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CONTRIBUTION Á L'ANALYSE DES INTERACTIONS DE LONGUE DISTANCE DANS LE LOCUS DES CHAINES LOURDES D'IMMUNOGLOBULINES

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ABBREVIATIONS

AID: Activation-Induced cytidine Deaminase.

ATM: Ataxia telangiectasia mutated.

BCR: B cell receptor.

bp: base pair.

BSAP: B cell Specific Activator Protein.

C : Constant region.

CD40L: CD40 ligand.

CSR: Class Switch Recombination.

D: Diversity segment.

DNA : deoxyribonucleic acid.

DNA-PK : DNA- dependent protein kinase.

DNase I: deoxyribonuclease I.

DSB: double-strand break.

ELISA: enzyme-linked immuno sorbentassay.

FDC: follicular dendritic cell.

FO : follicular cells.

GC: germinal center.

GT : germline transcription.

H2AX: Histone 2A family-member X.

HS: DNase I hypersensitive site.

IgH: immunoglobulin heavy chain.

IgL: immunoglobulin light chain.

IL: Interleukin.

J: Joining segment.

Kb: kilobase;

LCR: locus control region.

LPS: lipopolysaccharide.

MAR: matrix attachment region.

MMR: mismatch repair.

MZ: marginal zone.

NBS1: Nijmegen Breakage Syndrome protein.

NHEJ: non homologous end joining.

Pgk: Phsphoglycerate Kinase promoter.

Poly A: polyadenylation site.

RAG : recombination activation gene.

RNA : ribonucleic acid.

RS: recombination signals.

RT-PCR: reverse-transcriptase polymerase chain reaction;

S: Switch region.

SHM: somatic hypermutation.

STAT6 : Signal Transducer and Activator of Transcription factor.

TD : T dependent.

TdT:deoxynucleotidyl transferase.

TGF: Tumor growth factor.

TI : T independent.

TNF: tumor necrosis factor.

UNG: uracil deglycosylase.

V: Variable region.

XRCC4: X-ray Repair Cross-Complementing protein 4.

INTRODUCTION

The production of specific antibodies by mature B cells involves three separate types of Ig rearrangements. During early B cell development, IgH and IgL variable region exons are assembled through a process called V(D)J recombination. In response to antigen, mature B cells are capable to undergo two additional forms of genetic modifications that enable them to recognize and to respond to the cognate antigen. Somatic hypermutation (SHM) introduces a high rate mutations into the V region exons of both the IgH and IgL chains and allows the selection of B cells with receptors that have high affinity for a given antigen. IgH class switch recombination (CSR) adjoins a rearranged VDJ gene to one of the several downstream C_H genes through the deletion of intervening germline DNA sequences. This allows expression of an antibody with the same antigen binding specificity but with altered C_H effector function.

CSR is preceded by a process called germline transcription (GT). GT and subsequent CSR are controlled by distant cis-regulatory elements in the IgH locus. In order to understand the mechanism of interaction between these elements, we produced mutant mice bearing neomycin resistance gene (neo^r) in different sites of the IgH locus.

1. Immunoglobulin genes:

The immunoglobulins are the principal effectors in the humoral immune response. They are composed of a pair of heavy and a pair of light chains and within each chain, there is a V and a C region. In the mouse there are two sub-types for light chain constant regions: κ and λ, while there are different isotypes of C_H: μ, δ, γ3, γ1, γ2b, γ2a, ε, and α. The variable region of the immunoglobulin binds specifically to antigens and the constant region mediates the effector functions of an antibody (figure 1).

The Ig molecule is encoded by three different loci in both human and mice. The light chains are encoded by κ and λ loci, whereas the C_H is encoded by IgH locus.

1.1. Heavy chain genes:

The murine IgH locus consists of many V gene segments distributed throughout an approximate 1-megabase region beginning about 100 kb upstream of Cμ on chromosome 12. The heavy chain also consists of four J gene segments, and 13 known D gene segments are dispersed between the V_H and J_H gene segments. The entire murine C_H locus spans approximately 200 kb, and it consists of eight C_H genes that are organized as follows: 5'-JH-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3' (figure 2)

The human C_H gene family is mapped to the chromosome 14. Its organization differs from that of mouse in that a Cγ-Cγ-Cε-Cα unit is duplicated downstream of the Cμ- Cδ genes. There are also three pseudogenes with two of them being located in the same locus and one translocated to chromosome 9. The nine functional genes and the two pseudogenes are organized as follows : 5'- JH-Cμ-Cδ-Cγ3-Cγ1-ψCε-Cα1-ψCγ-Cγ2-Cγ4-Cε-Cα2-3' (Figure 2).

1.2. Light chains genes:

The murine Igκ locus is located on chromosome 6 and contains about 140 Vκ gene segments that can rearrange to 1 of 4 functional Jκ gene segments positioned

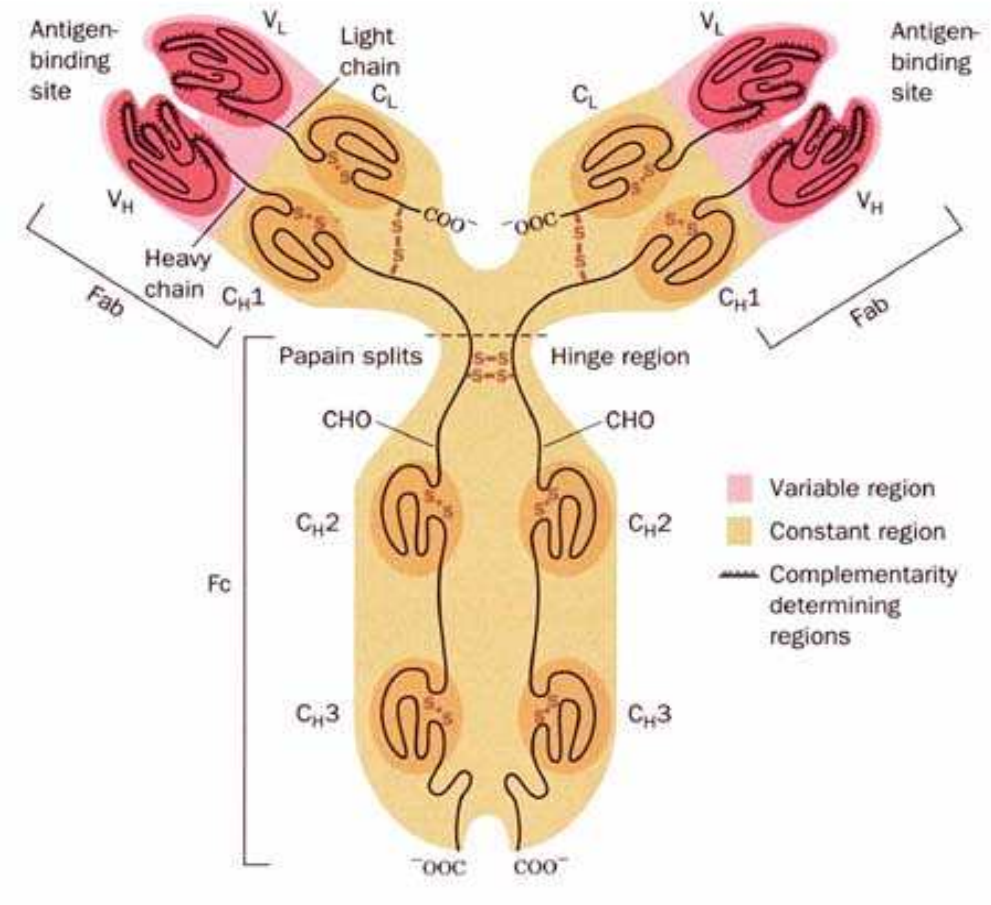


Figure 1. Immunoglobulin structure

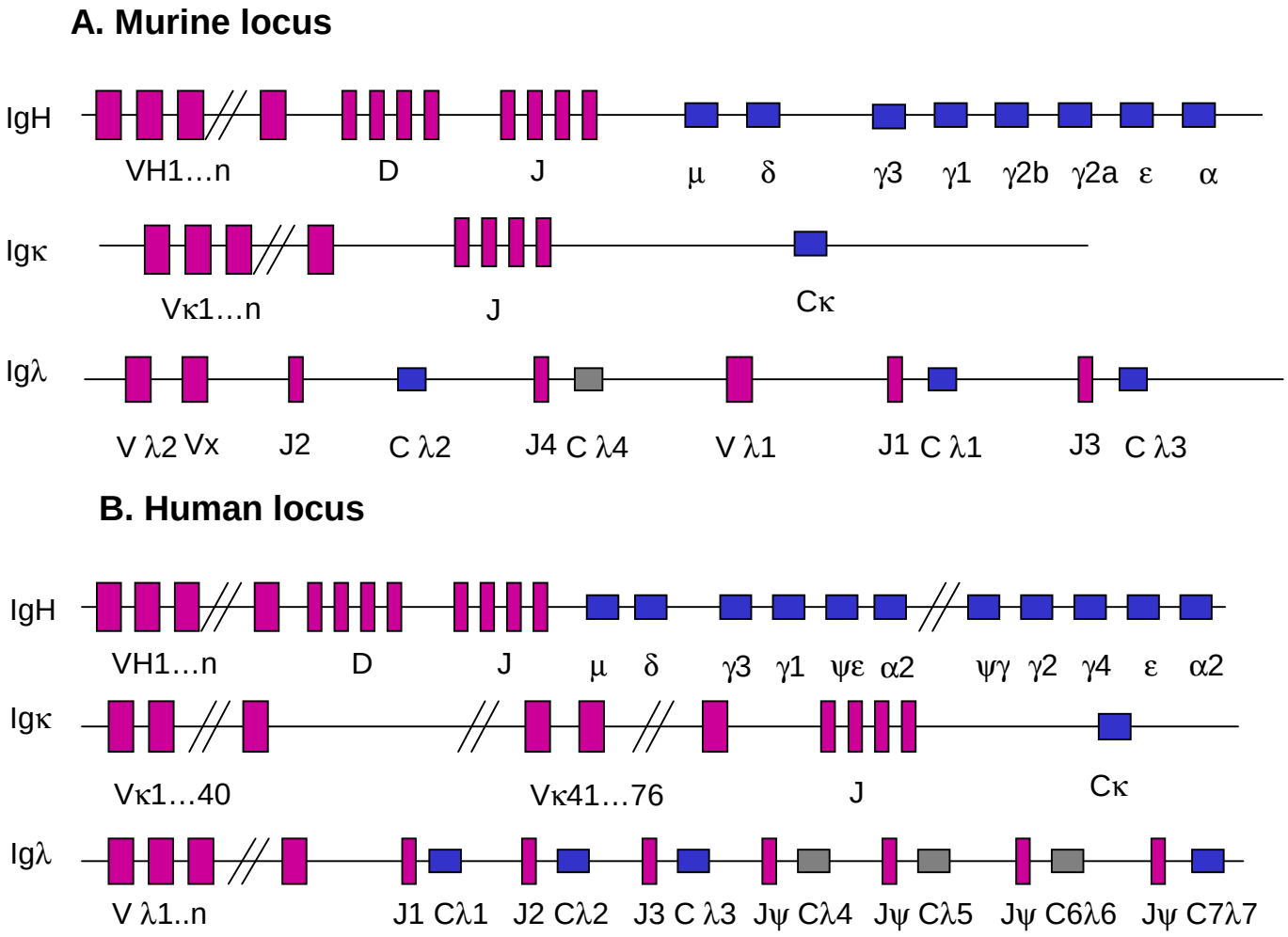


Figure 2. Immunoglobulin gene loci. Violet color represent the V region genes, the blue represent the constant region genes, and the grey represents the nonfunctional genes.

just upsream of a single Cκ gene. There is also one nonfunctional Jκ gene segment (Figure 2).

The murine Igλ locus is mapped on chromosome 16 and has only three functional Vλ gene segments. There are three functional and one nonfunctional Cλ genes. Two of the Vλ gene is positioned upstream of only the two 3'-most Jλ-Cλ units and therefore is restricted in potential rearrangements (figure 2).

1.3. VDJ recombination:

During B cell development the Ig gene loci go through several steps of rearrangements that allows assembly of functional antibody. V(D)J recombination assembles the gene segments coding for the V antigen-binding part of B and T cell receptors (figure 3). This process takes place in developing B cells in the bone marrow and does not depend on antigen. V(D)J recombination provides the immune system with the ability to respond to a very large number of antigens .

The V regions of the Ig are encoded by different V, D (for IgH chain), and J segments that are flanked by recombination signals (RS) sequences. RSs consist of a relatively conserved heptamer and nanomer, separated by a nonconserved spacer whose length is either 12 or 23 base pairs (Max et al., 1979; Sakanno et al., 1970). During V(D)J recombination only gene segments flanked by an RS of different length can rearrange efficiently (Lieber et al., 1994; Feeney et al., 2000; Olaru et al., 2005).

V(D)J recombination requires the recombination activating genes 1 and 2 (Rag1 and Rag2) (Schatz et al., 1989; Oettinger et al., 1990). Rag proteins are expressed in developing lymphocytes. Absence of these proteins lead to a complete block in B and T cells development (Mombaerts et al., 1992; Shinkai et al., 1992).

Rag proteins have the ability to recognize the RS sequences and cleave them to produce DSBs (McBlane et al., 1995; van Gent et al., 1995) (Figure 4). DNA cleavage products are treated differently. Signal ends are precisely joined, while

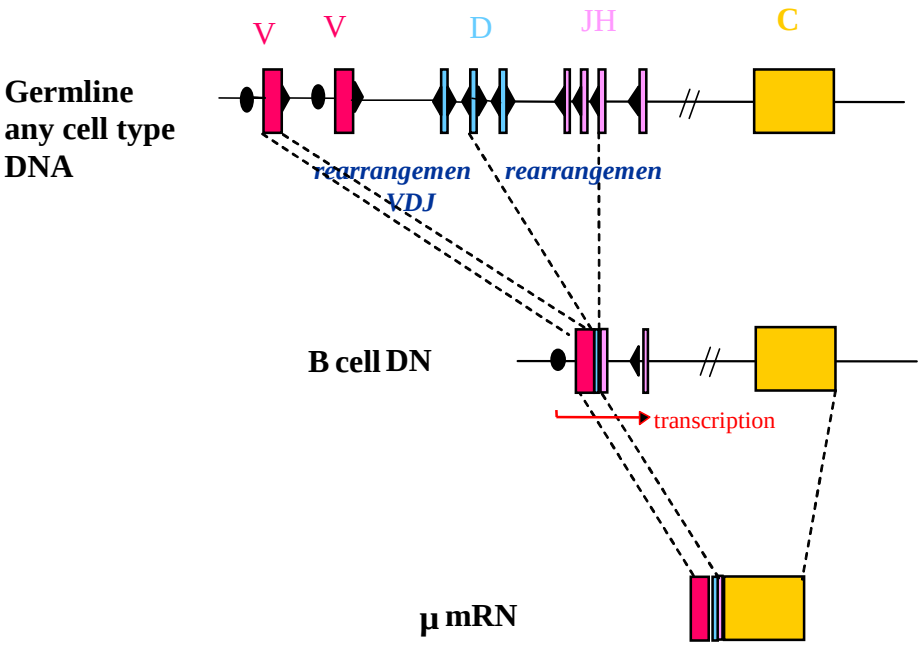


Figure 3. Organization and rearrangement of heavy-chain variable region. In the germ line, the IgH locus is organized with several variable (V_H) regions (each V region is preceded by a promoter (black oval)) followed by the diversity regions (D) (specific of the IgH locus), the joining regions (J_H), and the constant region of the μ heavy chain (Cμ). The V_H, D and J_H gene segments have recognition signals (black triangles) required for V(D)J recombination. In the first stage of rearrangement, a D segment is joined to a J_H segment with deletion of the intervening DNA. The DJ_H assembly is followed by a rearrangement that joins a V_H segment to the DJ_H leading to the formation of a V_HDJ_H heavy chain variable region.

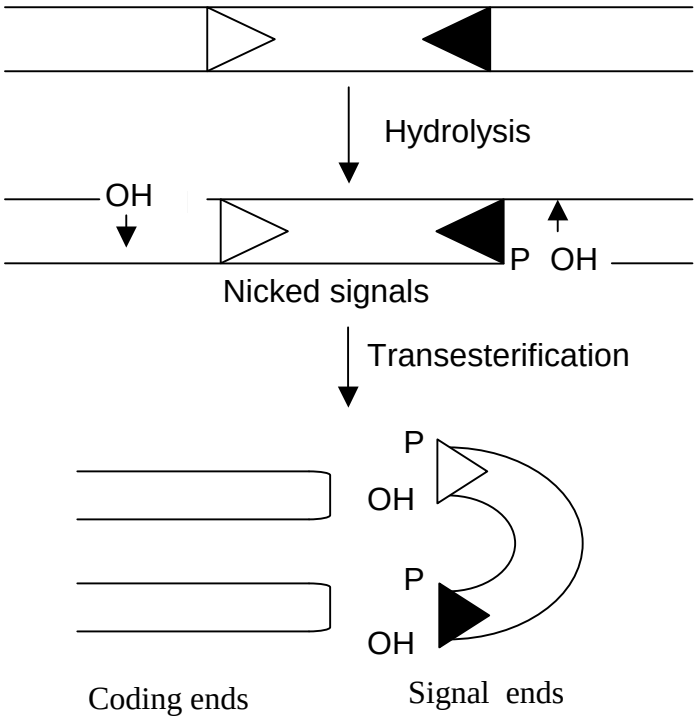


Figure 4. Rag mediated DNA cleavage. (Jones and Gellert, 2004).

coding ends are imprecisely joined via loss of nucleotides and addition of nontemplate “N” nucleotides at the junctions of segments thus increasing the diversity of Ig binding specificities (De and Rodgers, 2004; Chatterji et al., 2001; Alt and Baltimore, 1982). The DNA-binding proteins HMG1 and HMG2 promote Rag-mediated cleavage (Swanson et al., 2002a; van Gent et al., 1997).

NHEJ is the process by which the cleaved coding and RS segments generated by Rag proteins are joined and repaired. Several proteins are involved in NHEJ. Ku70-Ku80 proteins form DNA end binding heterodimers that recruit and activate DNA-PKcs. DNA-PKcs activates Artemis, which is necessary to cleave hairpins that are formed at coding ends of recombination junctions. XRCC4 and DNA ligase 4 form the ligation complex, which ligates DNA ends (Roth, 2003; Bassing et al., 2002; Jackson, 2002; Khanna and Jackson, 2001; Gellert et al., 2002) (figure 5).

2. B Cell Development:

Depending on the exposure to Ag, B cell development is divided into two phases. First, The Antigen-independent phase, which occurs in the bone marrow and results in the formation of immature B cells which express on their surfaces Ig that are able to respond to an antigen. The second phase is Ag-dependent and occurs in the secondary lymphoid organs, where a series of events take place following an encounter with Ag.

2.1. Antigen-independent phase:

B cells, like other cells of the immune system, develop from pluripotent haemopoietic stem cells which are normally found in the liver during mid-to-late fetal development and in bone marrow after birth. B cell differentiation in the bone marrow is characterized by successive rearrangements of the Ig genes that are necessary to produce a functional antigen receptor (figure 6).

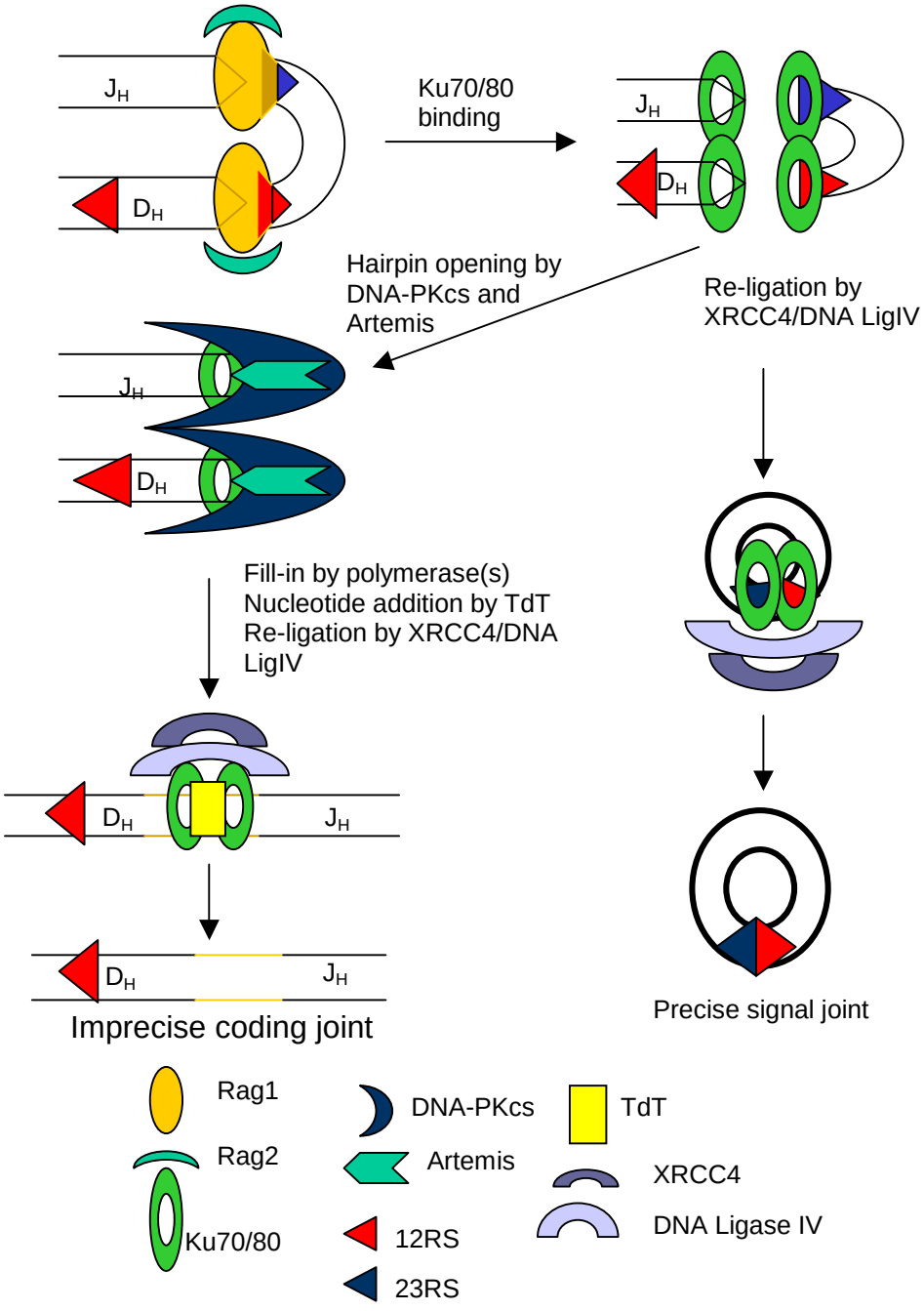


Figure 5. VDJ recombination and the NHEJ pathway. (Jung and Alt, 2004)

2.1.1. Pre-Pro-B cells:

The earliest committed B cell precursors are pre-pro-B cells. They express the surface markers B22O (CD45R), CD43 and c-kit (Hardy et al., 1991; Rollink et al., 1996a). They are characterized by low expression of Rag-1 and Rag-2 genes (Schatz et al., 1989; Oettinger et al., 1990)

2.1.2. Pro-B cells :

This stage is characterised by the initiation V(D)J rearrangement. The enzymes Rag and TdT are strongly expressed. These cells express on their surfaces B220, CD43, and c-kit.

Ig rearrangements start at the heavy chain locus with the juxtaposition of one of the D segments to one of the J segments on each chromosome (DJ_H recombination) (Ehlich et al., 1994; ten Boekel et al., 1995).

Pro-B cells are also the first B lineage cells to express a precursor form of the B cell receptor (BCR) . Pro-BCR is composed of Igα, Igβ, and the transmembrane protein calnexin (pro-BCR) (Nagata et al., 1997). Igα and Igβ are the signaling components of the BCR throughout B cell development, this feature depends on an immunoreceptor tyrosine-based activation motif (ITAM) .

2.1.3. Pre-B cells:

After DJ_H rearrangement, V_H becomes accessible to the V(D)J recombinase. V(D)J recombination enables pro-B cells to differentiate into pre-B cells which express μ heavy chain gene. Production of Igμ (the first isotype to be expressed) results in the assembly of the pre-BCR and marks the pre-B cell stage. The pre-BCR is composed of membrane Igμ, associated with pseudo-light chains, Vpre-B and λ5 (

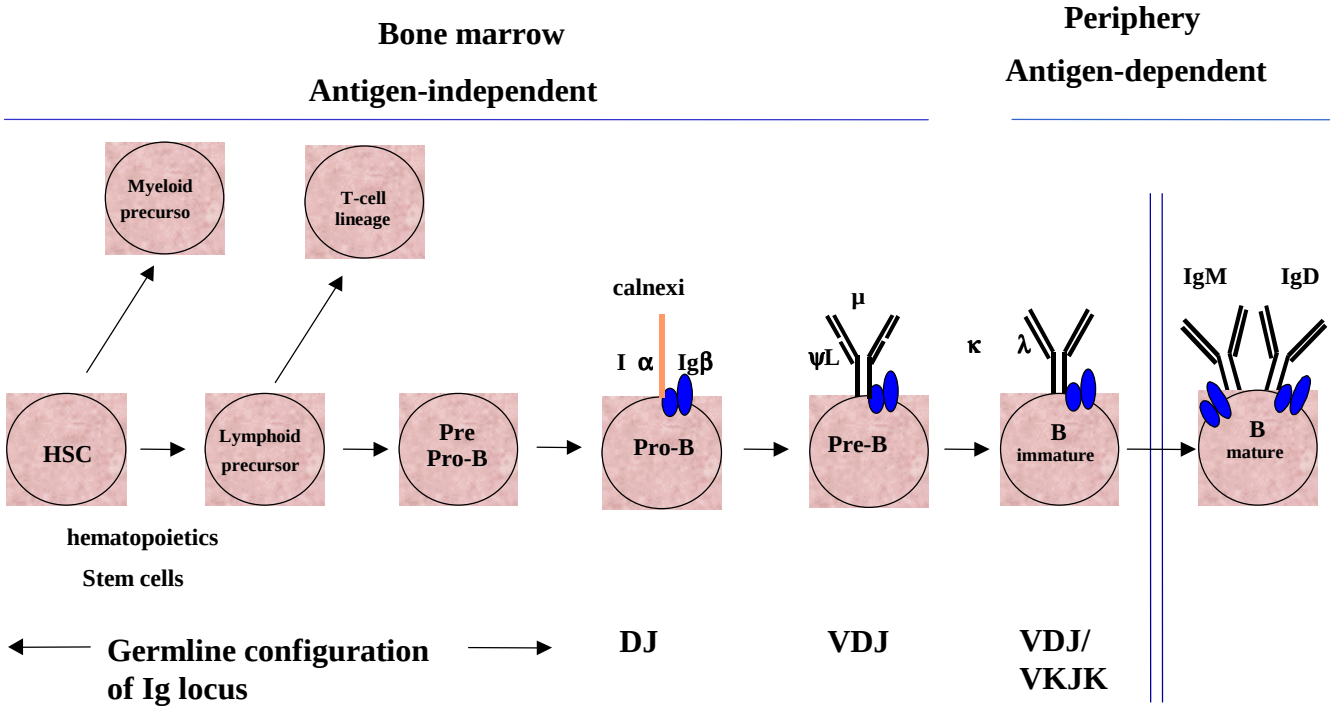


Figure 6. B cell development scheme. Developmental stages of B lymphopoiesis, rearrangements on both heavy and light chain loci and surface expression of pro-B, pre-B, or B cell receptors are shown. ψL stands for surrogate light chains (Meffre et al., 2000).

called surrogate light chains), Igα and Igβ (Karasuyama et al., 1990; Tsubata et al., 1990).

The pre-BCR is considered as a key check-point that regulates B cell development. It plays a role in the following processes: B cell differentiation, clonal expansion, and heavy chain allelic exclusion .

The "ordered model" suggests that gene rearrangements are initiated at the IgH locus, with $D>>J_H$ followed by $V_H>>DJ_H$ rearrangement. If a V_HDJ_H joint is productive (i.e in frame), a μ heavy chain is produced leading to a pre-B cell. The ordered model postulates that surface expression of μ-chain triggers the termination of rearrangements at the other IgH locus and the initiation of rearrangements at the light chain loci. Here again, an order of rearrangements (κ before λ) was postulated (Rajewsky, 1996).

Expansion of pre-B cells leads to light chain recombination. When the cell would have acquired a productive V_LL_L joint, it would express a light chain in addition to its μ heavy chain, and rearrangements would be terminated, leading to the replacement of surrogate light chains in the pre-BCR by Igκ or Igλ. The result is a transition from pre-B cells to immature B cells .

2.1.4. Immature B cells:

These cells are characterized by light chain expression and further production of monospecific BCRs composed of surface IgM (μ and κ or λ) and Igα and Igβ. Immature B cells are the first cells of the B lineage to express surface BCRs, they also express IgM but little or no IgD.

The immature B cell stage has critical importance to the immune system, since antigen-specific positive and negative selections occur during this stage. Negative selection allows the elimination of immature B cells expressing on their surfaces a BCR with high affinity for autoantigens. This event occurs during the transition of immature B cells from the bone marrow to the spleen (Rolink et al., 2004). Anergy is another fate of these self-reactive B cells . Anergic B cells are

short-lived, and have difficulty in developing from immature to long-lived B cells (Bell et al., 1994).

Immature B cells that emigrate to the periphery are refered to as transitional B cells. These cells are short-lived and small percentage of them reach the long-lived mature peripheral B cell compartment.

2.2. Antigen-dependent phase

2.2.1. Mature B cells

The transition of immature into mature B cells depend on BCR signaling and the ability to respond to locally produced growth factors.

Mature B cells are divided into follicular and marginal zone B cells, according to their surface markers and splenic localisation (Allman et al., 2004).

MZ B are specific to TI antigens and contribute to the early primary humoral response (Srivastava et al., 2005; Fuentes-Panana et al., 2004; Cariappa and Pillai, 2002).

FO B cells are specific to TD antigens. They are the main cellular constituent of the splenic and lymph node follicles.

Another subset of mature B cells known as B1 cells are long-lived B cells that are found in the pleural and peritoneal cavities, they have self-renewal capacity and express the surface marker CD5 (Cariappa & Pillai, 2002; Pillai et al., 2004). Like MZ B cells, they are involved in TI-antigenic response and they secrete natural antibodies important for innate immunity (Dono et al., 2004).

2.2.2. Plasma Cells:

Following an initial exposure to TD antigens, mature B cells differentiate into antibody-secreting cells called plasma cells. Two types of plasma cells develop, the short-lived plasma cells that represent an early reaction to antigen exposure and doesn’t require a GC reaction. These cells can undergo CSR but show no evidence

for SHM. The long-lived plasma cells are a product of GC reaction and can undergo CSR and SHM. These cells do persist in the spleen, but are thought to preferentially home to the bone marrow, where they reside for long periods (McHeyzer-Williams, 2003).

3. Somatic Hypermutation

Differentiation of B lymphocytes in the periphery achieves several goals. After the first encounter with an antigen mature B cells are able to undergo mutations at a high rate (10^{-4} to 10^{-3} per base pair per generation) into their assembled variable region exon sequences via a process called Somatic hypermutation (SHM). SHM allows production of antibodies with high affinity or avidity for the encountered antigen a process called “affinity maturation”.

3.1. Clonal Selection:

It is known that a number of antigens can activate B cells to proliferate and differentiate into antibody-secreting cells through a TD response. TD response allows the formation of unique structures called germinal centers (GCs) (figure 7). GCs provide specialized microenvronment where antibodies of high affinity and specificity are produced. GCs are considered as the site of B cell expansion, somatic hypermutation, isotype switching, affinity maturation, apoptosis, plasma cell commitment and memory cell formation (Cozine et al., 2005).

GCs are divided into two zones: the dark and the light zones. The dark zone contains densely packed large lymphoid cells called centroblasts, many of which are in cell cycle; only fine FDC processes penetrate this area. The light zone contains small lymphoid cells and less closely packed called centrocytes that are separated by a dense FDC network.

The presence of FDCs is important for the GCs since they optimise B-cell response and survival. FDCs exert their effect by the adhesion of CD54 and VCAM-1 on the FDC with CD11a/CD18 and VLA/4 on centrocytes (Koopman et al., 1991).

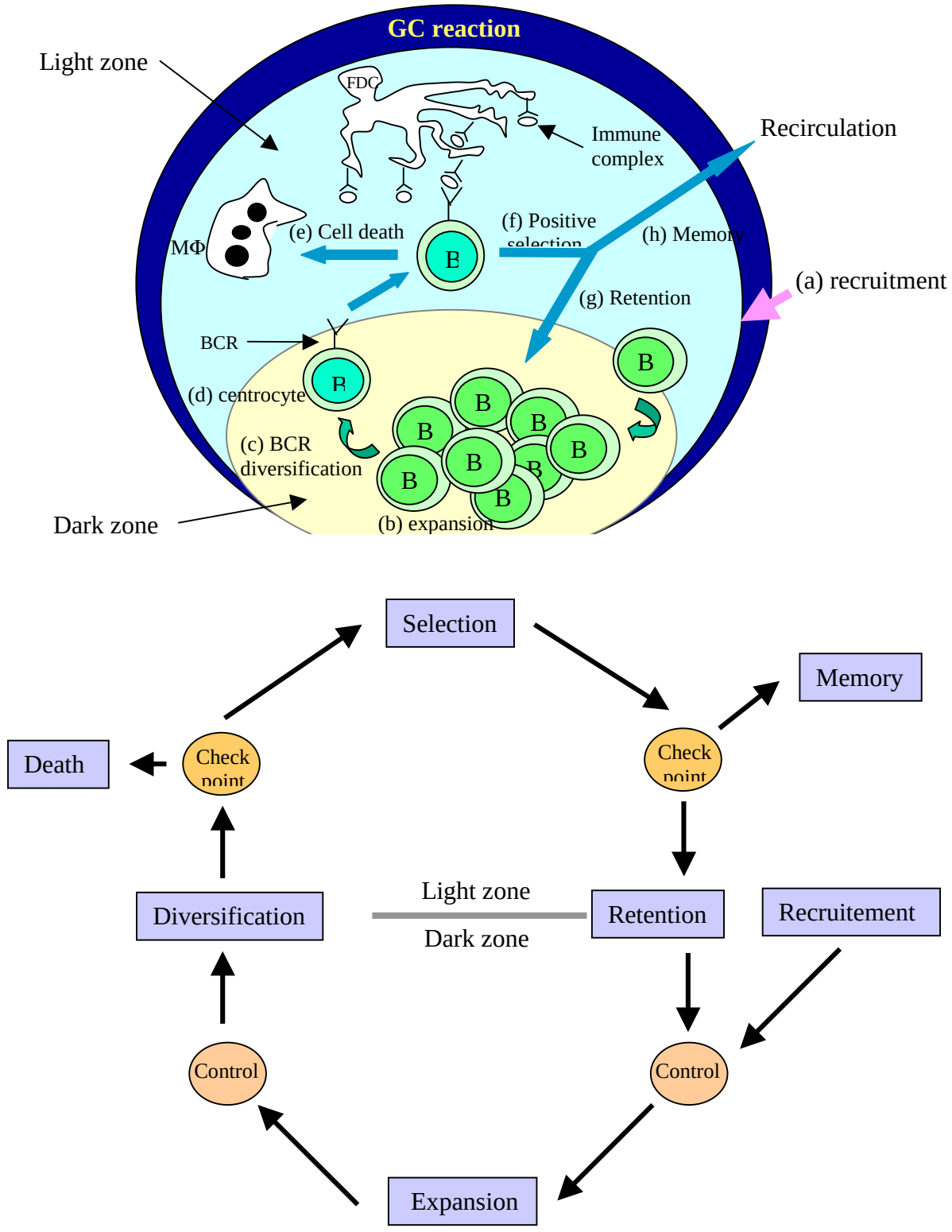


Figure 7. Germinal center reaction. The upper scheme shows the different zones of the GC, while the lower scheme shows the steps of B cells multiplication and selection. B, B lymphocytes; FDC, follicular dendritic cells; MΦ, macrophages (McHeyzer-Williams and Ahmed, 1999).

B cell-Th cell contact is also crucial for GC reaction and B cell activation. This contact is modulated by several cell surface molecules and cytokines. CD40 ligand-mediated signals are important for GC formation and differentiation of GC B cells. (Arpin et al., 1995). Long term survival of germinal center B cells can be achieved with either anti-CD40 monoclonal antibody or CD40 ligand (Holder et al., 1993).

Although the GC reaction is a TD antigen response, some TI antigens can induce GC formation, but these GCs promote low levels of SHM and are terminated early before maturation in the absence of T cell help (Toellner et al., 2002).

3.2. Mechanism of SHM:

The SHM is another manner for the B cell to further diversify the variable region of Ig. In contrast to V(D)J rearrangements, SHM is induced after antigen challenge and occurs inside the centroblasts of GCs. Somatic mutations consist of single nucleotide substitutions with rare deletions or insertions (Wilson et al., 1998). These mutations occurr in the V region, beginning ~150 to 200 bp downstream of the promoter, and extend ~1.5 kb further downstream, ending before the intronic enhancer (Eμ) and sparing the C region (Rada and Milstein 2001). The SHM shows preference for RGYW motives (R=A or G, Y=C or T, W=A or T) in the complementarity determining region (CDR), these motives referred as hotspots (Foster et al., 1999, Jacobs and Bross, 2001).

A number of experimental observations suggest that there is a connection between transcription and somatic hypermutation and both of them are cotrolled by cis-regulatory elements in the Ig locus.

Several studies showed that V_H promoters could be replaced by heterologous promoters, implying that promoters are not essential for hypermutation (Betz et al,. 1994). Results obtained using transgenic systems implied 3'Eκ, iEκ/MAR, IgH locus Eμ, and distal HS3b/HS4 enhancers as important elements for SHM targeting to transgenic constructs (Betz et al., 1994, Kodama et al., 2001, Lin et al,. 1998a; Terauchi et al., 2001). However, deletions of either 3'Eκ (vander Stoep et al., 1998)

or HS3b/HS4 IgH locus enhancers (Morvan et al., 2003) had no effect on SHM in mouse endogenous Ig loci.

SHM involves generation of single and double strand breaks (DSBs) (Papavasiliou and Schatz, 2000; Sale and Neuberger, 1998). Both blunt ended and resected DSBs can be detected in V_H regions. However, only resected DSBs are likely to be crucial for SHM (Zan et al., 2003). DSBs are probably repaired through the action of a subset of error-prone DNA polymerases and result in the production of point mutation (Gearhart and Wood, 2001).

Recent works showed that SHM required the activation-induced deaminase (AID). The exact role of AID in SHM is not yet clearly established. It is possible that AID is editing mRNA of some endonuclease that attacks blunt DNA ends, or it might directly deaminate DNA. (details about AID function will be discussed in Ig class switching section).

4. Class Switch Recombination (CSR)

Production of different isotypes of Abs is crucial for the generation of proper immune response. CSR allows the expression of antibodies that have the same antigen specificity but are of a secondary IgH isotype (IgG, IgA or IgE) and thereby have a different effector function. CSR involves a recombination between Sμ (donor site) and any downstream S region sequences, with deletion of the intervening DNA sequences. CSR has been shown to generate two products: the rearranged chromosome and an extrachromosomal circle containing deleted intervening sequences. Such recombination products can easily be explained by a simple cut-and-join mechanism (figure 8).

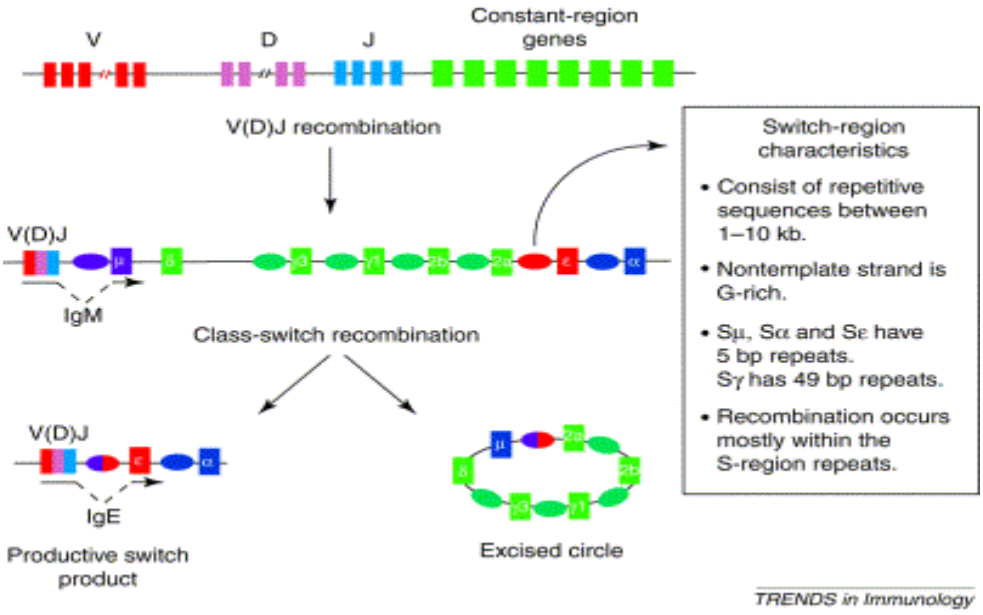


Figure 8. Mechanism of CSR. CSR occurs by recombination between Sμ and any downstream S region with deletion of intervening DNA sequence. Abbreviations: D, diversity; J, joining; S, switch; V, variable (Manis et al., 2002).

4.1. Effector Functions of Abs:

IgM can be secreted in the primary immune response upon antigen stimulation while IgG, IgE, and IgA are secreted in the secondary immune response. IgM is characterized by its ability to activate the complement efficiently.

IgGs are the most abundant Abs in serum. IgG subclasses have different specificities for antigens but all of them can cross the placenta .

IgG3 plays an important role in anti-bacterial responses and is very efficient in promoting phagocytosis.

IgG2a can activate the complement and is very efficient as a mediator of Ab-dependent cytotoxicity by binding to specific Fc receptors on macrophages.

IgG1 is the dominant isotype against parasitic and viral infections. It does not activate complement well but stimulates phagocytosis most efficiently.

IgE production is induced by parasitic infection. IgE also triggers allergy. Mast cells and basophils express high-affinity receptors for IgE (FcεRI) that can be bound by monomeric IgE in the absence of antigens. Specific antigens cause aggregation of the IgE-FcεR complex resulting in the secretion of histamine and other chemicals that mediate the immediate hypersensitivity response.

IgA plays a role in mucosal immunity. It provides an immune barrier by preventing the adherence and the absorption of antigens, neutralizing intracellular microbial pathogens directly within epithelial cells, and eliminating locally formed immune complexes by binding to the antigens and excreting them into the lumen (Zhang et al., 1995).

4.2. Factors Involved In CSR:

4.2.1. Germline Transcription(GT):

Germline transcription is a prerequiste for CSR. CSR to particular C_H gene is preceded by GT of that C_H gene.

Two models have been proposed to explain the exact role of germline transcription in CSR initiation: indirect and direct. In the term of indirect role, germline transcription is correlated with changes in chromatin structure, these changes increase the S region accessibility to factors that mediate CSR (Nambu et al., 2003; Kenter et al., 2003). The direct model argues that transcription plays a more primary role in the initiation of CSR, perhaps leading directly to the initiating event (Kenter et al., 2003; Manis et al., 2002).

Germline transcripts are normally found in activated B cells before undergoing CSR. These transcripts are sterile due to numerous stop codons and therefore are unable to encode proteins.

The transcripts run through the I exon and the S sequences and undergo polyadenylation downstream of the corresponding C_H genes. The primary germline transcript is spliced to generate a transcript containing an I exon fused with the C_H region. Splicing results in the excision of the intervening S sequences (figure 9). Experiments with knock in mice have shown that GT is biallelic (Deply et al., 2003).

The exact role of germline transcripts is not known. Gene targeting studies have shown that the I exon and/or its promoter are important for appropriate regulation of CSR (figure 10).

Deletion or replacement of I exons with a neomycin resistance gene (neo^r) suggested that the transcription of germline RNA is required for switch recombination (Zhang et al., 1993; Seidl et al., 1998). Other experiments indicated

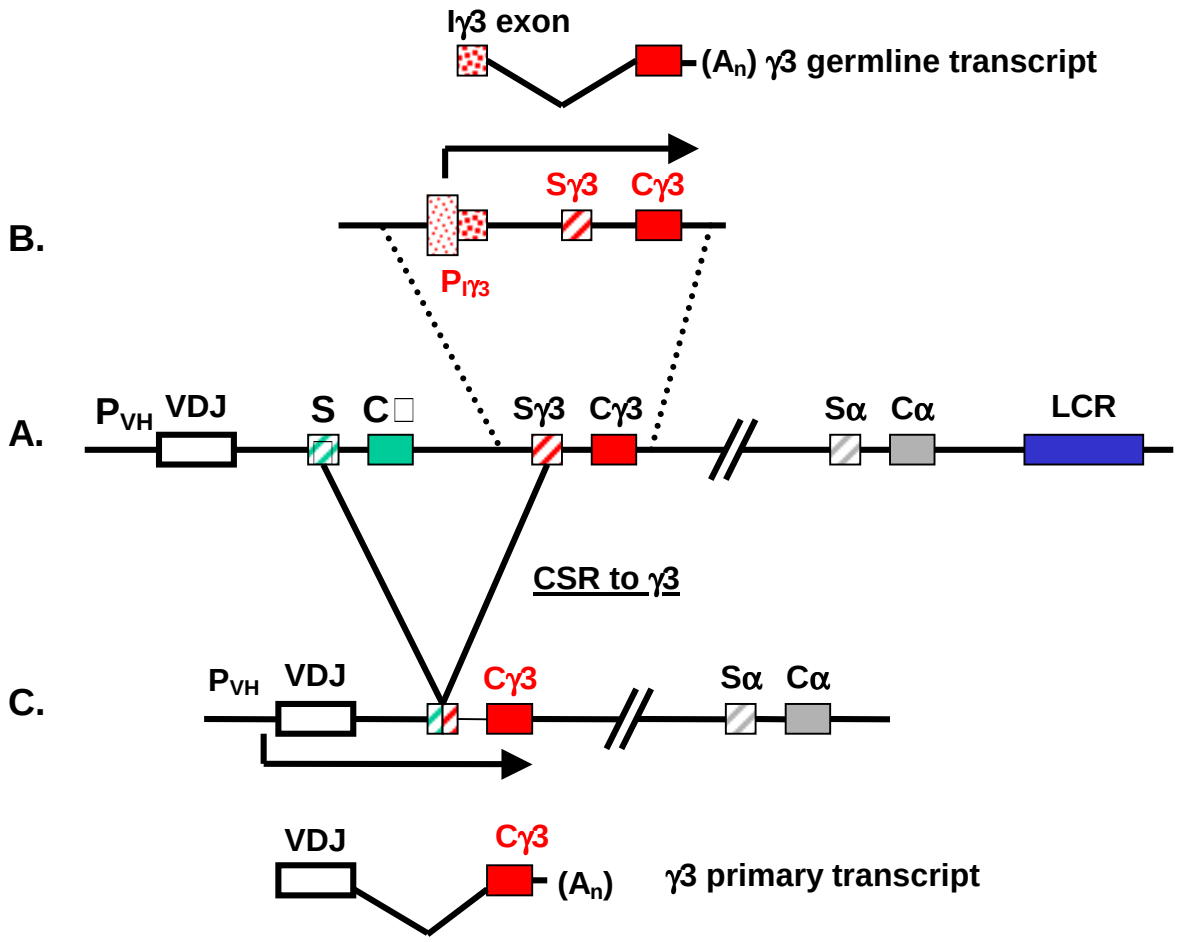


Figure 9. Germline transcription is a prerequiste to CSR.

- Scheme of rearranged μ gene. CSR of particular heavy-chain C_H genes (CSR to Cγ3 is shown here) is preceded by the induction of germline transcription of the C_H region targeted for CSR.
- Structure of the γ3 constant gene and the corresponding germline transcript. The later originates from the promoter (PIγ3) upstream of the Iγ3 exon, which precedes the Sγ3 region.
- CSR leads to deletion of the intervening DNA and transcription initiates from PVH leading to mature γ3 transcript.

that transcription alone is not sufficient for directing CSR, since replacement of the Iε exon by a heterologous strong promoter (a casette containing Eμ and a P_{VH} promoter) results in abundant unspliced transcripts but does not direct switch recombination to IgE (Bottaro et al., 1994).

Splicing of the germline transcript has been proposed to be important for CSR process, as deletion of a large portion of the Iγ1 exon including the splice donor site inhibited switching to IgG1 (Jung et al., 1993; Lorenz et al., 1995). Subsequently, it was shown that if Iγ1 exon is replaced by a metallothionine promoter, switching to IgG1does not occur. However, if the 114bp segment containing the Iγ1 splice donor site is inserted just downstream of the metallothionine promoter, switching to IgG1 is restorted (Hein et al., 1998).

It has been suggested that GT is necessary for the accessibility of S sequences to the recombinase machinery (Chaudhuri and Alt, 2004). Several studies showed that germline transcripts form RNA-DNA hybrids with the template strand (Reaban and Griffin, 1990, Reaban et al., 1994; Mizuta et al., 2003, Tian and Alt, 2000). Whereas the single-stranded, non-templated strand, form long and stable R-loops *in vivo* and *in vitro* (Yu et al., 2003) which may serve as substrates for activation-induced cystidine deaminase (Bransteiter et al., 2003; Chaudhri et al., 2003; Dickerson et al., 2003; Pham et al., 2003; Ramiro et al., 2003).

Whatever the exact mechanism, we can conclude that germline transcription generally precedes CSR, and that germline transcripts play an important role in directing CSR.

4.2.2. S Region :

CSR is targeted to S regions, these S regions are located upstream of each C_H gene (except for Cδ). CSR results from the fusion of an upstream S region to a downstream S region with the deletion of intervening DNA. Recombination points are found throughout individual S regions and can occur outside of the main S region. Therefore, unlike V(D)J recombination, CSR is a region-specific process,

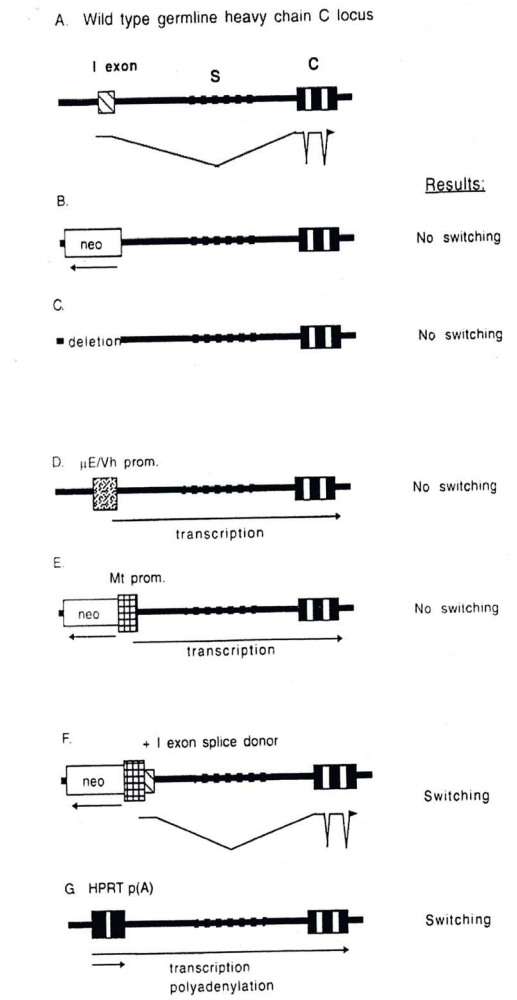


Figure 10. Summary of germline I exon knockout mutations and the results with regard CSR (Stavnezer, 1996).

occurs within introns and does not influence reading frames (Lee et al., 1998; Luby et al., 2001).

S regions are composed of repeats of pentamers (predominantly GAGCT and GGGGT) that are varying in length with some varying degrees of overall homology (table 1).

CSR break points generally lack homology or consensus junctions. This feature, together with the degeneracy of S region sequences, led to CSR models in which the S region recognition code might lie in higher-order structures, rather than primary sequences (Manis et al., 2002). In this regard, murine and human S regions are GC-rich and the nontemplate strand is highly G-rich. These properties may enable S sequences to form secondary structures such as R-loops, stem loops, G4 or as yet unidentified structures (Reaban and Griffin, 1990, Reaban et al., 1994; Daniel and Lieber, 1995; Mizuta et al., 2003, Tian and Alt, 2000; Dempsey et al., 1999).

Several targeted mutational studies have shown that the S regions are important for CSR but their precise function is still speculative. Deletion of the core tandem repeats of the Sμ sequence reduced greatly, but did not abolish, CSR in mice (Luby et al., 2001). Also complete deletion of all Sμ tandem repeats further reduced, but did not eliminate, CSR (Khamlichi et al., 2004).

The orientation of S region is also important for CSR. Inversion of Sγ1 sequence in the endogenous locus significantly decreases class switching to IgG1. On the other hand, G-rich single stranded DNA might be needed. This was confirmed by replacement of the Sγ1 region by G-rich or C-rich sequences. Only G-rich, but not C-rich sequence could support CSR (Shinkura et al., 2003). In addition, replacement of Sγ1 with synthetic or endogenous S regions with various lengths showed that the S region size is an important factor in determining the efficiency of CSR to IgG1 (Zarrin et al., 2005).

Table 1. Structural characteristics of murine switch regions (Chaudhuri and Alt, 2004).

Region	Non-template strand	Repeat length (bp)	Approximate total length of core (bp)
Sμ	GAGACTGAGCTGGGGTAGCT	10–40	3.2
Sγ3	GGGGACCAGGCTGGGGCAGCTCTNGGGGAGCTGGGGTAGGTTGGGAGTGT	49	2.5
Sγ1	GGTGACCCAGCAGAGCAGCTCCAGGGGCGCCAGGACAGGTGGAAGTGT	49	12.0
Sγ2b	AGGGGACCAGWCCTAGCAGCTRTGGGGGAGCTGGGGAWGGTGGGAATGTG	49	5.0
Sγ2a	GGGACCAGGCAGTACAGCTCTGGGTRGGGRNCAGGCAGTACAGCTCTGNGTG	52	2.5
Sε	GGGCTGGGCTGAGCTGRGCTGAGCTGRGCTGAGCTRARNT	40–50	1.0
Sα	ATGAGCTGGGATGRRCTGAGCTAGGCTGGAATAGGCTGGGCTGGGCTG GTGTGAGCTGGGTTAGGCTGAGCTGAGCTGGA	80	4.2

4.2.3. Activation-induced cytidine deaminase (AID):

The molecular mechanims of both CSR and SHM remained unknown until the discovery of AID. AID was found to be essential for these two processes in both humans and mice (Muramatsu et al., 2000; Revy et al., 2000).

AID's cDNA was isolated via a subtractive cloning screen from switch-stimulated and unstimulated murine B cell line (CH12F3-2) (Muramatsu et al., 1999). AID was foud to be expressed in germinal center B cells during an immune response (Muramatsu et al., 1999, 2000).

Ectopic expression of AID in non B cells, such as fibroblasts, hybridomas, and T cells enables them to undergo class switching and hypermutation (Okazaki et al., 2002; Martin et al., 2002; Yoshikawa et al., 2002). These results suggest that AID is the only B cell specific factor required for these two processes.

AID shares significant sequence homolgy with the RNA-editing cytidine deaminase APOBEC-1 (apolipoprotein B mRNA editing catalytic polypeptide1) (Muramatsu et al., 1999). APOBEC-1 functions as an RNA-editing enzyme, catalysing a C-to-U conversion at position 6666 of the ApoB mRNA (Chan et al., 1997).

The role(s) of AID in regulating CSR and SHM is still controversial (Honjo et al., 2005; Peterson-Mahrt, 2005). Two models have been proposed. One model predicts that AID edits one or more mRNA resulting in expression of endonuclease(s) that specifically induce DNA breaks in the S regions in antigen-activated B cells (Honjo et al., 2005, 2004).

It has also been proposed that AID might directly deaminate dC within the S regions which leads to the formation of uracil residues (dU). According to this model, the enzyme uracil DNA glycosylase (UNG) is needed for dU removal and generating abasic site which is then cleaved by an apyridimic (AP) endonuclease. Deamination and cleavage would occur on both DNA strands and thereby create staggered breaks that could initiate CSR (Chaudhuri et al., 2003; Ramiro et al., 2003) (figure 11).

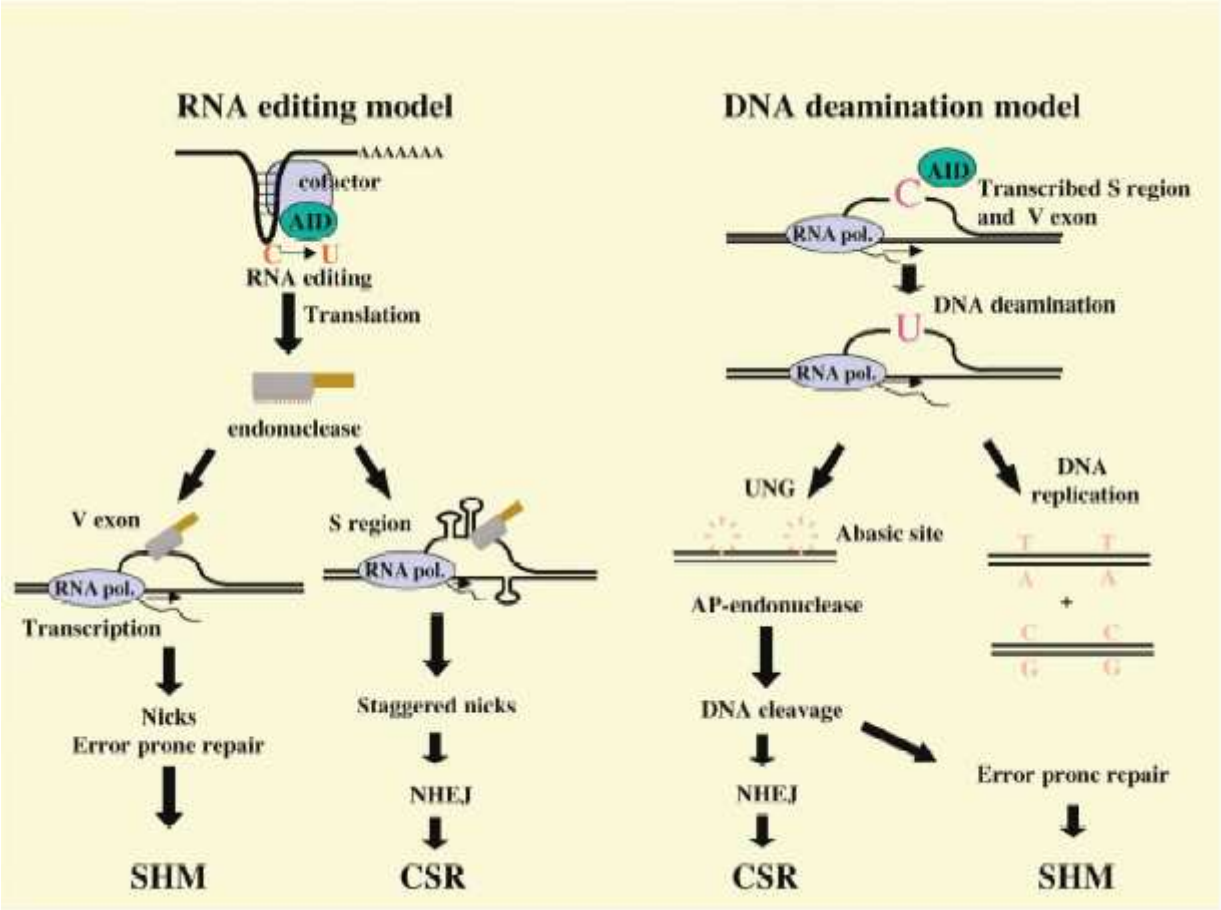


Figure 11. Two models are proposed for AID function (Honjo et al., 2004).

Whatever the mechanism, several studies showed that AID is important for both CSR and SHM. AID-defecient mice and *in vitro* stimulated B cells were unable to undergo CSR although they have germline transcripts for all isotypes (Muramatsu, et al., 2000). Defeciency in functional AID protein in humans is referred to as the hyper-IgM type 2 syndrome (HIGM2) .

However, recent studies have suggested that AID mutants differently affect CSR and SHM. AID that is mutated in its C-terminal region can promote SHM, but looses its ability to mediate CSR (Barreto et al., 2003; Ta et al., 2003). In contrast, mutations in the N-terminal region abolish SHM, but retain CSR (Shinkura et al., 2004). These finding imply that the C- terminal and N-terminal parts of AID could include specific binding sites for cofactors that are required for CSR and SHM.

As mentioned previously, transcription through S regions, in the physiological orientation, allows generation of ssDNA in the form of R loops (Tian and Alt, 2003; Yu et al., 2003), these R loops function as substrates for AID (Bransteitter et al., 2003; Chaudhri et al., 2003; Dickerson et al., 2003; Pham et al., 2003; Ramiro et al., 2003;). Recent studies showed that AID can deaminate the cytidines across the entire length of R loops with preference to WRC sequences (W=A or T and R=A or G) (Yu et al. 2005).

However, transcription through IgV exons in SHM doesn't generate R loops. In this case, it is thought that AID forms a complex with a ss-binding protein called replication protein A (RPA). AID-RPA complexes facilitate AID-induced DNA deamination of transcribed RGYW-containing substrates (Chaudhuri et al., 2004). AID-RPA complexes may have also a function in CSR (Zarrin et al., 2004).

In addition to AID, CSR is effected also by the base excision repair enzyme UNG. UNG-defecient B lymphocytes showed ten-fold reduction in CSR activity (Rada et al., 2002). Two possibilities have been suggested to explain the residual CSR activity: first, other uracil-DNA glycosylases may remove dU bases, and second, mismatch proteins may recognize dU:dG mismatches and thus create the DSBs.

4.2.4. DNA DSBs and end joining:

Several studies indicate that the CSR process involves DSBs generation (Chen et al., 2001; Rush et al., 2004; Wuerffel et al., 1997; Tian and Alt, 2000). These DSBs appear to be AID-dependent (Catalan et al., 2003; Schrader et al., 2005). Analysis of switch junctions derived from endogenous genes and inversional CSR constructs, reveal that short deletions, duplications, and mutations frequently occur at recombination sites (Chen et al., 2001; Rush et al., 2004). This suggests that staggered DSBs might be mediated by the CSR machinery.

The exact mechanism of DNA cleavage during CSR is not known. However, the excision repair nucleases XPF-ERCC1 and XPG can cleave the R loops structures and generate DSBs *in vitro* (Tian and Alt; 2000).

DNA DSBs formation enhances the phosphorylation of the histone H2AX (γ-H2AX). γ-H2AX forms nuclear foci at the C_H region in cells undergoing CSR and it is used as a marker for DSBs formation (Redon et al., 2002; Reina-San-Martin et al., 2003; Petersen et al., 2001; Celeste et al., 2002). Absence of γ-H2AX in AID deficient B cells reflects the participation of AID in DNA cleavage and generation of DSBs (Begum et al,. 2004). γ-H2AX deficient mice showed impaired CSR (Petersen et al.,2001). It has been proposed that phosphorylation of γ-H2AX would alter the overall structure of chromosomal domains through the C_H region, facilitating switch region synapsis (Reina-San-Martin et al., 2003).

Although UNG is important for CSR, its function in DSBs generation is controversial. Some studies showed that UNG is dispensable for DSBs generation and it might be involved in the repair step of CSR (Begum et al., 2004). In contrast, other studies suggested that DSBs formation is UNG-dependent (Schrader et al., 2005).

Completion of CSR seems to involve the joining of two broken S regions. S region breakpoints are very diverse and lack homology therefore, the NHEJ mechanism was proposed for rejoining of cut S regions (figure 12) (Manis et al., 2002; Manis et al., 1998).

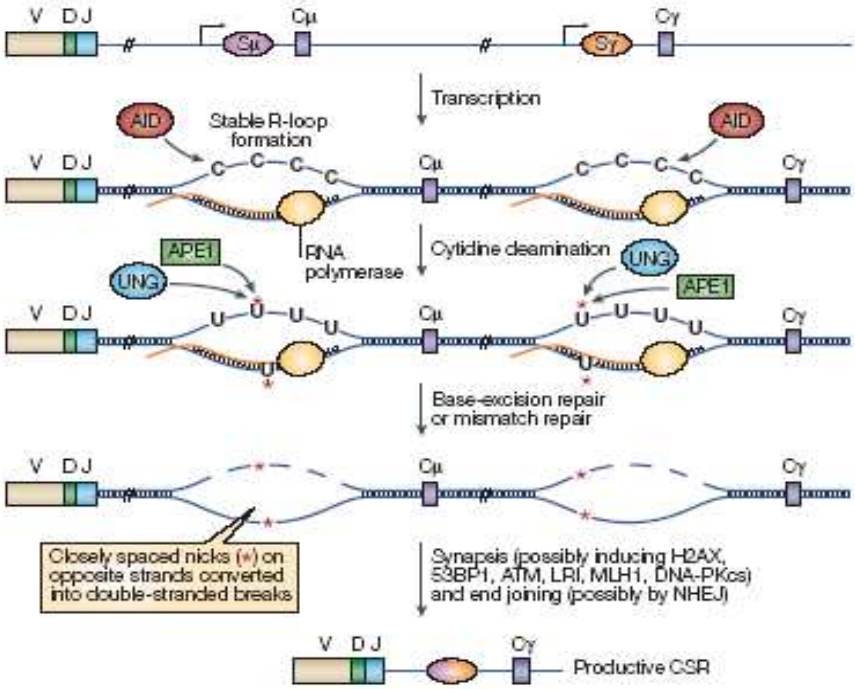


Figure 12. Summary of CSR model. The pink and orange oval at the recombination site represents deletion and mutations that are found at S junctions. AID: activation-induced cytidine deaminase, UNG: uracilglycosylase, APE1: apurinic/apyrimidinic endonuclease, DSBs: double strand breaks, H2AX: histone 2A family-member, 53BP1: p53 binding protein, LR1: lipopolysaccharide-responsive protein 1, ATM: ataxia telangiectasia mutated, MLH1: mutL homologue 1, and DNA-PKcs: catalytic subunit of DNA-dependent protein kinase (Chaudhuri and Alt, 2004).

Several proteins are invoved in NHEJ process, these proteins include:

- Ku70 and Ku80 which are involved in the formation of a DNA end-binding complex required for DSBs repair. Their deficiency result in severely impaired CSR (Casellas et al., 1998; Manis et al., 1998; Reina-San-Martin et al., 2003).

- DNA-PKcs which binds to Ku proteins to form DNA-PK complex. Deficiency in DNA-PK complex significantly reduced isotypes CSR except for IgG1 (Manis et al., 2002).

Another group of enzymes which is known to participate in CSR is the mismatch repair (MMR) proteins, including Msh2, Mlh1 and Pms2. MMR deficient mice display a 35-75% reduction in switching to IgG3, IgG1, IgG2b, and IgA, indicating that CSR is blocked only partially (Ehrenstein and Neuberger 1999; Schrader et al., 1999).

γ-H2AX is critical for the recruitment of specific DNA repair factors to site of DNA damage (Petersen et al., 2001; Begum et al., 2004).

53BP1 is a damage-sensing protein that presents at the sites of DNA lesion. 53BP1-deficient cells showed decrease in CSR although germline transcription and AID expression were normal (Manis et al., 2004).

NBS1 is a DNA repair protein localized with γ-H2AX in the IgH locus during CSR (Petersen et al., 2001).

ATM is required for S region synapsis and further for efficient CSR (Reina-San-Martin et al., 2004; Pan et al., 2002; Pan-Hammarstrom et al., 2003)

The DNA ligase IV/XRCC4 complex is recruited by DNA-PK complex during NHEJ. DNA ligase IV-deficient patients exhibit immunodeficiency with impaired antibody production (O'Driscoll et al., 2001). However, it is still unknown if DNA ligase IV is indeed the enzyme ligating S region DNA ends during CSR process.

5. Control of Germline Transcription In The IgH Locus

5.1. Regulation of transcription in the IgH locus:

5.1.1. Chromatin structure:

The cellular genes of eukaryotes are contained within densely packed chromatin, the basic unit of which is the nucleosome. The nucleosome is composed of about 200 bp of DNA wound around an octamer containing two molecules of each of the core histones H2A, H2B, H3, and H4. In addition, the linker histone H1 can be associated with the nucleosome, facilitating chromatin condensation and serving a regulatory function.

Nucleosomes are organized in higher-order chromatin structures that can be accessible or inaccessible for binding of trans-factors. Actively transcribed genes are known to be more sensitive to DNase I mediated degradation, which is a sign of an open, “loose” chromatin. Therefore, it is postulated that the first step in gene activation is chromatin opening. Several modifications of the core histones have been shown to modulate chromatin structure, these modifications are crucial for initiation of transcription (Forsberg and Bresnick, 2001). Histone acetylation is considered as one of the most common modifications. Histone acetylases (HATs) mediate acetylation of the conserved lysines within the amino terminal tails of core histones (Grunstein, 1997; Wolffe and Hayes, 1999). Histone deacetylase (HDACs) catalyze the opposing deacetylation reaction. Several studies showed that transcription activation is directly related to histone acetylation (Forsberg and Bresnick, 2001).

Chromatin structure is also manipulated by chromatin-remodeling complexes like SW1/SNF, these complexes use the energy of ATP hydrolysis to disrupt the chromatin and make it more accessible to DNA binding factors (Kingston and Narlikar, 1999).

Chromatin remodeling and histone modifications work together to enhance gene transcription through increasing the DNA accessibility to transcription factors,

which form nucleoprotein complexes that will produce further chromatin modifications.

5.1.2. DNA Methylation:

Methylation and demethylation of CpG dinucleotides have been shown to play a critical role in the regulation of tissue and developmental stage-specific genes (Bird and Wolffe, 1999). Tissue specific genes are mostly demethylated in the tissues where they are expressed while methylated in tissues where they are silent.

There are three possible mechanisms by which methylation can affect gene expression. One involves the methylated CpG residues interfering directly with the binding of specific transcription factors to DNA. A second possibility is that the direct binding of specific factors to methylated DNA mediates repression. Two such factors, MeCP1 and MeCP2 play a critical role. It is suggested that binding to MeCP2 may contribute to initiation or maintainanace of a repressive state (Siegfried and Cedar, 1997).

The third strategy by which methylation causes repression involves alteration of chromatin structure. Methylation induces a change in conformation to an inactive state by loss of DNase I hypersensitivity and of engaged RNA polymerase, and by the assembly of the inactivated promoter into a nucleosomal array (Siegfried and Cedar, 1997).

In this context, for activation of a repressed gene to occur two events must take place: firstly, the methylation must be removed; and secondly, changes in chromatin structure. Demethylation results in the removal of specific factors that bind methylated DNA, such as MeCP2, and thus allows the destabilization of the chromatin.

5.1.3. Transcription factors:

Gene transcription is a tightly regulated process, which is controlled by multiple DNA binding proteins, including transcription factors. A transcriptional activator is characterized by at least two domains: a DNA binding domain and a transactivation domain, the latter of which contacts proteins in the transcriptional machinery. Some activators lack a transactivation domain, but provide other protein-protein interactions, necessary to form an "activating surface" (Kimb and Maniatis, 1997). Transcription factors can bind similar DNA sequences, although they can display different tissue specificities. Some transcription factors can act as repressors interfering with activator proteins. This could be done by disruption of the activating surface composed of several activating factors. Some transcription factors exhibit dual activator/repressor functions (Kadonaga, 2004).

Several transcription factors appear to be involved in directing GT:

- STAT6 is a member of a family of transcription factors that are latent in the cytoplasm and activated by different cytokines and growth factors (Calo et al., 2003). STAT6 binding sites are found in murine Iε and Iγ1 promoters as well as in human Iε promoter (Delphin and Stavnezer, 1995; Hou et al., 1994). STAT6-deficient mice do not express IgE and have lower IgG1 levels, demonstrating its absolute requirement for isotype switching to IgE (Shimoda et al., 1996; Takeda et al., 1996). However, STAT6 alone cannot activate transcription, but requires help from other transcription factors or cofactors (Delphin and Stavnezer, 1995). Several studies indicate that STAT6 requires NF-κB for activation of murine Iε and Iγ1 and human Iε promoters (Shen and Stavenzer, 1998).

- NF-κB was first described as an activator of κ light chain enhancer (Sen and Baltimore, 1986). NF-κB activity is induced by a number of agents such as LPS, CD40, TNFα and BCR cross-linking. The binding sites for NF-κB are located in Iγ1, Iγ3, Iε and Iα promoters, in the S regions and the 3' IgH enhancers.

In addition to GL promoters, NF-κB regulates the activity of 3'IgH enhancers. It was demonstrated that NF-κB together with BSAP and Oct supress HS1,2 activity in pre-B cells and activate it after BSAP is downregulated in plasma cells (Singh and

Birshtein, 1996). NF-κB is also important for HS4 activity in mice and humans (Sepulveda et al., 2004). Furthermore, NF-κB binding sites were identified in murine HS3b enhancer (Gordon et al., 2003).

- The oct-1 and oct-2 have binding motifs in promoters of V genes, Eμ enhancer, and 3'IgH enhancers (Matthias et al., 1998). Activation of B cell-specific genes by Oct proteins requires the recruitment of a co-activator called OCA-B (Teitell, 2003). The OCA-B defecient mice have disrupted formation of GC and production of secondary Ig isotypes (Kim et al., 1996). Both Oct-1 and Oct-2 were shown to bind HS1,2 enhancer, while only Oct-1 can bind HS4 (Michaelson et al., 1996). It has been shown that Oct site is required for HS1,2 activity in plasma cell lines (Michaelson et al., 1996) and activated B cells. In addition, two Oct-binding sites were detected in HS3b (Gordon et al., 2003) and HS3a (Matthias and Baltimore, 1993).

- The Ets family of transcription factors share a common DNA-binding ets domain. Ets family member PU.1 synergizes with STAT6 in activation of human GL ε promoter. Elf-1 and PU.1 positively regulate the murine GL α promoter (Shi et al., 2001). PU.1 also regulates activity of HS1,2 enhancer and Eμ enhancer (Linderson et al., 2001; Nelsen et al., 1993). It was suggested that PU.1 might regulate chromatin accessibility in the IgH locus (Marecki et al., 2004).

- The E2A gene products are required for early B cell development. E2A proteins bind the so-called E boxes in multiple genes. In the IgH locus, they were shown to regulate the Eμ enhancer as well as 3'IgH enhancers. Multiple E-box sites are found within the HS3a, HS3b, and HS4. E2A proteins can positively regulate Iε promoter (Sugai et al., 2003). E2A proteins are also required for AID expression (Sayegh et al., 2003).

- BSAP is encoded by Pax5 gene, therefore it is often referred to as Pax5. Pax5 is considered as a crucial factor in maintaining B cell commitment during early B cell development (Urbanek et al., 1994; Nutt et al., 1997; Nutt et al., 1999; Rolink et al., 1999). On the other hand, down regulation of Pax5 is needed for plasma cell differentiation (Morrison et al., 1998).

In the IgH locus, Pax5 activates Iε and Iγ2b promoters while it suppresses Iα promoter (Max et al., 1995; Qiu and Stavnezer, 1998), and HS1,2 enhancer (Neurath et al., 1995; Singh and Birshtein, 1993). The activity of Pax5 is upregulated by CD40 and LPS stimulation (Anderson et al., 1996; Merluzzi et al., 2004; Thienes et al., 1997).

5.1.4. BCR signaling:

CSR and GT are also regulated by signals driven from B cells' BCR. BCR signalling directly affects the activation state of transcription factors which modulate GT and CSR in B cells. BCR signaling activates the cell cycle machinery through multiple signaling pathways which lead to the activation of various transcription factors such as NF-κB and AP-1 (Kurosaki et al., 2002).

Also activation of B cells with anti- δ dextran (resembles type 2 TI antigens) induces germline transcription of γ1, γ3, and γ2b (Zelazowski et al., 1995).

5.1.5. CD40/CD40L

CSR is induced by signals that result from direct contact of B cells with Th cells. The most important component that involved in this contact is CD40L. CD40L is 33-Kd glycoprotein which is expressed on activated Th cells (Stavnezer, 1996).

The CD40/CD40L interaction is critical for the activation of CSR induced by TD antigens. Mice lacking CD40 or CD40L respond to TD antigens by expressing IgM, but not IgG, IgA or IgE. The GC formation is also impaired (Xu et al., 1994). Absence of CD40L, CD40, or any downsream element that involves in CD40 signaling pathways result in X-linked hyper-IgM syndrome in humans (Durandy et al., 2001).

CD40 is a member of TNF receptor family, it has the ablility to activate NF-κB, Elf1, and AP-1 through TRAF (TNF-receptor associated factors) and MAPK/JNK pathways (Basaki et al., 2002).

CD40 signaling induces GL γ1 and low levels of ε transcripts in mice and synergizes with IL-4 inducing Ig class switching to IgG1 and IgE (Ferlin et al., 1996; Lin and Stavnezer, 1996; Lin et al., 1998; Strom et al., 1998). CD40 signaling alone activates GL γ2b transcription and induces CSR to IgG2b (Ström et al., 1998).

Other cell surface molecules contributing to B cells-Th cells contact are CD28-B7 (McAdam et al., 1998). Mice deficient in both B7 (B7-1 and B7-2) molecules failed to generate IgG1 and IgG2a and lacked germinal centers (Borriello et al., 1997). Another member of the B7 family, B7RP-1, specifically binds to the co-stimulator ICOS, which has homology with CD28 and is expressed on activated T cells. It has been shown that $ICOS^{-/-}$ mice had impaired CSR and GCs formation (McAdam et al., 2001).

It was also demonsrated that mice, which lack both IL-4 and IL-21 receptors are severely deficient in IgG response (Ozaki et al., 2002).

5.1.6. Cellular interactions with NK and dendritic cells:

It has been shown that NK cells play a role in B cell differentiation and class switching to Ig2a either in the presence or absence of IFN-γ (Gao et al., 2001). NK cells can exert their effect through direct cell-cell interactions and IFN-γ secretion (Gao et al., 2001; Gao et al., 2005).

CSR can also be induced in B cells through interactions with activated dendritic cells that express the TNF family member B lymphocyte stimulator protein (BlyS) and the proliferation-inducing ligand (APRIL) (Litinskiy et al., 2002).

5.1.7. Cytokines:

Several experimental data showed that CSR is regulated by B cell activators/or cytokines produced upon exposure to an antigen. Cytokines regulate

CSR by regulating GT since induction or suppression of GT by particular cytokines directly correlate with CSR to the same isotype after addition of a B cell activator.

Signal transduction pathways through surface cytokine receptors lead to the activation of cis-acting I-region promoters by specific transcription factors (Schebesta et al., 2002). For example, in vitro stimulation of B cells with LPS induces γ3 and γ2b germline transcription by recruiting NF-κB transcription factor, whereas LPS + IL-4 induces γ1 and ε GT by recruiting multiple transcription factors with synergistic or competing effects (figure 13).

As mentioned above, treatment of mouse splenic B cells with LPS results in the induction of germline γ3 and γ2b transcripts and subsequently switching to IgG3 and IgG2b.

Addition of IL-4 to LPS-activated B cells decreases germline γ3 and γ2b transcripts and switching to IgG3 and IgG2b. IL-4 + LPS induce germline γ1 and ε transcripts in the mouse and subsequent switching to these isotypes (Bergstedt-Lindqvist et al., 1984).

In contrast, addition of IFN-γ to LPS reduces germline γ1 and ε transcripts and switching to IgG1 and IgE in mouse B cells induced by LPS+ IL-4 (Berton et al., 1989). Addition of IFN-γ to LPS-activated B cells induces germline γ2a transcripts and subsequent switching to IgG2a in mouse B cells (Collins and Dunnick, 1993). IFN-γ also inhibits secretion of IgG3 by LPS-activated B cells (Coffman and Carty, 1986).

Addition of TGF-β increases germline α transcripts and subsequent switching to IgA (Coffman et al., 1989). TGF-β also enhances switching to IgG2b (Sonoda et al., 1992).

IL-5 is able to induce switching to IgG1 in the presence of 1L-4 (Snapper et al., 1997).

IL-10 strongly inhibits class switching to IgA cells in response to combined activation with LPS, IL-4, IL-5, anti-Ig-dextran, and TGF-β. IL-10 augments the

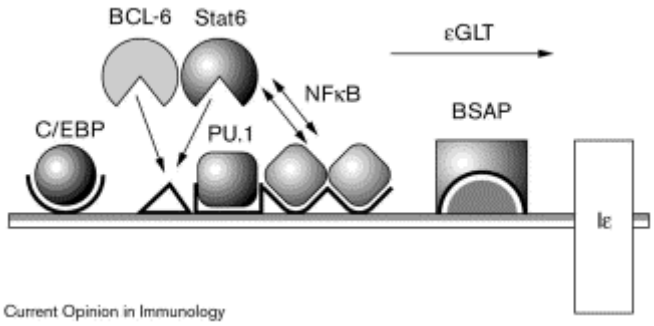


Figure 13. Structure of the Iε. (Oettgen, 2000).

generation of mIgG3 cells in the response to activation with LPS alone (Snapper et al., 1997).

5.2. Cis-Regulatory Elements in the IgH Locus:

Germline transcription precedes CSR to a given C_H gene in the same activated cells, and the induction of CSR by specific stimuli correlates directly with their ability to induce or suppress specific GT before CSR occurs. GT is regulated by B cells activators through regulatory elements within the IgH locus (figure 14).

5.2.1. Germline promoters:

All S regions, except Cδ, are preceded by a single noncoding exon (the I exon), and GT initiates from a promoter 5' of the I exon. I promoters are weak promoters that are presumbly transcribed by pol II. Multiple transcription initiation sites are feature of I promoters (Rothman et al., 1991; Xu and Stavnezer, 1992).

I promoters are most often silent in mature resting B cells and can be induced by stimuli, which activate B cells to go through CSR (Stavnezer, 2000). Induction of the I promoters by particular cytokines has been directly corrlated with subsequent switching to the same isotype and deletion of a particular I promoter abolishes switching to that isotype. In some instances, constitutively transcribed drug-resistance cassettes (e.g., the pgk-neo^r cassette) can replace I-region promoters in directing CSR to downstream S region, provided their transcriptional orientation is the same as that of the GL promoter (Seidle et al., 1998). However, replacement of the Iε promoter by Eμ-P_{VH} promoter cassette resulted only in a modest increase of constitutive switching together with a loss of cytokine-inducible Cε switching (Bottaro et al., 1994).

Several studies showed that GL promoters have multiple transcription factor binding sites that are shown to be necessary for promoters' activity (Stavnezer, 2000).

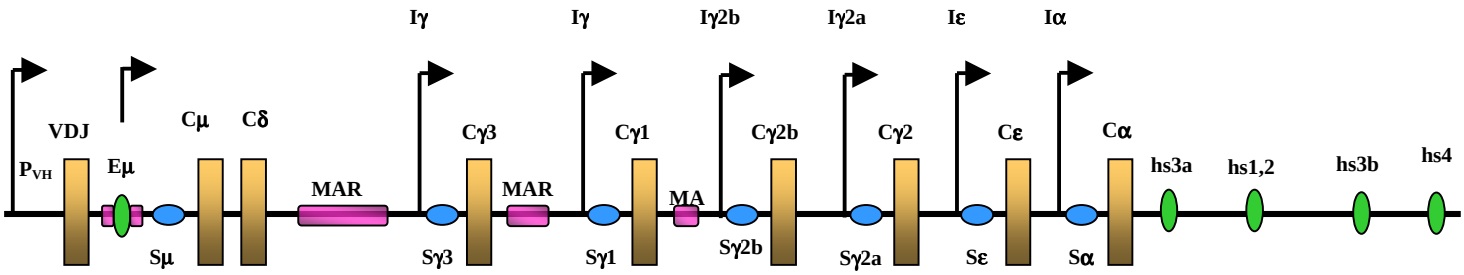


Figure 14. Structure of the mouse IgH locus. The mouse 3' regulatory region is located downstream of the Cα gene. S_x and I_x denotes switch sequences and germline I promoters respectively, upstream of constant regions. Arrows indicate the orientation of the germline transcripts. Also indicated are the matrix attachment regions (MARs) surrounding Eμ and within some IgH introns (Khamlichi et al., 2000).

5.2.2. Eμ enhancer and MARs:

Eμ is located in the intron between the J_H and Cμ gene (Banerji et al., 1983). It consists of the core enhancer (cEμ) flanked by 5' and 3' matrix attachment regions (MAR).

Several transgenic and transfection studies showed that Eμ is active in early B cell development and it has the ability to direct lymphoid-specific expression (Garcia et al., 1986; Gerlinger et al., 1986; Grossched and Baltimore, 1985; Grosschedl et al., 1984).

Complete Eμ deletion implicated that Eμ is important for V to (D)J recombination and expression of rearranged μ genes (Serwe and Sablitzky, 1993). In contrast, analysis of normal transformed B cell lines that have chromosomal Eμ deletions demonstrated that the endogenous IgH genes could be transcribed in the absence of Eμ (Klein et al., 1984; Wabl and Burrows, 1984, Aguilera., 1985; Eckhardt and Birshtein, 1985).

Other experiments have shown that large deletions in the Eμ region supress switch recombination at the Sμ region but permit intra-S region deletions of the downstream γ1 gene (Gu et al., 1993). In addition, deletion of Eμ decreased μ CSR, however, replacement of this enhancer with neo^r gene showed relatively normal switching (Bottaro et al., 1998).

The matrix attachment regions are lying in some introns within the IgH locus (Cockerill, 1990). The exact function of these regions is still controversial. They may be involved in chromatin accessibility and transcriptional activation (Forrester et al., 1999; Sakai et al., 1999; Fernandez et al., 2001). However, deletion of Eμ MARs led to no phenotype *in vivo* (Sakai et al., 1999).

5.2.3. IgH 3' enhancers:

Transcription is also regulated by a complex sequence with transcriptional enhancer activity that lies downstream of the Cα gene (Khamlichi et al., 2000) (figure 14). This 3' IgH regulatory region (3' IgH RR) acts as a locus control region

(LCR), that is, a region able to direct high level, tissue-specific expression of a linked gene in a position-independent, copy number-dependent manner (Madisen and Groudine, 1994).

It is important to stress that both Eμ and the 3' IgH RR are retained following CSR (whatever the targeted constant region) whereas the germline I promoters are deleted upon CSR.

The 3' IgH RR is ~40 Kb region and comprises four DNase-hypersensitive sites (HSs) which are lymphoid-specific transcriptional enhancers. These enhancers include from 5' to 3': HS3a, HS1,2, HS3b, and HS4. The HS1,2, HS3a, and HS3b are active at late B cell differentiation stage while HS4 seems to be active throughout B cell development (khamlichi et al., 2000).

Based on transient transfection experiments, it was concluded that the four enhancers are rather weak when they individually drive transcription of reporter gene. However, their combination displayed transcriptional synergies, especially when their normal palindromic arrangement was respected, and they also synergistically act as apowerful coenhancers of Eμ when driving transcription of reporter genes (Chauveau et al., 1998).

In a differentiated B cell line, spontaneous deletion of a large part of the 3' IgH RR was associated with a decreased transcription of an IgHα gene controlled by the sole Eμ. In a pre-B cell line, a large deletion including both HS3a and HS1,2 had no effect on μ expression. Targeted replacement of HS1,2 in a mature B cell line also affected transcription of a rearranged γ2a gene lacking Eμ (Gregor and Morrison, 1986; Saleque et al., 1999 ;Lieberson et al., 1995).

The function of the 3' IgH RR during B cell maturation has been approached by knockout experiments in ES cells. Targeted replacement of HS1,2 with a neo^r gene first suggested its major involvement in germline transcription of several downstream IgH constant region genes and CSR (Cogné et al., 1994). Essentially the same phenotype resulted from the replacement of HS3a with a neo^r gene (Manis et al., 1998). However, in both cases, further deletion of the neo^r gene with Cre/lox restores normal CSR (Manis et al., 1998), thus showing that HS3a and HS1,2 are both individually dispensable for this process. Given that the four enhancers act

synergistically for high-level transcription, one might speculate that deletion of only one weak enhancer will have no drastic effects on germline transcription and CSR.

On line with this interpretation, joint deletion of HS3b/HS4 in mice resulted in a drastic depression of CSR to most isotypes by decreasing germline transcription of the corresponding C_H genes (Pinaud et al., 2001) confirming a major role of the 3' IgH RR in the control of CSR by regulating transcription from germline promoters.

Finally, insertion of neo^r gene downstream of HS4 did not affect CSR and GT, which, together with the other data, strongly implies that the enhancer activity is contained within the HS3b or HS4 sequences, and possibly both (Manis et al., 2003).

The 3'LCR has the ability to modulate GT and CSR through long-range activation of germline promoters (Seidl et al., 1999). The LCR may exert its effect through mediating chromatin remodeling and opening of the locus (Madisen and Groudine, 1994) either by histone acetylation or demethylation of distal-controlled locus (Elefant et al., 2000; Santoso et al., 2000).

In addition to these cis-regulatory elements, data obtained from targeting of the IgH 3' enhancers (Cogné et al., 1994; Pinaud et al., 2001) indicated that γ1 locus may have additional regulatory elements (Xu and Stavnezer, 1992; Elenich et al., 1996; Cunningham et al., 1998; Adams et al., 2000).

6. Neo^r gene Effect:

In addition to being a selectable marker for transfection experiments, neo^r gene associated with its promoters (pgk or *hsv*-tk) is used in studying the role of regulatory elements within complex loci (Khamlichi et al., 2000).

Previous studies showed that neo^r gene insertion in certain loci led to the disruption of expression of other genes located near the insertion site. This disruption resulted in production of unexpected phenotypes (Olson et al., 1996).

Other studies using stable transfectants of mammalian cell lines suggested that neo^r gene; independent of its orientation; mediates a cis-acting negative effect on expression of neighboring genes (Artelt et al., 1991). This effect was postulated to be due to the higher density of CpG of the neo^r gene (Takeda et al., 1993; Artelt et al., 1991).

The human β-globin locus control region (LCR) is a complex regulatory element that controls the expression of all cis-linked globin genes. The LCR is composed of five DNase I hypersensitive sites located greater than 50 Kb upstream of the β-globin gene. Neo^r gene insertion was used as an approach to study the potential mechanism of interactions between the β-globin LCR and globin genes (Fiering et al., 1993). In a transgenic system, neo^r gene was inserted between 5'HS1 and 5'HS2 of the human β-globin LCR. The insertion completely suppressed the expression of the β-globin gene while neo^r gene expression was highly increased. These results were consistent with the promoter/enhancer competition model for LCR function and globin gene expression (Fiering et al., 1993).

The neo^r gene was also used to study the function of murine β-globin LCR elements. In this context, neo^r gene was used to replace both 5'HS2 and HS3 sites (Fiering et al., 1995; Hug et al., 1996). Replacement mutations resulted in severe reduction of globin genes' expression compared with the deletional mutations of both sites which had no significant effect on the expression of the locus. These data

suggested that neo^r inteferes with LCR activity and competes with the globin genes for an interaction with the LCR.

On the other hand, insertion of neo^r gene into the granzyme B gene, the most 5' gene in the granzyme B gene cluster, severly reduced the normal expression of multiple genes in the locus, even at distances greater than 100 Kb from the mutation (Pham et al., 1996). This observation suggested that neo^r can interact productively with the LCR and thereby disrupt normal interactions between this putative element and downstream genes.

In the Ig loci, neo^r gene was used to study the function of the enhancers Eμ (IgH locus) and Eκ (Igκ locus) in V(D)J rearrangement and expression of the loci. Replacement of Eκ with neo^r gene suppressed VκJκ rearrangements (Xu et al., 1996) while insertion of neo^r gene 3' of Eκ reduced these rearrangements (Takeda et al., 1993). These results have been explained by the ability of neo^r gene promoter to compete for binding of transactivating factors, and thus inhibits chromatin remodeling or germline transcription (Takeda et al., 1993; Sun and Storb, 2001).

In addition, Eμ replacement with neo^r gene resulted in dramatic decrease of J locus rearrangement, demethylation, and germline transcription. Insertion of this gene 5' of MAR sequence (upstream of core Eμ) also decreased recombination of J_H gene but didn't prevent demethylation of this gene. In contrast, replacement mutation of the 3' region downstream of core Eμ had no effect on the targeted allele (Chen et al., 1993). These results suggested that neo^r gene interferes with Eμ by disrupting DNA-protein complexes necessary for recombination enhancement. However, this effect appears to be position dependent (Chen et al., 1993).

Recently, it has been shown that insertion of neo^r gene upstream of Eμ resulted in alteration of D and J_H gene usage and blockade of transcription of the J_H region and the rearranged VDJ segments. Again, promoter competition mechanism was suggested to explain the effect of neo^r gene (Delpy et al., 2002).

In addition to its negative regulatory effect, neo^r gene can also positively affect the Ig gene expression. In this context, insertion of neo^r gene upstream of Jλ1 region upregulated Vλ1Jλ1 recombination (Sun and Storb, 2001). On the other hand,

targeted mutations in which neo^r gene was used to replace either Eμ/MAR or Iγ2b promoter showed that this gene was able to provide normal CSR (Sakai et al., 1999; Seidl et al., 1998).

In the case of the 3' IgH LCR, neo^r gene was used to study the mechanism by which the LCR effects its long-range control of germline promoters.

Indeed as mentioned previously, mutant mice were generated in which HS3a or HS1,2 were replaced with neo^r cassette. The mutant B cells were severely deficient in their ability to switch to IgG2b, IgG2a, IgG3, and IgE after appropriate stimulation, and these defects correlated with the corresponding defects in germline transcription and CSR to the affected C_H genes (Cogné et al., 1994; Manis et al., 1998). Therefore, the HS3a and HS1,2 replacement mutations inhibited CSR to different C_H genes spread over 150 kb.

The similarity of the HS3a and HS1,2 replacement mutation phenotypes, both of which were cis-acting, suggested that the inhibition of CSR resulted from effects of the inserted neo^r gene, potentially via competition of the neo^r gene promoter for control elements in the 3' IgH RR. These findings also led to the suggestion that CSR recombination could be regulated at least in part, by the relative ability of various germline C_H promoters, after activation, to compete for activities of this regulatory region (Cogné et al., 1994; Manis et al., 1998).

Additional insight into the potential mechanisms by which the 3' IgH regulatory region modulates germline transcription and CSR comes from recent studies of B cells in which the Cε or the Iγ2b exon were replaced with neo^r gene. The results of these experiments have shown that germline transcription and CSR to upstream C_H genes are impaired (with the exception of Cγ1) but the downstream C_H genes are not affected (Seidl et al.,1998).

Again, these findings support the existence of a long-range 3'IgH regulatory region required for germline transcription and CSR to multiple C_H genes. They suggest that neo^r gene insertion into the locus short-circuits the ability of this region to facilitate germline transcription of dependent C_H genes upstream, but not downstream, of the insertion site (figure 15).

Whatever the effect, all these data suggest that neo^r gene can exert its effect by a promoter competition mechanism through interaction with Ig enhancer and prevents promoter/ enhancer interaction.

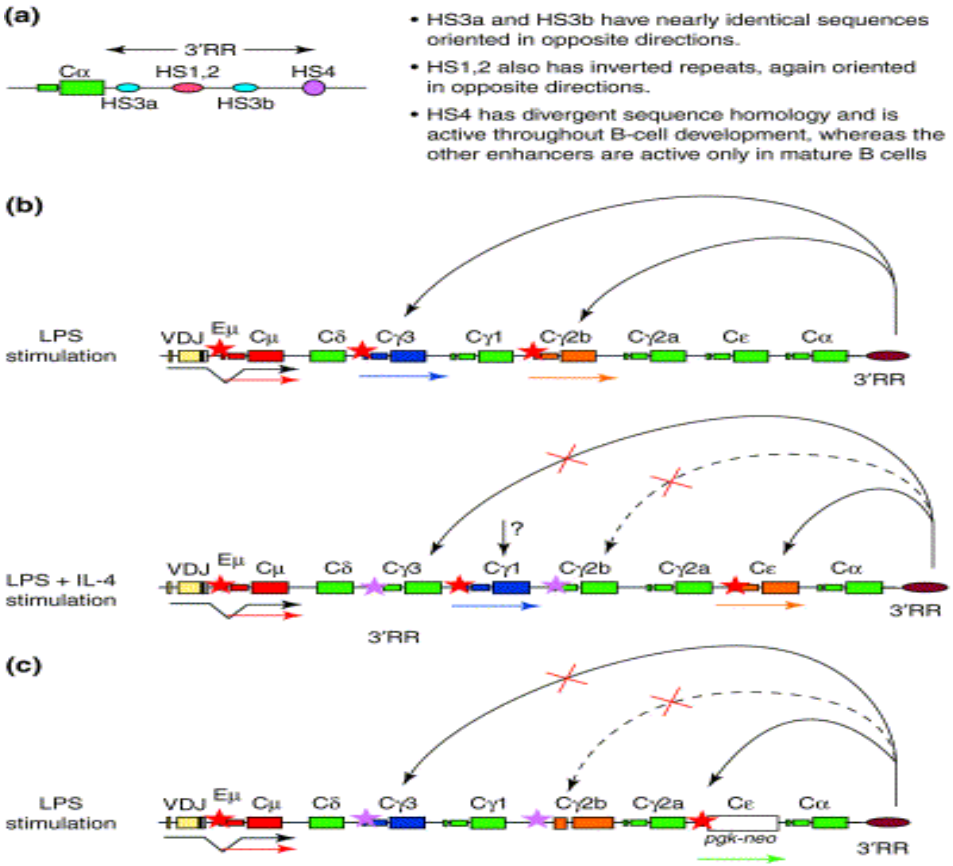


Figure 15. The 3' IgH regulatory region. (a) shows the structure of the 3'IgH RR. (b) CSR might be regulated by a promoter competition mechanism. In the context of such a model, adding IL-4 along with LPS would extinguish germline Cγ2b and Cγ3 expression by activating the Cε or Cγ1 promoters. The question mark indicates that Cγ1 transcription can be activated independently of the 3' IgH RR. (c) Placement of pgk neo^r promoter might, similarly, inactivate expression of upstream germline C_H genes. The red stars represent an activated C_H promoter that interacts with the 3'IgH RR to initiate germline transcription. The pink stars represent activated promoters that are unable to induce germline transcripts owing to promoter competition for the 3' IgH RR (Manis et al., 2002).

THE PROBLEM

As mentioned above, neo^r gene is often used as a tool to dissect complex loci and long range interactions between promoters and enhancers. The work described in this thesis includes analysis of mice bearing neo^r gene in different sites of the IgH locus.

Previous work has shown that deletion of HS3b/HS4 affects CSR to several isotypes with CSR to γ3 being one of the most drastically altered. This provides evidence that the 3' IgH LCR somehow interacts with Iγ3 lying some 150 Kb upstream of the LCR (Pinaud et al., 2001). On the other hand, insertion of neo^r gene, independently of its orientation, at specific sites within the IgH locus affects germline transcription of and CSR to upstream but not downstream isotypes (with the notable exception of Cγ1) (Seidl et al., 1998). However, in this work, neo^r gene was used to replace either Iγ2b promoter or Cε gene.

Relying on these data, we sought to insert neo^r gene downstream of Iγ3 exon by leaving intact all the necessary elements for proper germline transcription of γ3 gene (Iγ3, splice sites and constant gene). We ask 1) if and how neo^r gene insertion will affect transcription initiation from Iγ3 promoter and downstream germline promoters, 2) if there is any effect on μ gene expression since, among the germline promoters of the constant locus, Iγ3 promoter is the nearest to Eμ (which also spans Iμ) and 3) how could this long-range interaction between the LCR and Iγ3 be effected.

Also it has been shown that recombination of Sμ with any downstream S region is a critical step for CSR. In this context, deletion of the core tandem repeat sequences of Sμ significantly reduced CSR (Luby et al., 2001), although complete deletion of Sμ tandem repeats (most of Iμ-Cμ intron, leaving intact the necessary sequences for correct splicing of μ transcripts) further reduced, but did not abolish, CSR (Khamlichi et al., 2004). In this regard, we sought to analyze mice with neo^r gene replacing the deleted sequences of Sμ, to answer the following questions:1) will neo^r gene insertion affect transcripton initiation from Iμ, 2) will it affect germline

transcription and CSR, 3) how the long-range interaction of the LCR will be affected, and 4) what would be the effect of neo^r on V(D)J rearrangement.

Results

MANUSCRIPT N° 1

Germline transcription in mice bearing neo^r gene downstream of Iγ3 exon in the Immunoglobulin heavy chain locus

(Submitted to International Immunology)

MANUSCRIPT N° 2

Replacement of Sμ sequences by neomycin resistance gene impairs B cell development and μ gene expression

(In preparation)

DISCUSSION AND PERSPECTIVES

This section is divided into two parts. The first and the main part includes a discussion of the neo^r effect. The second part includes the perspectives of works to which I contributed.

I. neo^r effect:

Among other tools, neo^r can be used to gain insights into the mechanisms underlying long-distance gene activation by the LCRs of different loci. In this context, we designed two systems in which the long-range interaction between the 3'IgH RR and the germline promoters under its influence would be disrupted.

Data obtained from neo^r gene insertion in the β-globin locus suggested that the LCR controls the expression of β-globin genes through a promoter competition mechanism. Indeed, insertion of neo^r gene interferes with the LCR activity and competes with the globin genes for interaction with the LCR (Fiering et al., 1993; Fiering et al., 1995; Hug et al., 1996). Also, insertion of neo^r gene in granzyme B cluster disrupts the long-range effect of the LCR (Pham et al., 1996).

The neo^r gene exerted the same effects upon its insertion in the IgH locus. Models proposed to explain neo^r effect in this locus suggest the existence of a polarized and long-range 3' IgH RR effect required for germline transcription of multiple C_H genes and suggest that *neo*^{r} gene insertion into the locus short-circuits the ability of this region to facilitate germline transcription of dependent C_H genes upstream, but not downstream, of the insertion site (Cogné et al., 1994; Manis et al., 1998; Seidl et al., 1998; Pinaud et al., 2001).

Generally speaking, Three models have been proposed to accomodate the long-distance gene activation of complex loci by LCRs. These models have been abbreviated: looping, tracking and linking (figure 16).

The looping model underlies the idea that the LCR acts an integral unit to stimulate the transcription of individual genes by looping through the chromatin fiber

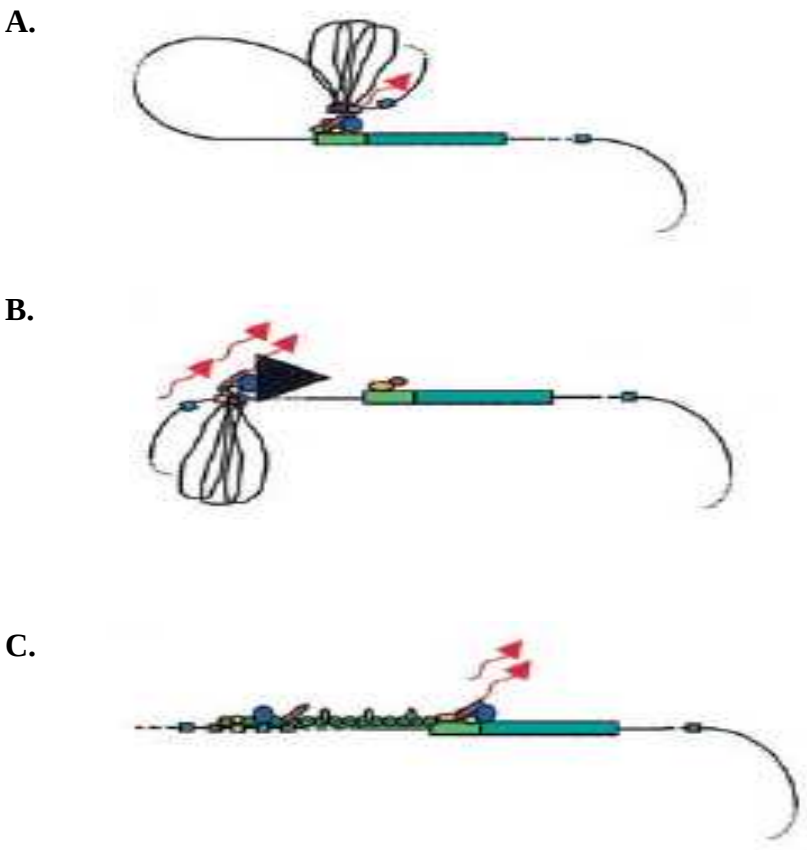


Figure 16. Models of LCR function in the globin gene. A. represents the Looping model, **B.** tracking, and **C.** the linking model. Globin gene is denoted by a green rectangular box with the promoter region indicated in a lighter green. The HS sites are indicated by small red boxes. Blue boxes are the positions of 5'HS5 and 3'HS1, representing insulator elements. The flanking DNA sequences are depicted as loops between HS cores . Transcripts are denoted by wavy arrows (Li et al., 2002).

to activate (or recruit) the transcriptional apparatus assembled at target promoters. Since the LCR would be limiting, only one promoter at a time could be transcribed (Bulger and Groudine, 1999; Li et al., 2002; Harju et al., 2002).

In the tracking model, the LCR binds transcription factors and forms an activation complex that migrates, or tracks, linearly along the DNA helix of the locus. When this transcription complex encounters the basal transcription machinery located at the target promoter, the complete transcriptional apparatus is assembled and transcription of that gene is activated (Li et al., 2002; Harju et al., 2002).

In the linking model, linking is envisioned to be the product of sequential stage-specific binding of transcription factors and chromatin "facilitators" throughout the locus to an array of chromatin elements that initially define the domain to be transcribed. Thus, the proteins bound to the locus to be transcribed are linked to one another by facilitating non-DNA binding factors, and these form a continuous protein chain from the LCR to the target genes (Bulger and Groudine, 1999; Li et al., 2002; Harju et al., 2002).

How then could neo^r insertion interfere with germline transcription of upstream I promoters ?

It is now known that histones acetylation and localization of a gene in a nuclear compartment permissive for transcription are important for chromatin opening and gene activation (Li et al., 2001). In this context, gene activation by the LCR should be preceded by nuclear localization of the locus away from the centeromeric heterochromatin (Li et al., 2001; Fu et al., 2002).

Recent studies showed that cis-regulatory elements other than the LCR may function to alter subnuclear location and chromatin structure through binding to factor-binding sites scattered throughout the locus. These elements may maintain the locus in an open chromatin/ acetylated configuration by disrupting or preventing its association with a nuclear compartment enriched in heterochromatin proteins and HDACs (Schubeler, et al, 2000). So one possibility is that neo^r interferes with these elements and lead to a new localization of the locus in the nucleus. Several studies suggest that the nucleus is divided into compartments in which specific proteins and specific sequences concentrate (Brown et al., 1997, 1999). Accordingly, neo^r

insertion may result in the localization of the locus in a nuclear compartment that contains DNA-binding proteins involved in the silencing of transcription from up stream but not from downstream promoters.

Another possibility is that neo^r insertion will cause a decreased accessibility of the affected C_H genes through changes in chromatin structure of affected C_H genes.

Indeed, it is now accepted that the chromatin structure is an essential component of the transcriptional regulation machinery.

Although the exact molecular mechanisms governing the opening of the chromatin compact structure and the accessibility to promoters and enhancers of *trans*-acting factors are still debatable, it is clear that changes in chromatin structure near transcriptionally active genes require cooperation between transcription factors, histones and other cofactors in order to remodel and displace nucleosomes (Kadonaga, 1998).

The 3' IgH RR is able to establish and maintain a transcriptionally active state over entire chromatin domains probably by recruiting histone acetyltransferase (HAT)-containing cofactors which might induce local histone acetylation further propagating throughout the chromatin domain under the influence of the LCR (Madisen et al., 1998).

Therefore, one way for the neo^r gene insertion to inhibit transcription initiation from upstream promoters is by blocking the propagation of such a signal emanating from the 3'IgH LCR to upstream (but not to downstream) promoters (tracking and linking models).

Alternatively, neo^r gene promoter may physically interact with the 3' IgH RR (looping model). The latter would then counteract the repressive chromatin structure by recruiting and/or directing (HAT)-containing molecules (such as transcriptional cofactors) to neo^r gene promoter.

On line with this interpretation, an enhancer shift model has been proposed (Arulampalam et al., 1997) which is a derivative of the looping model : with regard the germline transcription which is a prerequiste to CSR, the model postulates that

individual enhancers within the 3’ IgH RR would selectively interact with specific I promoters. Alternatively, the four enhancers may act as a unit on I promoters but would shift from an accessible I promoter to another following appropriate stimuli.

In this context, neo^r gene may outcompete affected I promoters because of its promoter strength, thus preferentially interacting with the 3’ IgH RR necessary for transcriptional activation. Consistent with this model, insertion of the *neo*^{r} gene in the IgH locus renders the *neo*^{r} gene LPS-inducible (Zhang et al., 1995 ; Manis et al., 1998).

Whatever the mechanism, the current findings are consistent with the hypothesis that promoter "competition" may be a general mechanism for modulating germline transcription and CSR activity in the IgH locus (Cogné et al., 1994). Thus, transcription from, at least a subset of I promoters is based on the ability of the local I region promoter to "interact" with the 3' IgH RR to achieve transcription. In the context of such a model, LPS treatment, for example, would lead to activation of the local Iγ2b and Iγ3 promoters, which, via interaction with the 3' IgH RR, would initiate transcription and lead to CSR.

Thus, the use of neo^r gene as a tool to dissect complex loci proves fruitfull for gaining insight into the basic mechanisms that underlie the function of distant regulatory regions, and helps elaborating additional approaches to elucidate such mechanisms. Such as the use of insulators which is underway in the group.

II. Perspectives:

In addition to neo^r effect studies, I contributed to two projects attempting to elucidate the functions of the cytoplasmic domains of the class-switched isotypes.

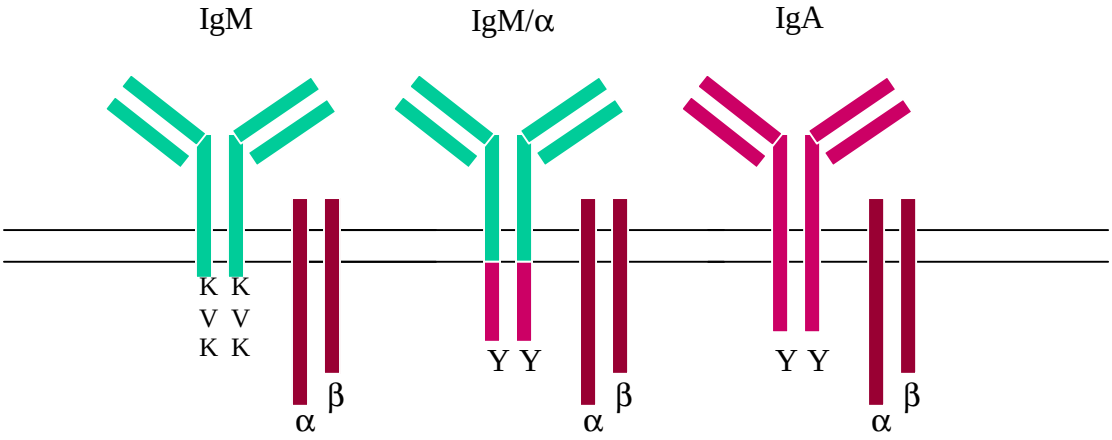
The B cell antigen receptor is composed of membrane bound immunoglobulin noncovalently associated with Igα/Igβ heterodimer. The cytoplasmic domains of IgM and IgD consist of three amino acids (KVK) while the other isotypes have more extended cytoplasmic domains, 28 amino acids for IgG and IgE and 14 amino acids for IgA with a highly conserved tyrosine. Mutational studies

of IgM confirmed the importance of Igα and Igβ cytoplasmic domains for signalling, efficient antigen internalization and presentation (Patel et al., 1993; Aluvihare et al., 1997; Bonnerot et al., 1995).

In contrast, the role of the cytoplasmic domains of class-switched isotypes and their conserved tyrosine is presently unclear. Several hypotheses have been proposed including: a role in signal transduction (qualitatively different of Igα/Igβ), antigen internalization, intra-cellular trafficking, antigen presentation and induction of immunological memory (Weiser et al., 1994; Knight et al., 1997).

In this context, we are generating a murine model in which the cytoplasmic domain of IgM is replaced by the cytoplasmic domain of IgA. The aim is to evalute the role of this cytoplasmic domain in B cell development specifically in the mature phase and also to study the potential pathological consequences that will result from expression of such chimeric antibody.

ES transfectants are being screened, and we hope getting soon positive(s) clone(s).

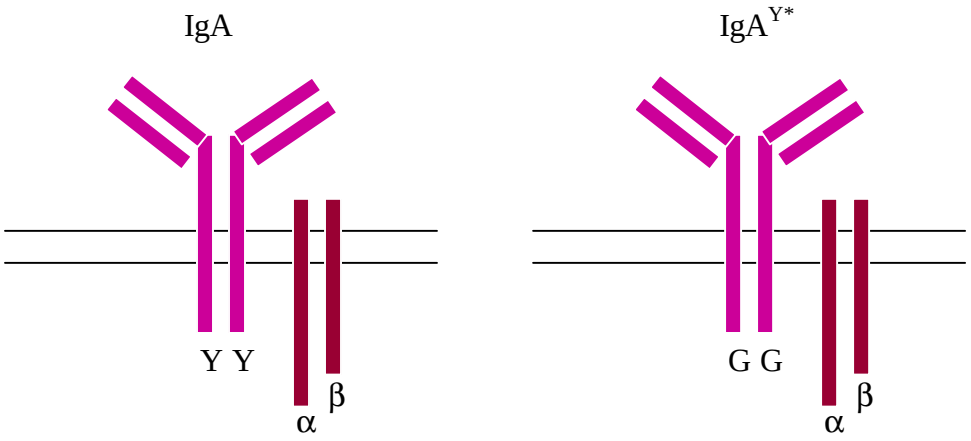


Previous work also showed that the cytoplasmic domain of γ heavy chain enabled internalization of the BCR independently of Igα/Igβ (Weiser et al., 1994;

Knight et al., 1997), but concluded to a partial dependence on the conserved tyrosine (Knight et al., 1997).

In order to study the exact role of the conserved tyrosine in the cytoplasmic domains of class-switched isotypes, I contributed to the generation of mutant mice that have IgA whose C-terminal tyrosine was replaced by glycine (IgA^{Y*}). These mice will be analyzed to answer several questions: does the tyrosine play a role in signal transduction, antigen internalization or receptor down-modulation? will the mutation affect Igα/Igβ association? what might be the pathological consequences on mucosal immunity? and what could be the effect on immunological memory?

We got germline transmission of this mutation and mice are now being analyzed.



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