Regulation of the murine Immunoglobulin GL γ1 and ε promoters.

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Stockholm 2002

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ABSTRACT

The immune system takes advantage of different genetic alterations to amplify its diversity. First at early stages of B cell differentiation when the antigen (ag) receptors are assembled from different gene segments. Secondly during the proliferative stage induced by ag challenge, when the Ig genes undergo two types of modifications: somatic hypermutation and class switch recombination (CSR). During CSR the effector function of an Ig molecule is altered, while the antigen specificity is remained, resulting in expression of IgG, IgA or IgE instead of the initial IgM. Switching to a given isotype requires induction of transcription over the targeted C_H-gene, while it is still in germline (GL) configuration. However, the specific function of GL transcripts in CSR is still unknown and regulation of GL transcription over the Cγ1 and Cε regions has been the main focus of this thesis.

Initially, we explored whether the CD40/CD40L interaction was sufficient for B cells to undergo CSR. In vivo distribution of an agonistic monoclonal rat anti-mouse CD40-antibody (ab) induced up-regulation of all Ig isotypes in mice completely devoid of T cells. This induction was to some extent independent of IL-4, since IgE synthesis was only partially inhibited by co-injection of anti-IL4. Anti-CD40 also induced GL ε transcripts and expression of IgE in B cells from IL-$4^{-/-}$ mice in vitro. We then investigated the molecular mechanisms of CD40 signalling in induction of GL transcripts and CSR. We found that signalling through CD40 stimulated GL γ1, γ2b and low levels of ε transcripts. In B cells activated by LPS or anti-CD40, we could detect CSR to Cγ2b. In addition, anti-CD40 together with IL-4 or IL-5 induced CSR to the γ1 region. We showed that NF-κB activated both by LPS and anti-CD40 bound to the γ1 promoter. However the total concentration of bound NF-κB varied, in addition to the subunit composition induced by the two stimuli.

Furthermore, we have investigated the dependency of proliferation for CSR and synthesis of GL transcripts. We examined what effect inhibition of DNA synthesis had on CSR. Cell cycle arrest was induced in cells stimulated with LPS+IL-4 by addition of the DNA synthesis inhibitors Hydroxyurea (HU) and Aphidicholin (AC). We found that CSR was completely abrogated. Addition of HU also reduced GL γ1 and ε transcripts in LPS plus IL-4 activated B cells. To enrich for B cell blasts in different cell cycle phases we used elutriator centrifugation and found that GL γ1 transcripts were expressed in G_1 and S phases, at lower levels in G_2/M but not in G_0. When investigating binding patterns of nuclear proteins to the GL γ1 promoter, we found two major LPS-induced DNA binding protein complexes. These complexes were shown by elutriation experiments to be expressed in G_1 and presumably S, but not G_0 or G_2/M. Furthermore, the complexes bound to an Ets consensus element. We concluded that there exists a relation between proliferation, Ig class switching and GL transcription.

The Ets consensus binding site in the γ1 promoter overlaps with a binding site for Ikaros, a protein important for development of all lymphoid cells, which was initially defined as a transcriptional activator but later has been suggested to act as a repressor. Ikaros forms spectacular clusters in the nuclei of activated B cells at loci of heterochromatin. There are a number of possible Ikaros binding sites in the γ1 and ε GL promoters. We showed that Ikaros could bind to several of the possible binding sites in the GL γ1 and ε promoters, presumably in a cooperative manner. Co-transfection of dominant negative Ikaros, unable to bind DNA, with γ1 and ε promoter-luciferase reporter constructs to a B cell line, led to modestly increased basal transcription levels from these promoters. However, retroviral transduction of wildtype or a dominant negative mutation of Ikaros into primary LPS activated B cells, had no effect on Ig class switching or GL transcription, even though the endogenous clusters were disrupted by introduction of the mutated Ikaros. Thus, Ikaros might not be directly involved in transcriptional regulation of specific genes but might exert its function at other levels. Colocalisation of Ikaros with methyltransferase, which serves to direct replication-coupled DNA methylation, might suggest a role in inheritable silencing of genetic material somehow selected for methylation.

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Back cover: Dr. Paul Ehrlich pictures that describe
"The side-chain theory", that he put forward 1897.

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MAIN REFERENCES

This thesis is based on the following papers referred to in the text by their Roman numerals:

I Ferlin W., G., Severinson, E., **Ström, L**., Heath, A., W., Coffman, R.,L., Ferrick, D., A. and Howard, M., C. CD40 signalling induces interleukin-4-independent IgE switching *in vivo*. *Eur. J. Immunol.1996. 26: 2911-2915*

II **Ström L**., Laurencikiene J., Miskiniene, A., and Severinson, E. Characterization of CD40-dependent Immunoglobulin class switching. *Scand. J. Immunol. 1999. 49:523-32*

III Lundgren M., **Ström, L**., Bergqvist, L.O., Skog, S., Heiden, T and Severinson, E. Cell cycle regulation of Immunoglobulin class switch recombination and germline transcription: Potential role of Ets family members. *Eur. J. Immunol. 1995. 25: 2042-2051.*

IV **Ström L**., Lundgren M., and Severinson E. The function of Ikaros as a regulator of Ig class switching. *Submitted. 2002*

ABBREVIATIONS

Ab	antibody
Ag	antigen
AID	activation-induced cytidine deaminase
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukaemia
αCD40	anti-CD40
AP-1	activator protein-1
APC	antigen presenting cell
bp	base pair
BCR	B cell receptor
BM	bone marrow
C/EBP	CCAAT/enhancer binding protein
C	constant
CD	cluster of differentiation
CD40L	CD40 ligand
CH	heavy chain constant region
CREB	cyclic AMP response element binding
CSR	class switch recombination
Ds	double strand
EBF	early B cell factor
EMSA	electrophoretic mobility shift assay
GC	germinal centre
GL	germline
GTF	general transcription factor
H	heavy chain
HAT	histone acetyl transferase
HDAC	histone deacetyl transferase
HIGM	hyper IgM syndrome
HMGI(Y)	high mobility group protein type I or Y
HP-1	heterochromatin protein-1
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
Inr	initiator
IRAK	IL-1R associated kinase
ITAM	immunoreceptor tyrosine-based activation motif
L	light chain
LCR	locus control region
JAK	Janus family of tyrosine kinases
LPS	lipopolysaccharide
MALT	mucosal associated lymphoid tissue
MHC	major histocompatibility complex
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa B
NHEJ	non-homologous end joining
pBCR	pre-B cell receptor
preB	precursor B cell
proB	progenitor B cell
R	receptor
RAG	recombination activating gene
RSS	recombination signal sequence
RT-PCR	reverse transcriptase polymerase chain reaction
S region	switch region
SH domain	src homology domain
SHM	somatic hyper mutation
Ss	single strand
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TD	T cell dependent
TdT	terminal deoxynucleotidyl transferase
TF	transcription factor
TGF	transforming growth factor
TI	T cell independent
TIR domain	Toll/IL1R domain
TLR	Toll like receptor
TNF	tumour necrosis factor
TRAF	TNF-receptor associated factor
VDJ	variable, diversity and joinin

INTRODUCTION

General Introduction

Life is a battle. Every day we have to fight against a continuous stream of microorganisms that could potentially be hazardous to our health or even life. To defend ourselves we have a remarkable immune system. The immune system consists of two main parts, the non-specific immediate or innate part and the adaptive or specific immune system. The innate part depends largely on barriers like the skin, mucous membranes and the acidic environment in the stomach. There are also specialised cells involved, that internalise, kill and digest microorganisms or break down foreign macromolecules (Goldsby et al, 1999). Lately, it has been shown that the non-specific immune system acts very rapidly via antibacterial peptides produced by the epithelial cells in for example the respiratory pathway via so called *Toll like receptors (TLR*)(Zarember & Godowski, 2002). TLR also signals to expression of CD80 and 86 on antigen presenting cells (*APCs)*, which play an important role in activation of *B and T cells*, the major players of the adaptive immune system (Medzhitov et al, 1997).

The adaptive or specific immune response takes more time to build up a response and acts via *antibody (ab)* secretion or cell mediated immunity. Each B or T cell has *specificity* to only one *antigen (ag)*. When and if they meet their corresponding ag they will be activated. One of the more important aspects of the specific immune system is its ability to distinguish between self and non-self or danger contra no danger (Matzinger, 1998). This is important to avoid immune reactions with the tissues of the body itself, which is the situation in autoimmune diseases. The B and T cells are educated in the bone marrow (BM) or the thymus respectively, where self-reactive clones can be eliminated before the mature lymphocytes are released to the periphery. The adaptive immune system evolved late, at the divergence of vertebrates between hagfish and lampreys (Agrawal et al, 1998). Upon antigen stimulation B cells start to proliferate and secrete abs. As a common event during an immune response, the effector function of an ab or *Immunoglobulin (Ig)* molecule is altered, by a process called *Ig class switching*. Hereby, the constant part of the Ig molecule is exchanged while the ag specificity is retained, resulting in expression of *IgG, IgA or IgE* instead of the initial *IgM and IgD*. This is an important process for many reasons, among others immunodeficient patients unable to perform Ig class switching suffer from severe, sustained

infections. Ig class switching is achieved via a complicated recombination mechanism referred to as *class switch recombination (CSR)*.

B cell activators along with specific *cytokines* direct CSR to different heavy chains. CSR can be stimulated in a T cell-independent or –dependent manner. In vitro the T cell independent pathway is mimicked by stimulation with bacterial *lipopolysaccharide (LPS*), which induces *proliferation*, maturation to IgM secretion as well as CSR, leading to production of *IgG2b* and *IgG3*. The T cell-dependent activation relies partly on the interaction between *CD40*, expressed on all B cells and *CD40Ligand (L)*, expressed on activated T cells, and partly on different cytokines secreted by the T cells. Cytokines alone do not induce CSR but can regulate the choice of Ig class to be expressed. For example, interleukin-4 *(IL-4)* induces switching to *IgG1* and *IgE* together with a co-stimulant such as LPS or CD40L. For CSR to occur the B cells need to enter a proliferating state. Switching to a given isotype requires induction of *germline (GL) transcripts*, specific for the targeted C_H-gene. Another requirement for CSR is a *recombinase machinery* responsible for the *double strand (ds) breakage* and joining. The factors involved in this process are largely unknown, although lately a putative RNA editing enzyme, called *activation-induced cytidine deaminase (AID)* has been identified, without which no CSR occurs (for reviews see (Honjo et al, 2002; Manis et al, 2002; Stavnezer, 1996)). This was a very brief overview. In the following text, I will try to elucidate the matters that are related to Ig class switching in greater detail, after all the main issue of this thesis.

Ig isotypes and subclasses

Expression of abs or Igs with specificity for every possible ag is an important part of an immune response. Already one of the earliest immunologists, Dr. P. Ehrlich proposed that "toxins" would attach to preformed receptors on the surface of cells and after attachment of large amounts of toxins the receptor would be released and new receptors formed, i.e. circulating antibodies (Ehrlich, 1897). This was actually a selection theory put forward long before the clonal selection theory proposed by Burnet (Burnet, 1957). The Burnet theory states that all possible specificities of abs are produced randomly, and that the clone with specificity for a certain ag will start to expand after encounter with that ag.

The Ig or ab molecule consists of four polypeptide chains, two identical heavy (H)

chains and two identical light (L) chains, held together by disulphide bonds as well as non-covalent associations (Fig. 1).

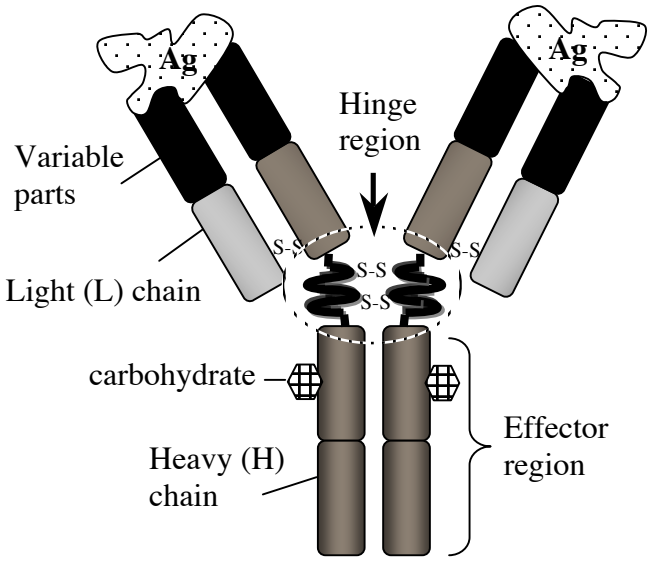

Figure 1. A schematic illustration of an Ig molecule with ag bound to its variable parts.

The light chains are either of the κ or the λ type. Ag binds to the amino terminal parts of the Ig-molecule, which are highly diverse and constituted by the variable (V), diversity (D) and joining (J) segments. The C-terminus is responsible for the effector functions of the Ig molecule and regarded as constant. Almost all vertebrates produce different isotypes of their Ig. Mouse, rat and human, the most well studied mammalian species, all have five classes of Ig heavy chains IgM, IgD, IgG, IgE and IgA, in addition they all have four subclasses of IgG. Each isotype exerts different biological activities like complement fixation, phagocytosis and mast cell degranulation. It also determines how the ag will be eliminated or where the Ig will be delivered. Different isotypes have different specialties. Due to the pentameric structure of IgM it has limited abilities to diffuse from one place to another. IgM abs tend to bind ag with relatively low affinity, to some extent compensated by their ten ag-binding sites and an efficient fixation of complement, the latter aiding in destruction or opzonisation of targets. In addition it has a short *in vivo* half-life and is less well adapted to secondary infections compared to IgG. IgG2a is efficient in defence against viruses and IgG3 is better at defence against bacterial infections etc. IgE is involved in protection against parasite infections and

binds to the Fcε receptor on mast cells which causes a degranulation reaction, an effect typical for allergic reactions (reviewed in (Snapper & Finkelman, 1999)). The different isotypes of Ig molecules produced in mice, are described in Table 1.

Table 1. Characteristics of murine Immunoglobulins.

	IgM	IgD	IgG1	IgG2a	IgG2b	IgG3	IgE	IgA
Form	pentamer	mono	mono	mono	mono	mono	mono	dimer
Serum level (mg/ml)	0,2	-	0,2	0,2	0,1	0,2	0, 0001	0.1
C´ activation	+++		+/-	++	++	+		+ *
Placental transfer			+	+	+	+		
Ag challenge (ex.)			Soluble protein	virus	Carbo-hydrate	Carbo-hydrate	Allergens parasites	
Presence in external secretions	Mucous etc	-	milk	milk	milk	milk	-	Mucous etc

*alternative pathway.

The Ig locus

The heavy chain gene locus spans approximately 200 kb and is found on chromosome 12 in mouse (Fig. 3). The κ and λ light chain gene loci are placed on chromosome 6 and 16, respectively. The V_H genes constitute the variable ag binding part of the Ig molecule and are rearranged through a precise procedure as will be discussed below. Ig transcripts are initiated from promoters located 5´ of each V gene (Staudt & Lenardo, 1991), which are relatively weak in the absence of enhancer activity. After the VDJ recombination is completed, the selected V-gene-promoter is within reach for the heavy chain intron enhancer Eμ, located between the last J gene segment and the μ_H chain. Downstream of the V, D and J gene segments the genes for the different C_H chains follow in the order: 5´- V-D-J-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3´ (Honjo & Kataoka, 1978; Shimizu et al, 1982). Each gene segment coding for a downstream heavy chain appears like a separate transcription unit and consists of in order from 5´ to 3´: a GL promoter, an I exon, a S region and the exons coding for the heavy chain. Each of these elements is of importance for different steps during the CSR and I will describe them in more detail in the following sections.

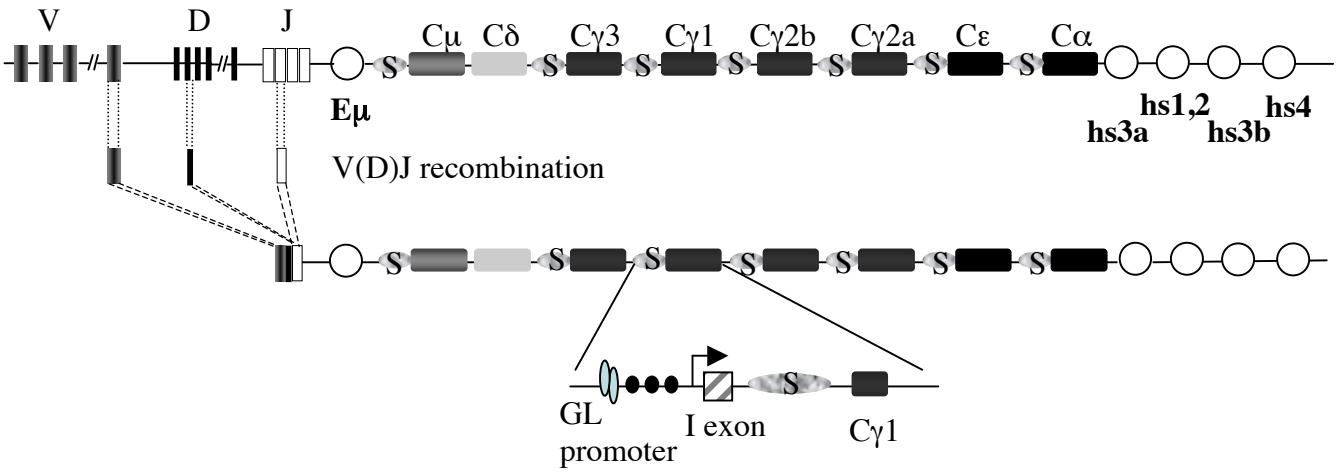

Figure 2. The Mouse Ig C_H gene locus with the Cγ1 transcription unit enlarged, including GL promoter, I exon, S region and Cγ1 exons. Upper graph: DNA in germline configuration. Lower graph: DNA configuration in mature virgin B cells.

B cell differentiation

What Ehrlich did not know was that ab formation and secretion is performed by the so called B lymphocytes. They are generated in the BM or the fetal liver during the embryonic development, from lymphoid stem cells. The earliest committed B cell precursors are called pro-B cells, which require contact with stromal cells to develop to pre-B cells (Rosenberg & Kincade, 1994). Pre-B cells depend on IL-7 for their further development. B cell maturation is also dependent on several transcription factors like PU.1, Ikaros, E2A, Early B cell factor (EBF) and Pax-5 or B-cell-specific activator protein (BSAP). The different stages of B cell maturation in the bone marrow and some of the proteins involved in this process are shown in Fig. 3 (for reviews see (Glimcher & Singh, 1999; Hardy & Hayakawa, 2001; O'Riordan & Grosschedl, 2000; Osmond et al, 1998)). During the ag independent phase of the B cell differentiation process, the V, D and J segments in the Ig gene locus are recombined in a stepwise fashion (Tonegawa, 1983). The products of the RAG1 and RAG2 (Oettinger, 1990) genes as well as the terminal deoxynucleotidyl transferase TdT (Landau et al, 1987), are part of a recombination machinery regulating this process. RAG1 and RAG2 recognize a precise primary sequence composed of a nonamer and a heptamer with a 12- or 23 base-pair spacer (Oettinger, 1999), so called recombination signal sequences (RSS). The very early progenitor

or pro-B stage cells have very few or no rearranged Ig genes. The above-mentioned five necessary transcription factors are believed to induce an early program of B cell gene expression including the genes λ5, VpreB, CD19, RAG1, RAG2, Igα and Igβ..

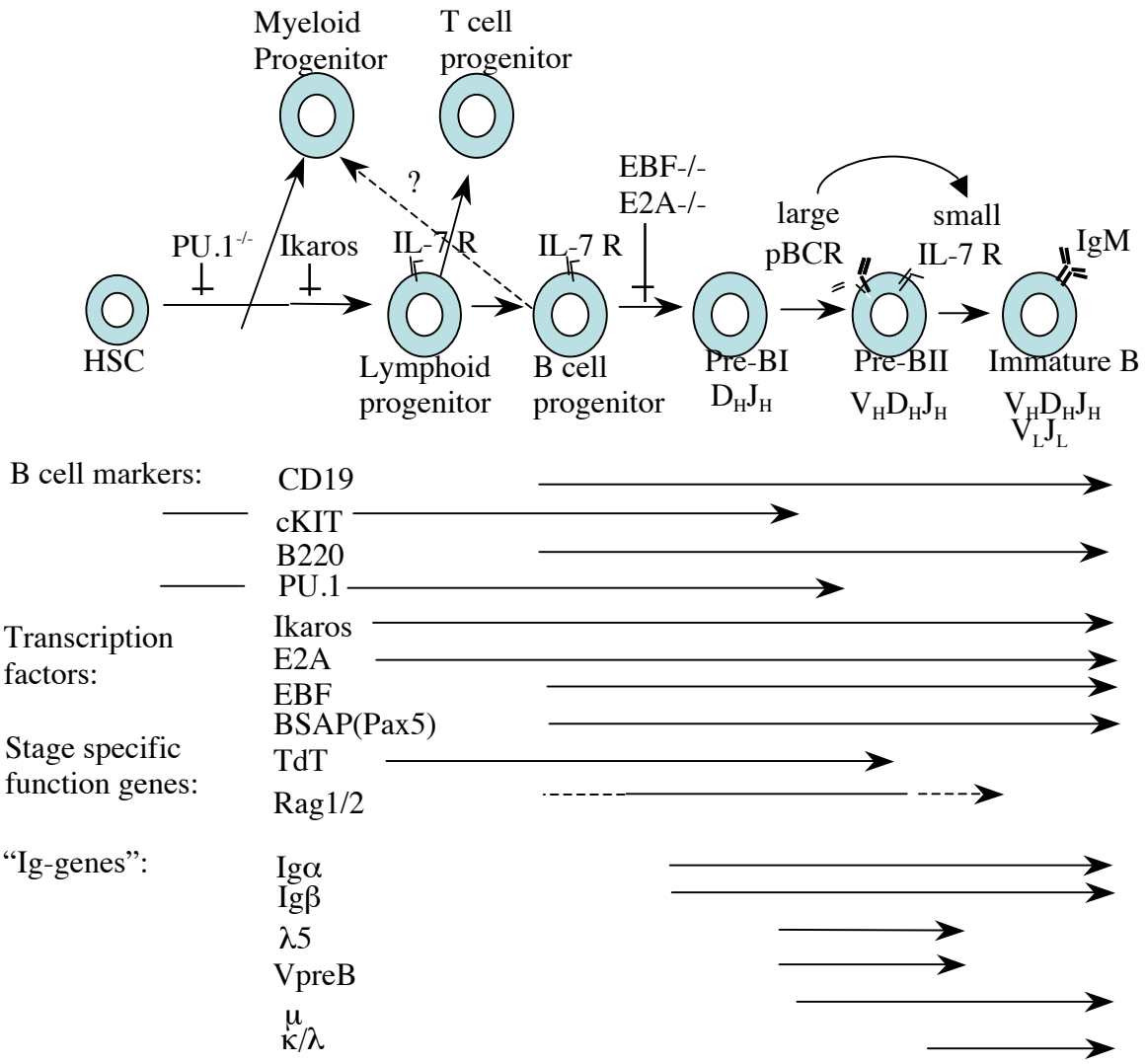

Figure 3. Antigen independent stages of B cell differentiation. Arrows denote the expression pattern of various transcription factors and genes for surface molecules expressed on B cells.

The next level in the B lineage are pre-BI cells, which have the IgH in D_HJ_H configuration and express the λ5 and VpreB proteins on the surface together with gp130 (Karasuyama et al, 1993; Ohnishi et al, 2000). Transcriptional regulation of the pre-B cells

remains to be clarified. However, the pre-BII cells start to proliferate and down regulate the recombination machinery so that further rearrangement of the heavy chain is repressed via signals from the pBCR (Mårtensson & Ceredig, 2000; Nussenzweig et al, 1987). VpreB1 and 2 have been genetically targeted and in these mice the pre-BII cells do not proliferate leading to reduced numbers of immature and mature B cells. However, the allelic exclusion process is still active (Mundt et al, 2001). If the heavy chain appears to be functional, rearrangement of the light chain starts, whereby IgM can be synthesized and expressed on the cell surface of immature B cells. Each B cell that have successfully reached this stage has shut down the second allele of IgH and IgL, thus each B cell clone will have specificity for only one ag. This phenomenon is known as allelic exclusion (Yancopoulos et al, 1986). B cells that do not react with auto-antigens can mature into resting B cells, expressing IgM and IgD on the surface. They will then migrate to secondary lymphoid organs like the spleen and lymph nodes where they are ready to interact with ag.

Ag encounters with B cells

When an ag enters the body it will be transported in different ways to the secondary lymphoid organs: via the lymphatic system to the lymph nodes, via the blood to the spleen or from various mucous membrane surfaces to mucosal associated lymphoid tissue (MALT). To the MALT belong Peyer´s patches in the intestines, tonsils and appendix. The ag is trapped there and can be attacked by the immune system. B cells are capable of recognizing soluble ag (Sela, 1966), while T cells will need the ag to be processed and presented together with the major histocompatibility complex (MHC) (Zinkernagel & Doherty, 1975) on APCs. The first signal required for activation of the B cells is delivered through the BCR. If the ag requires assistance from T cells to activate the B cells it is called T cell dependent or TD ag. The second signal is then mediated via T helper cells recognizing fragments of the ag presented on the surface of the B cell. The activated T cell expresses CD40L, which interacts with CD40 whereby a co-stimulatory signal is induced. Ag that are solid or repetitive can be independent of T cells (TI ag), and the second signal might then come from the ag itself or from non-thymus derived accessory cells. After ag challenge the B cells are induced to proliferation and secretion of the initial Ig isotype, IgM.

The Germinal Centre reaction.

The initial contact between B and T cells usually occurs in the T cell areas of the lymphoid tissue, where the B and T cells are trapped due to binding to ag. Further interactions are taking place after migration of the B cells to the B cell area, where so called germinal centres (GCs) are formed. The GC is a complex cellular microenvironment in follicles of the white pulp in the spleen and in lymph nodes, which supports further diversification after VDJ-recombination of the Ig genes. Thus, CSR and somatic hyper mutation (SHM), which introduces mutations in the V genes at high rate (Apel & Berek, 1990; Kosco-Vilbois et al, 1997) occur in the GCs. This is a specialized area for antigen retention and presentation and the place where T and B cells interact via CD40-CD40L and other co-stimulatory receptor ligand pairs like CD28-B.7 (Lenschow et al, 1996; McAdam et al, 1998) and the newly identified ICOS – B7RP-1(Hutloff et al, 1999; Yoshinaga et al, 1999). In support of this notion, knockout mice deficient in CD40, CD40L or CD28, all fail to produce GCs. The main function of the GC is to produce memory B cells: long-lived terminally differentiated plasma cells, that are isotype switched and somatically hypermutated (reviewed in (Kelsoe, 1996; McHeyzer-Williams et al, 2001; Tarlinton, 1998)). As a consequence of the rapid mutations in the GC a mechanism for elimination of newly created auto-reactive cells seems necessary and has been postulated to be induced due to lack of T cell help (Nossal et al, 1993). Formation of GCs is important for CSR but not absolutely required, since there are a number of reports about CSR taking place in the extrafollicular region (Toellner et al, 1996). It is also known that IgG3 synthesis after stimulation with TI ag takes place in the marginal zone, which is outside of GC (Guinamard et al, 2000; Oliver et al, 1997). In addition, lymphotoxin-$\alpha^{-/-}$ mice do not form GC but can however after receiving high doses of ag make isotype-switched ab at levels equal to wt mice (Matsumoto et al, 1996a; Matsumoto et al, 1996b). Most GC reactions described follows after challenge with a TD antigen. However, some TI ag can also induce GC formation (Liu et al, 1991; Sverremark & Fernandez, 1998).

Immunoglobulin class switching

Molecular basis for Ig class switching

As has been implicated in previous sections it is of importance to be able to express abs of all isotypes. In response to ag, B cells have the potential to change the effector function of the expressed ab while retaining the same ag binding specificity, via Ig class switching. Several models for Ig class switching have been postulated during the years after the identification of the different isotypes. Finally, an intra-chromosomal recombination-deletion model was proposed by Honjo and co-workers (Honjo & Kataoka, 1978), which was later confirmed by several groups (Iwasato et al, 1990; Matsuoka et al, 1990; von Schwedler et al, 1990). This is the process often referred to as CSR. Evidence for other mechanisms of CSR has been put forward, like inter-chromosomal recombination between homologous chromosomes during or immediately before mitosis and unequal crossing over between sister chromatids during cell division (Harriman et al, 1993; Wabl et al, 1985). In addition, switching without recombination has been suggested to occur via differential processing of a long primary transcript (Perlmutter & Gilbert, 1984) or trans-splicing of a V(D)J-Cμ transcript to a GL C_H transcript has been suggested (Shimizu et al, 1989). Here I will only describe "*The looping out and deletion model",* which at present appears as the most likely mechanism. Before a B cell is activated by ag, transcription starts from the VDJ locus and continues over Cμ and Cδ, consequently IgM and IgD are expressed. Simultaneously, GLμ transcripts are initiated from the Eμ, the enhancer for regulation of transcription from the V promoters, which can also act as a promoter for GLμ transcripts (Fig. 4:1) (Lennon & Perry, 1985). These transcripts are not up-regulated by stimuli, but are expressed at steady state levels (Li et al, 1994). After encounter with ag and depending on the type of co-stimuli the cell gets, induction of different downstream GL transcripts, like γ, ε or α will be induced (Fig. 4:2). For example, if the cell is activated via CD40 and the IL-4R, GL γ1 and GL ε transcripts will be synthesised. The role and function of these transcripts is not completely understood. They have been shown to be necessary, although not sufficient for CSR to occur. An accessibility model was proposed suggesting that CSR was directed to specific S regions

that had been induced to become accessible, as assayed by hypomethylation and GL transcripts (Stavnezer et al, 1984; Yancopoulos et al, 1986). This will be discussed further below. A recombinase of unknown identity will be activated (Fig. 4:3), and recombination between the Sμ and a selected downstream S region will be performed (Fig. 4:4). The recombination is initiated by introduction of ds breaks in both S regions. The intervening genetic material is thereafter deleted as a circle (Fig. 4:5). The ds DNA ends are ligated which will bring a new C_H gene adjacent to the V_H exon (Fig. 4:6). End joining has been suggested to be performed by the non-homologous end joining system (NHEJ), in which the DNA dependent protein kinase (DNA-PK/Ku) is known to be involved. DNA-PK is also required for CSR. Transcription from VDJ over the new C_H region can now lead to expression of the corresponding heavy chain (Fig. 4:7), while deleted intervening DNA segments will be rapidly degraded. An ab with a new type of effector function but with the same specificity can be synthesized and expressed. Some of the prerequisites for this process will be discussed in further detail below, like the structure of the S regions between which recombination occur, the GL transcripts and some of the known and postulated enzymes needed (Honjo et al, 2002; Manis et al, 2002; Stavnezer, 1996).

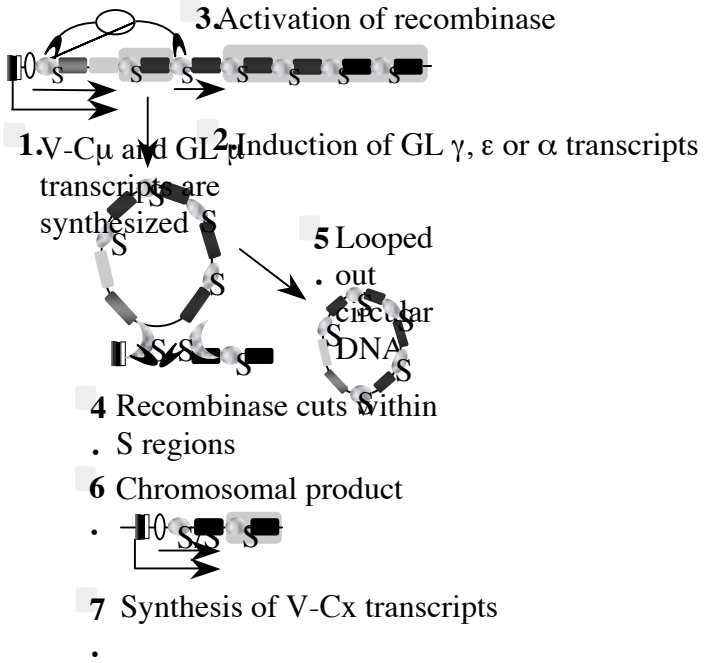

Figure 4. Class switch recombination (CSR) occurs by a recombination/deletion mechanism.

Structure of the Switch (S) regions

The actual recombination step during CSR occurs between the S region for μ and the S region of a selected downstream heavy chain, whereby the intervening DNA material is deleted as a circle. S regions are G-rich, simple tandem repetitive sequences 1(Sε)-10 (Sγ1) kb in length (Shimizu et al, 1982). Short 5-7 nucleotide repeats like, GAGCT, GGGGT and GGGCT are found in all S regions (Gritzmacher, 1989). Sμ, Sε and Sα are closely related, with 20, 40 and 80 base pairs (bp) repeat units respectively (Davis et al, 1980; Nikaido et al, 1981; Nikaido et al, 1982; Shimizu et al, 1982). The Sγ regions are less similar to Sμ and organized in 49-52 bp repeats (Gritzmacher, 1989; Kataoka et al, 1981; Mowatt & Dunnick, 1986; Nikaido et al, 1982; Stanton & Marcu, 1982). Another important feature of the S regions is the presence of frequent evolutionarily conserved palindromic sequences, which can form stem-loop structures at a denatured stage (Mussmann et al, 1997). This structure has been suggested to be the target motif for the recombinase (Kinoshita et al, 1998; Kinoshita & Honjo, 2000; Tashiro et al, 2001). In contrast to V(D)J recombination, switch recombination is not site-specific and recombination occurs all over the s regions (Dunnick et al, 1993). However in a given B cell, switch recombination is usually directed to the same S regions on both chromosomes (Radbruch et al, 1986).

Function of the S regions

That S regions are required for CSR was first demonstrated using artificial switch constructs. If these constructs lacked S sequences, CSR was completely abolished (Ott & Marcu, 1989). The specific function of the S regions in CSR has been described in various models. Even though several motifs have been found, that appear preferentially near switch junctions (Dunnick et al, 1993; Marcu et al, 1982), the degeneracy of S region sequences and heterogeneity of CSR sites suggested that the S region recognition code might lie in higher order structures rather than primary sequences. CSR is a unique type of recombination, since it is not homologous or site-specific but rather region-specific. The most important feature of the S regions might not be that they are repetitive or G rich but that they have palindromic

motifs (Tashiro et al, 2001). Since the latter are abundant in S regions, a stem-loop structure can be formed transiently at the denatured stage during transcription (Gnatt et al, 2001; Storb et al, 1998). Such a stem-loop structure could be functioning as a recognition target for the recombinase (Kataoka et al, 1981; Mussmann et al, 1997). Another higher order structure is the R-loop structure, formed by the nascent RNA transcript and DNA (Reaban & Griffin, 1990), that have been suggested to be induced after transcription of the S regions (Daniels & Lieber, 1995a; Tian & Alt, 2000). The recognition of a higher order structure would be consistent with the finding that primary sequences of S regions are not important for CSR, which was shown using artificial switch substrates (Kinoshita et al, 1998).

In attempts to identify the recombinase machinery or parts of it, proteins binding the S regions have been found. Thus Oct proteins bind to Sγ1 (Schultz et al, 1991). LR-1 is a LPS-inducible complex that binds to Sγ3, Sγ1 and Sα (Williams & Maizels, 1991). In addition, BSAP has been reported to bind to Sμ (as Sμ-B1), Sγ1 (Xu et al, 1992) and Sα (as Sα-BP)(Waters et al, 1989). Two DNA-binding protein complexes have been identified in mouse, which specifically interact with motifs in the Sγ tandem repeats. One of these protein complexes, initially termed SNIP, is indistinguishable from NF-κB p50 homodimer (Wuerffel et al, 1992), whereas the second protein complex termed SNAP, is most likely a hetero-oligomer containing the product of the E2A gene, E47, a helix-loop-helix transcription factor (Ma et al, 1997). SNIP/SNAP binding regions have been described in S regions also in humans (Pan et al, 1997). Based on in vivo cleavage site analysis, SNIP/SNAP sequences were proposed to be a recognition target for the recombinase (Kenter et al, 1993; Wuerffel et al, 1992). The possible importance for these proteins in CSR remains to be proven.

Germline (GL) transcripts

It is well established that the ability of a given cytokine to determine the target specificity of CSR, is correlated with induction of GL transcription from the GL promoters mentioned above. Transcription proceeds through the S regions and terminates downstream of the C_H exons. An accessibility model, suggesting that CSR is directed to specific S regions

which have been made accessible via transcription over the region, was proposed (Stavnezer et al, 1984; Stavnezer-Nordgren & Sirlin, 1986; Yancopoulos et al, 1986). Gene targeting experiments have been used to study the importance of the GL promoters and I exons. When the promoter and I exon of the γ1 gene were either replaced with a neo gene (in the reverse direction), or deleted, no switching to Cγ1 on the targeted allele could be detected, indicating that the I region or the transcription induced from these elements are important for regulation of CSR (Jung et al, 1993). The same conclusion could be drawn from experiments where the GLγ2b promoter and I exon were replaced with the neo gene in reverse orientation and Cγ2b class switching was almost entirely abolished. CSR to other isotypes was normal (Zhang et al, 1993). An alternative approach was applied where the GL ε promoter was replaced with the Eμ plus a V_H promoter to induce transcription over the Sε region constitutively in order to determine if this was sufficient to target the ε locus for class switching. This construct was introduced into a pre-B cell line, which can be induced by LPS plus IL-4 to express GL ε transcripts. LPS stimulation induced both GL ε transcripts and mature V-Cε transcripts in these cells, indicating that switching to ε can occur if the locus is actively transcribed (Xu et al, 1993). However, it could not be excluded that the pre B cell line is differently regulated than the mature B cells where class switching normally happen, or that inherent V_H recombination control elements were involved in regulation of CSR to Cε, rather than induction of transcription per se. In support of this are the results obtained when generating B cells from ES cells, with the same construct introduced. Despite efficient transcription over Cε, 10 to 100 fold less IgE was detected. This demonstrated that for optimal efficiency, the process requires the presence of the intact I region and/or GL promoter in cis, implying that transcription as such is not sufficient to cause efficient class switching (Bottaro et al, 1994). Lorenz et. al. showed that when an heterologous strong promoter, the metallothionein (MT) promoter, was introduced in place of the Iγ1 promoter only very modest levels of switching could be detected although transcription was induced over the region. Switching could however be restored if a 114-bp segment including the splice donor for the Iγ1 exon was inserted downstream of the MT promoter (Lorenz et al, 1995). In a later study it was shown

that the γ1 specific splice donor could be replaced with a splice site with bacterial origin with the same result, strongly suggesting that the processed transcript or the splicing mechanism in itself has a role in CSR (Hein et al, 1998). These data suggest that transcription per se is not sufficient for direction of switch recombination to specific S regions, but that somehow the processed transcripts seem to be important. The requirement for a spliced transcript was further supported in additional knock experiments. The Iα exon was replaced with a mini hypoxanthine phosphoribosyl transferase (HPRT) gene, which was transcribed and spliced to the normal Cα splice acceptor site. In these mice no reduction in switching to IgA was detected (Harriman et al, 1996). However, in the work of Seidl et al. most of the Iγ2b exon was replaced with the neo gene in the sense direction, leaving the endogenous GL γ2b promoter intact. Transcription over the Cγ2b region was induced and switching to Cγ2b at the same frequency as in wt cells could be detected. This controversy could possibly be explained with presence of cryptic splice donor sites in the neo gene yielding a transcript similar to a mature GLγ2b transcript (Seidl et al, 1998). In addition, in the same paper it was shown that a clean deletion without insertion of the neo gene, completely inhibits GL transcription of and CSR to the associated Cγ2b gene, showing that the deleted portion of the Iγ2b exon and splice donor site contain sequences necessary for efficient GL transcription and CSR.

This was a summary of experiments clearly showing that transcription over the S region and most probably spliced transcripts are important for efficient CSR to occur. The specific role of the GL transcripts in CSR is however still unknown, although several hypothesis have been presented. Probably the opening of the region via transcription is important. Formation of different structures composed of the DNA in the S region and the RNA transcript has been suggested to be formed. Thus, it has been shown that stable RNA-DNA hybrids exist, specifically at the S regions in the mouse genome and it was suggested that they act as targets for introduction of ds breaks (Daniels & Lieber, 1995b; Reaban & Griffin, 1990). Transcription through a switch region could also alter the methylation status of the targeted S region (Gu et al, 1993; Nakamura et al, 1996). Due to the presence of multiple stop codons in all open reading frames the likelihood of the mature GL transcripts to encode functional regulatory proteins is minimal, although possible.

Ds breaks

Although the mechanism for CSR starts to be understood, the recognition and cleavage of a particular S region remains an intriguing question. Wuerffel et al. showed, using a ligation mediated pcr method, that blunt 5´phosphorylated DNA ends were induced in S regions, using stimuli that induce CSR to the corresponding CH gene. They also detected a ds break that was rapidly induced in one of the tandem repeats in the Sγ3 region of B cells stimulated with LPS or anti-δ dex plus IL-5, i.e. under conditions inducing CSR to IgG3. GL γ3 transcripts were induced by anti-δ dex alone, but not CSR, indicating that the GL transcription in itself is not sufficient for break induction (Wuerffel et al, 1997). However, in a recent study by Honjo and co-workers where they used artificial CSR substrates, it was suggested that the S region breaks are induced by sequential nicking causing staggered ds cleavage (Chen et al, 2001). The cutting enzyme has not been identified, but it is not RAG 1 or 2, since CSR has been shown to be independent of those (Lansford et al, 1998; Rolink et al, 1996).

Activation-Induced cytidine deaminase (AID)

During a screen of cDNAs from switch-induced CH12F3-2 lymphoma cells, subtracted with cDNAs from uninduced cells the protein activation-induced cytidine deaminase (AID) was identified. AID is expressed in developing germinal centres and levels are upregulated by LPS, LPS plus IL-4 and LPS plus transforming growth factor (TGF)-β but not by CD40 stimulation (Muramatsu et al, 1999). $AID^{-/-}$ mice show elevated levels of IgM in serum, but are defective in production of all other isotypes and in addition no SHM occurs (Muramatsu et al, 2000). The IgM^{+} B cells in these mice can be stimulated to production of GL transcripts and their nonhomologous end joining (NHEJ) machinery is intact. AID deficiency does not seem to affect B cell maturation or other celltypes that are important for B cell activation. Since activation markers like CD69, CD86 and MHCII are upregulated normally upon ag challenge, the signal transduction pathway from the B cell receptor seems to be intact. All these data together suggest that AID plays a critical role in a step between GL transcription and joining of ds breaks common for both CSR and SHM (Nakagawa et al, 1999). Possibly it is involved in the initiation of ds breaks in the S regions by recognizing a characteristic

structure formed. It remains unclear whether AID functions directly or indirectly in mediating CSR and SHM (Longacre & Storb, 2000). Since AID is structurally homologous to APOBEC1, the catalytic part of an RNA editing enzyme (Teng et al, 1993), identifying a possible target transcript for the RNA editing activity will be important. It could be that AID is, if not the recombinase itself, at least an important part of the recombination machinery.

End joining

After the modifications of DNA are completed, the ds ends or nicks need to be joined for the cell to be able to complete DNA synthesis and survive. The joining systems of cleaved ends for CSR and SHM have been suggested to be distinct (Honjo et al, 2002). It has been well established that general ds breaks are repaired either by the non-homologous end joining (NHEJ) system or homologous recombination, depending on the cell cycle (Takata et al, 1998). Thus, NHEJ occurs during G_1 while homologous recombination plays its main role during G_2/M. Both ds (Papavasiliou & Schatz, 2000) and ss (Kong & Maizels, 2001) DNA breaks have been suggested to be induced during SHM. The ds breaks that occur in SHM seem to accumulate during the S/G_2 phase of the cell cycle, therefore they were proposed to be repaired by homologous recombination (Papavasiliou & Schatz, 2000). CSR breakpoints generally lack homology arguing against a primary role for homologous recombination. GL transcripts have been shown to be most abundant in cell populations enriched for cells in G_1/S (Paper III). If one assumes that GL transcripts need to be present during the process of CSR this may be an additional argument for NHEJ being used. Indeed, components of the NHEJ system have been shown to play a significant role in CSR (Casellas et al, 1998; Manis et al, 1998a; Rolink et al, 1996).

DNA-PK/Ku

Ku is a heterodimer consisting of Ku70 and Ku80 (or sometimes 86), which bind to DNA ds breaks, nicks, gaps and hairpins in a sequence-independent manner. When bound to DNA, Ku can recruit and activate the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which can then phosphorylate additional regulatory proteins (Blunt et al, 1996; Gottlieb & Jackson, 1993). Ku70/Ku80 plus DNA-PKcs together build the DNA dependent protein kinase, DNA-PK. Ku 70 and Ku 80 (86) deficient B cells have no ability to undergo CSR, neither in vivo nor in vitro (Casellas et al, 1998; Manis et al, 1998a; Zhu et al, 1996).

However, in the Ku deficiency also the B cell proliferation capacity is deficient, implicating that the lack of CSR could be an indirect effect. Interestingly, when the DNA-PKcs is non-functioning, as in the SCID mouse, there is no apparent lack of B cell proliferation and still a defect in CSR to all C_H genes is detected (Manis et al, 2002; Rolink et al, 1996). It has been suggested that the role for Ku should be to hold the DNA ends together and stimulate DNA end joining (Ramsden & Gellert, 1998). It is believed to protect the broken DNA ends and to recruit some of the many proteins required to complete repair, like DNA ligase IV (Lig.4). Experiments using Lig. $4^{-/-}$ and Lig. $4^{-/-}$xKu70$^{-/-}$ mice showed that Lig.4 is exclusively required for the Ku-dependent NHEJ pathway and that other DNA ligases cannot substitute in this function (Adachi et al, 2001). Ku 70 has been shown to interact with the nuclear matrix and it was proposed that the nuclear matrix is used as a scaffold in repair of ds breaks (Rodgers et al, 2002).

Mismatch repair enzymes

Mismatch repair (MMR) enzymes have a function in repair of mispaired bases due to DNA polymerase errors and DNA damage, thereby maintaining genetic stability. They recognize DNA distortions, they bind heteroduplex DNA and aid in removal of heterologous DNA at the ends of homologous recombining segments (Nakagawa et al, 1999). B cells from mice deficient in different MMR enzymes have decreased abilities to go through CSR (Ehrenstein & Neuberger, 1999; Schrader et al, 1999). CSR is not completely abolished in MMR deficient B cells which is in line with the fact that only those recombination intermediates containing heteroduplex DNA require MMR activity for resolution (reviewed in (Kenter, 1999)). Recently the MSh2-, Mlh1- and Pms 2-MMR proteins were all proposed to be involved in CSR, but that Msh2 appears to be involved at a different step from Mlh1 and Pms 2. Thus, Msh 2 may be involved in processing of the ends after ds break formation while Mlh1 and Pms 2 could be important for stabilisation of the recombination complex before DNA ligation (Schrader et al, 2002).

Other proteins implicated to be important for CSR

There are several other proteins in addition to the ones mentioned, that have been suggested to be of importance for CSR. As an example, SWAP-70 was identified in a

biochemical screen for nuclear proteins with potential to mediate CSR (Borggrefe et al, 1998). SWAP-70 is expressed in Mast cells and B cells, where upon activation its expression is rapidly increased (Borggrefe et al, 1999). SWAP-70 interacts with several other proteins including the BCR complex. However, a SWAP-70 deficient mouse showed only minor defects in the ability to perform class switching. More specifically, while the LPS plus IL4 induced IgG1 was largely unaffected, the IgE-response upon anti-CD40 plus IL-4 stimulation in these mice was reduced 5-8 fold and the in vivo IgE levels after parasite infection were 2-4 fold lower than in wt mice. A requirement for SWAP-70 in CD40 signalling, possibly in modulation of allergic responses was suggested (Borggrefe et al, 2001). Spo11, is a homolog of the novel type II topoisomerase (topo VI) that catalyses ds break formation. The mouse Spo11 was found to be upregulated in B cells by stimuli that cause induction of GL transcripts and it was suggested that Spo11 might participate in the initiation step of CSR (Tokuyama & Tokuyama, 2000). However, knockout mice with the gene for Spo 11 disrupted were perfectly able to perform Ig class switching (Klein et al, 2002).

VDJ contra CSR and SHM

During the successful differentiation and maturation of a B cell it is likely to go through three types of Ig gene alterations: 1. VDJ recombination, 2. IgH class switch recombination, and 3. Somatic hypermutation (SHM). The third genetic alteration of the Ig locus SHM, occurs concomitantly with CSR in GC. SHM induces mutations in the variable part of the Ig molecule and increases affinity for the ag the clone was selected for. The mechanism for SHM is largely, although not entirely unknown. DNA breaks are introduced in a transcriptionally associated manner and both ds breaks and nicks have been suggested. A model where mutations are introduced into the DNA during repair of ds breaks by homologous recombination has been put forward (Papavasiliou & Schatz, 2000). In addition AID, has been shown to be necessary for SHM to occur (Muramatsu et al, 2000). In Table 2, I have summarized some important similarities and differences between the different gene alterations.

Table 2. VDJ contra CSR and SHM

	VDJ	**CSR**	**SHM**
Organ/substructure	Bone Marrow	Germinal Centres	Germinal Centres
Celltype	Immature B and T	Mature B	Mature B
Ag dependent	no	yes	yes
Transcription dependency	yes	yes	yes
Recognition target	RSS (Site specific)	S regions	V regions
DNA breaks	ds breaks, blunt plus hairpin in G_0/G_1	ds breaks with staggered ends in G_1/S	ds breaks and/or ss nicks in S/G_2
RAG-1,2 dependent	yes	no	no
AID-dependent	no	yes	yes
Deletion circles formed	yes/no	yes	no
End joining	NHEJ	NHEJ	Error prone polymerase
Mismatch repair	?	yes	yes

Regulation of CSR

Cell cycle and cell division or proliferation

In addition to germline C_H gene expression, DNA synthesis appears to be necessary to allow B cells to undergo isotype switching. CSR occurs only in B cells that have been activated to proliferation. Inhibition of DNA synthesis by different inhibitors of DNA synthesis also inhibits GL transcription and CSR (Severinson Gronowicz et al, 1979b)(Paper III). Furthermore two to three rounds of cell division are required for IgG1 expression on the surface of activated B cells and another three rounds are required for IgE to be expressed (Hasbold et al, 1998; Hodgkin et al, 1996). Why several rounds of divisions are needed before IgG1 can be expressed is unknown, but it suggests that a serie of events need to be induced and that one or more of these require replication of DNA. One such event could be binding of certain transcription factors to promoter regions. In addition regulation of region accessibility, chromatin modifications like histone acetylation and DNA demethylation, synthesis of GL transcripts and formation of a recombinase machinery should be performed.

Modification of a RNA template for the putative RNA editing enzyme AID, necessary for both somatic hypermutation and CSR could be needed prior to pursuing its action. However, the requirement for DNA synthesis or cell proliferation does not indicate during which step of the cell cycle the CSR actually happens.

Transcriptional regulation in general

In eukaryotes the genetic material is transcribed by three types of RNA polymerases. The majority of the genes are transcribed by RNA polymerase II, which is responsible for synthesis of all mRNAs. One of the major pathways to control gene expression is via the initiation of transcription by RNA polII. PolII is recruited to the promoter of a gene selected for transcription by the general transcription factors (GTFs), a number of proteins that bind to the promoter and are necessary for the activity of RNA Pol II (Struhl, 1994). Most genes contain an A/T rich sequence 25-30 bp upstream of the transcription start site (Breathnach & Chambon, 1981). The consensus of this sequence is TATAAA and is called the TATA box. In many promoters, but not all, this is the sequence to which the GTF are recruited by the TATA binding protein (TBP). TBP is part of the transcription factor IID (TFIID) multiprotein complex. Two models for how the GTFs are assembled on the promoter have been put forward. In the first case the proteins are binding to each other sequentially on the promoter and in the second case a RNA PolII holoenzyme is formed before binding to the promoter (Lemon & Tjian, 2000). Most TATA less promoters appear to depend on the binding of TFIID as well, but uses a DNA element named the initiator (Inr) element for positioning of RNA pol II via Inr binding proteins like YY1 (Seidl et al, 1998; Seto et al, 1991; Smale & Baltimore, 1989).

Regulation of IgH GL transcripts

As mentioned earlier the Ig GL promoters are located 5´ to each S region. These are weak promoters that lack TATA boxes (Kuze et al, 1991; Lin & Stavnezer, 1992; Rothman et al, 1991; Xu & Stavnezer, 1992). They also share the presence of multiple start sites for transcription. To try to elucidate the requirements for activation of GL transcripts some of the GL promoters have been extensively studied, like the GL γ1, ε and α promoters. The GL γ3, γ2b and γ 2a promoters are less studied or not at all. It is known that LPS activates B cells

to switch to IgG2b and IgG3 (Severinson Gronowicz et al, 1979a), that CD40 cross-linking leads to IgG2b switch (Paper II) and that Interferon γ (IFNγ) induces IgG2a (Snapper & Paul, 1987). GL transcripts over respective region have been detected after stimulation with the different activators, but it is not known which transcription factors that are required. TGF-β stimulation of LPS activated murine B cells, leads to activation of the GL α promoter, and CSR to the Cα gene. Two tandem Ets-1 sites binding PU.1 and Elf-1 respectively have been shown in transient transfection assays to be important for activation of the promoter (Shi et al, 2001). In addition cyclic AMP response element binding protein (CREB) and Smad3 bind to adjacent sites at the promoter and synergize to mediate TGF-β induced transcription. The transcriptional response is further enhanced through cooperative binding of AML to Smad3/4 (Zhang & Derynck, 2000). In contrast, the γ1 and ε promoter regions have been quite extensively studied. They are relatively similar (Fig. 5), with several consensus sites for transcription factors in common, like STAT6 (signal transducer and activator of transcription), C/EBP and NF-κB (Delphin & Stavnezer, 1995; Iciek et al, 1997; Lin & Stavnezer, 1996; Lundgren et al, 1994; Mikita et al, 1998b; Warren & Berton, 1995). Recently the C/EBP site in the GL ε promoter has been redefined to be an AP-1 site (Shen & Stavnezer, 2001). The ε promoter has in addition a unique B cell specific activator protein (BSAP) site, which the γ1 promoter has not (Liao et al, 1994; Thienes et al, 1997).

These promoters can be activated through different pathways. For example, IL-4 activates the GL γ 1 and ε promoters via STAT6, while signalling through CD40-ligation can activate these promoters via NF-κB. LPS, on the other hand, has no effect on the promoter activity even though it can induce NF-κB binding (Lin & Stavnezer, 1996; Warren & Berton, 1995). For full activity of the promoters the different transcription factors are believed to synergize since mutations of any of their binding sites reduces activity of the promoter (Delphin & Stavnezer, 1995; Xu & Stavnezer, 1992). Less is known about negative modulation of these regions. C/EBPβ has been shown to inhibit transcription from the mouse GL ε and γ1 promoters. This inhibition is not dependent on binding of C/EBPβ to the promoters. The mechanism for inhibiotion was suggested to rather depend on disturbing the

interaction between the promoter and NF-κB (Mao & Stavnezer, 2001). The non-histone chromosomal protein HMG-I(Y)(Kim et al, 1995) as well as the protein encoded by the proto-oncogene BCL-6 both suppress ε promoter activity (Harris et al, 1999; Wang et al, 1996a). Furthermore, IFNγ a known negative regulator of Ig class switching and GL Ig transcription repress activity from a 51-bp fragment of the human ε promoter (Ezernieks et al, 1996). The transcription factor mediating this repression was not defined.

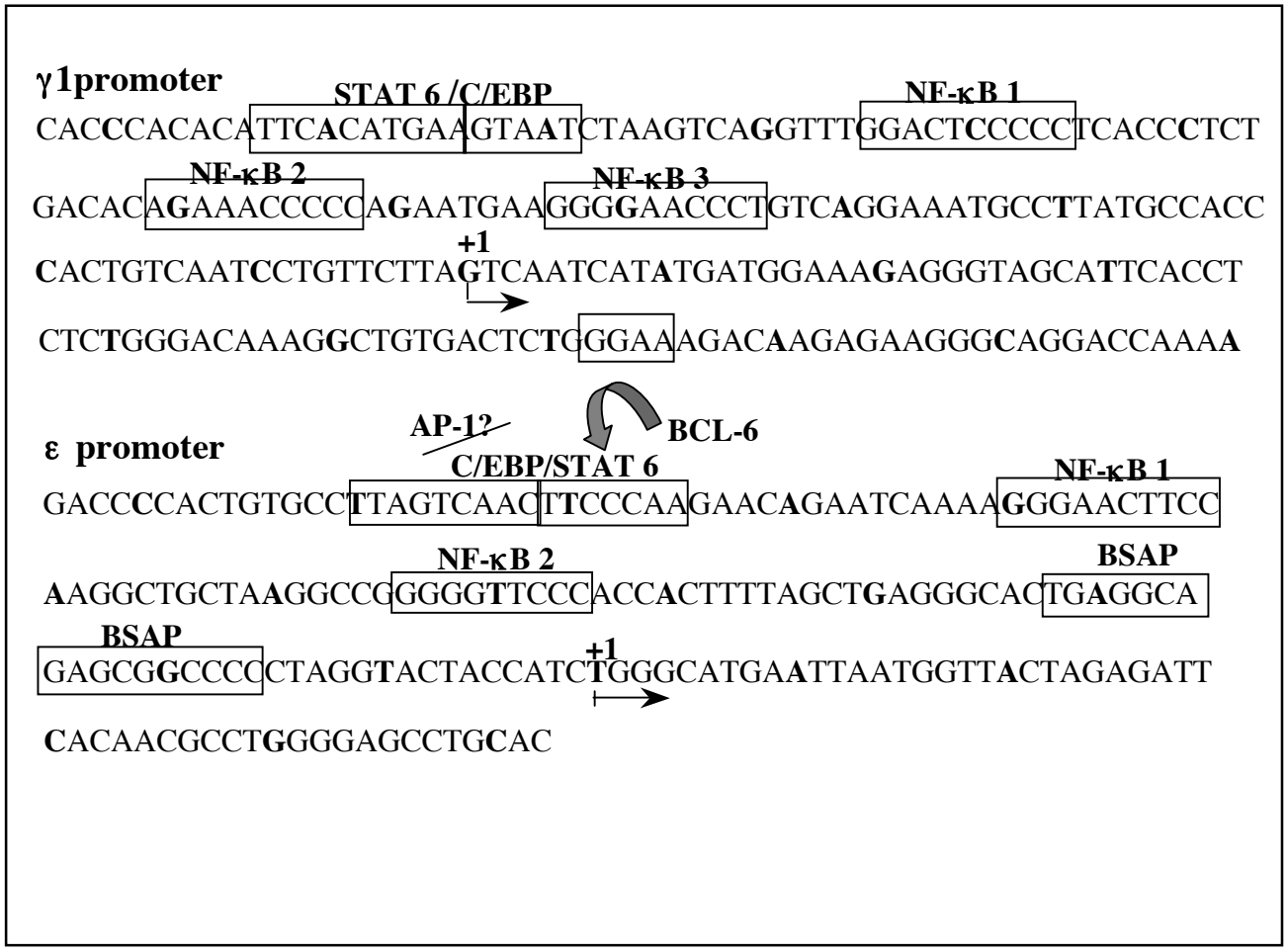

Figure 5. The GL γ1 and ε promoters with binding sites for known transcription factors boxed. BCL-6 can interfere with binding of STAT6 indicated with an arrow. The C/EBP site in the ε promoter has recently been suggested to be a binding site for AP-1.

Transcription factors regulating The Ig GL γ1 and ε promoters.

Under this section I will describe in more detail the transcription factors known to be involved in regulation of the GL γ1 and ε promoters, especially STAT6 and NF-κB. I will also introduce Ikaros, a protein that we have shown can bind to these promoters, at least in gel shift experiments (paper IV).

STAT6

STAT6 belongs to a family of proteins that are activated at the plasma membrane by the Janus kinase family members (JAKs). After activation STAT6 dimerises and translocates to the nucleus. There they bind specific promoters and are involved in activation of different target genes (Ihle, 1996). Seven STAT proteins have been identified in mammals. All STATs share functional domains, among them the SH2 domain, which plays an important role in association between STAT proteins and receptors. Differences in the SH2 domain determines selectivity of the interactions between different STATs and different receptors (Mikita et al, 1998a). The role of STAT6 in the regulation of γ1 and ε promoters is firmly established (Linehan et al, 1998; Warren & Berton, 1995). The transcriptional activation of γ1 and ε appears to involve STAT6 binding to the promoter (Delphin & Stavnezer, 1995; Köhler & Rieber, 1993; Lundgren et al, 1994). Work using gene-targeted mice shows that STAT6 is essential for IL-4 induced activation of IgE and optimal IgG1 responses (Shimoda et al, 1996; Takeda et al, 1996). Upon IL-4 stimulation, STAT6 is the primary STAT to respond and it is critical for the activation of many IL-4 responsive genes, like those of MHC II, CD23, GL ε and γ1, as well as IL-4Rα chain (Kaplan et al, 1996; Shimoda et al, 1996; Takeda et al, 1996). When the IL-4R is cross-linked due to binding of IL-4, the tyrosine kinases JAK1 and JAK 3 are phosphorylated and activated. Activation of JAKs induces phosphorylation of the IL-4Rα chain. STAT6 can then bind specific phosphorylated tyrosines on the IL-4Rα chain via an SH2 domain (Mikita et al, 1998a). It will thereby become phosphorylated and form a homodimer with a second phosphorylated STAT6 subunit, each binding a phosphotyrosine peptide with its SH2 domain. The dimer is then translocated to the nucleus, where it can bind to and initiate transcription (Nelms et al, 1999). The consensus binding site for STAT6 appears to be TTC-N_4-GAA. The exact mechanism whereby STAT6 activates transcription is

still largely unknown but it has been found that STATs associate with coactivator proteins. Thus, STAT6 can interact with CBP/p300 at its C terminal region (McDonald & Reich, 1999). In the γ1 and ε promoters, the STAT6 site is located immediately adjacent to a binding site for a basic region leucine zipper (bZip) which has been defined as a C/EBPβ–binding site, but lately redefined as an AP-1 site, required for IL-4 activation of the promoters (Shen & Stavnezer, 2001). In addition, STAT6 has been shown to interact with NF-κB, and together these transcription factors were shown to synergistically activate promoter constructs (Shen & Stavnezer, 1998).

NF-κB

NF-κB was initially described as an activator of the κ light chain enhancer (Sen & Baltimore, 1986). NF-κB consists of a family with five members: p50 (NF-κB1), p52 (NF-κB2), c-Rel, RelB and RelA (p65). The different family members are related by the high degree of homology shared in a 300 aa rel domain, which is essential for DNA binding, dimerization between family members and other transcription factors (for a review (Baeuerle & Baltimore, 1996; Wulczyn et al, 1996)). In pre-B cells the p50/p65 heterodimer is the dominant dimer, while in mature B cells it is the c-Rel /p50 and finally in plasma cells the p52/RelB (Liou et al, 1994). The dimerised complexes are kept inactive in the cytoplasm by association with IκB proteins. NF-κB is activated by a number of agents including LPS, cytokines, mitogens, DNA damaging agents and CD40 cross-linking. When the cells are activated IκB is phosphorylated and degraded in an ubiquitin dependent manner, which leads to release of the NF-κB dimer and translocation to the nucleus. Activated NF-κB appears to regulate a number of genes in various cell types. In mature B cells NF-κB binds to multiple target genes, such as the GL γ1 and ε promoters, and activate transcription (Baeuerle & Baltimore, 1996; Baldwin, 1996). A role for NF-κB/rel proteins in CSR has been established from knockout experiments. RelA is important for IgA and IgG1 production (Doi et al, 1997) as well as for class switching to IgG3 (Horwitz et al, 1999). B cells from $p50^{-/-}$ and $p52^{-/-}$ mice both show impaired CSR (Caamano et al, 1998; Franzoso et al, 1998; Sha et al, 1995). This is also true for B cells lacking the transactivating domain of c-Rel or being $c\text{-}Rel^{-/-}$ which both

have selective defects in CSR (Köntgen et al, 1995; Zelazowski et al, 1997). One of the reasons for the CSR defects could be related to the fact that NF-κB have been shown to be necessary for CD40-mediated activation of the GL γ1 and ε promoters (Delphin & Stavnezer, 1995; Iciek et al, 1997; Lin & Stavnezer, 1996).

C/EBPβ

C/EBPβ is a member of the CAAT-enhancer binding protein family of BZIP proteins together with C/EBPα, Ig/EBP, C/EBPδ and many others (Akira et al, 1990; Roman et al, 1990; Thomassin et al, 1992; Williams et al, 1991). There are C/EBP sites in Eμ, many V_H promoters, Iε, Iγ1 promoters and the intronic κ enhancer (Calame & Ghosh, 1995). In many of these elements C/EBP synergize with other transcription factors including the above mentioned NF- κB (LeClair et al, 1992) and STAT6 (Delphin & Stavnezer, 1995; Lundgren et al, 1994). C/EBPβ deficient mice have abnormal macrophage functions as well as B cell dysfunctions (Fattori et al, 1995; Tanaka et al, 1995).

AP-1 (Activator Protein-1)

AP-1 is a collective term referring to dimeric transcription factors composed of Jun, Fos or ATF (activating transcription factor), subunits that bind to a common DNA site, the AP-1 site. Studies in gene knockout mice and cell lines deficient in specific AP-1 components suggest that different AP-1 factors may regulate different target genes and thus execute distinct biological functions. AP-1 activity is regulated in part by the ability of the different family members to form hetero and homodimers in different combinations, but also through interactions with specific protein kinases and a variety of transcriptional coactivators. (for a review see (Karin et al, 1997)). Recently the former C/EBP binding site in the GL ε promoter was redefined as an AP-1 site and AP-1 was shown to synergistically activate the promoter together with STAT6 (Shen & Stavnezer, 2001).

BSAP

BSAP is one of the most critical B cell transcription factors and appears to function as a master B cell regulator. It is expressed mainly in the B cell lineage and has an influence during all stages of B cell maturation, up to the memory/plasma cell stages when it is no longer expressed (Barberis et al, 1990; Waters et al, 1989). Targeted deletion of the gene for BSAP (Pax5) ablates the B cell lineage (Urbanek et al, 1994) and allows cells expressing B cell specific markers to develop along the T, the NK or other lineages (Rolink et al, 1999). The mechanism behind this could be the dual role of BSAP, activating B cell specific genes and repressing lineage inappropriate genes (Horcher et al, 2001). There are binding sites for BSAP in several promoter and enhancer sequences of various B cell specific genes like λ5, VpreB (Nutt et al, 1997), HS1,2 (Singh & Birshtein, 1993) and Cε (Liao et al, 1994; Thienes et al, 1997). The binding site in the GL ε promoters is of high affinity type and over expression of BSAP in a B cell line that can be induced to switch to Cε led to elevated transcription from the GL ε promoter (Qiu & Stavnezer, 1998).

HMGI(Y)

It has been suggested that the murine GL ε promoter is repressed in resting B cells. This repression appears to be mediated in part by a binding site for HMGI(Y) (Kim et al, 1995). The mammalian HMGI(Y) family of "high mobility group" (HMG) non-chromosomal proteins is composed primarily of the isoforms HMG-I and HMG-Y(Johnson et al, 1988; Johnson et al, 1989) and the closely related HMGI-C nonhistone protein (Manfioletti et al, 1991). Differently from other HMG proteins the HMGI-(Y) proteins bind specifically in the minor groove of AT rich sequences of DNA in vitro (Solomon et al, 1986). Expression levels of HMGI-(Y) are elevated in proliferating cells (Giancotti et al, 1987). DNA binding domains of the protein are phosphorylatied by cell cycle dependent $p34^{cdc2}$-like kinases and this has been shown to decrease affinity for DNA (Elton et al, 1987; Giancotti et al, 1987; Nissen et al, 1991).

BCL-6

BCL-6 belongs to the Krüppel subfamily of zink-finger proteins (Ye et al, 1993). High expression levels of BCL-6 have been found only in certain B and T cells. Within the B cell lineage BCL-6 is expressed in mature GC B cells (Cattoretti et al, 1995). Mice deficient for

BCL-6 have normal numbers of B and T cells but they fail to form GCs (Fukuda et al, 1997). BCL-6 can bind to STAT6 binding sites in different IL-4 responsive promoters but it regulates only a few, including the GL ε promoter, upon which it acts as a repressor (Dent et al, 1997; Wang et al, 1996a; Ye et al, 1997).

Ikaros

Ikaros was originally identified as a potential regulator of enhancer and promoter elements critical for the expression of lymphoid-specific genes (Georgopolous et al, 1992; Hahm et al, 1994). The Ikaros gene encodes a set of at least 6 proteins, generated by alternative splicing. Ikaros belong to a family that contain Krüppel-type zink fingers organized in two functional domains, one for DNA binding and one for dimerization (Georgopopous et al, 1997; Hahm et al, 1994; Molnár & Georgopoulos, 1994). In the same family three other members have been identified: Aiolos (Morgan et al, 1997), Helios (Hahm et al, 1998), Eos (Honma et al, 1999) and Pegasus (Perdomo et al, 2000). The different family members can form hetero- and homo-dimers. These interactions influence the DNA binding ability and possibly activation/repression of transcription. Ikaros isoforms with at least three N-terminal zinc fingers (i.e. Ik-1, -2 and –3) are capable of binding to the core motif GGGAA with high affinity. Non-DNA binding isoforms can act as naturally occurring dominant negative factors to regulate the activity of the DNA binding forms (Georgopoulos et al, 1994; Sun et al, 1996).

A role for Ikaros in lymphopoiesis was demonstrated by gene targeting experiments. Animals with an Ikaros-null mutation lack fetal lymphocytes and adult B cells (Wang et al, 1996c). The B cell development is blocked before the pro-B cell stage. In addition they have a delayed T cell differentiation. Mice expressing a dominant negative version of Ikaros have a more severe phenotype (Georgopoulos et al, 1994). These mice have a complete block in lymphopoiesis, possibly caused by other Ikaros-like proteins or cofactors being inhibited. Ikaros binding sites have been described in the promoters of a number of genes, including RAG-1, IL-2 receptor, Igα, Vpre-B, λ5 and TdT (Georgopopous et al, 1997). For the latter three, the Ikaros binding site overlap with an Ets recognition sequence. On the TdT promoter, Ikaros compete for the same binding site with Elf-1, an Ets family member (Ernst et al, 1996; Trinh et al, 2001), and a mutation selectively disrupting binding of Ikaros prevented down-

regulation of the promoter during T cell differentiation. A study on the λ5 promoter has also indicated a role for Ikaros in gene silencing (Sabbattini et al, 2001). It has been observed that Ikaros is associated with several transcriptionally silent genes, and that these are detected in close vicinity to pericentromeric heterochromatin DNA in activated lymphocytes (Brown et al, 1999; Brown et al, 1997). Ikaros has also been shown to be part of multiple protein complexes like histone deacetylases, SWI/SNF ATP-dependent nucleosome remodelling complexes (Kim et al, 1999), clusters of DNA replication origins (Avitahl et al, 1999) and the putative co-repressors Sin3 and C terminal binding protein (CtBP) (Koipally & Georgopoulos, 2000; Koipally et al, 1999b).

Additional control regions in the Ig locus

Intron enhancer (Eμ) and IgH 3´enhancers

The expression of Ig is regulated via different control regions. I have previously described the V promoters located upstream of each V_H gene segment, as well as the μ intron enhancer (or Eμ) located between the last J segment and the Cμ gene (reviewed in (Henderson & Calame, 1998)). In the absence of Eμ, CSR or CSR-related deletions of downstream S regions still occur although at a lower level (Bottaro, 1998; Gu et al, 1993). This suggested that other enhancer elements were active in the region. In search for such, the first downstream enhancer was described in rat and called IgH 3´enhancer (Pettersson et al, 1990). Shortly thereafter the corresponding mouse IgH 3´enhancer was identified (Dariavach et al, 1991; Lieberson et al, 1991). Four enhancer elements have now been identified in this region, in part based on presence of DNase HS sites, and they are from 5´to 3´; HS3a, HS1,2,HS3b and HS4 (for a review see (Khamlichi et al, 2000)). HS1,2 corresponds to the IgH3´ previously mentioned. This region is conserved in humans and seems to harbour locus control region (LCR)-like properties (Madisen & Groudine, 1994). For positions in the IgH locus see Fig. 6. Replacing HS1,2 with the pgk-neo^r gene inhibited GL transcription of γ3, γ 2b, γ 2a and ε as well as CSR to the same C_H genes, while IgM and IgG1 levels were normal (Cogné et al, 1994). When the pgk-neo^r element was deleted, normal CSR was restored (Fiering et al, 1995). Similar phenotypes where observed when deleting or replacing the HS 3a element with the pgk-neo^r gene (Manis et al, 1998b). This indicates that neither HS1,2, nor

HS3a are required for CSR or it reflects a redundancy between the elements in the region. Interestingly, deletion of the HS3a-HS4 region blocked GL transcription to a similar set of CH genes as the pgk-neo^r insertion at the HS1,2 site (Pinaud et al, 2001). The Cγ1 and Cα have not been shown to be affected by the disruptions of the 3´enhancer elements. These C_H genes might be regulated differently as compared to the rest (Cogné et al, 1994; Pinaud et al, 2001). Interestingly, Dunnick and colleges showed that the γ1 gene includes two sets of DNase I HS sites, one that corresponds to the promoter region (site I) and one that is located just 5´of the S γ1 region (site II). The second HS site includes binding sites for STAT6 and NF-κB (Cunningham et al, 1998). DNase I HS sites are strongly correlated with regulatory elements (Gross & Garrard, 1988), and it was suggested that the γ1 gene includes its own LCR-like element, one candidate being the site II region (Adams et al, 2000).

Light chain enhancers

Not only the genes coding for the Ig heavy chains are regulated by enhancer elements but also the two variants of light chain genes, κ and λ. On the κ locus an intron enhancer, iEκ/MAR can be found between the last J segment and the first Cκ gene segment in addition to the κE3´enhancer situated 5´ from the last C gene segment (Meyer & Neuberger, 1989). Both the iEκ/MAR and the κE3´enhancers are critical for introducing somatic hypermutations to the Vκ segments (Betz et al, 1994; Graf et al, 1992). The iEκ becomes active at the pre-B to B cell transition and may function in activating κ locus recombination and transcription (Calame & Ghosh, 1995).

The murine Ig λ locus is organized into two rearrangement cassettes that each contains V λ and J λ gene segments as well as C λ coding exons. The λ-light chain enhancers are located 3´of C λ 4 (E λ 2-4) in the first cassette and 3´of C λ 1(E λ 1-3) in the second. These distal enhancer elements are likely to be the major regulators of Ig λ expression in mature B cell subsets (Brass et al, 1999).

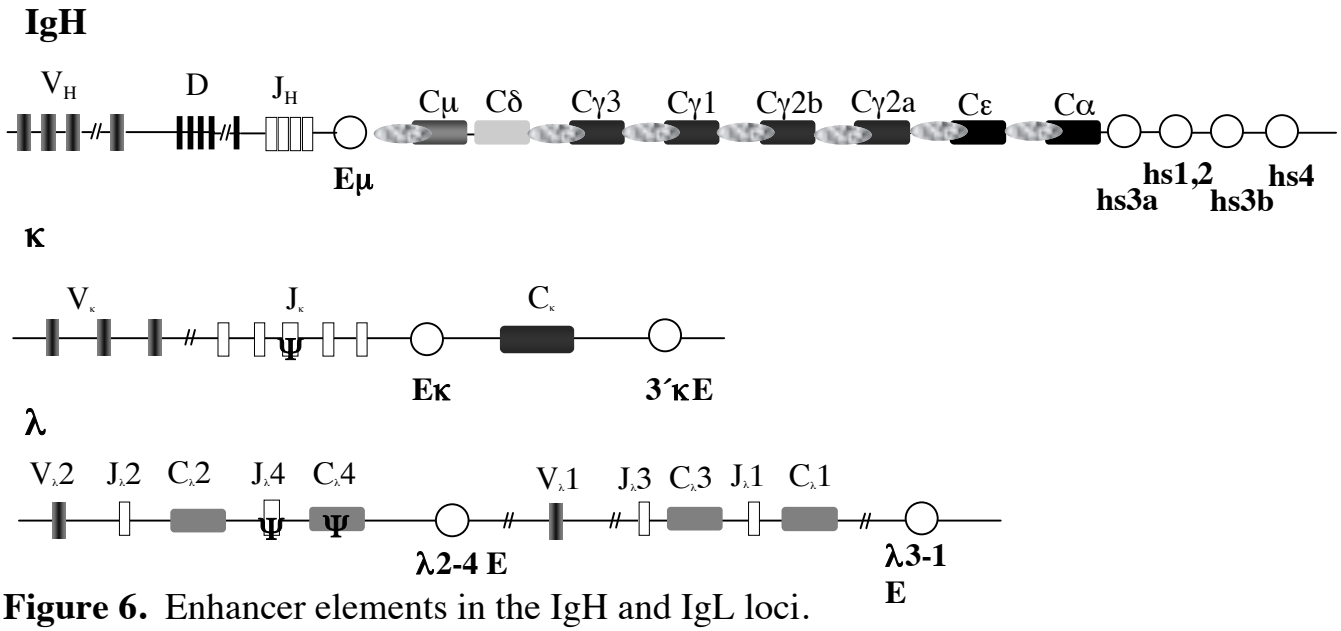

Figure 6. Enhancer elements in the IgH and IgL loci.

The influence of the chromatin state

As has been described above, the different genetic alterations that a B cell goes through during its differentiation process, all seem to depend on transcription. However, transcription *in vivo* and *in vitro* are two quite different events. In an intact cell the DNA is tightly packed with 146 bp of DNA winded up around a histone octamer, together forming the nucleosome. The nucleosome is "closed" by the associated heterochromatin protein-1 (HP-1) protein, thus forming what is known as chromatin (Hayes & Wolffe, 1993). The 11 nm nucleosome fibre is then assembled into a higher-order structure called the 30 nm filament, which in turn can assemble into more condensed higher-order structures. Decondensed chromatin is usually associated with genes that are accessible to interactions with the transcription or recombination machineries, and is likely to be a prerequisite for transcriptional activation. Transcriptional regulators are believed to exert their effects on transcription at least in part by modulating chromatin structure (Struhl, 1998). Different modulations of the chromatin can occur, like phosphorylation, methylation, ubiquitination and acetylation of the histone tails (Jenuwein & Allis, 2001; Kingston & Narlikar, 1999; Peterson & Workman, 2000). Acetylated histone tails are believed to reflect an active stage of a locus while modification by deacetylation and methylation is believed to lead to inactivation. Inactive chromatin is called heterochromatin while regions with active genes are most often positioned in

euchromatin. There are different types of so called coactivators that can perform modifications of the histone tails. Transcriptional coactivators are not necessary for basal level transcription but for enhanced transcription. Coactivators do not bind DNA directly, but connect gene-specific activators and the GTFs (Strahl & Allis, 2000).

Transcription through a locus can make it more accessible for recombinations and translocations etc. The series of events that can be imagined at a locus to be transcribed are: modifications of the nucleosomes, recruitment of gene specific transcription factors to the promoter, GTFs recruitment and initiation of transcription. Not much is known about the different modifications of chromatin that occur in the Ig locus. The chromatin status at the V(D)J locus is likely to determine how effective the rearrangement process will be. It was reported that acetylation of histone tails stimulated initiation of recombination initially inhibited by packaging of recognition sequences into nucleosomes (McBlane & Boyes, 2000). It has also been shown that demethylation of the V, D and J gene segments is necessary but not sufficient for successful rearrangement of the locus (Cherry et al, 2000; Engler & Storb, 1999). Demethylation is correlated with mutability of the V genes in the Igκ locus (Jolly & Neuberger, 2001). The C_H gene to which switching will occur is hypomethylated and in the promoter for GL transcripts DNase I hypersensitive sites are present (Berton & Vitetta, 1990; Schmitz & Radbruch, 1989; Stavnezer-Nordgren & Sirlin, 1986). Stimulation with LPS and IL-4, but not LPS alone, induces demethylation within 48 hours at an Msp I site 5´of the Sγ1 region of B-cell DNA (Burger & Radbruch, 1990). The late SV40 factor (LSF) binds both Sμ and Sα, and interacts both with histone deacetylases and the corepressor Sin3A. The reported repression of switching to IgA upon overexpression of a dominant negative version of LSF is proposed to be regulated via histone deacetylation of S region chromatin (Drouin et al, 2002).

Stimuli inducing CSR

Correlation between immunizing ags and the secreted isotypes detected suggested that Ig class switching is not a random, but a regulated process. Products included in supernatant from an activated T cell clone could induce IgG1 secretion and suppress IgG3 and IgG2b

secretion from LPS activated B cells. It was then suggested that humoral factors, later cytokines, participate in regulation of Ig class switching (Isaksson et al, 1982; Severinson et al, 1982). It could be concluded that the type of B cell activator along with specific cytokines directs CSR to different heavy chains. Here I will describe some of the known pathways for induction of Ig class switching.

BCR cross-linking

The BCR is expressed in the membrane together with its associated molecules Igα and Igβ (Hombach et al, 1988; Sakaguchi et al, 1988). The cytoplasmic tails of Igα and β contain ITAM motifs (immunoreceptor tyrosine-based activation motif), which are believed to be involved in signalling from the BCR as targets for src tyrosine kinases (Kurosaki, 1999). Proliferation and differentiation can be induced in resting B cells upon BCR ligation. However, additional costimulatory signals are usually required for efficient activation via the BCR. Signalling via the receptor for IL-4 or cross-linking of CD40 are pathways for activation that are well described, but co-stimulatory signals can also be provided via the CD19/CD21 complex or CD38 (Tsubata, 1999).

αIgM, αIgD, αδ-dex

By binding of anti-Ig to Sepharose-beads (Purkersson et al, 1988) or coupling of anti-δ to dextran (Snapper et al, 1992; Snapper et al, 1991), one can mimic a thymus-independent antigen and cause polyclonal activation of B cells. Anti-Ig Sepharose plus IL-4 induces GL γ1 transcripts and if IL-5 is included, also switching to IgG1 (Purkersson & Isakson, 1992). Anti-δ dextran can also induce GL γ1 transcripts even in the absence of IL-4. IL-4 increases the level of transcripts and if IL-5 is added switching to IgG1 can be detected (Mandler et al, 1993).

CD40

CD40 has the typical structure of a type I transmembrane protein and belongs to the tumour necrosis factor-receptor (TNF-R) family. It is a phosphorylated glycoprotein with 22 extra cellular cysteine residues, which are conserved between mice and humans. The intracellular part of the protein does not resemble any other characterized molecule. CD40 is

expressed constitutively on almost all mature B cells, on monocytes, follicular dendritic cells and dendritic cells as well as on some carcinomas (for review see (Banchereau et al, 1994; van Kooten & Banchereau, 2000)). Activation of B cells via cross-linking of CD40 leads to proliferation and in conjunction with cytokines allows isotype switching. In addition, it has in some reports been shown that CD40 activation alone can be sufficient to induce class switching (Maliszewski et al, 1993)(Paper I and II). CD40-deficient mice responded with IgM to TD antigens but no IgG, IgA or IgE was mounted. In addition GC formation was impaired. However, ab responses to TI ag including class switching were not affected (Kawabe et al, 1994). In humans mutations in the CD40 gene has been shown to cause an immune defect with lack of CSR, called HIGM3 (Ferrari et al, 2001).

Although CD40 in itself has no kinase domain, several signal transduction events are activated upon CD40 ligation. Among the earliest detectable events are activation of protein tyrosine kinases (PTK; including lyn, syk and JAK3), activation of phosphoinositide-3 kinase (PI-3 kinase) and phospholipase Cγ2 (Goldstein et al, 1997).

Mutagenesis of the intracellular part of CD40 has shown that CD40 interact with a new family of TNF-R associated factors or TRAFs. TRAF2, 3 and 5 at bind to one part of the cytoplasmic tail and TRAF6 binds at a separate position (Cheng et al, 1995; Ishida et al, 1996a; Ishida et al, 1996b; Rothe et al, 1995). The complex network of associated proteins and signalling pathways from CD40 are best described with a figure (see Fig.7). Signalling from CD40 leads to nuclear localization of NF-κB, NFAT, AP-1 (Francis et al, 1995) and possibly STAT3 and STAT6 via JAK3 (Hanissian & Geha, 1997; Karras et al, 1997).

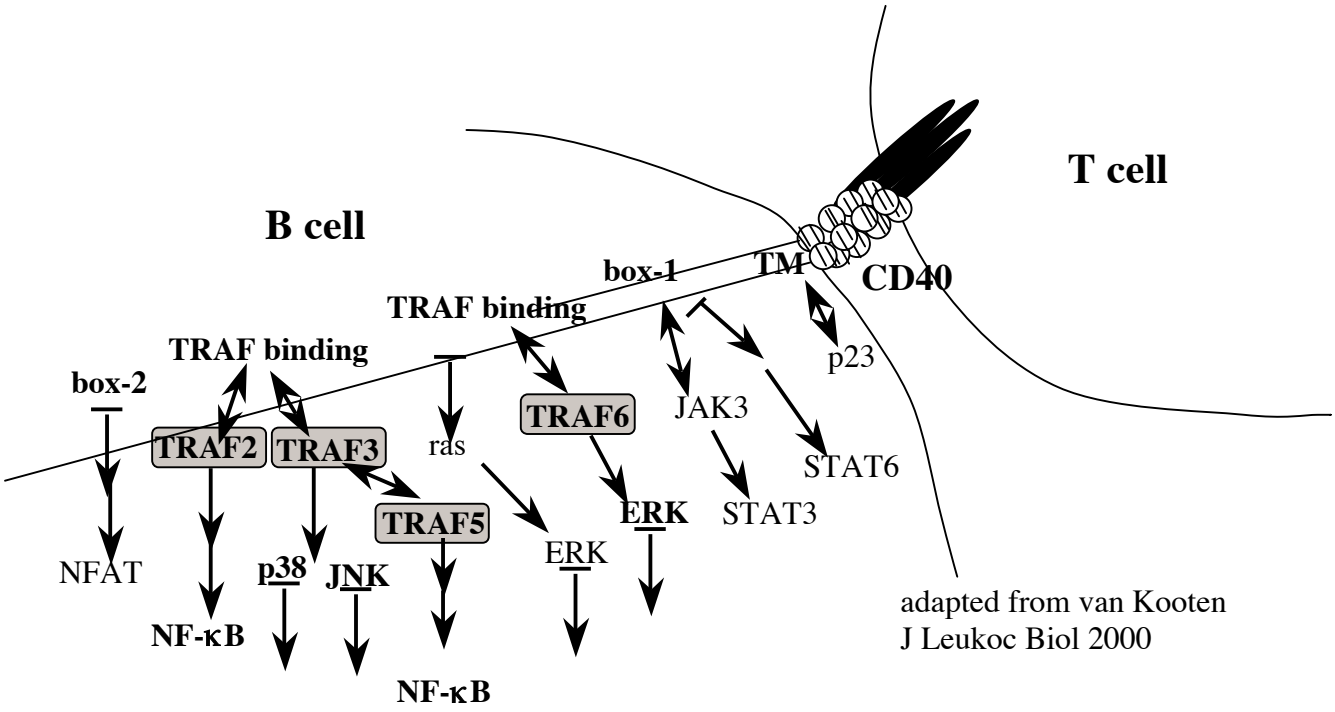

Figure 7. Proteins interacting with the cytoplasmic tail of CD40.

CD40L

CD40L is expressed mainly on activated mature T cells but not on resting T cells. Lately it has been shown to be present also on a number of other cells, like mast cells, basophils and eosinophils as well as on B cells, NK cells, monocytes/macrophages and DC under certain conditions (Carbone et al, 1997; Grammer et al, 1995; Mach et al, 1997; Pinchuk et al, 1996). CD40L is produced as a type II transmembrane protein and the three-dimensional organization is predicted to be similar to what has been predicted by modelling studies for TNF-α and LT-α. Crystallizations showed that the protein forms trimers (van Kooten & Banchereau, 2000).

The importance of the CD40-CD40L interaction has been proven by the immune deficiencies found in CD40 and CD40L knockout mice (Castigli et al, 1994; Kawabe et al, 1994; Xu et al, 1994), whose B cells can not undergo class switching in response to T cell dependent antigens. In addition, in humans the X-linked HIGM1 syndrome is caused by mutations in the CD40L (Allen et al, 1993; Aruffo et al, 1993; DiSanto et al, 1993; Fuleihan et al, 1993; Korthäuer et al, 1993). Activation through CD40 on B cells, by CD40 specific

mab or recombinant CD40L, in combination with cytokines leads to Ig class switching in a similar way as stimulation with LPS plus cytokines (Banchereau et al, 1994; Fujieda et al, 1995; Snapper et al, 1995).

Lipopolysaccharide (LPS)

LPS is part of the cell wall of gram-negative bacteria and is the most commonly used mitogen for mouse B cells in culture. It mimics the type of ag that act independently of T cells and induces extensive proliferation, secretion of IgM and isotype switching to IgG2b and IgG3 in mouse B cells, but not in human (Severinson Gronowicz et al, 1979a). The receptor for LPS was until recently unknown. CD14 has been known for some time to recognize LPS but since it is anchored in the cell membrane by a glycosylphosphatidyl-inositol (GPI) linkage, it was not believed to have sufficient ability in signalling. Another protein called LPS binding protein (LBP) recognizes the lipid-A part of LPS and has been shown to be important for the clearance of bacteria from the circulation mediated by CD14. Later it has been concluded that an LPS-binding and signalling complex is assembled when Toll-like receptor 4 (TLR4) interacts with LPS bound to CD14. TLR-4 interacts with CD14 via a leucine-rich repeat sequence in its extracellular domain. The presence of LBP further increases signalling (Hoffmann et al, 1999). Initially Toll was described in Drosophila being important for dorso-ventral axis formation during embryo-development. It was later described to be involved in an ancient host defence system (Lemaitre et al, 1996), with similarities from plants to vertebrates. Since 1997 10 different mammalian TLR have been identified (Aravind et al, 2001; Zhang & Ghosh, 2001) and growing. The TLR are characterized by a number of leucine rich extra cellular domain motifs, a single pass transmembrane domain and a cytoplasmic tail including a TIR motif (Toll/IL-1R domains), which is common for IL-1R and TLR (Gay & Keith, 1991). They recognize different and partly overlapping recognition motifs like lipopolysaccharides, ds RNA, unmethylated DNA, etc. They interact with the docking molecule MyD88 (Medzhitov et al, 1998) via TIR domains in the cytoplasmic domain of TLR and a TIR domain in MyD88. MyD88 recruits the kinase IRAK (IL-1R associated kinase) which once activated can phosphorylate TRAF6. TRAF6 is then part of the activation pathway for NF-κB and AP-1 (for a review see (Silverman & Maniatis, 2001)).

Cytokines

Cytokines belong to a large family of soluble proteins involved in regulation of growth and differentiation of different cells. To this family belongs IL-1-25 plus IFN-α, β, γ, TGF-β and TNF-α . Cytokines are mainly released from T helper (Th) cells. Th cells can be divided into two subsets according to their cytokine production (Mosmann et al, 1986). The Th1 cells produce mainly IFN-γ, IL-2 and TNF-β, while the Th2 cells secrete IL-4, IL-5, IL-10, IL-13 and GM-CSF. The ag concentration and the type of APC involved in activation will determine whether a Th1 or a Th2 response will take place (Secrist et al, 1995). Thus, a high allergen concentration favours a Th1 response while lower concentrations supports a Th2 dominated response. Cytokines act in an autocrine or a paracrine fashion on target cells with the proper receptors expressed on their surfaces (Tuyt et al, 1997). The Th2 subset provides help to B cells and promotes the production of IgM, IgE and some IgG isotypes. Th2 cells also support allergic reactions. The Th1 subset is associated with inflammatory responses such as delayed hypersensitivity and it also promotes differentiation of cytotoxic T cells. It has been suggested that the outcome of an infection can depend on the relative levels of Th1-like or Th2-like activities. The regulation of isotype specificity by cytokines is usually governed at the transcriptional level, by induction of transcription of C_H genes in GL configuration, prior to class switching. Here I am only describing the cytokines used in my studies in more detail.

Interleukin-4 (IL-4)

The IL-4 protein is a type I cytokine with an apparent molecular weight of 14,2 kD (Noma et al, 1986; Yokota et al, 1986). Early reports described IL-4 as being secreted by activated T cells only (Howard et al, 1982). Later it has been shown that also γ/δ T cells (Ferrick et al, 1995), mast cells, basophils and eosinophils can produce IL-4 (Dubucquoi et al, 1994; Plaut et al, 1989; Seder et al, 1991). IL-4 was initially found as a T cell-produced factor which synergized with anti-IgM antibodies in induction of B cell proliferation (Howard et al, 1982) and isotype switching to IgG1 in B cells activated with LPS (Isaksson et al, 1982). Later it was found to be a pleiotropic cytokine exerting its effects on B cells, T cells, mast cells and macrophages. It is crucial for development of Type I allergies and for protection against helminthic parasites. It induces upregulation of CD23 (Conrad et al, 1988), MHCII

(Noelle et al, 1984) as well as GL γ1 and ε transcripts. In addition, it induces class switching to IgG1 and IgE together with LPS or cross-linking of CD40 (Rothman et al, 1988; Sideras et al, 1985). In parallel, it represses GL γ2b and 3 transcripts as well as switching to IgG2b and 3 (Severinson et al, 1990). IL-4 mediates its transcriptional regulation by binding to the IL-4R causing association with the common γ chain (cγ or CD132), a component also of the IL-2, 7 and 13 receptors (Keegan & Pierce, 1994). The IL-4R complex lacks endogenous kinase activity, but receptor oligomerisation induces tyrosine phosphorylation of several intracellular proteins including the receptor itself. Induced differentiation largely depends on signalling via the JAK/STAT pathway, leading to nuclear translocation of the transcription factor STAT6 and binding to its target genes (Pernis et al, 1995; Ryan et al, 1996; Wang et al, 1996b), as discussed above under the STAT6 section. Another signalling pathway from the IL-4R is mediated via phosphorylation of insulin receptor substrate-2 (IRS-2), a docking protein which plays a major role in IL-4 induced proliferation (Welham et al, 1997). Mice homozygous for a mutation that inactivates the IL-4 gene were generated and showed normal T and B cell development but strongly reduced serum-levels of IgG1 and IgE. In addition, the IgG1 dominance in a T cell dependent immune response was lost and IgE was not detectable upon infection with a nematode parasite (Kuhn et al, 1991).

IL-5

IL-5 is expressed by the same subset of T cells as IL-4 and the MW of IL-5 is 12,3 kD. IL-5 is mainly exerting its effects on differentiation of eosinophils (Lopez et al, 1988). It can also, under certain conditions, costimulate growth and Ig secretion in B cells (Swain et al, 1988; Takatsu, 1998). It can also provide additional signals important for anti-Ig plus IL-4 induced switching to IgG1 and IgE (Mandler et al, 1993; Purkerson & Isakson, 1994; Purkersson & Isakson, 1992)(Paper II). IL-5 activates a number of kinases, including Btk and Jak2 (Hitoshi et al, 1993; Kouro et al, 1996; Ogata et al, 1998). It has been shown that IL-5 can induce CSR to Cγ1 together with cross-linking of CD38 in a STAT5a and b dependent manner (Horikawa et al, 2001). STAT5a and b are two well characterized molecules downstream of JAK2 (Leonard & O'Shea, 1998).

AIMS OF THE PRESENT STUDY

Why is it important to study Ig class switching? Persons suffering from different types of hyper-IgM syndromes are unable to change effector function of their antibodies meaning that they cannot perform class switching. These patients have normal or elevated serum IgM levels but lack IgG, IgA and IgE, resulting in a profound susceptibility to bacterial infections (Allen et al, 1993; Notarangelo et al, 1992). The Hyper IgE syndrome is another rare defect characterized by markedly elevated serum IgE levels, chronic dermatitis and lifelong recurrent severe infections (Buckley & Becker, 1978; Donabedian & Gallin, 1983). A far more common disease is extrinsic asthma typically associated with increased serum IgE levels, indicating a dysregulation of Ig class switching. This shows that CSR is an important feature of the immune system. Knowledge leading to the possibility of directing switching to a particular class could be most valuable.

The general goal of my studies was to clarify mechanisms of Ig class switching. In addition, I wanted to get increased understanding for the role of transcription in Ig class switching.

More specifically I wanted to:

Investigate the role of CD40 in class switching.

Understand the link between proliferation and class switching.

To identify proteins involved in regulation of the GL promoters.

RESULTS AND DISCUSSION

CD40 and its role in Ig class switching

When this work was initiated it was known that the interaction between B cells and T cells was important for induction of Ig class switching. In addition, CD40 had been defined as a critical mediator of T-B cell collaboration. In Paper I, we asked, whether the CD40/CD40L interaction was sufficient for B cells to undergo CSR. This was tested in mice resulting from the cross-breading of TCR$\beta^{-/-}$ mice and TCR$\delta^{-/-}$ mice (Mombaerts et al, 1994). In vivo distribution of an agonistic monoclonal rat anti-mouse CD40-ab induced massive B cell proliferation as well as up-regulation of all Ig isotypes in these mice that are completely void of T cells. This shows that the CD40-CD40L interaction is sufficient for induction of low levels of Ig class switching also when being the sole co-stimulatory pathway available. Since these mice completely lack T cells, the main producers of IL-4, this indicated that the response might be IL-4 independent. However, both basophils and mast cells can produce IL-4 (Brown et al, 1987; Piccinni et al, 1991; Plaut et al, 1989). In addition, a population of nonB/nonT cells that express IgE receptors have been reported to produce substantial amounts of IL-4 in schistosome-infected mice (Aoki et al, 1995). Thus, it is still possible that there is a source of endogenous IL-4 in these mice. To test if the induction of Ig secretion and presumably Ig class switching was independent of IL-4, anti-IL4 was injected together with anti-CD40. The IgE synthesis was only partially inhibited, suggesting that CD40 stimulation in these mice was sufficient for induction of low amounts of IgE. A more direct proof for the independence of IL-4 was provided when an IgE response could be induced also in IL4$^{-/-}$ mice. Anti-CD40 also induced GL ε transcripts in B cells from IL-4$^{-/-}$ mice in vitro, excluding the possibility of expansion of cells already switched in vivo by an alternative mechanism. Early in vivo studies in mice showed that IL-4 was absolutely required for IgE synthesis (Finkelman et al, 1986). Later studies have in agreement with paper I showed evidence for IL-4 independent switching to IgE. During the course of a retrovirus induced immunodeficiency disease (MAIDS), IL-4 deficient and control mice have comparably

elevated levels of serum IgE (Morawetz et al, 1996). Further, IgE is produced after infection with Plasmodium chabaudi (von der Weid et al, 1994) or Leishmania major (Noben-Trauth et al, 1996) in IL-4 deficient mice although the levels are only a small portion of those seen in wildtype (wt) mice. These data together suggest that IgE switching can occur in the absence of IL-4. However, the level of induced IgE only reaches the amount of IgE normally detected in non-immunized wt mice. The physiological role of these low amounts of IgE can clearly be questioned and the conclusion must be that IL-4 is still the major regulator of IgE responses.

In the next study we wanted to further characterize the role of CD40 signalling during the different molecular events behind induction of CSR. We used the same agonistic monoclonal rat anti-mouse CD40 antibody as in paper I, to mimic the CD40-CD40L interaction in vivo. This Mab was shown to induce increased DNA synthesis, as well as expression of MHC II and CD23, markers for early B cell activation (Heath et al, 1994). We found, in agreement with others, that signalling through CD40 stimulated GL γ1 and low levels of ε transcripts. Furthermore, we showed that anti-CD40 antibodies stimulated production of GL γ2b, but not γ2a or α transcripts. Initially, we used the digestion-circularisation (dc)-pcr method (Chu et al, 1992), to look for induction of CSR. With this method the composite S region created by CSR is digested out from the genome and immersedd under dilute conditions to favour circle formation. With primers positioned 5´ of Sμ and 3´ of Sγ1 one can then amplify a pcr-product across the restriction site as a sign of deletional rearrangement. In some experiments we detected low levels of Sμ/Sγ1 recombination after stimulation with anti-CD40 alone. This was possibly a result of the type of IL-4 independent switching similar to what we reported in paper I, but this result was inconsistent. For this reason we developed a more sensitive recombination specific rt-pcr. With this method one takes advantage of the fact that the Eμ enhancer, which regulates production of GL μ transcripts, remains active also after CSR has occurred (Li et al, 1994). Thereby, GL transcripts will be synthesised that span over the Iμ exon and continues through the new C_H exons targeted for switching (Fig 4), and detection of these can be used as a measure of CSR to the detected downstream C region. In B cells activated by LPS or anti-

CD40 we could detect CSR to Cγ2b but not to Cγ1. However, anti-CD40 together with IL-4 or IL-5 induced CSR to the Cγ1 region. The addition of IL-4 to LPS stimulated cells has long been known to cause a down-regulation of the IgG2b and IgG3 expression and presumably CSR to Cγ2b and Cγ3. The CSR to Cγ2b induced by anti-CD40 alone can in a similar manner be inhibited by addition of IL-4 (L.S unpublished data, Fig. 8).

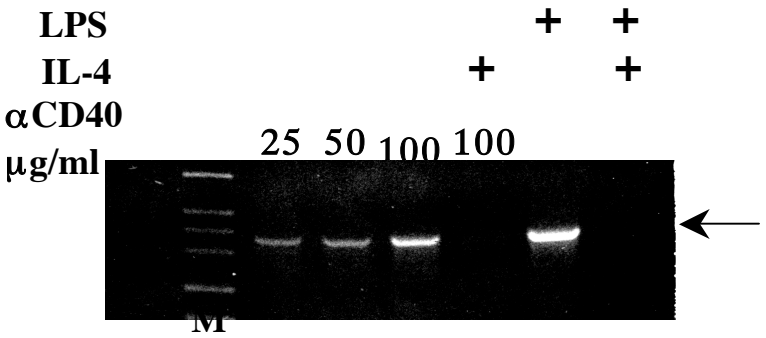

Figure 8. Anti-CD40 and LPS induces CSR to Cγ2b. The addition of IL-4 inhibits CSR to Cγ2b in both cases. B cells were stimulated as indicated for four days, RNA was extracted and cDNA synthesis performed. PCR was run with Iμ- and Cγ2b specific primers. The arrow points at the product of expected size, 424 bp. (-: no DNA, M: molecular weight marker).

Thus, CD40 signalling induces GL γ1, ε and γ2b transcripts, but CSR only to Cγ2b. This indicates that the recombinase machinery is functional after CD40 stimulation but that something more, possibly a third component, is required for switching to IgG1 and IgE. A similar conclusion was drawn by Mandler et al. who showed that anti-IgD coupled to dextran plus IL-4 stimulated production of GL γ1 transcripts, but not switching to IgG1 unless IL-5 was also present (Mandler et al, 1993). However, recent data demonstrate that all components necessary for CSR, except induction of GL transcription and expression of AID, are constitutively expressed in B cells, T cells and even fibroblasts. This was shown by over-expression of AID in NIH3T3 a fibroblast cell line, together with an actively transcribed, thus accessible, switch construct containing Sμ and Sα regions (Okazaki et al, 2002). This makes it difficult to explain the lack of CSR to Cγ1 since one would believe that the third required component would be B cell specific. However, it is still possible that there is a class or

subclass specific part present in the recombinase machinery. The group of Kenter has recently suggested the presence of at least four independent isotype-specific switching activities in cells that showed equal expression of AID. It was suggested that AID possibly recognizes various targeted S regions differently well via S region specific docking proteins (Ma et al, 2002; Shanmugam et al, 2000).

Both LPS stimulation and CD40 cross-linking are known to induce translocation of NF-κB to the nucleus. However, only stimulation via CD40 leads to induction of GL γ1 transcripts. In transfection assays it had earlier been shown that CD40 dependent activation of the GL γ1 promoter involved binding of NF-κB to three individual binding sites in the promoter (Lin & Stavnezer, 1996). LPS, on the other hand, was unable to induce activity from this promoter. We reasoned that this discrepancy could possibly be due to different subunits of the NF-κB family interacting with the promoter. We set out to test if there were differences in levels or composition of nuclear proteins binding the γ1 promoter after activation with the different stimuli. Using a probe covering the three NF-κB sites (–126/+25) the binding pattern was very similar testing nuclear extracts from cells activated with respective stimuli (L.S unbublished data). NF-κB activated via both LPS and CD40 could bind to all three individual NF-κB sites. However, the amount of bound NF-κB varied such that after anti-CD40 stimulation we could detect between 2-10 times more NF-κB than after LPS stimulation, on a per cell basis. In addition there were differences in subunit composition induced by the two stimuli. Thus, LPS stimulation led to relatively higher levels of p50 homodimers whereas in extracts from anti-CD40 stimulated cells the heterodimers p50/p65 and p50/cRel were more abundant. It has been shown that excess of p50 can reduce promoter activity, presumably by replacing binding of p50/p65 heterodimers, which have a higher transactivating potential with p50 homodimers (Franzoso et al, 1998; Kang et al, 1992). We proposed that the higher level of p50 homodimer seen in extracts from LPS activated cells compared with anti-CD40 activated cells were preventing activation of the GL γ1 promoter. Lin et al. performed a similar study and showed a somewhat different binding pattern of NF-κB to the γ1 promoter. Some of these differences can be explained by the usage of B cell lines instead of primary B cells, the time point chosen and also by the method for preparation

of nuclear extracts and running EMSA. It is known that formation of DNA-protein complexes is sensitive to salt concentration. Interestingly they could show that introduction of p50-c-Rel or p50-p50 fusion proteins could suppress transactivation induced by p50-RelA (Lin et al, 1998). It seems clear that the subunit composition of NF-κB dimers binding to DNA is important for activity of the promoters. This was also indicated from results using different knockout mice. Thus, B cells from mice lacking the transactivating domain of c-Rel can express both GL α and GL ε transcripts but perform switching only to Cα. Similarly, lack of the p50 subunit renders B cells deficient in CSR to all C_H genes except Cγ1, despite the ability to express γ1, γ3 and α GL transcripts. It is possible that LPS and anti-CD40 induce nuclear translocation of different NF-κB subunits because they induce phosphorylation of the IκB protein in different ways. CD40 exerts its signalling capacity through interactions with TRAF2, 3, 5 and 6 via direct binding of the TRAFs to the cytoplasmic tail of CD40 (Bradley & Pober, 2001). LPS has been shown to activate NF-κB via TLR4 and indirectly TRAF6 (Poltorak, 1998; Rhee & Hwang, 2000). It is possible that the difference in activation of different subunits of NF-κB reflects the differences in the types of NF-κB/IκB complexes that specific TRAFs preferentially activate.

Cell proliferation in relation to Ig class switching

Several lines of evidence suggest a coupling between proliferation and isotype switching. When DNA synthesis was inhibited, the switching to IgG secretion was decreased (Severinson Gronowicz et al, 1979b). The presence of mutations, duplications and deletions in recombined switch regions indicate a role of replication during the switch event (Dunnick et al, 1989). B cells have to go through several rounds of divisions before any CSR can be detected (Hodgkin et al, 1996). In paper III we studied the dependence of proliferation for CSR and synthesis of GL transcripts. The correlation between CSR and proliferation was assayed by addition of the DNA synthesis inhibitors Hydroxyurea (HU) which selectively interferes with ribonucleotide reductase and thereby inhibits DNA synthesis and Aphidicholin

(AC) which interacts directly with DNA polymerase α (Ikegami et al, 1978; Skoog & Nordenskjöld, 1971), followed by detection of recombination using dc-pcr. Cell cycle arrest in cells stimulated with LPS+IL-4 completely abrogated CSR. Furthermore, addition of HU severely reduced steady state levels of GL γ1 and ε RNA in LPS plus IL 4 activated B cells. To enrich for B cell blasts in different cell cycle phases we used elutriator centrifugation. Hereby, we found that GL γ1 transcripts were expressed both in G_1 and possibly S phase, but not in G_0 and were down regulated in G_2/M. Using EMSA, we looked at binding patterns of nuclear proteins to the GL γ1 promoter. We found two major LPS induced DNA binding protein complexes. Their level of binding correlated with the amount of LPS induced DNA synthesis. These complexes were shown by elutriation experiments to be expressed in G_1 and presumably S, but not G_0 or G_2/M, similar to the expression pattern of GL transcripts. Mapping of the binding showed that these complexes bind to an Ets consensus element, near the initiation sites used in proliferating cells (Berton & Vitetta, 1992).

In conclusion, we found a relation between proliferation, switching and GL transcripts. In addition, we identified cell cycle regulated protein complexes induced by LPS or LPS plus IL-4, that bind to a site in the GL γ1 promoter which is close to the major initiation site used upon activation. Later Hodgkin and colleges used a technique for simultaneous tracking of how many divisions stimulated cells had gone through and the level of surface Ig expressed. They could show that IgG1 appears after two to three cell divisions and reach maximum levels after six. For expression of IgE five to six divisions were needed (Hasbold et al, 1998; Hodgkin et al, 1996).

At which stage of the cell cycle CSR occurs is still unknown though. Since it is likely that during the time of CSR GL transcripts need to be present, these data suggest that CSR take place during G_1 or early S leaving time to repair DNA before completion of the S phase and duplication of the chromosomes.

The long probe used in Paper III sometimes caused technical problems in the EMSA experiments due to the difficulty for a long piece of DNA to migrate in the gel, especially after formation of several protein complexes on the DNA. To avoid some of these problems we designed a shorter probe. The two cell cycle regulated complexes binding to the long probe was mapped to bind within the +53/+76 region (Paper III), and the new probe used

reached from –28/+98. On this probe there was one major slow migrating complex formed together with two minor complexes (M.L and L.S unpublished data). The major complex turned out to be regulated in a similar fashion as complex 1 and 2 on the long probe. This complex is from now on called complex 1. In further experiments we investigated the nature of this complex. The major binding site for complex 1 appeared to be at +62/+64. The amount of complex 1 was not proportional to the amount of protein in the binding reaction, suggesting cooperative binding. Since the probe contains three independent GGA boxes, which was the motif found to be responsible for the binding of the cell cycle regulated complex, it is possible that more than one site is engaged. We found that complex 1 could be disrupted with sodium desoxycholate, indicating protein-protein interactions being involved in the complex formation. Furthermore, we showed that the typical complex formation on the –28/+98 GL γ1 promoter probe could be disturbed if the B cells were prevented from leaving the G_2/M stage by the drug Nocodazole before extract preparation, and was re-formed when cells entered G_1 after removal of Nocodazol. These results encouraged an attempt to correlate the expression of the complex with a function in regulation of the promoter activity. The region –147/+202 corresponding to the minimal γ1 promoter was cloned upstream of the Luciferase gene in the pXp2 plasmid. In a second construct the region around +60/+65 was deleted. Both were tested in transfection assays using the B cell line M12. The minimal promoter gave a significant response as compared to the empty vector control. However, no significant alteration of the promoter activity could be detected with the mutated construct (M.L and L.S. unpublished data). These results, although disappointing can be interpreted in different ways. Either the cell cycle regulated complex is not important for the activity of the GL γ1 promoter or the cell line used for these experiments regulates the promoter differently from primary cells. A third alternative is that this was not the proper assay to analyse the function of this complex.

Ikaros as a possible regulator of Ig class switching

The binding site for the cell cycle regulated complex (complex 1) discussed above, is also a potential high-affinity binding site for a DNA binding protein called Ikaros. Ikaros has been shown to be absolutely required for the development of B cells and in its absence T cell

differentiation is delayed (Georgopopous et al, 1997). It was found that Ikaros colocalize with transcriptionally silent genes at regions of heterochromatin in proliferating lymphocytes. Our transfection data with the mutated γ1 promoter indicated that the complex we had been studying was not important for activation of the promoter. Could it instead be involved in negative regulation of the promoter? We tested if complex 1 contained Ikaros by incubating nuclear extracts from nonactivated or activated B cells and actually the complex-formation was completely abolished as shown in Fig. 9.

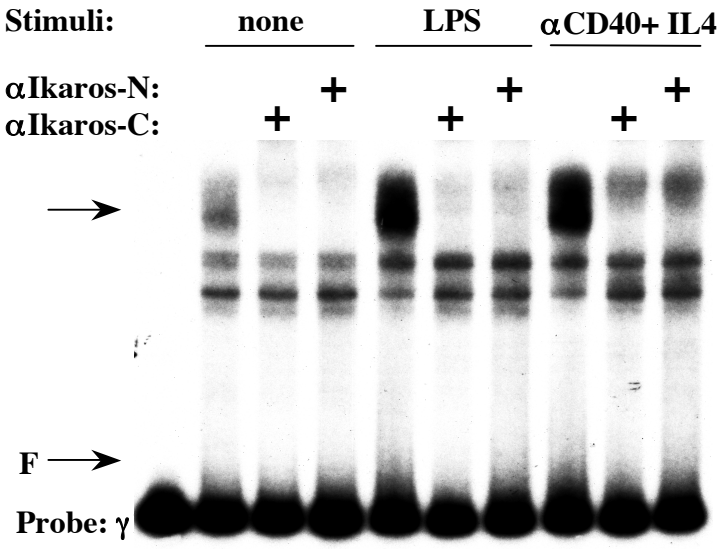

Figure 9. Ikaros in nuclear extracts from LPS and anti-CD40 stimulated B cells binds to the –28/+98 region of the GL γ1 promoter. Upper arrow points at "complex 1" and lower arrow at free probe.

This was the initiation of paper IV, presented here as a manuscript. When searching the sequences of the GL γ1 and ε promoter regions, several possible Ikaros binding sites, with the consensus GGGAA or being G-rich are revealed. We then amplified pcr-probes covering all possible Ikaros binding sites in the two promoters. A complex binding pattern was found, with two major slowly migrating complexes. Using an oligo binding Ikaros with high affinity

and comparing that with oligos covering one or two sites from the GL γ1 and ε promoter regions, we could conclude: Of the five possible sites in both promoters two seem to bind Ikaros with low affinity. Those are number two and five in the γ1 promoter and number one and two in the ε promoter. These sites overlap with NF-κB and complex 1 in the γ1 region and STAT6 and NF-κB in the ε promoter, respectively (see Fig. I in paper IV). Ikaros binds poorly to a single site-probe, while a very protein rich complex is formed when two or more sites are present simultaneously on the same DNA probe or oligonucleotide. Interestingly, an oligonucleotide containing one verified and one non-verified binding site inhibited complex formation to a much higher degree than an oligo with only one verified site at the same molar ratio. This shows that Ikaros binds cooperatively to the GL γ1 and ε promoters in vitro but it does not prove in vivo binding. Skok et al. has recently showed that one of the IgH alleles is associated with Ikaros clusters in activated B cells, which could indicate that Ikaros binds the locus in vivo (Skok et al, 2001). Where this binding occurs is not evident though, due to the resolution of the method used. In addition, Ikaros consensus sites can be found in many other regulatory regions within the IgH locus (Paper IV). Interestingly, it has been suggested, based on footprinting experiments in $p50^{-/-}$ mice, that Ikaros in the absence of p50 could bind to the Sγ3 region and induce chromatin remodelling of the region and thereby preventing CSR to Cγ3 (Wuerffel et al, 2001). To determine whether Ikaros is involved in regulation of GL γ1 and/or GL ε promoter activity, we co-transfected a dominant negative version of Ikaros, unable to bind DNA, with γ1 and ε promoter-luciferase reporter constructs to the B cell line M12. We reasoned that this would be a better strategy than mutating all the possible binding sites since there was some ambiguity as to which sites Ikaros was actually binding. The dominant negative version of Ikaros is believed to interfere with the interaction between DNA binding forms of endogenous Ikaros and the promoter. We showed that the basal transcription levels from these promoters were moderately but significantly elevated, while the αCD40+IL-4 stimulated transcription was less affected. This result was not so surprising since the promoters should be transcriptionally active after stimulation with anti-CD40+ IL-4. In our model, we hypothesised that Ikaros would bind the promoters in their inactive stage and upon activation with the correct stimuli the transcription factors known to be important

for activation of the promoters, STAT6 and NF-κB should be able to compete for overlapping sites. We postulated that Ikaros could repress basal transcription from these promoters.

Encouraged by these results we wanted to study the regulation of Ig class switching and GL transcription in primary B cells in vivo. We used retroviral constructs with either wt Ikaros or a mutated version with a point mutation at position 159, which changed asparagine to an alanine (Cobb et al, 2000). Ikaros with this mutation was unable to form the spectacular clusters normally associated with heterochromatin, when introduced into fibroblasts. We first tested if it would disrupt endogenous Ikaros clusters. We stimulated mouse B cells with LPS for 24 hours and then transduced them with wt or mutated retroviral Ikaros. After 48 or 72 hours the presence of Ikaros clusters was determined. In a majority of the cells expressing mutated Ikaros, no Ikaros clusters were formed by endogenous or ectopically expressed Ikaros (Fig. 5, Paper IV). The retrovirally expressed full length Ikaros colocalize with endogenous Ikaros in typical clusters. However, when analysing expression of IgG1 and IgE in both LPS and anti-CD40+IL4 stimulated cells, we could not detect any effect of neither mutated nor wt Ikaros. We then looked at induction of GL transcripts. In this way it should be possible to measure a more direct effect on promoter activity, since expression of Ig is regulated on many levels not only by activation of the GL promoters. However, looking at GL γ1 and ε transcripts with northern, no effect of full length or mutated Ikaros could be detected, indicating that Ikaros does not act as a transcriptional repressor or activator on these promoters. To investigate if the lack of transcriptional regulation by Ikaros was specific for the GL γ1 and ε promoters we looked at the transcriptional activity of genes that have been shown to colocalize with Ikaros at heterochromatin in their inactive stage. We performed rt-pcr on λ5, RAG-1 and CD8 and could not detect any transcription from these genes after disruption of the endogenous Ikaros clusters. These experiments indicate that Ikaros probably does not act as a transcriptional regulator on the genes we have studied here.

What is then the possible function for Ikaros in mature lymphocytes? Many different roles for Ikaros have been suggested. It has been shown to be involved in activation of transcription (Koipally et al, 2002; Sun et al, 1996), in repression or silencing of genes (Sabbattini et al, 2001; Trinh et al, 2001) and to interact with numerous chromatin remodelling complexes (Kim et al, 1999; Koipally & Georgopoulos, 2000; Koipally et al, 1999a; Koipally et al, 1999b). It is clearly necessary for development of all lymphoid and

erythroid lineages. It has though been difficult to study the role of Ikaros in mature B and T cells, since deletion of the Ikaros gene results in a complete arrest in B cell and fetal T cells. Postnatally, a reduced number of T cell precursors are found in the Thymus which develop into the TCR-α/β lineage (Wang et al, 1996c). Mice with a mutation in the DNA binding domain of Ikaros has a complete block in development of lymphocytes (Georgopoulos et al, 1994). Importantly, mice heterozygous for the dominant negative mutation rapidly develop T cell leukemias and lymphomas (Winandy et al, 1995). Furthermore, in leukemic cells from children with acute lymphoblastic leukemia (ALL) and subtypes of acute myeloid leukemias (AML), dominant negative isoforms of Ikaros lacking critical N-terminal zink fingers were expressed at high levels (Sun et al, 1999; Yagi et al, 2002). In patients with chronic myelogenous leukaemia (CML), expression of dominant negative isoforms of Ikaros correlated with blast crisis (Nakayama et al, 1999). Together this implicates a possible role for Ikaros as a tumour suppressor.

In a newly described novel mutation of the Ikaros gene, the β-galactosidase reporter was inserted into exon two, present in all Ikaros isoforms. Surprisingly, in contrast to previous mutant mice these express low levels of Ikaros, leading to the development of a B cell compartment. These B cells display several intrinsic defects including a lowered threshold of activation (Kirstetter et al, 2002). This resembles a previous report where Ikaros was shown to regulate T cell differentiation, selection and homeostasis by providing signalling thresholds for pre-TCR and TCR (Winandy et al, 1999). Thus, decreased levels of Ikaros allowed T cells to proliferate at suboptimal activation conditions, progress through G_1 and enter S phase more rapidly. Furthermore, Ikaros was shown to colocalize with components of the DNA replication machinery, like methyltransferase and cyclinA, which associate with and regulate the DNA replication megacomplex (Leonhardt et al, 1992). Methyltransferases serve to direct replication-coupled DNA methylation, which is important to keep the acquired pattern of active versus silent genes through cell divisions. Collectively, these data suggested a role in DNA replication for Ikaros (Avitahl et al, 1999).

One way to study functions of Ikaros is to make a conditional knockout of Ikaros. Another way to study the role of Ikaros at different stages of B cell differentiation could be the newly developed RNA interference (RNAi) technique. With this method one introduces 22 nt short ds RNAs to target homologous messenger RNAs for destruction, hereby inducing

specific gene silencing (Elbashir et al, 2001a; Elbashir et al, 2001b; Fire, 1999; Kennerdell & Carthew, 1998).

Another experiment that could be worth performing to elucidate the function of Ikaros binding to the GL promoters, is to mutate all the possible Ikaros binding sites in the GL ε promoter and use a construct including the HS1,2 enhancer downstream of the LUC gene as a reporter. Such a construct has allowed promoter activity to be detected in primary B cells (Laurencikiene et al, 2001). This could be valuable since it is known that regulation of class switching is different in transformed cell lines like M12, than in primary B cells. To study the GL γ1 promoter in this way would however not be suitable since IgG1 expression might be regulated also from an internal control element in addition to the downstream enhancers (Adams et al, 2000; Cogné et al, 1994).

CONCLUSIONS AND FUTURE PERSPECTIVES

This study has focused on Ig class switching in primarily three different aspects namely: The role of CD40 in class switching, the relation between proliferation and class switching and the definition of proteins involved in regulation of the GL γ1 and ε promoters. The results can be summarized as follows.

In the **first paper**, in vivo distribution of an agonistic monoclonal rat anti-mouse CD40-ab induced up-regulation of all Ig isotypes in mice completely devoid of T cells. This induction was to some extent independent of IL-4. IgE and GL ε transcripts could be induced also in $IL4^{-/-}$ mice. Thus, IgE switching can occur in the absence of IL-4, but IL-4 is still the most potent regulator of IgE responses.

In the **second paper,** we found that signalling through CD40 stimulated GL γ1, γ2b and low levels of ε transcripts. In B cells activated by LPS or anti-CD40 we could detect CSR to Cγ2b. In addition, anti-CD40 together with IL-4 or IL-5 induced CSR to Cγ1. Furthermore, NF-κB activated both by LPS and anti–CD40 could bind to the γ1 promoter. However, the total concentration of bound NF-κB, as well as subunit composition induced by the two stimuli varied. We proposed that the higher level of p50 homo-dimer seen in extracts from cells activated by LPS prevented the promoter from being activated by LPS.

In the **third paper,** we studied the relation between proliferation, CSR and synthesis of GL transcripts. We found that CSR was completely abrogated upon cell cycle arrest. In addition, steady state levels of GL γ1 and ε RNA in LPS plus IL-4 activated B cells were much reduced by cell cycle arrest. We found that GL γ1 transcripts were expressed in G_1 and S phases, at lower levels in G_2/M and not in G_0. In conclusion, we found a relation between proliferation, switching and GL transcription. In addition, cell cycle regulated protein complexes induced by LPS or LPS+ IL-4, binding a site in the GL γ1 promoter close to the major initiation site were identified.

In the **fourth study,** we identified several Ikaros binding sites in the γ1 and ε GL promoters with the consensus GGGAA. We showed that Ikaros could bind to some of these

sites in a cooperative manner. Co-transfection of a dominant negative version of Ikaros, unable to bind DNA, with γ1 and ε promoter-luciferase reporter constructs to the B cell line M12, showed that the basal transcription levels from these promoters were moderately elevated. However, retroviral transduction of a mutated non-DNA binding form of Ikaros to primary B cells had no effect on class switching or GL transcription, in spite of disrupting the endogenous Ikaros clusters. Silencing of genes has been suggested to correlate with association to Ikaros clusters at heterochromatin. Expression of a set of such genes, was not upregulated after disruption of the Ikaros clusters. These data implied that Ikaros might not be directly involved in transcriptional regulation of specific genes but might exert its function at other levels.

Despite the work of many scientists over many years there are still numerous questions that are not answered in the field of Ig class switching. What is the precise role of the GL transcripts? What interactions are happening on the GL promoters? Are different proteins binding at different times during the life of a cell or at different stages of the cell cycle, to allow or to prevent transcription? Or is transcription regulated not on the level of DNA binding proteins, but via coactivators or repressors and chromatin remodelling machineries? What is the nature of the recombinase and what is the recognition target for this enzyme? Is there one common or class and subclass specific recombinases?

It will be interesting to follow the progress of this field in the future. Will the putative RNA editing enzyme AID turn out to be the recombinase or part of the recombinase machinery? If so, will AID mean to CSR what the RAG proteins did to VDJ recombination?

In a wider perspective it is interesting to note that the type of processes that we have studied in the Ig locus might be of more general relevance. Large intergenic transcripts not encoding protein were identified in the β-globin locus. These transcripts were perfectly correlating with active domains of the locus and were suggested to be required for chromatin remodelling of chromosomal domains (Gribnau et al, 2000). It is possible that they resemble the GL transcripts induced over S regions targeted for CSR.

Furthermore, most human B cell lymphomas origin from GCs. Cellular oncogenes like BCL-2, BCL-6 and c-myc can be translocated into IgH S regions and come under the influence of IgH regulatory sequences, resulting in deregulated oncogene expression (Klein, 1999; Kuppers et al, 1999). It is possible that the CSR machinery is involved in such

translocations, therefore understanding the mechanisms of strand breakage and joining in CSR will increase the knowledge about how these malignancies occur.

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