar levels in $H2AX^{-/-}$ and $H2AX^{+/+}$ B cells but mature IgG_1 transcripts and DNA recombination were decreased in the knockout mice in a manner consistent with flow cytometry (Fig. 3-10B). We conclude that H2AX promotes efficient CSR, but it is not essential for the reaction, suggesting that H2AX independent DNA repair pathways can also process switch DNA lesions.

Figure 3-10 Switching in the absence of H2AX

(A) Cell surface expression of IgG1 in B cells from $H2AX^{-/-}$ mice and littermates assayed 72 hours after LPS+IL4 stimulation. An aliquot of the samples (shown below) was used to simultaneously measure cell cycle distribution at 72 hours.

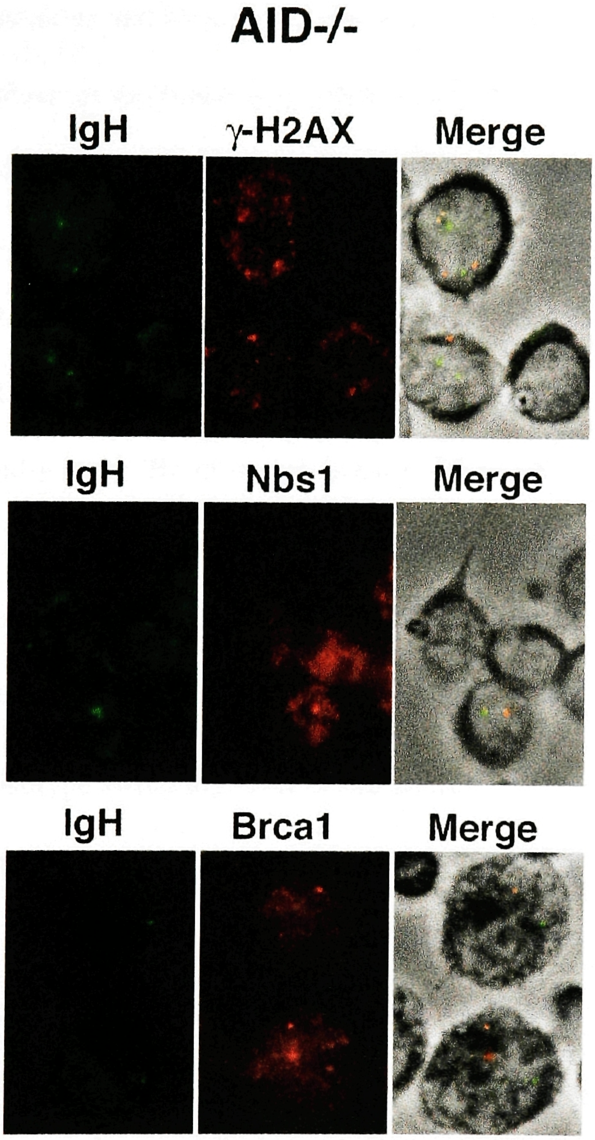
(B) Iγ1-Cγ1 transcripts, mature switch VDJ-Cγ1 transcripts and Sμ-Sγ1 DNA rearrangements in $H2AX^{-/-}$ and $H2AX^{+/+}$ B cells assayed at 0 and 72 hours after LPS+IL4 culture. DNA and RNA samples taken at 72 hours were diluted as indicated.


A
H2AX+/+
H2AX-/-
IgG1
15
2
B220
Cell counts
24 | 41 | 22
24 | 47 | 20
FL2-A
B
H2AX+/+
H2AX-/-
0hs
72hs
0hs
72hs
Iγ1-Cγ1
VDJ-Cγ1
Sμ-Sγ1
Actin

AID is genetically upstream of DNA DSBs during switching

To determine whether AID is required for switch region associated DNA repair focus formation, we assayed for co-localization of IgH with Nbs1, γ-H2AX, or Brca1 protein in stimulated B cells from AID$^{-/-}$ mice. In contrast to wild-type B cells, in which a significant fraction of Nbs1/γ-H2AX foci were detected at the IgH locus, only 4-7% of the cells containing IgH signals and Nbs1 or γ-H2AX foci showed co-localization (Fig. 3-11). In AID mutant cells, as in wild-type cells, all DNA probes failed to significantly co-localize with Brca1 (compare Fig. 3-9 and 3-11). AID$^{-/-}$ B cells stimulated with LPS+IL4 were indistinguishable from wild-type in terms of the percentage of cells with foci (Nbs1,γ-H2AX, Brca1, or Rad51), the average number of foci per cell and co-localization of these proteins with each other (Fig. 3-11). We conclude that AID is required specifically for CSR associated Nbs1/γ-H2AX focus formation but not for the recruitment of DNA repair factors to DSBs.

Figure 3-11 AID is required to locolize Nbs1 and γ-H2AX to switch DNA ds breaks AID$^{-/-}$ B cells were stimulated for 72 hours with LPS+IL4 and then the intracellular colocalization of Brca1, Rad51, Nbs1 and γ-H2AX with IgH locus was determined by ICC-FISH.


AID-/-
IgH
γ-H2AX
Merge
IgH
Nbs1
Merge
IgH
Brca1
Merge

Discussion

End-joining repair and switch recombination

At least two distinct biochemical pathways mediate DNA DSB repair in *Sacharomyces cerevisiae* and higher eukaryotes (Cromie et al., 2001). The gene products of the *RAD52* epistasis group function in DNA repair by homologous recombination, a process in which damaged chromosomes restore genetic integrity by physically pairing to a sister chromatid or homologue. Ku80 and Ku70 are part of a separate group of proteins that are essential for DSB repair by a mechanism that does not require DNA homology (Pastink et al., 2001). Consequently, Ku deficient yeast strains are unable to properly repair DSBs by end-joining (Boulton and Jackson, 1996; Milne et al., 1996). In mammalian cells, *Ku80* is similarly dispensable for repair by homologous recombination, but required for rejoining of endonuclease-induced DNA breaks (Liang et al., 1996), and V(D)J recombination(Rathmell and Chu, 1994; Taccioli et al., 1994).

Although isotype switching is known to proceed through looping out and deletion of DNA, the molecular details of the reaction remain to be determined. The similarity between different switch regions has led to the suggestion that short DNA stretches of identity could align and participate actively in the recombination process (reviewed in (Stavnezer, 1996)). However, if switch recombination were to proceed through a homology based DNA repair mechanism it would not be expected to be disrupted in Ku deficient mice. Indeed, the finding that Ku is required for switching suggests that switch recombination involves a non-homologous DNA DSB mechanism.

The requirement for Ku in switching and the detection of blunt 5' phosphorylated switch DNA ends suggest a model for switching that resembles V(D)J recombination. In this model switch donor and acceptor DNA tandem repeats are first cleaved by a switch specific endonuclease to produce DNA double-stranded breaks. The DNA ends would then be held together by Ku and possibly other proteins in a synaptic complex analogous to the one described for signal ends during the V(D)J recombination process (Agrawal and Schatz, 1997). An interesting prediction of this model is that switch region tandem repeats would not function in the DNA recombination process *per se*, but could act as recognition sites for a switch specific endonuclease, much as RSSs are targeted by RAG1 and RAG2 in V(D)J recombination. Alternatively, transcription of these tandem repeats could create DNA structures that would be recognized by a switch specific endonuclease.

The role of AID in switch recombination

It has been proposed that AID initiates somatic hypermutation and CSR by activating an endonuclease that produces lesions in either V genes or switch regions (Kinoshita and Honjo, 2001). In this model CSR breaks would be generated via nicking at staggered positions on both strands. We propose that such lesions become associated with Nbs1/γ-H2AX foci in the G1 phase of the cell cycle and that repair proceeds by NHEJ. In addition, we find that Nbs1/γ-H2AX foci are absent in AID$^{-/-}$ mice, suggesting that AID is either directly involved in producing the switch lesion, or activating the switch endonuclease, thereby placing AID upstream of H2AX phosphorylation, Nbs1 recruitment to sites of CSR, and DNA repair.

Chapter 4: Contribution of Receptor Editing to the Antibody Repertoire

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Introduction

The clonal selection theory anticipated that a random collection of immunoglobulins would include self-reactive specificities that require silencing (Burnet, 1959). The mechanisms by which such autoreactive B cells are tolerized were subsequently uncovered using mice transgenic for self-reactive immunoglobulins (Gay et al., 1993; Goodnow et al., 1989; Nemazee and Burki, 1989; Okamoto et al., 1992; Tiegs et al., 1993). In these mice, autoreactive B cells confronted with self-antigens were either eliminated, anergized, or altered by continued gene recombination, a process known as receptor editing. For example, nearly all B cells from mice carrying recombined anti-double stranded (ds) DNA or anti-MHC antibodies have their autoreactive specificities replaced by receptor editing (Gay et al., 1993) (Tiegs et al., 1993). There is also indirect evidence from Southern blotting and DNA sequence analysis that editing also occurs in non-transgenic B cells (Coleclough et al., 1981; Retter and Nemazee, 1998; Yamagami et al., 1999a; Yamagami et al., 1999b). However, the role of editing in shaping the antibody repertoire under physiological conditions is unknown.

To determine the extent of receptor editing we generated an Ig kappa polymorphism which facilitates detection of light chain gene replacements *in vivo*. We find that B cells are targeted for editing during a 2-hour delay in development at the pre-BII cell stage, and that approximately 25% of all antibody molecules are produced by gene replacement. These results suggest that receptor editing represents a major force in shaping the antibody repertoire.

Results and Discussion

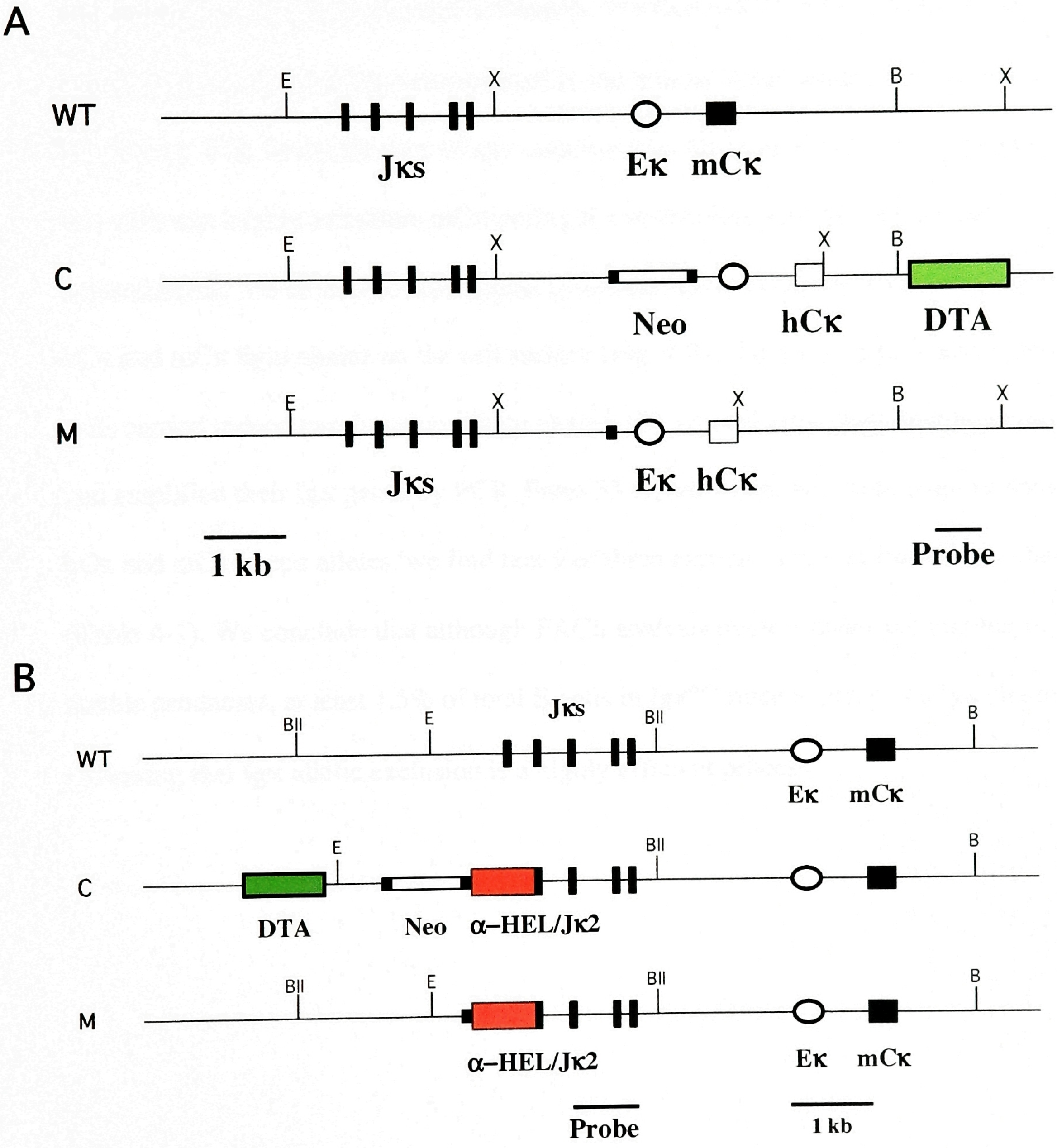
Generation of hCκ and αHEL knockin mice

To measure the extent to which editing occurs in developing B cells *in vivo*, we generated an allelic polymorphism of the mouse kappa constant region (mCκ) by replacing it with its human counterpart (hCκ) (Igκ$^{m/h}$ mice, Fig. 4-1A). These animals were subsequently crossed with mice carrying light chains pre-recombined at their physiological site (Igκ$^{3\text{-}83}$(Pelanda et al., 1996), Igκ$^{V\kappa4}$, Igκ$^{V\kappa8}$(Prak and Weigert, 1995) and Igκ$^{\alpha HEL}$ mice; Fig.4-1B).

Figure 4-1

A) The hCκ targeting vector was created from a 7 kb EcoRV-BamHI genomic piece spanning the Jκ segments and mCκ region. An XbaI restriction site was introduced at the 3' end of the hCκ gene, which was then joined by PCR to the 7 kb vector by PCR. A neomycin resistance gene (without polyA) flanked by *loxP* sites was then ligated into the XhoI site. To increase the likelihood of selecting for the right integrated clones, the polymerase II promoter driven diphtheria toxin gene (DTA, Lexicon Genetics) was subcloned 3’ of the targeting construct. hCκ-targeted ES cell clones could be distinguished from WT by the presence of a new 3 kb band after XbaI digestion. Mice carrying this mutant allele were crossed to cre recombinase transgenic mice (Lakso et al., 1996) to delete the neomycin resistance gene. E, EcoRV; B, BamHI; X, XbaI. WT, wild type genomic; C, construct; M, mutant allele.

B) The αHEL targeting vector was created from the same 7 kb EcoRV-BamHI genomic DNA fragment used to generate the hCκ construct. The neoR polyA- gene flanked by *LoxP* sites was ligated into the SphI-AvrII restriction sites. The Vκ22-33 promoter was then joined by PCR to the HyHEL-10-Jκ2 gene (Goodnow et al., 1988) This DNA was ligated 3’ of the neoR gene as PmeI-AvrII. The DTA gene was then subcloned 5’ of the targeting construct. Resistant colonies were screened by Southern blot by hybridizing BglII restricted genomic DNA with a probe amplified from the Jκ3-Jκ5 genomic region. αHEL-positive ES cell clones showed the presence of a new 5.6 kb band. The neoR gene was deleted by crossing αHEL mice with cre recombinase transgenic animals. BII, BglII; E, EcoRV; B, BamHI; WT, wild type genomic; C, construct; M, mutant allele.


A
E
X
B
X
WT
Jκs
Eκ
mCκ
E
X
X
B
C
Neo
hCκ
DTA
E
X
X
B
X
M
Jκs
Eκ
hCκ
1 kb
Probe
B
BII
E
Jκs
BII
B
WT
Eκ
mCκ
E
BII
B
C
DTA
Neo
α–HEL/Jκ2
BII
E
BII
B
M
α–HEL/Jκ2
Eκ
mCκ
Probe
1 kb

Efficiency of light chain allelic exclusion

To investigate whether the hCk gene could functionally replace its mouse homologue *in vivo*, we stained bone marrow and spleenic Igκ$^{m/h}$ B cells with anti-human and anti-mouse kappa monoclonal antibodies. We find that 43-44% of IgM^{+} B cells express light chains that have recombined in the human allele, while 44% express mouse kappa (Fig. 4-2). In the absence of any selective bias for or against hCκ, we conclude that this gene can largely substitute mCκ during B cell development. We notice that approximately 5% of total B cells appear to escape allelic exclusion by expressing both hCκ and mCκ light chains on the cell surface (Fig. 4-2). To investigate whether these cells carried indeed two functional light chains, we isolated individual double producers and amplified their Igκ genes by PCR. From 33 clones where we could amplify both the hCκ and mCκ kappa alleles, we find that 9 of them expressed two in frame light chains (Table 4-1). We conclude that although FACS analysis overestimates the number of double producers, at least 1.5% of total B cells in Igκ$^{m/h}$ mice express two Igκ chains, indicating that Igκ allelic exclusion is a highly efficient process.

Figure 4-2 Representation of the mouse Igκ locus: joining segments (Jκs, filled bars), kappa constant region (mCκ, filled rectangle), and targeted hCκ allele (open rectangle). Bone marrow immature (B220^{low}IgM^{high}) and spleen mature (B220^{high}IgM^{+}) Igκ$^{m/h}$ B cells were stained with monoclonal antibodies against the mCκ and hCκ proteins. Numbers represent percentage of total B220^{+}IgM^{+} cells.

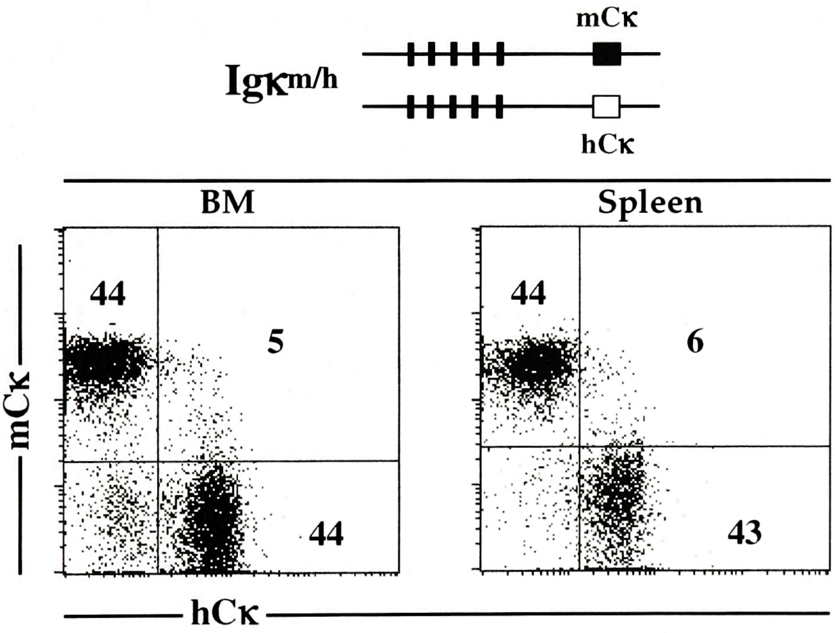

mCκ
Igκm/h
hCκ
BM
Spleen
mCκ
44
5
44
44
6
43
hCκ

Table 4-1 PCR Analysis of Vκ-Jκ rearrangements in single cells purified from Igκ$^{m/h}$ mouse spleens. Out of 179 samples analyzed, 80 cells showed PCR amplification of at least one Vκ-Jκ rearrangement and 33 showed two alleles. Of the samples where we could amplify both alleles, 17 showed a Vκ-Jκ germline configuration, indicating that they were false double producers. We found that 7 of the remaining 16 contained a productive rearrangement on one allele and a non-productive one on the other, while the remaining 9 (27% of the 33) showed two productive rearrangements.

Number of cells with two amplified kappa alleles*	Genotype	
	Vκ-Jκ/Germline	Vκ-Jκ/Vκ-Jκ
33	17	16
	Productive/Productive	9
	Productive/Non-Productive	7

* From 179 cells isolated from 3 mice in 3 independent experiments

Receptor editing in autoimmune mouse models

To determine whether the hCκ marker could be used to detect receptor editing *in vivo* we used mouse models in which the extent of editing is well defined (Chen et al., 1995a; Pelanda R et al., 1997; Sonoda et al., 1997). The hCκ allele was introduced into mice carrying antibodies specific for either single stranded (ss) ($\mathrm{IgH}^{3H9/+}\mathrm{Ig}\kappa^{V\kappa 8/h}$) or double stranded (ds) DNA ($\mathrm{IgH}^{3H9/+}\mathrm{Ig}\kappa^{V\kappa 4/h}$) (Chen et al., 1995a). Both antibodies arise spontaneously in autoimmune MRL/*lpr* mice and are reminiscent of those found in human lupus patients (Shlomchik et al., 1987). Their specificities, however, are modulated by the different light chains so that B cells carrying the anti-ds DNA receptors undergo extensive clonal deletion and light chain editing, while those carrying the ss-DNA form develop in the absence of editing and become anergic (Chen et al., 1997; Erikson et al., 1991). In agreement with previous work, we found little editing in B cells carrying the anti-ss DNA antibody, as almost all B lymphocytes in $\mathrm{IgH}^{3H9/+}\mathrm{Ig}\kappa^{V\kappa 8/h}$ mice expressed the pre-recombined mCκ allele (Fig. 4-3). In contrast, nearly all of the B cells in mice that carry the anti-ds DNA antibody do undergo receptor editing. 49% of the B cells in $\mathrm{IgH}^{3H9/+}\mathrm{Ig}\kappa^{V\kappa 4/h}$ mice expressed hCκ and the original Vκ4 allele was rarely found by mRNA analysis in the $\mathrm{mC}\kappa^{+}$ cells (Table 4-2). The remaining 10-12% of the B cells in $\mathrm{IgH}^{3H9/+}\mathrm{Ig}\kappa^{V\kappa 4/h}$ mice expressed Igλ. The choice between anergy (anti-ss DNA) and editing (anti-ds DNA) has been shown to be contingent upon enhanced DNA binding(Chen et al., 1997). Arginine substitutions in 3H9, which arise as somatic mutations in SLE patients and lupus-prone animals, create a positive potential in the heavy chain CDRs, thus increasing the overall affinity of the antibody for DNA (Li, 2001). In agreement with such studies, arginine substitutions at CDR2 and FR3 of 3H9 induce the anergic $\mathrm{IgH}^{3H9/+}\mathrm{Ig}\kappa^{V\kappa 8/h}$ B cells to recombine light chains at the hCκ allele (Fig. 4-3). We conclude that expression of hCκ correlates with previous measurements of receptor editing and that anergic B cells can be induced to undergo editing by increasing their antigen affinity.

Figure 4-3 Analysis of splenocytes from $\mathrm{IgH}^{3H9/+}\mathrm{Ig}\kappa^{V\kappa 8/h}$, $\mathrm{IgH}^{3H9/+}\mathrm{Ig}\kappa^{V\kappa 4/h}$, and $\mathrm{IgH}^{3H9\text{-}56/76R/+}\mathrm{Ig}\kappa^{V\kappa 8/h}$ mice. The configuration of the heavy (IgH) and kappa chain (Igκ) loci is indicated at the top of each column. Antibodies used for staining are indicated. Black stripes inside 3H9 depict arginine substitutions. Dot plots in the second row show cells pre-gated on B220. Antibodies were: anti-B220 APC; anti-IgM biotin revealed with SA-RED613; rat anti-human kappa FITC; rat anti-mouse kappa PE, anti-Igλ PE. Numbers indicate percentage of B220^{+} cells.

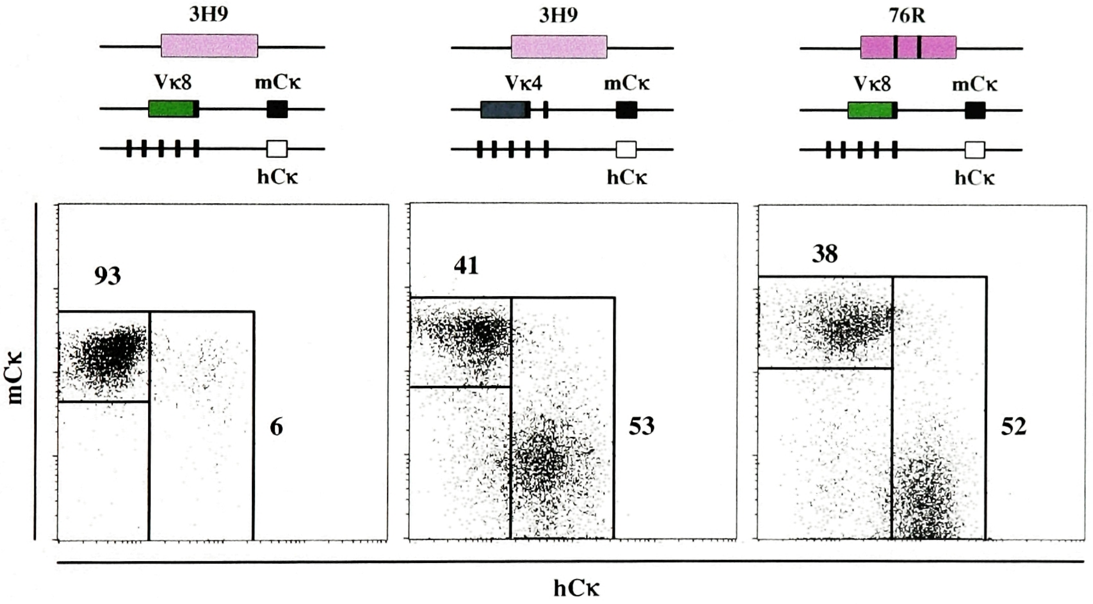

3H9
Vκ8
mCκ
hCκ
3H9
Vκ4
mCκ
hCκ
76R
Vκ8
mCκ
hCκ
93
6
41
53
38
52
mCκ
hCκ

Table 4-2 Receptor editing on targeted alleles. mCκ$^+$ cells from IgH$^{3H9/+}$Igκ$^{V\kappa 4/h}$, Igκ$^{\alpha HEL/h}$, and Igκ$^{V\kappa 4/h}$ mice were sorted and their Vκ-Jκ-mCκ genes isolated and sequenced.

Mouse	Number of samples	mCκ/Vκ transgene+	mCκ/Vκ transgene-
$\mathrm{IgH}^{3H9/+}\mathrm{Ig}\kappa^{V\kappa 4/h}$	42	1	41
$\mathrm{Ig}\kappa^{\alpha HEL/h}$	37	34	3
$\mathrm{Ig}\kappa^{V\kappa 4/h}$	36	34	2

Table I. Receptor editing on targeting alleles.
$\mathrm{mC}\kappa^{+}$ cells from $\mathrm{IgH}^{3H9/+}\mathrm{Ig}\kappa^{V\kappa 4/h}$, $\mathrm{Ig}\kappa^{\alpha HEL/h}$, and $\mathrm{Ig}\kappa^{V\kappa 4/h}$ mice were sorted and their Vκ-Jκ (mCκ) genes isolated and sequenced.

Receptor specificity either induces or bypasses light chain editing

To confirm that receptor editing in $\mathrm{Ig}\kappa^{m/h}$ mice is induced by changes in the antibody combining site, we engineered mice carrying the wild type B1-8 heavy chain and two variants that arise by somatic hypermutation during the anti-4-hydroxy-3-nitrophenylacetyl (NP) response (Allen et al., 1988; Sonoda et al., 1997). Replacement of tryptophan 33 by leucine in CDR1 of $\mathrm{IgH}^{B1\text{-}8}$ increases the affinity for NP 10 fold ($\mathrm{IgH}^{B1\text{-}8high}$), whereas the four amino acid changes found in hybridoma 3C52 decrease NP binding 4 fold ($\mathrm{IgH}^{B1\text{-}8low}$) (Allen et al., 1988). It has previously been shown that, in the absence of NP antigen, the B1-8 heavy chain in combination with the 3-83 light chain creates an apparently innocuous antibody which does not induce receptor editing (Pelanda et al., 1996). In agreement with such results, 99% of $\mathrm{IgH}^{B1\text{-}8/+}\mathrm{Ig}\kappa^{3\text{-}83/h}$ B cells carry 3-83-Jκ2-mCκ light chains and do not undergo receptor editing to downstream Jκs or at the hCκ allele (Fig.4-4 column 1). Similar results are obtained in $\mathrm{IgH}^{B1\text{-}8high/+}\mathrm{Ig}\kappa^{3\text{-}83/h}$ mice or by combining the wild type B1-8 with the αHEL light chain, which also creates an antibody with unknown specificity (Fig.4-4 columns 2 and 3). Contrastingly, both B1-8 variants in combination with the αHEL light chain convert this apparently innocuous antibody that is not edited ($\mathrm{IgH}^{B1\text{-}8high/+}\mathrm{Ig}\kappa^{\alpha HEL/h}$) into a receptor that induces a great deal of editing (Fig.4-4 columns 4 and 5). Approximately 38% of B cells in $\mathrm{IgH}^{B1\text{-}8high/+}\mathrm{Ig}\kappa^{\alpha HEL/h}$ mice and 64% in $\mathrm{IgH}^{B1\text{-}8low/+}\mathrm{Ig}\kappa^{\alpha HEL/h}$ mice expressed hCκ on their cell surface.

In addition to self-reactivity, it has been suggested that low levels of antibody expression, and possibly poor pairing of heavy and light chains may also induce receptor editing (Braun et al., 2000; Kouskoff et al., 2000). Since the structure of all three IgH^{B1-8} targeted genes is identical, different IgH expression levels are not likely to be responsible for the difference in editing seen between B1-8 and its two variants. In addition, the B1-8 mutations do not interfere with the assembly of the heavy chains with the αHEL light chain. This we determined by transiently expressing the heavy and light chains in A293 cells (Fig. 4-5). Thus it appears that the B1-8^{high} and B1-8^{low} heavy chains produce self-reactive antibodies when combined with αHEL. We conclude that Igκ$^{m/h}$ mice can be used to detect gene replacements triggered by changes in the antigen combining site.

Increased Igλ usage in the mouse B cell repertoire has often been used as a marker for receptor editing (Hertz and Nemazee, 1997; Retter and Nemazee, 1998; Tiegs et al., 1993). However, increased Igλ expression was only seen in IgH$^{B1-8high}$ mice (Fig. 4-4). The low level of surface Igλ expression in IgH$^{B1-8low}$ B cells is not due to inability of this heavy chain to pair with lambda light chains since IgH$^{B1-8low}$ Igλ is a naturally occurring antibody combination. Thus, surface Igλ expression does not always correlate with receptor editing.

Figure 4-4 Induction of receptor editing *in vivo* by changes in the antibody antigen-combining site.

Flow cytometry analysis of bone marrow cells (first row) and splenocytes (second and third row) from IgH$^{B1\text{-}8/+}$Ig$\kappa^{3\text{-}83/h}$, IgH$^{B1\text{-}8high/+}$Ig$\kappa^{3\text{-}83/h}$, IgH$^{B1\text{-}8/+}$Ig$\kappa^{\alpha HEL/h}$, IgH$^{B1\text{-}8high/+}$Ig$\kappa^{\alpha HEL/h}$, and IgH$^{B1\text{-}8low/+}$Ig$\kappa^{\alpha HEL/h}$ animals. The configuration of the Ig loci is indicated schematically on top of each column. Antibodies used for staining are indicated on the appropriate axis. Black stripes inside B1-8^{high} and B1-8^{low} genes depict single amino acid mutations. Dot plots in the first and third row, show cells that were pre-gated as B220^{+}. Numbers indicate percentage of B220^{+} cells.

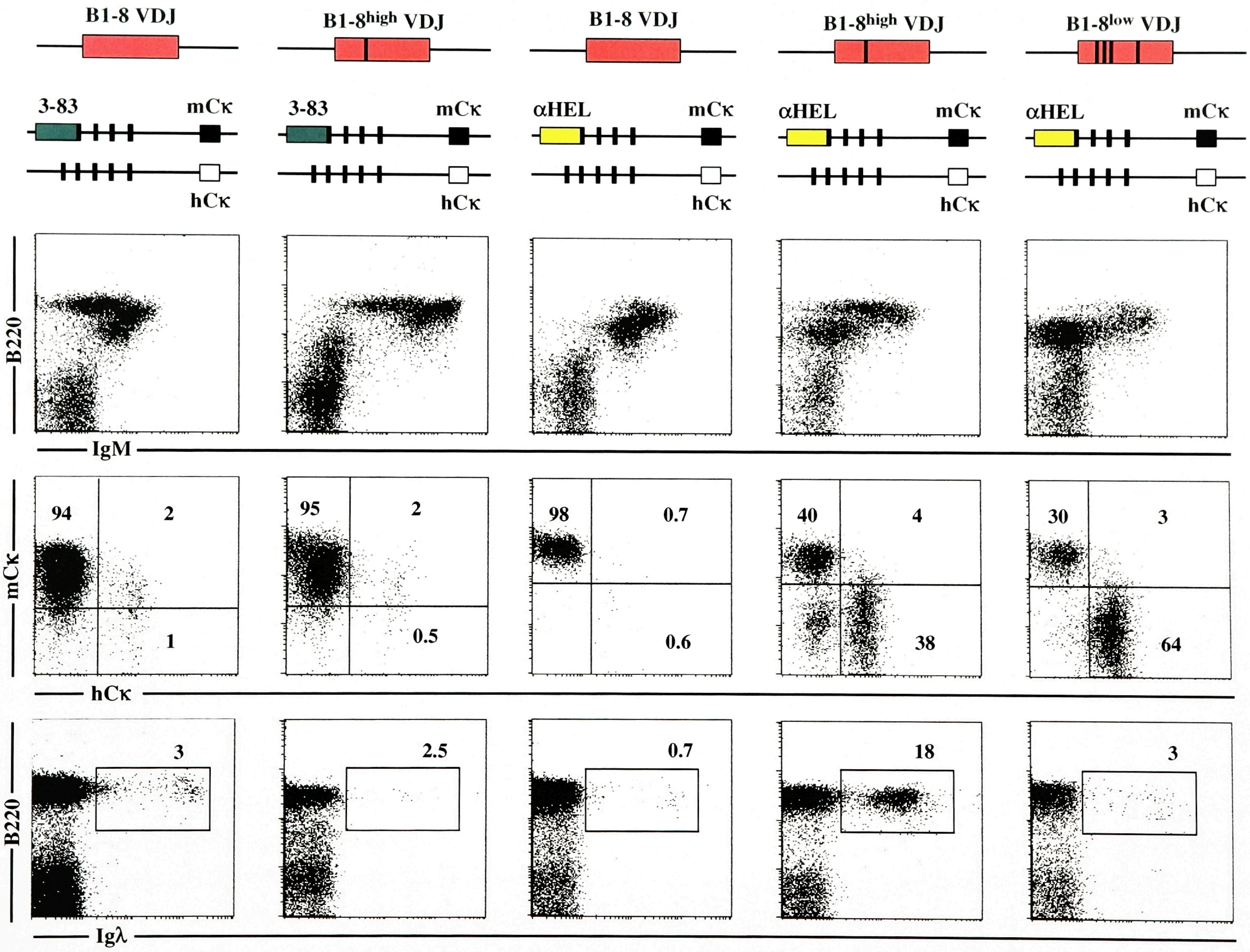
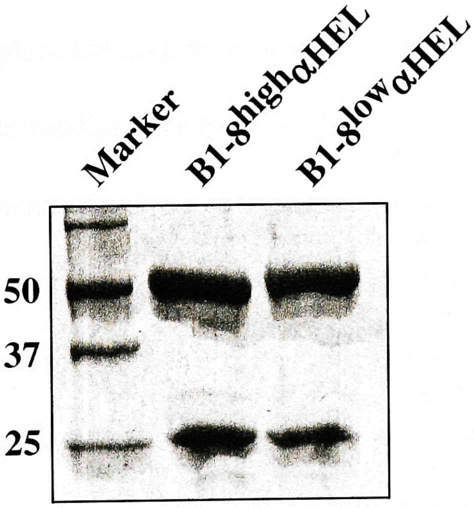

B1-8 VDJ
B1-8high VDJ
B1-8 VDJ
B1-8high VDJ
B1-8low VDJ
3-83
mCκ
hCκ
αHEL
B220
IgM
mCκ
hCκ
B220
Igλ
94
2
1
95
2
0.5
98
0.7
0.6
40
4
38
30
3
64
3
2.5
0.7
18
3

Figure 4-5 Assembly of B1-8^{high} and B1-8^{low} heavy chains with VκαHEL light chains. B1-8^{high}/αHEL, and B1-8^{low}/αHEL antibodies were transiently expressed in A293 cells in the pRK expression vector. Supernatants were then collected and antibodies purified by protein G affinity column chromatography and analyzed by SDS-PAGE. Numbers represent molecular weights (kDa) of protein standards.


Marker
B1-8highαHEL
B1-8lowαHEL
50
37
25

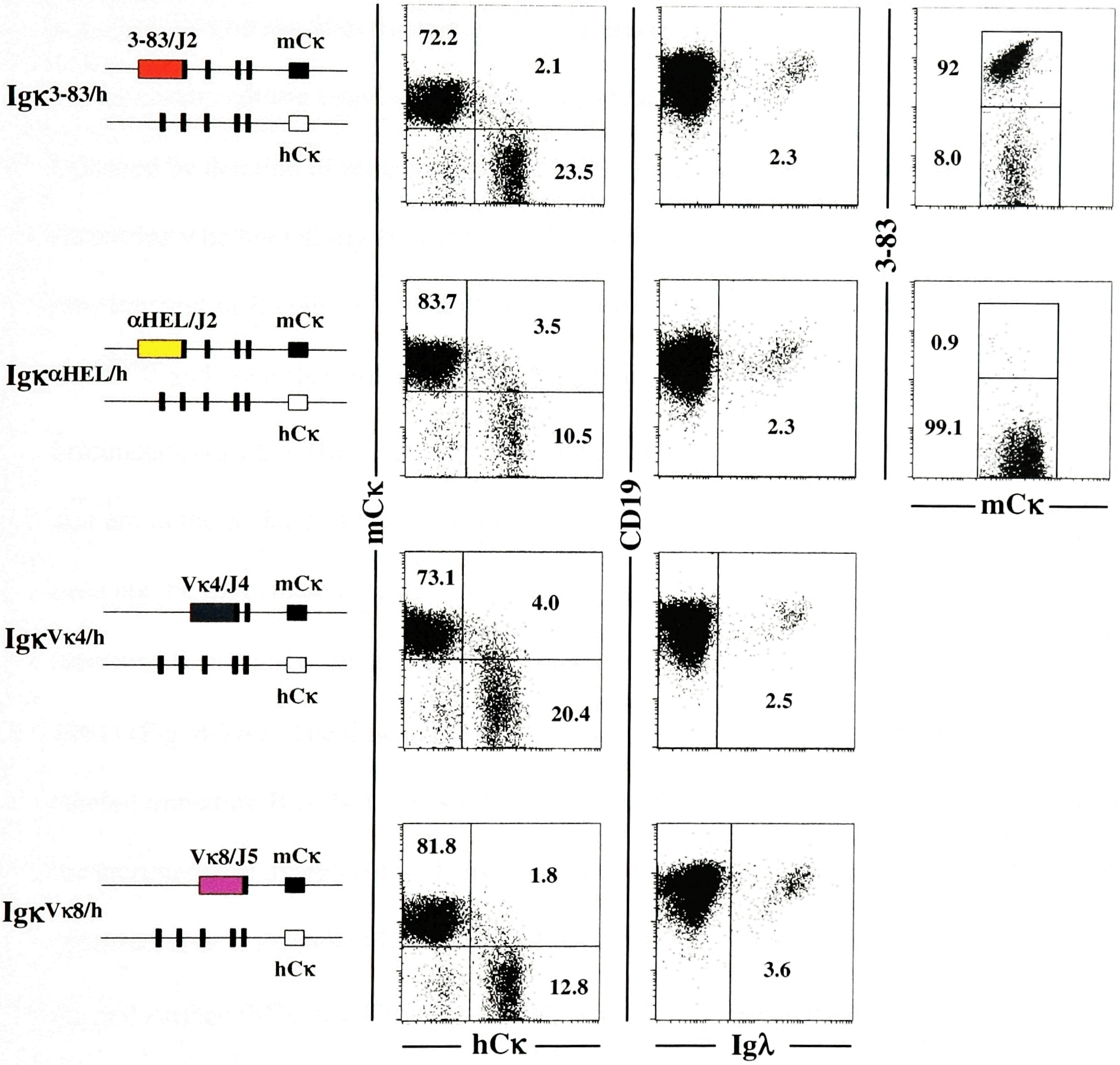
Extent of Receptor Editing in Igκ$^{\mathrm{V}\kappa\mathrm{x/h}}$ mice

To determine the extent of receptor editing for a single light chain combined with any random heavy chain, we introduced the hCκ allele into mice expressing the pre-recombined 3-83 light chain (Pelanda R et al., 1997). In these mice, Ig heavy chain gene recombination is not constrained. Therefore, 3-83 light chains, expressed at the pre-BII cell stage, are paired with the full spectrum of mouse heavy chains, an unknown number of which would be expected to induce receptor editing. To detect B cells that undergo receptor editing and replace the original mouse 3-83 allele, we used anti-Vκ3-83 and anti-hCκ monoclonal antibodies. We find that 3-83 light chains are frequently replaced during B cell development. Analysis of bone marrow Igκ$^{3\text{-}83/\mathrm{h}}$ B cells shows that 25% (±2) of B lymphocytes in these mice express hCκ either alone or in combination with mCκ, and 3% (±1) express λ chains (Fig. 4-6 row 1). The extent of editing on the mCκ locus was determined by loss of 3-83 staining. We found that 8% of the mCκ^{+} lymphocytes were products of receptor editing. Therefore, approximately 35% of newly formed B cells in Igκ$^{3\text{-}83/\mathrm{h}}$ mice replace the targeted light chain.

To estimate the extent of receptor replacement occurring in other light chain knock-in mouse models, we combined the hCκ allele with three additional pre-rearranged VκJκ genes: VκαHEL, Vκ4R, and Vκ8R (Prak and Weigert, 1995). B cells that undergo receptor editing and replace the original mouse allele were enumerated by flow cytometry and mRNA analysis from purified mCκ^{+} cells. We found that approximately 17% of the B cells in Igκ$^{\alpha\mathrm{HEL/h}}$ and 33% of the B cells in Igκ$^{\mathrm{V}\kappa4/\mathrm{h}}$ mice substituted their light chains during B cell development (Fig. 4-6 row 2 and 3). Igκ$^{\mathrm{V}\kappa8/\mathrm{h}}$ mice can only delete Vκ8R by RS recombination, nevertheless 18% (±1) of B cells in these mice replaced the targeted gene and expressed hCκ or Igλ on the cell surface (Fig. 4-6 row 4). Despite differences in the level of Ig kappa chain editing the amount of Igλ expression was similar in all strains (Fig. 4-6 and Table 4-2).

Altogether, our data from four separate Igκ knock-in mouse strains shows that approximately 25% (± 7) of the light chains found on the surface of developing B cells *in vivo* originate from receptor editing. Whether all of these replacements are induced by self-reactivity is presently unknown. Nevertheless, extrapolating from these experiments, we conclude that receptor editing makes an important contribution to the normal antibody repertoire.

Figure 4-6 Extent of receptor editing in Igκ targeted mice. Diagrams show the Igκ locus for each mouse strain. Dot plots show mCκ, hCκ, Igλ and Igκ3-83 expression in immature bone marrow B cells gated on B220^{low} IgM^{+} cells. Total editing for Igκ$^{\alpha HEL/h}$ was 22% (± 3), n = 8 mice; Igκ$^{3\text{-}83/h}$ mice was 24% (± 3), n=6 mice ; Igκ$^{V\kappa 4/h}$ 33% (± 3), n=8 mice; Igκ$^{V\kappa 8/h}$ 18% (± 1) n=6 mice.


3-83/J2
mCκ
Igκ3-83/h
hCκ
αHEL/J2
mCκ
IgκαHEL/h
hCκ
Vκ4/J4
mCκ
IgκVκ4/h
hCκ
Vκ8/J5
mCκ
IgκVκ8/h
hCκ
mCκ
72.2
2.1
23.5
83.7
3.5
10.5
73.1
4.0
20.4
81.8
1.8
12.8
hCκ
CD19
2.3
2.3
2.5
3.6
Igλ
3-83
92
8.0
0.9
99.1
mCκ

Editing occurs during a 2-hour small preBII cell arrest

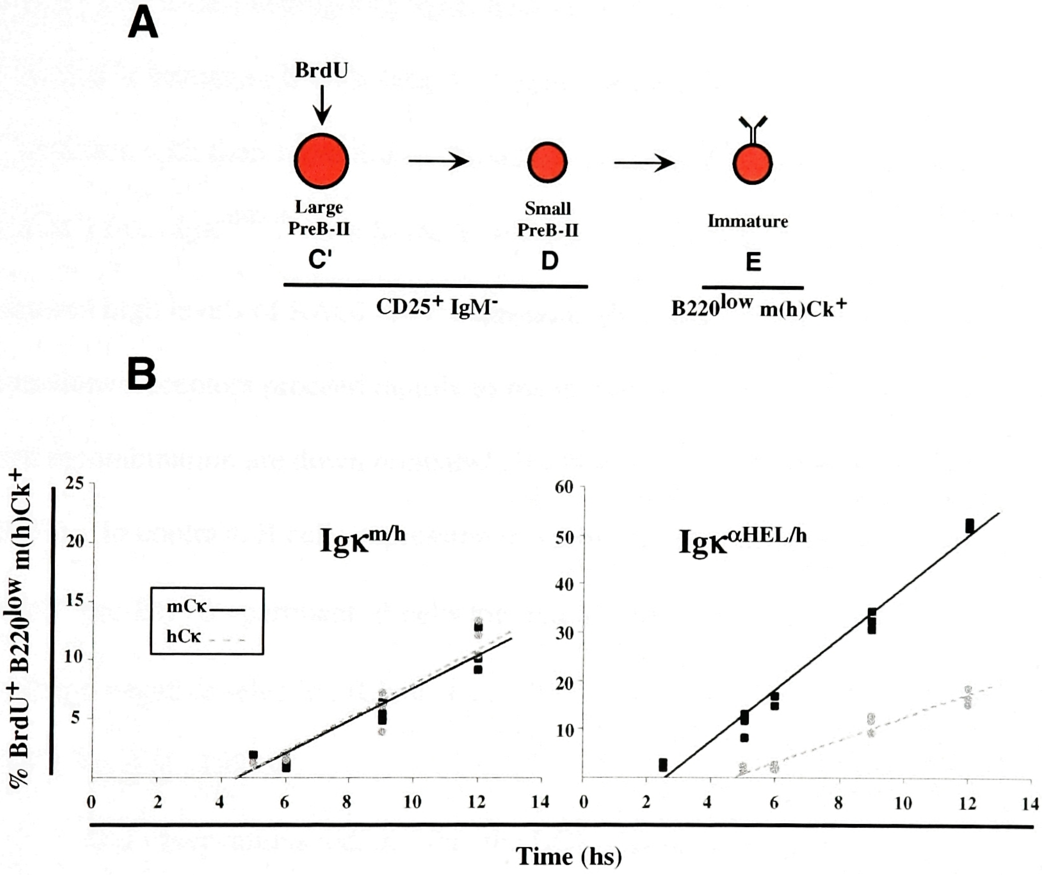
Two mechanisms have been proposed to explain receptor editing in transgenic models (Chen et al., 1997; Gay et al., 1993; Pelanda R et al., 1997; Tiegs et al., 1993). Editing might be specifically induced by self-reactive or otherwise abnormal receptors. Alternatively, editing could reflect random premature Vκ-Jκ recombination in pro-B cells followed by deletion of remaining autoreactive B cells (Novobrantseva et al., 1999). To determine whether editing is random or specifically induced, we compared the kinetics of development of B cells that do or do not undergo light chain gene replacements *in vivo.* Igκ$^{\alpha\mathrm{HEL/h}}$ and controls were injected with a single dose of the thymidine analogue bromodeoxy-uridine (BrdU), which is incorporated into the DNA of large pre-BII cells that are in the S phase of the cell cycle (Opstelten and Osmond, 1983). Large pre-BII cells are the immediate precursors of small pre-BII cells, non-cycling cells that actively rearrange their light chain genes and become immature B cells (IgM^{+}) (Hardy et al., 1991) (Fig. 4-7A). The time elapsed between BrdU injections and the first appearance of labeled immature B cells corresponds to the minimum time spent in the small pre-BII cell compartment. In agreement with previous data, BrdU labeled immature B cells first appeared after 4.5 hours in Igκ$^{\mathrm{m/h}}$ control mice regardless of the Igκ allele expressed on the cell surface (hCκ or mCκ) (Fig. 4-7B). Therefore, it normally takes a minimum of 4.5 hours for cells to go from a germline Igκ locus in large pre-BII cells to cell surface expression of a functional Igκ in immature B cells. In contrast, immature B cells expressing the pre-recombined αHEL light chain (mCκ^{+}) emerged 2.6 hours after BrdU injection in Igκ$^{\alpha\mathrm{HEL/h}}$ mice (Fig. 4-7B). The time difference between the pre-recombined VκαHEL B cells and their wild type counterparts is consistent with the completion of G2 and mitosis phases of the cell cycle by large pre-BII cells and suggests that B cells with innocuous receptors spend little or no time in the small pre-BII stage.

In contrast to unedited VκαHEL expressing B cells (mCκ$^+$), all of the edited B cells from the same mice (hCκ$^+$) are developmentally delayed, and the first edited cells appeared in the immature compartment after 4.5 hours (Fig. 4-7B, similar results were obtained with Igκ$^{3\text{-}83/h}$ and Igκ$^{V\kappa 8/h}$ animals). This difference in kinetics indicates that B cells undergoing editing are specifically delayed in the small pre-BII cell compartment for at least 2 hours. Thus, the rate of transit through the small pre-BII cell compartment correlates with receptor editing. We conclude that editing is not the result of random premature recombination in pro-B cells followed by selection at later stages. Instead, editing is induced in specific B cells during a 2-hour developmental delay at a stage when they are normally recombining their light chain genes. The observation that cells carrying "perfect" receptors (for instance IgH$^{B1\text{-}8/+}$Igκ$^{\alpha HEL/h}$ Fig. 4-5) do not undergo editing further reinforces this conclusion.

Figure 4-7 Kinetics of receptor editing *in vivo.*

A) Strategy for labeling developing B lymphocytes. BrdU was injected intraperitoneally into $\mathrm{Ig\kappa}^{m/h}$ and $\mathrm{Ig\kappa}^{\alpha HEL/h}$ mice. BrdU is uptaken by cycling large preBII cells. These become small preBII, which actively recombine the light chain genes creating a complete BCR and becoming immature B cells.

B) linear regression analysis showing percentages of immature BrdU^{+} B cells (Y-axis) $\mathrm{mC\kappa}^{+}$ (black squares) or $\mathrm{hC\kappa}^{+}$ (gray circles) plotted against time (X-axis). $\mathrm{Ig\kappa}^{m/h}$ (left) and $\mathrm{Ig\kappa}^{\alpha HEL/h}$ mice (right) were injected with 0.5 mg of BrdU and sacrificed after 2.5, 5, 6, 9, and 12hs (three mice per time point).


A
BrdU
Large PreB-II
C'
Small PreB-II
D
Immature
E
CD25+ IgM-
B220low m(h)Ck+
B
% BrdU+ B220low m(h)Ck+
Igκm/h
mCκ
hCκ
IgκαHEL/h
25
20
15
10
5
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Time (hs)

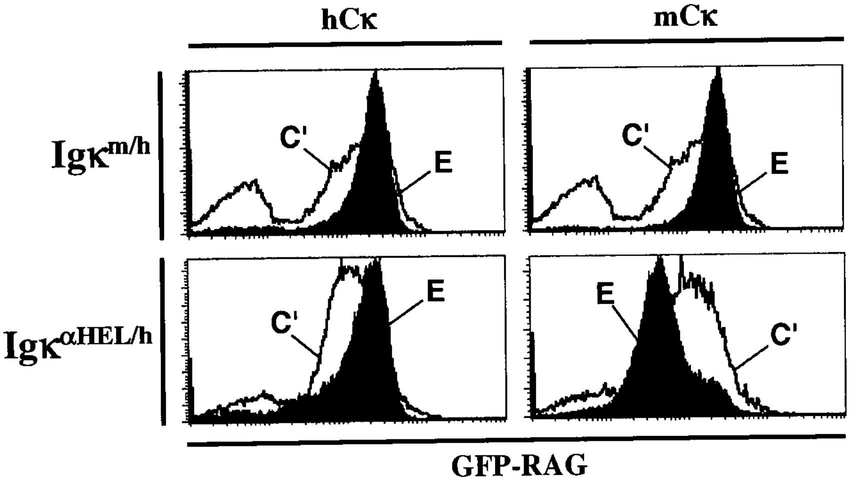
Edited cells express higher levels of RAG-GFP

To examine RAG expression in B cells undergoing editing *in vivo* we combined the Ig$\kappa^{\alpha\mathrm{HEL/h}}$ allele with a RAG2-GFP indicator transgene (Yu et al., 1999). Like RAG2, GFP expression is first induced in pro-B cells undergoing heavy chain recombination and it decreases in large cycling pre-BII cells; GFP and RAG are normally re-induced in small pre-BII cells undergoing light chain gene rearrangements, and GFP remains elevated in immature B cells (Fig. 4-8) (Grawunder et al., 1995; Yu et al., 1999). Consistent with their rapid transit through the small pre-BII stage, unedited B cells (mCκ^+) from Ig$\kappa^{\alpha\mathrm{HEL/h}}$ mice failed to re-induce RAG2 (Fig. 4-8). In contrast, edited cells showed high levels of RAG2-GFP expression (Fig. 4-8). Thus, B cells expressing functional receptors proceed rapidly to the immature B cell stage where RAG expression and recombination are down regulated (Hartley et al., 1993; Melamed and Nemazee, 1997). In contrast, B cells expressing receptors targeted for editing are arrested in the RAG$^+$ pre-BII compartment. B cells that fail to edit in this compartment are likely to undergo negative selection (Chen et al., 1995b; Grawunder et al., 1995; Pelanda R et al., 1997; Yu et al., 1999).

Our observations indicate that the BCR regulates receptor editing by controlling the rate of B cell development. B cells with self-reactive antibodies and those cells that have not yet expressed a receptor are delayed in the RAG$^+$ small pre-BII cell compartment, where normal light chain rearrangements takes place. This is in distinction with current models that favor a return of self-reactive immature B cells to the preBII stage (Melamed and Nemazee, 1997). B cells expressing innocuous receptors transit rapidly from this stage to the immature compartment where RAG gene expression and

V(D)J recombination are down regulated (Hartley et al., 1993). This model clarifies how allelic exclusion is maintained in B cells despite high levels of receptor editing: B cells that deposit non-self reactive antibodies on their cell surface rapidly turn off V(D)J recombination.

Figure 4-8 Histograms showing expression of RAG2-GFP in fraction C' (line), and E (filled) of Igκ$^{m/h}$ (upper histograms) and Igκ$^{\alpha HEL/h}$ (lower histograms) mouse B cells expressing either mCκ (left column) or hCκ (right column) light chains. Cells were stained with antibodies against B220, CD25, mCκ, and hCκ.


hCκ
mCκ
Igκ$^{m/h}$
Igκ$^{\alpha HEL/h}$
C'
E
C'
E
C'
E
E
C'
GFP-RAG

Chapter 5: OcaB Regulates Transcription, Recombination, and Editing of Vκ genes

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Introduction

An essential feature of the immune system is the ability to recognize and destroy foreign antigens while maintaining self-tolerance (Burnet, 1959; Lederberg, 1959). In the B cell compartment, tolerance is established by deletion and receptor editing during B cell development in the bone marrow (Chen et al., 1995b; Gay et al., 1993; Goodnow, 1996; Hartley et al., 1991; Nemazee and Burki, 1989; Radic et al., 1993; Tiegs et al., 1993), and by silencing auto-reactive cells that escape to the periphery (Erikson et al., 1991; Goodnow et al., 1989). Receptor editing is a major force in shaping the antibody repertoire, accounting for up to 25% of all immunoglobulins (Casellas et al., 2001). To date, however, little is known about the mechanisms that regulate receptor editing *in vivo*. Here we report that OcaB, a transcriptional co-activator also known as Bob-1 or OBF-1, is essential for normal expression, editing and allelic exclusion of Ig genes. We show that OcaB modulates editing by directly regulating Igκ gene transcription.

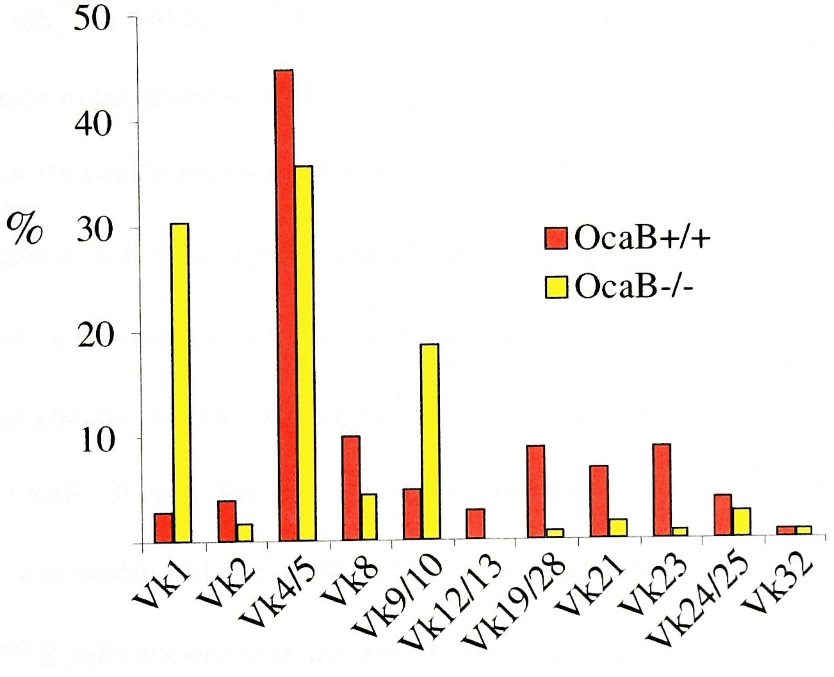
Results

Skewed Vκ repertoire in OcaB deficient animals

OcaB was isolated on the basis of its ability to enhance transcription of immunoglobulin heavy chain genes (IgH) *in vitro* (Gstaiger et al., 1995; Luo et al., 1992; Luo and Roeder, 1995; Strubin et al., 1995). Paradoxically, OcaB$^{-/-}$ mice showed no apparent deficiency in IgH transcription, only cellular immune defects including decreased numbers of immature B cells (Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996). At the immature stage of B cell development the BCR is first expressed and central tolerance is established by receptor editing and clonal deletion.

To determine whether reduced numbers of immature B cells in OcaB$^{-/-}$ mice is associated with an alteration in the antibody repertoire we isolated IgM^{-} preBII cells from OcaB$^{-/-}$ and control animals and compared their Vκ genes by cloning and sequencing. We found that the Vκ4/5 family is the most frequently used Vκ family in wild type and OcaB$^{-/-}$ mice (Fig. 5-1 and (Thiebe et al., 1999)). However, OcaB$^{-/-}$ B cells differed from controls in the high level of Vκ1, Vκ9/10 and the low level of Vk12-13, 19-28, and 21usage (Fig. 5-1). We conclude that developing B lymphocytes exhibit a skewed Vκ repertoire in the absence of OcaB.

Figure 5-1 Abnormal Vκ repertoire and receptor editing in OcaB$^{-/-}$ mice. Vκ family usage (% of total sequences) in OcaB$^{+/+}$ (red bars) and OcaB$^{-/-}$ (yellow bars) animals was determined by genomic Vκ-Jκ PCR from B220^{+}IgM^{-}CD25^{+}Igκ$^{h/h}$ preBII cells.

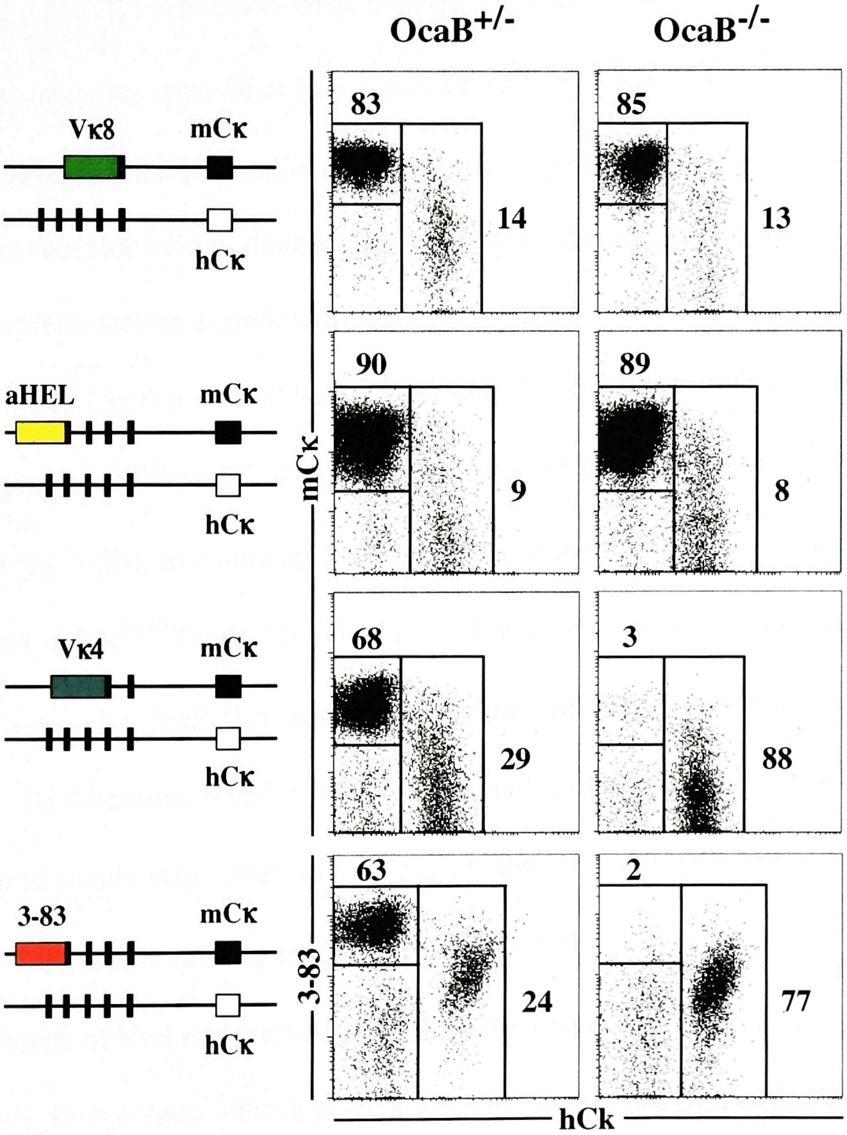

50
40
30
20
10
%
🟥 OcaB+/+
🟨 OcaB-/-
Vk1
Vk2
Vk4/5
Vk8
Vk9/10
Vk12/13
Vk19/28
Vk21
Vk23
Vk24/25
Vk32

Abnormal receptor editing of a subset of kappa genes in OcaB$^{-/-}$ mice

The Vκ repertoire is shaped to a large extent by light chain receptor editing (Casellas et al., 2001). To determine whether OcaB is required for normal editing, we measured secondary gene replacements using an indicator allele encoding the human kappa constant region (hCκ), which can be distinguished from mouse Cκ (mCκ) by flow cytometry (Casellas et al., 2001). Editing of four pre-recombined Igκ genes (Vκ8, VκαHEL, Vκ4, and Vκ3-83) representing different Vκ families was examined (Fig. 5-2).

Igκ$^{V\kappa 4/h}$, and Igκ$^{3\text{-}83/h}$ B cells showed a marked shift in hCκ indicator gene expression in the absence of OcaB (Fig. 5-2). In OcaB wild-type controls only 30% (±3, n = 5) of Vκ4-mCκ alleles were replaced by the hCκ indicator allele, whereas in OcaB$^{-/-}$ mice almost all B cells expressed hCκ^{+} light chains (Fig. 5-2, row 3). Similarly, in Igκ$^{3\text{-}83/h}$ mice, replacement of Vκ3-83, as determined by loss of staining with a monoclonal antibody specific for Vκ3-83, increased from 25% in wild type (±3, n = 6) to 80% (±2, n = 5) in OcaB$^{-/-}$ B cells (Fig. 5-2, row 4). In contrast, the extent of Vκ8 and VκαHEL editing was unaffected by the absence of OcaB. Analysis of bone marrow Igκ$^{V\kappa 8/h}$ and Igκ$^{\alpha HEL/h}$ B cells showed that, irrespective of OcaB expression, 13-14% (±2, n = 5) and 8-9% (±3, n = 3) of newly formed B cells edited their receptors (Fig. 5-2, rows 1 and 2). We conclude that OcaB regulates receptor editing for some but not all Vκ genes.

Figure 5-2 Receptor editing in Igκ targeted mice measured by mCκ (or 3-83) and hCκ expression on B220^{low}IgM^{high} bone marrow B cells from Igκ$^{V\kappa 8/h}$, Igκ$^{\alpha HEL/h}$, Igκ$^{V\kappa 4/h}$, Igκ$^{3\text{-}83/h}$ mice (diagrams on the left depict the targeted loci for each mouse strain). Left and right dot plots are representative of 7 independent experiments on OcaB$^{+/-}$ and OcaB$^{-/-}$ mice respectively. Numbers indicate percentage of total immature B cells.


OcaB+/-
OcaB-/-
Vκ8
mCκ
hCκ
83
14
85
13
aHEL
mCκ
hCκ
90
9
89
8
Vκ4
mCκ
hCκ
68
29
3
88
3-83
mCκ
hCκ
63
24
2
77
mCκ
3-83
hCk

Diminished mature transcription of affected Vκ genes

OcaB co-activates transcription by a mechanism that requires extensive contacts between its short α-helical segment and the POU domain of Oct-1 (Chasman et al., 1999). Leu^{32} resides in the α-helix domain of OcaB (Chasman et al., 1999), and Leu^{32}Pro mutation specifically abrogates OcaB/Oct-1 interactions, thereby abolishing OcaB transcriptional activation (Gstaiger et al., 1996). To examine whether the effect of OcaB on receptor editing depends upon its transcriptional enhancing activity, we carried out complementation experiments with retroviruses carrying either wild type OcaB, or the Leu^{32}Pro OcaB mutant (OcaB L32P) (Fig. 5-3a). Retroviral expression of OcaB in developing Ig$\kappa^{V\kappa4/h}$OcaB$^{-/-}$ B cells reconstituted editing of Vκ4-mCκ to near normal levels (Fig. 5-3b). In contrast, OcaB L32P was unable to restore surface expression of Vκ4-Jκ4 in Ig$\kappa^{V\kappa4/h}$OcaB$^{-/-}$ B cells (n = 2; Fig. 5-3b). We conclude that transcriptional co-activation by OcaB is required for regulation of Vκ gene editing.

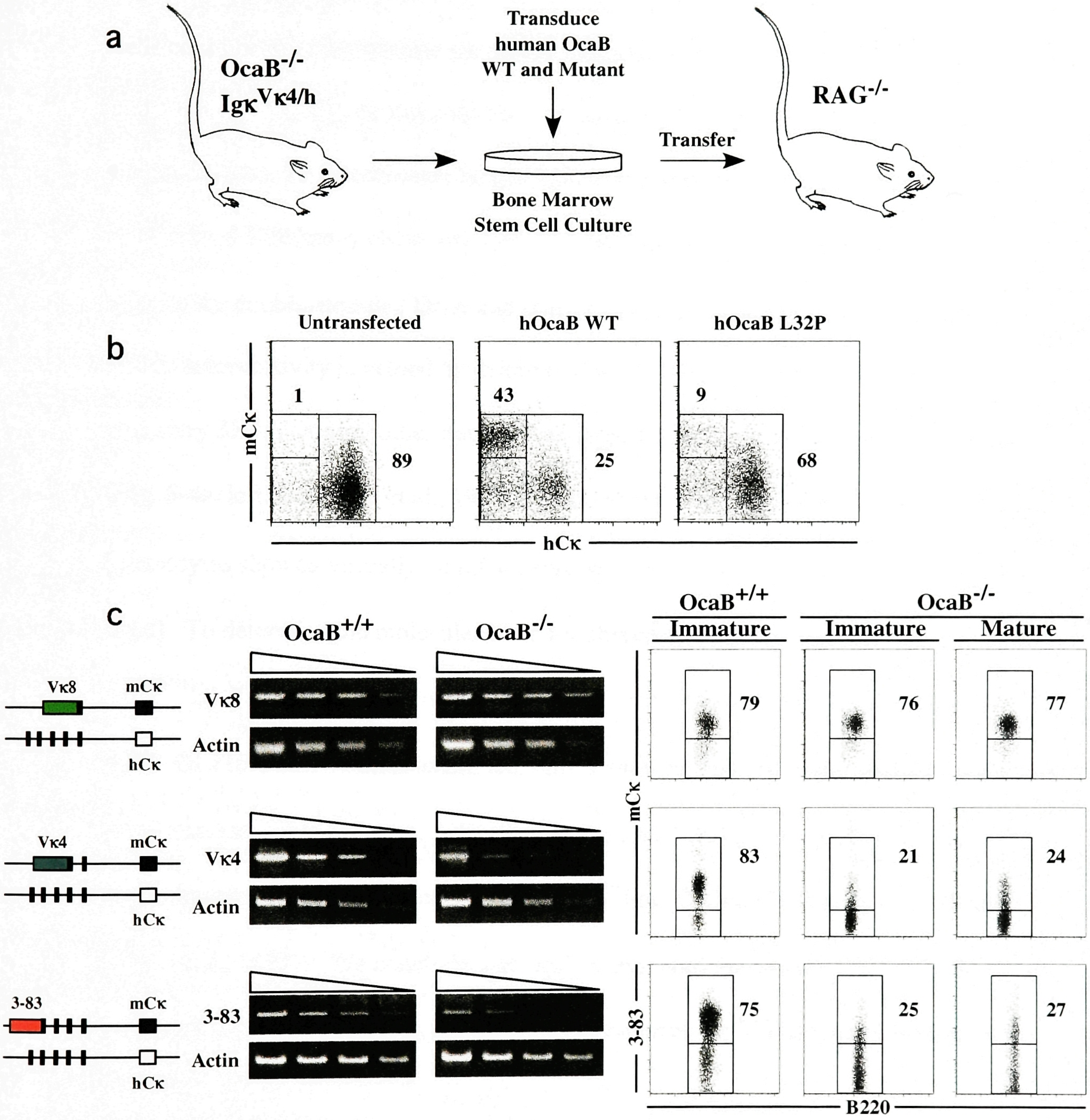
To determine whether OcaB is required for transcription of Vκ genes, we measured steady state levels of pre-recombined Vκ4 and Vκ3-83 mRNAs in OcaB$^{-/-}$ and OcaB$^{+/+}$ control B cells by semi-quantitative RT-PCR. We found a 5-10 fold reduction in the amount of Vκ4 and Vκ3-83 mRNA in the absence of OcaB (Fig. 5-3c, left). In contrast, Vκ8, whose editing is not altered in the absence of OcaB, showed indistinguishable mRNA levels in the presence or absence of OcaB (Fig. 5-3c, left). To determine whether decreased levels of Vκ4 and Vκ3-83 gene expression result in altered Igκ protein production we measured intracellular mCκ in developing B cells from Ig$\kappa^{V\kappa4/h}$, Ig$\kappa^{3\text{-}83/h}$, and Ig$\kappa^{V\kappa8/h}$ wild type and OcaB$^{-/-}$ mice by FACS. We found low levels of Vκ4- and Vκ3-83-mCκ light chains in OcaB$^{-/-}$ mice compared to controls, while Vκ8 production was unaffected (Fig. 5-3c, right). We conclude that OcaB deficiency results in diminished transcription and expression of pre-recombined Vκ4 and Vκ3-83 but not Vκ8 light chain genes.

Figure 5-3 OcaB regulates transcription of Vκ genes.

A) Reconstitution strategy using retroviruses that direct the expression of wild type and L32P mutant OcaB.

B) Flow cytometry analysis of spleen cells from RAG mice reconstituted with uninfected (left), OcaB retrovirus infected (middle), or L32P OcaB retrovirus infected (right plot) OcaB$^{-/-}$Igκ$^{V\kappa 4/h}$ bone marrow cells. Numbers represent percentage of gated B220^{+}GFP^{+} splenocytes.

C) Vκ gene expression as determined by RT-PCR on sorted populations of Igκ$^{V\kappa 8/h}$, Igκ$^{V\kappa 4/h}$, or Igκ$^{3\text{-}83/h}$ immature bone marrow B cells from OcaB$^{+/+}$ or OcaB$^{-/-}$ mice. Serial five-fold dilutions of cDNA were normalized to the actin control. Right, immature IgM^{+}B220^{low} B cells from the same mice were stained for intracellular mCκ or 3-83 expression. Percentages of total gated B cells are indicated.


a
OcaB-/-
Igκ Vκ4/h
Transduce
human OcaB
WT and Mutant
Bone Marrow
Stem Cell Culture
Transfer
RAG-/-
b
Untransfected
hOcaB WT
hOcaB L32P
mCκ
1
89
43
25
9
68
hCκ
c
Vκ8
mCκ
hCκ
Vκ4
3-83
OcaB+/+
OcaB-/-
Vκ8
Actin
Vκ4
Actin
3-83
Actin
OcaB+/+
OcaB-/-
Immature
Immature
Mature
mCκ
79
76
77
83
21
24
3-83
75
25
27
B220

Absence of Vκ4-Jκ4 gene replacements at targeted loci

Increased expression of the hCκ indicator allele in Igκ$^{V\kappa4/h}$ and Igκ$^{3\text{-}83/h}$OcaB$^{-/-}$ B cells could be due to excessive secondary recombination (i.e. RS recombination, (Retter and Nemazee, 1998)), or alternatively to a specific block in transcription and editing at the mCκ allele. To discriminate between these two possibilities we introduced a pre-recombined 3H9 heavy chain into Igκ$^{V\kappa4/h}$OcaB$^{-/-}$ mice. 3H9/Vκ4 antibodies exhibit high affinity for double-stranded DNA and B cells carrying these antibodies cannot develop unless autoreactivity is vetoed by deletion of the Vκ4 gene (Chen et al., 1997). B cells that carry 3H9/Vκ4 antibodies normally undergo high levels of Vκ4 gene replacement (Fig. 5-4a, left and (Chen et al., 1997)). In the absence of OcaB, IgH$^{3H9/+}$Igκ$^{V\kappa4/h}$ splenocytes showed virtually no mCκ expression (< 1% of B220^{+} cells, n = 5; Fig. 5-4a, right). To determine the molecular basis for altered mCκ expression in IgH$^{3H9/+}$Igκ$^{V\kappa4/h}$OcaB$^{-/-}$ cells, we derived hybridomas and analyzed them by genomic PCR. Of 216 OcaB$^{-/-}$ clones examined, only 1 showed deletion of the Vκ4-Jκ4 gene by a cis rearrangement to Jκ5 (Fig. 5-4b). In contrast, Vκ4-Jκ4 gene replacements occurred in approximately 85% of hybridomas from IgH$^{3H9/+}$Igκ$^{V\kappa4/+}$OcaB$^{+/+}$ B cells (Fig. 3b and (Chen et al., 1997)). We conclude that OcaB is essential for replacement of the Vκ4-Jκ4 gene by V(D)J recombination, but that it is not required for V(D)J recombination on the hCκ allele.

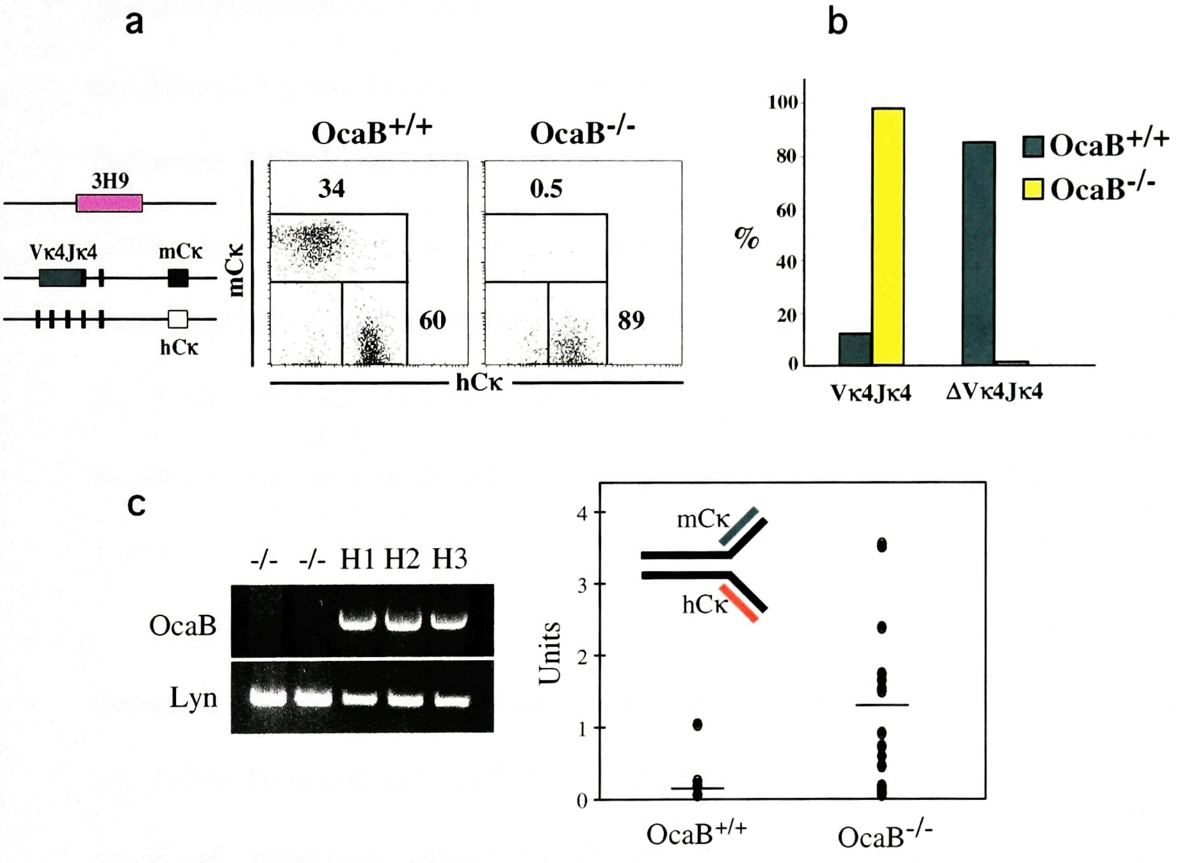
In IgH$^{3H9/+}$Igκ$^{V\kappa4/h}$OcaB$^{-/-}$ hybridomas the myeloma partner provides a wild type OcaB allele after fusion (Fig. 5-4c, left). Since these hybridomas retained the Vκ4-Jκ4 gene at the mCκ locus, we examined whether wild type OcaB could complement the Vκ4 transcriptional defect and induce secretion of mCκ^{+}hCκ^{+} antibodies. To this aim, we assayed culture supernatants from IgH$^{3H9/+}$Igκ$^{V\kappa 4/h}$OcaB$^{-/-}$ hybridomas for antibodies that share mCκ and hCκ. We found that most IgH$^{3H9/+}$Igκ$^{V\kappa 4/h}$OcaB$^{-/-}$ hybridomas secreted antibodies with two different light chains (mCκ^{+} and hCk^{+}) while control Igκ$^{m/h}$ B cells did not (Fig. 5-4c, right). Moreover, these double producers expressed the forbidden Vκ4-3H9 anti-DNA specificity as determined by ELISA and flow cytometry (not shown). Since nearly all hCκ^{+} OcaB$^{-/-}$ B cells have retained the Vκ4-Jκ4 gene in its original configuration, our data shows that IgH$^{3H9/+}$Igκ$^{V\kappa 4/h}$OcaB$^{-/-}$ lymphocytes fail to establish Igκ allelic exclusion. We conclude that decreased expression of Vκ4 in OcaB$^{-/-}$ mice leads to paralysis of the mCκ targeted allele with regard to secondary Vκ-Jκ recombination. Thus, OcaB is required for normal transcription, editing and allelic exclusion of Vκ genes.

Figure 5-4 OcaB is required for Vκ4-Jκ4 gene replacements and allelic exclusion.

(A) Flow cytometry analysis of splenocytes from IgH$^{3H9/+}$Igκ$^{V\kappa4/h}$OcaB$^{+/+}$ (left plot) and IgH$^{3H9/+}$Igκ$^{V\kappa4/h}$OcaB$^{-/-}$ (right plot) animals. Diagram on the left represents the heavy and light chain targeted loci in both strains. Numbers specify percentage of B220^{+} splenic B cells.

(B) Characterization of the Vκ4-mCκ targeted locus (depicted) in OcaB$^{+/+}$ and OcaB$^{-/-}$ hybridomas determined by genomic PCR assays (as in (Prak and Weigert, 1995)).

(C) Left, OcaB expression in IgH$^{3H9/+}$Igκ$^{V\kappa4/h}$OcaB$^{-/-}$ splenic B cells before (-/-) and after fusion to the myeloma cell line (samples H1 to H3). Right, light chain double producers from Igκ$^{m/h}$ and IgH$^{3H9/+}$Igκ$^{V\kappa4/h}$OcaB$^{-/-}$hybridomas measured by α-hCκ and α-mCκ specific ELISA.


a
3H9
Vκ4Jκ4
mCκ
hCκ
OcaB+/+
OcaB-/-
mCκ
34
60
0.5
89
hCκ
b
100
80
60
40
20
0
%
OcaB+/+
OcaB-/-
Vκ4Jκ4
ΔVκ4Jκ4
c
-/-
-/-
H1
H2
H3
OcaB
Lyn
4
3
2
1
0
Units
mCκ
hCκ
OcaB+/+
OcaB-/-

Germline transcription and primary recombination in OcaB$^{-/-}$ mice

The findings aforementioned are derived from animals carrying pre-rearranged light chains introduced at their physiological site. To rule out the possibility of a knock-in related artifact, we investigated transcription and recombination of Vκ4 and Vκ3-83 in OcaB$^{-/-}$ animals carrying wild type kappa loci. To explore this we first measured Vκ germline transcription in OcaB$^{-/-}$ and OcaB$^{+/+}$ preBII cells. Germline transcripts initiate upstream of V genes before or concomitantly with V(D)J recombination (Schlissel and Baltimore, 1989; Yancopoulos and Alt, 1985). IgM^{-}CD25^{+} small preBII cells from OcaB$^{-/-}$ and OcaB$^{+/+}$ animals were cell sorted and germline transcripts from VκαHEL, Vκ3-83, and Vκ4 were amplified by RT-PCR using gene specific primers (as depicted in Fig. 5-5A). We found that transcription of Vκ3-83 and Vκ4 was markedly diminished in the absence of OcaB, while germline transcription of VκαHEL appeared unaffected (Fig. 5-5A).

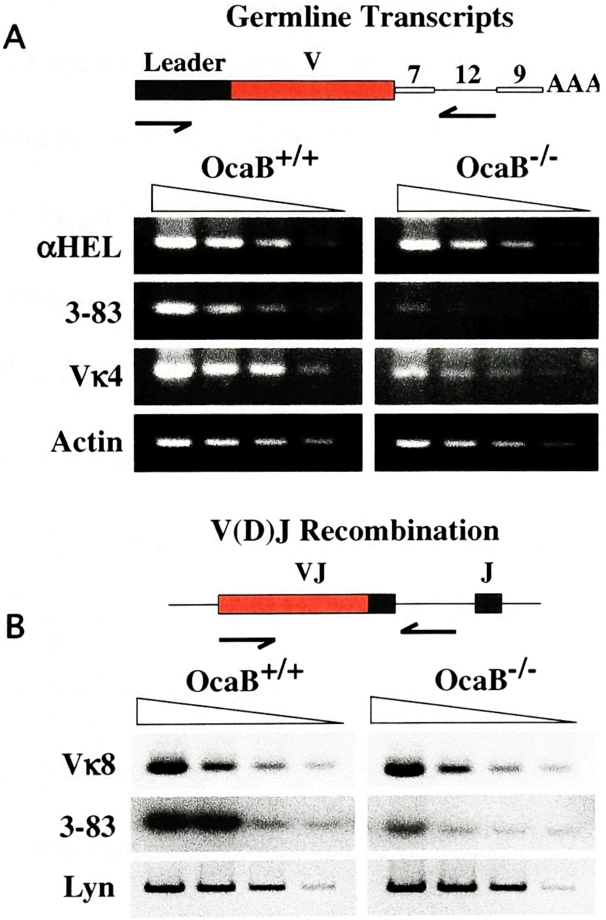
Some studies propose a direct correlation between transcription efficiency and frequency of Ig gene rearrangement (Stiernholm and Berinstein, 1995; Yancopoulos and Alt, 1985). To investigate whether reduced germline transcription in OcaB$^{-/-}$ mice specifically influences primary recombination of Vκ genes we amplified by PCR Vκ3-83-Jκ and VκαHEL-Jκ light chains from IgM^{+}B220^{low} immature B cells (outlined in Fig. 5-5B). Interestingly, we found that diminished sterile transcription correlated with a five-fold reduction of Vκ3-83 rearrangements in OcaB$^{-/-}$ B cells compared to wild-type controls (Fig. 5-5B). Recombination of Vκ8 was unaffected in OcaB$^{-/-}$ animals compared to wild type controls (Fig. 5-5B).

We conclude that germline transcription of Vκ genes is impaired in OcaB$^{-/-}$ animals bearing wild-type kappa loci.

Figure 5-5 Germline transcription and primary recombination of Vκ genes in OcaB deficient mice.

(A) Sterile transcription of αHEL, 3-83, and Vκ4 genes was measured by RTPCR from sorted Igκ$^{h/h}$OcaB$^{+/+}$ (left) or Igκ$^{h/h}$OcaB$^{-/-}$ (right) small preBII cells. cDNA was serially diluted five fold and PCR amplified with gene specific or Actin control primers.

(B) Vκ-Jκ recombination of Vκ8, and 3-83 was assessed by genomic PCR from IgM^{low}B220^{low} immature B cells sorted either from Igκ$^{h/h}$OcaB$^{+/+}$ (left) or Igκ$^{h/h}$OcaB$^{-/-}$ (right) animals. As in (A), isolated genomic DNA was serially diluted five fold and PCR amplified in the presence of Vκ, Jκ2, as well as Lyn specific primers.


A
Germline Transcripts
Leader
V
7
12
9
AAA
OcaB+/+
OcaB-/-
αHEL
3-83
Vκ4
Actin
V(D)J Recombination
VJ
J
B
OcaB+/+
OcaB-/-
Vκ8
3-83
Lyn

Normal accessibility of unedited Vκ genes

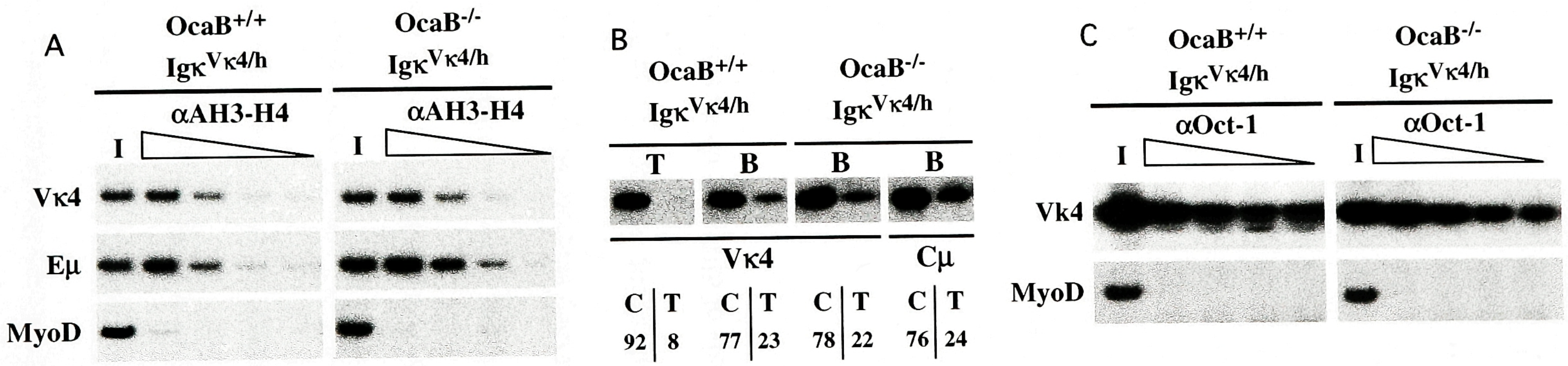
Ig gene transcription and V(D)J recombination is influenced by DNA methylation of CpG islands (Engler et al., 1993; Lichtenstein et al., 1994; Whitehurst et al., 2000), and by histone acetylation (Kwon et al., 2000; McMurry and Krangel, 2000; Stanhope-Baker et al., 1996). While methylation is believed to inhibit binding of trans-acting factors to DNA, acetylation of N-terminal tails of histones appears to unfold higher order chromatin into an open configuration thereby facilitating gene expression (Grunstein, 1997). To investigate whether diminished Vκ4 transcription and absence of secondary recombination in OcaB$^{-/-}$ mice was the result of repressed or otherwise inaccessible chromatin, we measured histone acetylation and CpG methylation of the paralyzed Vκ4 allele in Igκ$^{V\kappa4/h}$OcaB$^{+/+}$ and Igκ$^{V\kappa4/h}$OcaB$^{-/-}$ B cells. We found no difference in histone H3/H4 acetylation or CpG methylation of the pre-recombined Vκ4 locus between OcaB$^{-/-}$ and OcaB$^{+/+}$ B cells as measured by chromatin immunoprecipitation and methylation-sensitive single nucleotide primer extension (Ms-SNuPE) (Gonzalgo and Jones, 1997) respectively (Fig. 5-6a and b). Furthermore, despite the low levels of transcription, Oct-1 was bound to the Vκ4 promoter octamer in OcaB$^{-/-}$ and OcaB$^{+/+}$ B cells, as determined by anti-Oct-1 chromatin immunoprecipitation (Fig. 5-6c). We conclude that low levels of transcription and lack of editing in Igκ$^{V\kappa4/h}$OcaB$^{-/-}$ B cells cannot be ascribed to differences in histone acetylation or CpG methylation and that the Vκ4 promoter is occupied by Oct-1 in the absence of OcaB.

Figure 5-6 Analysis of Oct-1 interaction and accessibility of Vκ genes *in vivo*

(A) H3/H4 acetylation measured by ChIP on OcaB$^{+/+}$Igκ$^{V\kappa 4/h}$ and OcaB$^{-/-}$Igκ$^{V\kappa 4/h}$ B lymphocytes. Five-fold serial dilutions of immunoprecipitated DNA were amplified with Vκ4, Eμ, and MyoD specific primers. "I" represents input DNA before immunoprecipitation.

(B) DNA methylation by Ms-SNuPE assay. Methylation status at the first CpG site of Vκ4 CDR3 from T or B cells (indicated in capitals on top of each column) isolated from OcaB$^{+/+}$Igκ$^{V\kappa 4/h}$ and OcaB$^{-/-}$Igκ$^{V\kappa 4/h}$ mice. The extent of methylation at the assayed CpG site equals the percentage of T conversion (% = T X 100/(C + T), depicted below each column). The Cμ gene, which is actively transcribed in both mouse strains, was used as a positive control (last column).

(C) Immunoprecipitation of the Vκ4 or MyoD promoter using anti-Oct-1 antibodies from OcaB$^{+/+}$Igκ$^{V\kappa 4/h}$ and OcaB$^{-/-}$Igκ$^{V\kappa 4/h}$ splenic B cells.

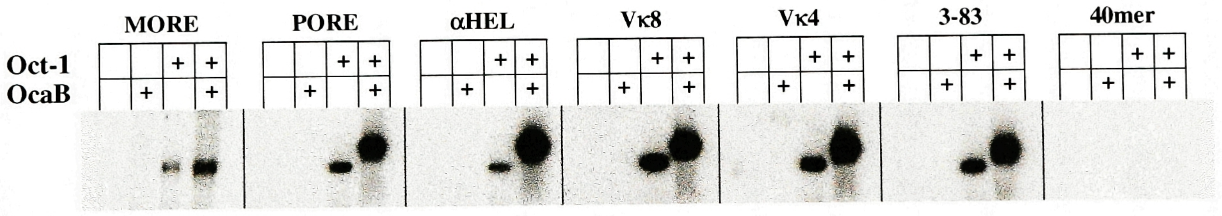

A
OcaB+/+ Igκ Vκ4/h
OcaB-/- Igκ Vκ4/h
αAH3-H4
I
Vκ4
Eμ
MyoD
B
OcaB+/+ Igκ Vκ4/h
OcaB-/- Igκ Vκ4/h
T
B
Vκ4
Cμ
C | T
92 | 8
77 | 23
78 | 22
76 | 24
C
OcaB+/+ Igκ Vκ4/h
OcaB-/- Igκ Vκ4/h
αOct-1
I
Vk4
MyoD

OcaB efficiently interacts with kappa promoters

X-ray crystallography shows that OcaB interacts with Oct-1 in the context of a PORE octamer motif, whereas binding of Oct-1 to MORE octamer sequences inhibits OcaB interaction (Remenyi et al., 2001; Tomilin et al., 2000). Non-recruitment of OcaB to Vκ8 and VκαHEL promoters could provide a rationale for their apparent normal transcription and editing in OcaB$^{-/-}$ cells. To investigate this possibility we performed electrophoretic mobility shift assays (EMSA). In contrast to results obtained with heavy chains (Tomilin et al., 2000), we found that all Vκ promoters tested are functionally analogous to POREs in that they support Oct-1/OcaB interaction (Fig. 5-7). Thus, the difference in expression and editing between Vκ genes in OcaB$^{-/-}$ mice cannot be explained simply on the basis of differential OcaB recruitment to Vκ promoters.

Figure 5-7 OcaB interaction with Vκ promoters

EMSA for Oct-1 and/or OcaB binding to αHEL, Vκ8, Vκ4, or 3-83. MORE and PORE oligonucleotides were previously described (Tomilin et al., 2000). The 40mer oligonucleotide coded for a random DNA sequence and served as a negative control.


MORE
PORE
αHEL
Vκ8
Vκ4
3-83
40mer
Oct-1
OcaB
+

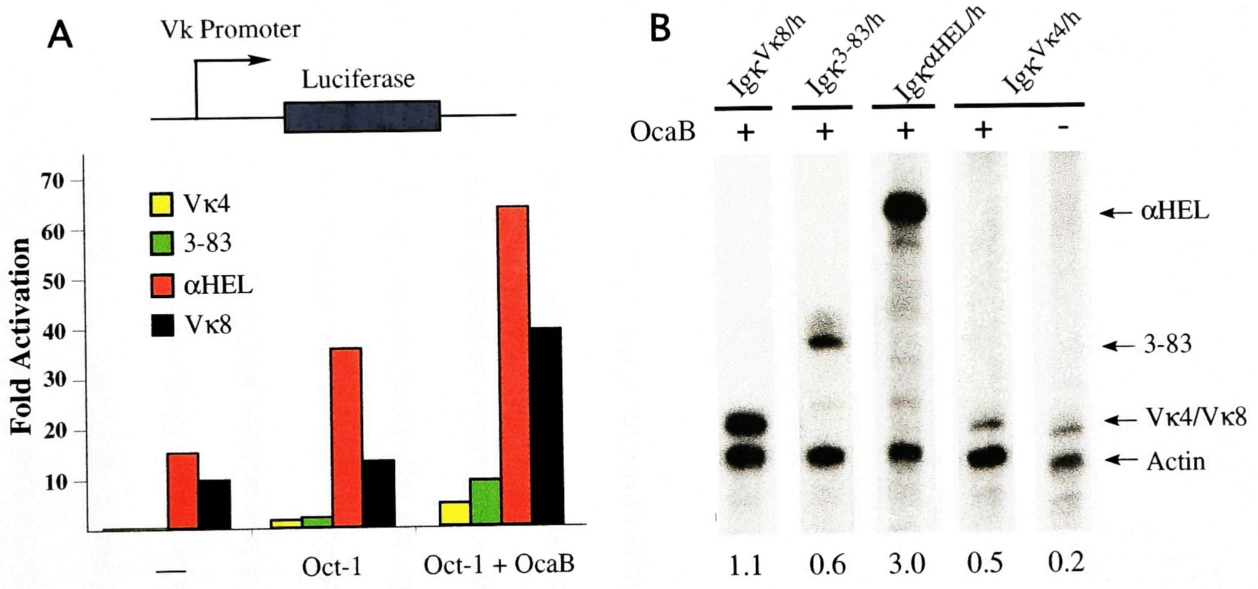
Heterogeneity of Vκ transcription *in vivo*

To examine why expression of some but not all Vκ genes is impaired in the absence of OcaB, we compared transcriptional activity of affected Vκ4, and Vκ3-83 promoters with unaffected Vκ8 and VκαHEL promoters in transiently transfected 293T cells. These cells express low levels of endogenous Oct-1 but not OcaB. We found that the VκαHEL and Vκ8 promoters supported low levels of luciferase expression when transfected alone into 293T cells and this basal activity was increased upon expression of additional Oct-1 by co-transfection (Fig. 5-8a). In contrast, Vκ4 and Vκ3-83 promoters showed no baseline activity and only low levels of transcription were seen with added Oct-1 (Fig. 5-8a). Transcription from all promoters was enhanced upon co-transfection with OcaB, but Vκ4 and Vκ3-83 promoter activity remained 5-15 fold below that of VκαHEL and Vκ8 (Fig. 5-8a). Thus, both sets of Vκ promoters are OcaB responsive. However, the baseline transcription activity of Vκ promoters that are affected by the absence of OcaB *in vivo* (Vκ4 and Vκ3-83) is considerably lower than those that are not (VκαHEL and Vκ8). To examine whether Vκ promoters are transcriptionally heterogeneous under physiological conditions we measured their transcriptional activity in B cells by RNAse protection assay. In agreement with the transfection experiments, we found that both VκαHEL and Vκ8 mRNA levels were higher than Vκ4 and Vκ3-83 mRNA levels (Fig. 5-8b). We conclude that, under physiological conditions, transcription efficiency varies between different Vκ promoters.

Figure 5-8 Heterogeneity of Vκ transcription *in vivo.*

(A) Luciferase reporter plasmids (50ng) containing the Vκ4, Vκ3-83, VκαHEL, or Vκ8 promoters were transfected into 293T cells either alone (left) or with Oct-1 (middle) or Oct-1 and OcaB (right). Fold activation (Y axis) refers to the luciferase activity using untransfected cells as baseline (not shown). 1ng of Renilla plasmid control (Clonetech) was cotransfected for standardization in all experiments.

(B) RNAse protection assay performed with RNA isolated from splenic CD19^{+} B cells from Igκ$^{V\kappa 8/h}$, Igκ$^{3\text{-}83/h}$, Igκ$^{\alpha HEL/h}$, Igκ$^{V\kappa 4/h}$, and Igκ$^{V\kappa 4//h}$OcaB$^{-/-}$ mice. Gene specific and actin control radiolabeled antisense probes were hybridized simultaneously and samples were run on a 5% denaturing gel (8.3M urea). Numbers at the bottom of each column represent fold increase of Vκ gene with respect to normalized actin control determined by phosphorimaging analysis (fold = (Vκ– background)/(Actin – background)).


A
Vk Promoter
Luciferase
Fold Activation
70
60
50
40
30
20
10
🟨 Vκ4
🟩 3-83
🟥 αHEL
■ Vκ8
—
Oct-1
Oct-1 + OcaB
B
IgκVκ8/h
Igκ3-83/h
IgκαHEL/h
IgκVκ4/h
OcaB
+
+
+
+
-
αHEL
3-83
Vκ4/Vκ8
Actin
1.1
0.6
3.0
0.5
0.2

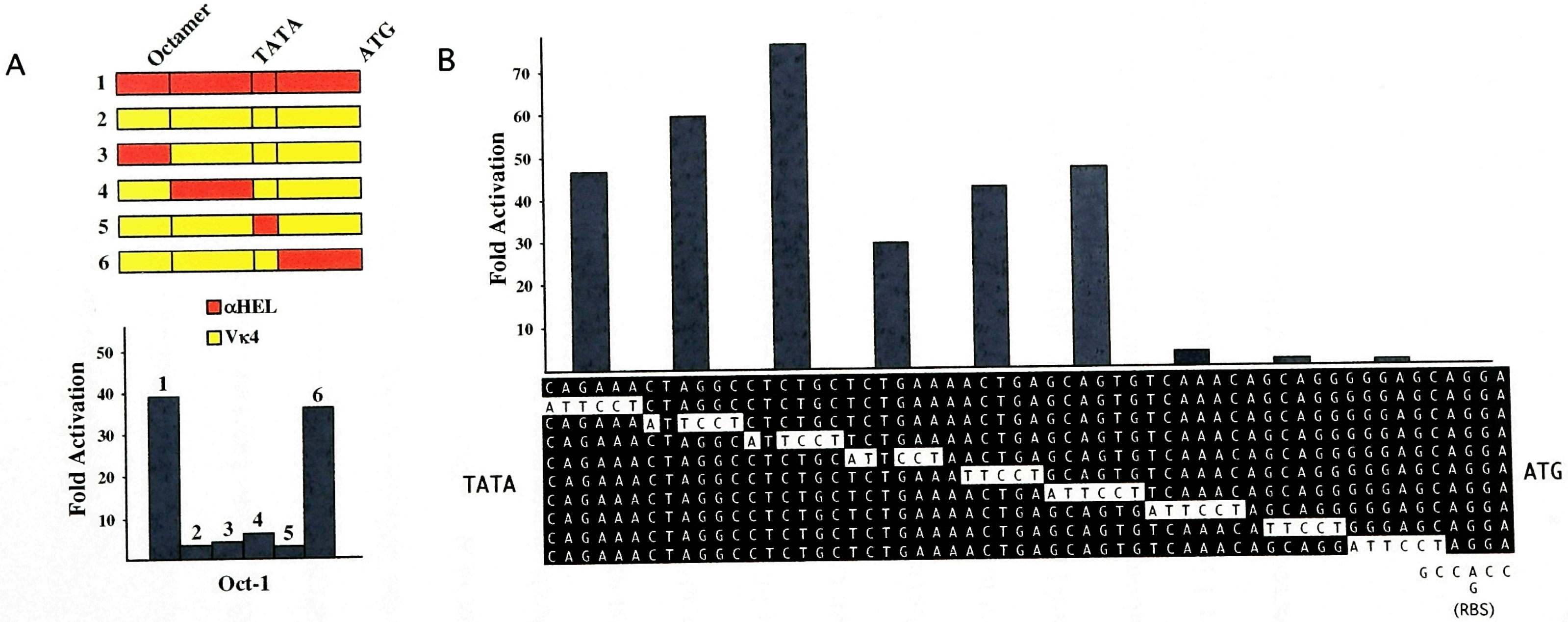
OcaB independent promoters bypass OcaB deficiency by means of additional transcriptional elements

To define the promoter region critical for high baseline VκαHEL transcription, we performed promoter swap experiments between VκαHEL and Vκ4 (Fig. 5-9a). We found that transcriptional differences between these promoters could not be attributed to either the octamer or the TATA box but were dependent on a cis element situated between the TATA box and the ATG (Fig. 5-9a, construct 6). Linker-scanning analysis of this region showed that a 12 base-pair element found immediately upstream of the ATG was responsible for the high transcription activity of the VκαHEL promoter (Fig. 5-9b). This same element was found to be present in the Vκ8 but absent in the Vκ3-83 promoter (not shown). We conclude that baseline Vκ transcription is regulated in part by a cis element immediately upstream of the ATG.

Figure 5-9 A cis-transcriptional element circumvents OcaB absence in unaffected Vκ genes

(A) αHEL complementation of Vκ4 promoter. Luciferase reporter vectors containing the αHEL (red, #1), Vκ4 (yellow, #2), or hybrid αHEL/Vκ4 promoters (#s 3 to 6 as depicted) were co-transfected into 293T cells with Oct-1.

(B) Linker-scanning analysis. The nucleotide sequence spanning the TATA/ATG intervening region is shown as well as the locations where substitutions were introduced (ATTCCT). The corresponding fold activation for each of the mutant plasmid is shown. RBS = ribosomal binding site consensus sequence.


A
Octamer
TATA
ATG
1
2
3
4
5
6
αHEL
Vκ4
Fold Activation
10
20
30
40
50
1
2
3
4
5
6
Oct-1
B
Fold Activation
10
20
30
40
50
60
70
TATA
CAGAAACTAGGCCTCTGCTCTGAAAACTGAGCAGTGTCAAACAGCAGGGGGAGCAGGA
ATTCCTCTAGGCCTCTGCTCTGAAAACTGAGCAGTGTCAAACAGCAGGGGGAGCAGGA
CAGAAAATTCCTCTCTGCTCTGAAAACTGAGCAGTGTCAAACAGCAGGGGGAGCAGGA
CAGAAACTAGGCATTCCTTCTGAAAACTGAGCAGTGTCAAACAGCAGGGGGAGCAGGA
CAGAAACTAGGCCTCTGCATTCCTAACTGAGCAGTGTCAAACAGCAGGGGGAGCAGGA
CAGAAACTAGGCCTCTGCTCTGAAATTCCTGCAGTGTCAAACAGCAGGGGGAGCAGGA
CAGAAACTAGGCCTCTGCTCTGAAAACTGAATTCCTTCAAACAGCAGGGGGAGCAGGA
CAGAAACTAGGCCTCTGCTCTGAAAACTGAGCAGTGATTCCTAGCAGGGGGAGCAGGA
CAGAAACTAGGCCTCTGCTCTGAAAACTGAGCAGTGTCAAACATTCCTGGGAGCAGGA
CAGAAACTAGGCCTCTGCTCTGAAAACTGAGCAGTGTCAAACAGCAGGATTCCTAGGA
CAGAAACTAGGCCTCTGCTCTGAAAACTGAGCAGTGTCAAACAGCAGG
ATG
GCCACC
G
(RBS)

Discussion

The role of OcaB in Ig gene transcription

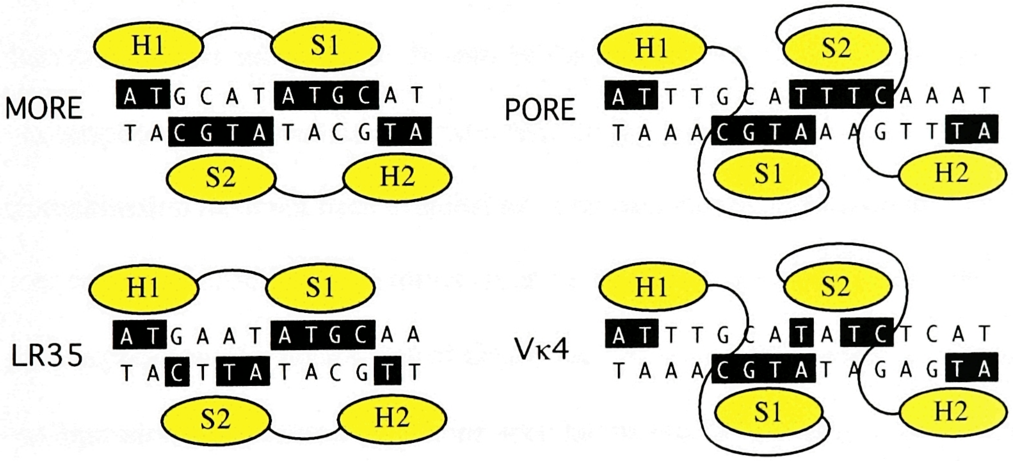
We have shown that OcaB interacts with Vκ gene promoters and regulates their transcription in vivo. These functions, which are essential for receptor editing and allelic exclusion, were not previously appreciated because of the heterogeneity in Vκ transcription and the difficulty in detecting editing in vivo(Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996).

Why is OcaB-dependent transcription restricted to Vκ genes? Recent crystallographic studies indicate that due to structural constrains imposed by Oct-1dimerization, OcaB is unable to interact with VH octamer/heptamer elements (Remenyi et al., 2001; Tomilin et al., 2000). In support of this, no differences were found in VH repertoire between OcaB$^{-/-}$ and OcaB$^{+/+}$ animals (Schubart et al., 2000). Thus heavy chain promoters must have developed alternative means to enhance transcription.

In contrast to heavy chains, we show that light chain octamer motifs efficiently associate with Oct-1 and OcaB by gel shift assays and *in vivo* by Oct-1 immunoprecipitation. Furthermore, based on sequence similarity with the consensus PORE motif, Oct-1 is expected to dimerize over light chain octamers in such a way as to permit OcaB interaction (Fig. 5-10, and (Remenyi et al., 2001; Tomilin et al., 2000)).

Figure 5-10 Light chain Octamer sequences are identical to PORE motifs.

Top: scheme revealing the overall arrangement of the POU subdomain within the Oct-1:MORE and Oct-1:PORE crystal structures. Bottom: Predicted LR36 heavy chain and Vκ4 light chain Octamer motifs bond by Oct-1. The POU$_S$ domain (S) and the POU$_H$ (H) are indicated. The POU subdomains belonging to one polypeptide chain have the same numbering and are connected by a linker.

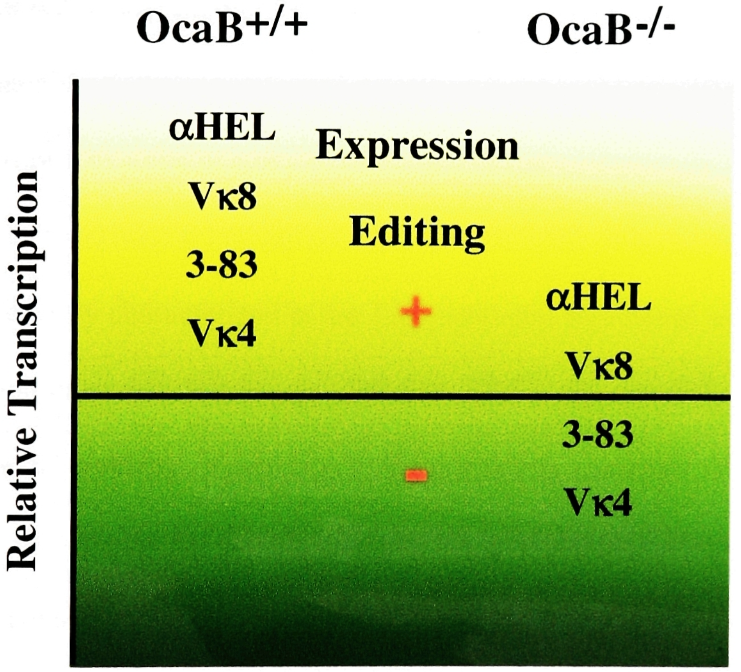

MORE
H1
S1
S2
H2
ATGCATATGCAT
TACGTATACGTA
PORE
H1
S2
S1
H2
ATTTGCATTTCAAAT
TAAACGTAAAGTTTA
LR35
H1
S1
S2
H2
ATGAATATGCAA
TACTTATACGTT
Vκ4
H1
S2
S1
H2
ATTTGCATATCTCAT
TAAACGTATAGAGTA

The role of transcription in recombination

Ig gene expression was proposed to be essential for V(D)J recombination based on the direct correlation between the onset of germline transcription and antibody gene recombination (Blackwell, 1986; Schlissel and Baltimore, 1989; Yancopoulos and Alt, 1985). In support of this model, targeted deletion of transcription regulatory elements impairs both expression and recombination of TCR and Ig genes in vivo (Chen et al., 1993; Serwe and Sablitzky, 1993; Whitehurst et al., 2000; Xu et al., 1996). Additionally, V(D)J recombination can be induced in non-lymphoid cells upon expression of the RAG1 and RAG2 plus the E2A transcription factor, although the target for E2A has not been determined (Romanow, 2000). In spite of these observations, the requirements for transcription, as opposed to chromatin remodeling and promoter loading in recombination have not been established. Our data clearly demonstrates that OcaB, a transcriptional coactivator, controls recombination by regulating the efficiency of Vκ gene expression. In the absence of OcaB, the Igκ chromatin domain is in an accessible configuration as measured by histone acetylation and DNA methylation. furthermore, without OcaB Oct-1 and the basal transcriptional machinery are recruited to light chain gene promoters and the Igκ genes are transcribed but at a suboptimal level. Based on these findings we propose that transcription and an open chromatin domain are insufficient to activate Ig gene recombination, instead a specific transcription threshold is required to ensure receptor editing and maintain allelic exclusion. In the absence of OcaB weak promoters fall below this threshold while more potent promoters, equipped with additional transcription elements, retain sufficient activity to avert recombinational paralysis (Fig. 5-11).

Figure 5-11 Threshold model

The relative transcription of light chain genes under physiological conditions (left) varies based on the quantity or quality of transcriptional elements present within their promoters. Above a particular threshold (middle line), expression and secondary recombination are normally achieved (+). In OcaB deficient animals however, the overall transcription of Vκ genes is decreased (left). Light chain genes found below the critical threshold (-) cannot drive sufficient transcription to ensure normal BCR expression or receptor editing in the advent of self-reactivity.


OcaB+/+
OcaB-/-
Relative Transcription
αHEL
Vκ8
3-83
Vκ4
Expression
Editing
+
-
αHEL
Vκ8
3-83
Vκ4

We have shown that differential transcription among Vκ genes is due to promoter heterogeneity, namely the presence or absence of co-stimulatory elements. Sequencing analysis and *in vitro* experiments reveals a variety of transcriptional elements found whithin Vκ promoters, such as the pentadecamer, κY, EBF, and E-boxes (reviewed in (Bemark et al., 1998a)). Although these elements cannot confer specificity on their own, they clearly synergize the action of TATA and octamer binding factors (Aranburu et al., 2001; Atchison et al., 1990; Bemark et al., 1998b; Falkner and Zachau, 1984; Schwarzenbach et al., 1995)). We have found that αHEL and Vκ8 promoters share a novel enhancing element positioned downstream of the transcriptional starting site. This element allows Vκ8 and VκαHEL promoters to bypass OcaB deficiency *in vivo* and is sufficient to coactivate transcription from Vκ4 in 293T cells. A similar downstream element, recently characterized in the T1κ promoter, was found to increase Igκ transcription in transfection assays (Pelletier, 1997). We conclude that other transcriptional elements, in addition to the previously characterized octamer and TATA boxes, are responsible for driving efficient Vκ gene expression and light chain editing in OcaB$^{-/-}$ B cells (Fig. 5-11).

Bibliography

Agrawal, A., and Schatz, D. G. (1997). RAG1 and RAG2 form a stable postcleavage synaptic complex with DNA containing signal ends in V(D)J recombination, Cell *89*, 43-53.

Allen, D., Simon, T., Sablitzky, F., Rajewsky, K., and Cumano, A. (1988). Antibody engineering for the analysis of affinity maturation of an anti- hapten response [published erratum appears in EMBO J 1989 Aug;8(8):2444], Embo J *7*, 1995-2001.

Allman, D., Li, J., and Hardy, R. R. (1999). Commitment to the B lymphoid lineage occurs before DH-JH recombination, Journal of Experimental Medicine *189*, 735-40.

Alt, F. W., Yancopoulos, G. D., Blackwell, T. K., Wood, C., Thomas, E., Boss, M., Coffman, R., Rosenberg, N., Tonegawa, S., and Baltimore, D. (1984). Ordered rearrangement of immunoglobulin heavy chain variable region segments, Embo J *3*, 1209-19.

Aranburu, A., Carlsson, R., Persson, C., and Leanderson, T. (2001). Transcription factor AP-4 is a ligand for immunoglobulin-kappa promoter E-box elements, Biochemical Journal *354*, 431-8.

Arrhenius, S. (1907). Immunochemistry. The applications of the principles of physical chemistry to the study of the biological antibodies, Macmillan *New York*, 309.

Atchison, M. L., Delmas, V., and Perry, R. P. (1990). A novel upstream element compensates for an ineffectual octamer motif in an immunoglobulin V kappa promoter, EMBO Journal *9*, 3109-17.

Bauer, D. C., and Stavisky, A. B. (1961). On the different molecular forms of antibody synthesized by rabbits during the early response to a single injection of protein and cellular antigens, Proceedings of the National Academy of Sciences *47*, 1667-1680.

Behring, E., and Kitasato, S. (1890). Ueber das Zustandekommen der Diphtherie-Immunitat und der Tetanus-Immunitat bei Thieren, Deutsche Medizinische Wochenschrift *16*, 1113-1114.

Bell, S. E., and Goodnow, C. C. (1994). A selective defect in IgM antigen receptor synthesis and transport causes loss of cell surface IgM expression on tolerant B lymphocytes, Embo J *13*, 816-26.

Bemark, M., Liberg, D., and Leanderson, T. (1998a). Conserved sequence elements in K promoters from mice and humans: implications for transcriptional regulation and repertoire expression, Immunogenetics *47*, 183-95.

Bemark, M., Olsson, H., Heinegard, D., and Leanderson, T. (1998b). Purification and characterization of a protein binding to the SP6 kappa promoter. A potential role for CArG-box binding factor-A in kappa transcription, Journal of Biological Chemistry *273*, 18881-90.

Bernard, O., Gough, N. M., and Adams, J. M. (1981). Plasmacytomas with more than one immunoglobulin kappa mRNA: implications for allelic exclusion, Proc Natl Acad Sci U S A *78*, 5812-6.

Besmer, E., Mansilla-Soto, J., Cassard, S., Sawchuk, D. J., Brown, G., Sadofsky, M., Lewis, S. M., Nussenzweig, M. C., and Cortes, P. (1998). Hairpin coding end opening is mediated by RAG1 and RAG2 proteins, Mol Cell *2*, 817-28.

Blackwell, T. K. (1986). Recombination between immunoglobulin variable region gene segments is enhanced by transcription, Nature *324*, 585-9.

Bolland, S., and Ravetch, J. V. (1999). Inhibitory pathways triggered by ITIM-containing receptors, Adv Immunol *72*, 149-77.

Boulton, S. J., and Jackson, S. P. (1996). Identification of a Saccharomyces cerevisiae Ku80 homologue: roles in DNA double strand break rejoining and in telomeric maintenance., Nucleic Acids Research *24*, 4639-48.

Brack, C., and Tonegawa, S. (1977). Variable and constant parts of the immunoglobulin light chain gene of a mouse myeloma cell are 1250 nontranslated bases apart, Proc Natl Acad Sci U S A *74*, 5652-6.

Braun, U., Rajewsky, K., and Pelanda, R. (2000). Different sensitivity to receptor editing of B cells from mice hemizygous or homozygous for targeted Ig transgenes [In Process Citation], Proc Natl Acad Sci U S A *97*, 7429-34.

Burnet, M. F. (1959). The Clonal Selection Theory of Acquired Immunity, Vanderbilt University Press and Cambridge University Press, 209.

Casellas, R., Nussenzweig, A., Wuerffel, R., Pelanda, R., Reichlin, A., Suh, H., Qin, X. F., Besmer, E., Kenter, A., Rajewsky, K., and Nussenzweig, M. C. (1998). Ku80 is required for immunoglobulin isotype switching, Embo J *17*, 2404-11.

Casellas, R., Shih, T. A., Kleinewietfeld, M., Rakonjac, J., Nemazee, D., Rajewsky, K., and Nussenzweig, M. C. (2001). Contribution of Receptor Editing to the Antibody Repertoire, Science *291*, 1541-1544.

Chasman, D., Cepek, K., Sharp, P. A., and Pabo, C. O. (1999). Crystal structure of an OCA-B peptide bound to an Oct-1 POU domain/octamer DNA complex: specific recognition of a protein-DNA interface, Genes & Development *13*, 2650-7.

Chen, C., Nagy, Z., Prak, E. L., and Weigert, M. (1995a). Immunoglobulin heavy chain gene replacement: a mechanism of receptor editing, Immunity *3*, 747-55.

Chen, C., Nagy, Z., Radic, M. Z., Hardy, R. R., Huszar, D., Camper, S. A., and Weigert, M. (1995b). The site and stage of anti-DNA B-cell deletion, Nature *373*, 252-5.

Chen, C., Prak, E. L., and Weigert, M. (1997). Editing disease-associated autoantibodies, Immunity *6*, 97-105.

Chen, H. T., Bhandoola, A., Difilippantonio, M. J., Zhu, J., Brown, M. J., Tai, X., Rogakou, E. P., Brotz, T. M., Bonner, W. M., Ried, T., and Nussenzweig, A. (2000). Response to RAG-mediated VDJ cleavage by NBS1 and gamma-H2AX, Science *290*, 1962-5.

Chen, J., Young, F., Bottaro, A., Stewart, V., Smith, R. K., and Alt, F. W. (1993). Mutations of the intronic IgH enhancer and its flanking sequences differentially affect accessibility of the JH locus, EMBO Journal *12*, 4635-45.

Cherry, S. R., Beard, C., Jaenisch, R., and Baltimore, D. (2000). V(D)J recombination is not activated by demethylation of the kappa locus [In Process Citation], Proc Natl Acad Sci U S A *97*, 8467-72.

Chu, C. C., Paul, W. E., and Max, E. E. (1992). Quantitation of immunoglobulin mu-gamma 1 heavy chain switch region recombination by a digestion-circularization polymerase chain reaction method., Proc Natl Acad Sci U S A *89*, 6978-6982.

Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994). High sensitivity mapping of methylated cytosines, Nucleic Acids Research *22*, 2990-7.

Coleclough, C., Perry, R. P., Karjalainen, K., and Weigert, M. (1981). Aberrant rearrangements contribute significantly to the allelic exclusion of immunoglobulin gene expression, Nature *290*, 372-8.

Constantinescu, A., and Schlissel, M. S. (1997). Changes in locus-specific V(D)J recombinase activity induced by immunoglobulin gene products during B cell development, J Exp Med *185*, 609-20.

Corcoran, A. E., Riddell, A., Krooshoop, D., and Venkitaraman, A. R. (1998). Impaired immunoglobulin gene rearrangement in mice lacking the IL-7 receptor, Nature *391*, 904-7.

Corcoran, L. M., Karvelas, M., Nossal, G. J., Ye, Z. S., Jacks, T., and Baltimore, D. (1993). Oct-2, although not required for early B-cell development, is critical for later B-cell maturation and for postnatal survival, Genes & Development *7*, 570-82.

Cromie, G. A., Connelly, J. C., and Leach, D. R. (2001). Recombination at double-strand breaks and DNA ends: conserved mechanisms from phage to humans, Molecular Cell *8*, 1163-74.

Cummingham, B. A., Gottlieb, P. D., Konigsberg, W. H., and Edelman, G. M. (1968). The covalent structure of a human gamma G-immunoglobulin. V. Partial amino acid sequence of the light chain, Biochemistry *7*, 1983-94.

Cyster, J. G., and Goodnow, C. C. (1995). Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection, Immunity *2*, 13-24.

Cyster, J. G., Hartley, S. B., and Goodnow, C. C. (1994). Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire [see comments], Nature *371*, 389-95.

Cyster, J. G., Healy, J. I., Kishihara, K., Mak, T. W., Thomas, M. L., and Goodnow, C. C. (1996). Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45, Nature *381*, 325-8.

Davis, M. M., Kim, S. K., and Hood, L. E. (1980). DNA sequences mediating class switching in alpha-immunoglobulins, Science *209*, 1360-5.

Downs, J. A., Lowndes, N. F., and Jackson, S. P. (2000). A role for Saccharomyces cerevisiae histone H2A in DNA repair, Nature *408*, 1001-4.

Dreyer, W. J., and Bennett, J. C. (1965). The molecular basis of antibody formation: a paradox, Proc Natl Acad Sci U S A *54*, 864-9.

Early, P., and Hood, L. (1981). Allelic exclusion and nonproductive immunoglobulin gene rearrangements, Cell *24*, 1-3.

Early, P., Huang, H., Davis, M., Calame, K., and Hood, L. (1980). An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH, Cell *19*, 981-92.

Edelman, G. M., and Poulik, M. D. (1961). Studies on the structural units of the g-globulins, Journal of Experimental Medicine *113*, 861-884.

Edmundson A, Ely K, Abola E, Schiffer M, and N, P. (1975). Rotational allomerism and divergent evolution of domains in Ig light chains, Biochemistry *14*, 3953-3961.

Ehrlich, P. (1900). On immunity with special reference to cell life, Proceedings of the Royal Society, London *66*, 424-448.

Engler, P., Weng, A., and Storb, U. (1993). Influence of CpG methylation and target spacing on V(D)J recombination in a transgenic substrate, Molecular & Cellular Biology *13*, 571-7.

Erikson, J., Radic, M. Z., Camper, S. A., Hardy, R. R., Carmack, C., and Weigert, M. (1991). Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice, Nature *349*, 331-4.

Feddersen, R. M., and Van Ness, B. G. (1985). Double recombination of a single immunoglobulin kappa-chain allele: implications for the mechanism of rearrangement, Proc Natl Acad Sci U S A *82*, 4793-7.

Fugmann, S. D., Lee, A. I., Shockett, P. E., Villey, I. J., and Schatz, D. G. (2000). The RAG proteins and V(D)J recombination: complexes, ends, and transposition, Annual Review of Immunology *18*, 495-527.

Fulcher, D. A., and Basten, A. (1994). Reduced life span of anergic self-reactive B cells in a double- transgenic model, J Exp Med *179*, 125-34.

Garrot, A. E. (1923). Inborn Errors of Metabolism, Oxford University Press, London *2nd Ed.*

Gay, D., Saunders, T., Camper, S., and Weigert, M. (1993). Receptor editing: an approach by autoreactive B cells to escape tolerance, J Exp Med *177*, 999-1008.

Gearhart, P. J., Sigal, N. H., and Klinman, N. R. (1975). Production of antibodies of identical idiotype but diverse immunoglobulin classes by cells derived from a single stimulated B cell, Proc Natl Acad Sci U S A *72*, 1707-11.

Giachino, C., Padovan, E., and Lanzavecchia, A. (1995). kappa+lambda+ dual receptor B cells are present in the human peripheral repertoire, J Exp Med *181*, 1245-50.

Gong, S. (1996). Regulation of an early developmental checkpoint in the B cell pathway by Ig beta, Science *272*, 411-4.

Gonzalgo, M. L., and Jones, P. A. (1997). Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE), Nucleic Acids Research *25*, 2529-31.

Goodnow, C. C. (1996). Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires, Proceedings of the National Academy of Sciences of the United States of America *93*, 2264-71.

Goodnow, C. C., Crosbie, J., Adelstein, S., Lavoie, T. B., Smith-Gill, S. J., Brink, R. A., Pritchard-Briscoe, H., Wotherspoon, J. S., Loblay, R. H., Raphael, K., and et al. (1988). Altered immunoglobulin expression and functional silencing of self- reactive B lymphocytes in transgenic mice, Nature *334*, 676-82.

Goodnow, C. C., Crosbie, J., Jorgensen, H., Brink, R. A., and Basten, A. (1989). Induction of self-tolerance in mature peripheral B lymphocytes [see comments], Nature *342*, 385-91.

Gottlieb, T. M., and Jackson, S. P. (1993). The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen, Cell *72*, 131-42.

Grawunder, U., Leu, T. M., Schatz, D. G., Werner, A., Rolink, A. G., Melchers, F., and Winkler, T. H. (1995). Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement, Immunity *3*, 601-8.

Grawunder, U., Wilm, M., Wu, X., Kulesza, P., Wilson, T. E., Mann, M., and Lieber, M. R. (1997). Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells [see comments], Nature *388*, 492-5.

Grosschedl, R., and Baltimore, D. (1985). Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements, Cell *41*, 885-97.

Grunstein, M. (1997). Histone acetylation in chromatin structure and transcription, Nature *389*, 349-52.

Gstaiger, M., Georgiev, O., van Leeuwen, H., van der Vliet, P., and Schaffner, W. (1996). The B cell coactivator Bob1 shows DNA sequence-dependent complex formation with Oct-1/Oct-2 factors, leading to differential promoter activation, EMBO Journal *15*, 2781-90.

Gstaiger, M., Knoepfel, L., Georgiev, O., Schaffner, W., and Hovens, C. M. (1995). A B-cell coactivator of octamer-binding transcription factors, Nature *373*, 360-2.

Harada, K., and Yamagishi, H. (1991). Lack of feedback inhibition of V kappa gene rearrangement by productively rearranged alleles, J Exp Med *173*, 409-15.

Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D., and Hayakawa, K. (1991). Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow, J Exp Med *173*, 1213-25.

Hartley, S. B., Cooke, M. P., Fulcher, D. A., Harris, A. W., Cory, S., Basten, A., and Goodnow, C. C. (1993). Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death, Cell *72*, 325-35.

Hartley, S. B., Crosbie, J., Brink, R., Kantor, A. B., Basten, A., and Goodnow, C. C. (1991). Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens, Nature *353*, 765-9.

Hashimoto, S., Mohrenweiser, H. W., Gregersen, P. K., and Chiorazzi, N. (1994). Chromosomal localization, genomic structure, and allelic polymorphism of the human CD79 alpha (Ig-alpha/mb-1) gene, Immunogenetics *40*, 287-95.

Hawley, R. G., Lieu, F. H., Fong, A. Z., and Hawley, T. S. (1994). Versatile retroviral vectors for potential use in gene therapy, Gene Therapy *1*, 136-8.

Healy, J. I., Dolmetsch, R. E., Timmerman, L. A., Cyster, J. G., Thomas, M. L., Crabtree, G. R., Lewis, R. S., and Goodnow, C. C. (1997). Different nuclear signals are activated by the B cell receptor during positive versus negative signaling, Immunity *6*, 419-28.

Hernandez, N. (2001). Small nuclear RNA genes: a model system to study fundamental mechanisms of transcription, Journal of Biological Chemistry *276*, 26733-6.

Hertz, M., and Nemazee, D. (1997). BCR ligation induces receptor editing in IgM+IgD- bone marrow B cells in vitro, Immunity *6*, 429-36.

Hesse, J. E., Lieber, M. R., Mizuuchi, K., and Gellert, M. (1989). V(D)J recombination: a functional definition of the joining signals, Genes Dev *3*, 1053-61.

Hilschmann, N., and Craig, L. C. (1965). Amino acid sequence studies with Bence-Jones proteins, Proceedings of the National Academy of Sciences *53*, 1403.

Honjo, T., and Kataoka, t. (1978). Organization of immunoglobulin heavy chain genes and allelic deletion model, Proc Natl Acad Sci U S A *75*, 2140-4.

Hsieh, C. L., and Lieber, M. R. (1992). CpG methylated minichromosomes become inaccessible for V(D)J recombination after undergoing replication, Embo J *11*, 315-25.

Inaoki, M., Sato, S., Weintraub, B. C., Goodnow, C. C., and Tedder, T. F. (1997). CD19-regulated signaling thresholds control peripheral tolerance and autoantibody production in B lymphocytes, J Exp Med *186*, 1923-31.

Iwasato, T., Shimizu, A., Honjo, T., and Yamagishi, H. (1990). Circular DNA is excised by immunoglobulin class switch recombination., Cell *62*, 143-149.

Kabat, E. A., National Institutes of Health (U.S.), and Columbia University. (1991). Sequences of proteins of immunological interest, 5th edn (Bethesda, MD, U.S. Dept. of Health and Human Services Public Health Service National Institutes of Health).

Kim, U., Qin, X. F., Gong, S., Stevens, S., Luo, Y., Nussenzweig, M., and Roeder, R. G. (1996). The B-cell-specific transcription coactivator OCA-B/OBF-1/Bob-1 is essential for normal production of immunoglobulin isotypes, Nature *383*, 542-7.

Kinoshita, K., and Honjo, T. (2001). Linking class-switch recombination with somatic hypermutation, Nature Reviews Molecular Cell Biology *2*, 493-503.

Kleinfield, R., Hardy, R. R., Tarlinton, D., Dangl, J., Herzenberg, L. A., and Weigert, M. (1986). Recombination between an expressed immunoglobulin heavy-chain gene and a germline variable gene segment in a Ly 1+ B-cell lymphoma, Nature *322*, 843-6.

Kouskoff, V., Lacaud, G., Pape, K., Retter, M., and Nemazee, D. (2000). B cell receptor expression level determines the fate of developing B lymphocytes: receptor editing versus selection [In Process Citation], Proc Natl Acad Sci U S A *97*, 7435-9.

Kwan, S. P., Max, E. E., Seidman, J. G., Leder, P., and Scharff, M. D. (1981). Two kappa immunoglobulin genes are expressed in the myeloma S107, Cell *26*, 57-66.

Kwon, J., Morshead, K. B., Guyon, J. R., Kingston, R. E., and Oettinger, M. A. (2000). Histone acetylation and hSWI/SNF remodeling act in concert to stimulate V(D)J cleavage of nucleosomal DNA, Molecular Cell *6*, 1037-48.

LaBella, F., Sive, H. L., Roeder, R. G., and Heintz, N. (1988). Cell-cycle regulation of a human histone H2b gene is mediated by the H2b subtype-specific consensus element, Genes & Development *2*, 32-9.

Lakso, M., Pichel, J. G., Gorman, J. R., Sauer, B., Okamoto, Y., Lee, E., Alt, F. W., and Westphal, H. (1996). Efficient in vivo manipulation of mouse genomic sequences at the zygote stage, Proc Natl Acad Sci U S A *93*, 5860-5.

Lawton, A. R. d., Asofsky, R., Hylton, M. B., and Cooper, M. D. (1972). Suppression of immunoglobulin class synthesis in mice. I. Effects of treatment with antibody to -chain, J Exp Med *135*, 277-97.

Lederberg, J. (1959). Genes and Antibodies, Science *129*, 1649-1653.

Li, H. (2001). Editors and editing of anti-DNA receptors, Immunity *15*, 947-57.

Liang, F., Romanienko, P. J., Weaver, D. T., Jeggo, P. A., and Jasin, M. (1996). Chromosomal double-strand break repair in Ku80-deficient cells., Proceedings of the National Academy of Sciences U S A *93*, 8929-8933.

Lichtenstein, M., Keini, G., Cedar, H., and Bergman, Y. (1994). B cell-specific demethylation: a novel role for the intronic kappa chain enhancer sequence, Cell *76*, 913-23.

Lorenz, M., Jung, S., and Radbruch, A. (1995). Switch transcripts in immunoglobulin class switching, Science *267*, 1825-8.

Lorenz, M., and Radbruch, A. (1996). Developmental and molecular regulation of immunoglobulin class switch recombination., Current Topics in Microbiology & Immunology *217*, 151-169.

Luo, Y., Fujii, H., Gerster, T., and Roeder, R. G. (1992). A novel B cell-derived coactivator potentiates the activation of immunoglobulin promoters by octamer-binding transcription factors, Cell *71*, 231-41.

Luo, Y., and Roeder, R. G. (1995). Cloning, functional characterization, and mechanism of action of the B-cell-specific transcriptional coactivator OCA-B, Molecular & Cellular Biology *15*, 4115-24.

Ma, Y., Pannicke, U., Schwarz, K., and Lieber, M. R. (2002). Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination, Cell *Published online*.

Manis, J. P., Gu, Y., Lansford, R., Sonoda, E., Ferrini, R., Davidson, L., Rajewsky, K., and Alt, F. W. (1998). Ku70 is required for late B cell development and immunoglobulin heavy chain class switching, J Exp Med *187*, 2081-9.

Matsuoka, M., Yoshida, K., Maeda, T., Usuda, S., and Sakano, H. (1990). Switch circular DNA formed in cytokine-treated mouse splenocytes: evidence for intramolecular DNA deletion in immunoglobulin class switching., Cell *62*, 135-142.

McBlane, J. F., van Gent, D. C., Ramsden, D. A., Romeo, C., Cuomo, C. A., Gellert, M., and Oettinger, M. A. (1995). Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps, Cell *83*, 387-95.

McMurry, M. T., and Krangel, M. S. (2000). A role for histone acetylation in the developmental regulation of VDJ recombination. [see comments], Science *287*, 495-8.

Meffre, E., Casellas, R., and Nussenzweig, M. C. (2000). Antibody regulation of B cell development, Nature Immunology *1*, 379-85.

Melamed, D., and Nemazee, D. (1997). Self-antigen does not accelerate immature B cell apoptosis, but stimulates receptor editing as a consequence of developmental arrest, Proc Natl Acad Sci U S A *94*, 9267-72.

Milne, G. T., Jin, S., Shannon, K. B., and Weaver, D. T. (1996). Mutations in two Ku homologs define a DNA end-joining repair pathway in

Saccharomyces cerevisiae., Molecular & Cellular Biology *16*, 4189-98.

Monroe, R. J., Seidl, K. J., Gaertner, F., Han, S., Chen, F., Sekiguchi, J., Wang, J., Ferrini, R., Davidson, L., Kelsoe, G., and Alt, F. W. (1999). RAG2:GFP knockin mice reveal novel aspects of RAG2 expression in primary and peripheral lymphoid tissues, Immunity *11*, 201-12.

Moshous, D. (2001). Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency, Cell *105*, 177-86.

Mostoslavsky, R., Singh, N., Kirillov, A., Pelanda, R., Cedar, H., Chess, A., and Bergman, Y. (1998). Kappa chain monoallelic demethylation and the establishment of allelic exclusion, Genes Dev *12*, 1801-11.

Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y., and Honjo, T. (2000). Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. [see comments], Cell *102*, 553-63.

Nemazee, D. A., and Burki, K. (1989). Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes, Nature *337*, 562-6.

Nielsen, P. J., Georgiev, O., Lorenz, B., and Schaffner, W. (1996). B lymphocytes are impaired in mice lacking the transcriptional co-activator Bob1/OCA-B/OBF1, European Journal of Immunology *26*, 3214-8.

Nissonoff, A., Wissler, F. C., and Lipman, L. N. (1960). Properties of the major component of a peptic digest of rabbit antibody, Science *132*, 1770-1771.

Nossal, G. J. V., Szenberg, A., Ada, G. L., and Austin, C. M. (1964). Single cell studies on 19S antibody production, Journal of Experimental Medicine *119*, 485-502.

Novobrantseva, T. I., Martin, V. M., Pelanda, R., Muller, W., Rajewsky, K., and Ehlich, A. (1999). Rearrangement and expression of immunoglobulin light chain genes can precede heavy chain expression during normal B cell development in mice, J Exp Med *189*, 75-88.

Nussenzweig, A., Chen, C., da Costa Soares, V., Sanchez, M., Sokol, K., Nussenzweig, M. C., and Li, G. C. (1996). Requirement for Ku80 in growth and V(D)J recombination., Nature *382*, 551-5.

Nussenzweig, M. C., Shaw, A. C., Sinn, E., Danner, D. B., Holmes, K. L., Morse, H. C. d., and Leder, P. (1987). Allelic exclusion in transgenic mice that express the membrane form of immunoglobulin mu, Science *236*, 816-9.

Nutt, S. L., Urbanek, P., Rolink, A., and Busslinger, M. (1997). Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult

B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus, Genes & Development *11*, 476-91.

Oettinger, M. A., Schatz, D. G., Gorka, C., and Baltimore, D. (1990). RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination., Science *248*, 1517-23.

Okamoto, M., Murakami, M., Shimizu, A., Ozaki, S., Tsubata, T., Kumagai, S., and Honjo, T. (1992). A transgenic model of autoimmune hemolytic anemia, J Exp Med *175*, 71-9.

Opstelten, D., and Osmond, D. G. (1983). Pre-B cells in mouse bone marrow: immunofluorescence stathmokinetic studies of the proliferation of cytoplasmic mu-chain-bearing cells in normal mice, J Immunol *131*, 2635-40.

Osmond, D. G. (1993). The turnover of B-cell populations [published erratum appears in Immunol Today 1993 Feb;14(2):68] [see comments], Immunol Today *14*, 34-7.

Papavasiliou F, Casellas R, Suh H, Qin Xf, Besmer E, Pelanda R, Nemazee D, Rajewsky K, and Nussenzweig Mc (1997). V(D)J recombination in mature B cells: a mechanism for altering antibody responses [see comments]. Comment in: Science 1997 Oct 10;278(5336):238-9, Science *278*, 298-301.

Pastink, A., Eeken, J. C., and Lohman, P. H. (2001). Genomic integrity and the repair of double-strand DNA breaks, Mutation Research *480-481*, 37-50.

Pauling, L. (1940). A theory of the structure and process of formation of antibodies, Journal of the American Chemical Society *62*, 2643-2657.

Paull, T. T., and Gellert, M. (1999). Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex, Genes Dev *13*, 1276-88.

Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M., and Bonner, W. M. (2000). A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage, Current Biology *10*, 886-95.

Pelanda R, Schwers S, Sonoda E, Torres Rm, Nemazee D, and Rajewsky K (1997). Receptor editing in a transgenic mouse model: site, efficiency, and role in B cell tolerance and antibody diversification, Immunity *7*, 765-75.

Pelanda, R., Schaal, S., Torres, R. M., and Rajewsky, K. (1996). A prematurely expressed Ig(kappa) transgene, but not V(kappa)J(kappa) gene segment targeted into the Ig(kappa) locus, can rescue B cell development in lambda5-deficient mice., Immunity *5*, 229-239.

Pelletier, M. R. (1997). Efficient transcription of an immunoglobulin kappa promoter requires specific sequence elements overlapping with and downstream of the transcriptional start site, Nucleic Acids Research *25*, 3995-4003.

Pernis, B., Chiappino, G., Kelus, A. S., and Gell, P. G. (1965). Cellular localization of immunoglobulins with different allotypic specificities in rabbit lymphoid tissues, J Exp Med *122*, 853-76.

Petersen, S. (2001). AID is required to initiate Nbs1/gamma-H2AX focus formation and mutations at sites of class switching, Nature *414*, 660-5.

Phillips, K., and Luisi, B. (2000). The virtuoso of versatility: POU proteins that flex to fit, Journal of Molecular Biology *302*, 1023-39.

Pike, B. L., Boyd, A. W., and Nossal, G. J. (1982). Clonal anergy: the universally anergic B lymphocyte, Proc Natl Acad Sci U S A *79*, 2013-7.

Porter, R. R. (1959). The hydrolysis of Rabbit g-globulin and antibodies with crystalline papain, Biochemical Journal *73*, 119-126.

Prak, E. L., and Weigert, M. (1995). Light chain replacement: a new model for antibody gene rearrangement, J Exp Med *182*, 541-8.

Radic, M. Z., Erikson, J., Litwin, S., and Weigert, M. (1993). B lymphocytes may escape tolerance by revising their antigen receptors, J Exp Med *177*, 1165-73.

Rathmell, W. K., and Chu, G. (1994). A DNA end-binding factor involved in double-strand break repair and V(D)J recombination., Mol Cell Biol *14*, 4741-8.

Reichlin, A. (2001). B cell development is arrested at the immature B cell stage in mice carrying a mutation in the cytoplasmic domain of immunoglobulin beta, Journal of Experimental Medicine *193*, 13-23.

Remenyi, A., Tomilin, A., Pohl, E., Lins, K., Philippsen, A., Reinbold, R., Scholer, H. R., and Wilmanns, M. (2001). Differential dimer activities of the transcription factor Oct-1 by DNA-induced interface swapping, Molecular Cell *8*, 569-80.

Retter, M. W., and Nemazee, D. (1998). Receptor editing occurs frequently during normal B cell development, J Exp Med *188*, 1231-8.

Revy, P., Muto, T., Levy, Y., Geissmann, F., Plebani, A., Sanal, O., Catalan, N., Forveille, M., Dufourcq-Labelouse, R., Gennery, A., *et al.* (2000). Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). [see comments], Cell *102*, 565-75.

Ritchie, K. A., Brinster, R. L., and Storb, U. (1984). Allelic exclusion and control of endogenous immunoglobulin gene rearrangement in kappa transgenic mice, Nature *312*, 517-20.

Rolink, A., Melchers, F., and Andersson, J. (1996). The SCID but not the RAG-2 gene product is required for S mu-S epsilon heavy chain class switching., Immunity *5*, 319-330.

Romanow, W. J. (2000). E2A and EBF act in synergy with the V(D)J recombinase to generate a diverse immunoglobulin repertoire in nonlymphoid cells, Molecular Cell *5*, 343-53.

Roth, D. B., Menetski, J. P., Nakajima, P. B., Bosma, M. J., and Gellert, M. (1992). V(D)J recombination: broken DNA molecules with covalently sealed (hairpin) coding ends in scid mouse thymocytes, Cell *70*, 983-91.

Rusconi, S., and Kohler, G. (1985). Transmission and expression of a specific pair of rearranged immunoglobulin mu and kappa genes in a transgenic mouse line, Nature *314*, 330-4.

Sandel, P. C., and Monroe, J. G. (1999). Negative selection of immature B cells by receptor editing or deletion is determined by site of antigen encounter, Immunity *10*, 289-99.

Sawchuk, D. J., Weis-Garcia, F., Malik, S., Besmer, E., Bustin, M., Nussenzweig, M. C., and Cortes, P. (1997). V(D)J recombination: modulation of RAG1 and RAG2 cleavage activity on 12/23 substrates by whole cell extract and DNA-bending proteins, J Exp Med *185*, 2025-32.

Schatz, D. G., and Baltimore, D. (1988). Stable expression of immunoglobulin gene V(D)J recombinase activity by gene transfer into 3T3 fibroblasts, Cell *53*, 107-15.

Schatz, D. G., Oettinger, M. A., and Baltimore, D. (1989). The V(D)J recombination activating gene, RAG-1., Cell *59*, 1035-1048.

Schlissel, M. S., and Baltimore, D. (1989). Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription, Cell *58*, 1001-7.

Schubart, D. B., Rolink, A., Kosco-Vilbois, M. H., Botteri, F., and Matthias, P. (1996). B-cell-specific coactivator OBF-1/OCA-B/Bob1 required for immune response and germinal centre formation, Nature *383*, 538-42.

Schubart, D. B., Rolink, A., Schubart, K., and Matthias, P. (2000). Cutting edge: lack of peripheral B cells and severe agammaglobulinemia in mice simultaneously lacking Bruton's tyrosine kinase and the B cell-specific transcriptional coactivator OBF-1, Journal of Immunology *164*, 18-22.

Serwe, M., and Sablitzky, F. (1993). V(D)J recombination in B cells is impaired but not blocked by targeted deletion of the immunoglobulin heavy chain intron enhancer, EMBO Journal *12*, 2321-7.

Shimizu, A., Takahashi, N., Yaoita, Y., and Honjo, T. (1982). Organization of the constant-region gene family of the mouse immunoglobulin heavy chain, Cell *28*, 499-506.

Shlomchik, M. J., Aucoin, A. H., Pisetsky, D. S., and Weigert, M. G. (1987). Structure and function of anti-DNA autoantibodies derived from a single autoimmune mouse, Proceedings of the National Academy of Sciences of the United States of America *84*, 9150-4.

Snapper, C. M., Finkelman, F. D., and Paul, W. E. (1988). Differential regulation of IgG1 and IgE synthesis by interleukin 4, J Exp Med *167*, 183-96.

Sonoda, E., Pewzner-Jung, Y., Schwers, S., Taki, S., Jung, S., Eilat, D., and Rajewsky, K. (1997). B cell development under the Condition of Allelic Inclusion, Immunity *6*, 225-233.

Stanhope-Baker, P., Hudson, K. M., Shaffer, A. L., Constantinescu, A., and Schlissel, M. S. (1996). Cell type-specific chromatin structure determines the targeting of V(D)J recombinase activity in vitro, Cell *85*, 887-97.

Staudt, L. M., and Lenardo, M. J. (1991). Immunoglobulin gene transcription, Annual Review of Immunology *9*, 373-98.

Staudt, L. M., Singh, H., Sen, R., Wirth, T., Sharp, P. A., and Baltimore, D. (1986). A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes, Nature *323*, 640-3.

Stavnezer, J. (1996). Immunoglobulin class switching., Current Opinion in Immunology *8*, 199-205.

Stavnezer, J., Radcliffe, G., Lin, Y. C., Nietupski, J., Berggren, L., Sitia, R., and Severinson, E. (1988). Immunoglobulin heavy-chain switching may be directed by prior induction of transcripts from constant-region genes, Proc Natl Acad Sci U S A *85*, 7704-8.

Stavnezer-Nordgren, J., and Sirlin, S. (1986). Specificity of immunoglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching, Embo J *5*, 95-102.

Stiernholm, N. B., and Berinstein, N. L. (1995). A mutated promoter of a human Ig V lambda gene segment is associated with reduced germ-line transcription and a low frequency of rearrangement, Journal of Immunology *154*, 1748-61.

Strubin, M., Newell, J. W., and Matthias, P. (1995). OBF-1, a novel B cell-specific coactivator that stimulates immunoglobulin promoter activity through association with octamer-binding proteins, Cell *80*, 497-506.

Taccioli, G. E., Gottlieb, T. M., Blunt, T., Priestly, A., Demengeot, J., Mizuta, R., Lehmann, A. R., Alt, F. W., Jackson, S. P., and Jeggo, P. A. (1994). Ku80: product of the XRCC5 gene and its role in DNA repair and V(D)J recombination., Science *265*, 1442-5.

Thiebe, R., Schable, K. F., Bensch, A., Brensing-Kuppers, J., Heim, V., Kirschbaum, T., Mitlohner, H., Ohnrich, M., Pourrajabi, S., Roschenthaler, F., *et al.* (1999). The variable genes and gene families of the mouse immunoglobulin kappa locus, Eur J Immunol *29*, 2072-81.

Tiegs, S. L., Russell, D. M., and Nemazee, D. (1993). Receptor editing in self-reactive bone marrow B cells, J Exp Med *177*, 1009-20.

Tiselius, A., and Kabat, A. (1939). An electrophoretic study of immune sera and purified antibody preparations, Journal of Experimental Medicine *69*, 119-131.

Tomilin, A., Remenyi, A., Lins, K., Bak, H., Leidel, S., Vriend, G., Wilmanns, M., and Scholer, H. R. (2000). Synergism with the coactivator OBF-1 (OCA-B, BOB-1) is mediated by a specific POU dimer configuration, Cell *103*, 853-64.

Tonegawa, S. (1983). Somatic generation of antibody diversity, Nature *302*, 575-81.

Tonegawa, S., Brack, C., Hozumi, N., and Schuller, R. (1977). Cloning of an immunoglobulin variable region gene from mouse embryo, Proc Natl Acad Sci U S A *74*, 3518-22.

Torres, R. M., Flaswinkel, H., Reth, M., and Rajewsky, K. (1996). Aberrant B cell development and immune response in mice with a compromised BCR complex [see comments], Science *272*, 1804-8.

Tsao, B. P., Chow, A., Cheroutre, H., Song, Y. W., McGrath, M. E., and Kronenberg, M. (1993). B cells are anergic in transgenic mice that express IgM anti-DNA antibodies, Eur J Immunol *23*, 2332-9.

Tsukamoto, A., Weissman, I. L., and Hunt, S. V. (1984). Allelic exclusion in rat kappa immunoglobulin chains: extent of Jk rearrangement in normal B lymphocytes, Embo J *3*, 975-81.

von Schwedler, U., Jack, H. M., and Wabl, M. (1990). Circular DNA is a product of the immunoglobulin class switch rearrangement., Nature *345*, 452-456.

Wasserman, R., Li, Y. S., and Hardy, R. R. (1997). Down-regulation of terminal deoxynucleotidyl transferase by Ig heavy chain in B lineage cells, J Immunol *158*, 1133-8.

Weigert, M., Gatmaitan, L., Loh, E., Schilling, J., and Hood, L. (1978). Rearrangement of genetic information may produce immunoglobulin diversity, Nature *276*, 785-90.

Weigert, M. G., Cesari, I. M., Yonkovich, S. J., and Cohn, M. (1970). Variability in the lambda light chain sequences of mouse antibody, Nature *228*, 1045-7.

Whitehurst, C. E., Schlissel, M. S., and Chen, J. (2000). Deletion of germline promoter PD beta 1 from the TCR beta locus causes hypermethylation that impairs D beta 1 recombination by multiple mechanisms, Immunity *13*, 703-14.

Wirth, T., Staudt, L., and Baltimore, D. (1987). An octamer oligonucleotide upstream of a TATA motif is sufficient for lymphoid-specific promoter activity, Nature *329*, 174-8.

Wu, T. T., and Kabat, E. A. (1970). An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity, J Exp Med *132*, 211-50.

Wuerffel, R. A., Du, J., Thompson, R. J., and Kenter, A. L. (1997). Ig Sgamma3 DNA-specifc double strand breaks are induced in mitogen- activated B cells and are implicated in switch recombination, J Immunol *159*, 4139-44.

Xu, Y., Davidson, L., Alt, F. W., and Baltimore, D. (1996). Deletion of the Ig kappa light chain intronic enhancer/matrix attachment region impairs but does not abolish V kappa J kappa rearrangement, Immunity *4*, 377-85.

Yamagami, T., ten Boekel, E., Andersson, J., Rolink, A., and Melchers, F. (1999a). Frequencies of multiple IgL chain gene rearrangements in single normal or kappaL chain-deficient B lineage cells, Immunity *11*, 317-27.

Yamagami, T., ten Boekel, E., Schaniel, C., Andersson, J., Rolink, A., and Melchers, F. (1999b). Four of five RAG-expressing JCkappa-/- small pre-BII cells have no L chain gene rearrangements: detection by high-efficiency single cell PCR, Immunity *11*, 309-16.

Yancopoulos, G. D., and Alt, F. W. (1985). Developmentally controlled and tissue-specific expression of unrearranged VH gene segments, Cell *40*, 271-81.

Yancopoulos, G. D., DePinho, R. A., Zimmerman, K. A., Lutzker, S. G., Rosenberg, N., and Alt, F. W. (1986). Secondary genomic rearrangement events in pre-B cells: VHDJH replacement by a LINE-1 sequence and directed class switching., EMBO *5*, 3259-3266.

Yu, W., Misulovin, Z., Suh, H., Hardy, R. R., Jankovic, M., Yannoutsos, N., and Nussenzweig, M. C. (1999). Coordinate regulation of RAG1 and RAG2 by cell type-specific DNA elements 5' of RAG2, Science *285*, 1080-4.

Zhu, C., Bogue, M. A., Lim, D.-S., and Hasty, P. R., D. B. (1996). Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates, Cell *86*, 379-89.

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