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A

A Study of Immunoglobulin Heavy Chain 3' Enhancer Function Within Chromatin

by

XUERONG SHI

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2000

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

A Study of Immunoglobulin Heavy Chain 3' Enhancer Function Within Chromatin

by

XUERONG SHI

Advisor: Professor Laurel Eckhardt

The immunoglobulin heavy chain (IgH) locus is controlled by multiple regulatory elements including IgH promoter and intronic enhancer (Eμ) located within the IgH transcription unit, and 3'IgH enhancers (Hs3a, Hs1,2, Hs3b, and Hs4) residing downstream of IgH coding sequences. Eμ plays a central role in VDJ recombination and activates IgH gene transcription at the earlier B cell stages. The 3' IgH enhancers are believed activate/maintain IgH gene transcription at the later B cell stages and may also play a role on heavy chain class switch recombination.

To assess directly the regulatory activity of the first identified 3'IgH enhancer, Hs1,2, we deleted this enhancer from the IgH locus of an Ig-secreting cell line that already lacked Eμ. We found that Hs1,2 was essential for IgH gene transcription in this cell line.

To study further the regulatory activity of this and the other 3'IgH enhancers in the context of chromatin, we created IgH mini-loci (Igγ2b transgenes under control of the 3'IgH enhancers) to manipulate these 3' enhancers. The mini-loci were used to stably transform both a surface-Ig positive cell line and an Ig-secreting cell line. After stable expression of the loci had been achieved in the respective cell lines, we induced enhancer deletions within the mini-loci. We found that the hs3a/hs1,2 and the hs3b/hs4 pairs were functionally redundant in the surface Ig^+ cell line but that the hs3b/hs4 pair played a dominant role in maintaining reporter gene expression in the Ig-secreting cell line. Our results provide evidence to support the importance of the 3'IgH enhancers in IgH transcription at the later B cell stages, and support and extend emerging models of the

IgH locus in which complex and shifting modes of control regulate this locus over the course of B lymphocyte development and antigen-stimulated differentiation.

Finally, we exploited the possibility that the 3'IgH enhancers are able to interact with one another and/or with IgH promoters through an association with the nuclear matrix. We assayed most of a 34 kb region encompassing the 3'IgH enhancers for the presence of matrix attachment regions (MARs). While our assays confirmed the presence of MARs adjacent to the intronic enhancer, Eμ, we saw no evidence of MARs in the 3'IgH enhancer region.

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CHAPTER 1 INTRODUCTION

I. Overview of B cell development and differentiation in immune system

B and T lymphocytes are the cells responsible for antigen-specific immune responses in all vertebrates, including humans. B-lymphocytes develop within the bone marrow and leave the marrow expressing a unique antigen-binding membrane receptor. The B cell receptor (BCR) is an antibody molecule, a membrane-bound glycoprotein. The basic structure of the antibody molecule consists of two identical heavy polypeptide chains and two identical light polypeptide chains (see **Figure 1-1**). The chains are held together by disulfide bonds. The amino-terminal ends of each heavy and light chain pair form a cleft within which antigen binds. The variable regions of both heavy and light chains determine the specificity of an antibody for antigen binding. When a B cell encounters the antigen for which its membrane-bound antibody is specific, the cell begins to divide rapidly and differentiate into a memory B cell or effector cell (plasma cell). The memory B cells continue to express membrane-bound antibody with the same specificity as the original parent cell. Plasma cells do not produce membrane-bound antibody but produce the antibody in a form that can be secreted instead (Goldsby et al., 2000).

1. Stages of B lineage lymphocytes in development and differentiation

B lymphoid cells are originated from hematopoietic stem cells. Stem cell commitment to lymphoid differentiation is a highly sophisticated process. Several genes that appear to regulate this process have been identified. Inactivation (gene "knockout") of the gene encoding PU.1, which is a member of Ets family proteins, led to loss of detectable progenitors of B cells, T cells, monocytes, and granulocytes, but didn't affect erythroid precursors (Scott et al., 1994). *Ikaros*, a gene encoding a family of zinc-finger DNA–binding proteins is required for lymphoid commitment. Inactivation of Ikaros led to the loss of all lymphoid cells and lymphoid progenitors, but had no effect on all other hematopoietic lineages (Georgopoulos et al., 1994). Early B cell factor (EBF) is expressed in B cell precursors, and regulates expression of the *mb-1* gene. The *mb-1* gene

encodes Igα that is part of the pre-B cell receptor complex. Knockout of EBF didn't have effect on the survival of the earliest, lineage-specific B cell precursors but the surviving B lineage cells were not capable of undergoing IgH rearrangement (Lin and Grosscheld, 1995). Similarly, Knock-out of the *E2A* gene, which encodes the ubiquitously expressed helix-loop-helix transcription factors E12 and E47, blocked development of B cell precursors or expression of the recombinase gene *RAG-1* that is involved in Ig V(D)J or TCR V(D)J rearrangement (Bain et al., 1994; Zhuang et al., 1994)

B lymphocyte development and differentiation can be divided into several stages: progenitor B cells, precursor B cells, surface-Ig^+ cells and plasma cells according to Ig gene rearrangement status and expression. While progenitor B cells develop and differentiate into precursor B cells and then into early surface-Ig^+ B cells (virgin B cells) in the adult bone marrow and the fetal liver, the primary lymphoid organs, surface-Ig^+ B cells mature and differentiate into plasma B cells upon antigen activation in the periphery, and in secondary lymphoid tissues.

Progenitor (pro) B cells, which develop from hematopoietic stem cells, don't express immunoglobulin heavy chain (IgH) and light chain (IgL) proteins, but their IgH and IgL chain variable gene segments, and J_H segments (Yancopoulos and Alt, 1985; Blackwell et al., 1986; Schlissel and Baltimore, 1989) as well as constant region genes (Nelson et al., 1983, 1985; Lennon and Perry, 1985) may actively be transcribed before any V(D)J rearrangement. These germline transcriptions may be related to DNA recombination such as V(D)J joining in an Ig locus. It has been shown that Jκ-Cκ germline transcription is correlated with κ locus rearrangement in transformed pre-B cell lines (Schlissel and Baltimore, 1989; Lennon and Perry, 1990).

Precursor (pre) B cells, which are the earliest cells of B lineage and developed from progenitor B cells, produce Ig heavy chain proteins (μ chains) after a successful IgH gene VDJ rearrangement, but light chains are not produced by these cells since VJ rearrangement in Ig light chain locus begins later (Yancopoulos and Alt, 1986). The newly synthesized μ chains assemble with BiP/GRP78 (Ig binding protein/glucose

regulated protein) (Hass and Wabl, 1983; Munro and Pelham, 1986) and other resident endoplasmic reticulum (ER) proteins. These Ig heavy chain molecules are retained with Bip in the ER until the light chains dissociate the Bip-μ heavy chain interaction. Two genes, *λ5* and *Vpre-B*, which encode proteins that associate with Ig μ chain proteins at the early stage of B cell development, were identified by several researchers (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987). The *λ5* gene (Jκ-and Cκ-like sequences), and the *Vpre-B* gene (a V_λ-like sequence) encode λ5 and Vpre-B proteins, respectively, which are able to associate to form a surrogate L chain or pseudo-light chain (ψLC) (Karasuyama et al., 1990; Misener et al., 1990; Tsubata and Reth, 1990). It is believed that the ψLC proteins (λ5 and Vpre-B) can disrupt the Bip-μ heavy chain interaction in the ER of pre-B cells, allowing the μ chains to form a complex with the ψLC proteins. The μ-ψLC complex can then leave the ER and is eventually transported to the cell surface to mediate transmembrane signals through association with Igα/β signal transducers (Kayasuyama et al., 1996; Reth and Wienands, 1997). This may serve as a strategic checkpoint in which the cell verifies that IgH rearrangement has been productive. It has been shown that surrogate light chain is important for pre-B cell survival and important for IgH allelic exclusion. In *λ5*-deficient mice, production of pre-B cells and B cells were dramatically reduced, and IgH allelic exclusion was affected (Kitamura et al., 1992; Loffert et al., 1996). Synthesis of Ig μ chain protein may actively inhibit further IgH rearrangement, leading to heavy chain allelic exclusion (Weaver et al., 1985; Nussenzweig et al., 1987; Muller et al., 1989). When pre-B cells are induced to undergo light chain V-J rearrangement and subsequently produce Ig light proteins, they develop and differentiate into the sequential stage, surface-Ig^+ cells.

Surface-Ig^+ cells are the cells in which both Ig heavy and light proteins are synthesized, subsequently associate into an Ig complex, and are then transported to the cell surface as an antigen receptor. Naïve B cells that express membrane-bound Ig (mIgD and mIgM) with a single antigen specificity leave the bone marrow, and are carried to the secondary lymphoid organs, such as spleen and lymph nodes.

Plasma B cells are terminally differentiated B-lymphocytes and have an abundant cytoplasm and features typical of a secretory cell. Usually, the mature plasma cells do not produce sIgM.

2. B lymphocyte development and differentiation in the germinal centers (GCs)

The interaction between antigen and B cell Ig receptor in the secondary lymphoid organs signals the virgin immature B cells to proliferate and differentiate into either a plasma cell or memory cell. B cell activation induced by T cell-dependent antigens requires the delivery of contact-dependent help. The formation of T-B cell conjugates facilitates the interaction between the signaling molecules such as CD40 and B-7 that are expressed on the B cell surface, and CD40L and CD28 expressed on the T cell surface. In a primary response (first exposure to antigen), virgin B cells undergo clonal selection, subsequent clonal expansion (Jerne, 1955), and differentiation into memory cells or plasma cells.

While T-independent antibody responses do not require the formation of germinal centers, T-dependent antibody responses generally do. Germinal centers (GCs) are microenvironments formed during primary immune responses of B cells to T cell-dependent antigens. GCs are highly specified structures that develop around the follicular dendritic cells (FDC). In addition to B cells, GCs contain antigen-presenting cells such as follicular dendritic cells (FDCs) and T helper cells. Within GCs, there are three zones: mantle zone, dark zone and light zone. Virgin B cells that have not yet been antigenically selected reside in the mantle zone. In the dark zone, B cells intensely proliferate after activation by T-dependent antigen, and somatic hypermutation (point mutations are introduced into the Ig heavy and light chain variable region genes) is coupled with B cell proliferation. In the light zone, B cell proliferation reduces and affinity selection predominates (MacLennan, 1994; Liu and Arpin, 1997). Somatic hypermutations led to generation of three kinds of mutants including high-affinity, low-affinity, and autoreactive mutants in the light zone of GCs. The fate of these three types of somatic mutants is determined by interaction between the antibody receptor on these B cell

mutants and the low-level antigen on the surface of the antigen-presenting cells, follicular dendritic cells (FDCs). Autoreactive mutants and low-affinity mutants are deleted (apoptosis). High-affinity mutants survive through capturing antigen, processing it and presenting it to the T cells. After interaction with B cells, the T cells are induced to express CD40 ligand (CD40L) and produce cytokines, including IL-4 and IL-10, which are crucial for B cell survival and proliferation during primary immune responses. Eventually, high-affinity B cells differentiate into memory B cells if CD40L signaling is prolonged or into plasma cells when CD40L signaling is removed (Arpin et al., 1995; Callard et al., 1995).

Somatic hypermutation (Maizels, 1995; Neuberger and Milstein, 1995) increases diversity of antibodies. Affinity maturation results in the generation of antibodies with high affinity and specificity for antigen binding. It has been shown that T-B cell membrane interactions through surface molecules are required for somatic hypermutation (Huang et al., 1999; Denepoux et al., 2000).

II. Organization and recombination of Ig gene loci in mouse

Ig heavy chain, and light chain (κ, λ) loci and T cell receptor loci (TCR-α, β, γ and δ) have a similar organization: multiple variable (V) and diversity (D) segments, which are present only at Ig heavy chain locus, and TCR β and δ loci, and several joining (J) segments. The constant regions of an immunoglobulin and TCR molecule are encoded by separate exons that lie downstream of the variable region gene segments. All of the gene segments in Ig and TCR loci are separated from each other by non-coding sequences (introns). The Ig heavy chain (IgH), and light κ and λ gene loci are mapped on chromosomes 12, 6, and 16 in the mouse, respectively. In the human genome, they map to chromosomes 14, 2, and 22, respectively. **Figure 1-2** shows germ-line organization of Ig heavy chain and light chain gene loci in the mouse.

1. Organization of Ig heavy and light chain loci

Ig κ-chain multigene family in the mouse contains approximately 85 Vκ gene segments, each with an adjacent leader sequence a short distance upstream. There are five Jκ segments (one of which is a pseudogene that is incapable of encoding protein) and a single Cκ gene segment. The Vκ and Jκ gene segments encode the variable region of the κ light chain, and the Cκ gene segment encodes the constant region. Since there is only one Cκ gene segment, there are no subtypes of κ light chains. The κ-chain locus in humans, which is similar to that in the mouse, contains approximately 40 Vκ gene segments, five Jκ gene segments, and a single Cκ gene segment (Goldsby et al., 2000).

The Ig λ-chain multigene family in the mouse germ line consists of two Vλ gene segments, four Jλ gene segments, and four Cλ gene segments. The Jλ4 and Cλ4 gene segments are defective. As in the κ locus, the Vλ and the three Jλ gene segments encode the variable region of the light chain, and each of the three functional Cλ gene segments encodes the constant region of the one of the three λ-chain subtypes (λ1, λ2, and λ3). In humans, there are about 30 Vλ gene segments, four Jλ segments, and four Cλ segments (Goldsby et al., 2000).

The organization of Ig heavy chain genes is similar to, but more complex than, that of the κ and λ light chain genes in the mouse. An additional gene segment, designated D_H for diversity, encodes part of the heavy chain variable region. As with light chain genes, each V_H gene segment has a leader sequence a short distance upstream from it. There are approximately 134 V_H gene segments in the murine locus. Downstream from the 13 D_H gene segments are four functional J_H gene segments, followed by a series of C_H gene segments: Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, and Cα. Each C_H gene segment encodes the constant region of an Ig heavy chain isotype. The IgH chain locus in humans, which is similar to that in the mouse, contains approximately 51 V_H, 27 D_H, and six J_H gene segments, and has two C_H clusters lying downstream of Cμ and Cδ: Cγ3, Cγ1, Cε2, Cα1 and Cγ2, Cγ4, Cε2, Cα2, but the first Cε2 is a pseudogene (Goldsby et al., 2000).

2. V(D)J recombination in Ig heavy and light chain gene loci

B-lymphocytes undergo V(D)J rearrangement at the earliest stages of their development. IgH chain variable region is assembled from three individual dispersed gene segments, V_H, D_H, and J_H. VDJ joining requires two distinct somatic recombination events or steps to complete gene assembly, following an intrinsic order of gene assembly (Alt et al., 1984). One of the D segments assembles randomly with one of the J segments at first, resulting in a DJ joining, then followed by VD joining in which one of the V segments assembles with the DJ complex, forming a final VDJ coding segment. Unlike IgH VDJ recombination, Ig κ and λ light chain genes are formed in a single step by direct V to J joining. Ig V(D)J arrangement brings the newly assembled V(D)J exon in proximity to the exons of the C gene segments. Diversity is generated through the well-known combinational assembly of numerous minigene elements and by nucleotide additions and deletions at the gene segment ends prior to joining (Tonegawa, 1983).

Ig and TCR V(D)J recombination is initiated by the lymphocyte-restricted DNA recombinases, RAG-1 and RAG-2 (Schatz et al., 1989; Oettinger et al., 1990). *RAG*-deficient mice do not have mature B and T cells. In these mutant mice, V(D)J recombination is not detected (Mombaerts et al., 1992; Shinkai et al., 1992). RAG-1 and RAG-2 proteins mediate V(D)J recombination through recognizing a homologous recombination signal sequence (RSS), which flanks individual V, D, and J gene segments , and is composed of a highly conserved palindromic heptamer motif, a spacer, usually of 12 or 23 base pairs, and a conserved nonamer.

In addition to RAG proteins (recombinases), some ubiquitous factors are involved in the V(D)J recombination process. For instance, the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), and the DNA-binding subunits of DNA-PK, Ku70, and Ku80/Ku86 are involved in this process. Inactivation of DNA-PKcs led to a severe defect in V(D)J recombination in mice (Shin et al., 1997). Moreover, *Ku86*-targeted mice show a defect in both signal and coding joint formation in lymphocytes (Zhu et al., 1996). These results suggest that DNA-PKcs plays a role in the formation of both signal and coding joints. It has been shown that terminal deoxynucleotidyl transferase (TdT), which is responsible for addition of nucleotides to protein coding

segment ends, also plays a role in V(D)J recombination, but its role is not fundamental to the V(D)J recombination process (Gilfillan et al., 1993 & 1995).

The fact that DJ rearrangements are found on both chromosomes in most B lymphocytes, whereas productive VDJ joins are generally found on only one chromosome raises a possibility that the functional VDJ rearrangement results in heavy chain gene allelic exclusion (Alt et al., 1984). Studies in transgenic mice showed that V(D)J rearrangement in the endogenous heavy chain locus was significantly inhibited by transgenes directing expression of a μ or δ heavy chain to the B cell surface (Weaver et al., 1985; Nussenzweig et al., 1987; Manz et al., 1988; Muller et al., 1989). Secreted IgM, however, did not inhibit rearrangement or expression of endogenous heavy chain genes (Nussenzweig et al., 1987; Manz et al., 1988). These results support the critical role of the membrane-bound μ or δ heavy chain in the normal mediation of heavy chain allelic exclusion.

Ig μ chain is associated with λ5 and Vpre-B proteins (μ-ψLC complex) before Ig light chain proteins have been produced (Karasuyama et al., 1990; Misener et al., 1990; Tsubata and Reth, 1990). In earlier B cell stages, surface μ heavy chain also associates with two other transmembrane proteins (Igα and Igβ), leading to formation of the pre-B cell receptor (pre-BCR) in pre-B cells and BCR in B cells. Participation of Ig μ protein in the pre-BCR suggests that IgH allelic exclusion might be mediated by the pre-BCR (Bauer and Scheuermann, 1993). There is strong evidence to support this proposal from gene targeting studies. Ig heavy chain allelic exclusion was inhibited in B cells derived from gene-targeted mice that produce secreted but not membrane μ heavy chain (the μ chain membrane exon was targeted) (Kitamura et al., 1992). Gene-targeted mice also supported the role of the pre-BCR in mediating Ig heavy chain allelic exclusion. In the λ5- deficient mice, production of pre-B cells and B cells were strikingly reduced, and productive Ig heavy chain rearrangement on the both alleles was observed in developing B cells that are unable to express a functional pre-BCR (μ-ψLC-Igα/β complex) (Kitamura et al., 1992; Loffert et al., 1996). These results strongly indicate that

expression of the pre-B cell receptor inhibits further rearrangement of Ig heavy chain variable region genes, leading to allelic exclusion.

The fact that Ig heavy chain rearrangement and expression occurs prior to light chain rearrangement suggests that the heavy chain protein may regulate light chain rearrangement. Several observations have shown that Ig μ chain protein promotes Ig light chain gene rearrangement and expression (Ritchie et al., 1984; Reth et al., 1987; Tsubata et al., 1992; Shapiro et al., 1993). Other studies, however, have shown that production of Ig μ chain protein is not necessary for Ig light chain expression (Blackwell et al., 1989; Schlissel and Baltimore, 1989; Ehlich et al., 1993; Grawunder et al., 1993). These results suggest that Ig heavy chain protein plays an important role but it is not essential for Ig light chain production.

Studies of transgenic mice showed that high-level expression of a functional transgenic κ or λ chain generally inhibited the rearrangement of endogenous light chain genes (Brinster et al., 1983; Hagman et al., 1989; Neuberger et al., 1989). This suggests that productive Ig light chain gene rearrangements may play a crucial role in mediating light chain gene allelic exclusion.

Like Ig heavy chain rearrangement, Ig light chain gene rearrangement appears ordered. It has been shown that most of the κ light chain genes are nonproductively rearranged in murine λ-expressing cells (Zou et al., 1993; Gorman et al., 1996). In κ-expressing cells, however, the majority of λ light chain genes remain in the germline configuration (Zou et al., 1993). Preferential κ versus λ gene rearrangement has also been found in people.

In human and murine λ-expressing but not κ-expressing cells, Cκ or the entire Jκ-Cκ region is usually deleted. This type of deletion is mediated by an RS/kde element (Recombining Sequence in mouse / kappa deleting element in human) (Durdik et al., 1984; Siminovitch et al., 1985). RS/kde is located ~ 25 kb downstream of Cκ gene segment which also places it downstream of the κ light chain 3' enhancer (3'Eκ). RS/kde

contains an RSS sequence with a 23 bp spacer. The RS/kde can recombine with a Vκ region or with an independent RSS that is located in the JκCκ intron, just upstream from the intronic enhancer (iEκ), leading to deletion of the Cκ or the entire Jκ-Cκ region including both iEκ and 3'Eκ. Deletion of iEκ and 3' Eκ mediated by RS/kde may promote λ rearrangement. It has been shown that replacement of either iEκ or 3'Eκ by a *loxP* sequence in mice dramatically reduces VκJκ rearrangement and alters κ:λ ratio from naturally 10:1 or greater to 1:1 or 2:1 (Gorman et al., 1996; Xu et al., 1996). This suggests that deletion of the regulatory elements in κ locus permits λ gene rearrangement.

Ig V(D)J recombination may be regulated by Ig enhancers. Gene targeting studies show that Ig heavy chain intronic enhancer, Eμ, is important for efficient VDJ rearrangement but is not absolutely required (Chen et al., 1993; Serwe and Sablizky, 1993). Ig κ light chain intronic enhancer (iEκ), and 3' enhancer (3'Eκ) contribute to the process of κ chain VJ rearrangement but are not strictly necessary (Takeda et al., 1993; Gorman et al., 1996; Xu et al., 1996).

3. Ig heavy chain isotype switching

As mentioned above, the virgin B cells expressing membrane-bound IgM or IgD molecule migrate to the secondary lymphoid organs from the primary lymphoid organs (adult bone marrow and fetal liver). They mature and differentiate into plasma B cells in response to antigens in the periphery, and in the secondary lymphoid tissues. Activated B cells can produce other isotypes of antibodies, upon activation driven by T cell dependent antigen, but with the same specificity for antigen binding.

In most mammals, there are five isotypes of antibodies, IgM, IgD, IgG, IgE and IgA, determined by the constant region portion of the heavy chain (C_H). Murine antibodies are divided into eight different isotypes, IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE and IgA, expressed from Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, and Cα region segments, respectively. The heavy chain proteins can be produced as secreted form or membrane-bound form depending on the final location of the antibody.

Like V(D)J recombination taking place at RSSs (rearrangement signal sequences), class switch recombination (CSR) occurs at class switching regions (termed S regions). S regions are composed of tandem repetitive DNA sequences, and are located upstream of each constant gene segment except for Cδ (Davis et al., 1980). Each switch region has a few sequence motifs such as GAGCT and GGGGT. These motifs are repeated multiple times within the S regions (Nikaido et al., 1981; Marcu et al., 1982). CSR between Sμ and a downstream S region led to deletion of the intervening sequences including the Cμ and Cδ gene segments, and led to a downstream C_H gene such as Cγ, Cε, or Cα becoming juxtaposed to the expressed variable region of Ig heavy chain gene (Shimizu and Honjo, 1984). CSR can also take place between two downstream S regions, resulting in the sequential production of two or more isotypes by one B cell during its differentiation (Nikaido et al., 1980; Yoshida et al., 1990).

It has been shown that induction of CSR to a particular C_H gene is strongly correlated with the transcriptional activation of the C_H gene in its germline configuration (Stavnezer and Sirlin, 1986; Yancopoulos et al., 1986; Lutzker et al., 1988; Rothman et al., 1990; Xu et al., 1993). The germline transcripts initiating upstream and terminating downstream of the C_H genes are produced prior to CSR. These germline transcripts are not translated. The production of germline transcripts is under control of inducible promoters (I promoters), which are located upstream of each switch sequence and induced by certain mitogens and cytokines. So far, several I promoters have been well characterized: Iε and Iγ1 promoters are induced by LPS plus IL-4 (Rothman et al., 1991; Xu and Stavnezer, 1992), the Iα promoter by LPS plus TGF-β (Lin and Stavnezer, 1992), and the Iγ2b and Iγ2a promoters by LPS alone (Lutzker et al., 1988). Replacement of the transcriptional start site and promoter of the germline transcriptional element of either IgG1 or IgG2a gene segment inhibited switching to those isotypes (Jung et al., 1993; Zhang et al., 1993). It is possible that transcription through the S region may open this DNA region to facilitate the CSR process even though the precise role of germline transcription in CSR remains obscure.

Germline transcription of the IgH constant genes is regulated by extracellular signals such as stimulation by LPS and soluble cytokines including IL-4 (interleukin-4), IFN-γ (interferon-γ), and TGF-β (tumor growth factor β), and binding of CD40 ligand to CD40 receptor (Stavnezer, 1996). For instance, patients with X-linked immunoglobulin deficiency fail to produce CD40 ligand on activated T cells, leading to a block in Ig isotype switching in B cells (Fuleihan et al., 1995). IL-4 is important for generation of certain Ig isotypes. Gene targeting studied showed that inactivation of the IL-4 gene led to absence of IgE and a decrease in IgG1 (Kuhn et al., 1991). It has also been shown that IFN-γ stimulates switching to IgG2a and inhibits IL-4 induction of IgG1 and IgE. In contrast, TGF-β induces switching to IgA.

Recently, plasmid-based recombination assays showed that plasmid-based CSR did not require the inducers of CSR, but the recombination activity on the plasmids strictly correlated with isotype-specific switching at the endogenous genes. The switch plasmids underwent CSR only in activated splenic B cells or in cell lines capable of switching their endogenous genes. DNA sequence analysis of recombinant S-S junctions derived from switch plasmids indicated direct S-S joining. These data suggest that switching activities in the cells are constitutively expressed, and isotype-specific factors may regulate CSR independent of inducers of CSR (Stavnezer et al., 1999; Shanmugam et al., 2000).

Gene-targeting studies show that CSR may be regulated by Ig heavy chain enhancers including the intronic enhancer (Eμ) and 3' IgH enhancers (Gu et al., 1993; Cogne et al., 1994).

III. Ig enhancers and their activity in Ig gene expression and recombination

1. Murine Ig Heavy chain gene intron enhancer and its activity

Over 10 years ago, the first cellular enhancer was identified. It resided within the J_H-Cμ intron of the murine IgH locus and was identified by three laboratories (Banerji et al., 1983; Gillies et al., 1983; Neuberger, 1983). Like viral enhancers, the intron enhancer,

termed Eμ, functions in a position- and orientation-independent manner, but in addition, Eμ is tissue-restricted, functioning only in lymphoid-cells. Eμ is matrix attachment region (MAR)-associated. Its core region is flanked by MARs on both sides (Cockerill et al., 1987).

MARs are approximately 200 bp DNA regions mediating attachment of chromatin to the nuclear matrix, and are composed of AT-rich sequences (Cockerill and Garrard., 1986). MARs have been shown to be associated with regulation of DNA replication, transcription, and RNA processing. Overwhelming evidence shows that transcription factor binding sites, promoter and enhancer regions, or other regulatory regions in a locus may be nuclear matrix associated. Association of promoter or enhancer with MAR has been found in many loci (reviewed by Getzenberg, 1994). A number of experiments support a role for MARs in the regulation of gene transcription (Blasquez et al., 1989; Stief et al., 1989; Phi-Van et al., 1990; Webb et al., 1991; Forrester et al., 1994; Oancea et al., 1997).

Studies of gene targeted (knock-out) or transgenic mice have shown that Eμ promotes VDJ rearrangements in developing B-lymphocytes. Replacement of a 1 kb DNA fragment, which contains both Eμ and its flanking MARs, by a neomycin-resistant gene (*neo*^{r}), resulted in a dramatic *cis*-acting inhibition of both VDJ rearrangement (a reduction to 12%) and germline transcription of Cμ in most of cell lines from the targeted mice, and insertion of the *neo*^{r} gene into the MAR sequence 5' of the Eμ core region also dramatically decreased VDJ recombination (a reduction to 18%) and germline transcription (Chen et al., 1993). In chimeric mice with ES cells lacking Eμ (core region and two flanking MARs) on one of two IgH alleles, VDJ recombination was impaired (15-30% relative to the wild type alleles) on the mutant allele, but it was not blocked completely (Serwe & Sablitzky, 1993). Similarly, in the absence of Eμ, VDJ rearrangements were not detected in the lymphoid tissues from independent transgenic mice (Fernex et al., 1994).

Chimeric mice with ES cell lines that have targeted deletion of the core enhancer region of Eμ (cEμ, a 220 bp HinfI/HinfI fragment), 5'MAR, 3'MAR, or both 5'and 3'MARs showed that the core enhancer was necessary and sufficient to mediate VDJ recombination and IgH gene transcription in the mutant B cells, and both the 5' and 3' MAR sequences were dispensable for these processes (Sakai et al., 1999a). D_H to J_H rearrangements occurred at normal levels in 5'MAR- or 3'MAR-deficient mice. V_H to D_HJ_H rearrangements also occurred at normal levels on the 5'MAR- or 3'MAR-deficient allele. Moreover, chimeric mice generated with 5'MAR- or 3'MAR-deficient ES cell lines exhibited a normal ratio of IgM^a (targeted allele)- vs. IgM^b-expressing B cell numbers in the periphery. These results show that the MAR sequences do not have essential independent functions in mediating VDJ recombination and IgH gene transcription processes. There were significant levels of D_H to J_H rearrangement, however, on the targeted allele, but V_H to D_HJ_H rearrangement was dramatically reduced in the cEμ-deficient mice. Moreover, hybridoma analyses showed that only 2/21 cEμ-mutated alleles, as opposed to all 21 wild-type alleles in these cells, had $V_HD_HJ_H$ rearrangements. Flow cytometric analyses revealed a significant reduction in IgM^a-expressing splenic B cells with a cEμ-mutated IgH^a allele. These results show that cEμ is necessary and sufficient V_H to D_HJ_H recombination and IgH gene transcription. The 5' and 3' MAR elements are, however, not able to mediate V_H to D_HJ_H recombination and IgH transcription in the absence of cEμ. Both D_H to J_H and V_H to D_HJ_H rearrangements, however, occurred at normal levels on the both 5' and 3'MARs-deficient alleles. The relative numbers of IgM^a (targeted allele) vs. IgM^b B cells, as well as the level of surface expression of the targeted allotype, were identical to the wild type controls. These results indicate that cEμ is sufficient to mediate VDJ recombination at the IgH locus and IgH gene transcription in the mutant B cells.

Eμ is necessary for the initial activation of IgH gene transcription and necessary for maintenance of gene transcription at a high level in the earliest stages of B cell development, but it is dispensable at later stages for gene transcription. In several B-lymphoid cell lines, Eμ has been lost due to a spontaneous deletion, but heavy chain synthesis is not affected and persists at a high level equivalent to their parental cell line

(Wabl and Burrows, 1984; Klein et al., 1984; Aguilera et al., 1985: Eckhardt and Birshtein, 1985; Zaller and Eckhardt, 1985). For example, our laboratory and others found that in an Igγ2a- producing mouse myeloma cell line 9921 derived from a γ2b-producing line (MPC11), class switch rearrangement from γ2b to γ2a resulted in deletion of Eμ. Nevertheless, the outcome was a γ2a-producing cell that produced heavy chain at levels approximately equal to that of its γ2b- producing parent (Eckhardt and Birshtein, 1985; Zaller and Eckhardt, 1985). However, Eμ has been shown to be required for efficient transcription of a cloned IgH gene when introduced back into B-lymphoid cells (Banerji et al., 1983; Gillies et al., 1983; Neuberger, 1983; Grosschedl and Marx, 1988; Zaller and Eckhardt, 1985; Aguilera et al., 1985). Our laboratory reported that the Igγ2a gene cloned from 9921 cells was not itself enhancer-independent. When reintroduced into myeloma cells, the cloned Igγ2a gene was poorly expressed unless Eμ was added to it, showing the transfected Igγ2a gene was dependent upon Eμ for activation of its expression (Zaller and Eckhardt, 1985). Similarly, Klein et al. (1985) tried to test whether Eμ was no longer necessary for maintenance of transcription, cloned the relevant portion of the Eμ-deleted variant IgH gene and then transfected it into myeloma and hybridoma cells. They found that the reintroduced gene segment was not expressed unless it was linked again to Eμ. In addition, two studies addressed whether enhancer dependence of a transfected gene changed after activation (Grosscheld and Marx, 1988; Porton et al., 1990). Unlike endogenous IgH genes, transfected IgH genes required the continual presence of Eμ to maintain their expression. The observations that Eμ-deficient myeloma variants continue to maintain wild-type levels of IgH gene expression following spontaneous deletion of Eμ, as mentioned above, raised the possibility that there were other regulatory elements in the IgH locus, but not present in cloned IgH genes, that could compensate for Eμ deletion.

Eμ may play a role in the IgH gene class switching process. Gu et al (1993) generated a mutant mouse strain in which J_H-Eμ region including all the J_H segments, and Eμ (core region and its flanking MARs) was deleted from the IgH locus through CRE/*loxP*-mediated gene targeting. By analysis of Ig isotype switch recombination in heterozygous

mutant B cells activated by lipopolysacchride (LPS) plus IL-4, class switch recombination at the μ gene switch region was strongly suppressed on the mutant chromosome (30% that of the wild-type chromosome), whereas the switch region of the γ1 gene was efficiently rearranged (93% that of the wild-type). These data demonstrate that the J_H-Eμ mutation interferes with switch recombination at Sμ (class switch region at μ gene) in cis, and the regulation of Sγ1 (class switch region at γ1 gene) rearrangement in switch recombination is independent from that of Sμ and not under control of the Eμ element on the same chromosome. Because of the deletion of the J_H-Eμ, it is obviously impossible for B cells to generate any functional VDJ/DJ gene rearrangement on the mutant chromosome (Chen et al., 1993; Serwe & Sablitzky, 1993). B cells from normal animals, however, generally have a VDJ-Eμ complex on the productive chromosome and a DJ-Eμ complex on the nonproductive chromosome (Alt et al., 1987). Ig isotype switch recombination occurs on both productively and nonproductively rearranged IgH locus (Radbruch et al., 1986). The data from J_H-Eμ mutant mice, therefore, suggest that the VDJ/DJ-Eμ complex plays an important role in conferring on Sμ accessibility for switch recombination. Chimeric mice with ES cell lines that have targeted deletion of the core enhancer region of Eμ (a 220 bp HinfI/HinfI fragment), 5'MAR, 3'MAR, or both 5'and 3'MARs showed that the core enhancer was necessary and sufficient for providing the functions of Eμ required for efficient class switch recombination at the IgH locus in the mutant B cells, and both the 5' and 3' MAR sequences were dispensable for the process (Sakai et al., 1999b).

2. Murine Ig heavy chain gene 3' enhancers and their activity

As described earlier, Eμ-deficient myeloma variants continue to produce wild-type levels of IgH following spontaneous deletion of Eμ. It was also noted, by those studying B lymphoid malignancies, that reciprocal chromosomal translocations between oncogene *c-myc* and the IgH locus were common in mouse plasmacytomas and human Burkitt's lymphomas. These chromosome translocations often juxtaposed *c-myc* with IgH 3'sequences and led to de-regulated *c-myc* expression (Cory, 1986; Magrath, 1990; Spancer and Groudine, 1991). These two types of observations eventually led to

discovery of 3' IgH enhancers in the murine IgH locus. Four 3' enhancers, Hs3a, Hs1,2, Hs3b and Hs4, located downstream of Cα coding sequences have been identified. These four enhancers span a 34 kb region, and are over ~ 170 kb from the J gene segment in the germline configuration (see **Figure 1-3**).

Hs3a, formerly referred to as Cα3' lies 1kb downstream of IgH Cα gene segment exon 3 (Matthias and Baltimore, 1993). Hs1,2, formerly 3'α E, maps approximately 16 kb downstream of Cα (Lieberson et al., 1991; Dariavach et al., 1991). Hs3b (formerly Hs3) and Hs4 lie approximately 13kb and 17kb downstream of Hs1,2, respectively (Madisen and Groudine, 1994; Michaelson et al., 1995). Hs3a and Hs3b share 97% DNA sequence homology with each other, and are inverted with respect to one another (Chauveau and Cogne, 1996). It has been noted that Hs1,2 is at the center of an approximately 25kb region of dyad symmetry flanked by Hs3a and Hs3b (Chauveau and Cogne, 1996; Saleque et al., 1997).

Like Eμ, all the 3'IgH enhancers are lymphoid-specific, but they function differently at different stages of B cell development when assayed in a transient transfection model. Hs3a is active in several Ig-secreting plasma cell lines, such as S194 and J558 when tested with a CAT reporter (Matthias and Baltimore, 1993), but it is not active in a pre-B cell line (18-81) when tested with an IgVλ1-driven luciferase reporter (Saleque et al., 1997). Hs3a is DNase I hypersensitive in plasma cell lines (S194, and MPC11), but not in a pre-B cell line (18-81). Furthermore, Hs3a and Hs3b show significant transcriptional synergy (IgVλ1-driven luciferase reporter) only in plasma cells (Saleque et al., 1997). Hs1,2 is not active in pre-B cells, but is active in plasma cell lines (Dariavach et al., 1991; Lieberson et al., 1991; Singh and Birshtein, 1993&1996). In the surface-Ig positive cell lines, it is generally inactive (Singh and Birshtein, 1993&1996). Like Hs3a, Hs1,2 is DNase I hypersensitive in plasma cell lines (Giannini et al., 1993; Madisen and Groudine, 1994). Like Hs3a and Hs1,2, Hs3b is active in plasma cell lines but not in pre-B cell lines (Madisen and Groudine, 1994; Saleque et al., 1997), and is DNase I hypersensitive in plasma cell lines (Madisen and Groudine, 1994). Unlike other 3'IgH enhancers, Hs4 is active in pre-B, surface-Ig positive, and plasma cell lines and its activity is relatively

strong when tested in the transient assays (Madisen and Groudine, 1994; Michaelson et al., 1995). Similarly, Hs4 is DNase I hypersensitive from pre-B through plasma cell lines (Giannini et al., 1993; Madisen and Groudine, 1994). Transient transfection assays show that the group of 3'IgH enhancers becomes active in surface-Ig^+ cells (but not active in pre B cells) and functions as a highly synergistic unit. In Ig-secreting cells, the individual 3' enhancers have detectable activity, but they are less interdependent (Ong et al., 1998). Compared to Eμ, activity of the individual 3'enhancers is weak. For instance, the activity of Hs1,2 is approximately 25% that of Eμ in Ig-secreting cells (Lieberson et al., 1991). All the 3' enhancers together, however, can synergize to yield a strong activity equivalent to that of Eμ in surface-Ig positive cell lines when tested by the luciferase reporter gene in transient transfection assays (Ong et al., 1998). In plasmacytoma cells, Hs1,2, Hs3b and Hs4 together can synergize to an activity greater than Eμ when tested by an IgVλ1-driven *c-myc* reporter gene in transient assays, but the combination of only Hs1,2 and Hs4, or Hs3b and Hs4 still can synergize to levels equivalent to that induced by Eμ (Madisen and Groudine, 1994).

The regulatory activity of the 3'IgH enhancers in endogenous IgH gene transcription is not fully understood, but several observations support a role for these enhancers in endogenous IgH gene transcription. One line of evidence comes from an observation that natural deletion of an approximately 34 kb DNA region including Hs3a, Hs1,2, Hs3b and Hs4 enhancers downstream of Cα gene segment in a mouse myeloma variant, LP1.2, dramatically reduced levels of Igα mRNA compared to the parental cell line (Gregor and Morrison, 1986; Michaelson et al., 1995). It is suggested that the decreased transcription rate of the Igα gene in LP1.2 could result from the deletion of positive regulatory sequences from the 3' flanking regions. The possibility that a negative element is introduced into the locus by the same deletion, however, cannot be excluded.

As described in more detail in **Chapter 2** of this thesis, we have replaced Hs1,2 with *neo*^{r} in a myeloma cell line, 9921, which lacked Eμ within the expressed γ 2a-gene as a result of an aberrant class switch recombination. In the mutant 9921 cells, the γ2a gene was totally silenced upon deletion of Hs1,2, suggesting that Hs1,2 is essential for IgH

gene transcription in the absence of Eμ (Lieberson et al., 1995). As the experiment involved insertion of *neo*^{r} in the place of Hs1,2, it isn't excluded that *neo*^{r} itself might disrupt IgH gene expression . It has been shown that some dramatic phenotypes initially observed upon replacement of a regulatory element with *neo*^{r} (or simply upon *neo*^{r} insertion into a regulatory region) are eliminated following subsequent deletion of *neo*^{r} from the gene locus (Kim et al., 1992; Fiering et al., 1993; Xu et al., 1996; Manis et al., 1998).

Chromosomal translocations between the IgH locus and *c-myc* leads to one *c-myc* allele becoming juxtaposed to IgH sequences through a reciprocal 5'→5' chromosomal translocation event in mouse plasmacytoma and human Burkitt's lymphoma cells (Cory, 1986; Magrath, 1990; Spencer and Groudine, 1991). The translocation breakpoints involved on both chromosomes are highly variable, but the breakpoints of *c-myc* are often within either its first non-coding exon or first intron and the breakpoints in the IgH gene locus are usually within a class switch region (Molding et al., 1985; Kelly and Siebenlist, 1986). This links the *c-myc* coding regions with the IgH 3' region including all the 3'IgH enhancers. The upstream non-coding region of *c-myc* is then linked to IgH 5' region, including Eμ. The rearranged *c-myc* allele is transcriptionally active in these cell types (Yang et al., 1985; Croce, 1987), whereas the germline *c-myc* alleles are silenced, as they are in normal mature B cells (Bernard et al., 1983). Transfection studies of a truncated *c-myc* gene (Feoet al., 1984) and the characterization of a naturally occurring deletion 5' of the *c-myc* gene (DelSenno et al., 1986) showed that activation of the translocated *c-myc* gene is a consequence of its association with IgH sequences rather than merely due to disassociation from its physiological 5' control region. This suggests that IgH 3' enhancers play an important role in the de-regulated expression of the translocated *c-myc* gene.

The 3'IgH enhancers may function as an LCR (locus control region) (Madisen and Groudine, 1994). The first LCR discovered was a cluster of hypersensitive sites (HS sites) in the β-like globin locus (Tuan et al., 1985; Forrester et al., 1986; Grosveld et al., 1987). The human β-globin locus consists of five erythroid-specific genes, ε, Gγ, Aγ, δ, and β,

which are expressed sequentially during development. Five DNase I hypersensitive sites (HSs), which span a 20 kb region, are located upstream of the β-globin locus. These hypersensitive sites consist of the β-globin LCR. Individual HSs contain binding sites for ubiquitous and erythroid-restricted trans-acting factors within their core regions. It has been shown that β-globin transgenes without the β-globin LCR are expressed at low levels and in an integration site-dependent manner ("chromosome site variegation") in transgenic mice. When linked to the β-globin LCR, however, they are expressed in an integration site-independent (transgenes are expressed regardless of the integration sites in the host genome) and copy number dependent (expression is directly related to copy number) manner. Moreover, transgenes per copy expressed at the same levels as that of an endogenous mouse β-globin locus (Grosveld et al., 1987). It is believed that LCRs are able to establish an "open" chromatin structure that is necessary for proper expression of a linked transgene without effect of integration sites (Festenstein et al., 1996).

The functional significance of the β-globin LCR in the endogenous locus has been established. A natural deletion of a 35 kb region including HS2-5 of the β-LCR (Hispanic thalassemia in human) led to the failure to transcriptionally activate the cis-linked globin genes in erythroid cells. In the mutants, the entire beta-globin locus and sequences approximately 100 kb 5' and 3' of the adult beta-globin gene were DNase I-resistant and did not form characteristic distant hypersensitive sites (Forrester et al., 1990). Similarly, when the functional components of the β-LCR (20 kb, including HS2-5), as defined by transfection and transgenic studies, were deleted from the human endogenous beta-globin locus using homologous recombination, transcription of all beta-globin genes (ε, γ, and β genes) was abolished in every MEL (murine erythroleukemia) cell carrying the deletion. However, formation of the remaining hypersensitive site(s) of the LCR and the presence of a DNase I-sensitive structure surrounding the beta-globin locus were not affected by the deletion (Reik et al., 1998). In this experiment, human beta-globin locus (chromosome 11) was first introduced into the chicken pre-B cell line DT40, which exhibits highly efficient gene targeting, by cell fusion. After the functional components of the β-LCR were deleted in the chicken/human hybrid, the mutant human beta-globin chromosome was then transferred back into MEL cells by cell fusion. The murine beta-

globin locus control region (β-LCR), however, appeared less important than the human β-LCR in similar experiments. The murine β-LCR (HS1-6) was deleted from its native chromosomal location using homologous recombination in ES cells (homozygous mutants). The approximately 25 kb deletion eliminated all the sequences and structures homologous to those defined as the human β-LCR. In differentiated ES cells (differentiated in liquid culture), and human erythroleukemia cells (K562) containing the mouse β-globin LCR-deleted chromosome (mouse β-globin LCR-deleted ES cell/human K562 hybrids), DNase I sensitivity of the beta-globin domain was established and maintained, developmental regulation of the locus was intact, although beta-like globin RNA levels were reduced to 5%-25% of wild type. The data showed that in the native murine beta-globin locus, the LCR was necessary for normal levels of transcription, but other elements were sufficient to establish the open chromatin structure, transcription, and developmental specificity of the locus (Epner et al., 1998).

In addition to the β-globin locus, LCRs have then been found associated with the human CD2 gene, which produces a protein present on most thymocytes and probably on all peripheral T cells (Greaves et al., 1989), the human α-globin gene (Higgs et al., 1990), the macrophage-specific lysozyme gene (Bonifer et al., 1990), the p56lck gene (lymphocyte-specific protein tyrosine kinase) (Abraham et al., 1991), and the TCR α/δ locus (Diaz et al., 1994).

Hs1,2, Hs3b and Hs4 enhancers together are able to induce a *c-myc* reporter to express in a copy number-dependent and position-independent manner when stably transfected into plasmacytoma cells (Madisen and Groudine, 1994). More recent studies, however, show that they function as a partial LCR, not a classical one. In transgenic mice harboring a V_H promoter-β-globin reporter gene linked to four IgH 3' enhancers (Hs3a, Hs1,2, Hs3b, and Hs4), transgene expression was strictly confined to the B cell lineage in all transgenic founder lines. Furthermore, reporter gene activity was chromosome site-independent but not strictly copy number-dependent (Chauveau et al., 1999). Similarly, when a CAT reporter gene driven by a heavy chain variable gene promoter (pV_H) was linked to the palindromic part of the 3' IgH regulatory region including Hs3a, Hs1,2, and

Hs3b, it was expressed in all clones, regardless of integration sites but copy number-dependent expression was not observed (Chauveau et al., 1998).

It has been suggested that the 3'IgH enhancers regulate Ig heavy chain class switch recombination. In gene targeted mice, replacement of either Hs3a or Hs1,2 with *neo*^{r} resulted in severe impairment of IgH class switch recombination to several isotypes on the targeted chromosomes. Mice whose B cells lacked either Hs3a, or Hs1,2 were deficient in serum Ig G2a and IgG3, while cultured splenic B cells from these mice failed to undergo switching to IgG2a, IgG2b, IgG3 and IgE. (Cogne et al., 1994; Manis et al., 1998). These mutant phenotypes were eliminated, however, and class switch recombination returned to normal when the *neo*^{r} in the place of the enhancer was deleted subsequently by Cre/*loxP*-mediated recombination (Manis et al., 1998). These data show that Hs3a and Hs1,2 are not essential for IgH isotype switch recombination. In related studies, a pre-B cell line (70Z/3) that had spontaneously deleted a region including both Hs3a and Hs1,2 was fused to a plasmacytoma cell line (NSO). The enhancer-deleted allele of the pre-B cell underwent spontaneous class switch recombination, resulting in a switch from μ to γ1 at a frequency comparable with that of most hybridomas (Saleque et al., 1999). These studies suggest that spontaneous class switching in a plasma cell line requires neither Hs3a nor Hs1,2.

It cannot be excluded, however, that Hs3a/Hs1,2, and Hs3b and/or Hs4 might be functionally redundant in regulation of IgH class switch recombination. Alternatively, Hs3b and/or Hs4, and/or other regulatory elements that have not been identified and are located farther 3' of the IgH locus are responsible for the class switch process. A line of indirect evidence to support the notion that the 3'IgH region might be responsible for IgH class switch recombination (CSR) comes from observations that CSR is position-dependently inhibited by PGK-*neo*^{r} cassettes inserted into the IgH constant regions. As described earlier, germline C_H transcripts initiate from a promoter upstream of a non-coding I exon, proceed through the switch (S) region and terminate downstream of the associated C_H exons. As described earlier, induction of CSR to a particular C_H gene is strongly correlated with germline transcription of the gene. Insertion of a PGK-*neo*^{r}

cassette at two sites (replacing Hs3a and Hs1,2 with the PGK-*neo*^{r} cassette) downstream of Cα inhibited, in cultured B cells, germline transcription of and CSR to a subset of C_H genes (including Cγ3, Cγ2b, Cγ2a, and Cε) that lie as far as 120 kb upstream (Cogne et al., 1994; Manis et al., 1998). In other experiments, the PGK-*neo*^{r} cassette was inserted in place of sequences in the Iγ2b region (most of the Iγ2b exon was replaced by a PGK-*neo*^{r} gene inserted in the same transcriptional orientation as the endogenous Ig locus. Insertion/deletion extended from immediately 3' of the majority of transcription initiation sites in Iγ2b to a region beyond its donor splice site). This insertion inhibited germline transcription of and CSR to the upstream Cγ3 gene, but had no major effect on the downstream Cγ2b, Cγ2a, Cε and Cα genes. Moreover, replacement of the Cε exons (1-4 exons) with a PGK-*neo*^{r} cassette in the opposite transcriptional orientation also inhibited, in cultured B cells from the Ig-locus disrupted mice, germline transcription of and CSR to the upstream Cγ3, Cγ2b, and Cγ2a genes and to the Sε (Seidl et al., 1999). As with the PGK-*neo*^{r} insertions 3' of Cα, the Cγ1 and Cα genes were less affected by these mutations both in cultured B cells and in mice, whereas the Cγ2b gene appeared less affected in vivo. These results support the existence of a long-range 3' IgH regulatory region required for germline transcription of and CSR to multiple C_H genes, and suggest that PGK-*neo*^{r} cassette insertion into the locus short-circuits the ability of this region to facilitate germline transcription of dependent C_H genes upstream of the insertion.

3. Murine Ig κ light chain gene enhancers and their activity

Two enhancer elements have been described at the Ig kappa light chain locus (Igκ), which are located on either side of the unique Cκ region and are referred to as the intronic enhancer, iEκ, residing 0.5kb upstream of Cκ (Queen and Baltimore, 1983; Picard and Schaffner, 1984), and the 3' enhancer, 3'Eκ, lying 9 kb downstream of Cκ (Meyer and Neuberger, 1989). Like IgH enhancers, both are lymphoid-specific, and the Eκ is MAR-associated (Cockerill and Garrard, 1986). Transient transfection assays showed that transcriptional activity of 3'Eκ is much stronger than that of iEκ. In κ-producing lymphoma (Daudi) cells, the activity of 3'Eκ is ~27-fold that of iEκ (luciferase reporter

gene driven by Vλ3 promoter). Similarly, in λ-producing lymphoma (MN-60) cells, the activity of 3'Eκ is ~16-fold that of iEκ (Asenbauser et al., 1999).

It has been shown that both iEκ and 3'Eκ enhancers play an important role in VκJκ rearrangement. In iEκ-deficient mice in which iEκ is replaced by the *neo*^{r} gene, B cells heterozygous for the mutation undergo VκJκ recombination exclusively on the intact Igκ locus but not on the mutated allele (Takeda et al., 1993). Homozygous mutant mice exhibit no rearrangement in their Igκ loci. However, all the B cells express λ chain bearing surface Ig. These findings suggest that iEκ is essential for VκJκ rearrangement but is not necessary for λ chain rearrangement. Furthermore, *neo*^{r} insertion at the 3' end of the iEκ also shows some suppressive effect on VκJκ recombination (Takeda et al., 1993). In another experiment, MAR/iEκ (740bp, iEκ with its associated MAR) was similarly replaced by a *loxP* flanked *neo*^{r} cassette at first, and then followed by *neo*^{r} deletion using the Cre/*loxP* approach (Xu et al., 1996). The MAR/iEκ-deleted B cells from gene-targeted homozygous mice greatly impaired VκJκ rearrangement, and altered κ:λ ratio from the usual 10:1 to 1:1. When the enhancer was replaced by *neo*^{r}, all VκJκ recombination ceased. When the enhancer was simply deleted plus no *neo*^{r} remained in its place, VκJκ recombination was greatly impaired but was not eliminated. Moreover, MAR/iEκ sequences did not appear necessary for efficient transcription of the rearranged κ gene on κ-expressing B cells lacking MAR/iEκ. The surface level of Ig κ light chain on these κ-expressing B cells was equivalent to that on κ-expressing B cells derived from normal mice. This finding is consistent with the earlier observation that MAR/iEκ is not necessary for normal κ gene transcription in transgenic studies involving a gene with iEκ and 3'Eκ, alone or in combination (Betz et al., 1994). Furthermore, λ-expressing B hybridomas derived from iEκ-deficient B cells displayed little κ rearrangement. This is in contrast to λ-expressing B cells from normal mice. In 97% of λ-expressing B cells from normal mice, the κ locus of at least one allele has undergone either nonproductive rearrangement or has been deleted through RS/kde recombination. Results of mice lacking MAR/iEκ show that MAR/iEκ is significantly important but not necessary for VκJκ rearrangement, and is not required for normal κ gene transcription in B cells.

Targeting the 808-bp fragment of the 3'Eκ enhancer yielded similar results (Gorman et al., 1996). In this study, 3'Eκ was replaced by the *neo*^{r} cassette at first, and the *neo*^{r} was then removed by the Cre/*loxP* system. In mice homozygous for 3'Eκ deficiency, κ-expressing B cells were dramatically reduced. λ-expressing B cells were increased, however, in these mice. This altered κ:λ ratio from the usual 10:1 to 2:1. Analysis of heterozygous 3'Eκ-deficient mice showed that reduction of κ rearrangement took place on the targeted allele. Moreover, 3'Eκ deletion did not dramatically affect κ expression in B cells that lacked 3'Eκ and yet assembled an Ig κ light chain gene. This finding is not in accordance with the earlier observation that 3'Eκ was both necessary and sufficient for high-level expression of κ transgenes (Meyer et al., 1990; Blasquez et al., 1992; Betz et al., 1994). Results of 3'Eκ-deficient mice suggest that like iEκ, 3'Eκ is critical but not essential for efficient VκJκ recombination, and is not required for normal κ transcription in B cells. Observations that neither iEκ nor 3'Eκ plays an essential role in regulating transcription of rearranged κ light chain genes suggest that these two enhancers may be functional redundant: the remaining enhancer is able to compensate functionally for the deleted one.

Studies of transgenic mice have shown that the Ig iEκ and 3'Eκ are required for V region hypermutation in the Ig κ light chain locus. Betz et al. (1994) found that both the iEκ/MAR and 3'Eκ regions were essential for full somatic hypermutation. When 3'Eκ and iEκ/MAR were deleted from the transgenes, hypermutation decreased to 17.7% and 6%, respectively, relative to wild type. Similarly, Goyenechea et al. (1997) showed that in modified transgenes, the recruitment of hypermutation was substantially impaired by deletion of the MAR that flanks the iEκ. Decreased mutation was also obtained if iEκ, the core region of 3'Eκ or 3'Eκ plus flanking region were removed individually. 3'Eκ-deficient mice (homozygous), however, showed that there was no absolute requirement for the 3'Eκ with respect to somatic hypermutation of the endogenous κ locus (65% of wild type) (van der Stoep et al., 1998).

4. Murine Ig λ light chain locus enhancers

In the mouse Ig λ light chain locus, there are two 3' enhancers: λ2-4E resides 15kb downstream of Cλ4, and λ3-1E is located 35kb downstream of Cλ1 (Eccles et al., 1990; Hagman et al., 1990) (see **Figure 1-2** for orientation of Cλ genes). Both enhancers are lymphoid-specific and functionally independent, and function in the absence of the transcription factor NF-κB, which is necessary for kappa enhancer function. Rearrangement of Vλ2 to Jλ3-Cλ3 or Jλ1-Cλ1 genes deletes λ2-4E. λ3-1E is 90% homologous to λ2-4E sequence (Hagman et al., 1990).

It has been observed that a λ2 transgene under the control of the λ2-4E undergoes mutation in both the Peyer's patches (PP) and splenic B cell populations, but at a frequency lower than endogenous light chain genes (Klotz and Storb, 1996). Kong et al. (1998) also showed that a rearranged λ1 transgene under the control of the λ2-4E (Vλ1-Jλ1-Cλ1/λ2-4E) was driven to undergo active hypermutation in Peyer's patch cells. Analysis of transgene DNA derived from the Peyer's patch cells showed that the level of mutation of the Vλ1-Jλ1 region was 3.8 mutations/kb (69 mutations were identified in a total of 19372 sequenced bases). In contrast, there is no evidence of hypermutation in mice carrying the rearranged λ1 transgene under the control of the 3'Eκ (Vλ1-Jλ1-Cλ1/3'Eκ). These results suggest that Ig λ light chain enhancers may be required for somatic hypermutation in the λ locus.

5. Human Ig locus enhancers and their activity

Enhancers analogous to those found in murine Ig loci have been identified in human Ig loci: the intronic IgH enhancer, Eμ (Rabitts et al., 1983), and three 3' IgH enhancers (Hs1,2, Hs3 and Hs4) located downstream of each Cα gene segment in the heavy chain gene locus (Chen and Birshtein, 1997; Mills et al., 1997; Pinaud et al., 1997); the iEκ and 3'Eκ in the κ light chain locus (Emorine et al., 1983; Judde and Max, 1992), and one 3' enhancer residing downstream of the last constant gene segment Cλ7 in the λ light chain locus (Spandidos and Anderson, 1984; Blomberg et al; 1991).

The human Eμ is located ~5 kb upstream of Cμ gene and active in mouse lymphoid cells. The human Eμ shares ~70% homology with the murine Eμ within a 633 bp EcoRI/HindIII fragment (Rabbitts et al., 1983). The corresponding sequences downstream from each of the two human Cα genes in the IgH locus are nearly identical to each other. Within 25 kb downstream of both the Cα genes, 3'IgH enhancers, Hs1,2, Hs3, and Hs4 are located. Like the murine Hs1,2, both the human Hs1,2s are flanked by long inverted repeats. Furthermore, the two Hs1,2-like regions generally appear to be inverted with respect to each other. Within a 135-bp core homology region, the human Hs1,2s are approximately 90% identical to the murine homologue and include several motifs previously demonstrated to be important for function of the murine Hs1,2. Certain functional elements in the murine enhancer, including a B cell-specific activator protein (BSAP) site, however, do not appear to be conserved in human Hs1,2s. The human homologues of two other murine 3'IgH enhancers, Hs3 and Hs4, show lower overall sequence conservation, but for at least two of the functional motifs in the murine Hs4 (a κB site and an octamer motif) the human Hs4 homologues are exactly conserved (Chen and Birshtein, 1997; Mills et al., 1997; Pinaud et al., 1997).

Hu et al (2000) investigated the regulation of germline Cα1 and Cα2 promoters, upstream of the class switch (S) and intervening (I) regions, by different human 3' enhancer fragments in cell lines representing various developmental stages. They found that both Cα1 Hs1,2 and Cα2 Hs1,2 fragments showed equally strong enhancer activity on the germline Cα1 and Cα2 promoters in both orientations when reporter genes carrying these elements were transiently transfected into a number of surface-Ig^+ B cell lines but not in a human pre-B cell line or a human T cell line (Jurkat). Hs3 showed no enhancer activity by itself in any of the cell lines, whereas a modest effect was noted using Hs4 in the three surface-Ig^+ B cell lines (Dg75, Cl-01, and HS Sulton). However, the combination of the Cα2 Hs3-Hs1,2-Hs4 fragments, which together form a potential locus control region, displayed a markedly stronger enhancer activity than the individual fragments, with a differential effect on the Cα1 and Cα2 promoters. These results suggest that like the murine 3'IgH enhancers, the human 3' IgH enhancers are able to synergize to yield a stronger enhancer activity. These results also suggest that the human 3'

enhancer regions may *cis*-up-regulate the germline Cα promoter activity that directs IgA isotype switch in memory B cells. As mentioned earlier, IgH chain class switch recombination is correlated to germline transcription at C_H genes (Stavnezer and Sirlin, 1986; Yancopoulos et al., 1986; Lutzker et al., 1988; Rothman et al., 1990; Xu et al., 1993).

The human iEκ is located ~ 0.5 kb upstream of Cκ gene and shares 81% homology with the murine iEκ within a 126 bp core region (Emorine et al., 1983). The human 3'Eκ is located 12 kb downstream of the human Cκ. The core enhancer region of the human 3' Eκ is highly homologous to the murine 3' Eκ. In addition to two regulatory elements homologous to the functional motifs of the murine 3'Eκ, however, a third positive regulatory element associated with an 11/12-bp direct repeat in the human 3'Eκ is found (Judde and Max, 1992).

To study the control of human Ig κ light chain gene rearrangement, transgenic mice that carry a germ-line human κ mini-locus (HK) derived from a 45 kb germline fragment from the κ locus and containing the Jκ-proximal Vκ (Vκ4), the Vκ-Jκ intergenic region, the five Jκ segments and the Cκ were generated (Caveliver et al., 1997). The human κ mini-locus included the human iEκ, but not the 3'Eκ. Rearrangement of the human κ transgene was found to occur and was lymphoid specific and restricted to the B cell lineage. On the average one copy of the transgene was rearranged per B cell for both of two independent transgenic lines. One transgenic line had 5 copies of HK, and the other had 20 copies of HK. This result suggests that the human 3'Eκ is not absolutely essential for VκJκ recombination in the endogenous human κ locus.

Monocytoid B cell lymphoma (MBCL) is a malignancy of mature B cells carrying somatically mutated V region genes. In two of the five analyzed monocytoid B cell lymphomas, normal hypermutated V_H gene rearrangements were found but hypermutated and functional Vκ gene rearrangements were not found. A further analysis of these two lymphomas revealed that the Cκ gene and both enhancers, iEκ and 3'Eκ, had been deleted in *cis* to the rearranged Vκ region genes by rearrangement of the RS/kde

(Kuppers et al., 1996). These results suggest that iEκ and/or 3'Eκ alone is essential for Vκ gene somatic hypermutation in the human endogenous κ locus.

The human 3' λ enhancer (1.2 kb SstI/SstI fragment, HuEλ) originally identified resides 11.7kb downstream of Cλ7, the most 3' lambda constant region gene, in the λ locus and is independent of NF-κB, similar to the mouse lambda enhancers (Blomberg et al., 1991). HuEλ is active in both mouse and human B cell lines; interestingly, the mouse lambda enhancers are active in mouse lines but not in a human B cell line. DNA sequence comparison of the mouse and human lambda enhancers indicates a higher degree of homology (average of 72.5%) within the 111 bp PstI/SstI enhancer core region located at the 3' of 1.2 kb HuEλ than for the remaining flanking sequence compared (average of 42%) (Blomberg et al., 1991). Further studies showed that a 311 bp fragment, which is the complete enhancer, (111 bp enhancer core region plus 200 bp sequence immediately downstream of the original 1.2 kb HuEλ) had maximal activity in transient CAT assays in human pre-B, κ-producing, and λ-producing lymphoma cell lines. The 111 bp core region retained 1/3 to ½ the activity of the complete enhancer in the cell lines. The 200 bp sequence when assayed alone in either orientation, however, resulted in no significant CAT conversion in human λ-producing lymphomas. This result suggests that the 200 bp sequence does not have enhancer activity but can augment the activity of the 111 bp enhancer core region. Furthermore, the activity of the complete enhancer was greater than that of the original 1.2 kb HuEλ (2.5-fold) in human λ-producing lymphomas (Glozak and Blomberg, 1996). Interestingly, a 565 bp StuI/StuI fragment (311 bp complete enhancer plus 81 bp upstream and 173 bp downstream of the enhancer) had approximately the same activity as the 311 bp "complete enhancer" when tested in human κ- and λ-producing cells. As compared with the complete enhancer, however, the 565 bp fragment had only ¼ to 1/8 activity in a human pre-B cell line. Further analysis showed that negative elements flanked the 311 bp complete enhancer (Glozak and Blomberg, 1996). These results suggest that the presence of developmentally regulated negative elements flanking the human lambda enhancer prevents or reduces its activity at very early stages in B cell development. Motifs that are shared with the murine lambda enhancers such as λA, λB, and E-box motifs (λE1,λE2, and λE3), as well as a unique

motif, HELP (human enhancer lambda protein), have been identified in the HuEλ (Glozak and Blomberg, 1996).

Asenbauer et al (1999) have identified three DNase I-hypersensitive sites, Hs1, Hs2, and Hs3, located 6 kb, 9.8 kb, and 13.3 kb downstream of Cλ7, respectively. The human lambda light enhancer (HuEλ), originally identified by Blomberg et al. (1991), was contained within Hs3. The three HSs synergized in transcriptional activation of the luciferase reporter gene driven by either SV40 or Vλ3 promoter in κ- and λ-producing lymphoma cells (~5-fold that of Hs3 alone). Furthermore, in λ-producing lymphoma cells, only Hs2 and Hs3 together could synergize the same activity as that of all the 3 HSs together (93%). All the 3 sequence elements together constituted a powerful tissue-specific enhancer that is a much stronger transcriptional activator than the kappa enhancers, iEκ and 3'Eκ, alone or in combination in κ- and λ-producing lymphoma cells.

IV. Transcription factors and their binding sites in Ig enhancers

The activities of enhancers in Ig loci are regulated by transcription factors. Several key transcription factor families, including C/EBP proteins, E2A proteins, Ets family proteins, Octamer proteins, Pax proteins, NF-κB/Rel proteins, and TFE3 proteins, have been discovered. Some of them are B cell-specific gene activator, but others are ubiquitously expressed. What follows is a brief summary of the functions associated with these transcription factors.

C/EBP (CCATT/E-site binding protein) family proteins bind to " E " sites (motifs) named for their discoverer, Anne Ephrussi (Ephrussi et al., 1985) in the Eμ, iEκ, and several V_H promoters (Roman et al., 1990). These " E " sites are functionally important for the activity of these enhancers or promoters. NF-IL6 is a member of the C/EBP family. NF-IL6 mRNA level is virtually undetectable in the pro-B and pre-B cell lines but is readily detected in plasma cell lines. In surface-Ig positive cell lines, however, NF-IL6 mRNA level is undetectable or very low. NF-IL6 mRNA levels are significantly induced when normal splenic B cells are stimulated by LPS (Cooper et al., 1994). These

results suggest that NF-IL6 functions in surface-Ig positive and plasma cells. The role of NF-IL6 *in vivo* has been investigated. NF-IL6-deficient mice (homozygous) were generated by gene targeting (Tanaka et al., 1995). NF-IL6-deficient mice were highly susceptible to infection. Furthermore, the tumor cytotoxicity of macrophages from these mice was severely impaired. However, cytokines involved in macrophage activation, such as TNFα and IFNγ, were induced normally in the mutant mice. These results demonstrate the crucial role of NF-IL6 in macrophage bactericidal and tumoricidal activities.

E2A family proteins bind to the μE5 and μE2 sites in Eμ, the μE5 site in all the 3'IgH enhancers, κE2 site in the iEκ, and μE2/5 sites in the λ enhancers. One gene encodes three E2A family activators that are present in B cells: E12, E47, and E2-5. They are differential splice products of the *E2A* gene. E2A family proteins contain a basic helix-loop-helix domain, which is responsible for protein dimerization and DNA binding. As described earlier, disruption of the *E2A* gene (gene "knockout") led to a block in B lymphoid development before detectable IgH rearrangement or expression of the recombinase gene *RAG-1*, which is involved in Ig V(D)J or TCR V(D)J rearrangement (Bain et al., 1994; Zhuang et al., 1994).

Ets family proteins bind to the two functionally important sites μA (π) and μB in both the Eμ and 3'IgH enhancer (Hs1,2), one Ets-binding site in 3'Eκ, and one Ets-binding site in λ enhancers. PU.1 is a member of this family proteins and an important gene expression activator in lymphoid development and differentiation. As mentioned earlier, knockout of the gene encoding PU.1 resulted in specific loss of any detectable progenitors of B cells, T cells, monocytes, and granulocytes, but had no effect on erythroid precursors (Scott et al., 1994).

Octamer proteins bind to the OCTA site in the Eμ, all the 3'IgH enhancers, and iEκ as well as Ig heavy and light chain gene promoters. Two transcription factors, Oct-1 and Oct-2, can bind to the octamer element in B cells (Singh et al., 1986; Staudt et al., 1986). These factors are members of a family of homeodomain proteins known as the POU

family. Oct-1 is ubiquitously expressed. Oct-2 is tissue-restricted, however, primarily in lymphoid cells and the central nervous system. Both Oct-1 and Oct-2 function by interacting with a common B cell- factor, OCA-B, which stimulates B cell-specific transcription (Pierani et al., 1990; Luo et al., 1992).

It has been shown that Oct-2 is not essential for Ig gene expression, but surface-Ig positive B cells from Oct-2 locus knock-out mice were unable to differentiate into Ig-secreting cells when cultured in vitro with LPS (Corcoran et al., 1993). Gene targeting studies have also shown that OCA-B, the B cell-specific co-activator that interacts with both of Oct-1 and Oct-2, is not required for initial transcription of immunoglobulin genes nor for B cell development, *in vivo*. It is essential., however, for the response of B cells to antigens and for normal production of Ig isotypes, and is required for the formation of germinal centers (Kim et al., et al., 1996; Schubart et al., 1996).

BSAP/Pax proteins bind to the BSAP-binding sites in the 3'IgH enhancers, Hs1,2, and Hs4. BSAP (B cell-specific activator protein) is coded by the *Pax*-5 gene in mammals, and is present in pro-B, pre-B, and surface-Ig positive B cells, but not in plasma cells, T cells, and other cell types (Barberis et al., 1990; Singh and Birshtein, 1993). Transient transfection assays showed that mutation of even one BSAP site in Hs1,2 led to an increase in Hs1,2 activity in surface-Ig positive B cell lines, while a reduction was observed in plasma cell lines (Singh and Birshtein, 1993; Neurath et al., 1994). This implies that BSAP acts as a repressor of Hs1,2 in surface-Ig positive B cells. It has been shown that BSAP regulates activity of Hs1,2 in such cells by blocking activation by NF-αP (blocking binding of NF-αP to its cognate site on Hs1,2), a member of the Ets family present in both surface-Ig positive B cells and plasma cells (Neurath et al., 1995).

There are multiple BSAP binding sites (a high affinity and several weaker) within Hs4. Site-mutated analysis of the high affinity BSAP binding site within Hs4 showed a 2.5 fold increase in transcriptional activity after mutation relative to wild type enhancer in pre-B cells (18-81) and an 83% loss of activity in surface-Ig positive B cells (A20) in

transient CAT assays (Michaelson et al., 1996). These results suggest that BSAP is a repressor and an activator for Hs4 transcriptional activity in pre-B cell line and in surface-Ig positive cell line, respectively.

As described earlier, induction of IgH isotype switching to a particular C_H gene correlates with the transcriptional activation of the same gene in germline configuration. Induction of correctly spliced germline transcripts is necessary to target a switch region for recombination and switching. BSAP binding sites have been found 5' to or within almost all IgH S regions examined, including Sμ, Sγ1, Sγ2a, Sγ3, Sε, and Sα. BSAP may play an essential role, therefore, in induction of C_H germline transcription required for IgH gene class switch recombination. For instance, BSAP binds to the murine and human germline Cε promoters (Iε promoters). Transient transfection models showed that mutation or deletion of the BSAP binding site in the Iε promoter decreased transcription of CAT or luciferase reporter gene driven by the Iε promoter in surface-Ig positive cell line (Liao et al., 1994; Thienes et al., 1997). In addition, BSAP has been implicated as a regulatory factor for many other B cell-specific genes, including *λ5* and *Vpre-B* genes. *Pax-5* deficient mice showed a complete block at the pro-B cell developmental stages, with absence of pre-B, surface-Ig positive, and plasma cells (Urbanek et al., 1994).

NF-κB/Rel family proteins bind to the κB sites in the iEκ, and 3'IgH enhancers (Hs1,2, and Hs4). The κB sites are active in transcription only in surface-Ig positive and plasma cells that were expressing the κ gene, but not active in pre-B cells and other non-lymphoid cells. Therefore, it appeared that the transcription factor binding to these sites, NF-κB, was responsible for the developmentally regulated activity of iEκ. Compared to wild type Hs1,2, mutation of the κB site within Hs1,2 (site-directed mutagenesis) led to a dramatic increase in Hs1,2 activity (~ 4-fold) in surface-Ig positive cells (A20, and M12) in transient CAT assays. Mutation of the κB site within Hs1,2, however, resulted in a reduction by approximately ½ in transcriptional activity in plasma cell line (S194) (Michaelson et al., 1996). These results suggest that NF-κB-like factors repress Hs1,2 activity in surface-Ig positive cell line but activate its activity in plasma cell line. In the case of Hs4, κB site mutation of the enhancer led to 78% reduction in transcriptional

enhancement as compared with the wild type enhancer in plasma cells (S194). Similarly, κB site mutation resulted in a 43% and a 95% reduction in Hs4 activity relative to the wild type enhancer in pre-B cell line (18-81) and in surface-Ig positive cells (A20), respectively (Michaelson et al., 1996). These results suggest that κB binding is a critical activator for Hs4 transcriptional activity in all the stages of B cell development.

In non-B cells, NF-κB is responsible for the inducible expression of a wide variety of genes including cytokines, lymphokines, adhesion molecules and acute phase proteins. NF-κB is a member of the Rel family of proteins, and consists of two subunits p50 (NF-κB1) and p65 (RelA). Other members of the Rel family include p52 (NF-κB2) and RelB. Both p50 (NF-κB1) and p52 (NF-κB2) are synthesized from their cytoplasmic precursors p105 and p100, respectively. p105 and p100 are not able to bind to the κB sites. IκB family proteins (IκBα and IκBβ) can inactivate NF-κB (p50/p65 heterodimer) by association with the p65 subunit of the NF-κB heterodimer, forming an NF-κB- IκB complex. NF-κB, however, can be activated, by dis-association from the IκB proteins, by inducers.

NF-κB is thought to regulate a wide variety of genes involved in immune function and development. The p50 subunit of NF-κB knockout mouse showed no developmental abnormalities, but exhibited multifocal defects in immune responses involving B lymphocytes and nonspecific responses to infection. B cells did not proliferate in response to bacterial lipopolysaccharide (LPS), and were defective in basal and specific antibody production (Sha et al., 1995). In contrast, p65 subunit knockout (RelA) led to embryonic lethality at 15-16 days of gestation, concomitant with a massive degeneration of the liver by programmed cell death or apoptosis (Beg et al., 1995). Mice homozygous for the disrupted RelB locus showed no defect in embryo development (Burkly et al., 1995; Weih et al., 1995). These mice, however, showed a loss of thymic dendritic cells, displayed other defects such as a lack of erythropoiesis in bone marrow, myeloid hyperplasia in bone marrow, and lung and liver inflammation, and had impaired cellular immunity (Weih et al., 1995). *c-rel* deficient mice lacked any defects in embryonic development. They, however, displayed defects in proliferation of B and T cells in

response to antigens, and in IL-2 synthesis (Kontgen et al., 1995). Taken together, these results show that the functions of the inducible Rel/ NF-κB family members are varied and distinct and are involved in functional processes ranging from immune cell activation and function to embryonic development of the liver.

TFE3/USF proteins bind to the μE3 site in Eμ, all the 3'IgH enhancers, and V_H promoters, and to the κE3 site in the iEκ. This family of proteins contains bHLHZIP (basic helix-loop-helix zip) domains that mediate DNA binding via the basic regions and protein dimerization via the HLH and ZIP domains. TFE3 is LPS inducible in B cell lines and splenic B cells. Studies of *TFE3*-deficient mice have shown that TFE3 is required for efficient IgH isotype switching (antigen-dependent activation) *in vivo*, but is not essential for Ig transcription (Merrell et al., 1997).

YY-1 binds to the μE1 site in the Eμ and 3'IgH enhancer (Hs1,2), and to the NF-E1 site in the 3'Eκ. YY-1 is a zinc finger protein that can be an activator, a repressor or an initiator of transcription. For instance, mutational analyses show that YY-1 is an activator in Eμ. It appears, however, to be a repressor in 3'Eκ. Like TFE3/USF family proteins, YY-1 is widely expressed in many lineages including B cells.

In summary, in vitro transcription assays have suggested that each of these transcription factors can influence Ig gene expression. Gene knockout experiments have implicated some of these transcription factors in the development and/or function of B lymphocytes. The connection between their effects on Ig genes and on B cell development and function, however, remains unclear.

V. An overview of this thesis

The goal of the work described in this thesis is to better understand the functional importance of the enhancers lying at the far 3' end of the murine IgH locus. To this end, we tested the effect of deleting one of these enhancers (Hs1,2) from the endogenous locus of an Ig-secreting cell line (**Chapter 2**, Lieberson et al., 1995). In the latter study, Hs1,2

was replaced with a reporter gene (*neo*^{r}) that might have itself perturbed IgH gene expression. As a result, we attempted (unsuccessfully) to generate a "clean" deletion of Hs1,2 (**Chapter 3**). We used a stable transfection system to establish "mini-loci" that placed an immunoglobulin heavy chain gene under the control of all four identified 3'IgH enhancers. These mini-loci were incorporated into the genome of both a surface-Ig positive cell line and an Ig-secreting cell line. We then used the Cre/*loxP* system as a means of deleting enhancer pairs from these loci to assess their contribution to IgH transcription. As described in **Chapter 4**, we found that enhancer function changed with developmental stage. As another approach to assessing 3' enhancer function, we asked whether any of these enhancers was associated with matrix attachment site or regions (MARs, **Chapter 5**). Finally, we generally discuss the results from the experiments in this thesis (**Chapter 6**).

CHAPTER 2

Hs1,2 Was Essential For IgH Chain Gene Transcription

(published results, Liberson et al., 1995)

CHAPTER 2

Hs1,2 Was Essential For IgH Chain Gene Transcription

The 3'IgH enhancers, Hs3a, Hs1,2, Hs3b, and Hs4, are believed to play an important role in IgH chain gene expression. For instance, in several B-lymphoid cell lines from pre-B cell types to plasmacytomas, Eμ has been lost due to a spontaneous deletion, but heavy chain synthesis is not affected and persists at a high level equivalent to their parental cell lines (Wabl and Burrows, 1984; Klein et al., 1984; Aguilera et al., 1985; Eckhardt and Birshtein, 1985; Zaller and Eckhardt, 1985). These results suggest that the 3'region is responsible for maintaining or activating IgH chain gene expression in the absence of Eμ. Another observation also showed importance of the 3'region of IgH locus in IgH chain gene expression. In a mouse myeloma variant, LP1.2, natural deletion of an approximately 34 kb DNA region including all the 3'IgH enhancers downstream of Cα gene segment dramatically reduced levels of Igα mRNA (reduction to 10%) compared to the wild-type cells (Gregor and Morrison, 1986; Michaelson et al., 1995). This suggests that the 3'region is required for high-level IgH chain gene transcription. Furthermore, chromosomal translocations between the IgH locus and *c-myc* leads to one *c-myc* allele becoming juxtaposed to the 3'IgH enhancer sequences in mouse plasmacytoma and human Burkitt's lymphoma cells (Cory, 1986; Magrath, 1990; Spencer and Groudine, 1991). In these tumor cells, the translocated c-myc gene was deregulated and was transcribed at high levels. Normal *c-myc* genes are expressed at low levels and transcription is cell cycle controlled. Deregulated expression of the c-myc oncogene, therefore, suggests that the 3'region of IgH is capable of activating *c-myc* gene transcription.

When MPC11 (an Igγ2b-producing cell line) underwent class switching from γ2b production to γ2a production, Eμ was deleted. In its daughter cell line, 9921, Eμ no longer existed, but IgH chain gene (γ2a) transcription was not affected upon deletion of Eμ. High-level IgH chain gene expression in the absence of Eμ in 9921 suggested that another enhancer(s) within the locus was regulating this gene's expression. As we had

recently identified an enhancer ~ 70 kb downstream of the γ2a gene in the cell line (Hs1,2), we designed an experiment to ask whether this enhancer was required for expression of this γ2b gene.

To target Hs1,2 in the natural IgH locus in 9921 cells, we generated a targeting construct (vector) named RVA, with DNA fragments subcloned from upstream and downstream regions of Hs1,2, PGK-*neo* (neomycin resistant gene, a drug marker) designed for positive selection, and HSV-*tk* (herpes simplex virus thymidine kinase) designed for negative selection that allows to eliminate random non-homologous recombinants. Both positive and negative selections in a targeting system (a targeting vector) can increase high efficiency of homologous recombination events. It has been shown that this high efficiency depends on not only the content of homology between targeting and targeted DNA sequences but also usage of both positive and negative selections in the targeting vector. HSV-*tk* was, however, found not to operate well in 9921 cells. While the drug gancyclovir should kill HSV-tk^+ cells, it didn't do so in HSV-tk^+ transformants of 9921.We had to abandon using the HSV-*tk* negative selection system, therefore, in the targeting vector.

We stably introduced the targeting vector, RVA, into 9921 cells and then fed cells with G418 (an analogue of neomycin) to select for G418-resistant clones. We screened 325 G418-resistant transformants by using genomic Southern blotting. Of them, one homologous recombinant was identified. Due to the fact that there are 3-4 copies of *c-myc* translocated chromosomes with the 3'region of IgH locus (the 3' region of IgH locus including the 3'enhancers is *cis*-linked to the *c-myc* oncogene locus) in 9921 cells, we had to determine whether this Hs1,2 deletion had occurred on the functional IgH locus or on one of the *c-myc* translocated chromosomes. Cell fusion of 9921 and CHO cells (Chinese hamster ovary cells) was used as a means to separate these chromosomes. After the chromosomes had been separated by cell fusion, we determined that Hs1,2 deletion had occurred on the functional Ig locus, not on a translocated *c-myc* chromosome.

γ2a mRNA in Hs1,2-deleted 9921 (homologous recombinant) cells was measured by northern blotting analysis. Compared to γ2a mRNA level produced by wild-type 9921 cells, γ2a mRNA was not detectable in the Hs1,2-deleted 9921 cells. This showed that Igγ2a transcription ceased upon deletion of Hs1,2 in the IgH locus in 9921. The result of this experiment suggested that Hs1,2 is essential for IgH chain gene transcription.

For more detailed information about this experiment, see published results (Lieberson, R., Ong, J., Shi, X. and Eckhardt, L.A. 1995. Immunoglobulin gene transcription ceases upon deletion of a distant enhancer. EMBO. J. 14:6229-6238).

CHAPTER 3

An attempt to delete Hs1,2 from a natural IgH locus, leading to a "null" mutation

INTRODUCTION

In the case of the Hs1,2 (formerly referred to as 3'α E) deletion in 9921 cells in our previous report (**Chapter 2**, Lieberson et al., 1995), the Hs1,2 deletion in mice (Cogne et al., 1994) and the Eμ deletion in mice (Chen et al., 1993), the targeted DNA sequences had been replaced with a selectable marker, which is inserted in the place of deleted genomic DNA sequences. It is possible that substitution of the respective enhancers with a marker gene (in our case it is *neo*^{r}) does not constitute a true " null " mutation. The laboratory of Dr. Mark Groudine has reported that insertion of a marker gene, either *neo*^{r} or *hygro*^{r}, within the locus control region (LCR) of the β-like globin gene locus can, by itself, inactivate expression of the β-like globin gene, and the subsequent deletion of the *neo*^{r} gene from the LCR can restore gene expression (Kim et al., 1992; Fiering et al., 1993). The importance of the "null" mutation can be argued by recent observations. It has been shown that some dramatic phenotypes initially observed upon replacement of a regulatory element with *neo*^{r} are eliminated following subsequent deletion of *neo*^{r} from the gene locus (Xu et al., 1996; Manis et al., 1998). These results suggest that the *neo*^{r} gene could, by itself, disrupt the gene expression when inserted into some regulatory elements of a gene. Although the *neo*^{r} gene sequence does not always have a negative effect on gene expression when it is inserted in place of deleted DNA sequence, we thought it important to measure the effect of a true "null" mutation on IgH gene expression. Ideally, Hs1,2 enhancer is replaced by PGK-*neo*^{r} gene and the *neo*^{r} gene is then subsequently deleted using CRE/*loxP* system, leading to a true "null" mutation. In the "null" mutation status, IgH gene transcription would be silenced if Hs1,2 is essential for IgH gene transcription. Alternatively, IgH gene transcription would be restored if Hs 1,2 is not essential for IgH expression, suggesting that other elements such as the 3' enhancers Hs3a, HS3, and HS4 contribute to IgH gene transcription independently or together with Hs1,2.

In this unsuccessful attempt, we tried to develop a system in which the Hs1,2 is replaced with a pGK-*neo-loxP* cassette using several gene targeting vectors . Once the Hs1,2 had been replaced, the *neo*^{r} would then be subsequently deleted in the place of the deleted enhancer to assess necessity of the Hs1,2 for IgH gene transcription in the "null" mutation. We screened 2017 wells (1-3 clones per well on the average) of *neo*^{r} stably transfected clones using genomic Southern blotting or PCR, but none of these transformants appeared to be homologous recombinants. 6-thioxanthine was, however, found to be useful for a negative selection in a positive-negative selection system, which is used to enrich efficiency of recovering homologous recombinants in the gene targeting experiments.

MATERIAL AND METHODS

Cell lines

9921 is an Igγ2a/κ-producing Ig class switch variant that arose spontaneously from the cell line 971. 971 is, in turn, isolated from mutagenized 45.6.2.4 cells. This lineage is described in Eckhardt and Birshtein (1985). 45.6.24 is a tissue culture-adapted subline of the IgG2b/κ-producing BALB/c mouse tumor MPC 11 (Laskov and Scharff, 1970). 9921 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, catalogue no. 12100-061, Life Technologies; Gaithersburg, MD) with 10% Bovine Calf Serum (BCS, catalogue no. SH30072.03, HyClone Laboratories, Logan, UT). A20 was maintained in RPMI 1640 medium (catalogue no. 31800-089, Life Technologies) with 10% BCS. All media contained 100U/ml penicillin/streptomycin (catalogue no. 15140-122, Life Technologies) and 2 mM L-glutamine (catalogue no. 21051-016, Life Technologies). All cells were maintained at 37°C in an atmosphere of 7 % CO_2.

Plasmid constructs

RVA-*loxPneo* (15.3 kb). RVAΔ*neo*, which consists of both 7 kb 5' and 1.3 kb 3' flanking sequences and the negative selection marker, HSV-*tk* (Lieberson et al., 1995), was linearized by SalI. A 2.0 kb NotI 2*loxPneo* fragment was inserted at the SalI site. The latter fragment contained a PKG-*neo* resistant gene and two *loxp* sites at the ends and

was isolated from the plasmid p*loxP2neo* (a gift from Dr. F.W. Alt, Center for Blood Research, Harvard Medical School, Boston, MA) (see Figure 3-1B.1).

4.4-RVA-*loxPneo* (19.7). RVAΔ*neo* was cut with EcoRI and a 4 kb EcoRI fragment isolated from pIgA3 that was kindly provided by Dr. Sherie Morrison (UCLA, Gregor and Morrison, 1986) was inserted upstream of the 7 kb 5' flanking sequence. The resulting construct, 4.4+RVAΔneo, was linearized with SalI and a 2.0 kb NotI 2*loxPneo* fragment described above was inserted. This construct was made by Adrienne Alaie-Petrillo, a graduate student in our lab (see Figure 3-1B.2).

PBS-SK-SV2*gpt* (5.8 kb). A 2.8 kb PvuII/EcoRI fragment containing the bacterial xanthine-guanine phosphoribosyl transferase gene (*xgpt*) under control of the eukaryotic SV40 promoter/enhancer was isolated from pSV2*gpt* (Mulligan and Berg, 1980&1981). This SV2*gpt* fragment was inserted into PBS-SK$^+$ (catalog no. 21120, Stratagene, La Jolla, CA) digested with both HincII and EcoRI.

PBS-SK-ES*gpt* (6.6 kb). A 3.6 kb PvuII/EcoRI fragment containing *xgpt* under control of the SV40 promoter and IgH intronic enhancer (Eμ) was isolated from pES*gpt* (Zaller et al., 1988). This ES*gpt* fragment was inserted into PBS-SK$^+$ (catalog no. 21120, Stratagene, La Jolla, CA) cut with both HincII and EcoRI.

pSV2*gpt*-RVA-*neo* (16.2 kb). PBS-SK-SV2*gpt* was linearized by SmaI and a 10.4kb SmaI/SmaI fragment was inserted. The latter fragment contained both 7 kb 5' and 1.3 kb 3' flanking sequences and the positive selection marker (*loxP-neo*) and was isolated from RVA-*loxPneo* (see Figure 3-1B.3).

pES*gpt*-RVA-*neo* (17.0 kb). PBS-SK-ES*gpt* was linearized by SmaI and the same 10.4kb SmaI/SmaI fragment described above was inserted (see Figure 3-1B.4).

Stable transfections of 9921 cells

The linearized plasmid DNAs were introduced into 9921 cells by electroporation. 20 μg of XhoI-linearized RVA-*loxPneo*, 4.4-RVA-*loxPneo*, pSV2*gpt*-RVA-*neo*, or pES*gpt*-RVA-*neo* were combined with a 1 ml suspension of 10^7 9921 cells and the mixture dispensed into a 0.4 cm (width) electroporation cuvette (Bio-Rad, Hercules, CA). An electric pulse was delivered at 960 μF and 250 V by a Bio-Rad Gene Pluser ™ electroporator and Capacitance Extender ™ (Bio-Rad, Hercules, CA). The cells were

then diluted in non-selective medium and plated at 500 cells/well in 96-well culture plates. After 48 hrs, medium supplemented with selective drugs was added. In some cases, 1.5mg/ml G418 (catalog no. 11811-031, GibcoBRL, Grand Island, NY) was included in the medium to select for *neo*^{r} transformants. 2μM 6-thio-xanthine (catalog no. T-8125, Sigma Chemical Company, St. Louis, MO) was added in some cases to select against cells carrying the *xgpt* gene. MHX was, however, used to select for *xgpt*-expressing transformants. MHX was composed of Mycophenolic Acid (catalog no. 11814-019, GiBcoBRL, Grand Island, NY), Hypoxanthine (catalog no. H-9636, Sigma Chemical Company), and Xanthine (catalog no. X-3627, Sigma Chemical Company). In MHX-containing medium, 6 μg/ml of Mycophenolic Acid, 15μg/ml of Hypoxanthine, and 250μg/ml of Xanthine were included. Colonies were visible ~ 2 weeks after transfection. In most experiments, transformants arose in 30-40% of the individual wells on each culture plate.

Southern blot analyses

Agarose gel electrophoresis, transfer to membrane and DNA hybridization were performed essentially as described previously (Radomska et al., 1994), with minor modifications. Briefly, ~ 25 μg restriction enzyme-digested DNA was loaded into each lane of a 0.7% agarose gel. Size-fractionated DNA was then transferred to nylon transfer membrane (catalogue no. N00HY00010, Micron Separations Inc, Westborough, MA). Blots were pre-hybridized and hybridized at 65°C in buffer containing 7.5X Denhardt's solution, 3X SSC, 100μg sonicated salmon sperm DNA and 0.5% SDS. Probes were labeled by the random primer method using a MegaPrime ™ labeling kit (catalog no. RPN 1605, Amersham, Arlington Heights, IL). To remove non-specially bound probe, blots were washed once in 1X SSC and 0.1% SDS at 65°C for 30 min or more, as necessary. The probe used was the 3' junction fragment, which is a 500 bp EcoRI/XbaI fragment derived from the 3' end of the 3.7 kb XbaI fragment that spans Hs1,2 (see **Figure 3-1A**) (Lieberson et al., 1995).

Oligonucleotides and PCR

Two oligonucleotides were used to identify homologous recombinants mediated by the targeting vectors. The forward primer, neo 818, anneals to the *neo*^{r} sequence. The reverse primer, 3'frva, anneals to the DNA sequence immediately downstream of 3' flanking DNA sequence in the targeting vectors. The sequences of the neo 818, and 3'frva are 5'-AAGACAGAGGAAGACGAAGGGAG-3', and 5'-TGGCTACCCGTGATATTGGGAA-3', respectively. 1~2 μg genomic DNA was amplified at 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM each dNTPs, 2.0U AmpliTaq (catalog no. N801-0060, Perkin Elmer, Foster City, CA) with each primer at a concentration of 0.25μM. Polymerase chain reaction (PCR) was in a GeneAmp 9600 (Perkin Elmer, Norwalk, CT), for 40 cycles consisting of 94°C for 1 min, 65°C for 2 min and 74°C for 5 min. There was a final extension at 74°C for 7 min. The PCR product was visualized by gel electrophoresis, and verified by Southern blot, using a probe, 1.4kb EcoRI/BamHI fragment containing *neo*^{r} gene isolated from plasmid p*loxP*2*neo* (see above).

Enzyme-linked immunosorbent assays (ELISA)

Microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 10μg /ml affinity-purified Fc fragment-specific rabbit anti-mouse IgG2a (catalog no. 315-005-008, Jackson ImmunoResearch Labs, West Grove, PA). Coated wells were then incubated with 50μl samples of cell lysates or culture supernatant. Cell lysates were prepared by lysis of 1x 10^6 cells in 50μl 0.5% Nonidet P-40 lysis buffer (Zaller and Eckhardt, 1985). γ2a heavy chains were assayed with alkaline phosphatase–conjugated rabbit anti-mouse IgG2b (catalog no. 315-005-008, Jackson ImmunoResearch Labs, West Grove, PA), using *p*-nitrophenol phosphate as the enzyme substrate (catalog no. 104-105, Sigma, St. Louis, MO). The absorbance at 405 nm was measured in an ELISA plate reader (Bio-Rad, Hercules, CA).

RESULTS

The first targeting vector we used was RVA-*loxPneo* (**Figure 3-1B.1**). RVA-*loxPneo* is a construct in which a 7 kb 5' flank DNA sequence is derived from the DNA sequence 5' of the Hs1,2 and a 1.4 kb 3' flank DNA sequence is derived from the DNA sequence 3' of the Hs1,2 in the IgH gene locus of 9921 cells. Between these two flanks is a 2.0 kb *neo*^{r} gene sequence flanked by two *loxP* sites. The *neo*^{r} gene driven by PGK (Phosphoglycerate Kinase) promoter is used to mark the cells. Outside the region of homology is an HSV-*tk* gene (herpes simplex virus thymidine kinase gene). HSV-*tk* gene is originally designed to allow negative selection in a positive-negative selection system so that random, non-homologous transformants are unable to divide. This selection exploits the fact that DNA replication can be blocked in cells that produce HSV-TK (herpes simplex virus thymidine kinase) through use of HSV-*tk* substrate analogues such as gancyclovir and FIAU. These analogues are not recognized by mammalian *tk* gene product. We found, however, that this negative selection didn't work well in 9921 cell line (the transformants retaining HSV-*tk* gene were not killed by negative selection with gancyclovir). This strategy of enriching for homologous recombination events, therefore, had to be abandoned in our experiments. XhoI-linearized RVA-*loxPneo* was stably introduced into 9921, an IgG2a-expressing cell line which lacks IgH intronic enhancer, Eμ. After selection by neomycin analogue, G418, we harvested 892 wells of the G418-resistant clones. 315 wells of the clones were screened for homologous recombinants by genomic Southern blot with probe 3' junction sequence (see **Figure 3-1A**). The rest of the wells of the clones were screened by PCR.

The RVA-*loxPneo*-mediated homologous recombinants can be identified by Southern blot. As diagramed in Figure 3-1, a homologous recombination event between RVA-*loxPneo* DNA sequences and their counterparts in 9921 cell genome should result in a novel 3.7 kb BamHI fragment that would hybridize with a 500 bp 3' junction probe. In addition to the novel BamHI associated with the homologous recombination event, a 6.9 kb BamHI fragment, which is from non-targeted copy of the IgH chromosome, is also detected by the probe. However, no homologous recombinants were identified in the first 315 wells of clones. **Figure 3-2A** showed example data from this genomic Southern analysis. As we expected, a 3.7 kb BamHI fragment (gene-targeted) and a 6.9 kb BamHI

fragment (non-targeted) were detected by the 3'junction probe in the clone B48. B48 (a positive control) is an Hs1,2-deleted 9921 cell line in our previous experiment (see **Chapter 2**, Lieberson et al., 1995). No 3.7 kb gene targeted band was, however, detected in *neo*^{r} resistant transformants (e.g. clones A41 through A47, **Figure 3-2A**). In PCR screening, B48, as described above, was used as a positive control for PCR (a 2.3 kb PCR product was obtained by using neo 818, a forward primer, and 3'frva, a reverse primer). Eight samples of positive control DNA isolated from B48, were used to verify PCR product each time. PCR products were visualized by electrophoretic analysis, and confirmed by the Southern blot. No homologous recombinants were, however, identified by PCR in the clones recovered from 892 wells of *neo*^{r} resistant transformants. **Figure 3-2B** shows example data from PCR screening. As we expected, a 2.3 kb PCR product was visualized by gel electrophoresis in B48. No targeted PCR product was, however, detected in 9921 (the negative control) nor in any of the homologous recombinant candidate clones (e.g. H68 through H76, **Figure 3-2B**). The 2.3 kb gene-targeted PCR product derived from B48 was confirmed by Southern blot with 1.4 kb *neo*^{r} probe (data not shown, see Material and Methods).

It has been shown that efficiency of homologous recombination is correlated to the content of homology between targeting vector and targeted DNA sequences (Smith and Kalogerakis, 1990; Kumar and Simons, 1993; Scheerer and Adair, 1994). To increase efficiency of homologous recombination events, a 4.4 kb DNA fragment located immediately downstream of the Cα gene was inserted upstream of the 7 kb 5'flanking DNA sequence of RVA-*loxPneo*, leading to generation of a construct, 4.4-RVA-*loxPneo* (see **Figure 3-1B.2**). Like RVA-*loxPneo*, XhoI-linearized 4.4-RVA-*loxPneo* was stably introduced into 9921 cells. We recovered 374 wells of G418-resistant clones. The clones were screened for homologous recombinants by PCR. Once again, no homologous recombinants were identified among these clones.

Gancyclovir and FIAU (pyrimidine derivative) are nucleotide analogues recognized by herpes simplex virus thymidine kinase. They are popularly used to eliminate random, non-homologous recombinants that retain the HSV-*tk* gene, leading to enrichment of

homologous recombinants in a *neo-tk* positive-negative selective system. Similarly, 6-thio-xanthine is a nucleotide analogue recognized by the bacterial XGPT (xanthine-guanine phosphoribosyl transferase) and can be used to select against cells expressing the *xgpt* gene. XGPT encoded by the *xgpt* gene incorporates 6-thio-xanthine into DNA when DNA is replicated during cell division. The cells that have the *xgpt* gene integrated into their genome, therefore, take up the 6-thio-xanthine, leading to a block in cell division and eventual cell death. Those that are homologous recombinants (the *xgpt* gene is not integrated into the genome of cells), however, won't take up this nucleotide analogue and should not be eliminated when in medium containing 6-thio-xanthine. No published studies have exploited this negative selection system in gene targeting studies. In an attempt to enrich for homologous recombination events via negative selection mediated by 6-thio-xanthine, we replaced the HSV-*tk* gene with either the SV2*gpt* gene (driven by SV40 promoter) or ES*gpt* gene (driven by both SV40 promoter and Eμ), resulting in generation of two additional targeting constructs, pSV2*gpt*-RVA-*neo* and pES*gpt*-RVA-*neo* (see **Figure 3-1B3&4**). Both XhoI-linearized pSV2*gpt*-RVA-*neo* and pES*gpt*-RVA-*neo* were independently introduced into 6-thio-xanthine-resistant 9921 cells by stable transfection. After selection by medium supplemented with both G418 (1.5mg/ml) and 6-thio-xanthine (2μM), we had 369 wells of pSV2*gpt*-RVA-*neo* transfected clones and 382 wells of pES*gpt*-RVA-*neo* transfected clones. Both types of clones were screened by PCR. No homologous recombinants were, however, identified in these 751 wells of clones.

However, as we expected, 6-thio-xanthine was found to be useful for negative selection. We analyzed 109 clones that had been selected for G418 only (56 clones come from the pES*gpt*-RVA-*neo* transformants, and 53 clones from the pSV2*gpt*-RVA-*neo* transformants), and found that when these clones were placed in 6-thio-xanthine, 73 clones died, and 36 clones survived (20 clones came from pES*gpt*-RVA-*neo* transformants and 16 clones from pSV2*gpt*-RVA-*neo* transformants). We then tested whether the surviving clones had, in fact, lost XGPT activity. Medium supplemented with MHX (Mycophenolic Acid, Hypoxanthine, and Xanthine) was used to select for clones that express the *xgpt* gene. As we expected, almost all of the 36 clones that were

6-thio-xanthine-resistant, as described above, died in MHX medium (only one clone survived). Furthermore, we tested 20 MHX-resistant clones (13 pES*gpt*-RVA-*neo* and 7 pSV2*gpt*-RVA-*neo* clones), and found only one of them survived in 6-thio-xanthine-containing medium. These results suggest that 6-thio-xanthine is able to eliminate the clones that express the *xgpt* gene.

We assumed that, prior to *neo*^{r}-deletion, Hs1,2-deleted homologous recombinants-mediated by the targeting vectors used in this study (including RVA-*loxPneo*, 4.4-RVA-*loxPneo*, pSV2*gpt*-RVA-*neo*, and pES*gpt*-RVA-*neo*) would exhibit the same phenotype as the Hs1,2-deleted 9921 cell line that was generated in our previous study (see Chapter 2, Lieberson et al., 1995). That is, the Igγ2a gene would be transcriptionally silenced upon replacement of Hs1,2 by *neo*^{r} . Igγ2a gene silence in cells can be directly determined by ELISA. We assessed all of the 2017 wells of clones by ELISA assays, therefore, using their cell supernatant as an additional screen for possible homologous recombinants. All the clones except for one produced IgG2a. Further genomic Southern blot analysis, however, showed that this clone was not a homologous recombinant. It could be a natural mutant instead.

DISCUSSION

The homologous recombination technique for gene targeting has been used to delete (knockout) a DNA sequence in the Ig gene loci (Cogne et al., 1993; Gu et al., 1993; Serwe and Sablitzky, 1993; Chen et al., 1993; Lieberson et al., 1995; Xu et al., 1996; Gorman et al., 1996; Manis et al., 1998). It provides researchers a means of introducing a well-defined, predetermined change in chromosomal genes. When the linearized targeting vectors, which bear regions of homology to the targeted sequences of interest, are introduced into 9921 cells, they could anneal with 9921 cell genome at the IgH gene locus and, following a double crossover event taking place between the identical parts of both targeting vector DNA sequences and their endogenous counterparts, Hs1,2 and its flanking region could be replaced by a *neo*^{r} gene, leading to generation of homologous recombinants. Usually, the *neo*^{r} gene serves to mark cells that have taken up the replacement vector. Cell clones that successfully incorporate this vector into their

genome are resistant to the neomycin analogue, G418. The most difficult part in this approach is that the frequency of the homologous recombination events is rather low, particularly in mammalian cells, compared to that of random, non-homologous recombination events.

We used different approaches or strategies including increasing the content of homology between targeting vectors and target DNA sequences, and providing negative selection against random, non-homologous recombinants to delete one of the 3'IgH enhancer, Hs1,2. Negative selection with the HSV-*tk* gene did not work in 9921 cells. As a result, we tried an alternate negative selection scheme involving the *xgpt*. Our data showed that 6-thio-xanthine was capable of eliminating 9921 cells that expressed the *xgpt* gene (19/20 clones). This would enrich for homologous recombinants. We isolated 751 wells containing clones that were both G418- and 6-thioxanthine-resistant. These clones no longer expressed *xgpt* gene, but they were not homologous recombinants. We did not directly analyze these clones to confirm that they retained the *xgpt* gene. We think it unlikely, however, that all of these clones had lost the *xgpt* gene but retained the *neo*^{r} gene. It is possible that the *xgpt* transgene in these clones was not transcribed because of "position effect variegation". Indeed, not all stable transformants are able to express their transgenes since the transgenes are influenced by chromatin structure surrounding them at integration sites, unless they are under control of an LCR (locus control region). It is possible, therefore, that not all the G418-resistant clones expressed the *xgpt* transgene. Naturally, G418-resistant clones that did not express the *xgpt* transgene would not be killed by negative selection with 6-thioxanthine. Similarly, not all gancyclovir resistant clones are homologous recombinants in other gene targeting studies using HSV-*tk* (Mombaerts et al., 1991; Gu et al., 1993; Serwe and Sablitzky, 1993). For instance, only two clones appeared to be homologous recombinants among 249 *neo*^{r}- and gancyclovir-resistant clones in a gene targeting study involving the T cell antigen receptor β subunit locus in mouse embryonic stem cells (Mombaerts et al., 1991).

We screened more than 2000 wells containing G418-resistant clones, but no homologous recombinants were identified. One of the possible reasons for this

unsuccessful attempt could be that the 3' regulatory region of the IgH locus was hard for the targeting vectors to access. It has been shown that frequencies of homologous versus non-homologous recombination range from 1/100,000 (Miller and Temin, 1983; Lin et al., 1985) to 1/1000 (Smithies et al., 1985; Thomas and Capecchi, 1987), and even as high as 1/100 (Gu et al., 1993; Serwe and Sablitzky, 1993) and 1/10 (Doetschman et al., 1987). These observations indicate that efficiency of homologous recombination events varies from locus-to-locus and there are cell type differences as well. It has been reported that gene targeting in embryonic stem cells is significantly more efficient than in other cell types (Arbones et al., 1994). It is, therefore, perhaps not surprising that there is a quite low frequency of homologous recombination events in the 9921cell line.

CHAPTER 4

Activity of 3'IgH enhancers changes with B cell developmental stage

INTRODUCTION

The first transcriptional enhancer identified within the IgH locus mapped to an intronic region between the J_H (joining) gene segments and Cμ, the first in a tandem array of C_H (heavy chain constant region) coding segments (Banerji et al., 1983; Gillies et al., 1983; Neuberger, 1983). While initially identified by virtue of its ability to augment transcription from IgH promoters, this enhancer, Eμ, was later implicated in regulation of IgH variable region gene assembly (VDJ joining), as well (Chen et al., 1993; Serwe and Sablitzky, 1993). Early observations that Ig secreting cell lines occasionally lacked Eμ but nevertheless expressed the affected IgH allele at high levels led to the suggestion that there were additional transcriptional control elements within the IgH locus (Aguilera et al., 1985; Eckhardt and Birshtein, 1985; Klein et al., 1984; Wabl and Burrows, 1984; Zaller and Eckhardt, 1985). IgH chromosome translocations with the chromosome carrying the oncogene *c-myc* supported this hypothesis since the latter oncogene was expressed in a B-lymphocyte-specific manner after translocation and yet often lay on the reciprocal translocation product from Eμ (reviewed in Cory, 1986; Greenberg et al., 1989).

Several years ago, we and others identified a second enhancer region 3' of Cα, the last in the tandem array of C_H genes (**Figure 4-1**) (Dariavach et al., 1991; Lieberson et al., 1991). Discovery of this enhancer, Hs1,2, was rapidly followed by the discovery of other enhancer regions 3' of the IgH locus so that today, there are 3 other regions identified in the mouse locus (Hs3a, Hs3b, and Hs4) (reviewed in Arulampalam et al., 1997). Hs3a and Hs3b are 97% identical in sequence (Chauveau and Cogne, 1996; Saleque et al., 1997). There are homologues to the hs3 elements, to Hs4, and to Hs1,2 in the human IgH loci, with a set of these enhancers (hs3, Hs1,2, and Hs4) lying downstream of each of the two human Cα genes (Chen and Birshtein, 1997; Mills et al., 1997). Conservation of these control elements, along with the functional implications of the observations

described above, attests to their probable importance to IgH gene expression.

We and another group attempted an analysis of the function of Hs1,2 by enhancer knock-out studies in a cell line and in mice, respectively (Cogne et al., 1994; Lieberson et al., 1995). In both studies, Hs1,2 was replaced by a marker gene encoding neomycin resistance (*neo*^{r}). In the cell line study, the contribution made by Hs1,2 to the activity of an IgH promoter lying over 70kb away was analyzed. Eμ was already missing from the manipulated IgH locus so that the function of Hs1,2 could be studied in the absence of possible compensatory effects mediated by Eμ. In the mouse studies, Eμ remained within the manipulated locus. In both studies, however, a pronounced effect was seen upon replacement of Hs1,2 with *neo*^{r}: a complete loss of IgH promoter activity in the cell line and a striking effect on heavy chain class switching in mice. Subsequent knock-out studies in mice, however, demonstrated that the effect on class-switching was not the result of Hs1,2 deletion but, rather, was due to insertion of the *neo*^{r} gene into this region of the IgH locus (Manis et al., 1998). Similarly, replacement of Hs3a with *neo*^{r} had an effect on class-switching that virtually disappeared when the *neo*^{r} gene was subsequently deleted from the locus (Manis et al., 1998). Interestingly, simple insertion of *neo*^{r} at sites between particular C_H genes and the 3' IgH enhancers resulted in inhibition of class-switching to the C_H genes (Seidl et al., 1999). These findings suggest that the *neo*^{r} gene somehow disrupts necessary communication between the enhancers and the constant region genes during the process of heavy chain class switching. It is possible, but has not yet been directly demonstrated, that the pronounced effect of Hs1,2 deletion/*neo*^{r} insertion on IgH promoter activity in the Ig-secreting cell line (where Eμ was absent) was similarly due to a *neo*^{r} - mediated disruption in communication between the remaining 3' enhancers and the affected IgH promoter.

In the present study, we have taken an alternate approach to analyzing 3' IgH enhancer activity within the context of chromatin. Because both we and others have found the 3' region of the endogenous IgH locus unusually difficult to target (efficiencies are 1/100 to 1/1000 that achievable in the Eμ region, for example), we have used, instead, IgH "mini-loci" that lack Eμ as our targets for 3' IgH enhancer deletions. The design of these

experiments arose from the earlier finding that three of the murine 3' IgH enhancers (Hs1,2/Hs3b/Hs4), when linked as a unit to the *c-myc* oncogene, behaved much like a locus control region (LCR), ensuring that the resultant *c-myc* transgene was expressed at high levels and in a copy number-dependent fashion in a transfected cell line, regardless of chromosomal position (Madisen and Groudine, 1994). More recent studies in mice, using an IgH promoter/β globin gene linked to the full complement of murine 3'IgH enhancers (Hs3a/Hs1,2/Hs3b/Hs4), again revealed an LCR-like activity for these enhancers with position-independent (but not copy number-dependent) expression of the transgene (Chauveau et al., 1999). We reasoned that since the LCR-like activity of the 3'IgH enhancers allowed an associated transcription unit to behave much like the endogenous IgH locus, we could use just such a "mini-locus" to study the relative contributions of the individual 3' IgH enhancers to IgH transcription. We introduced transgenes consisting of an IgH transcription unit linked to the 3'IgH enhancers (Hs3a/Hs1,2/Hs3b/Hs4= "hs1-4") into both a plasmacytoma (Ig-secreting cell line) and a surface Ig^+ cell line. After stable expression of the integrated transgenes had been established, we then deleted either Hs1,2/Hs3a or Hs3b/Hs4 from the transgenes of individual transformants and measured the effect on IgH expression levels. We used B lymphoid cell lines representing two different functional stages since our earlier transient transfection studies (where reporters were not integrated into chromosomes) had revealed a pronounced difference in the behavior of the 3' IgH enhancers at these two stages (Ong et al., 1998).

As described below, we found that the transgenes were expressed in a site-independent but not a copy-number dependent fashion, in agreement with the recent transgenic mouse experiments (Chauveau et al., 1999). Levels of expression were not noticeably affected by the relative positions of the four 3'IgH enhancer regions within the IgH reporter genes. Working with several independent, single-copy transformants of each cell line, we found that deletion of Hs3b/Hs4 from the transgene had a pronounced effect on IgH mRNA levels in the Ig-secreting cell line but not in the surface Ig^+ cell line, suggesting a shift in enhancer activity as B lymphocytes differentiate into Ig-secreting plasmacytes.

MATERIAL AND METHODS

Cell lines

9921 is an IgG2a-producing class-switch variant that was derived, through an intermediate, from the IgG2b-producing plasmacytoma MPC11 (Eckhardt and Birshtein, 1985). In the course of the heavy chain class switch, Eμ was deleted from the γ2a heavy chain transcription unit in 9921. MPC11 used in these studies is a tissue culture adapted subline of the BALB/c mouse tumor MPC11 and is formally designated 45.6.2.4. (Laskov and Scharff, 1970). **A20** is a surface IgG-positive cell line also derived from the BALB/c mouse and was obtained from the American Type Culture Collection (TIB-208Kim et al., 1979).

9921 and MPC11 were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies; Gaithersburg, MD; catalogue no. 12100-061) with 10% Bovine Calf Serum (BCS; HyClone Laboratories, Logan, UT; catalogue no. SH30072.03). A20 was maintained in RPMI 1640 medium (Life Technologies; catalogue no. 31800-089) with 10% BCS. All media contained 100U/ml penicillin/streptomycin (Life Technologies; catalogue no. 15140-122) and 2 mM L-glutamine (Life Technologies; catalogue no. 21051-016). All cells were maintained at 37°C in an atmosphere of 7% CO_2.

Plasmid Constructs

pBS185 is a *cre* recombinase gene expression vector (GibcoBRL, Grand Island, NY, catalog no. 10347-011) in which *cre* is expressed under control of the cytomegalovirus (CMV) promoter. Cre recombinase mediates *loxP* site-specific DNA recombination.

pEGFP-C1 encodes a red-shifted variant of green fluorescence protein (catalog no. 6084-1, Clontech, Palo Alto, CA). It was co-transfected with pBS185 in order to isolate cells that had taken up DNA as made evident by their fluorescence.

The following enhancer fragments and plasmids were used in the construction of the IgH mini-loci diagramed in **Figure 4-2**:

Hs1,2: a 3.6 kb XbaI/HindIII fragment isolated from lambda phage clone M2 containing

BALB/c genomic DNA (Dariavach et al., 1991).

Hs3a: a 1.1 kb XbaI fragment, also isolated from lambda phage clone M2.

Hs3b,4: a 2.6 kb NotI/HindIII fragment from plasmid pHS3.4 which contains a fusion of Hs3b and Hs4 (Madisen and Groudine, 1994). Hs3b and Hs4 DNA were originally isolated from the 129 mouse strain.

V297: a 2.2 kb XbaI fragment containing the MPC11 (and 9921) IgH variable region, isolated from plasmid p297γ2b (Zaller and Eckhardt, 1985).

Cγ2b: a 5.2 kb XbaI genomic DNA fragment containing BALB/c mouse Cγ2b isolated from plasmid pγ2b-R1.4 (Zaller and Eckhardt, 1985).

psk-2*loxp*neo (5.0 kb). PBS-SK$^+$ (catalog no. 21120, Stratagene, La Jolla, CA) was cut by both SpeI and HindIII, and was then religated. The resulting plasmid was linearized by NotI and then inserted by a 2.0 kb NotI 2*lox*pneo fragment, which contains a PKG-neo resistant gene and two *loxp* sites at the ends and was isolated from the plasmid p*loxp*2neo (a gift from Dr. F.W. Alt, the Children's Hospital., the Center for Blood Research, Harvard Medical School, Boston, MA).

psk-Hs123a (7.7 kb). PBS-SK$^+$ (catalog no. 21120, Stratagene, La Jolla, CA) was cut by both XbaI and HindIII, and was then inserted by both Hs3a and Hs1,2 fragments.

pSH3.4(5.6 kb). It was provided by Dr. Mark Groudine (see above). PBS-KSII was inserted by 1.2kb XbaI/SacI Hs3b and 1.4 kb PstI/ HindIII Hs4 fragments (Madisen and Groudine, 1994).

psk-2*loxp*Hs123a(8.3 kb). psk-2*loxp*neo was cut by both BamHI and EcoRI removing a 1.4 kb neo^r gene, and was then flushed and inserted by a 4.7 kb NotI/ HindIII fragment containing a fusion of Hs3a and Hs1,2, which was isolated from psk-Hs123a.

psk-2loxpHs3b4(6.1 kb). psk-2loxpneo was cut by both BamHI and EcoRI removing the neo^r gene, and was then flushed and inserted by a 2.6 kb NotI/ HindIII fragment containing a fusion of Hs34 and Hs4 isolated form pHS3.4.

psk-γ2b (psk-v_{297}γ2b, 10.5 kb). PBS-SK$^+$ (catalogue no. 21120, Stratagene, La Jolla, CA) was linearized by XbaI, and was then inserted by both V_{297} and γ2b fragments. The right orientation of psk-γ2b was confirmed with BamHI.

The resulting mini-loci carried either Hs3a/Hs1,2 flanked by loxP sites (**γ2b-hs1-4*loxP*Hs123a**) or Hs3b4 flanked by loxP sites (**γ2b-hs1-4*loxP*Hs3b4**).

γ2b-hs1-4*loxP*Hs123a (pksγ2bHs3b4loxp123a, 18.3 kb): pSH3.4 was linearized by XhoI, and then inserted by a 7.5 kb NotI/SpeI fragment containing both V_{297} and γ2b fragments, which is isolated from psk-V_{297}γ2b, by blunt-end ligation. The resulting plasmid, pHS3.4-V_{297}γ2b, was linearized by NotI, and then inserted by a 5.2 kb NotI fragment containing a fusion of Hs3a and Hs1,2 flanked by two *loxp* sites at the ends, which is isolated from psk-2*loxp*Hs123a. Hs3a/Hs1,2 was located upstream of Hs3b/Hs4. The order of enhancers was confirmed by HindIII.

γ2b-hs1-4*loxP*Hs3b4 (pskγ2bHs123aloxp3b4, 18.3 kb): psk-Hs123a was linearized by XhoI , and then inserted by a 7.5 kb NotI/SpeI fragment containing both V_{297} and γ2b fragments which is isolated from psk-V_{297}γ2b, by blunt-end ligation. The resulting plasmid, psk-Hs3a-3'αE-V_{297}γ2b, was linearized by NotI, and then inserted by a 3.1 kb NotI fragment containing a fusion of Hs3b and Hs4 flanked by two *loxp* sites at the ends, which is isolated from psk-2*loxp*Hs3b4. The Hs3b/Hs4 is located upstream of Hs3a/Hs1,2. The order of enhancers was confirmed by HindIII.

Stable transformations:

Linearized plasmid DNAs were introduced into both 9921 and A20 cells by electroporation. 20 μg of PvuI-linearized γ2b-hs1-4*loxP*Hs123a, PvuI-linearized γ2b-hs1-4*loxP*Hs3b4, or NotI-linearized, enhancerless psk-γ2b were introduced into 9921 and A20 cells along with XhoI-linearized psk-2loxPneo (molar ratio of the γ2b constructs: *neo*^{r} drug selection plasmid was 1:1). DNAs were combined with a 1 ml suspension of 10^7 9921 or A20 cells and the mixture was dispensed into a 0.4 cm (width) electroporation cuvette (Bio-Rad, Hercules, CA). An electric pulse was delivered at 960 μF and 250V by a Bio-Rad Gene Pulser™ electroporator and Capacitance Extender™ (Bio-Rad, Hercules, CA). The cells were then diluted in non-selective medium and plated at 500 cells/well in 96-well culture plates. After 48 hours, medium supplemented with 1.5 mg/ml G418 (GibcoBRL, Grand Island, NY) was added to the cultures to select for stable transformants expressing the *neo*^{r} gene. Colonies were visible ~ 2 weeks after

transfection. In most experiments, transformants arose in 30-40% of the individual wells on each culture plate.

Transient transfections and cell sorting:

30 μg pBS185 (*cre*-expressing plasmid) and 2.5 μg pEGFP-C1 (green fluorescence protein-expressing plasmid) were simultaneously introduced, by electroporation, into 9921 and A20 clones that carried a single copy of one of the γ2b mini-loci. DNAs were combined with a 1 ml suspension of $5x10^6$ 9921 or A20 cells and the mixture dispensed into a 0.4 cm (width) electroporation cuvette (Bio-Rad, Hercules, CA). An electric pulse was delivered at 960 μF and 290V by the same electroporator and capacitance extender described above. The cells were then incubated at 37°C in an atmosphere of 7 % CO2. Forty-eight hours after transfection, the cells were centrifuged and cell pellets resuspended in 1ml staining buffer (1x PBS, 0.1% glucose and 1mg/ml bovine serum albumin). Fluorescent cells (expressing the EGFP gene) were identified and bulk sorted with a FACS Vantage™ (Becton Dickinson, San Jose, CA). The sorted cells were then manually subcloned into 96-well plates (25 cells plated in a 96-well culture plate). Clones were visible in 20-30 individual wells/plate ~ 3 weeks after sorting.

Southern blot analyses:

Agarose gel electrophoresis, transfers to membranes, and DNA hybridizations were performed essentially as described previously, with minor modifications (Radomska et al., 1994). Briefly, ~25 μg restriction enzyme-digested DNA were loaded into each lane of an 0.7% agarose gel. DNA, size-fractionated by gel electrophoresis, was transferred to a nylon membrane (Micron Separations Inc, Westborough, MA, catalogue no. N00HY00010). Blots were pre-hybridized and hybridized at 65°C in buffer containing 7.5X Denhardt's solution, 3X SSC, 100g sonicated salmon sperm DNA and 0.5% SDS. Probes were labeled by the random primer method using a MegaPrime™ labeling kit (Amersham, Arlington Heights, IL, catalogue no. RPN1605). To remove non-specifically bound probe, blots were washed in 1X SSC and 0.1% SDS at 65°C for 30 minutes to 2 hours, as needed.

Probes included pJ11HE and Hs3b (probes A and B, respectively, **Figure 4-2**). pJ11HE is a 1.5kb HindIII/EcoRI fragment isolated from pJ11, a plasmid containing the J_H gene region of BALB/cJ liver DNA (Marcu et al., 1980). Hs3b is a 1.2 kb XbaI fragment isolated from plasmid pHS3.4 (Madisen and Groudine, 1994).

Northern blot analyses:

Total cellular RNA was isolated by Trizol reagent (catalogue no. 15596-026, GibcoBRL, Grand Island, NY) according to the manufacturer's instructions. Approximately 25 μg total RNA was analyzed/sample. RNA was denatured with formamide and sized-fractionated by electrophoresis through 1% formaldehyde-agarose gels. The RNA was transferred to nylon (as for genomic Southerns, see above). Blots were pre-hybridized and hybridized to ^{32}P-labeled DNA probes at 37°C for 24 hrs in a buffer solution of 50% formamide, 2.5X Denhardt's solution, 5X SSC, 0.1% SDS, 50 mM NaPO4 (PH 7.4), 50μg/ml poly[A], and 60μg/ml sonicated salmon sperm DNA. To remove non-specifically bound probe, blots were washed in 1X SSC, 0.1% SDS at 45°C for 30 minutes to 2 hours, as needed.

γ2b mRNA levels were assessed with a 0.3 kb SacI fragment containing the CH3 domain of Cγ0b (Tilley and Birshtein, 1985). This probe does not cross-hybridize to γ2a transcripts. γ2a transcripts were detected with an analogous probe consisting of the CH3 domain of Cγ2a (Tilley and Birshtein, 1985). To verify the integrity of RNA samples and to compare RNA levels, blots were stripped and rehybridized to a β-actin probe (Ambion, Austin, TX, catalogue no. 7323) or a GAPDH probe (Ambion, Austin, TX, catalogue no. 7330).

Enzyme-linked immunosorbent assays (ELISA)

Microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 2 μg /ml purified rat anti-mouse γ2b (Pharmingen, San Diego, CA, catalog no. 02041D). Coated wells were then incubated with 50μl cell lysate which was prepared by lysis of 10^6 cells in 50μl 0.5% Nonidet P-40 lysis buffer (Zaller and Eckhardt, 1985). Mouse γ2b heavy

chains were then detected with alkaline phosphatase -conjugated rabbit anti-mouse γ2b (catalog no. 61-0322, ZYMED, S. San Francisco, CA), using ρ-nitrophenol phosphate as the enzyme substrate (Sigma, St. Louis, MO, catalog no.104-105). Absorbance at 405 nm was measured in an ELISA plate reader (Bio-Rad, Hercules, CA).

RESULTS

The murine 3'IgH enhancers mediate position-independent but not copy number-dependent expression of an IgH reporter gene in Ig-secreting and surface-Ig$^+$ B lineage cells

To test the ability of the 3'IgH enhancers to activate IgH expression regardless of chromosomal context, we compared expression levels of a γ2b reporter gene lacking enhancers with two reporters carrying different configurations of the 3'IgH enhancers. The reporter genes carrying enhancers are diagramed in **Figure 4-2** (γ2b-hs1-4*loxP*Hs123a and γ2b-hs1-4*loxP*Hs3b4). The enhancerless γ2b reporter was an enhancerless version of γ2b-hs1-4*loxP*Hs3b4.

Each linearized plasmid construct was co-transfected with a loxP*neo*^{r} marker gene into 9921 (an IgG2a-secreting cell line) and A20 (surface IgG-positive cell line) cells, and, immediately following transfection, the cells were dispensed into 96-well plates. G418-resistant clones were recovered in 30-40% of the wells in each plate so that each growing well represented, on average, a single transformation event (Poisson statistic). Isolated clones were tested by genomic Southern for uptake of the γ2b reporter gene (data not shown) Clones carrying a reporter gene were then analyzed by Elisa for expression of the γ2b transgene. As summarized in **Table 4-1**, >96% of clones carrying either of the two γ2b-hs1-4 reporters expressed the transfected γ2b gene. More specifically, of 118 individual clones recovered, only three did not express the transgene. The latter three might have taken up a damaged transcription unit although this was not tested directly. In contrast, when clones carrying the γ2b transgene without enhancers were analyzed, none of 25 clones recovered expressed γ2b. Addition of the 3'IgH enhancers, therefore, ensured γ2b expression in as many as 115 out of 118 chromosomal positions represented

by these independent transformants.

Southern blot analyses using probe A and HindIII digestion of genomic DNA (**Figure 4-2**) allowed us to determine the number of copies of the transgenes integrated into the genome of each transformant. Note that with this probe and enzyme, each transformant should have a novel HindIII fragment due to random integration into the genome. The probe also detects HindIII fragments derived from the endogenous IgH loci of 9921 and A20 (one fragment per genome for 9921; two fragments per genome for A20; data not shown). Using this information, along with densitometry measurements of the autoradiographs from Southern blots, we assigned copy numbers for the transformants. **Figure 4-3** shows a northern blot of mRNA isolated from representative clones carrying different transgene copy numbers. γ2b mRNA levels were determined with a γ2b-specific probe (CH3-γ2b; see Materials and Methods) and RNA amounts loaded in each lane were normalized in reference to β-actin mRNA levels. As is evident from the northern blot and from a graph of these data (**Figure 4-4**), there is a trend upward in gene expression with copy number, but there is clearly not a strict correlation between copy number and expression level (e.g. expression of 5 copies in 5-copy transformants is not 5 times that of a single copy in single-copy transformants). Moreover, when we directly analyzed single-copy transformants by using Northern bloting (see **Figures 4-5&4-6**), there was considerable variation in expression levels (up to 4 or 5-fold differences among clones; **Table 4-2**). While the γ2b-hs1-4 transgenes could generally ensure expression at any chromosomal site, the chromosomal site still had an effect on the overall level of transgene expression. The 3'IgH enhancers, therefore, contain a "partial" LCR activity.

It should also be noted from **Table 4-2** that γ2b transgene expression was always lower than that of a γ2b gene in its natural chromosomal position (**Table 4-2** provides transgene expression levels relative to the endogenous γ2b gene of the MPC11 plasmacytoma, see legend). To investigate the possibility that negative feedback regulation was taking place between the endogenously-encoded γ2a gene of 9921 and the γ2b-hs1-4 transgenes, we compared γ2a expression among six 9921 clones that did not carry a γ2b-hs1-4 transgene and eight 9921 clones that did. When the eight 9921-γ2b-

hs1-4 clones were directly compared to a single clone of 9921, the 9921-γ2b-hs1-4 clones produced close to the same amount of endogenous γ2a as the untransfected 9921 clone (average of 87%). In addition, there was no inverse relationship between γ2b and γ2a mRNA levels in the 9921-γ2b-hs-4 clones (data not shown). The co-efficient of variation across these nine cell lines was 23%. This was less than the co-efficient of variation (36%) for six independent 9921 clones lacking the γ2b-hs1-4 transgene. There was no evidence, therefore, that expression of γ2b was negatively influencing expression of endogenously-encoded γ2a, arguing against negative feed-back as an explanation for low transgene expression. The more likely explanation is that the γ2b-hs1-4 transgenes lack some of the control elements and/or the appropriate configuration necessary for establishing wild-type levels of gene expression in novel locations. In this respect, the 3'IgH enhancers again are best described as having only "partial" LCR activity.

Transgene expression is unaffected by enhancer order

The natural order of the known elements within the 3'IgH enhancer region is Hs3a, Hs1,2, Hs3b, Hs4 (5' to 3' relative to IgH gene transcription). The first three of these enhancers lie within a large palindromic region (~25kb) within the mouse chromosome, with Hs1,2 at the axis of symmetry. Hs3a and Hs3b are 97% homologous but are inverted relative to one another (Chauveau and Cogne, 1996; Saleque et al., 1997). In the mini-loci prepared for our experiments, Hs3a and Hs3b flank Hs1,2 in one construct (γ2b-hs1-4*loxP*Hs123a) as in the endogenous locus. In this construct, however, the homologous sequences making up Hs3a and Hs3b are not inverted with relation to one another. In the other construct (γ2b-hs1-4*loxP*Hs3b4), Hs3a and Hs3b flank Hs4 (again, Hs3a and Hs3b constitute tandem, rather than inverted, repeats). We compared the expression levels of these two differently-configured transgenes when stably integrated into the genomes of transfected cell lines.

As shown in **Table 4-2**, single-copy transformants expressed either transgene at similar levels, ranging from 10% to 50% endogenous locus expression levels. The average expression level of both of the γ2b transgenes in 9921 cells (Ig-secreting cells) was 26%

of that of a natural γ2b locus in the IgG2b-secreting cell line MPC11 and in A20 cells, the averages were 22% (γ2b-hs1-4*loxP*Hs123a) and 24% (γ2b-hs1-4*loxP*Hs3b4) of MPC11 γ2b expression levels. Clearly, in the context of these reporter constructs, the order of the enhancers had little effect on overall enhancer activity.

Deletion of either Hs3a/Hs1,2 or Hs3b/Hs4 has little effect in surface Ig$^+$ cells

Having established that we could achieve measurable expression from a single copy of either γ2b-hs1-4 mini-locus in multiple transformants, we chose several single transformants of the A20 cell line (surface Ig$^+$ cells) for further analysis of enhancer function. Since each transgene contained *loxP* sites flanking either the Hs3a/hs1,2 or the Hs3b/Hs4 enhancer pair, we were able to induce deletion of these pairs without changing the chromosomal location of the transgene. In this way, we could ask whether one or the other enhancer pair was critical to gene expression when in a chromosomal context.

A20 transformants carrying the γ2b-hs1-4*loxP*Hs123a transgene were transiently transfected with a *cre*-expressing plasmid (pBS185) and a plasmid expressing green fluorescence protein (pEGFP-C1) at a molar ratio of ~8:1. Cells that had taken up the EGFP plasmid were identified and isolated by flow cytometry (see Materials and Methods, data not shown). It was expected that most of these cells would have also incorporated the *cre*-expressing plasmid. The sorted cells were cloned by limiting dilution and growing clones recovered three weeks later. Southern analyses were recovered.

Four independent transformants yielded subclones with deletion of Hs3a/Hs1,2, as determined by Southern blot (**Figure 4-7**). Genomic DNA from the A20-γ2b-hs1-4*loxP*Hs123a transformants was digested with HindIII and Southern blots hybridized with probe B (see **Figure 4-2**). As expected, a 7.9kb fragment was detected which is derived from the transgene. Two additional HindIII fragments were also seen and are derived from the IgH loci of A20. The 7.9kb transgene fragment migrates very close to one of these two endogenous gene fragments but is clearly distinguishable from it (see

Figure 4-7 "B" lanes = before deletion; right panel, ΔHs123a). After *loxP*-mediated deletion of *Hs3a*/Hs1,2, the 7.9kb transgene fragment should be lost and a new HindIII fragment formed, its size dependent upon the site of transgene integration (see maps, **Figure 4-2**). As expected, therefore, in the transformant subclones that had undergone loxP-mediated enhancer deletion, the 7.9kb HindIII was missing and a new HindIII fragment was detected in the deletion subclone of each independent transformant (**Figure 4-7**, "A" lanes = after deletion; right panel, ΔHs123a). In two of the deletion clones (P19 and P71), the new HindIII fragment co-migrated with one of the endogenous HindIII fragments. This was obvious from the difference in relative signal intensity of these two endogenous fragments in A20 (and in the transformants prior to enhancer deletion) as compared to the deletion sub-clones (P19 deletion subclone, "B" lane in **Figure 4-7**, has a new HindIII fragment that co-migrates with the larger endogenous fragment; P71 subclone has a new HindIII fragment that co-migrates with the smaller endogenous fragment).

These Southern blots were also hybridized with probe A (see map, **Figure 4-2**) to confirm that enhancer deletion left the rest of the transgene intact. This probe hybridizes to a HindIII fragment that spans most of the γ2b transcription unit and extends into the adjacent DNA at the site of transgene integration. As shown in **Figure 4-2**, this fragment is predicted to be at least 8.2kb in size and to differ among transformants (because of differences in integration site). As shown in **Figure 4-7**, a fragment greater than 8.2kb was detected in each transformant before enhancer deletion ("B" lanes), and the same fragment, unmodified, was detected in each enhancer-deletion subclone ("A" lanes).

γ2b mRNA levels were determined by northern blot in both the initial transformants and in their enhancer-deletion subclones. As shown in **Figure 4-8**, γ2b mRNA levels changed very little after enhancer deletion (compare B and A lanes; right panel, ΔHs123a). In two transformant pairs (P17 and P19), transgene expression increased slightly after enhancer deletion while in the other two transformant pairs (P69 and P71), transgene expression decreased slightly (graph of quantitation data obtained from blot in **Figure 4-8** is provided in **Figure 4-11A**).

The same kind of analysis was done for five A20 transformants carrying the γ2b-hs1-4*loxP*Hs3b4 transgene and deletion subclones that had deleted the Hs3b/Hs4 enhancer pair. Southern blots confirmed enhancer deletion without disruption of the γ2a transcription unit (**Figure 4-7**, left panel, ΔHs3b4). Northern blots again revealed no dramatic effect on transgene expression upon deletion of this enhancer pair (**Figures 4-8&4-11A**). Again, there appeared to be modest reduction after Hs3b/Hs4 deletion when comparing some of the transformant pairs (compare B and A lanes for clones P34 and P34.4) and an increase in γ2a expression in others (P29). Overall, the enhancer deletion results in A20 transformants suggested that neither pair was essential to transgene expression after its integration into the chromosome.

Deletion of Hs3b/Hs4 but not Hs3a/Hs1,2 has a dramatic effect on transgene expression in Ig-secreting cells

9921 transformants carrying the γ2b-hs1-4loxPHs123a and γ2b-hs1-4loxPHs3b4 mini-loci were similarly co-transfected with the cre-expressing plasmid and EGFP, fluorescent cells sorted by flow-cytometry, cloned by limiting dilution, and individual enhancer-deletion clones recovered. As described for the A20 transformants, both types of 9921 transformant, before and after enhancer deletion, generate a 7.9kb HindIII fragment that hybridizes with probe B (**Figure 4-9**, "B" lanes). This fragment is lost after enhancer deletion, being replaced by a new HindIII fragment, its size dependent upon integration site. In 9921, there is only one size endogenous gene fragment detected with this probe, and it migrates slightly above the 7.9kb transgene fragment. The difference in signal intensity for the endogenous and the 7.9kb transgene fragment results from the fact that this cell line carries four copies of the 3'IgH enhancer region: one is associated with the expressed γ2a locus and the others are associated with translocated copies of the IgH locus that juxtapose this region with the oncogene c-myc (Stanton et al., 1984). Enhancer deletion explains the difference in signal intensity for the 7.9kb fragment (before) versus the new HindIII fragment that replaces it (after deletion): in the 7.9kb fragment, both Hs3a and Hs3b are present and homologous to this probe while in the new fragment, only Hs3a or Hs3b is present.

Again, probe A was used on HindIII-digested DNA to confirm that the γ2b transcription unit remained unchanged after enhancer deletion. As expected, DNA from individual transformants yielded unique HindIII fragments that did not change after enhancer deletion (**Figure 4-9**, lower blot, compare A&B lanes).

As shown in **Figure 4-10**, the effect of Hs3b/Hs4 deletion differed dramatically from that of Hs3a/Hs1,2 deletion. In all three independent transformants carrying γ2b-hs1-4loxPHs3b4, cre-mediated deletion of Hs3b/Hs4 resulted in a precipitous drop in γ2b mRNA expression. In two clones, no γ2b mRNA was detectable and in the third, it was reduced to 8% initial levels (**Figure 4-11B**). In contrast, deletion of Hs3a/Hs1,2 from γ2b-hs1-4loxPHs123a transformants had no such effect. As in both types of deletion in A20 transformants, loss of Hs3a/Hs1,2 had a barely discernible effect on transgene expression in the Ig-secreting cell line, 9921 (**Figures 4-10&4-11B**).

DISCUSSION

Two mini-loci consisting of a γ2b transcription unit and two different arrangements of the 3' IgH enhancers (Hs1-4) were consistently expressed in over 100 transformants analyzed involving both a surface Ig+ cell line (A20) and an Ig-secreting cell line (9921). Without the 3'IgH enhancers, the same γ2b transcription unit was inactive in all of 25 independent transformants analyzed. While early studies of the individual 3'IgH enhancers showed that most had no activity in surface Ig^+ cells and that Hs4 had only minimal activity in these cells, transient transfection studies with the group of enhancers revealed pronounced synergistic activity (Ong et al., 1998). In the present study, the latter finding is supported and extended by the finding that an IgH gene lacking the intronic enhancer Eμ and carrying only the 3'IgH enhancers is efficiently expressed in surface Ig^+ cells. The 3'IgH enhancer region, therefore, begins to play a role in IgH gene expression well before B cells differentiate into Ig-secreting plasmacytes.

A classical LCR, such as the β-globin LCR, confers not only chromosome position independent but also copy-number dependent expression upon a cis-linked reporter gene. Moreover, the mRNA produced by each transgene copy is equivalent in level to that produced by the natural endogenous gene (Grosveld et al., 1987). When we assayed γ2b mRNA levels in several transformants with different numbers of γ2b transgenes, γ2b transgene transcription was found not to be strictly copy-number dependent. Further analysis of independent single-copy transformants confirmed this observation. The 32 single-copy transformants carrying one or the other of the two IgH mini-loci produced γ2b mRNA at varying levels, ranging from 10% to 50% of that produced by the endogenous γ2b gene of a related plasmacytoma line (MPC11).

Site-independent but not copy-number dependent expression of the γ2b mini-loci suggests that the 3'IgH enhancers contain partial LCR activity. This finding is not consistent with a previous observation that the 3'IgH enhancers (including Hs1,2, Hs3b, and Hs4) functioned as a full LCR when tested with the *c-myc* reporter gene (Madisen and Groudine, 1994). This discrepancy might be attributed to the difference in the reporter genes used in the two experiments. It has been reported previously that the nature of the reporter gene used can affect LCR function (Guy et al., 1996). In the present study, the reporter was an IgH gene, presumably the gene that the 3'IgH enhancers are meant to regulate. Our observation is consistent with those of two other studies (Chauveau et al., 1998; Chauveau et al., 1999). In those studies, 3'IgH enhancers also conferred integration-independent but not strictly copy-number dependent expression upon the cis-linked transgenes in both cultured cells and transgenic mice. It remains to be determined what sequences can together constitute an independently acting and chromatin-insulated IgH locus. It is possible that DNA sequences that lie within the 3' region of the IgH locus, but which have no enhancer activity, are required for full LCR function. These sequences would have been missing from our IgH mini-loci and from the transgene constructs of others.

Regardless of the order of the 3'IgH enhancers, the γ2b transgenes in both mini-loci transcribed at equivalent levels. This observation suggests that the configuration of 3'IgH

enhancers is not important for their activity with respect to IgH transcription. We did find, however, that there was a striking difference in the way that enhancer pairs functioned at the two developmental stages studied. The Hs3a/Hs1,2, and Hs3b/Hs4 pairs were functionally redundant with respect to their effects on IgH gene transcription in the surface-Ig$^+$ cell line. In contrast, Hs3b/Hs4 was essential for IgH gene transcription in the Ig-secreting cell line (9921) while Hs3a/Hs1,2 was not.

It is not clear how this change in function is achieved. Certainly, it has already been documented that there are changes in the available pool of transcription factors as B lymphocytes undergo development and then differentiation into Ig-secreting cells. BSAP (B cells-specific activator protein), for example, which binds to sites within Hs1,2 and Hs4, is present in pre-B and surface-Ig$^+$ B cells but is absent in Ig-secreting cells (Barberis et al., 1990; Singh and Birshtein, 1993). Given that the Hs1,2/hs3a pair was able to sustain transgene expression after Hs3b/Hs4 deletion in surface Ig$^+$ cells but not in Ig-secreting cells, it might be suggested that BSAP was in some way mediating enhanced Hs1,2 activity in the Ig$^+$ cells. Transient transfection data, however, suggest the opposite. In such assays, BSAP has been shown to repress Hs1,2 activity in these cells (Singh et al., 1993, 1996; Neurath et al. 1994, 1995). NF-κB has also been shown to repress the activity of this enhancer in surface Ig$^+$ cells (Michaelson et al., 1996). We and others have previously shown that Hs1,2 has little or no activity when assayed alone in these cells (Ong et al., 1998; Singh and Birshtein 1993&1996; Michaelson and Birshtein, 1996). What may be a critical difference, however, is that the enhancer deletion experiments we describe in the present study are assaying the activity of enhancer pairs. While neither Hs3a nor Hs1,2 had appreciable activity when assayed individually by transient transfection, the pair showed pronounced synergy when assessed together in mouse splenic B cells, yielding an activity equivalent to that of Eμ (Stevens et al., 2000). While available transcription factors may suppress Hs1,2 activity in these cells, therefore, its ability to synergize with another enhancer element, e.g. Hs3a, is not compromised. This could be achieved through recruitment of additional transcription factors that bridge the two enhancers and prohibit repressor binding or even by converting repressive factors into activators through a change in binding context. NF-κB provides an example of a

transcription factor that can repress or enhance the activity of the same enhancer, depending upon cell stage and, therefore, presumably upon binding context (presence/absence of other DNA-binding proteins; Michaelson et al., 1996).

Hs3a/Hs1,2 serves as a highly active control element in surface Ig^+ cells, but why does this pair not sustain expression of a transgene in Ig-secreting cells? Each of these elements has low, but significant activity when assayed by transient transfection into Ig-secreting cell lines (Ong et al., 1998; Singh and Birshtein 1993&1996; Michaelson and Birshtein, 1996) and preliminary data from our laboratory show that they have at least additive if not slightly greater than additive activity when assayed as a pair in these lines (Ong, Stevens, Roeder, and Eckhardt, unpublished data). This points up the other critical feature of the present study – the transgenes are assayed in the context of chromatin. Proteins that can bind enhancers when available on extra-chromosomal plasmids are not necessarily capable of penetrating a locus embedded in chromatin. One possibility is that while there are transcription factors available in the Ig-secreting cell capable of binding Hs3a/Hs1,2, this enhancer pair is unable, alone, to recruit the necessary chromatin-remodeling co-activators to allow transcription factor access to the transgene. As DNase-hypersensitive sites have been associated with each of the 3' enhancers and such sites are one measure of chromatin structure, it would be interesting to analyze the transgenes in both surface Ig^+ cells and Ig-secreting cells for the presence of these sites before and after enhancer deletion. In this way, it might be possible to determine whether loss of transgene expression after hs3b/hs4 deletion in the Ig-secreting cells is due to loss of an "open" chromatin structure and impeded factor access to the remaining enhancers (Hs3a/Hs1,2).

Chapter 5

3' IgH enhancers are not matrix attachment region (MAR)-associated

INTRODUCTION

The mammalian genome is divided into many looped DNA domains anchored to the nuclear matrix or scaffold. The nuclear matrix constitutes the framework scaffolding of the nucleus and is composed of a variety of proteins such as matrins (Nakayasu and Berezney, 1991; Hakes and Berezney, 1991), lamins (Georgatos et al., 1994) and topoisomerase II (Berrios et al., 1985). Matrix attachment regions (MARs), which are usually approximately 200 base pairs in length and occur once on the average every 30 kb of eukaryotic DNA, are DNA sequences that mediate attachment of DNA looped domains to the nuclear matrix. There is no known consensus sequence that is characteristic of a MAR, but some motifs, such as origins of replication, TC-rich sequences, topoisomerase II sites and AT-rich sequences, have been identified (Gautam et al., 1997). The nuclear matrix may be composed of proteins that are restricted to a specific cell type or a specific physiological stage of a cell type (Dickinson et al., 1992; Herrscher et al., 1995; Zong and Scheuermann, 1995). This suggests that MARs could be tissue-specific or stage-specific.

Promoter, enhancer or other regulatory regions of a gene might be nuclear matrix-associated. For instance, MARs have been found close to or within the promoter or enhancer regions of many gene loci such as mouse Ig heavy chain gene (Cockerill et al., 1987), mouse Ig κ light chain gene (Cockerill and Garrard, 1986), human H4 histone gene (Dworetzky et al., 1992), β-globin gene locus (Jarman and Higgs, 1988; Greenstein, 1988), chicken lysozyme gene (Phi-Van and Stratling, 1988), mouse CD8a gene (Banan et al., 1997), chicken α-globin gene (Farache et al., 1990), human interferon-β gene (Bode and Maass, 1988; Klehr et al., 1991), human HPRT gene (Sykes et al., 1988) , three root-specific tobacco gene (Hall et al., 1991) and several developmentally regulated genes of Drosophila (Gasser and Laemmli, 1986). Evidence showed that MARs might correlate to gene expression or activities of transcriptional enhancers. For instance, when

a reporter gene was flanked with the 5' MAR of the chicken lysozyme gene, its expression was greatly increased and was chromosome site-independent (Stief et al., 1989). The importance of MARs has also been shown in expression of the immunoglobulin kappa light chain gene. In stable transfection, deletion of the intronic MAR sequence leads to a fourfold decrease in expression of the rearranged kappa light chain gene (Blasquez et al., 1989). In the transgenic mice, in the absence of the MARs, the Ig μ transgene is impaired in its ability both to undergo transcription activation and to overcome the variability associated with chromosomal position effect, despite the presence of the intronic enhancer, but the transgene that contains only MARs and lacks the core enhancer expresses at very low levels (Forrester et al., 1994). Similarly, IgH μ gene transcription in different kinds of gene-targeted recombinants showed that MARs flanking core Eμ region were required for efficient expression of IgH μ gene. Removal of Eμ core region plus part of 5'MAR sequence reduced IgH μ mRNA level to 51% of wild type. Additional deletion of 3'MAR sequence, and S region led to a reduction to 19%. Deletion of Eμ core region, both 5'and 3'MAR sequences, and S region, however, resulted in a reduction to 1.7% relative to wild type (Oancea et al., 1997).

The functional murine immunoglobulin heavy chain (IgH) locus contains a variable region (VDJ joining) gene, 8 constant region genes, Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε and Cα, and an intronic enhancer, which is located in the intron between the V region gene and Cμ gene, as well as four 3' IgH enhancers that reside at the downstream of the Cα gene. In the murine IgH locus, the intronic enhancer region and three other regions between Cδ and Cγ3, Cγ3 and Cγ1, and Cγ1 and Cγ2b, respectively, have all been shown to be MAR-associated, but no MAR-associated regions have been found from Cγ2b through Cα gene segment (Cockerill, 1990). In the BCg3R-1d transfected cell line, which is a derivative of the BCL_1B_1 murine B cell lymphoma, a 0.5 kb region immediately 5' of the IgH promoter is also MAR-associated (Webb et al., 1991). The IgH intronic enhancer (Eμ) (Banerji et al., 1983; Gillies et al., 1983; Neuberger, 1983), and four 3'IgH enhancers, Hs3a (Matthias and Baltimore, 1993), Hs1,2 (Lieberson et al., 1991; Dariavach et al., 1991), Hs3b (Madisen and Groudine, 1994) and Hs4 (Madisen and Groudine, 1994; Michaelson et al., 1995) are important for IgH gene expression and

probably class switching. The 3'IgH enhancers span an approximately 34 kb region that is over 170 kb from the J_H region in the germline IgH locus, but the mechanism how these enhancers work with the IgH promoter over such a long distance remains unclear. One hypothesis for that is that the 3' enhancers might be folded back close to the IgH promoter. One candidate of evidence to support this folding back model would be that MARs exist within the 3' region of the IgH locus, bringing the 3' IgH enhancers close to the IgH promoter with the help of nuclear matrix proteins.

In the present study, we have searched a 26 kb DNA region that includes four 3' IgH enhancers downstream of Cα gene segment for MARs. As described below, no MAR was identified. Our data show that unlike the IgH intronic enhancer, the four 3' IgH enhancers are not MAR-associated regulatory elements although it remains possible that MARs exist somewhere else farther downstream of the IgH locus.

MATERIAL AND METHODS:

Cell line

9921 is a γ2a-expressing variant that lacks the intronic enhancer (Eckhardt and Birshtein, 1985). 9921 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies; Gaithersburg, MD; catalogue no. 12100-061) with 10% Bovine Calf Serum (BCS) (HyClone Laboratories, Logan, UT; catalogue no.SH30072.03). A20 was maintained in RPMI 1640 medium (Life Technologies; catalogue no. 31800-089) with 10% BCS. All media contained 100U/ml penicillin/streptomycin (Life Technologies; catalogue no. 15140-122) and 2 mM L-glutamine (Life Technologies; catalogue no. 21051-016). All cells were maintained at 37°C in an atmosphere of 7 % CO_2.

Isolation of nuclei for matrix preparation

All the steps used for nuclei isolation were performed at 4° C. 1 x 10^8 of 9921 cells were washed once with 30 volumes of PBS (phosphate-buffered saline), resuspended in RSB (10 mM Tris-HCl pH7.4, 10mM NaCl, 3mM $MgCl_2$) with 0.5 mM PMSF (cat. No. P-7626, Sigma, Louis, MO), 10 μg/ml aprotinin (cat. No. 236624, Roche Molecular Biochemicals, Indianapolis, Indiana), 5 μg/ml leupeptin (cat. no. L-2884, Sigma), and 0.5

mM EDTA. The cells were incubated on ice for 10 min, and then homogenized with a Dounce homogenizer B. After centrifugation at 3,500 rpm for 10 min (clinical centrifuge), the pelleted nuclei were washed twice with RSB-0.25M sucrose (10-15 volumes relative to the pellet), suspended in an equal volume (relative to the pellet) of RSB-2M sucrose, and centrifuged through a cushion of RSB-2M sucrose (~ 25 ml) at 24,000 rpm for 30 min (SW 28 horizontal rotor, ultracentrifuge). Isolated nuclei were washed once in RBS-0.25 M sucrose by centrifugation at 3,500 rpm for 10 min (eppendorf centrifuge) and resuspended in RBS-0.25 M sucrose plus 1mM $CaCl_2$.

Nuclear matrix isolation

Nuclei (4mg/ml nucleic acid determined by spectrophotometer, 1 OD_{260} = ~ 50μg nucleic acid) in RBS-0.25 M sucrose plus 1mM $CaCl_2$ were digested in a final concentration of 200U/ml DNase I (cat. No. 18047-019, GibcoBRL) for 1.5 hours at 23° C. The nuclei were pelleted after centrifugation for 10 min at 3,500 rpm at 4°C (eppendorf centrifuge) washed with 10-15 volumes of RBS-0.5 M sucrose (relative to pellet), and resuspended in 10-15 volumes of RBS-0.25 M sucrose. An equal volume of cold solution containing 20mM Tris-HCl at pH 7.4, 4M NaCl and 20mM EDTA was added. After 10 min at 0° C and then centrifugation at 5,000 rpm for 15 min at 4°C (eppendorf centrifuge), pellets were washed twice by suspension in a cold solution containing 10mM Tris-HCl at pH 7.4, 2M NaCl, 10mM EDTA, 0.5 mM PMSF and 0.25mg/ml BSA, and spun down at 9,000 rpm for 15 min at 4°C (eppendorf centrifuge). The resulting nuclear matrices were washed with RBS-0.25 M sucrose containing 0.25mg/ml BSA (centrifugation at 9000 rpm at 4° C for 15 min using eppendorf centrifuge), resuspended in 10-15 volumes of the same buffered solution, and then stored for up to 6 months at – 20°C after combining with an equal volume of glycerol. Concentration of the matrices was determined by Bradford Assay (Bio-RAD Protein Assay, Cat. No. 500-0006, Bio-RAD Laboratories, Hercules, CA).

DNA fragments used in nuclear matrix-binding assays (see **Figure 5-1**)

Fragments 1, 9 and 25 were isolated from pIgA3, a plasmid that contains a 4.4 EcoRI fragment and that was kindly provided by Dr. Sherie Morrison (UCLA; Gregor and Morrison, 1986).

Fragments 2, 10, 11, 12, 16 and 17 were isolated from pRV5', a plasmid containing a 7.1 EcoRI/HindIII fragment with an additional, internal, HindIII site (Lieberson et al., 1995).

Fragments 3,13, 14, 18, 19, 22, 23 and 26 were isolated from pBR325-H5.8, which contains a 5.8 kb HindIII fragment isolated from the phage pλm2 (Dariavach et al., 1986).

Fragments 7, 8, 15, 20 and 24 were isolated from p6.2.11 (Gregor and Morrison, 1986).

Fragments 5 and 6 were isolated from pHS3b4, which contains a fusion of Hs3b and Hs4 and was kindly provided by Dr. Mark Groudine (Fred Hutchinson Cancer Research Center, Seattle, WA; Madisen et Groudine, 1994).

Fragment 4 was isolated from pλm2Xba4.2 in which a 4.2 XbaI fragment was subcloned from pλm2 (Dariavach et al., 1991) into pBS plasmid DNA.

Fragment 16 was isolated from a plasmid in which a 4.4 kb EcoRI fragment isolated from pIgA3 and a 7.1 kb EcoRI fragment isolated from pRV5' (see above) were together subcloned.

Eκ-MAR was isolated from TM, a genomic clone of the expressed Ig κ locus from the murine IgA-secreting cell line, S107, kindly provided by Dr. Sherie Morrison (Gregor and Morrison, 1986).

Eμ-MAR was isolated from p297γ2b that contains a functional γ2b gene with Eμ between V_H and C_H sequences (Zaller and Eckhardt, 1985).

Assay of DNA binding to nuclear matrices

Matrices were washed three times in washing buffer (50 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-HCl at pH7.4, 0.5 mM PMSF and 0.25 mg/ml BSA) by centrifugation for 30 seconds at 10, 000 rpm at 4°C in a microcentrifuge and resuspended in Assay Buffer (50 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl at pH7.4, 0.5 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.25 mg/ml BSA). 5 μg of matrices protein in 10μl was added to 90 μl

Assay Buffer with DNA fragments (20-30 ng/ml ^{32}P-end-labeled DNA fragments each and 200 μg/ml unlabeled, sonicated E. coli DNA). After incubation on a shaker for 2 hours at 23° C, 500 μl of assay buffer without DNA was added, and matrices were recovered by centrifugation at 10,000 rpm for 2 min at 4°C. After washing in 1 ml of final washing buffer (50 mM NaCl, 2 mM EDTA, 10mM Tris-HCl at pH7.4 and 0.25mg/ml BSA), the protein-DNA complexes were solubilized in 15-20 μl of Solubilizing Buffer (2 mM EDTA, 40 mM Tris-acetate, 0.4 mg/ml proteinase K (cat. No. 24568-2. EM Science. Gibbstown. NJ). 0.5% SDS and 5 μg/ml sonicated sperm DNA) and incubated overnight at 37°C. The resulting matrix-bound DNA fragments were resolved by electrophoresis on 3% polyacrylamide gels in 1X TBE buffer,180 Volts, for 2-3 hours alongside a sample of probe DNA representing 25 % of that used in the initial reaction. The gel was dried and autoradiographed.

RESULTS

Nuclear matrices isolated from an IgH-secreting cell line, 9921, were examined for their ability to retain DNA fragments containing the 3'IgH enhancers and some of their adjacent regions. **Figure 5-1** provides an overview of all of the DNA fragments examined. Together these fragments constitute approximately 26 kb out of a 41 kb IgH 3'region. To ensure that no MARs were overlooked as a result of restriction enzyme cleavage within MARs, several different overlapping restriction enzyme fragments were used (e.g, fragments 4, 14, 18, 19, 22, 23, and 26 cover Hs1,2 region). Kappa light chain MAR (Eκ-MAR), Ig heavy chain intronic enhancer MAR (Eμ-MAR), or both were used as internal references in each assay. Eκ-MAR is a 2.9 kb HindIII/BamHI fragment containing MAR sequence, Eκ (intronic enhancer), and Cκ gene segment. The HindIII site of this fragment is immediately followed by the MAR DNA sequence (~254 bp, Cockerill and Garrard, 1986). Eμ-MAR is a 1.0 kb fragment containing the core region of Eμ and its two flanking MAR elements each ~300 bp in length (Cockerill et al., 1987).

When ^{32}P labeled DNA fragments are incubated with nuclear matrices, only the DNA fragments that have MARs will be retained after disruption of non-specific binding by

washing. As we expected, Eμ-MAR and Eκ-MAR were both retained by the nuclear matrices (**Figures 5-2** and **5-3**, see Eμ and Eκ bands in B lanes), confirming previous observations (Cockerill et al., 1987; Cockerill, 1990). None of the other labeled fragments bound the nuclear matrix (**Figures 5-2** and **5-3**). These data show no evidence of MARs in the region downstream of Cα gene segment within the IgH locus. Unlike Eμ, Hs3a, Hs1,2, Hs3b, and Hs4 appear not to be MAR-associated.

DISCUSSION

It has been shown that 2.9 kb Eκ-MAR bound to a greater degree than the similarly sized 2.3 kb Eμ-MAR (Cockerill et al., 1987; Cockerill, 1990), but this difference was distorted when a 1.0 kb Eμ-MAR was compared to 6.8 kb Eκ-MAR, even though the Eκ-MAR had greater binding affinity (Cockerill, 1990). Binding efficiency decreased steadily as fragment length increased above about 1 kb. However, for fragments below 1 kb, binding might decrease with decreasing size as well (Cockerill and Garrard, 1986). These observations indicated that DNA fragment size could affect MARs' ability to bind to matrices. To ensure that no MARs were overlooked resulting from the influence of DNA fragment size, differently sized fragments cloned from the same DNA region (see **Figure 5-1A**) were examined in matrices-DNA binding assays. A 1.0 kb Eμ-MAR and a 6.8 kb fragment containing Eκ-MAR still yielded a high binding affinity in matrix-binding assays (Cockerill, 1990). All of the DNA fragments except fragment 18 and 19 in our experiments ranged from 1.0 kb to 4.2 kb, and regions covered by fragments 18 and 19 (each < 1.0 kb) were also contained in another fragment of larger size. Given the considerable overlap in fragments, we think it unlikely that any MARs were overlooked in our measurements.

Our data showed that no MARs immediately flanked any of the four 3'IgH enhancers, Hs3a, Hs1,2, Hs3b and Hs4, indicating that unlike IgH intronic enhancer, Eμ, these 3' IgH enhancers were not MAR-associated. It is generally believed that Eμ is mainly responsible for the initial VDJ rearrangement process (Chen et al., 1993; Serwe and Sablitzky, 1993; Fernex et al., 1994) and for the initial activation of IgH gene

transcription once a functional VDJ recombination succeeds (Banerji et al., 1983; Gillies et al., 1983; Neuberger, 1983; Zaller and Eckhardt, 1985; Grosschedl and Baltimore, 1985; Grosschedl and Marx, 1988; Porton et al., 1990), whereas the 3' IgH enhancers might have a regulatory role later in IgH gene expression (Gregor and Morrison, 1986; Madisen and Groudine, 1994; Lieberson et al., 1995), and in the IgH class switching process (Cogne et al., 1994). So far, there have been no reports to show that the unique functions of the IgH intronic enhancer (Eμ) are due to the presence of its MAR, but this remains a possibility. Interaction between MAR elements and matrices could make the activity of Eμ different from that of the 3' IgH enhancers, even though motifs within the core region of Eμ might also make contributions to its special activity. In fact, some observations have suggested that MAR elements are necessary for Eμ activity. In gene targeted mice, replacement of the 1 kb region containing core enhancer Eμ and two flanking MARs results in a cis-acting block in VDJ recombination (a reduction to 12% relative to that of wild type) in most B cells derived from the Eμ/MARs-deficient mice (Chen et al., 1993). Surprisingly, the same effect is observed in transformants derived from pGK-*neo*^{r} cassette insertion into the MAR sequences 5' of Eμ (a reduction to 18%) (Chen et al., 1993).

Previous studies showed that Hs1,2 was at the center of an approximately 25kb region of dyad symmetry, which is composed of inverted repeats and tandem repeats (Chauveau and Cogne, 1996; Saleque et al., 1997). **Figure 5-1B** shows the pattern of this dyad symmetry. Hs3a (IR1) and Hs3b (IR1') are part of the dyad symmetry and are virtually identical (97% homology). IR2 and IR2' are inverted repeats and share 74% homology. IR3 and IR3' are 83.5% homologous. TR1 and TR2 are tandem repeats and share 89% homology. TR2 and TR3 are 91% homologous.

The area located approximately between PstI and XbaI immediately upstream of Hs3b is possibly homologous to the immediate 3' region of Hs3a based on symmetry in this region, although the DNA sequence of this region is not known. Our physical search for MARs did not cover the areas **a**, **b**, **c** and **d** as depicted in **Figure 5-1A&B**. Area **a** and most of area **b**, however, are contained within the large inverted repeat region and were

likely indirectly assessed. So far, no reports have shown that areas **c** and **d** share high homology or are repeats of some of the DNA sequences we have examined in the experiments. We cannot exclude the possibility, therefore, that MARs exist in these two regions. It is also possible that MARs lie farther downstream of the IgH locus, a possibility that will require further studies.

CHAPTER 6 GENERAL DISCUSSION

The work involved in this thesis is to evaluate the activity of the 3' IgH enhancers (Hs3a, Hs1,2, Hs3b, and Hs4), which are situated far 3' of the murine immunoglobulin heavy chain locus, in IgH gene transcription. IgH gene transcription at high levels is under control of the IgH intronic enhancer (Eμ), and 3'IgH enhancers alone or in combination. Eμ is necessary for the efficient assembly of IgH genes and may be necessary for maintenance of gene transcription at high levels in the earlier stages of B cell development, but it is dispensable at later stages for IgH gene transcription. At the B cell later stages, high-level transcription of the IgH gene can be maintained in the absence of Eμ. In several B-lymphoid cell lines, Eμ has been lost due to a spontaneous deletion, but heavy chain synthesis is not affected and persists at a high level equivalent to their parental cell lines (Wabl and Burrows, 1984; Klein et al., 1984; Aguilera et al., 1985; Eckhardt and Birshtein, 1985; Zaller and Eckhardt, 1985).

As described in **Chapter 1**, there are several lines of evidence to support the hypothesis that the 3'IgH enhancers play a role in IgH gene transcription and are responsible for continued IgH expression in Eμ-deleted mutants. Natural deletion of an approximately 34 kb DNA region including all the 3'IgH enhancers downstream of Cα gene segment in a mouse myeloma variant, LP1.2, dramatically reduced levels of Igα mRNA compared to the parental cell line (Gregor and Morrison, 1986; Michaelson et al., 1995). Furthermore, chromosomal translocations between the IgH locus and *c-myc* leads to one *c-myc* allele becoming juxtaposed to the 3'IgH enhancer sequences through a reciprocal 5'→5' chromosomal translocation event in mouse plasmacytoma and human Burkitt's lymphoma cells (Cory, 1986; Magrath, 1990; Spencer and Groudine, 1991), resulting in the de-regulated transcription of the translocated *c-myc* genes. This suggests that the 3'IgH enhancer region has taken control of expression of the translocated *c-myc* gene. While these observations implicate the 3'IgH enhancers in control of both Ig and translocated *c-myc* expression, they do not directly prove their involvement.

The most informative approach to evaluate the regulatory activity of these 3'IgH enhancers in a natural IgH locus is to knockout (delete) the individual enhancers from an IgH locus that lacks Eμ in a cell line such as 9921, and then to measure the effect of deletion on IgH gene transcription.

We made a gene targeting vector specified to delete one of the 3'IgH enhancers, Hs1,2, through replacement of the enhancer by *neo*^{r} in a homologous recombination event. We generated an Hs1,2-deficient transformant of 9921 by gene targeting (Lieberson et al., 1995). The 9921 cell line naturally lacks Eμ, but its IgH gene transcription is still at high levels. In the Hs1,2-deleted 9921 cells, which lack both the Eμ and Hs1,2, the IgH gene was found to be no longer expressed. This observation indicates that Hs1,2 plays an essential role in maintaining IgH gene transcription in the absence of Eμ.

However, we cannot conclude that Hs1,2 is the sole element within the IgH locus responsible for maintaining IgH gene expression of 9921 since the other individual 3'IgH enhancers identified in the vicinity of Hs1,2 might be necessary for but not sufficient enough for gene expression. In addition, we should not disregard the possibility that insertion of an active transcription unit, *neo*^{r} gene, within the particular chromosomal position may somehow negatively affect IgH expression. In the Hs1,2-deleted 9921 mutant, therefore, we should consider any potential effects on IgH gene transcription caused by insertion of the *neo*^{r} gene rather than by deletion of Hs1,2.

It has been shown that integration of a *neo*^{r} gene into a chromosomal locus can, by itself, inactivate the targeted gene's expression (Kim et al., 1992; Fiering et al., 1993). One study shows that insertion of a *neo*^{r} gene into the LCR region of the β-globin locus disrupts β-globin gene expression. Removal of the *neo*^{r} gene, however, restores wild-type expression of the β-globin gene in MEL (murine erythroleukemia) hybrid cells (Fiering et al., 1993). While we were attempting to replace Hs1,2 in 9921 cells with a *loxp*-flanked *neo*^{r} gene, another group succeeded in doing this in the genome of mice. Manis et al (1998) showed that when either Hs3a or Hs1,2 was replaced by a *neo*^{r} gene cassette, Ig heavy chain gene class switching recombination (CSR) was blocked in the B lymphoid

cells derived from the Hs3a or Hs1,2-deficient mice (homozygous). When the *neo*^{r} gene inserted in the place of the deleted 3'IgH enhancers was subsequently deleted from the IgH locus, CSR returned to normal. Inactivation of the targeted genes as a result of an insertion of the *neo*^{r} gene has been interpreted that *neo*^{r} successfully competes with the targeted gene for interaction with the regulatory elements because of its preferential position (Fiering et al., 1993; Manis et al., 1998). In these mice, Eμ was present, so the contribution made by Hs3a or Hs1,2 to IgH gene transcription was not directly measurable.

The most importance for a successful gene targeting experiment is high efficiency of homologous recombination events. It has been shown that high efficiency is correlated with the content of homology between the targeting and targeted DNA sequences (Smith and Kalogerakis, 1990; Kumar and Simons, 1993; Scheerer and Adair, 1994), and efficiency of screening depends on usage of both positive and negative selection in the targeting system as well. We flanked the *neo*^{r} gene with a *loxP* site on both sides to remove it from the IgH locus after the *neo*^{r} had successfully replaced Hs1,2. We constructed several different targeting vectors with the modified *neo*^{r} gene, attempting to increase efficiency of homologous recombination in the gene targeting experiments and improve the efficiency of screening for these events. Our approach to achieving this was to increase the content of homology between the targeting vector and targeted DNA, and to introduce a negative selection system into the targeting systems by using SV2*gpt* (driven under an SV40 promoter and enhancer) or ES*gpt* (driven under SV40 promoter and Eμ) instead of HSV-*tk*. HSV-*tk* negative selection system was found not to operate well in the 9921cell line (The *tk*-transformants were not killed by gancyclovir).

We screened more than 2000 wells of stable transformants obtained after transfections with the different targeting vectors (1-3 clones per well on the average). No homologous recombinants were, however, identified. Our lab had also unsuccessfully tried to delete all of the 3'IgH enhancers in a natural IgH locus using a similar targeting vector. It has been shown that frequencies of homologous versus non-homologous recombination vary from locus to locus and cell-type to cell-type. For instance, gene targeting in embryonic

stem (ES) cells is significantly more efficient than in other cell types (Arbones et al., 1994). A gene targeting study showed that frequency of homologous recombinants was as high as 1/34 (one homologous recombinant out of 34 G418 and gancyclovir double-resistant clones) when Eμ region was targeted in mouse embryonic stem (ES) cells (Gu et al., 1993). The frequency of homologous recombinants, however, decreased to $1.1x10^{-6}$ ~ $2x10^{-7}$ when the same region was targeted in the surface-Ig^+ cell line (igm692) (Oancea et al., 1997; Wiersma et al., 1999). 3' region of IgH locus was harder to be targeted relative to Eμ region. It has been shown that frequency of homologous recombinants was 3/275 (three homologous recombinants out of 275 G418 and gancyclovir double-resistant clones) when 3'IgH enhancer (Hs1,2) was targeted in ES cells (Cogne et al., 1993). These results indicate that targeted DNA sequences and cell types can influence efficiency of homologous recombination. Therefore, we attributed our lack of success to the extremely low efficiency of homologous recombination because of difficult induction of homologous recombination by targeting vectors in the 3'region of IgH locus in culture cells.

In transient transfection models, each of 3'IgH enhancers is individually capable of activating a reporter gene in Ig-secreting cells while only Hs4 can do this in surface-Ig+ cell lines. The group of 3'IgH enhancers is highly synergistic in surface-Ig^+ cells while their activities are generally additive in Ig-secreting cells (Ong et al., 1998). We wanted to measure the regulatory ability of these 3'IgH enhancers in the context of chromatin, however, because transient transfection assays cannot measure the remodeling function of these regulatory elements. The most straightforward approach for these goals is to delete these enhancers directly from a natural IgH locus, and then measure effects of their deletion on IgH gene transcription. We had difficulties to delete these enhancers from an endogenous IgH locus. We created our IgH "mini-locus", therefore, to manipulate these 3'IgH enhancers. This is another approach to evaluate the activity of these enhancers in the context of chromatin, and it would be easier to handle the enhancers in an IgH "mini-locus" than in a natural IgH locus.

We constructed two different IgH "mini-loci" using all the 3'IgH enhancers and an IgH transgene. The IgH "mini-loci" were designed to delete a pair of 3'IgH enhancers from the IgH transgene using Cre/*loxP* system after transgene integration into the host cell's genome. The mini-loci were stably introduced into surface-Ig$^+$ and Ig-secreting cell lines to measure effects of deletion of each pair of 3'IgH enhancers on IgH transgene transcription, and to measure whether activity of these 3'IgH enhancers changed with B cell developmental stage. In this experiment, we first found that the IgH transgene under control of these 3'IgH enhancers was expressed in a chromosome site-independent but not a strictly copy number dependent manner in both surface-Ig$^+$ and Ig-secreting cell lines. Furthermore, the single copy transgenes derived from both cell lines generated different levels of mRNA, ranging from 10% to 50% that of an endogenous IgH gene. In contrast, the transgene lacking 3'IgH enhancers did not express in any of the 25 transformants. These results suggest that the 3'IgH enhancers have a regulatory ability and function as a partial LCR, leading to the cis-linked transgene transcription. Second, we found that the natural order of 3'IgH enhancers was not required for this partial LCR activity. This result suggests that configuration of 3'IgH enhancers is not important for their function in this model system. Third, we found that Hs3b/Hs4 was essential for IgH expression in an IgH-secreting B cell line while Hs3a/hs1,2 wasn't. In contrast, Hs3a/Hs1,2 and Hs3b/Hs4 were functionally redundant in a surface-Ig$^+$ B cell line. These results suggest that activity of the 3'IgH enhancers changes with B cell developmental stage.

In these experiments, we deleted both Hs3a plus Hs1,2 (Hs3a/Hs1,2) from an IgH mini-locus in 9921 cells. In this case, deletion of the Hs3a/Hs1,2 enhancer pair did not affect transgene γ2b transcription. This result suggests that neither Hs3a nor Hs1,2 is essential for transgene expression. It also suggests that inactivation of Igγ2a expression in our previous Hs1,2-deficient 9921 (Chapter 2) is due to insertion of PGK-*neo*^{r}, rather than deletion of Hs1,2 from the IgH locus. If Hs1,2 is not essential for IgH gene expression, its absence should not have had an effect on IgH gene transcription. The IgH gene in the endogenous locus was silenced, however, upon replacement of Hs1,2 with

neo^r . This indicates, therefore, that insertion of *neo*^{r} in the place of the deleted Hs1,2, not deletion of Hs1,2 itself, is responsible for silencing the IgH gene.

In our experiments, we also found that deletion of Hs3b and Hs4 had no effect in the surface Ig$^+$ cell line while deletion of these elements dramatically reduced mini-locus expression in Ig-secreting cells. This indicates that Hs3a and Hs1,2 can, together, sustain Ig expression in the surface-Ig$^+$ cell line but not in the Ig-secreting cell line. Transient transfection assays have shown that Hs3a, and Hs1,2, individually have no detectable activity in surface-Ig$^+$ cells, while each one has detectable activity in Ig-secreting cells (Singh and Birshtein, 1993 & 1996; Ong et al., 1998). Transient transfection assays have also shown, however, that Hs3a and Hs1,2, together, yield a high synergistic activity equivalent to that of Eμ in splenic cells (Stevens et al., 2000). In both surface-Ig$^+$ and Ig-secreting cell lines, Hs3a and Hs1,2 together have synergistic activity when tested by transient transfection assays (Ong and Eckhardt, unpublished results). These results suggest that this pair of two 3'IgH enhancers is active in both surface-Ig$^+$ cells and Ig-secreting cells. In the context of chromatin, however, we found that Hs3a/Hs1,2 was able to sustain transgene expression after hs3b/Hs4 deletion in the surface-Ig$^+$ cells but not in the Ig-secreting cells. In other words, Hs3a/Hs1,2 is active in the surface-Ig$^+$ cells but not in the Ig-secreting cells in the context of chromatin. The transient assays demonstrate that there are transcription factors available to bind to and mediate Hs3a/Hs1,2 function in Ig-secreting cells but it is apparent that these factors are not able to gain access to these enhancers in the Hs3b/Hs4-deleted min-locus. It could be of interest to know whether these Hs3a/hs1,2 binding factors are able to bind these 2 enhancers when Hs3b/Hs4 are present or if hs3a/hs1,2 are not active even before Hs3b/Hs4 deletion.

Transient transfection data show that the activity of Hs1,2, when measured alone, is inhibited by repressors in the surface-Ig$^+$ cells. BSAP (B cell-specific activator protein), which is coded by *Pax*-5 genes and binds to BSAP-binding sites in the 3'IgH enhancers (Hs1,2, and Hs4), is present in pro-B, pre-B, and surface-Ig positive B cells, but not in plasma cells (Barberis et al., 1990; Singh and Birshtein, 1993). BSAP is a repressor for Hs1,2 activity but an activator for Hs4 activity in surface-Ig positive cells in transient

transfection assays (Singh and Birshtein, 1993; Neurath et al., 1994; Michaelson et al., 1996). As described earlier, BSAP repressed Hs 1,2 activity by blocking binding of NF-αP to its cognate site within Hs1,2. NF-αP is a member of the Ets family of transcription factors and is present in both surface-Ig$^+$ and Ig-secreting B cells. Like BSAP, NF-κB has also been shown to repress the activity of Hs1,2 in the surface-Ig$^+$ cells but it is an activator for Hs1,2 activity in the Ig-secreting cells when tested in transient transfection assays (Michaelson at al., 1996). In the context of chromatin, however, BSAP and NF-κB appear not to repress Hs1,2 activity in surface-Ig positive cells. An observation from our lab shows that BSAP doesn't affect IgH gene expression when a gene expressing high levels of BSAP is stably introduced into an Ig-secreting cell line (9921, Alaie-Petrillo and Eckhardt, unpublished results). It is possible, therefore, when Hs1,2 is together with another enhancer such as Hs3a, binding of additional transcription factors that bridge these two enhancers can inhibit repressors such as BSAP, and NF-κB binding or even convert repressors into activators through a change in binding context, leading to Hs1,2 activity becoming active in the surface-Ig$^+$ cells.

It has been shown that the octamer binding sites within the 3'IgH enhancers have a more critical role in transcriptional activity in a surface-Ig$^+$ cell line (M12) than in Ig-secreting cell lines (MPC 11, S194, MOPC 31) in transient transfection assays (Tang and Sharp, 1999). Transcriptional activity of the 3'IgH enhancers (Hs1,2, Hs3b, and Hs4) with mutant octamer sites decreased to 24% that of these 3'IgH enhancers with wild type octamer binding sites in the surface-Ig$^+$ cells when tested with a luciferase reporter gene. Transcriptional activity of the 3'IgH enhancers with mutant octamer sites was equivalent to that of the wild type 3'IgH enhancers, however, in Ig-secreting cells. Further analysis showed that Oct-2 rather than Oct-1 has a unique function when interacting with the 3'IgH enhancers in the surface-Ig$^+$ cells.

In a recent collaboration between our lab and that of R. Roeder, we showed that T cell-mediated stimulation of B cells increased 3'IgH enhancer function but not in the absence of OCA-B (Stevens et al., 2000). This suggests that a role for OCA-B, a B-cell specific cofactor for Oct-1 and Oct-2, in 3'IgH enhancer function, as well. Based on the

apparent importance of Oct-2 and OCA-B in the function of the 3'IgH enhancers in the surface-Ig$^+$ cells, one possibility is that Hs3a/Hs1,2 and Hs3b/Hs4 are functionally redundant in surface-Ig$^+$ B cells because all if these enhancers contain octamer sites. In Ig-secreting cells, 3'IgH enhancer function is not clearly dependent upon Oct-2 octamer-binding factor and may instead, be dependent upon factors binding to other sites within the enhancers. Our data would suggest that these other factors are differentially expressed in surface-Ig$^+$ versus Ig-secreting cells, and that they preferentially ensure Hs3b/Hs4 but not Hs3a/Hs1,2 function in Ig-secreting cells.

As discussed above, our results with the IgH mini-locus suggest that *neo^r* insertion in place of Hs1,2 was responsible for silencing the endogenous γ2a gene in 9921 cells. The effect of *neo^r* gene on the endogenous locus brings up question of whether our co-transfected *neo^r* gene was affecting mini-locus expression (the *neo^r* gene was co-transfected with IgH mini-locus in the experiments). The observation that the transgene without 3'IgH enhancers was not expressed, however, suggests that the co-transfected *neo^r* gene doesn't have a positive effect on transgene expression. Moreover, the finding that almost all the transgenes in different integration sites were expressed suggests that the co-transfected *neo^r* doesn't have a negative effect on the IgH mini-loci in our experiments, either. One possibility that the co-transfected *neo^r* doesn't have a negative effect on the transgene expression is that the *neo^r* gene does not integrate in the same site as each IgH transgene or the *neo^r* integrates in the same site as the transgene but does not influence expression of the transgene that is under control of the four 3'IgH enhancers. Pair enhancer deletion from the IgH mini-loci showed that the co-transfected *neo^r* gene did not affect expression of the transgene under control of a pair of enhancers if it is assumed that the *neo^r* gene integrates in the same site as each IgH transgene. If the *neo^r* has a negative effect on expression of the transgene with a pair of enhancers, when Hs3/Hs1,2 is deleted from the mini-loci, transgene expression should be silenced because the insertion of *neo^r* can block Hs3b/Hs4 function to IgH promoter, as it did in Hs1,2 deleted-9921 cells (**Chapter 2**). After Hs3a/Hs1,2 deletion, however, we found that the transgene under control of Hs3b/Hs4 was still expressed in both surface-Ig$^+$ and Ig-secreting cell lines. This indicates that the co-transfected *neo^r* gene does not have a

negative effect on Hs3b/Hs4 activity even though it integrates in the same site as the transgene. Similarly, if the co-transfected *neo*^{r} gene has a negative effect on Hs3a/Hs1,2 function, the transgene should be silenced when Hs3b/Hs4 is deleted form the mini-locus in both surface-Ig$^+$ and Ig-secreting cell lines. In contrast, the transgene with Hs3a/Hs1,2 was expressed in surface-Ig$^+$ cells but not in Ig-secreting cells. These results, therefore, show that the co-transfected *neo*^{r} does not have a negative effect on either pair enhancers, Hs3a/Hs1,2 and Hs3b/Hs4 in our experiments.

Our finding that all of the 3'IgH enhancers functioned as a partial LCR is not consistent with a previous observation that three 3'IgH enhancers, Hs1,2, Hs3b and Hs4, in combination functioned as a full LCR when tested with the stably transfected human *c-myc* reporter gene (Madisen and Groudine, 1994). Our finding is, however, in accordance with two other observations. When a reporter gene driven by an IgH V gene promoter (pV_H) is linked to the palindromic part of the 3' IgH regulatory region including Hs3a, Hs1,2, and Hs3b, the reporter gene is expressed in all clones, regardless of integration site, but copy number-dependent expression is not observed (Chauveau et al., 1998). Similarly, in transgenic mice harboring a V_H promoter-β-globin reporter gene linked to four IgH 3' enhancers, Hs3a, Hs1,2, Hs3b, and Hs4, transgene expression is strictly confined to the B cell lineage in all transgenic founder lines. Furthermore, reporter gene activity is chromosome site-independent but not strictly copy number-dependent (Chauveau et al., 1999). In an earlier study (Madisen and Groudine, 1994), the *c-myc* reporter gene was driven by its own *c-myc* promoter, and expression was measured in an IL-6-dependent Ig-secreting plasmacytoma. Moreover, only three 3'IgH enhancers rather than all the four 3'IgH enhancers were used in that study. It is possible, therefore, that the difference in promoter used (our study and the transgenic mouse study used an IgH promoter) and/or the addition of a fourth enhancer region (Hs3a) in our study and the transgenic mouse study is responsible for the different results.

The β-globin LCR confers high levels of position-independent, copy number-dependent expression onto a β-globin transgene in transgenic mice (Grosveld et al., 1987). In transgenic mice that carry the β-globin LCR in cis with the β-globin gene promoter

driving a lacZ reporter gene, however, expression of the lacZ transgene in fetal liver cells was found to show strong position effects, varying as much as 700-fold per transgene copy. These position effects occurred although the whole β-globin gene including the downstream enhancer was incorporated as part of the lacZ reporter gene. But when the lacZ reporter gene was replaced by β-globin, the LCR functioned properly (Guy et al., 1996). This result shows that the heterologous reporter gene can affect proper function of an LCR. This finding may raise another possibility that the different results in the earlier study (Madisen and Groudine, 1994) and in our study is due to difference in reporter gene coding sequences (we used Igγ2b reporter gene while the earlier study used the *c-myc* reporter gene).

In an endogenous IgH locus, the 3'IgH enhancers span an approximately 34 kb region, but in the IgH 'mini-locus" (constructs) in the experiments done by us and others, all of the four 3'IgH enhancers were contained within about 7~8 kb of DNA. Some DNA sequences that are necessary for full LCR activity could be lost in the constructs. Loss of these DNA sequences could lead to partial function of the 3'IgH enhancers. This is supported by an observation from the human CD2 LCR. Transgenic mice carrying a hCD2 mini-gene attached only to the 3'CD2 transcriptional enhancer exhibited variegated expression when the transgene was integrated in the centromere. In contrast, mice carrying a transgene with additional 3' sequences, a 0.5 kb region with no enhancer activity, showed no variegation even when the latter integrated in centromeric positions (Festenstein et al., 1996). This result suggests that the short region with no enhancer activity functions in the establishment, maintenance, or both of an open chromatin domain, and is essential for full LCR activity.

Another possibility that partial LCR function of 3'IgH enhancers was observed in our experiment is that the terminally developed and differentiated culture cell line may not provide a proper environment for correct LCR function. It has been shown that correct function of the β-globin LCR requires passage through a nonerythroid cellular environment (Vassilopoulos et al., 1999). When a 155 kb β-globin locus YAC (yeast artificial chromosome) was introduced into MEL 585 (murine erythroleukemia) cells by

lipofection, β-globin expression was strongly influenced by the position of integration of the β-globin YAC into the MEL cell genome. When the β-globin YAC was first microinjected into L-cells and then transferred into MEL cells by fusion, however, β-globin expression was independent of position of integration of the transgene, as expected for normal LCR function. These results suggest that normal activation of the LCR requires interaction with the transcriptional environment of an uncommitted, nonerythroid cell. It has been proposed, therefore, that activation of the β-globin LCR may represent a multistep process initiated by the binding of ubiquitous transcription factors early during the differentiation of hematopoietic stem cells and completed with the binding of erythroid-specific factors in the committed erythroid progenitors (Vassilopoulos et al., 1999). The possibility that the 3'IgH enhancers function as a partial LCR because of an improper environment, however, is contradicted by results in transgenic mice. As in the cell culture lines, the 3'IgH enhancers still display characteristics of a partial LCR in transgenic mice (Chauveau et al., 1999).

The most informative approach to measure LCR function of the 3'IgH enhancers, therefore, would be to include the entire region spanning all of the 3'IgH enhancers in a transgene to prevent omission of DNA sequences that may not have enhancer activity but are necessary for LCR function. This transgene should use an IgH gene as reporter to eliminate influence from a heterologous reporter gene because, as described earlier, a heterologous reporter gene could affect proper LCR function, and should be introduced into mice to provide a proper environment for the LCR to function correctly. If 3'IgH enhancers are found to function as a partial LCR even under the conditions described above, it is possible that they combine with other regulatory elements, not yet identified within the IgH locus, to form powerful LCR that maintains IgH gene transcription in the absence of Eμ. It would be desirable to search for these regulatory elements farther downstream of the 3'IgH enhancers.

Enhancers/LCR may recruit transcription factors, which directly interacts with the gene's promoter, leading to gene transcription (human β-globin LCR, Dillon et al., 1997) or may recruit chromatin disrupters (such as DNA helicases), which establish an open

chromatin structure that allows the promoter to function effectively, resulting in gene expression (human CD2 LCR, Festenstein et al., 1995).

One of possibilities that Hs3a/Hs1,2, behaves differently in the surface-Ig$^+$ cells and in the Ig-secreting cells in our experiments in **Chapter 4** could be due to chromatin remodeling mediated by 3'IgH enhancers. Presumably, all the four 3'IgH enhancers together are able to establish an "open" chromatin structure for the transgene expression in both surface-Ig$^+$ cells (A20) and Ig-secreting cells (9921). After deletion of Hs3b/Hs4 from the IgH mini-locus, Hs3a/Hs1,2 is still able to sustain this open chromatin structure established by all the 3'enhaners, leading to transgene transcription, in the surface-Ig+ cells. In the Ig-secreting cells, however, Hs3a/Hs1,2 is not capable of maintaining this open chromatin structure after deletion of hs3b/Hs4, leading to the transgene becoming silenced. In contrast, Hs3b/Hs4 can sustain the open chromatin structure after Hs3a/Hs1,2 deletion from the transgene in both surface-Ig$^+$ and Ig-secreting cells. We can examine this hypothesis by looking for formation of DNase I hypersensitive sites in the transgenes before and after deletion of Hs3a/Hs12, or Hs3b/Hs4 in the surface-Ig$^+$ and Ig-secreting cell lines. In this way, it would be possible to determine if loss of transgene expression is correlated to loss of the open chromatin structure.

We used A20 and 9921 cell lines as representatives of different B cell stages, surface-Ig$^+$ and plasma cell stages, respectively, in studying developmental change in activity of the 3'IgH enhancer in the context of chromatin (**Chapter 4**). Transient transfection assays shows that 3'IgH enhancers (Hs3a, Hs1,2, Hs3b, and Hs4), alone or in combination, behave in a similar pattern in three surface-Ig$^+$ cell lines, A20, Raji, and Namalwa. In these three cell lines, individual 3'IgH enhancers except Hs4 have no detectable activity but all enhancer together yield a highly synergistic activity that is much stronger than Eμ (Ong et al., 1998). This result suggests that A20 is reliable to be representative of the surface-Ig$^+$ cell lines. It has also been shown that the 3'IgH enhancers (Hs1,2, Hs3b, and Hs4) behave in a similar pattern in two Ig-secreting cell lines, MPC11 and TEPC 1165, when assayed in transient models (Madisen and Groudine, 1994). Hs1,2 has an equivalent activity in three Ig-secreting cell lines, MPC11, HOPC1,

and S194 in transient transfection assays (Dariavach et al., 1991, Singh and Birshtein, 1993). These results suggest that MPC11 can be used as a representative of the Ig-secreting cell lines. 9921 cell line is a derivative of MPC11, a natural γ2b-producing cell line. 9921 should share a similar cellular environment with MPC11, and can be representative of the Ig-secreting cell lines.

How sequences located in a long distance from the promoter regulate gene transcription still remains obscure. There are two different models, looping model and tracking model, to interpret this phenomenon. In the looping model (Ptahne, 1988), it is proposed that these regulatory sequences (enhancers) directly contact with promoters via a protein or protein complex that simultaneously binds two different sites (promoter and enhancer) in the gene locus. Observation that the steady-state RNA levels of the two β-globin genes are dependent on their relative distance from the LCR suggests that the human β-globin LCR functions in this way (Dillon et al., 1997). In the tracking model (Wasylyk et al., 1983), it is proposed that sequence motifs within the enhancers serve as points of entry for transcription factors or disrupter of chromatin (such as DNA helicases) that then migrate along the DNA to the promoter, generating an open chromatin structure that allows entry of other factors required for transcription. The observation that a short region without enhancing ability within human CD2 LCR can serve as a point of entry for transcription factors or chromatin disrupter to establish an open chromatin domain for full LCR activity suggests that human CD2 LCR might work in this way (Festenstein et al., 1996).

The 3'IgH enhancers span an approximately 34 kb region that is over 170 kb from the J_H region in the germline IgH locus, but how these enhancers work with the IgH promoter over such a long distance remains unclear. One hypothesis is that the 3' enhancers loop together with the promoter via nuclear matrix proteins at matrix attachment regions (MARs). We have searched for MARs in a 34 kb 3'regulatory region within the IgH locus and have found none. It is possible that sequence motifs such as the octamer, which is found within the promoter and all the 3'IgH enhancers (or μE5, shared by the 3'IgH enhancers), allow these 3'IgH enhancers to loop together through protein

complexes consisting of Oct-1, Oct-2, OCA-B, or E2A family proteins, to form a very powerful regulatory unit, which activates IgH gene transcription in the absence of Eμ.

It is believed that IgH gene expression is controlled by two distinct units, Eμ and the 3'IgH enhancers, at different B cell developmental stages. In the early stages, IgH gene transcription is established by Eμ. In the later stages, however, IgH gene transcription is maintained by the 3'IgH enhancers even in the absence of Eμ. This enhancer shift might be important for ensuring gene expression at the later stages when there is the possibility that Eμ will be deleted (by accident) during IgH class switch recombination. Experiments in this thesis provide evidence to support the importance of the 3'IgH enhancers in IgH gene transcription at the later B cell stages, reveal functional redundancy of individual 3'IgH enhancers in the IgH gene transcriptional regulation, and prompt continued exploration of the complex nature of IgH regulation by the 3'IgH enhancers.

FIGURES AND TABLES

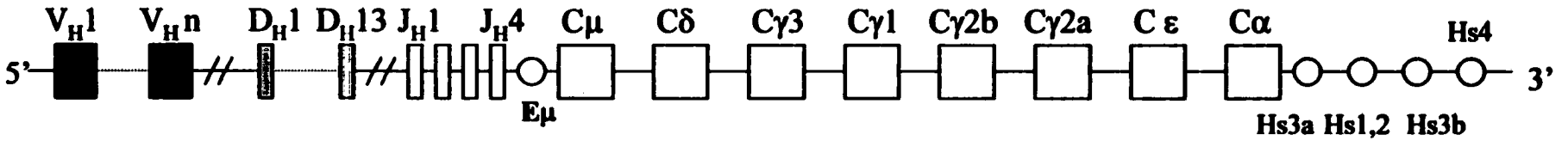
Figure 1-1. Schematic diagram of structure of immunoglobulin (Ig). An immunoglobulin (Ig) molecule consists of two identical heavy polypeptide chains and two identical polypeptide chains. Each heavy chain and light chain in an Ig molecule contains an amino-terminal variable (V) region and constant (C) region. Both heavy and light chains are held together by disulfide bonds. The variable regions of both heavy and light chains determine the specificity of an antibody for antigen binding.

Figure 1-1

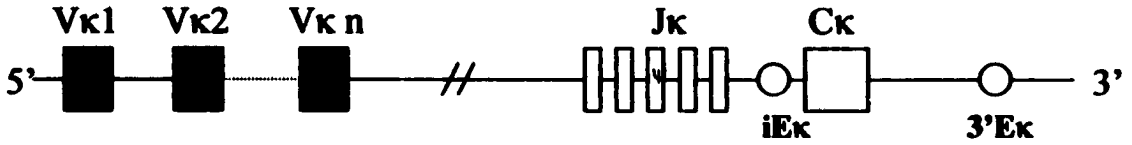
Figure 1-2. Organization of immunoglobulin germline gene segments in the murine: (a) heavy chain locus, (b) κ light chain locus, (c) λ light chain locus. The heavy chain is encoded by V, D, J, and C gene segments. The κ and λ light chains are encoded by V, J, and C segments. The relative distances the various gene segments are not indicated. Boxes denote coding sequences and circles represent enhancer regions.

Figure 1-2

(a). Heavy-Chain DNA



(b). κ-chain DNA



(c). λ-chain DNA

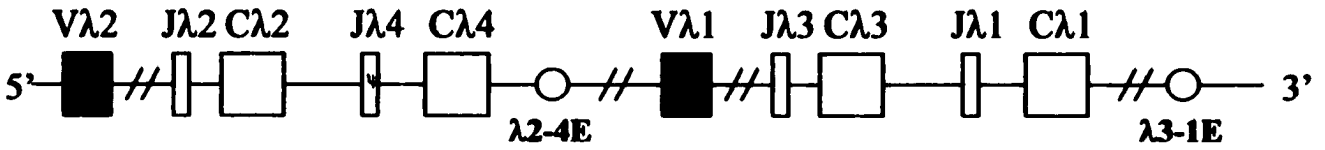


Figure 1-3. Location of 3'IgH enhancers in the murine IgH locus. An IgH variable region gene (P-VDJ) is situated upstream of μ constant region coding sequences (Cμ). The 3'IgH enhancers, Hs3a, Hs1,2, Hs3b, and Hs4, reside downstream of the Cα gene. Black boxes denote coding sequences and open circles represent enhancer regions. Relative distances between elements are not indicated. For reference, the enhancer Hs3a lies170 kb downstream of Eμ. The 3'IgH enhancers span a ~34 kb region.

Figure 1-3

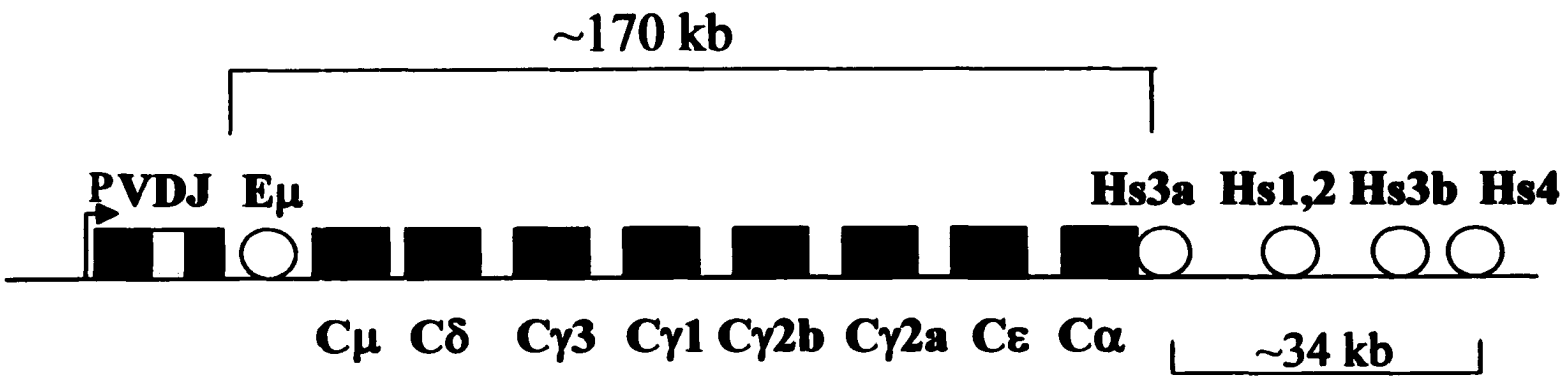


Figure 3-1. Diagram of the 3' region of the murine IgH locus and targeting vectors. **(A) The 3' region of IgH locus.** Black boxes represent 3' IgH enhancers. The restriction enzymes in the region are indicated for ease of reference but they don't represent all of the sites in the region. B: BamHI, Ap: ApaII, E: EcoRI, H: HindIII, Pst: PstI, S: SacI, Sau: Sau3AI, X: XbaI.

(B) Targeting vectors used to replace Hs1,2 with PGK-neor cassette. Black bars represent homologous DNA sequences between the targeting vectors and the targeted DNA in the 3' region. PGK-neor and HSV-tk/Esgpt/SV2gpt were designed for positive and negative selection, respectively, in a positive and negative selection system.

Figure 3-1

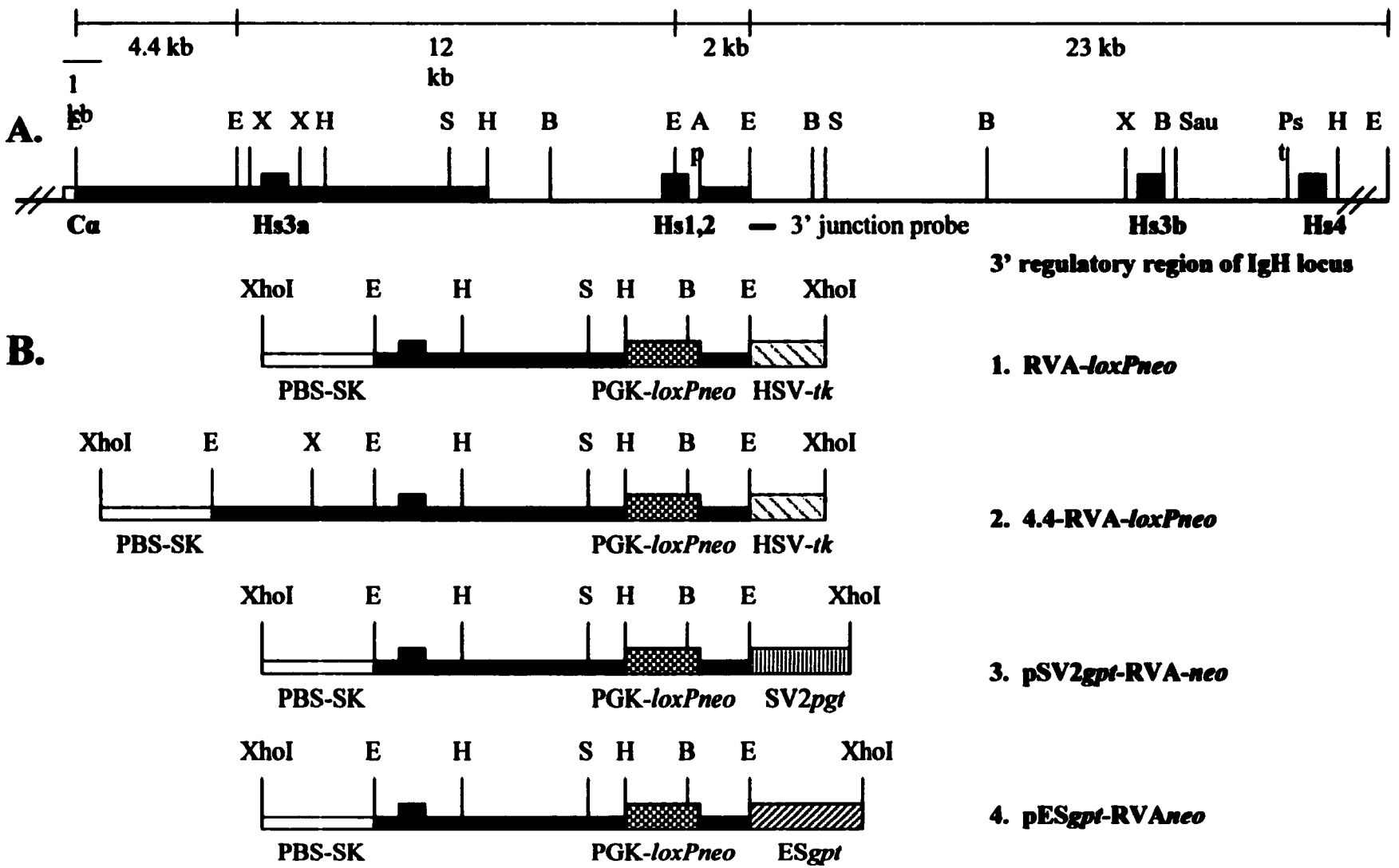


Figure 3-2. Screening for homologous recombinants using genomic Southern blot analysis and PCR testing of gene-targeted candidate clones (G418 resistant clones). DNA isolated from 9921 cells and B48 cells were used for negative and positive controls, respectively. B48 is the subclone of 9921 that carries a neor gene in place of Hs1,2 downstream of the γ2a gene.

(A) **Genomic Southern blot analysis of 9921 transformants.** DNA derived from representative clones were digested with BamHI and hybridized with the 3' junction probe (see **Figure 3-1**). Fragments derived from the endogenous IgH loci of 9921 are indicated by an arrow labeled with 6.9 kb. The gene-targeted fragment in B48 is indicated by an arrow labeled with 3.7 kb.

(B) **PCR product analyzed using gel electrophoresis.** Gene-targeted PCR product was synthesized by using forward primer, neo 818, and backward primer, 3'frva (see Material and Methods). The 2.3 kb gene-targeted PCR product derived from B48 is indicated by an arrow labeled with PCR. The kb ladder used as a DNA marker is indicated by arrows labeled with different sizes.

Figure 3-2

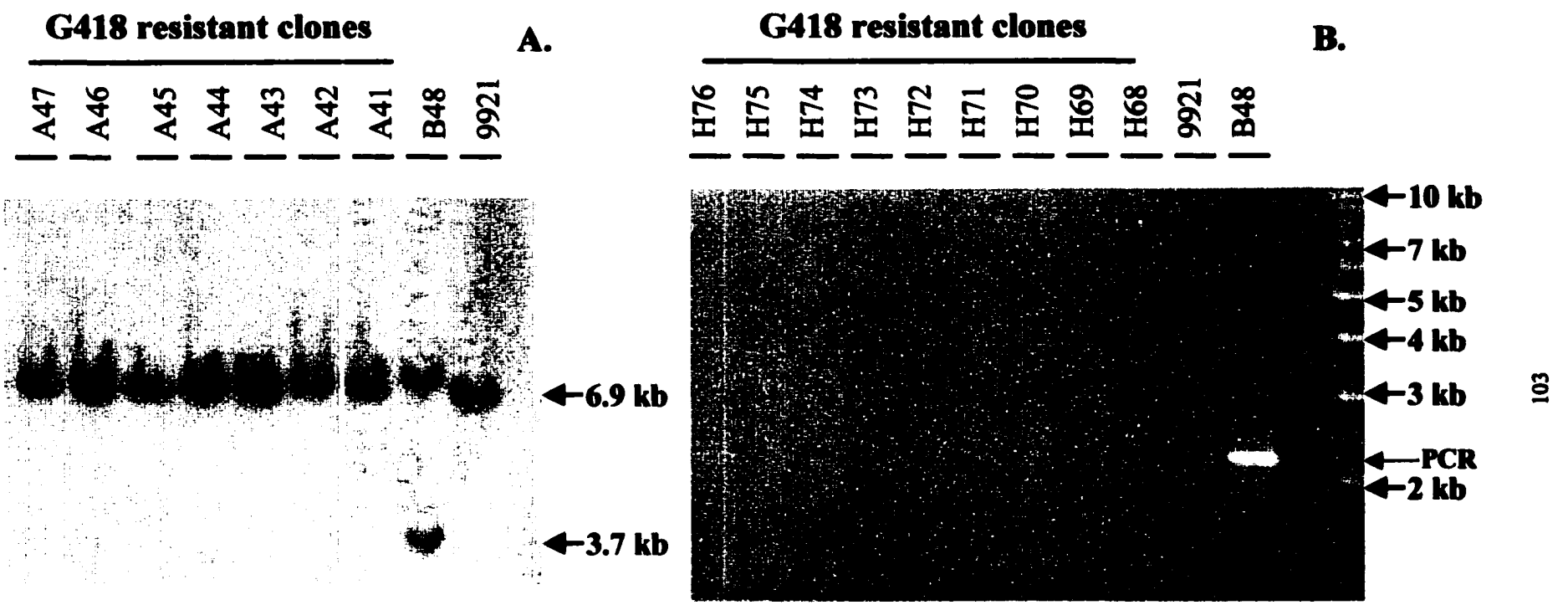


Figure 4-1. Diagram of the murine IgH locus after assembly of an IgH variable region gene (P-VDJ) upstream of μ constant region coding sequences (Cμ). Black boxes denote coding sequences and open circles represent enhancer regions. Relative distances between elements are not indicated. For reference, the enhancer Hs3a lies170 kb downstream of Eμζ

Figure 4-1

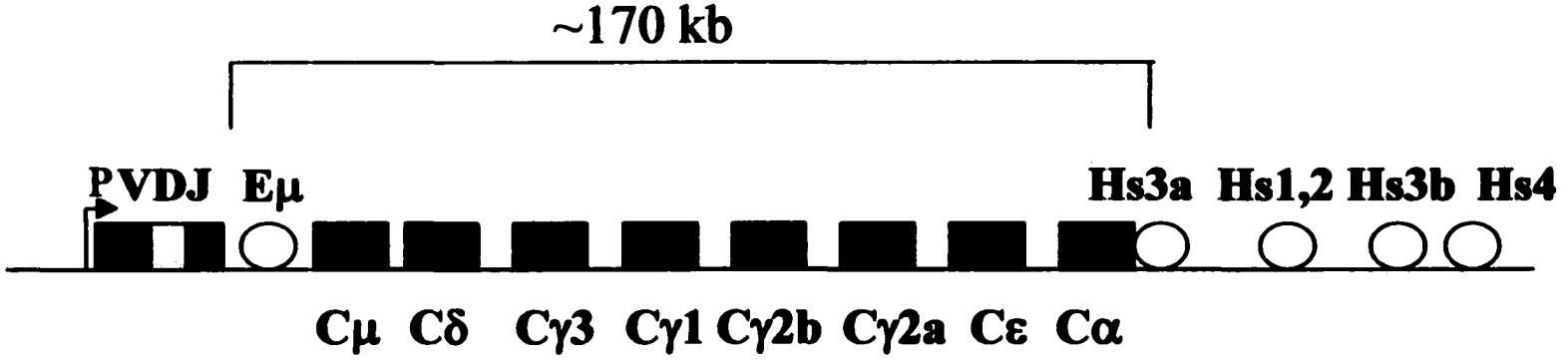


Figure 4-2. IgH mini-loci before and after enhancer deletions

γΠΔζ γ2b-hs1-4 locus with *loxP* sites surrounding the enhancers Hs3a and Hs1,2 (γ2b hs1-4*loxP*hs123).

LoxP sites are indicated with wide arrows. The γ2b gene in this mini-locus consists of VH coding sequences (identical to that expressed in MPC11 and 9921, see Materials and Methods) and γ2b constant region sequences (Cγ2b). DNA probes used in Southern analyses of transformants are indicated (probe A and probe B). Probe B is homologous to both Hs3a and Hs3b, as shown. The bacterial gene for ampicillin resistance is shown (ampr) although this gene is not expressed in eukaryotic cells. Plasmids were linearized with PvuI (P) before introduction into cells. This site was usually destroyed in the course of integration into the genome (slashes in P). Thick lines surrounding the mini-locus denote genomic DNA at the site of mini-locus integration.

HindIII restriction sites within the mini-locus (H) were used to analyze transformants both for transgene copy number and for *cre*-mediated enhancer deletions (see text). Bracket above the HindIII fragment detected with probe B indicates fragment size (7.9kb). Arrow above the HindIII fragment detected with probe A indicates that the size of this fragment will be equal to or greater than 8.2kb, depending upon integration site (see text).

The predicted changes in mini-locus structure, subsequent to *cre*-mediated enhancer deletion, are shown below the intact mini-locus map (γ2b hs1-4*loxP*Δhs123).

γπΔζ γ2b-hs1-4 locus with *loxP* sites surrounding the enhancers Hs3b and Hs4 (γ2b hs1-4*loxP*Hs3b4). Designations are as described in A.

Figure 4-2

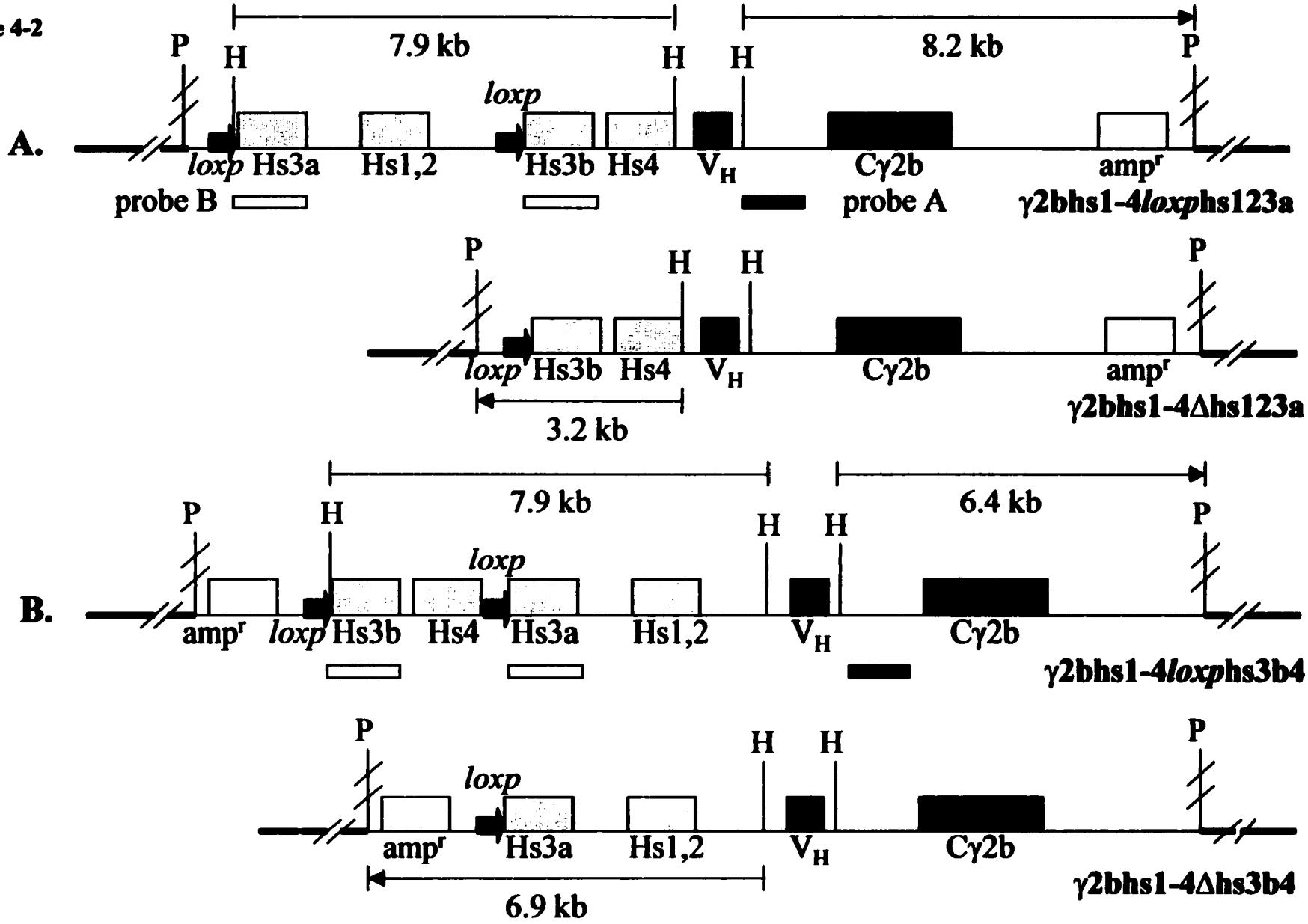


Figure 4-3. Northern blot analysis of 9921 transformants with different numbers of γ2b transgenes. mRNA samples were isolated from representative clones. The same blot was sequentially hybridized with a γ2b probe and a β-actin probe (for sample loading normalization).

Figure 4-3

Figure 4-4. Comparisons of transgene copy number with transgene expression level. γ2b mRNA levels were assessed by northern blot (sample loading normalized with β-actin). Copy number was determined by Southern analysis as described in the text. The data for eleven individual clones are shown (asterisks). A line connects the average γ2b mRNA values for 1, 2, 4, 5, and 7 copy-number transformants.

Figure 4-4

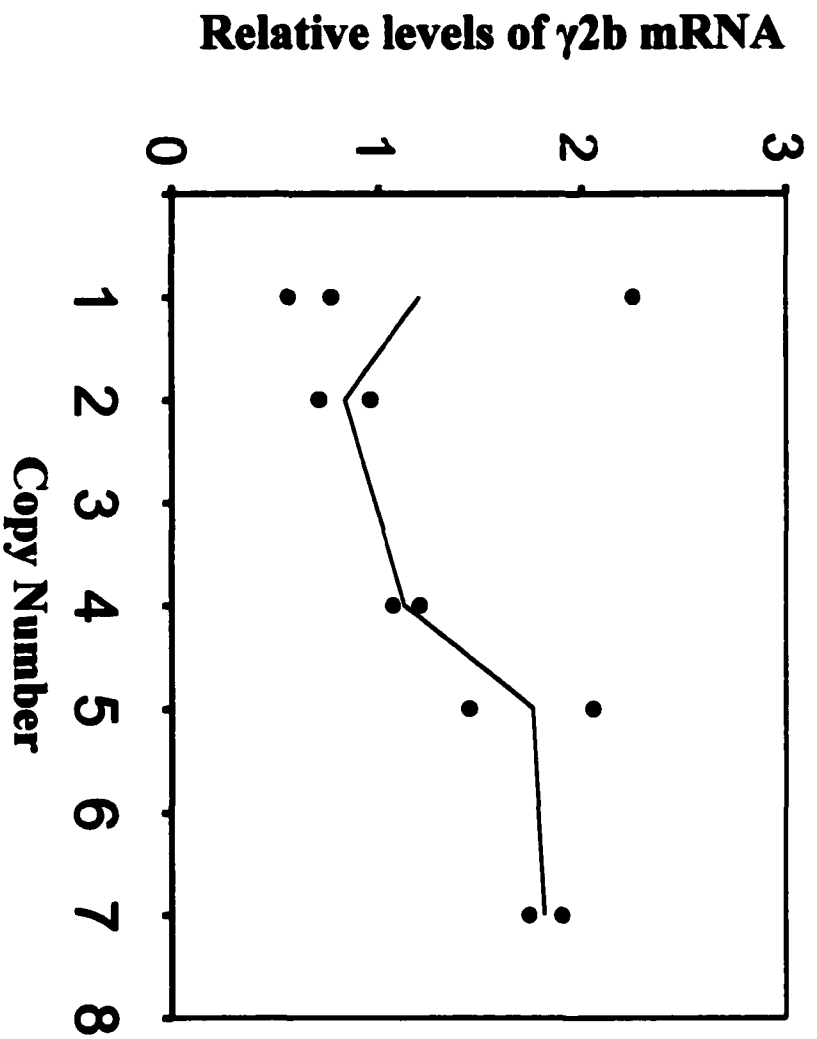


Figure 4-5. Northern blot analysis of 9921 transformants with a single copy of the γ2b transgene. mRNA samples were isolated from representative clones. The same blot was sequentially hybridized with a γ2b probe and a β-actin probe (for sample loading normalization). γ2bhs1-4*loxp*Hs123a and γ2bhs1-4*loxp*Hs3b4 are two IgH mini-loci with different arrangement of the 3'IgH enhancers (see Material and Methods)

Figure 4-5

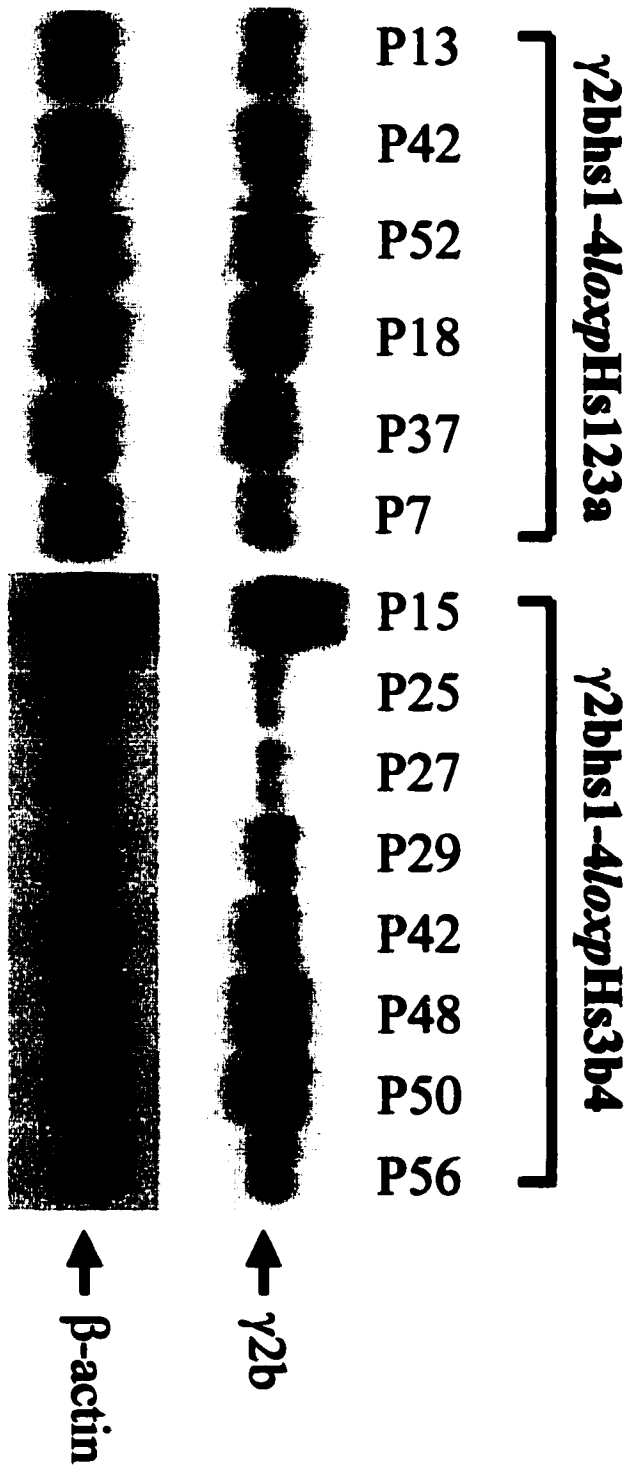


Figure 4-6. Northern blot analysis of A20 transformants with a single copy of the γ2b transgene. mRNA samples were isolated from representative clones. The same blot was sequentially hybridized with a γ2b probe and a β-actin probe (for sample loading normalization). γ2bhs1-4*loxp*Hs123a and γ2bhs1-4*loxp*Hs3b4 are two IgH mini-loci with different arrangement of the 3'IgH enhancers (see Material and Methods)

Figure 4-6

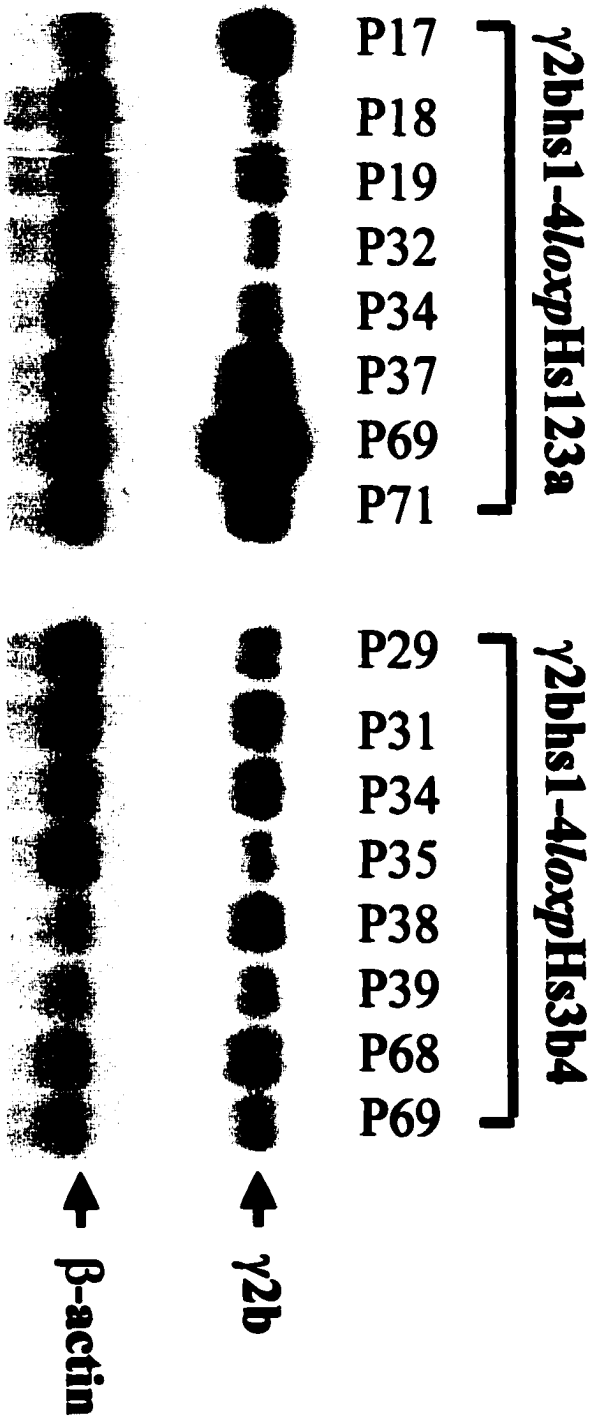


Figure 4-7. Genomic Southern blot analyses of A20 transformants before and after enhancer deletion.

Right panel is of transformants before (B) and after (A) deletion of Hs1,2/Hs3a (ΔHs123a). Individual transformant pairs are given unique designations (P71, P69, etc.). ***Left panel*** is of transformants before (B) and after (A) deletion of Hs3b/Hs4 (ΔHs3b4). Transformants P34 and P34.4 were recovered from one culture well in the initial transfections with the γ2bhs1-4*loxP*Hs3b4 mini-locus. As is evident with probe A, however, these subclones represent independent integration events (compare size of fragment detected in the P34 transformant pair with that detected in the P34.4 transformant pair).

Upper blot: Hybridized with Probe B. Fragments derived from the endogenous IgH loci of A20 are indicated with arrows. Because of faster migration of the outer-most lanes of the gel, the 7.9kb transgene fragment detected by probe B appears slightly larger than its actual size.

Lower blot: The same blot shown in the upper panel was "erased" and then re-hybridized with Probe A. Fragments derived from the endogenous IgH loci of A20 migrate below the region shown on this blot.

Figure 4-7

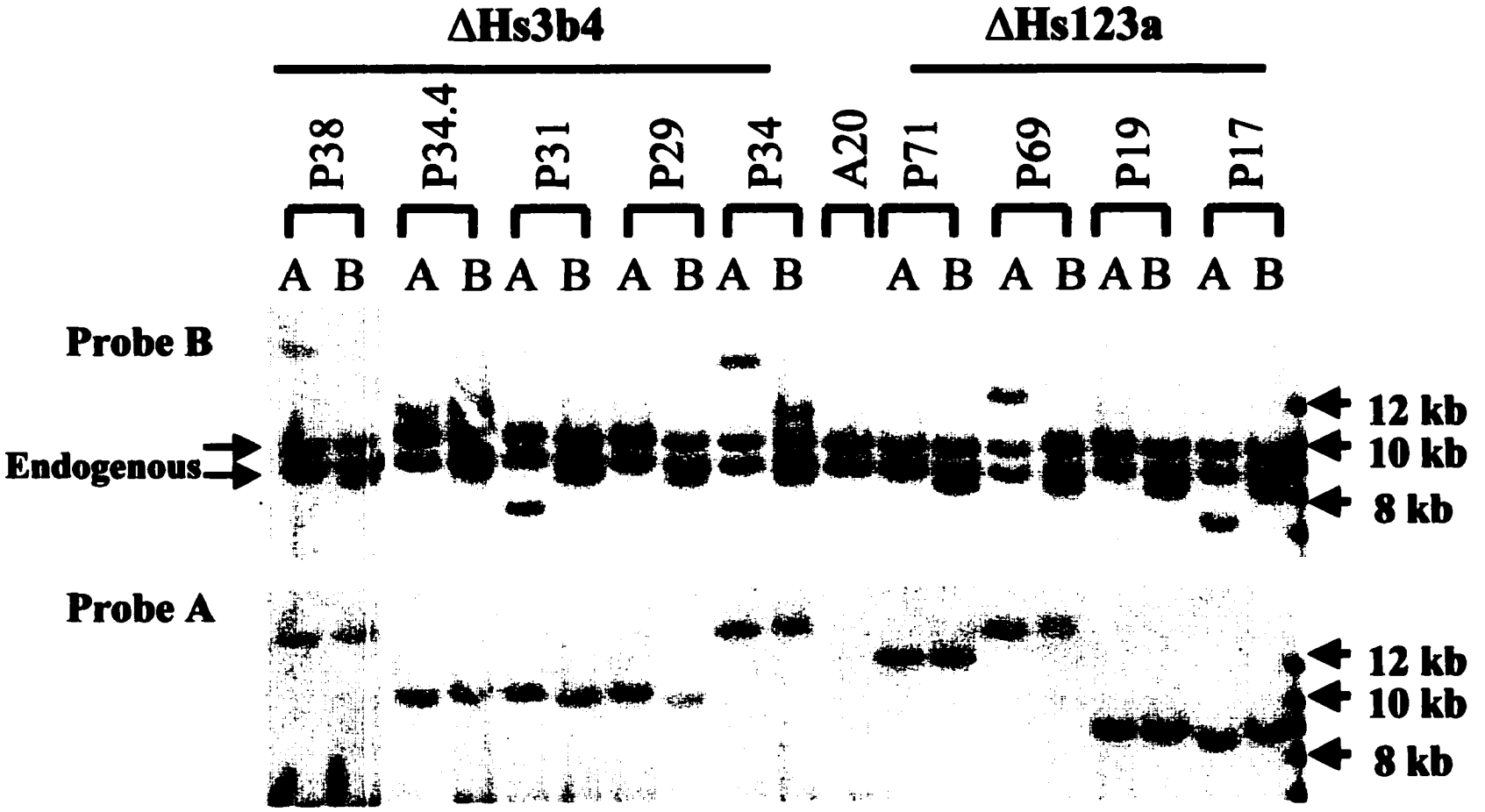


Figure 4-8. γ2b transgene expression in A20 clones before and after enhancer deletion. Representative northern blot of mRNA isolated from A20 clones before (B) and after (A) deletion of enhancers. The same blot was sequentially hybridized with a γ2b probe and a GAPDH probe (for sample loading normalization). The left panel shows transformant pairs with the mini-locus that allows *cre*-mediated deletion of Hs3b/Hs4 (ΔHs3b/Hs4) while the right panel is of pairs that carry the mini-locus that yields Hs1,2/Hs3a deletions (ΔHs123a).

Figure 4-8

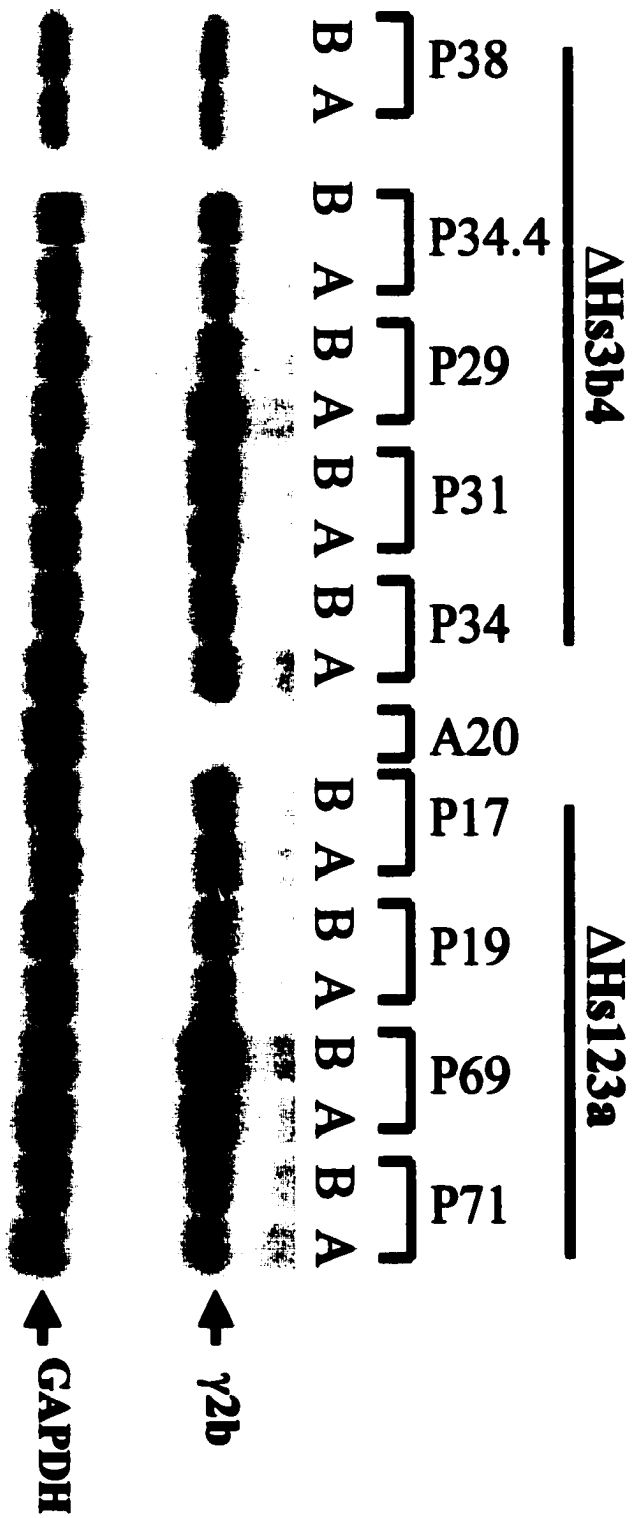


Figure 4-9. Genomic Southern blot analyses of 9921 transformants before and after enhancer deletion.

Right panel is of transformants before (B) and after (A) deletion of Hs3b/Hs4 (ΔHs3b4). Individual transformant pairs are given unique designations (P50, P42, etc.).

Left panel is of transformants before (B) and after (A) deletion of Hs1,2/Hs3a (ΔHs123a).

Upper blot: Hybridized with Probe B (see **Figure 4-2**). Fragments derived from the endogenous IgH loci of 9921 are indicated with an arrow. Because of faster migration of the outer-most lanes of the gel, the 7.9kb transgene fragment detected by probe B appears larger than its actual size.

Lower blot: The same blot shown in the upper panel was "erased" and then re-hybridized with Probe A. Fragments derived from the endogenous IgH loci of 9921 migrate below the region shown on this blot.

Figure 4-9

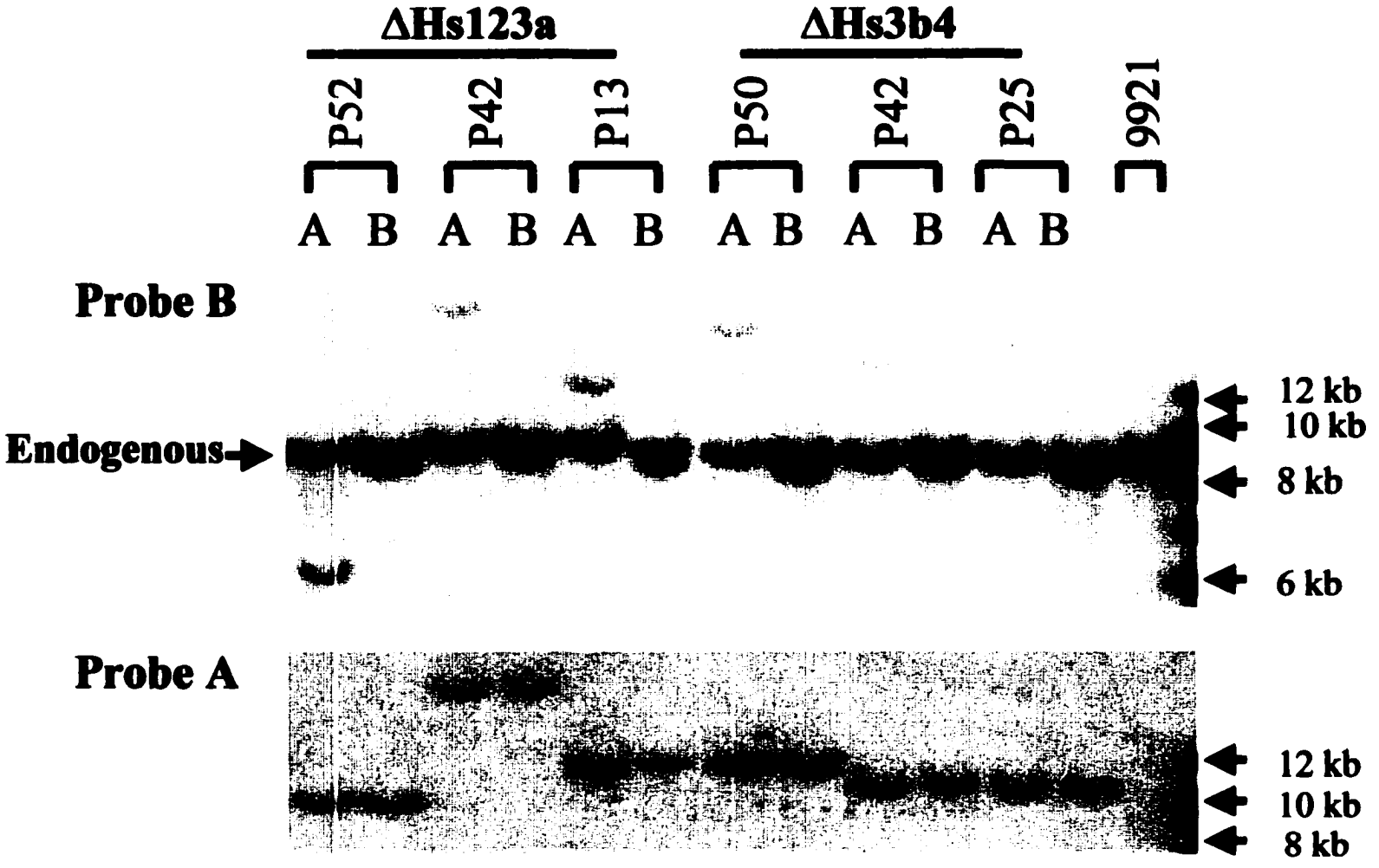


Figure 4-10. γ2b transgene expression in 9921 clones before and after enhancer deletion. Representative northern blot of mRNA isolated from 9921 clones before (B) and after (A) deletion of enhancers. The same blot was sequentially hybridized with a γ2b probe and a GAPDH probe (for sample loading normalization). The left panel shows transformant pairs with the mini-locus that allows *cre*-mediated deletion of Hs3b/Hs4 (ΔHs3b/Hs4) while the right panel is of pairs that carry the mini-locus that yields Hs1,2/Hs3a deletions (ΔHs123a).

Figure 4-10

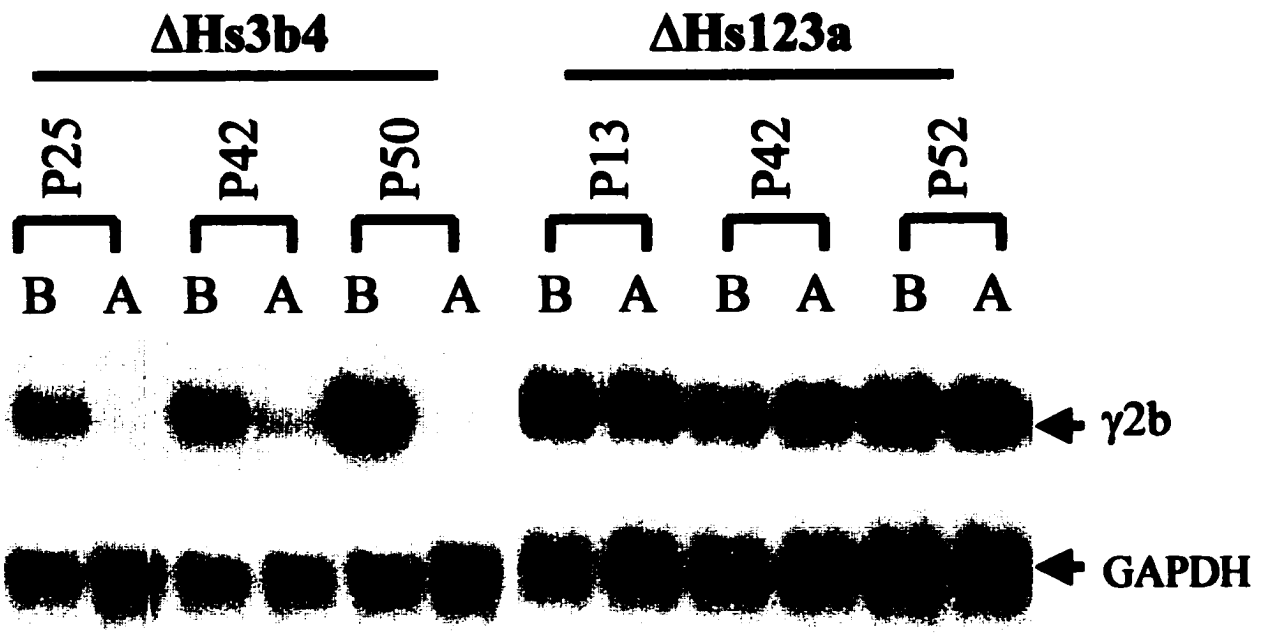


Figure 4-11. Quantitation of northern blot data presented in Figures 4-8 & 4-10.

(A) Data generated from A20 transformant pairs.

(B) **Data generated from 9921 transformant pairs**. Black bars are values for transformants before enhancer deletion and shaded bars are for their subclones, after deletion. Several different exposures of the autoradiographs shown in **Figures 4-8 & 4-10** were quantified by densitometry (Molecular Dynamic Densitometer SI; scanned images analyzed with ImageQuant). Values for γ2b mRNA were normalized with gapdh mRNA values. The normalized γ2b mRNA levels were then compared for each transformant pair, with the value for the transformants before enhancer deletion (black bars) set to 100%.

Figure 4-11

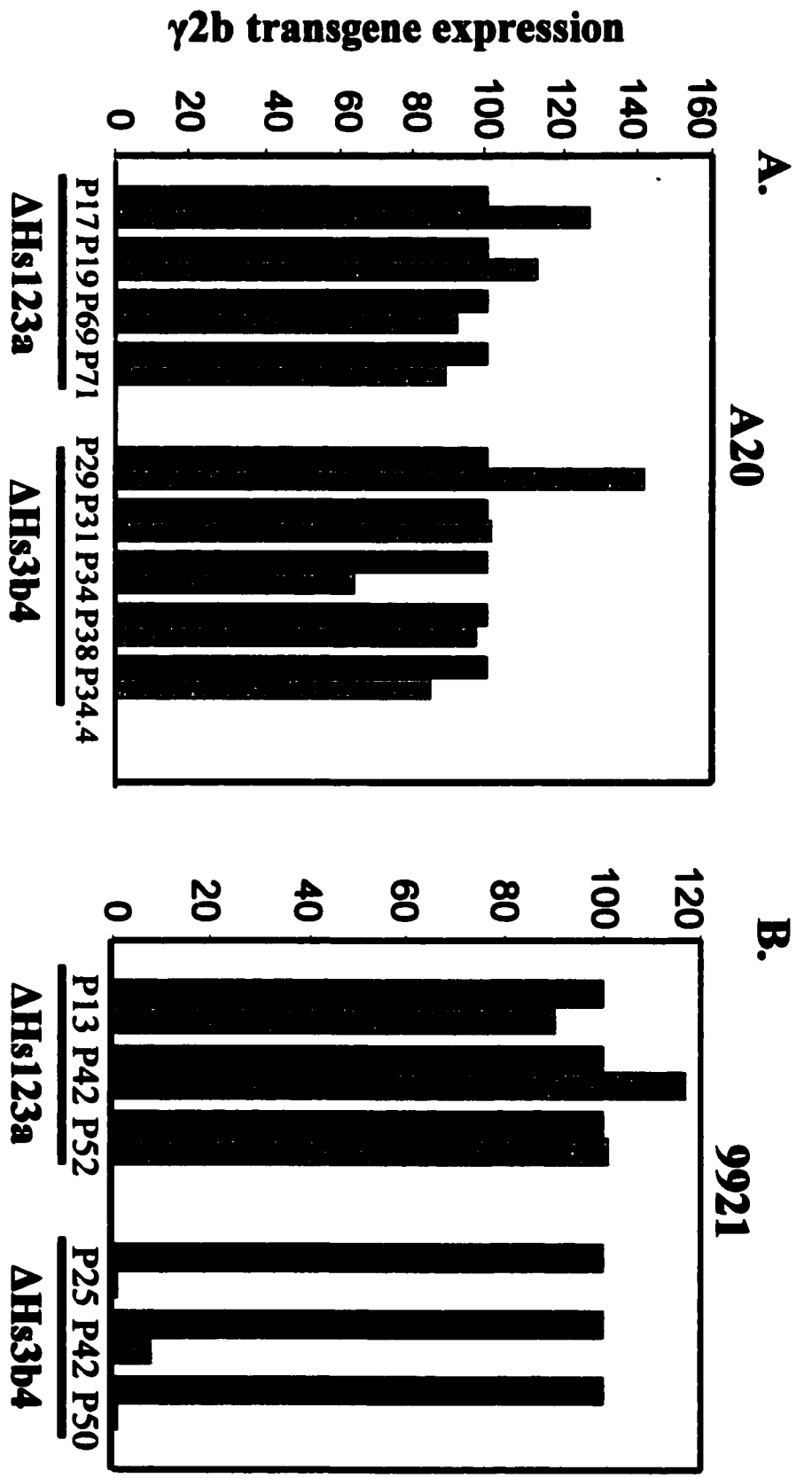


Figure 5-1. Diagram of the 3' region of the murine IgH locus. Black boxes represent 3' IgH enhancers. The restriction enzymes in the region are indicated for ease of reference but they don't represent all of the sites in the region. B: BamHI, Bg: BglII, BgI: BglI, E: EcoRI, H: HindIII, Pst: PstI, S: SacI, Sm: SmaI, Sau: Sau3AI, X: XbaI.

(A) DNA fragments cloned from the 3' region and used in matrix-DNA binding assays. The solid lines labeled with a number represent DNA fragments used to search for existence of MARs (Matrix Attachment regions). The dotted lines with a small letter represent DNA sequences that haven't been assessed directly for MARs.

(B) DNA sequence repeats within the 3' region. Several pairs of inverted repeats (IRs), and tandem repeats (TRs) consist of a symmetric region with virtually identical enhancers, Hs3a and Hs3b at its termini and Hs1,2 at the center. DNA sequences between the two vertical arrows in this region have been sequenced (Chauveau and Cogne, 1996). The solid and dotted lines represent DNA sequences that have been and haven't been assessed for MARs, respectively.

Figure 5-1

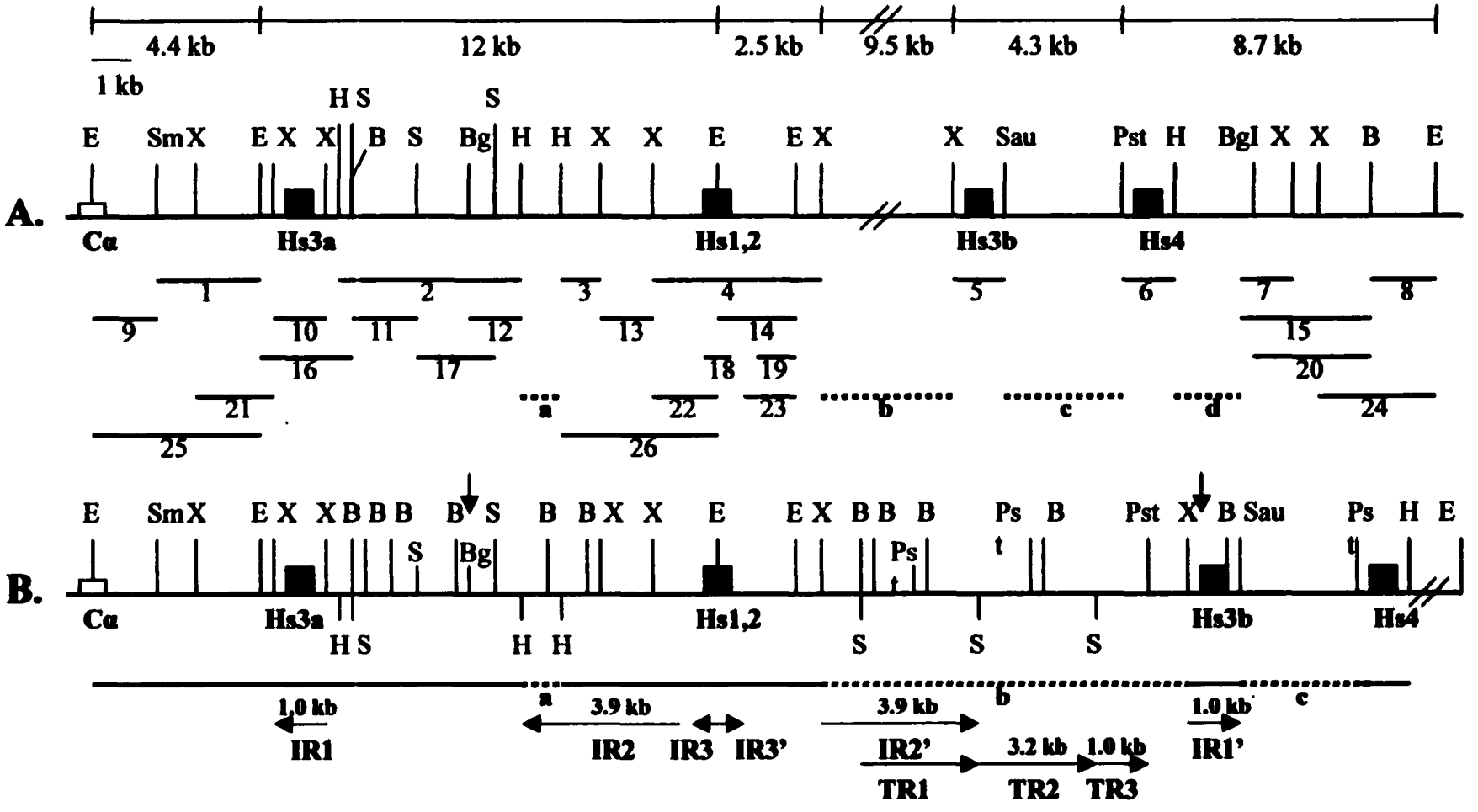


Figure 5-2. DNA-nuclear matrix binding assay. Lanes M1, M2, M3 and M4 are a mixture of the ^{32}P-labeled DNA fragments tested for matrix attachment regions (MARs). Lanes B1, B2, B3 and B4 are the DNA fragments retained by the nuclear matrix proteins after incubation of the DNA fragments with the matrices, followed by washing to disrupt non-specific binding. The series of numbers represent different DNA fragments that were cloned from the 3' region of the murine IgH locus (see **Figure 5-1A**). Eμ and Eκ are DNA fragments containing Eμ/MARs and Eκ/MAR, respectively.

Figure 5-2

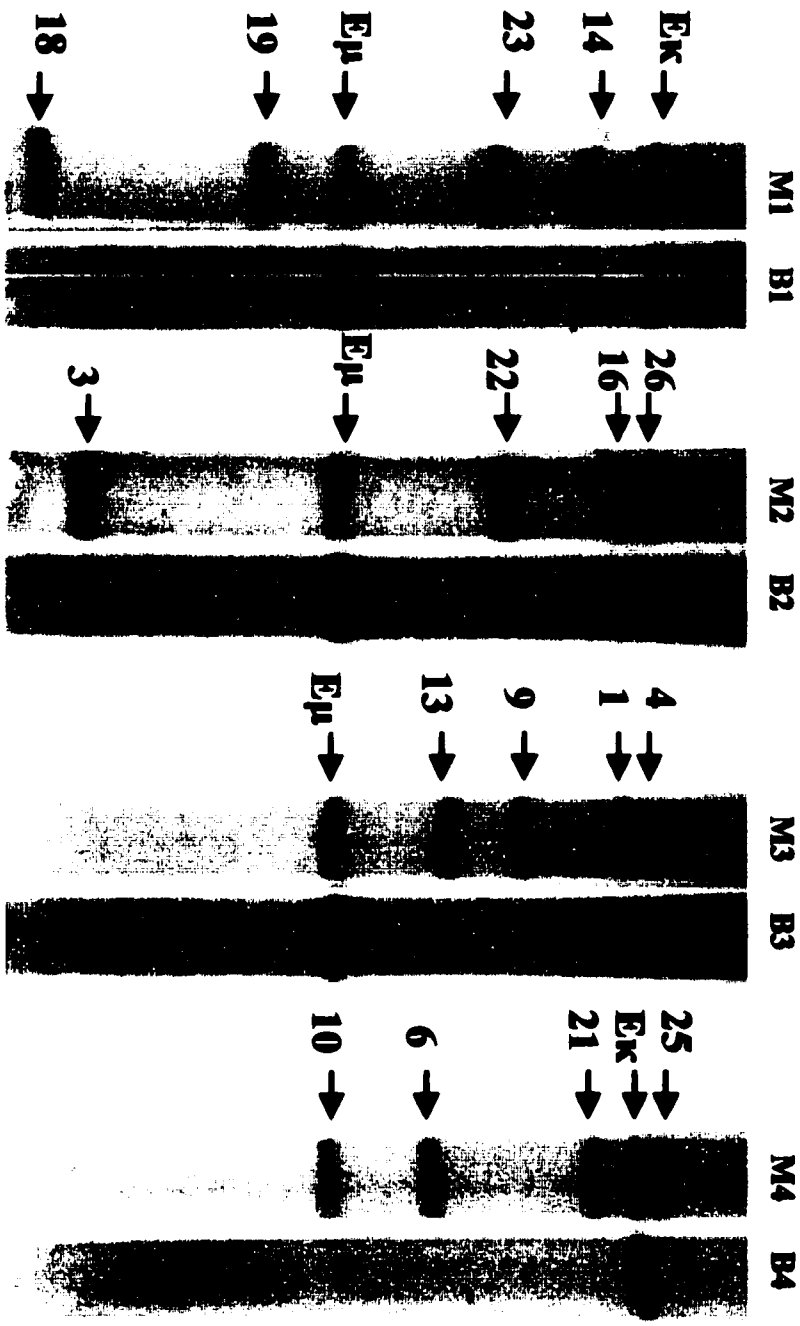


Figure 5-3. DNA-nuclear matrix binding assay. Lanes M5, M6, M7 and M8 are a mixture of the 32P-labeled DNA fragments tested for matrix attachment regions (MARs). Lanes B5, B6, B7 and B8 are the DNA fragments retained by the nuclear matrix proteins after incubation of the DNA fragments with the matrices, followed by washing to disrupt non-specific binding. The series of numbers represent different DNA fragments that were cloned from the 3' region of the murine IgH locus (see **Figure 5-1A**). Eμ and Eκ are DNA fragments containing Eμ/MARs and Eκ/MAR, respectively.

Figure 5-3

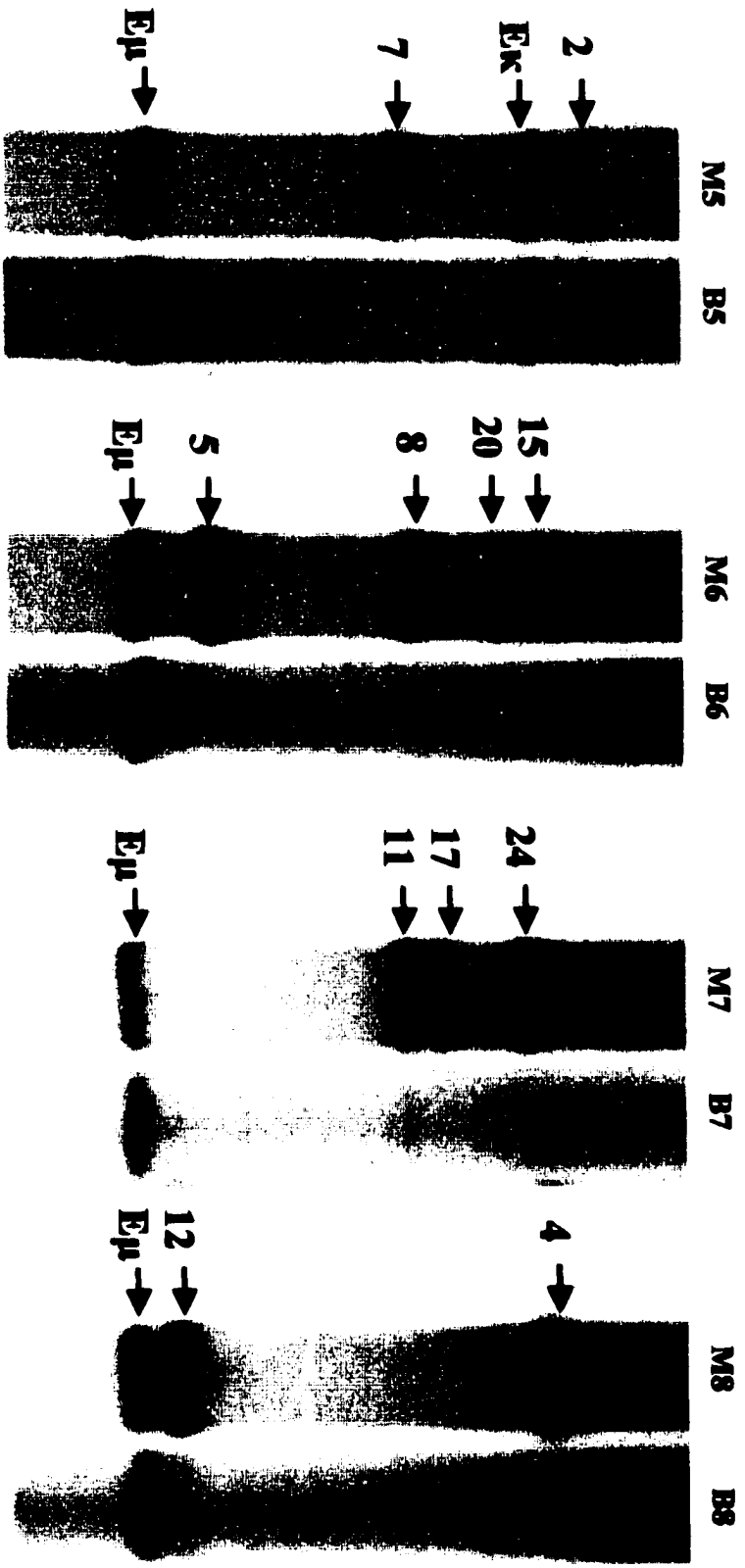


Table 4-1. Frequency of transgene expression in A20 and 9921 transformants

Table 4-1.

Cell lines	Constructs	Number of clones with γ2b transgene	Number of clones expressing γ2b transgene	Percent clones expressing γ2b transgene
9921	γ2bhs1-4*loxp*hs123a	26	25	96%
	γ2bhs1-4*loxp*hs3b4	36	35	97%
	psk-γ2b	13	0	0%
A20	γ2bhs1-4*loxp*hs123a	28	27	96%
	γ2bhs1-4*loxp*hs3b4	28	28	100%
	psk-γ2b	12	0	0%

Table 4-2. γ2b mRNA levels in single-copy transformants

Table 4-2.

Constructs	Cell lines	γ2b mRNA levels in individual clone (% endogenous locus)[a]								
γ2bhs1-4*lox*phs123a	9921	P1[b]	P7	P11	P13	P18	P37	P42	P52	Mean
		11	17	48	30	32	23	20	26	26
	A20	P17	P18	P19	P32	P34	P37	P69	P71	Mean
		24	11	19	12	13	25	46	22	22
γ2bhs1-4*lox*phs3b4	9921	P15	P25	P27	P29	P42	P48	P50	P56	Mean
		15	10	13	30	29	50	40	24	26
	A20	P29	P31	P34	P35	P38	P39	P68	P69	Mean
		16	19	24	12	42	25	28	25	24

[a]γ2b mRNA levels produced by transgenes were compared, by northern blot, to γ2b mRNA levels in a γ2b-producing plasmacytoma (MPC11). GAPDH was used to normalize sample loading.

[b]Individual transformants are given unique designations: P1, P7, etc.

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