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# SHIFTING CONTROL OF IMMUNOGLOBULIN HEAVY CHAIN GENE EXPRESSION

by

**JANE ONG**

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


**Shifting Control of Immunoglobulin Heavy Chain Gene Expression**

by

**Jane Ong**

Adviser: Professor Laurel Eckhardt

Regulation of IgH gene expression is tightly controlled during B cell development. Crucial to this regulation is a group of B-cell specific enhancers; the intronic (Eμ) and several 3' enhancers {Cα3'E (hs3A), 3'αE (hs1,2), hs3B, hs4}, found embedded both upstream and downstream of the constant gene exons of the murine heavy chain gene locus.

To define the activities and inter-relationships of the enhancer elements in the IgH locus, constructs housing various enhancer combinations placed at a distance from a luciferase reporter gene controlled by a genuine $V_H$ promoter were created and transiently transfected into B-cell lines representing various stages of development. The results from these transfections show that the intronic enhancer, Eμ, is active throughout B-cell development at relatively constant levels. In contrast, the 3' IgH enhancers when assayed alone, show very little activity at any stage of B cell differentiation. However, and more importantly, when the 3' IgH enhancers are combined, they have little or no activity in pre-B cells, are strongly active in B cells and moderately active in plasma cells. Therefore, unlike Eμ, the 3' IgH enhancers show a clear change in activity over the course

of B-cell development, with their relative contribution to overall IgH expression being low in early B-cell development, very high at the surface $Ig^+$ stage and roughly similar to Eμ at the plasma cell stage.

Further examinations using different enhancer pairs showed that the functions attributed to each pair changes over the course of B cell differentiation. More enhancer pairs exhibit synergies in Raji cells, very few in S194 and intermediate numbers in M12.4.1 cells.

Taken together, these data comprise the first comprehensive examination of all the known IgH enhancer elements throughout B-cell development.

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Finally, I want to thank my parents (Ong Geok Ho & Ch'ng Siew Ngo), my brothers (Robert & Charlie), my sisters (Joyce, Helen, Katherine & Susy) and all of my nieces and nephews! from the very bottom of my heart for all their sacrifices, their enduring love, their support and their belief in me throughout the years. To them, I owe the most.

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# Chapter 1

## Overview Of B-Cell Development In Mice

## 1.1 - General Introduction

The immune system can be divided functionally into the innate and the adaptive immune system which collectively are responsible for protecting the host against various infectious agents and abnormal cells. Innate immunity, mediated by phagocytes including neutrophil polymorphs, monocytes and macrophages, act as a first line of defense against a wide range of constituents on bacterial surfaces. Binding to those pathogens trigger the phagocytes to engulf and digest them. If these first defenses are breached, adaptive immunity, mediated by lymphocytes is activated and produces a specific reaction to a given infectious agent which initially eradicates that agent, and then maintains specific immunity to prevent later reinfection. Innate immunity differs functionally from the adaptive immunity in that innate immunity is less specific than the adaptive immunity, and does not improve in strength or reactivity with repeated exposures to a given antigen. The lymphocytes of the adaptive immune system are of two kinds; B cells which mediate the humoral response and T cells which govern the cell-mediated response. The humoral response involves production of antibodies, which specifically recognize and bind to infectious agents, while the cell-mediated response is responsible for the actual destruction and removal of virally infected or otherwise abnormal cells. T cells participate in both the cell-mediated and humoral responses, playing a crucial role in activating B cells in the humoral immune response.

An antibody molecule consists of two duplicate immunoglobulin heavy chains

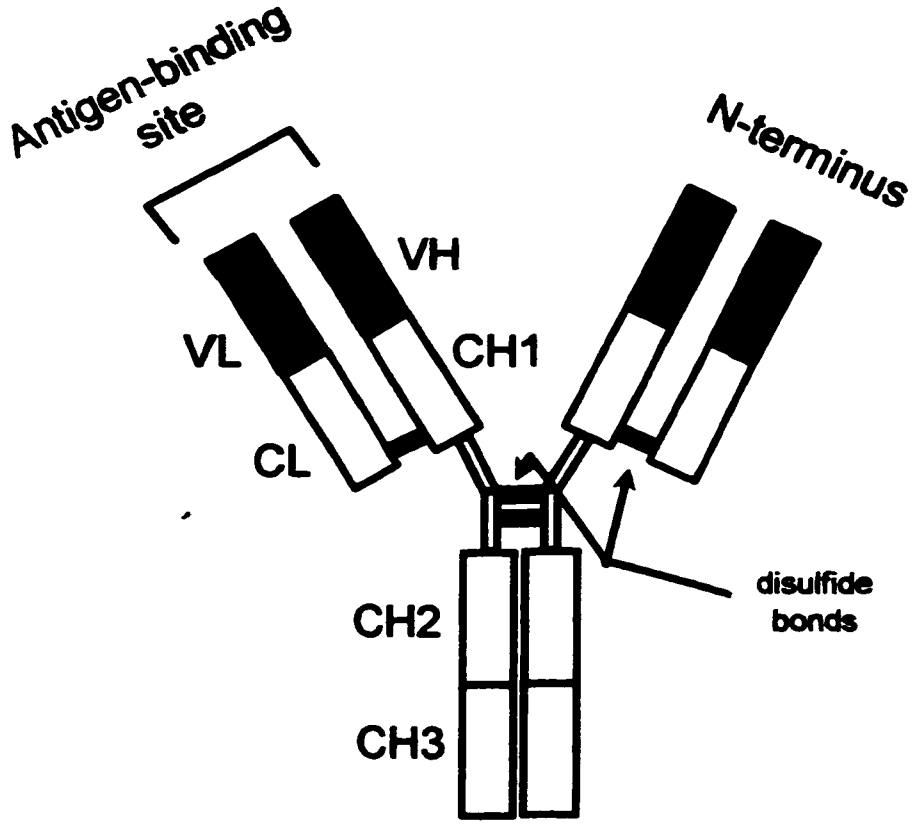
(IgH) and two duplicate immunoglobulin light chains (IgL) held together by disulfide bonds (see Fig. 1). The amino terminus of each chain is a region that varies in amino acid sequence (V-region) with the remaining portion being relatively constant in amino acid sequence (C-region). The V-region of each heavy and light chain pair form the antigen binding site of the molecule (indicated by shaded region in Fig. 1), while the constant region of the heavy chain confers specific effector functions of the molecule.

In the murine system, there is only one IgH chain locus but there are two different types of IgL chain loci; kappa (κ) and lambda (λ). So although there are two chromosomes encoding heavy chains and a total of four chromosomes encoding light chains (κ and λ are on different chromosomes), only one heavy chain and one light chain are finally expressed. This restriction, known as allelic exclusion, means that only one heavy and one light chain gene is expressed in a given cell, while the other gene is either left unexpressed in their germline configuration, or in an appropriate (out of frame) rearrangement. Allelic exclusion indicates an important property of B cells known as 'clonal selection'; a given B cell only expresses a single type of antibody molecule. In other words, after initial rearrangement of heavy and light chain loci, a given B cells will recognize a particular antigenic determinant dictated by the specific combination of heavy and light chains. In this manner, expansion and selection of B cells will continue to improve recognition and eradication of a specific antigen without the risk of production of antibodies of inappropriate specificities.

The murine heavy chain gene on chromosome 12 consists of multiple gene segments - the variable (150 - 200 $V_H$ genes within a region of approximately ~ 2Mb

**Figure 1.**

Schematic representation of an immunoglobulin molecule. It is consisted of two heavy (designated by $V_H$, $C_H$'s) and two light (designated by $V_L$, $C_L$) chains linked together by disulfide bonds. The variable region of both heavy and light chain, together form the antigen binding site of the molecule (N-terminus; shaded region), while the constant region of the molecule (C-terminus; open rectangles) determines the effector function of the molecule.

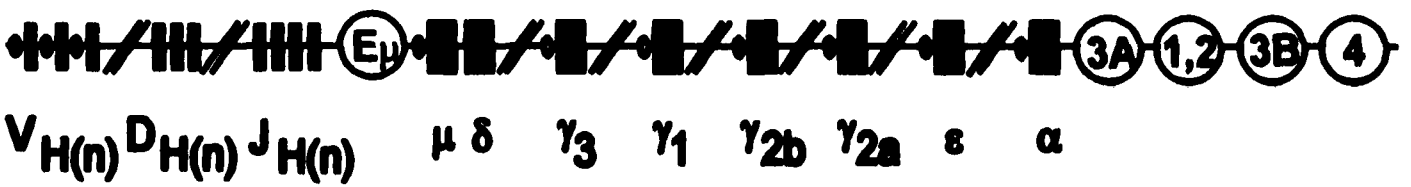


DNA on murine IgH locus (Angelin-Duclos and Calame, 1998), the diversity (≈ 12 $D_H$ genes), the joining (4 functional $J_H$ genes), followed by a stretch of constant gene exons - μ, δ, $\gamma_3$, $\gamma_1$, $\gamma_{2b}$, $\gamma_{2a}$, ε and α. Also found within the gene are enhancers - the intronic Eμ (between $J_H$ and $C_\mu$ gene exon) and the 3' IgH enhancers, downstream of $C_\mu$ gene exon (see Fig. 2). The murine κ light chain gene, on chromosome 6, resembles that of the IgH chain in that, it consists of many variable gene segments (≈ 160 $V_\kappa$ genes), the joining gene segments ( 4 functional $J_\kappa$ genes) but only one constant region gene ($C_\kappa$ gene). Also present are enhancers - κ intronic enhancer (between $J_\kappa$ and $C_\kappa$ genes), and the 3' κ enhancer (downstream of the $C_\kappa$ genes) (see Fig. 2). The murine λ light chain gene, on chromosome 16 on the other hand, represents the smallest gene family and is organized in a different way, containing 2 separated clusters of one $V_\lambda$, $2J_\lambda$ and $2C_\lambda$ gene segments. Each of these clusters contains a homologous enhancer element located 3' of the most 3' $C_\lambda$ segments. The $E_\lambda$2-4 enhancer is located 16 kb downstream of $C_\lambda$4 and $E_\lambda$3-1 enhancer located 35 kb downstream of $C_\lambda$1 (see Fig. 2).

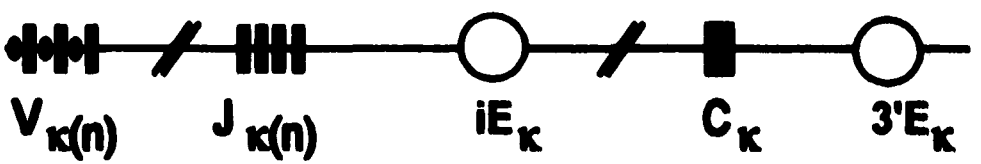
All the cells of the immune system arise from pluripotent hematopoietic stem cells in the bone marrow through two main lines of differentiation; the lymphoid lineage - producing B and T lymphocytes, and the myeloid lineage - producing phagocytes and other cells such as erythrocytes, polymorphonuclear leukocytes and blood platelets. Both B and T cells originate from lymphoid precursors in the bone marrow, but while B cells mature and differentiate in the bone marrow, T cells migrate to the thymus where they mature and differentiate. B cell development is a multistage differentiation process that ultimately generates antibody-secreting plasma cells. It begins with cells expressing the

**Figure 2.**

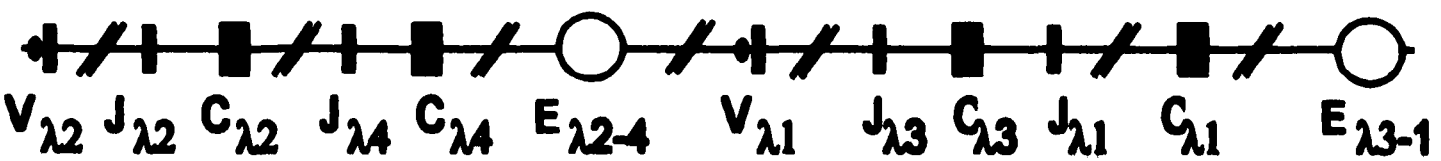
Schematic maps of the murine immunoglobulin heavy, kappa (κ) & lambda (λ) light chain loci in germline DNA configurations. The bigger filled rectangle boxes represent the constant region exons, the narrower filled rectangle boxes represent either the variable, diversity and joining regions of heavy or light chain loci. Circles denote the various enhancer elements found within the respective loci. Grey ovals refer to the switch recombination sites upstream of each constant region exon except $C_\delta$ gene exon while the black ovals represent the promoter upstream of each variable region gene segment.




Heavy chain
germ-line DNA




κ
germ-line DNA




λ
germ-line DNA

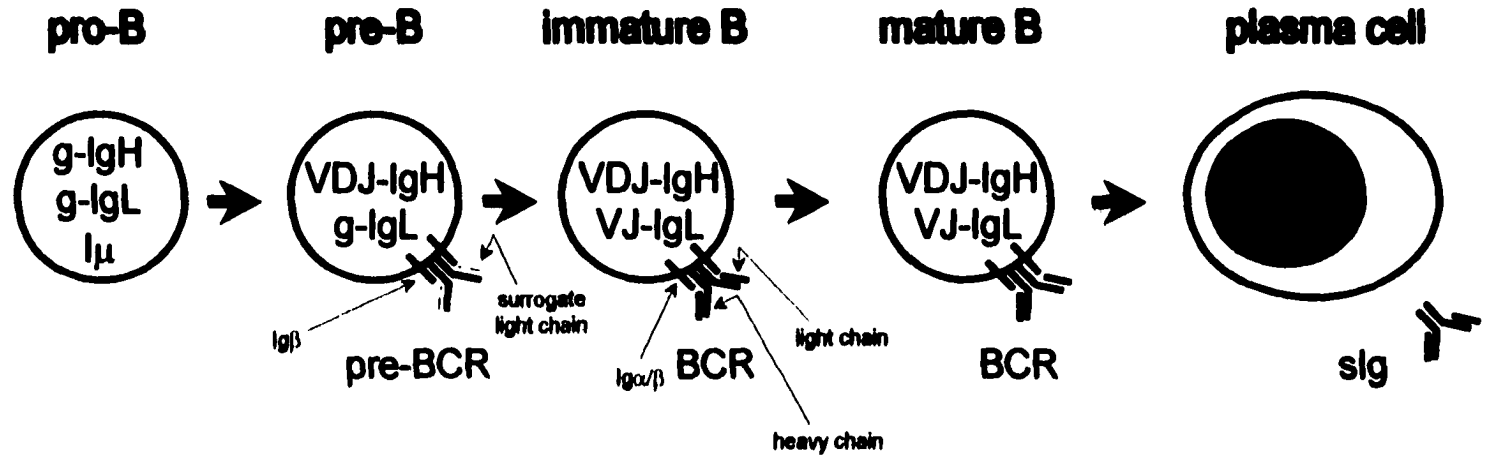
surface antigen characteristic of B cells, such as B220, associating with the stromal cells. B220 is an isoform of the transmembrane tyrosine phosphatase CD45, found in all hematopoietic cells, its precise function in B cell development is still unknown. Association with stroma cell is necessary because it promotes the binding of their surface Kit receptor tyrosine kinase to stem cell factor (SCF) on the stromal cell surface, activating the kinase and inducing proliferation.

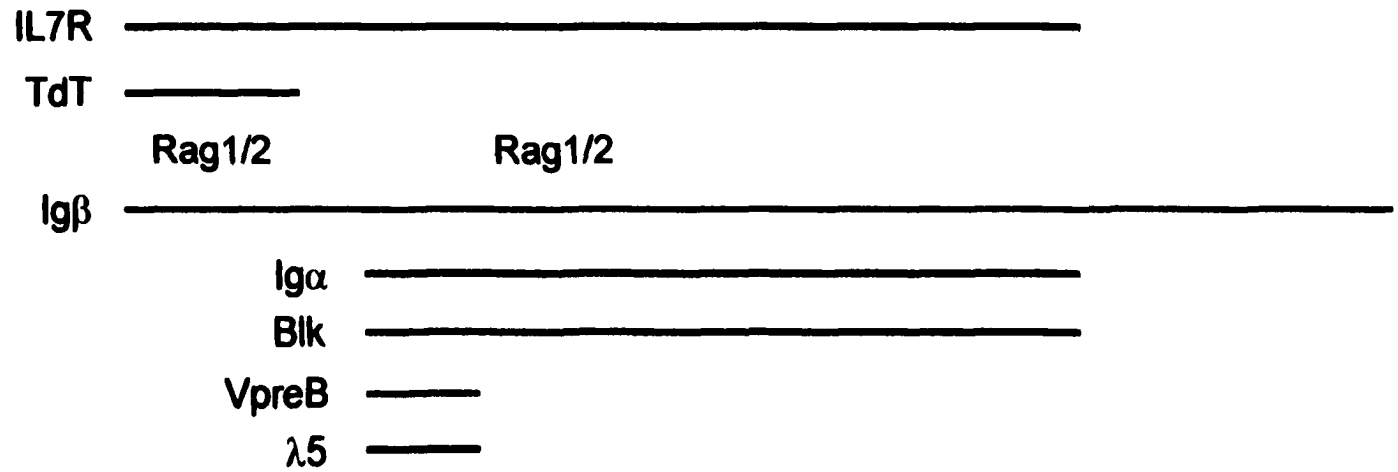
These B cell progenitors (pro B cells, Fig. 3), carry their Immunoglobulin (Ig) genes in germline configuration, but produce sterile transcripts Iμ, from the IgH chain locus, which are thought to help initiate the rearrangement process by 'opening' the chromatin to allow access of the various components of the recombinase machinery. Pro B cells also contain transcripts encoding Terminal Deoxynucleotide Transferase (TdT), IL-7 receptor and Igβ (B29) (reviewed in Liberg and Sigvardsson, 1999). TdT, involved in increasing antigen receptor diversity by adding nucleotides in a template independent manner into the recombination junction of IgH, and T cell receptor (TCR) genes; IL-7 receptor, for binding to soluble IL-7 secreted from the stromal cells, required for pro-B cell growth and maturation; and Igβ (B29), an essential part of the B cell receptor complex (BCR) where it acts as a signal transducer. Subsequent differentiation of these cells results in activation of the recombinase activating genes (RAG1 and RAG2), allowing for initiation of recombination events in IgH loci by the late pro B cell stage (see Fig. 3).

VDJ recombination events on the IgH gene loci occur in an orderly fashion. First, one of the $D_H$ gene segments is rearranged to one of the $J_H$ gene segments (reviewed in

**Figure 3.**

Schematic diagram showing the stages of B cell development starting with pro-B cell and ending up with plasma cell stage, with emphasis on the recombination status of both the heavy and light chain loci: g, germline; VDJ or VJ, the rearranged heavy chain and light chain loci respectively and Iμ, the sterile μ transcript. Right below that is the expression pattern of some of the genes related to B cell development discussed in the text.



Melchers et al., 1999). This occurs on both heavy chain alleles in late pro B cells stage, thus at this stage, heavy chain genes are not allelically excluded. Next, one of the $V_H$ gene segments are rearranged to the DJ complex. In mouse B cell development, approximately 50% of all B cells become VDJ rearranged on one allele and DJ rearranged on the other, while the other 50% have VDJ rearranged on both alleles. In the VDJ/DJ rearranged B cells, the VDJ heavy chain allele is productively rearranged while in the majority but not all VDJ/VDJ rearranged cells, only one allele is productively, the other non-productively rearranged (Melcher et al., 1999). The VDJ rearrangement process involves recognition of specific recombination signal sequences flanking the Ig gene segments (reviewed in Rubin & Thompson, 1998). Recombination signal sequences (RSS) consist of conserved heptamer and nonamer motifs separated by 12 or 23 bp spacers. VDJ recombination only occurs between sites with different spacers (the 12/23 rule). Two proteins, RAG1 and RAG2, bind to the RSS and together carry out VDJ recombination. The steps include a single strand nicking of the DNA at the cleavage site and a nucleophilic attack by the resultant 3' hydroxyl on an adjacent phosphodiester bond of the opposing strand. The result is a double strand break with a sealed "hairpin" loop on the coding end. The sealed coding ends, for example the $D_H$ gene segment, are then joined together to the sealed coding end of the $J_H$ gene segment with the help of DNA-dependent protein kinase (DNA-PK), a serine/threonine kinase implicated in repair of DNA double strand breaks. TdT adds nucleotides in a template - independent manner into the recombination junction of IgH gene, creating additional diversity in antigen binding receptor. If the VDJ joining is in frame, the μ heavy chain protein can be expressed and exhibited on the surface.

These cells are now designated 'pre B cells'.

Once at the pre B stage, the cells produce μ heavy chain protein, and additional B cell-specific proteins such as Blk, Igα, λ5 and VpreB (Fig. 3). λ5 and VpreB become non-convalently linked, forming a surrogate light chain that can associate with the rearranged IgH chain, form an Ig-like signalling complex. This surrogate receptor subsequently associates with two other transmembrane molecules, Igα and Igβ, forming a pre B cell receptor complex (preBCR). The preBCR is critical to early pre B cell survival and progression to subsequent developmental stages; homozygous disruption of the λ5 gene, the Igβ gene, or the transmembrane domain of the IgH gene, all lead to developmental blockade during or immediately after IgH rearrangment (Erlich et al., 1993; Gong et al., 1996; Kitamura et al., 1992). Pre B cells expressing a preBCR on their cell surface enter proliferation and generates multiple signals, inhibiting further germline transcription and rearrangment of IgH loci. This positive selection for functional IgH rearrangement allows for reactivation of RAG genes and initiation of IgL chain recombination.

Rearrangement at κ light chain loci may begin before those at the λ light chain gene loci reviewed in (Melchers et al., 1999). However, it is apparent in κ light chain gene rearrangement-deficient ($J_\kappa C_\kappa^{-/-}$) or κ light chain protein deficient ($C_\kappa^{-/-}$) mice that rearrangment at λ light chain loci can occur independently of those at κ light chain locus. In mice, there is a skewing of B cells toward the κ chain isotype - as many as 95 % of B cells produce κ light chains (Takemori and Rajewsky, 1981). After successful completion of IgL rearrangment, a similar complex containing IgH, IgL, Igα and Igβ, the B cell receptor complex (BCR) is expressed on the cell surface which initiates a distinct

signaling cascade resulting in transcriptional repression of the RAG genes and loss of all VDJ recombination activity in the cell. These cells are then referred to as immature B cells (Fig.3).

DNA germline rearrangement is not restricted to immunoglobulin heavy and light chains of B cells. Early in development, T cells also undergo rearrangement of germline DNA segments of their α, β, γ and δ loci in order to construct functional T cell receptor (TCR) transcription units in a manner analogous to that of Ig assembly in B cells. Formation of the variable region exon antigen receptor genes (generally referred to as VDJ recombination) is restricted to immature lymphoid cells. Clearly the process is tissue specific since rearrangement is confined largely to Ig genes in B cells and to TCR genes in T cells.

At the immature B cell stage, the cells undergo selection against 'self specificity'. Immature B cells that express receptors that recognize ubiquitous self cell-surface antigens such as those of the major histocompatibility complex (MHC), are deleted from the repertoire. These B cells are believed to undergo programmed cell death or apoptosis. Although most of the B cells recognizing self MHC are destined to die in the bone marrow, some continue to express RAG1 and RAG2, allowing for ongoing rearrangement of their endogenous light chain genes. The rearrangement of their endogenous light chain can then replace the "autoreactive" light chain with one that does not recognize self antigen, a process that is known as receptor editing. Immature B cells that bind soluble self antigens are rendered unresponsive or anergic to the antigens and lose surface IgM. They migrate to the periphery where they express IgD but remain

anergic. Immature B cells that do not encounter antigen in the bone marrow at this early stage of development mature normally, and migrate from the bone marrow to the lymphoid follicles of lymph nodes and spleen and other peripheral lymphoid tissues as mature B cells bearing both IgM and IgD on their cell surface (Fig. 3).

When a mature B cell encounters an antigen for which it carries a specific BCR, the cell responds by activation, proliferation, and differentiation into clonal population of effector cells capable of secreting antibodies with a specificity identical to that of the surface receptor. Depending upon the particular antigen and the responding B cell, these activating events may or may not require participation of T cells.

In T-cell independent activation events, B cells are activated simply by exposure to the antigen. There are two classes of T-independent antigens. Antigens in the first class, the T-independent type 1 (TI-1) antigens such as bacterial lipopolysaccharide, contain an intrinsic activity that can directly induce the proliferation of B cells. At high concentration, these molecules cause the proliferation and differentiation of all B cells, regardless of their antigen specificity; this is known as polyclonal activation. When B cells are exposed to much lower TI-1 antigen concentration, only those B cells whose Ig receptors bind these TI-1 molecules become activated. The second type of T-independent antigen (TI-2) consists of molecules such as bacterial cell wall polysaccharides that have highly repetitive structures. These TI-2 antigen contain no intrinsic B cell stimulating activity and only activate mature B cells most probably via multiple cross-linking of the BCR which then leads to antibody production.

In T-cell dependent B cell activation events, binding of T cell dependent antigens

such as protein antigen, triggers B cell to internalize the antigen and to return the processed antigen to the cell surface as peptides bound to MHC II molecules. This antigen presenting B cell then binds to specific helper T cells. Once bound, the helper T cell is triggered to express CD40 ligand (CD40L) and begin secreting IL4 and other cytokines. CD40L on the T cell then interact with CD40 molecules on the B cell, resulting in B cell proliferation. This is followed by the B cells differentiating into plasma cells aided by IL4, IL5 and IL6. The importance of CD40/CD40L interactions for B cell activation is illustrated by experiments in which this interaction is blocked experimentally with antibodies against CD40 or CD40L, or genetically in the human disease X-linked hyper-IgM syndrome, in which T-dependent antibody responses are defective (Allen et al., 1993; Aruffo and Seed, 1987; Liu et al., 1989). While proliferation of B cells occurs first in the T cell areas, some proliferating cells migrate to the centre of the lymphoid follicles to establish germinal centres (GC).

GCs are complex structures that form in lymphoid tissues such as spleen and lymph nodes in response to antigenic challenge. There are three histologically distinct microenvironments within the germinal centre: a mantle zone containing small resting B cells that are not specific for antigen, surrounding an interior dark (containing dividing centroblasts) and light (containing non-dividing centrocytes) zones. In the dark zone, antigen triggers activation of the densely packed B cells to undergo intensive proliferation, coupled with somatic hypermutation, isotype switching and receptor editing.

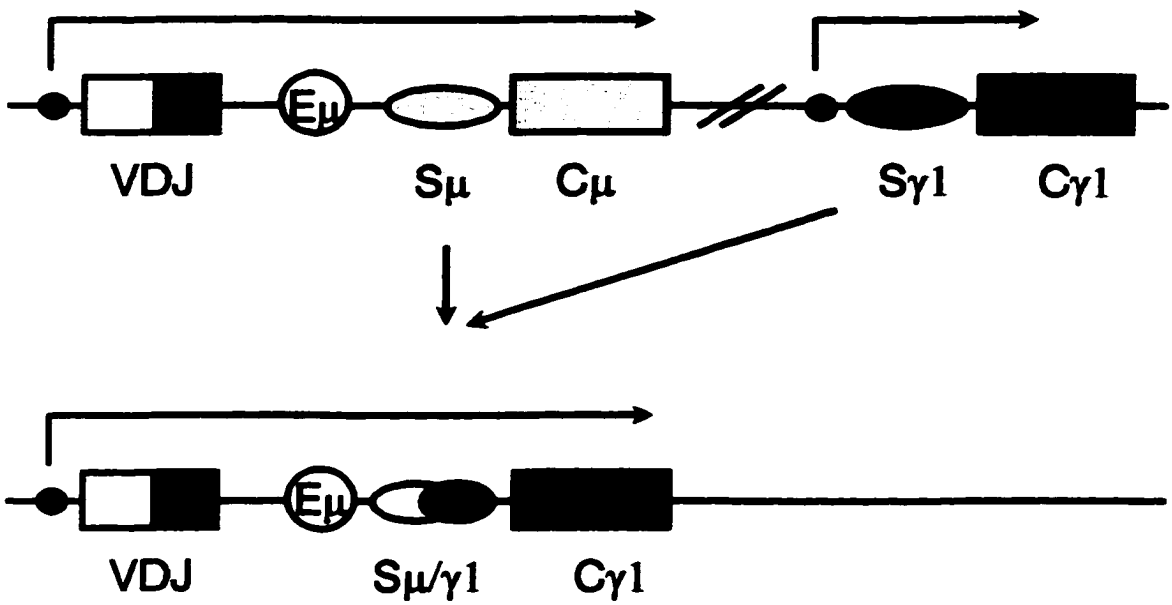
Somatic hypermutation of Ig genes of B cells within the GC dark zone

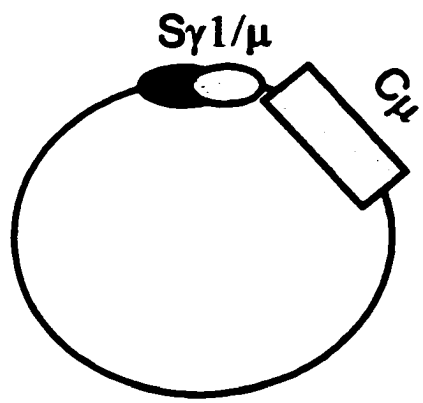
dramatically increased the mutation rate (up to $10^{-3}$ mutations per base per cell division) within the gene segments encoding both the IgH and IgL variable genes (reviewed in Lieberg et al., 1998). This process increases the diversity within the antigen binding pocket of the Ig receptor, altering the binding specificity. Isotype switching is yet another mechanism to increase the diversity of the antibody molecule. In an activated B cell, signals from engagement of both transmembrane and intracellular receptor can initiate low levels of transcription of germline $C_\gamma$, $C_\alpha$, or $C_\varepsilon$ loci from promoters upstream of the isotype switch region. These germline transcripts are driven by cytokine signals and are an essential part of the mechanism leading to switch recombination. In mice, IL4 was demonstrated to preferentially induce switching to IgG1 and IgE, IFN-γ induced switching to IgG2a and IgG3 while TGF-β induced switching to IgG2b and IgA. Isotype switching involves recognition of specialized, repetitive DNA regions upstream of each $C_H$ exon except $C_\delta$ by a hypothetical "switch recombinase", double strand break and religation of the switch region of IgM ($S_\mu$) with an S region from another $C_H$ and other isotypes. The extrachromosomal excised segment is religated and is lost with progressive cell divisions of the responding B cell clone (see Fig. 4).

B cell clones emerging from within the dark zone then enter the light zone where they contact antigen-bound to follicular dendritic cells (FDCs) and antigen-specific T-helper cells. Centroblasts stop dividing, becoming centrocytes and are subjected to selection for high affinity antibodies. Diminished binding for antigen leads to programmed cell death and rapid clearance by resident macrophages. Improved binding of antigen results in positive selection. Positive selection may lead to re-entry into the

**Figure 4.**

Diagram illustrating the events occurring in class-switching process, from IgM to IgG1. Circle refers to intronic enhancer (Eμ), ovals, Sμ, Sγ1, the switch signals upstream of Cμ and Cγ1 respectively and the promoters, represented by a small circle upstream of the rearranged VDJ region and the switch region of γ1. The arrow above the small circle indicates the direction of transcription with respect to the appropriate promoters.



dark zone and retention in the GC reaction for another cycle of expansion, diversification and selection or exit from GC micoenvironment and differentiation into either memory cells or plasma cells, where they migrate to the medullary cords of the lymph node or to bone marrow (Fig. 3).

Overall, B cell development appears to be governed by the stringent selection of cells with an appropriate Ig receptor on their cell surface. An understanding of the molecular mechanisms that modulate the expression of these Ig genes would enhance our understanding of the dynamics of B cell development.

In this thesis, the focus is on gaining a better understanding of the transcriptional control of the IgH locus over the course of B cell development, emphasizing the function of the various enhancers associated with the IgH locus.

# Chapter 2

## Materials & Methods

## 2.1 - Cell Lines

The following cell lines were utilized. Cells representative of the Ig secreting stage - S194 (BALB/c, plasmacytoma α,κ; a gift of Dr. Birshtein's laboratory), P3X63Ag8 (IgG1) (American Type Culture Collection), 9.9.2.1 (BALB/c, myeloma, γ2a,κ); pre-B cells: 18-81 (a gift of Dr.Birshtein's laboratory), 18-8 (a gift of Dr. Radomska); murine surface Ig positive B-cells - A20 (IgG) and M12.4.1 (IgG2a) (gifts from Dr. Birshtein's laboratory); human surface Ig positive B-cells: Namalwa and Raji cells (gifts from Dr. Roeder's laboratory). P3X63Ag8, 9.9.2.1, Namalwa and Raji cells were maintained in DME (Life technologies - cat # 12100-061), 15 % BCS (Bovine Calf Serum) (HyClone Laboratories, Logan, UT; cat # SH30072.03) and the remainder of the cell lines were maintained in RPMI 1640 medium (Gibco - BRL, Grand Islands, NY; cat # 31800-089), 20 % BCS (HyClone Laboratories, Logan, UT; cat # SH30072.03), 1 % penicillin-streptomycin (Life Technologies; cat# 15140-122), 2 mM L-Glutamine and 50 μM β-mercaptoethanol (Sigma, cat # M-7522 ). All cells were maintained @ $37^0$ C in an atmosphere of 7-8 % $CO_2$.

## 2.2 - Vector Constructions

### 2.2(a) - Enhancer Synergies Constructs

**pGL2BV**, the basic construct, was made by inserting the 140 bp Kpn I/Bgl II $V_H$ promoter fragment (obtained as a PCR product from the construct $V_{9.7.1}$ E-P ( Zaller, 1985) into the Kpn I/Bgl II sites on the luciferase basic vector pGL2 (Promega). All the other test constructs were derived from this basic construct by inserting the appropriate enhancer element(s) downstream of the reporter gene. (Fig.11; construct # 1 )

**pGL2BV(Eμ)** was made by isolating the 1 kb (Eμ) fragment as a Bam HI/Sal I digest of the vector pBS-Eμ, and then ligating the isolated fragment to Bam HI/Sal I digested pGL2BV. (pBS-Eμ was made by ligating the 1 kb Xba I digested (Eμ) fragment to the Xba I digested pBS vector)

**pGL2BV(hs3A)**, was made by removing the 1 kb (hs3A) fragment (accession no. U65625) from the plasmid pBS-(hs3A) by Bam HI/Sal I digestion, and then inserting it into the Bam HI/Sal I sites of pGL2BV. (pBS-(hs3A) was the product of subcloning the 1 kb germline derived Xba I fragment (hs3A) into an Xba I digested pBS vector).

**pGL2 BV(hs1,2)**, was made by isolating the 4 kb (hs1,2) fragment (accession no. X96607) as a Bam HI/Sal I digest from pλM2-(hs1,2), and ligating it to Bam HI /Sal I digested pGL2BV. (pλM2-(hs1,2), was made by subcloning the 4 kb (hs1,2) Xba I fragment from the phage clone pλM2 (as described in Lieberson, 1995) into an Xba I digested pBS vector (Blue-Script- Stratagene)).

**pGL2BV(hs3B)**, and **pGL2BV(hs4)** were both generated from the vector **pGL2BV(hs4,3B)**.

**pGL2BV(hs4,3B)**, was made by isolating the 2.5 kb (hs3B,4) BssH II fragment from the HS3.4 plasmid (a gift from Dr. Groudine's laboratory Madisen and Groudine, 1994), and ligating it to a Sal I- digested vector pGL2BV, after converting the Sal I cohesive ends to blunt ends with Klenow.

**pGL2BV(hs3B)** was generated by digesting pGL2BV (hs4,3B) with Spe I/Xho I to remove the (hs4) fragment. The remaining fragment was made blunt using Klenow and religated.

**pGL2BV(hs4)** was made by digesting pGL2BV(hs4,3B) with Sac I to remove the (hs3B) fragment (accession no. S74164). The remaining fragment was then religated.

**pGL2BV(hs3A,1,2,3B,4)** was made by ligating a blunt-ended 2.5 kb BssH II fragment containing both hs3B and hs4, with pGL2BV(hs3A,1,2) (described below) cut with Sal I and blunt-ended with Klenow.

**pGL2BV(hs4,3B,3A,1,2,Eμ)** was made by digesting the pGL2BV(hs3A,1,2,Eμ) vector with Bam HI, the ends made flush with Klenow, and then ligated to a 2.5 kb blunt-ended BssH II-digested (hs3B,4) fragment obtained from the HS3.4 plasmid.

**pGL2BV(hs3A,1,2,Eμ)** was generated by linearizing the pGL2BV(hs3A,1,2) construct with Sal I and then ligating it to the 1 kb Sal I fragment of Eμ) isolated from pESgpt (Zaller, 1987).

**pGL2BV(hs1,2,3B)** and **pGL2BV(hs1,2,4)** were both made from **pGL2BV (hs1,2,4,3B)**.

**pGL2BV(hs1,2,4,3B)** was made by ligating Xho I-digested pGL2BV(hs4,3B) made blunt with Klenow, to a 4 kb Xba I (hs1,2) fragment isolated from pλM2-(hs1,2) similarly blunt-ended.

**pGL2BV(hs1,2,3B)** was generated by digesting pGL2BV (hs1,2,4,3B) (described earlier) with Spe I/Xho I to remove the (hs4) fragment. The resulting fragment was blunt-ended and religated.

**pGL2BV(hs1,2,4)** was subsequently made by digesting pGL2BV (hs1,2,4,3B) with Sac I to remove the (hs3B) fragment. The remaining fragment was religated.

**pGL2BV(hs3A,1,2)**, the 5 kb (hs3A,1,2) fragment was isolated from the vector pBS(hs3A,1,2) by Bam HI/Sal I digestion and ligated to Bam HI/Sal I- digested pGL2BV. (pBS(hs3A,1,2), was made by a 3 piece ligation consisting of 1 kb Xba I (hs3A) fragment, 4 kb Xba I (hs1,2) fragment, and an Xba I digested pBS vector).

**pGL2 BV(hs1,2,Eμ)** was generated by ligating Sal I-digested pGL2 BV(hs1,2) with the 1 kb Sal I (Eμ) fragment isolated from pESgpt (Zaller, 1987).

**pGL2BV(Eμ, hs3A)** was made by ligation of Sal I-digested pGL2 BV, the 1 kb Bam HI/Sal I (hs3A) fragment and the 1 kb Sal I (Eμ) fragment from pESgpt.

**pGL2BV(Eμ, hs3B)** and **pGL2BV(Eμ, hs4)** were both generated from **pGL2BV(Eμ, hs4,3B)** .

**pGL2BV(Eμ,hs4,3B)** was made by ligating blunt-ended Xho I digested pGL2BV(hs4,3B) to a 1 kb Sal I-digested (Eμ) fragment (isolated from pESgpt), also blunt-ended.

**pGL2BV(Eμ, hs3B)** was generated by digesting pGL2BV(Eμ,hs4,3B), described

above, with Spe I/Xho I to remove the (hs4) fragment. The remaining fragment was made blunt with Klenow and then religated.

**pGL2BV(Eμ, hs4)** was made by digesting the pGL2BV(Eμ,hs4,3B) vector with Sac I enzyme to remove the (hs3B) fragment. The remaining fragment was religated.

**pGL2BV(hs 3B,3A)** and **pGL2BV(hs4,3A)** were both generated from the initial vector, **pGL2BV(hs 3B,4,3A)**.

**pGL2BV(hs 3B,4,3A)**, was made by blunt-ending the Bam HI digested pGL2BV(hs3A) construct, and then ligating to the 2.5 kb BssH II digested fragment containing (hs3B,4) which was also blunt-ended.

**pGL2BV(hs 3B,3A)** was generated by digesting pGL2BV(hs 3B,4,3A) with Spe I/Xho I to remove the (hs4) fragment. The remaining fragment was made blunt with Klenow, and religated.

**pGL2BV(hs4,3A)** was made by digesting pGL2BV(hs 3B,4,3A) with Sac I to remove the (hs3B) fragment. The remaining fragment was re-ligated.

**pGL2BV(hs3A,3A)** was made by ligating Bam HI/Sal I digested pGL2BV with a 2kb Bam HI/Sal I digested (hs3A,3A) fragment from pBS(hs3A,3A). (pBS (hs3A,3A) was made by subcloning the 1 kb Xba I-digested (hs3A) fragment into an Xba I-digested pBS vector and screening for multimers).

**pGL2BV(Eμ,Eμ)**, was made by ligating Sal-I digested pGL2BV(Eμ) with the 1 kb Sal I (Eμ) fragment isolated from pESgpt.

**pGL2BV(hs1,2,1,2)**, was made by the ligation of a Klenow filled-in Sal I digested pGL2BV(hs1,2) with a blunt-ended 4 kb Xba I (hs1,2) fragment isolated from

pλM2-(hs1,2).

**pGL2BV(hs3B,3B)** was made by ligating a blunt-ended Sac I digested 1.2 kb (hs3B) fragment to a blunt-ended Not I digested pGL2BV(hs3B).

**pGL2BV(hs4,4)** was made by ligating blunt-ended Spe I-digested pGL2BV(hs4) to a blunt-ended 1.4 kb Sac I (hs4) fragment isolated from HS3.4.

Since all the above constructs were generated without taking the enhancers' relative positions into consideration, we generated two versions of the test construct pGL2BV(hs1,2,4,3B)- pGL2BV(hs1,2,3B,4), and pGL2BV(hs1,2,3B,4)*, and one version of the test construct pGL2BV(hs3A,1,2,3B,4)- pGL2BV(hs3A,1,2,3B,4)* to investigate the effect of having "correct" enhancer orientations on the expression of a transfected reporter gene (Fig. 32; constructs 46-50). The difference between pGL2BV(hs1,2,3B,4) (construct 46), and pGL2BV(hs1,2,3B,4)* (construct 47) is the orientation of hs1,2,3B,4 with respect to the luciferase reporter gene. In construct 46, hs1,2,3B,4 is oriented such that hs1,2 is the farthest away from the reporter gene, while in construct 47, hs1,2 is closest to the reporter gene. As for pGL2BV(hs3A,1,2,3B,4) (construct 49) and pGL2BV(hs3A,1,2,3B,4)(construct 50*), the difference here lies in the orientation of hs3A and hs3B. In construct 49, hs3A and hs3B are oriented in the same direction while in construct 50*, hs3A are oriented opposite to hs3B as it would normally be in the chromosomal context.

**pGL2BV(hs1,2,3B,4)** was made by digesting pGL2BV(hs4,3B) with Not I, blunting with Klenow and then ligating to a flush-ended 4 kb Xba I-digested (hs1,2) fragment (isolated from pλM2-(hs1,2)).

**pGL2BV(hs1,2,3B,4)*** was made by digesting the above pGL2BV(hs1,2,3B,4) construct with Sal I then religating. This was followed by screening for the insert positioned 5' (hs1,2,3B,4) 3' with respect to the luciferase reporter gene.

**pGL2BV(hs3A,1,2,3B,4)*** was made in 2 steps. First, an intermediate construct **pλM2-(hs 3A,1,2)** was made by cutting pλM2-(hs1,2) with Sal I, blunt-ending with Klenow and ligating with a blunt-ended 1 Kb Xba I-digested (hs3A) fragment. Next, the (hs3A,1,2) fragment was isolated as Bam HI/Sph I digestion from pλM2-(hs 3A,1,2), the fragment was then blunt-ended and ligated to blunt-ended Not I- digested pGL2BV(hs4,3B).

**pGL2BV(hs1,2,□, Eμ)** was made by digesting pGL2BV(hs1,2,Eμ) (described earlier) with Xho I, blunt-ending and ligating to a 5 kb Hind III digested and blunt-ended fragment isolated from the vector pSK7.0 kb 5'F. (the 5 kb Hind III fragment belongs to the 5' flanking sequences used in the homologous recombination experiment and which was found to house no enhancer-like element(s)). □ = spacer DNA.

**pGL2BV(hs1,2,□, Eμ)*** (construct 43*), was made through the same way that **pGL2BV(hs1,2,□, Eμ)** was generated. The only difference here is that in pGL2BV(hs1,2,□, Eμ)*(construct 43*), one of the Hind III restriction sites was regenerated during the blunt-end ligation.

To study the importance of placing the enhancer a distance away from, rather than adjacent to, the promoter, the construct p3αEEμ700 (TK), obtained from Dr Van Ness's laboratory (Fulton & Van Ness, 1994), were modified in the following manner;

**p3αEEμ700 (-TK)**, was made by removing the TK promoter from the original

p3αEEμ700 (TK) vector with Xho I/Bgl II digestion, blunt-ending, and religating. (p3αEEμ700 (TK), contains the core sequences of hs1,2 (abbreviated (3αE)) and Eμ placed upstream of the TK promoter from pTK vector)

**pTK**, a generous gift from Dr. Van Ness's laboratory, consists of a 105 bp HSV-TK promoter inserted upstream of the luciferase reporter gene of pGL2Basic vector.

To show that the $V_H$ promoter chosen to drive the luciferase reporter gene, was functional but much weaker then the ubiquitous TK promoter, the following constructs were generated.

**p3αEEμ700 ($V_H$)**, was generated by substituting the TK promoter of p3αEEμ700 (TK) with the $V_H$ promoter. p3αEEμ700 (TK), was digested with Xho I, blunt-ended, then digested with Bgl II, and ligated to the 140 bp $V_H$ promoter fragment (obtained by digesting pGL2BV with Sma I/Bgl II).

**pGL2B-TK(hs1,2,Eμ)** was made by digesting pGL2BV(hs1,2,Eμ) with Sma I/Bgl II to remove the $V_H$ promoter fragment, and ligating it to a TK promoter fragment obtained by digesting the pTK vector with Sma I/Bgl II.

## 2.2(b) - Gene Targeting Constructs

**RVB** ( homologous recombination vector used to generate a larger deletion of the IgH locus) was made as follows:

**Generation of the 5' flanking sequences:**

**pSK7.0 kb 5'F** (5' flanking sequences) vector was assembled through the generation of two intermediate vectors: **λM2-Sac 2.6** and **λM2-H34.9**.

**λM2-Sac 2.6** was put together by taking the 2.6 kb Sac I fragment from the 5' end of the 12 kb Eco RI fragment (isolated from the murine IgH genome) and cloning it into the pBS-M13 vector (Stratagene) also cut with Sac I. The 2.0 kb fragment of DNA needed for the pSK7.0 kb 5'F vector was then isolated from the construct λM2-Sac 2.6 as an Eco RI/ Hind III digest.

**λM2-H34.9** was assembled by taking the 5 kb Hind III fragment from the 5' end of the 12 kb Eco RI fragment (isolated from the murine IgH genome) and cloning it into the pBS-M13 also cut with Hind III. The 5 kb fragment of DNA needed for the assembly of the pSK7.0 kb 5'F was then isolated from the λM2-H34.9 as a Hind III digest.

**pSK7.0 kb 5'F**, was finally made by taking the 2.0 kb Eco RI/Hind III digested fragment (described above) and the 5.0 kb Hind III fragment (described above) and ligating them to an Eco RI/Hind III digested pBS-SK$^+$ vector (Stratagene).

**Generation of the 3' flanking sequences with the TK gene:**

**pTK 0.2 p/b**, the 3' flanking sequences obtained from the far end of the 26 kb Eco RI fragment of the mouse IgH genome, was assembled by digesting the pUN-pst vector (a gift from Dr. Morrison's laboratory; Gregor & Morrison, 1986) with Bam HI/Pst I to isolate the 2.0 kb 3' flanking fragment. This 2.0 kb fragment was then made flush with

Klenow and ligated to the pMCI-TK vector (a gift from M. Cappechi; Mansor et al., 1988) cut with Hind III and also made flush with the Klenow. The colonies were screened for the appropriate orientation of the 2.0 kb 3' flanking sequences with respect to the TK gene.

**Isolation of the 1.9 kb neomycin gene fragment:**

**pGKneo**, a neomycin-resistance gene fragment driven by the PGK promoter, was isolated from the pKJ-1 vector (a gift from Fred Alt's laboratory; Chen et al., 1993) as an Eco RI/Hind III digest and then made blunt-ended with Klenow.

**Assembly of the construct RVB:**

To make the final construct RVB, an intermediate construct RVBΔneo, was created. **RVBΔneo** was made by digesting pSK7.0 kb 5'F with Xho I, and then ligating to the 3' flanking sequences (which includes the TK gene, isolated from the pTK 0.2 p/b construct as an Xho I/Sal I digest). This RVBΔneo construct was then digested with Sal I, Klenowed and ligated to a Klenowed neomycin fragment, pGKneo (isolated as described above).

**pBS-(0.3 kb Bam HI/Pst I)** was made by isolating the 0.3 kb fragment of DNA (obtained after Bam HI/Pst I digest of PUN-pst), and ligating it to a Bam HI/Pst I digested pBS vector.

**pλM2-(hs1,2)** was made by subcloning the 4 kb Xba I fragment of (hs1,2) from

the phage clone into an Xba I-digested pBS vector.

## 2.3 - Transient Transfections

All cells were grown to log phase and then transiently transfected with DNA either by the DEAE-dextran supplemented electroporation method as described in Gauss et al., 1992 (S194 and P3X63Ag8 cells), or simply via DEAE-Dextran transfections as described in the product information from Promega (18-81, 18-8, M12.4.1, A20, Namalwa and Raji cells), or by calcium phosphate method (Rat-1 cells).

In electroporation experiments, $4x10^6$ cells were used for each transfection. In general, cells were seeded at 5 x $10^5$ cells per ml a day before transfection. On the day of transfection, into each 0.4 cm (width) electroporation cuvette (Bio-Rad, Hercules, CA), was added 125 ng of β-galatosidase expression vector (control plasmid), followed by the appropriate amount of test plasmid (the molar ratio of the control plasmid to the test plasmid maintained at a constant ratio of 1:7 respectively). To that was added 10 μg of DEAE-dextran (Promega). The total amount of DNA transfected per cell line (3 ug / transfection) was kept constant throughout via the addition of the appropriate amount of carrier DNA (pBS digested with Bam HI). Then, cells were added to the cuvette, an electric pulse was delivered at 960 μF and 300 V (S194), or 280 V (P3x63Ag8) by a Bio-Rad Gene Pulser™ electroporator and Capacitance Extender™ ( Bio-Rad, Hercules, CA).

The cells were then placed in a 37$^0$ C incubator with 7-8 % $CO_2$. Transfections were harvested after 18 - 24 hours. For those cells transfected through the DEAE-Dextran method, the DNA to be introduced was first diluted to a final volume of 326 µl with PBS. Then, 17 µl of 10 mg/ml DEAE-dextran solution was added and the samples were immediately vortexed. 4x10$^6$ cells were pelleted, resuspended in the DEAE-dextran/DNA mixture and then were placed in the incubator for 30 minutes. After the incubation, the cells were removed and added to 3.5 ml of (0.08 mM) chloroquine-supplemented media followed by a 2 hour incubation at 37$^0$ C. The cells were then finally pelleted and resuspend in 5 ml of complete media and plated onto a six-well plate for 48 hours prior to harvesting.

## 2.4 - Luciferase Assay

At harvest, all cells were collected, washed 2 X with phosphate buffered saline, and transfered to 1.5 ml microfuge tubes. The cell pellets were then lysed using 200 µl of 1 X Reporter Lysis Reagent (Promega, Madison, WI), incubated for approximately 20 minutes at room temperature, vortexed and then centrifuged at maximum speed for 10 minutes at 4$^0$ C. The resulting pellets were discarded and the supernatant (lysate) saved. An aliquot of the lysate (10 µl) was transfered to 0.5 ml microfuge tubes. 50 µl of the Luciferase Assay Reagent (Promega) were added, mixed thoroughly and counted for 1

minute in a scintillation counter (Beckmen LS6000). Duplicate samples were counted and the average determined.

Luciferase values were normalized for transfection efficiency by dividing each by the amount of activity obtained from the β-galactosidase reporter gene control. The fold enhancement (± SD) for each test construct was determined by dividing the normalized luciferase values obtained for a given sample by the normalized luciferase activity generated by an enhancerless construct. At least six independent transfections, using at least two different plasmid preparations, were performed with each test construct. In some cases, a p value is given for he significance of differences in activity among enhancer constructs in the same cell line or among cell lines with the same enhancer construct. The p value was obtained by Student's t-test. p value $\leq 0.05$ or less was regarded as evidence that the datasets had significantly different means.

## 2.5 - β-galactosidase Assay

β-galatosidase assays were performed using the colorimetric subsrate o-nitrophenyl-β-D-galactopyranoside (ONPG) in β-galatosidase assays kit from Promega. Briefly, 150 µl of the β-galatosidase reagent was added to 150 µl of the cell lysates. The mixture was incubated at $37^0$ C for 30 minutes and 500 µl of 1 M $Na_2CO_3$ added to stop the reaction. The resulting mixture was transferred into a disposable cuvette (Bio-Rad,

Cat # : 223-9955). Absorbance at 420 nm was measured with a spectrophotometer (DU series 600, Beckman).

## 2.6 - Stable Transfections

For the RVB knock-out experiments, plasmid DNA was introduced into the cells using an electroporator. Briefly, $1x10^7$ 9.9.2.1 cells were resuspended in 1ml of DME medium and mixed with 20 μg of Xmn I-cut RVB or 20 μg of Xho I-cut rvb, and introduced into a 0.4 cm (width) electroporation cuvette (BTX, San Diego, CA). An electric pulse was delivered at 960 μF and 250 V by a Bio-Rad Gene-Pulser electroporator™ and Capacitance Extender™ (Bio-Rad Hercules, CA). The cells were diluted with DME medium containing 15% BCS and were plated in a 96 well plate at a concentration of 1000 cells per well. 48 hours post-transfections, medium supplemented with 1.5 mg/ml Geneticin (G418, GIBCO labs, Grand Island, NY) was added to select for stable transformants expressing the $neo^r$ gene. Colonies were visible within 2 weeks after transfections. In most experiments, transformants arose in 60-75 % of the individual wells on the culture plate.

To determine if the TK gene driven by a kappa promoter in the presence of Eμ would be a more efficient expression vector over a pGK promoter driven TK gene in 9.9.2.1 cells, stable transformants generated from cotransfection of 1 μg of Eco RI

linearized pKONeo and 10 μg of Bam HI linearized pKHTK were obtained as described above. 48 hours post-transfection, 1.5 mg/ml of geneticin was added to the media. Neomycin resistant clones, confirmed through southern blotting and PCR assays, were isolated and screened for the presence of TK gene using PCR. Representative clones positive for both neomycin and TK genes were isolated and subjected to varying FIAU and Gancyclovir drug concentrations to see the effect of the drugs on the survival of such clones.

## 2.7 - Southern Blot Analyses

Genomic DNA was isolated from transformants as described in Lieberson et al., 1995 with slight modifications. Briefly, cells were collected from confluent 24 well plates and lysed with 150 μl lysis buffer (10 mM Tris pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5 % w/v sarcosyl, proteinase K (1 mg/ml)]. DNA from confluent wells was precipitated by adding 300 μl of 75 mM NaCl in absolute alcohol. After precipitation, the DNA was dissolved in water and subjected to the appropriate restriction enzyme digest.

DNA was size fractionated on 0.8 % agarose gels overnight at constant voltage, then transfered onto Nytran membranes (Micron Separations Inc, MA). Blots were pre-hybridized and hybridized at 65$^0$ C in buffer containing 7.5 X Denhardt's solution, 3 X SSC, 100 ug sonicated salmon sperm DNA and 0.5 % SDS. Probes were labeled by the

random primer method using a MegaPrime™ labeling kit (Cat # RPN1605, Amersham, Arlington Heights, IL) as recommended by the manufacturer.

Probes used included pJ11 (a Bam HI/Eco RI fragment derived from the $J_H$ gene region of BALB/cJ liver DNA and subcloned into pBR322 (Marcu et al., 1980), neo (a Pvu II fragment isolated from $pSV_2$neo; Southern and Berg, 1982), 2 kb and 0.3 kb Bam HI/Pst I fragments isolated from pUN-pst (Gregor & Morrison, 1986), and the Pst I fragment of TK gene isolated from pKHTK.

## 2.8 - Polymerase Chain Reaction (PCR)

Plasmid and genomic DNA from tissue culture cell lines were used as templates. Genomic DNA isolation was as for Southern blot analyses. All reactions for PCR were done at $4^0$ C unless otherwise indicated. Typical PCR reactions were carried out in 40 µl total volume and contained 4 µl 10 X PCR buffer (10 X = 100 mM Tris-Cl, pH 8.3, 500 mM Kcl, 15 mM $MgCl_2$), 2 ul 4 mM dNTP's (Pharmacia, Piscataway, NJ, Cat # 27-2035-01), 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer and 0.25 µl (1.25 U) Amplitaq DNA polymerase (Perkin Elmer, Lot # 0668). 1-2 µl genomic DNA (or 1 ng of plasmid DNA as positive control) was added and PCR was performed in a thermal cycler (Gene Machine II). 30 cycles: $94^0$C (1 min), $60^0$C (1 min), $72^0$ C ($2^1/_2$ min) were carried out and followed by a final incubation at $72^0$C for 7 minutes. The sequences of primers

specifically amplifying the neo gene were:

Neo 160 (Upper): 5' CAA GAT GGA TTG CAC GCA GGT TCT CC 3'

Neo 933 (Lower): 5' GTC AAG AAG GCG ATA GAA GGC GAT GC 3'

Expected size fragment: 773 bp

For the amplification of TK gene were:

TK #1 (Upper): 5' ACC CGA GCC GAT GAC TTA CTG G 3'

TK #2 (Lower) 5' CGC ACC GTA TTG GCA AGT AGC 3'

Expected size fragment: ≈ 600 bp

For RVB screening,

Neo 818 (Upper): 5' TGG CTA CCC GTG ATA TTG CTG AA 3'

p/b (Lower): 5' CAG CTC TTT ATG TTG TCA TCC C 3'

Expected size fragment if homologously recombined : ≈ 2 kb

## 2.9 - Enzyme Linked Immunosorbant Assay (ELISA)

The IgG-specific ELISA was performed in 96 well microtiter plates (Dynatech Laboratories, Inc., VA) coated overnight with 10 μg/ml of affinity-purified, Fc-fragment specific rabbit anti-mouse IgG (Jackson Immuno Research Lab, Inc., Lot # 40182). The coating antibodies were removed and 200 μl of blocking solutions (3 % BSA in PBS solution) added to the wells, and incubated at room temperature for 2 hours. The plates

were rinsed at least 3 X with washing solution (1 X PBS + 0.05 % Tween). Then, 50 µl of serially - diluted cell supernatant obtained from 48 hour cultures of $1X10^6$ cells were added and incubated for 1 hour at room temperature. This was followed by another rinse step. IgG was detected with secondary antibody (alkaline phosphatase-conjugated rabbit anti-mouse IgGγ2a (Zymed Labs, Lot # 61-0222)) using *p*-nitrophenol phosphate as the enzyme substrate (ca t# 104 phosphatase substrate tablets, Sigma, St. Louis, MO). The absorbance at 405 nm was measured in an ELISA microplate reader (Bio-Rad, Model 450).

## 2.10 - Double-Stranded Plasmid DNA Sequencing

The sequencing of both the 3' end of the 4.0 kb Xba I fragment of (hs1,2) and the ≈0.3 kb Bam HI/Pst I fragment were determined according to the procedure recommended by manufacturer (Sequenase Kit, United States Biochemical Corp., Cleveland, Oh., Cat # 70770). 3-5 µg of plasmid DNA was denatured by adding 6 µl of freshly prepared 1 N NaOH. A 24 µl total volume mixture was incubated at room temperature for 5 minutes. The DNA was precipitated with 3 µl of 2 M $NH_4OAc$ (pH 4.5) and 70 µl of 100 % ethanol at -70° C for 20 minutes. 70 % ethanol-washed and dried DNA pellet was resuspended in 7 µl of $H_2O$. 1 µl of 0.5 µM primer and 2 µl of 5 X sequencing buffer were added. The mixture was heated to 65° C for 2 minutes and then

allowed to cool slowly by sequential incubations: at 37$^0$C for 15 - 30 minutes and at room temperature for 15 - 45 minutes.

1 µl 0.1 M DTT, 2 µl sequencing labelling mix (1:5 dilution), 0.5 µl α-$^{35}$S dATP (600 Ci/mmol; Amersham, Arlington Heights, Ill., Cat # SJ304) and 2 µl Sequenase in sequenase dilution buffer (1:8 dilution) were added to the annealed primer and DNA. The mixture was incubated at room temperature for 5 minutes and the reaction was terminated by aliquoting 3.5 µl of it into four tubes, each containing 2.5 µl of pre-warmed (37$^0$C) ddATP, ddCTP, ddGTP or ddTTP. The tubes were kept for additional 5 minutes at 37$^0$C and then 4 µl of stop buffer (sequencing dye in 80 % formamide) was added.

Sequencing reactions (3 µl) were resolved by electrophoresis at 45-60 amperes (1800-2000 V) on pre-run (at least 20 minutes at 45 mAmps) 6 % (19:1) polyacrylamide/ 8 M urea gel in 1 X TBE (8.9 mM tris-base, 8.9 mM boric acid, 0.2 mM EDTA, pH 8.0).

# Chapter 3

## Hs1,2 is crucial for high levels γ2a gene expression in 9.9.2.1 cells

## 3.1 -Introduction

Intronic enhancer Eμ, the first B-cell specific enhancer identified, is functionally active through all stages of B cell development. It plays an important role in VDJ recombination events which occur early in B cell development, but becomes dispensable at the Ig-secreting as indicated by a number of cell lines that lack Eμ, yet maintain high levels of IgH gene expression (Aguilera et al., 1985; Banerji et al., 1983; Chen et al., 1993; Eckhardt and Birshtein, 1985; Gillies et al., 1983; Klein et al., 1984; Neuberger, 1983; Serwe and Sablitzky, 1993; Wabl and Burrows, 1984; Zaller and Eckhardt, 1985). For example, the myeloma cell line 9.9.2.1 which expresses γ2a heavy chain and κ light chain was derived from MPC11 cells through a class-switching event that deleted the intronic enhancer from the IgH locus. Despite the loss of Eμ, 9.9.2.1 continued to express γ2a at levels equal to those in the parental MPC11 cells, suggesting that Eμ was dispensable at this stage (Eckhardt and Birshtein, 1985; Zaller and Eckhardt, 1985). The continued expression of the γ2a gene furthermore, suggested the presence of additional regulatory elements within the IgH locus that could compensate for the loss of Eμ.

In 1986, a mutant cell line, LP1.2, was discovered. This cell line had lost approximately 34 kb of its genomic DNA downstream of the Cα constant gene exons and was producing IgA at 1/10 the amount produced by its parental cell line. This suggested the presence of positive regulatory elements downstream of the Cα constant gene exons, within the 34 kb of genomic DNA that was deleted in LP1.2. It also demonstrated that

Eμ alone was not sufficient for IgA expression in LP1.2 (Gregor and Morrison, 1986).

In 1991, a transcriptional enhancer was identified 3' of the murine IgH locus (Dariavach et al., 1991; Lieberson et al., 1991). This enhancer formerly referred to as 3'αE, corresponds to DNAse hypersensitive sites 1 and 2 (hs1,2) identified by in vivo DNAse mapping studies downstream of the Cα gene exons (Giannini et al., 1993) (Fig. 5). Unlike Eμ, hs1,2 has been shown to be active only at late stages of B cell development as measured through both transient transfection assays and transgenic mouse studies (Arulampalam et al., 1994; Dariavach et al., 1991; Fulton and Van Ness, 1994; Madisen and Groudine, 1994; Singh and Birshtein, 1993).

We wanted to know the role of hs1,2 in IgH transcription in a myeloma that lacked Eμ. The cell line 9.9.2.1, provides an excellent opportunity to study directly the effects of the 3' IgH enhancer elements on γ2a expression in the absence of Eμ. We decided to employ homologous recombination techniques to selectively remove hs1,2 from the IgH locus and examine the effect on γ2a expression.

Rebecca Lieberson, in our laboratory, initiated these experiments, constructing targeting vectors RVA and RVB (rvb) (Figs. 6 & 7). Cells transfected with RVA should undergo homologous recombinantion and specifically eliminate hs1,2, while those that undergo homologous recombination with RVB or (rvb), should delete hs1,2, hs3B as well as hs4. I joined this project, contributing to it in the following ways:

1) Testing the efficiency of the thymidine kinase gene as a "negative" selectable marker in 9.9.2.1 cells

2) Designing of primers for PCR screening of transformants

**Figure 5.**

Schematic map of the immunoglobulin heavy chain gene locus illustrating the relative positions of the enhancers. Filled boxes represents constant region exons (e.g. Cα , Cμ), as well as the assembled variable region exon (VDJ). Circles represents the various enhancers - Eμ, hs3A($C_\alpha$3'E ), hs1,2(3'αE ), hs3B, and hs4. Each variable region segment associated with its own promoter, denoted by a small black oval while the grey oval upstream refers to each switch recombination signal upstream of each constant gene exon with the exception of Cμ.

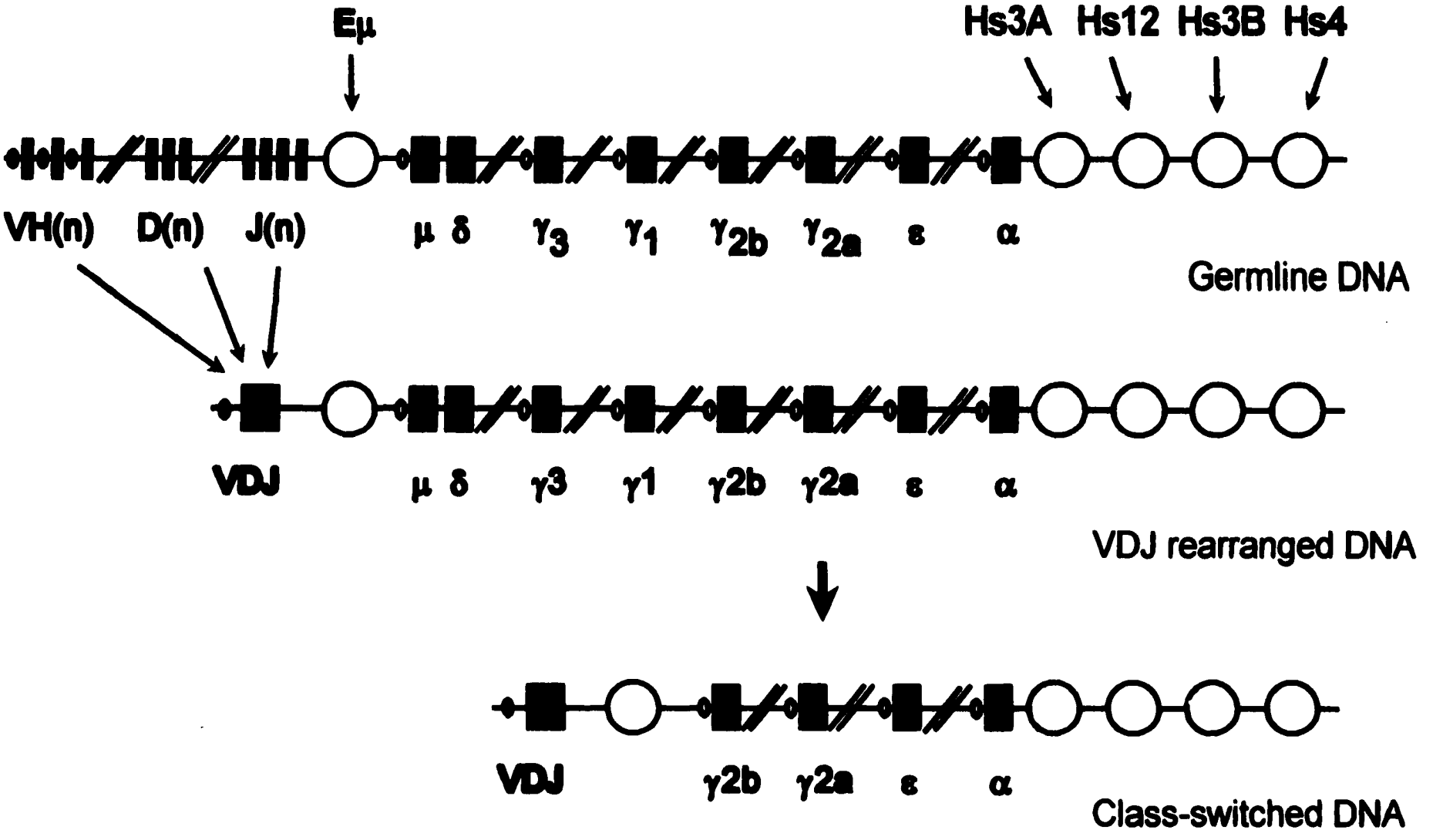


**Figure 6.**

Targeted deletion of the 3'α E (hs1,2) region in cell line 9.9.2.1. The first two schematic diagrams illustrate the characteristics of IgH locus of 9.9.2.1. Filled boxes represent the constant region exons and rearranged variable region (VDJ); grey small ovals, switch recombination signals upstream of each constant gene exons; the black small oval, the promoter upstream of the VDJ region; circles, the various enhancers. The distance between 3'αE and the VDJ region is ≈ 70 kb. The region downstream of the Cα constant gene exons were amplified detailing the homologous 5' and 3' flanking region of the genomic DNA used in the construction of the targeting construct RVA (as described in the material and method section). The 5' flanking DNA region containing hs3A enhancer and the 3' flanking region of targeting construct RVA outlined as elongated filled rectangular boxes; Neo and TK genes represented by open boxes, P1 and P2 represents the primers used for PCR assay described in material and methods section. The bottom-most diagram shows the predicted structure of the genomic locus after homologous recombination with RVA. The specific restriction site Eco RI was included to illustrated what the correct recombinant would look like upon the loss of 3'αE.

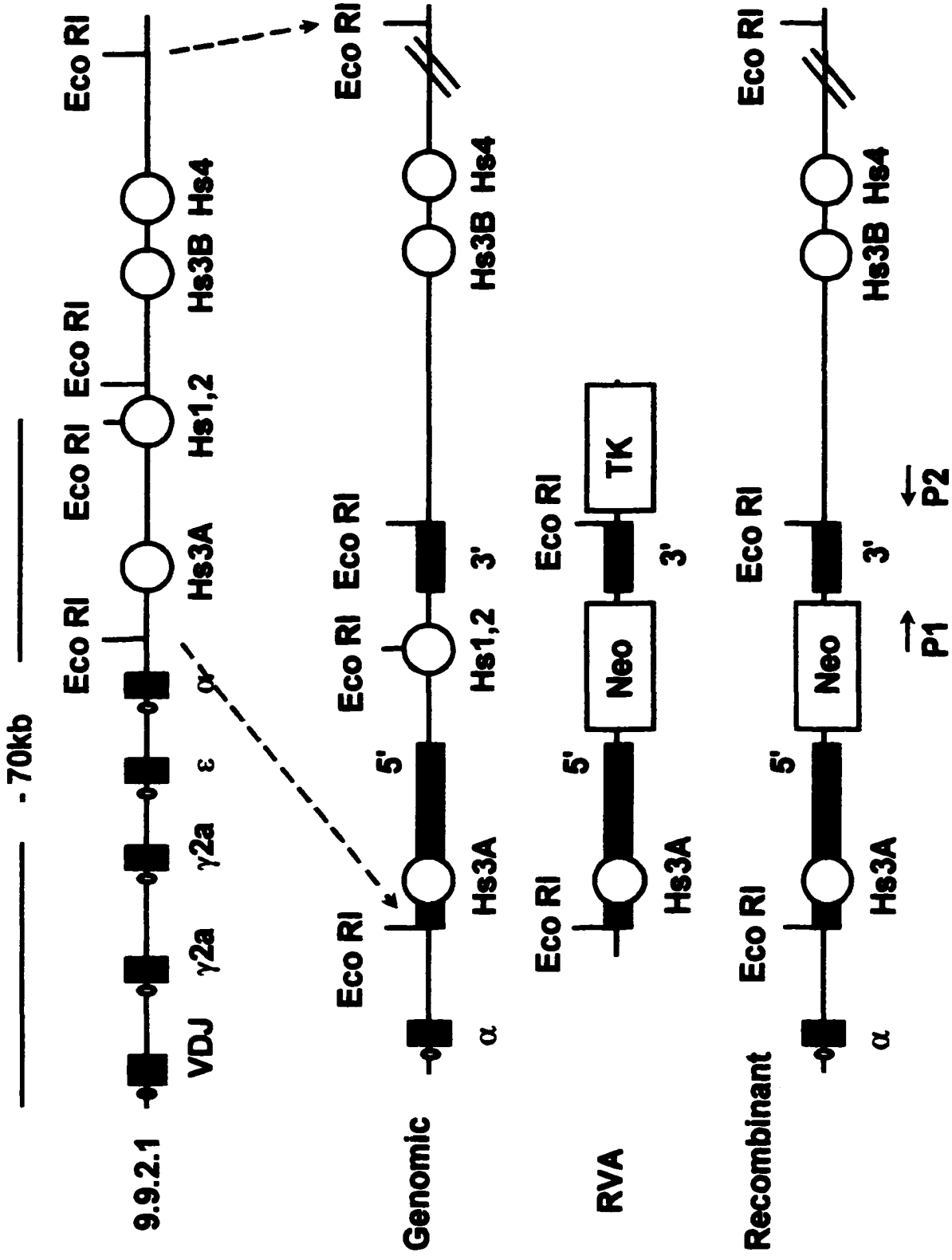
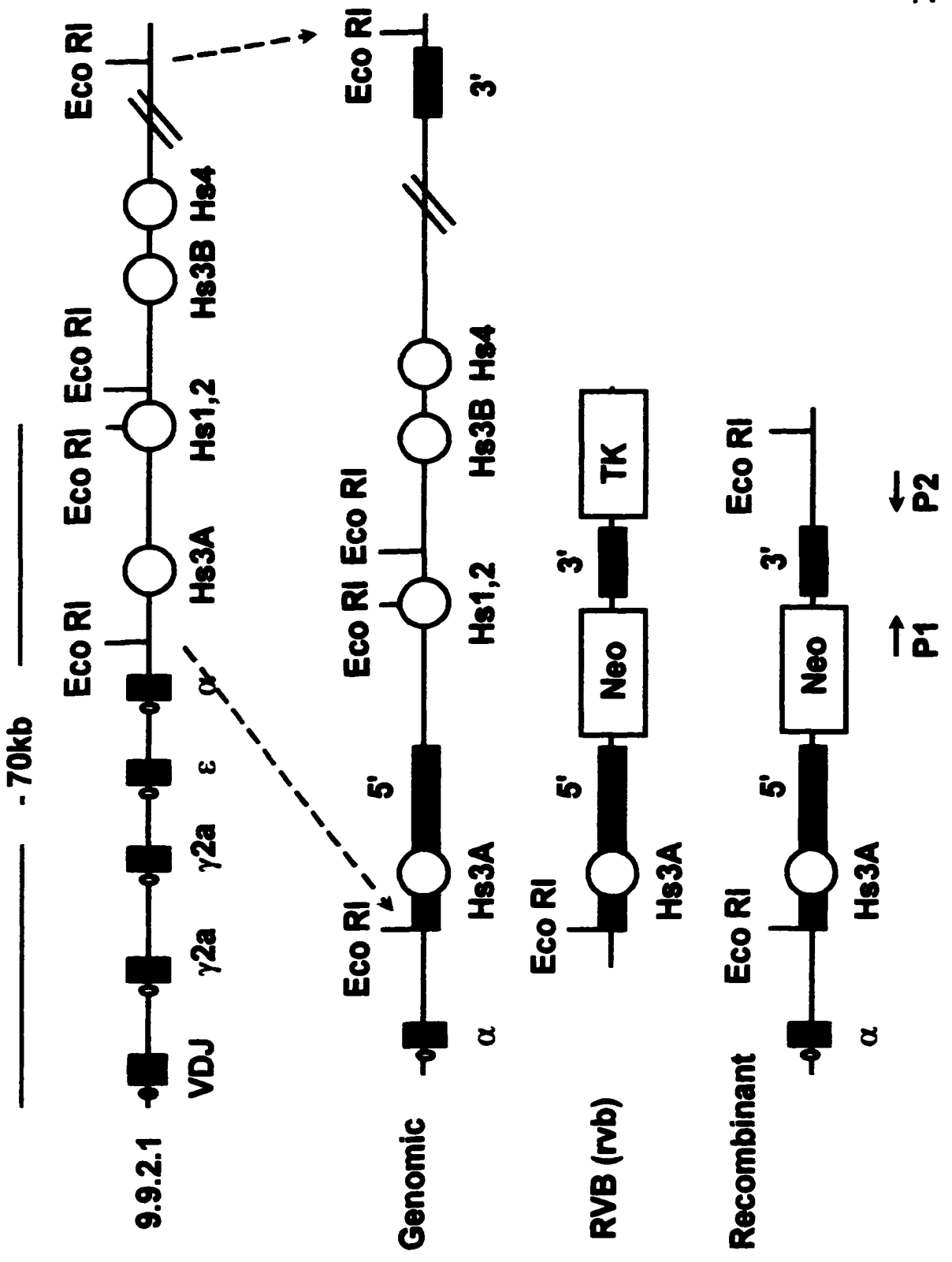


**Figure 7.**

Targeted deletion of the [3'α E (hs1,2), hs3B and hs4] region in cell line 9.9.2.1. The first two schematic diagrams illustrate the characteristics of IgH locus of 9.9.2.1. Filled boxes represent the constant region exons and rearranged variable region (VDJ); grey small ovals, switch recombination signals upstream of each constant gene exons; the black small oval, the promoter upstream of the VDJ region; circles, the various enhancers. The distance between 3'αE and the VDJ region is ≈ 70 kb. The region downstream of the Cα constant gene exons were amplified detailing the homologous 5' and 3' flanking region of the genomic DNA used in the construction of the targeting construct RVB (as described in the material and method section). The 5' flanking DNA region containing hs3A enhancer and the 3' flanking region of targeting construct RVB outlined as elongated filled rectangular boxes; Neo and TK genes represented by open boxes, P1 and P2 represents the primers used for PCR assay described in material and methods section. The bottom-most diagram shows the predicted structure of the genomic locus after homologous recombination with RVB. The specific restriction site Eco RI was included to illustrated what the correct recombinant would look like upon the loss of [3'α E (hs1,2), hs3B and hs4].



3) Transfection of 9.9.2.1 cells and screening with a targeting vector designed to delete a large region 3' of the IgH coding sequences (RVB or (rvb))

4) Assisting in screening of 9.9.2.1 cells transfected with the "hs1,2 deletion" targeting vector, RVA

Deletion of hs1,2 was accomplished and a paper describing this work was published in 1995. A discussion of these experiments, referring to figures in the published paper, follows a brief description of the particular projects outlined above.

# Chapter 3

## Results

## 3.2 - Thymidine kinase does not function as a negative selectable marker in 9.9.2.1 cells

To test the effectiveness of both neomycin ($neo^R$) and thymidine kinase (TK) genes as positive and negative selectable markers respectively, in 9.9.2.1 cells, we first co-transfected with linearized expression vectors pKOneo and HSV-TK using electroporation. Transformants were cultured in media supplemented with the drug-geneticin to select for neomycin expressing clones. Neomycin resistant clones were collected within 2 weeks, genomic DNA isolated, and tested by both PCR and Southern blot analyses for the presence of the neomycin reporter gene as well as for the TK gene. Neomycin and TK reporter gene positive transformants were then cultured in media supplemented with varying concentration of Gancyclovir (from 2.5 μM to 20 μM) and checked for their survival over the course of 2 weeks. $Neo^R$ clones that expresses the HSV-TK gene will phosphorylate the nucleoside analog Gancyclovir. This phosphorylated substrate will then be incorporated into the growing DNA chain during S-phase resulting in cell death. Since nucleoside analogs are approximately 1000 fold poorer substrates for non HSV-TK gene, the murine TK gene will not be able to phosphorylate Gancyclovir and thus, no cell death results, consistent with our observations. Additionally, transformants containing transfected HSV-TK remained unaffected even in the highest concentration of Gancyclovir (20 μM), indicating the failure of HSV-TK gene as a negative selectable marker in our cell line, 9.9.2.1. We next

tested another TK expression vector (pKHTK). In this vector, the TK gene is covalently linked to Eμ, theoretically to enhance the expression of the HSV-TK gene in B lineage cells 9.9.2.1 cells were cotransfected with pKOneo and pKHTK. Five independent $neo^R$ transformants were chosen and tested for the presence of the TK gene using PCR. These $neo^R$ and TK positive transformants were later subjected to varying concentrations of FIAU (more sensitive and less toxic than Gancyclovir) and their survival checked over a period of 2 weeks. Again, we saw no effect on naive as well as transformed 9.9.2.1 cells despite increasing FIAU concentrations from 1 X to 10 X (1000 X = 0.2 mM).

## 3.3 - Design of primers for PCR screening

As a negative selectable marker the TK gene was ineffective in our positive - negative - selection (PNS) method for homologous recombination, thus, we had to devise an alternative assay that allowed us to quickly screen large numbers of $neo^R$ transformant clones. We chose PCR as our method, and therefore needed to design the appropriate primers that allowed the synthesis of a distinct PCR product for transformants that had undergone homologous recombination at the target site (see Figs. 6 & 7).

Primer 1 is the same in both RVA and RVB transformants; it is derived from the $neo^R$ :

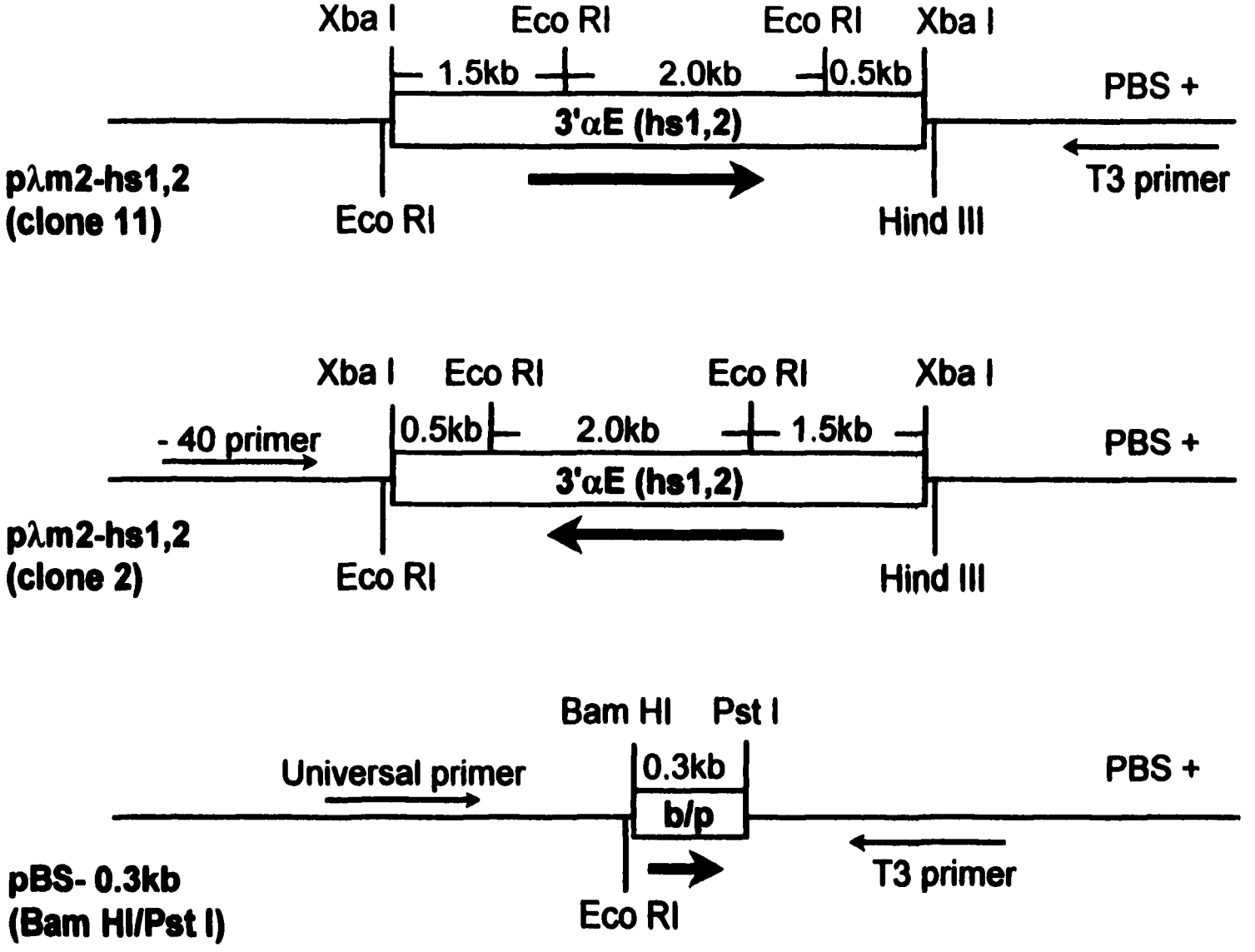
Neo 818 (Upper): 5' TGG CTA CCC GTG ATA TTG CTG AA 3'

Primer 2 differs in Figs. 6 & 7, and is derived from sequences immediately adjacent to the 3' flanking sequences utilized in the targeting vectors RVA or (RVB/rvb) respectively.

To generate these primers, we subcloned the relevant DNA fragments into the cloning vector pBS (Strategene). As indicated in Fig. 8, to generate P2 for RVA transformants, two different plasmids were used, pλm2-hs1,2 (clone # 11, and clone # 2). Depending upon the orientation of the hs1,2 fragment into pBS, we used either a T3 primer (clone # 11) or -40 primer (clone # 2) to obtain the required sequences for designing the P2 primer. To design P2 for RVB or (rvb) transformants, pBS-0.3kb (b/p) was sequenced using the T3 primer and the Universal primer.

**Figure 8.**

Constructs used for sequencing the 3' end of 3'αE and the 0.3 kb Bam HI/Pst I fragment of DNA. pλm2-hs1,2, clone #11 and #2 depending upon the orientation of hs1,2 into pBS + vector, primers T3 or -40 was used to obtain the required sequences for designing the P2 primer in RVA knock-out experiments. To design the P2 primer for use in the detection of homologous recombinant using RVB targeting construct, construct pBS-0.3 kb Bam HI/Pst I was sequenced using the T3 primer and the Universal primer. The thick arrow indicates the orientation of the fragments to be sequenced and the thin arrow refers to the position and orientation of the primers relative to the fragments of interest. Xba I, Eco RI, Bam HI as well as Pst I refer to the restriction sites of the region of interest.



### 3.3(a) - DNA sequencing of the 3' end of the 4kb Xba I fragment of hs1,2

The sequence shown is of the lower strand with respect to IgH gene transcription.

Clone # 2 (-40 primer) (5' → 3'):

1

TTGGCTAGAGGATCACAGAGTGATGTTTTCCTTCTTGGCCAGTTTCAGGC

51

CAGGGGACTCAGGGTAAGAGACAGAAAGTCAGGAAGGTAGGAAAGACAGG

101

GAGACATAATAAAGGTAAGACAGAGGAAGACGAAGGGAGAAACTGCAAGT

151

CTGCAGGGGGAGACAAGGAGAAGCCTGACAGAGACAGACAGCGTTCCGGA

201

AAAGACAGAGATGAAGGA TCAGAGGTGGAGATGTGTAGAGACAGAGAGA

251

GATTAATGGGAGCACAGAGACAGGTGTGACAGAGAATAGGGAGAACA

Clone # 11 (T3 primer) (5' → 3'):

1

TTTTCCTTTTT..CCAGTTTCAGGCCAGGGGACTCAGGGTAAGAGACAGA

51

AAGTCAGGAAGGTAGGAAAGACAGGGAGACATAATAAAGGTAAGACAGAG

101

GAAGACGAAGGGAGAAACTGCAAGTCTGCAGGGGGAGACAAGGAGAAGCC

151

TGACAGAGACAGACAGCGTTCCGGAAAAGACAGAGATGAA.GTCCAGAGG

201

TGGAGATGTGTAGAGACAGAGAGAGATTAATGGGAGCACAGAGACA

P2 (Fig. 6) RVA (lower): 5'AA GAC AGA GGA AGA CGA AGG GAG 3' (underlined)

## 3.3(b) - DNA sequencing of the ≈ 300bp Bam HI/Pst I fragment of the ≈ 2.5 kb pUNpst fragment

Universal primer, upper strand sequence:

1

GGTACCCGGGGATCCCCGCTAAGGTCCGATGTCTTTTCCACACTAGTTGA

51

AAGTCAGATGATTTGAAACAGTAAACAAATTACAGTTATGAGGTAGCAAA

101

AAAAATAATTTTACGGTT***GGGATGACAACATAAAGAGCTG***TATTAAAAGG

151

TCACAACGTAAGGAAGATTGAGAACCACTGAGCTAAAGCGACCAGGAGTA

201

CATGCCTGGGTTTTGATGATCAAGTTGAGAGTTCAGGCTTGTACTGCAGG

251

CATGCAAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTT CGTAA

T3 primer, lower strand sequence:

1

CTGCAGTACAAGCCTGAACTCTCAACTTGATCATCAAAACCCAGGCATGT

51

TCTCCTGGTC(c/t)TTAGCTCAGTGGTTCTCAATCTTCCTTACGTTGTGAC

101

CTTTAATACAGCTCTTTATGTTGTCATCCCAACCGTAAAATTATTTTTTT

151

TGCTACCTCATAACTGTAATTTGTTTACTGTTTCAAATCATCTGACTTTC

201

AACTAGTGTGGAAAAGACATCGGAC

P2 (Fig. 7) b/p (Lower): 5' CAG CTC TTT ATG TTG TCA TCC C 3' (underlined) and complementary sequences (in ***bold italics***).

## 3.4 - Deletions with RVB or (rvb)

While deletion of hs1,2 in 9.9.2.1 was in progress, we decided to do a parallel deletion using RVB. This would result in a much larger deletion of ≈ 27 kb of genomic DNA. Our reasoning was that if the smaller deletion (hs1,2) resulted in no substantial change in γ2a expression levels, the larger might prove more effective.

Transfection into 9.9.2.1 was done with RVB constructs (Fig. 7). Over 2000 $neo^R$ transformants were screened using PCR and Southern blot analyses for possible homologous recombinants. We found only one candidate which upon further analyses turned out to be non-informative due to a faulty targeting construct: we discovered that the 5' flanking sequence was reversed during construction. A new, proper, construct was generated (rvb) and transfected into 9.9.2.1 cells. Out of the 497 $neo^R$ transformants analysed however, none had successfully recombined at the target site.

## 3.5 - Screening 9.9.2.1 cells transfected with RVA constructs

As transformants of 9.9.2.1 with RVA were generated, I helped in screening for homologous recombinants using Southern blot analyses. The PCR products from RVA transformants using P1 and P2 (Fig. 6) were not reliably detectable by agarose gel staining with EtBr, so we shifted to Southern screening. Other laboratories (e.g. Alts's

lab) were encountering similar difficulty with PCR screening and had devised a protocol for rapid DNA preparation and Southern blotting. We used this method. After screening 450 $neo^R$ transformants, we found one that was properly recombined at the target site.

Southern blot analyses with Bam HI, Sac I and Xmn I - digested genomic DNA obtained from the homologous recombinant (9.9.2.1 Δ 3'αE) with probe b (3' junction probe), probe c (3'αE) and probe a (5' junction probe) (Figs. 1 & 2 (a,b,c) of Lieberson et al., 1995) confirmed the loss of hs1,2 from the genome. ELISA assays on cytoplasmic lysates and culture supernatants obtained from (9.9.2.1 Δ 3'αE) along with northern blot analyses showed the mutant cells no longer made γ2a mRNA and protein [see Fig.3 (a) (b) of Lieberson et al., 1995], suggesting the importance of hs1,2 in maintenance of γ2a gene expression.

# Chapter 3

## Discussion

## 3.6 - Discussion

The murine IgH locus possesses enhancers both upstream and downstream of the constant gene exons. Eμ, the first B-cell specific transcriptional enhancer identified, has been demonstrated to play an important role in the VDJ recombination process (Chen et al., 1993; Serwe and Sablitzky, 1993). Discovery of cell lines like 9.9.2.1 that lack Eμ yet maintain high levels of IgH gene expression, suggested that Eμ was dispensable at the plasma cell stage and that other regulatory elements might exist within the locus that can compensate for the loss of Eμ (Aguilera et al., 1987; Klein et al., 1984; Zaller and Eckhardt, 1985). Chromosomal translocations, characteristic of almost all mouse myelomas, place the 3' region of the IgH locus upstream to the *c-myc* coding gene exons, resulting in the de-regulation of *c-myc* gene expression (reviewed in Cory, 1986). This further supports the idea that positive regulatory elements exist within the 3' IgH region. More evidence comes from the LP1.2 mutant cell line which is missing ≈ 34 kb of its genomic DNA downstream of the Cα gene exons, and generates only 10% of the IgA levels in the parental cell line (Gregor and Morrison, 1986; Michaelson et al., 1995). These notions were later confirmed by the discovery of hs1,2 approximately 12.5 kb downstream of the Cα membrane exons, within the 34 kb fragment of genomic DNA that was lost in LP1.2 (Dariavach et al., 1991; Lieberson et al., 1991). It was postulated that hs1,2 might be crucial for IgA expression in LP1.2 since the loss of it results in ≈ 10 fold reduction in IgA gene expression and Eμ alone was not sufficient to support high levels

of IgA expression.

Using the cell line 9.9.2.1, which is missing Eμ as a result of a class-switch event from MPC11 (γ2b) to 9.9.2.1 (γ2a), we decided to examine the role of hs1,2 in IgH gene expression. Using homologous recombination, a targeting construct, RVA, was transfected into 9.9.2.1 cells. Approximately 450 $neo^R$ transformants were chosen and analysed for homologous recombinantion using Southern blot analyses. We found one clone that tested positive for the loss of hs1,2 from the expressed IgH chromosome. 9.9.2.1Δ3'αE, as it is referred to, no longer expresses the γ2a gene as confirmed by northern and ELISA assays.

These data would suggest that hs1,2 plays an important role in γ2a gene expression in 9.9.2.1 cell line. How it does so is unclear. Hs1,2 could act alone or it could act in concert with the other 3' IgH enhancers namely hs3A, hs3B and hs4 for efficient γ2a gene expression. Alternatively, the insertion of $neo^R$ gene into the IgH locus might modify the chromatin structure such that it disrupts the γ2a gene expression altogether, just like it did in the human β-globin locus (Fiering et al., 1993; Kim et al., 1992).

The human β-globin locus spanning approximately 70 kb on chromosome 11, consists of five functional β-globin like genes arranged in order of their expression during ontogeny: 5'- ε, Gγ, Aγ, δ, β - 3'; ε is expressed during embryonic period, Gγ and Aγ during fetal period and δ & β, from late fetal period onwards. Located approximately 6 - 22 kb upstream of the ε-globin gene is a regulatory region that controls the timing of replication, chromatin structure and transcription of the entire β-globin domain. Locus control region (LCR) as it is referred to, is composed of five individual DNAse I

hypersensitivity sites (Reviewed in Martin et al., 1996). Full LCR activity requires that more than one hypersensitivity site be present as exemplified in an Hispanic $(\gamma\delta\beta)^0$ thalassemia patient which lost an approximately 35 kb region 5' of 5'HS1 of the LCR, including hypersensitivities II, III, IV and V. The result was failure to activate the intact cis-linked β-liked globin genes in either fetal or adult erythroid cells (Review in Martin et al., 1996). These data suggested a required interaction of some sort between the full 5' HSs and the downstream functional genes. This notion was further explored by (Kim et al., 1992). Using homologous recombination technique, they successfully inserted a hygromycin selectable marker between hypersensitivity sites 1 and 2 of a human lymphoid / MEL hybrid cell line (N-MEL) containing a single normal human chromosome 11. These cells are transformed erythroid cells that can be induced to differentiate into erythrocytes and, during differentiation, express the adult globin genes. Uniduced N-MEL cells exhibit no human β-globin gene expression but upon induction with hexamethylene bisacetamide (HMBA), human β-globin transcripts were detectable. The targeted clones when analyzed, showed no detectable human β-globin gene expression before and after induction with HMBA. To ensure that lack of human β-globin was not due to extinction of the gene by de novo methylation of the genomic DNA in cell culture, targeted clones were treated with 5' azacytidine. Treatment with 5' azacytidine will cause extinction cells to revert back to normal cells and hence production of human β-globin transcript. Thus uninduced parental N-MEL cells, upon treatment with 5' azacytidine resulted in increase level of human β-globin expression both before and after HMBA induced differentiation. Hygromycin - targeted clones however, when induced

with 5' azacytidine showed no human β-globin expression both before and after HMBA induction. Interestingly, when they analyzed the treated clones for the effect of 5' azacytidine on the hygromyicn selectable marker, they found that the induced targeted clones showed a much higher level of hygromycin expression than the uninduced clones. This demonstrated that the failure of human β-globin gene expression in targeted clones is not the result of methylation event in a subset of cells from the original population but is due to the targeted insertion of hygro$^R$ gene (Kim et al., 1992).

To confirm this hypothesis, Fiering et al. (1993) repeated the above experiment but this time modified the targeting construct to include two FRT targets flanking the Friend virus long terminal repeat enhancer/promoter (F-LTR) driven neo$^R$ gene. This allowed for later deletion of the neo$^R$ gene once targeted into the locus of the N-MEL cells. Consistent with earlier findings, targeted clones showed no detectable human β-globin gene expression after induction of differentiation but showed significant increased in neo$^R$ gene instead. In the presence of FLP recombinase expressing vector however, the FLP recombinase of Saccharomyces cerevisiae catalyses recombination between the two FRTs resulting in deletion of the neo$^R$ gene. Clones that successfully deleted the neo$^R$ gene were then analyzed for the presence of human β-globin. Interestingly, targeted clones that lost the neo$^R$ gene began to exhibit normal levels of human β-globin indicating the insertion of the neo$^R$ gene between HS1 and HS2 had disrupted the LCR such that it no could longer support human β-globin gene expression. They reasoned that the supression of β-globin gene by insertion of the neo$^R$ gene, the result of promoter competition between the F-LTR and β-globin gene for the interaction with the LCR.

While we were knocking-out hs1,2 in a cell line that lacked Eμ, Cogne et al were assessing the role of hs1,2 in developing mice, where Eμ was still present. Using homologous recombination technique, they successfully generated knock-outs of hs1,2 on either one copy of the IgH locus (hs1,2$^{+/-}$) or on both copies of the IgH locus (hs1,2$^{-/-}$) in ES cells. These mutant ES cell clones were then injectd into blastocysts of RAG-2 $^{-/-}$ mice and then implanted into foster mothers. Chimeric mice with lymphocytes derived from ES clone resulted from successful implantation and were confirmed to carry lymphocytes with targeted knock-out of hs1,2 by Southern blot analyses. The effects of this knock-out on IgH gene expression was then determined. They showed that spleen cells obtained from (hs1,2 $^{+/-}$) and (hs 1,2 $^{-/-}$) chimeric mice, had surface IgM $^{+}$ B cells, demonstrating that hs1,2 was not essential for VDJ recombination. In contrast, spleen cells from (hs 1,2 $^{-/-}$) mice showed defects in isotype switching to γ2b, γ2a, γ3, ε and partially to γ1. Additionally, these mice had a severe deficiency in serum IgG3 and IgG2a.

Class-switching has been correlated with germline transcription of the targeted $C_H$ genes. LPS treatment of splenic B cells induces expression of germline $\gamma_{2b}$ and $\gamma_3$ transcripts, while LPS and IL-4 treatment induces $\gamma_1$ and ε transcripts. Treatment with (LPS +TGF-β) promotes transcription and switching to α while IFN-γ induces $\gamma_{2a}$ transcripts. Analyses for induction of germline $C_H$ transcripts in activated normal (hs1,2 $^{+/-}$) and (hs1,2$^{-/-}$) B cells was thus performed. Consistent with the above findings, they reported that while LPS and (LPS + IL4) treatment of normal (hs1,2$^{+/-}$) B cells generates ($\gamma_{2b}$ and $\gamma_3$) and $\gamma_1$ germline as well as ($\gamma_{2b}$ and $\gamma_3$) and $\gamma_1$ containing transcripts respectively, while (hs1,2$^{-/-}$) B cells showed no detectable $\gamma_{2b}$, $\gamma_3$ and $\gamma_1$ germline

transcripts. Additionally, (hs1,2$^{-/-}$) B cells exhibited a decrease in ε germline transcript. These results suggested a role for hs1,2 in class-switching events to certain isotypes. (Cogne et al., 1994).

Because insertion of the neo$^{R}$ gene into the LCR of the human β-globin locus had been shown to have an adverse effect on the expression of functional human β-globin gene (Fiering et al., 1993), the authors noted that the defects in class-switching and serum Ig levels observed for the (hs1,2 $^{-/-}$) chimeric mice might not have resulted necessarily from loss of hs1,2 but rather from insertion of the neo$^{R}$ gene into the IgH locus. We also pointed out that the same argument could apply to the phenotype of the 9.9.2.1Δ3'αE mutant cell line.

To test this notion, modified hs1,2 targeting constructs housing loxP sites on each end of the neo$^{R}$ gene could be made. These loxP sites are targets for a bacterial enzyme Cre that catalyses site-specific recombination between them, allowing for the removal of the neo$^{R}$ gene, leaving behind a loxP site in the IgH locus. Thus, using the Cre-loxP system, it is possible to replace hs1,2 by a neo$^{R}$ gene as positive selection marker first, followed subsequently by the removal of the neo$^{R}$ gene from the locus using Cre-enzyme.

Several loxP neo targeting vectors have been tested in our laboratory, but none as yet has generated the desired recombinant. There has been progress in generation of similar recombinants in mice, however. Manis et al., 1998, successfully generated "clean" hs1,2 and hs3A knock-out mice. They reported that in both knock-outs, mice homozygous for the insertion of the neo$^{R}$ gene into the IgH locus showed defects in IgH class-switching as well as reduced serum Ig levels for certain isotypes (i.e. γ3, γ2b, γ2a

and ε). Upon removal of the neo$^R$ gene however, the phenotype previously observed was lost, suggesting that the insertion of neo$^R$ gene into the locus caused the observed disruption of IgH class-switching and that deletion of hs1,2 or hs3A, individually, had no detectable effect on Ig gene expression or class-switching. This was further supported by findings from Siedl et al., 1999, in which they showed that the insertion of pGK-neo cassettes into various sites within the immunoglobulin heavy chain locus resulted in the inhibition of IgH gene expression to constant region upstream of the insertion site with the exception of G1 and A. For example, insertion of neo$^R$ gene in place of sequences in the I$\gamma_{2b}$ inhibits germline transcription of and class-switching recombination to upstream C$\gamma_3$ gene, but no major effect downstream of C$\gamma_{2a}$, Cε gene (see Fig. 5 for the order of the constant gene exons). Replacement of Cε exons with a neo cassettes in opposite transcriptional orientation also inhibits, in culture, germline transcription of and class-switching recombination to the upstream Cγ3, C$\gamma_{2b}$, C$\gamma_{2a}$ genes. Neo$^R$ gene insertion 3' of Cα, C$\gamma_1$ and Cα genes were less affected by these mutations both in culture and mice. They reasoned that the existence of a long-range 3' IgH regulatory region is required for germline transcription and class-switching recombination to multiple $C_H$ genes and that the neo$^R$ cassette insertion into the locus disrupts the ability of this region to facilitate germline transcription of dependent $C_H$ genes upstream of the insertion. Similarly, hs3A, and hs1,2 replacement mutation phenotypes both of which were cis-acting, suggested that the inhibition of class-switching recombination resulted from effects of inserted neo$^R$ gene, potentially via competition of the inserted promoter for control elements in the putative 3' IgH class-switching recombination regulatory locus. These findings also led to

the suggestion that class-switching recombination could be regulated, at least in part, by the relative ability of various germline $C_H$ promoters, after activation, to compete for activities of this putative regulatory region. The partial effect observed for G1 and the lacked of effect on IgA would suggest other mode of regulation existed for the regulation of these two isotypes (Seidl et al., 1999).

Consistent with above, it would make sense why replacement of hs1,2 by the $neo^R$ gene in cell line 9.9.2.1 would result in cessation of $\gamma_{2a}$ gene. The interesting question would be to see the effect of the $neo^R$ gene insertion in 9.9.2.1Δ3'αE, on ε and α germline transcription in the absence of Eμ.

# Chapter 4

## Shifting Control of Immunoglobulin Heavy Chain Gene Expression

## 4.1 - Introduction

In the last decade and a half, a large amount of data has been generated concerning the immunoglobulin (Ig) heavy chain locus and its regulation during B cell development. The intronic enhancer (Eμ), the first tissue specific transcription enhancer to be identified, plays an important role in the VDJ recombination process early in B-cell differentiation (Chen et al., 1993; Serwe and Sablitzky, 1993), and is necessary for the expression of μ heavy chain transcripts in pre-B cells (Wabl and Burrows, 1984) both in vivo and when linked to transfected reporter genes (Banerji et al., 1983; Gillies et al., 1983; Neuberger, 1983; Porton et al., 1990). In contrast, at later stages of B-cell development, Eμ becomes dispensable, shown by a number of Ig-secreting cell lines that lack Eμ, yet maintain high levels of heavy chain gene expression (Aguilera et al., 1987; Eckhardt and Birshtein, 1985; Klein et al., 1984; Wabl and Burrows, 1984; Zaller and Eckhardt, 1985). While IgH transcription in these cells did not require Eμ, other studies suggested there might be other regulatory elements that might be important (Grosschedl and Marx, 1988; Porton et al., 1990).

Analyses of chromosomal translocations characteristic of human Burkitt's lymphomas, mouse and rat myelomas indicated that the translocations placed the c-myc gene under the transcriptional control of the IgH locus (reviewed in Klein and Klein, 1985) . This results in the deregulated expression of the translocated c-myc allele in a B-cell specific manner as demonstrated in cell fusion studies (Greenberg et al., 1989). It was

suggested by several groups that juxtaposition of c-myc with a B-cell specific control element(s) within the IgH locus was the explanation for abnormal c-myc expression in these tumour lines. Although Eμ was initially considered an ideal candidate, it was subsequently noted that in many mouse myelomas, the translocated c-myc and Eμ lay on opposite translocation products reviewed in (Cory, 1986).

In support of the notion that additional transcriptional control elements might be present within the IgH locus, a subclone of the mouse myeloma cell line Δ 15.8, LP1.2 was discovered that retained an undisrupted IgH gene, complete with Eμ sequences, but with a large DNA deletion downstream of $C_\alpha$ exons. The result of the deletion was a reduction in IgA gene expression to approximately 10 % wild-type (Gregor and Morrison, 1986) . While it is possible that the downregulation of alpha gene expression in LP1.2 was due to transfer of a transcriptionally repressive element(s) closer to the alpha gene as a result of the deletion, another interpretation was that the deleted DNA sequences housed one or more positive regulatory elements. Support for the latter interpretation came when several laboratories showed that a number of positive regulatory elements exist downstream of $C_\alpha$ exons (Dariavach et al., 1991; Lieberson et al., 1991; Madisen and Groudine, 1994; Michaelson et al., 1995), all which are missing in LP1.2 (Michaelson et al., 1995).

Heading the list of the growing number of DNA segments exhibiting enhancer activity was hs1,2 formerly 3'αE) (Dariavach et al., 1991; Lieberson et al., 1991). This enhancer region is located approximately 16 kb downstream of the $C_\alpha$ exons in the murine IgH locus, and lies approximately 200 kb 3' of the $J_H$ gene segments where $V_H$

gene assembly takes place. As measured through both transient transfection assays and transgenic mouse studies, hs1,2 was found to be inactive at the pre-B cell stage and in resting B-cells, began to function upon antigen or mitogen activation of B-cells, and was maintained in an active state in Ig-secreting cells. In transient transfection assays, hs1,2 appeared to be less powerful than Eμ in some plasmacytoma lines, while in others, the two had equivalent activity (Arulampalam et al., 1994; Dariavach et al., 1991; Fulton and Van Ness, 1994; Lieberson et al., 1991; Madisen and Groudine, 1994; Singh and Birshtein, 1993). In any case, it appeared that while Eμ was active very early in B cell development, hs1,2 became active much later.

Hs3A (formerly $C_\alpha$3'E), was subsequently discovered immediately 3' of the $C_\alpha$ exons and was identified as a weak enhancer in Ig-secreting cells (Matthias and Baltimore, 1993) . DNase I protection studies on the IgH gene locus showed four DNase I hypersensitivity sites in the 3' region of the IgH locus: two of these (hs1 and hs2), mapped within 3'αE, a third, (hs3) mapped 13 kb downstream of hs1,2 and the forth (hs4), was 17 kb downstream of hs1,2. The DNA fragment surrounding hs3 was shown to serve as a transcription enhancer only in plasmacytomas by transient transfection analyses and the region is now denoted as hs3B. A DNA fragment surrounding hs4 was active in cell lines derived from all stages of B cell differentiation up to, and including, plasmacytomas (Giannini et al., 1993; Madisen and Groudine, 1994; Michaelson et al., 1995).

More recent data hinted that hs3A and hs3B were significantly related in sequence and functions (Chaveau and Cogne, 1996; Saleque et al., 1997). DNA sequence analyses

done on the entire length of the 1 kb Xba I fragment containing hs3A and the 1.2 kb sequence of hs3B showed not only that they were ≈ 94 % homologous except for a 100 bp gap found within the hs3A fragment, but also that they were oriented opposite to each other and localized at approximately equal distances away from the hs1,2 enhancer element (Chaveau and Cogne, 1996; Saleque et al., 1997). Because of these unique features, it was postulated that hs3A, hs1,2 and hs3B functioned together as a unit through the formation of a huge palindromic structure with (hs1,2) as the core, hs3A and hs3B flanking on each side.

While a number of studies have addressed the role of individual IgH enhancers in an isolated setting, few have examined their function as a group. Targeted disruption of Eμ drastically reduced VDJ recombination, severely affecting later B-cell differentiation (Chen et al., 1993; Serwe and Sablitzky, 1993). Interestingly, DJ joining is normal in these mice and some low level VDJ recombination can still occur, suggesting a weak functional compensation by other transcriptional elements within the IgH locus. As described in chapter 3, deletion of hs1,2 by homologous recombination in the cell line 9.9.2.1, a $\gamma_{2a}$ - expressing plasmacytoma which lacks Eμ (Eckhardt and Birshtein, 1985; Zaller and Eckhardt, 1985) , resulted in cessation of $\gamma_{2a}$ gene expression even though the other downstream enhancers, hs3A, hs3B and hs4 remained intact (Lieberson et al., 1995). While this result suggest that none of the remaining enhancers is sufficient to drive $\gamma_{2a}$ gene expression, it doesn't rule out the possibility that one or more is essential to this expression, in the presence of hs1,2. Similar arguments hold true for hs1,2 deficient splenocytes in chimeric mice (Cogne et al., 1994). Surface expression of IgM and

switching to $IgG_1$ and IgA appeared normal in these splenocytes while switching to other isotypes was impaired (Cogne et al., 1994). While hs1,2 was not essential for their expression, there is insufficient evidence to conclude which (if any) of the remaining enhancers was responsible or required for IgM, $IgG_1$, and IgA gene expression.

As described in chapter 3, in both the 9.9.2.1 cell line and in mice, the deleted hs1,2 was replaced by a neomycin$^r$ selectable marker gene. "Clean" knockouts of hs1,2 as well as of hs3A, were subsequently performed in mice using the Cre-loxP system (Manis et al., 1998). The findings from the latter studies show conclusively that neither hs3A nor hs1,2 is essential for IgH expression when all other IgH enhancers, including Eμ, are intact since both IgH expression and class-switching could occur in the absence of either one of them. What is still not clear from these knock-out experiments is which elements, if any, are individually essential to IgH expression or class-switching. Alternatively, after VDJ joining, the several elements may exhibit significant functional redundancy.

As an approach to these questions, we designed a series of reporter constructs with a $V_H$ promoter and enhancers positioned 3' of the reporter gene, to study the activity of these enhancers in various combinations over the course of B cell development.

# Chapter 4

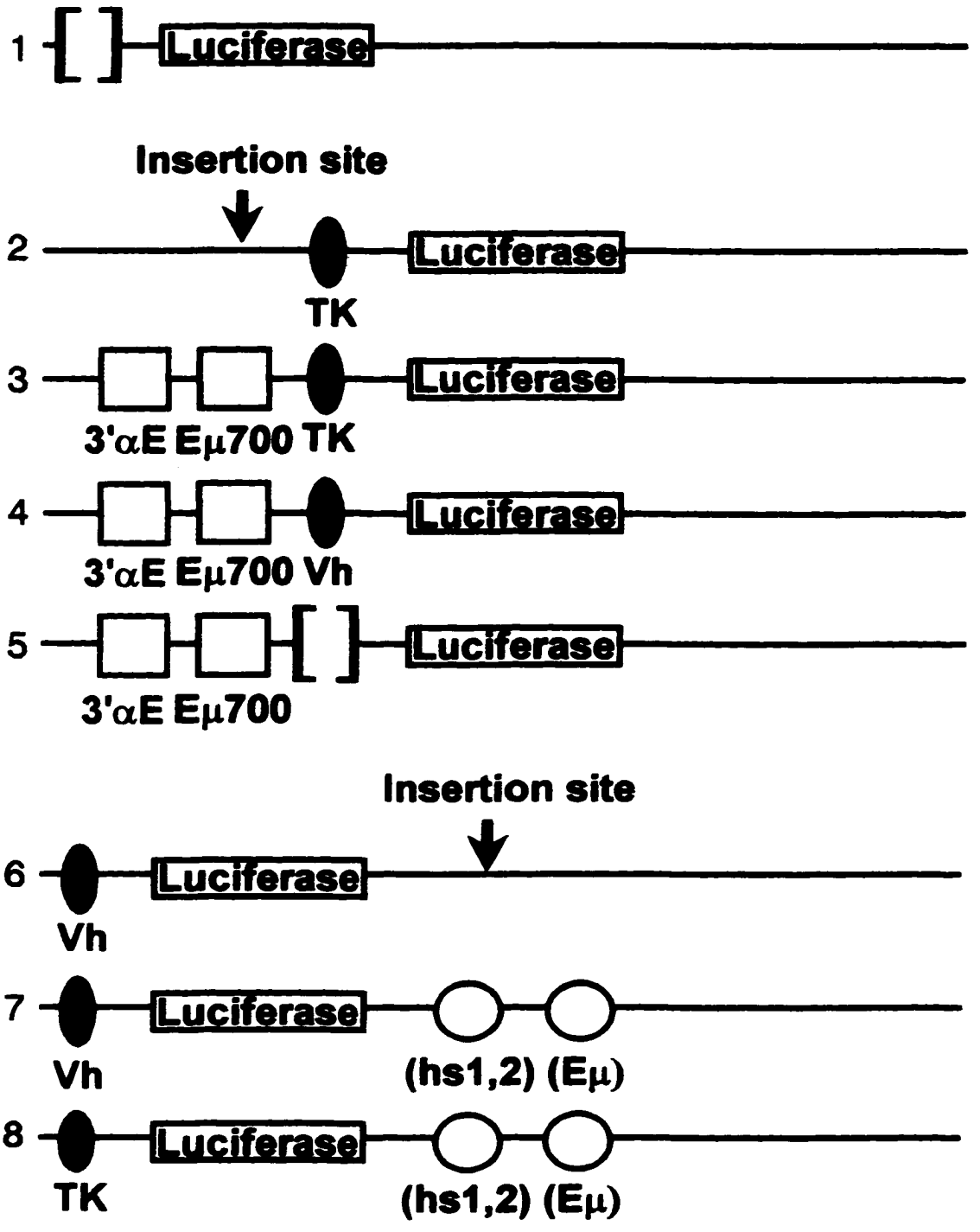
## Results

## 4.2 - Nature of the promoter and the placement of enhancer(s) relative to it, are crucial for accurately assessing enhancer activities

Enhancers are cis-acting DNA sequences that increase transcription from promoters in a manner that is independent of their orientation and distance relative to the RNA start site (Khoury and Gruss, 1983). The murine IgH locus illustrates this vividly - the intronic enhancer Eμ, lies within the second intron of the transcription unit approximately 4kb away from the rearranged variable region promoter. The 3' IgH enhancers are over 200kb away from the $J_H$ gene segments where $V_H$ gene assembly takes place (Fig. 5). Most published studies examining the activities of enhancer elements have used constructs with heterologous promoters or with the proposed enhancers placed immediately upstream of the promoter (Fulton and Van Ness, 1993; Fulton and Van Ness, 1994; Garcia et al., 1986; Madisen and Groudine, 1994; Matthias and Baltimore, 1993; Singh and Birshtein, 1996). To test directly whether positioning or the type of promoters is important for transcriptional activity, we utilized the constructs shown in Fig. 9. Starting from the promoterless luciferase reporter, pGL2 Basic (construct 1; Fig. 9), two different types of promoters were inserted: a genuine IgH heavy chain promoter, $V_H$ (construct 6; and abbreviated (BV) in the bar graphs) or the thymidine kinase promoter (TK) (construct 2; and abbreviated (pTK) in the bar graphs). To each of these promoter constructs then, were added enhancer combinations consisting of core regions of 3'αE and Eμ (designated 3'αEEμ700) or the "full-length" 4 kb 3'αE and the 1 kb Eμ

**Figure 9.**

Schematic diagrams of the test constructs used for different promoter and placement of enhancer analyses. Construct #1, indicate the promoterless vector, represented by [ ] placed upstream of the luciferase reporter gene. Construct #2, pTK, consisting of TK promoter driving the luciferase reporter gene and the site for enhancer insertion upstream of the TK promoter as indicated by the thick arrow. Constructs #3-5 compare the effect of various promoters on the activity of luciferase reporter gene in the presence of 3'αE Eμ700 enhancer combination. Constructs #6, BV, $V_H$ promoter is represented by the filled ovals placed upstream of the luciferase reporter gene and the site for enhancer insertion downstream of it as indicated by the arrow. Construct #7 and 8, used to study the effect of different promoter on luciferase reporter gene containing (hs1,2,Eμ) enhancer combinations placed downstream of it. Enhancers are represented by circles or squares placed either downstream or upstream of the luciferase gene.

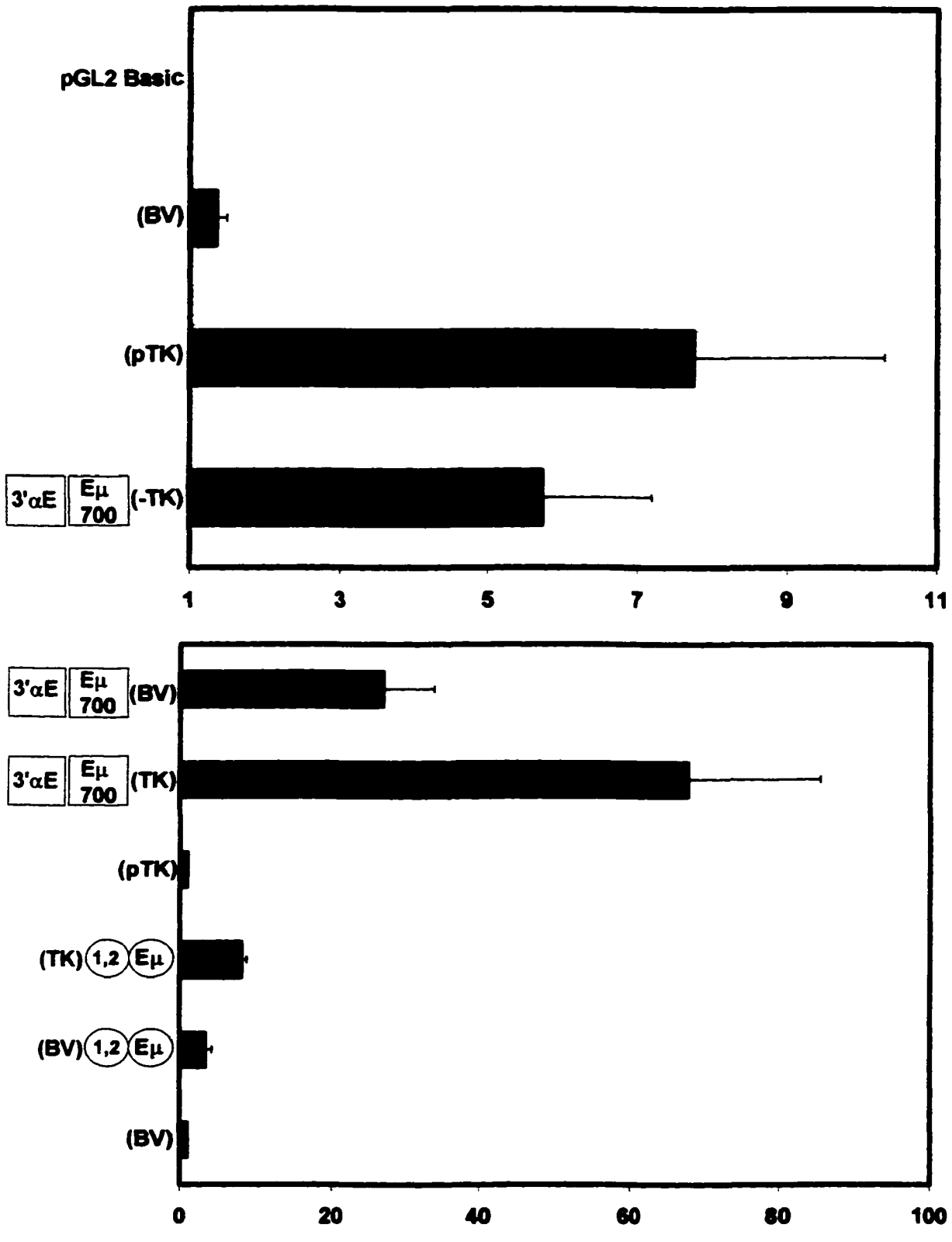


fragments (designated hs1,2,Eμ), either immediately adjacent to the promoter (constructs 3 & 4; Fig. 9) or downstream of the luciferase reporter gene (constructs 7 & 8; Fig. 9). Finally, in order to test if the enhancers when placed immediately adjacent to the promoter, could substitute for promoter activity, we placed the core enhancer pairs (3'αEEμ700) immediately upstream of the luciferase reporter in the absence of any promoter (construct 5; Fig. 9).

These constructs were then transiently transfected into the plasmacytoma cell line S194 with β-galactosidase reporter gene as an internal control, and assayed for function. The results obtained are shown in Fig. 10 (a & b). The activity of the promoterless pGL2 Basic construct was used as a reference point (assigned an arbitrary value of 1), and all other activities are fold enhancement relative to this background level. In Fig. 10 (a), the activities of both (pTK) and (BV) constructs are compared, showing that the TK promoter is substantially more active than the $V_H$ promoter, ≈ 8 fold versus ≈ 1.5 fold activity relative to pGL2 Basic construct, respectively. As shown in Fig. 10 (b), the insertion of either the core enhancer sequences (3αEEμ700) or the full-length enhancers (hs1,2,Eμ), stimulated promoter function in all cases. However, the amount of activity varied depending upon their position relative to the promoter. When inserted downstream of the luciferase gene, (hs1,2,Eμ) activated the $V_H$ promoter <5 fold (BV (1,2,Eμ)] and the TK promoter <10 fold relative to their respective enhancerless constructs ((TK)(1,2,Eμ)). If core sequences of these enhancers, were placed immediately adjacent to the promoter, their activation increased significantly to ≈25 fold with $V_H$ promoter ((3αEEμ700)(BV)), and ≈65 fold with TK promoter ((3αEEμ700)(TK)) (fold enhancement again, relative to

**Figure 10.**

Enhancer activities as observed in S194 cell line. The fold enhancement above the enhancerless construct was determined by dividing the normalized luciferase values obtained for each construct by the values generated by the either the promoterless (pGL2 Basic) or the enhancerless constructs (pTk or BV). Error bars show the standard of deviation of the values obtained in each case. At least six independent transfections, using at least two different plasmid preparations, were performed with each construct.



their respective enhancerless constructs). Importantly, even when there was no promoter present ((3αEEμ700)(-TK)), there were significant levels of transcription generated by enhancer combinations (3αEEμ700) alone (construct 5; Fig. 9) suggesting that (3αEEμ700) can not only serve as an enhancer but also as a promoter should it be placed upstream of the reporter gene [3'αEEμ700(-TK), see Fig. 10 (a)].

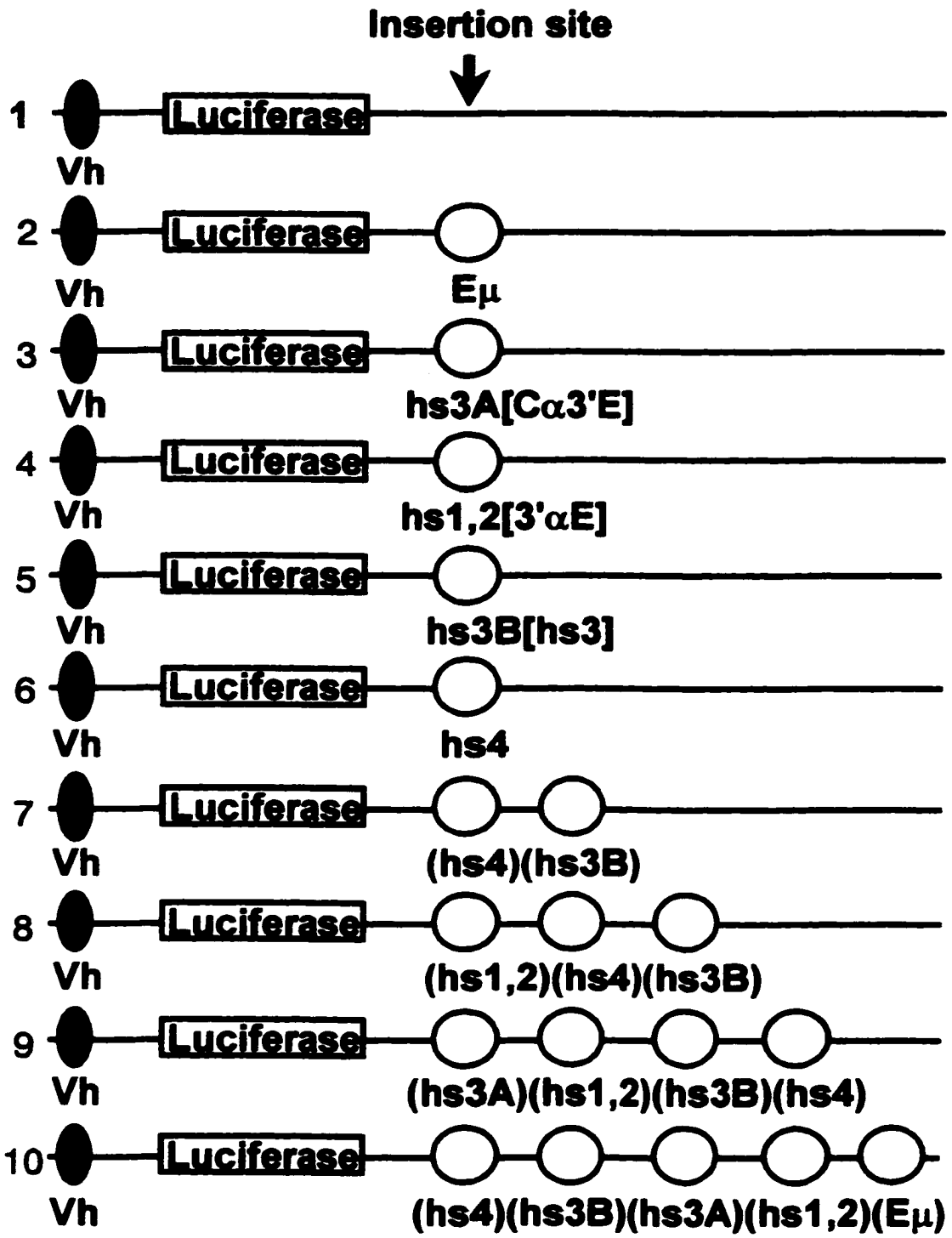
## 4.3 - The relative contributions of Eμ and the 3' enhancers change during B cell development

Eμ is located approximately 4 kb away from the rearranged variable gene promoter region (see Fig. 5). Stable transformants of pre-B cells carrying transfected Ig genes with and without Eμ, indicate the importance of Eμ in the activation and maintenance of such transfected gene expression (Grosschedl and Marx, 1988; Porton et al., 1990; Zaller and Eckhardt, 1985). Earlier studies demonstrating Eμ as an enhancer were done with Eμ inserted either upstream and adjacent to a heterologous promoter, or downstream in a reporter gene driven by a heterologus promoter (Banerji et al., 1983; Gillies et al., 1983) (Mercola et al., 1983). Certainly, this is not an accurate representation of the situation encountered in a normal IgH locus.

We inserted Eμ at the insertion site of the previously described $V_H$ promoter construct-pGL2BV (construct 6; Fig. 9; or construct 1; Fig. 11), resulting in

**Figure 11.**

Schematic diagrams of the test constructs used for enhancer combination analyses. The $V_H$ promoter is represented by the filled ovals placed upstream of the luciferase reporter gene. Enhancers are represented by circles downstream of the luciferase gene, a distance away from the $V_H$ promoter. Construct # 1 is the enhancerless construct, also diagramed in Fig. 9; constructs #2-6 housed individual enhancers only; constructs # 7-10 housed combinations of enhancers as indicated. The actual DNA fragments used as enhancers are described in Materials and Methods.



pGL2BV(Eμ) (construct 2; Fig. 11), trying to mimic the IgH locus as closely as possible. We then transiently transfected cell lines representing non-B cells, pre-B cells, B cells (mouse and human) and plasma cells. The results of 6-17 independent experiments, presented as fold enhancement above the enhancerless construct, are shown in Figs. 12 (a & b), 13 (a & b), 14 (a & b), 15 (a & b) and 16. Although the fold enhancement by Eμ varies slightly between individual cell lines, it is apparent that Eμ is functional throughout all stages of B cell development; activating promoter activity approximately 5 - 20 fold.

As had been shown previously, Eμ is active in cell lines representing all stages of B cell differentiation. Since Ig expression is controlled by both Eμ and the 3' IgH enhancers concurrently, we examined the relative contribution of each unit (Eμ vs 3' IgH enhancers) and then asked what its relative contribution to IgH expression was, when all IgH enhancers were present. This can be expressed as the percent activity of each (Eμ and the combined 3' elements; construct 2 & 9, Fig. 11) compared with activities of all enhancers combined (construct 10; Fig. 11), summarized as follows and also in legend to Table I.

(Eμ)/ (hs4,3B,3A,1,2,Eμ) X 100 % = % relative contribution of Eμ to overall activity

(Hs3A,1,2,3B,4)/(hs4,3B,3A,1,2,Eμ) X 100 % = % relative contribution of 3'IgH enhancers to overall activity

Table I summarizes the data expressed in this fashion. Referring to Table I, at the pre-B cell stage and the plasma cell stage, the relative contribution of Eμ is high compared to

**Figure 12.**

Enhancer activities as observed in two pre-B cell lines (18-81, 18-8). The fold enhancement above the enhancerless construct was determined by dividing the normalized luciferase values obtained for each construct by the values generated by the enhancerless construct. Error bars show the standard of deviation of the values obtained in each case. At least six independent transfections, using at least two different plasmid preparations, were performed with each construct.

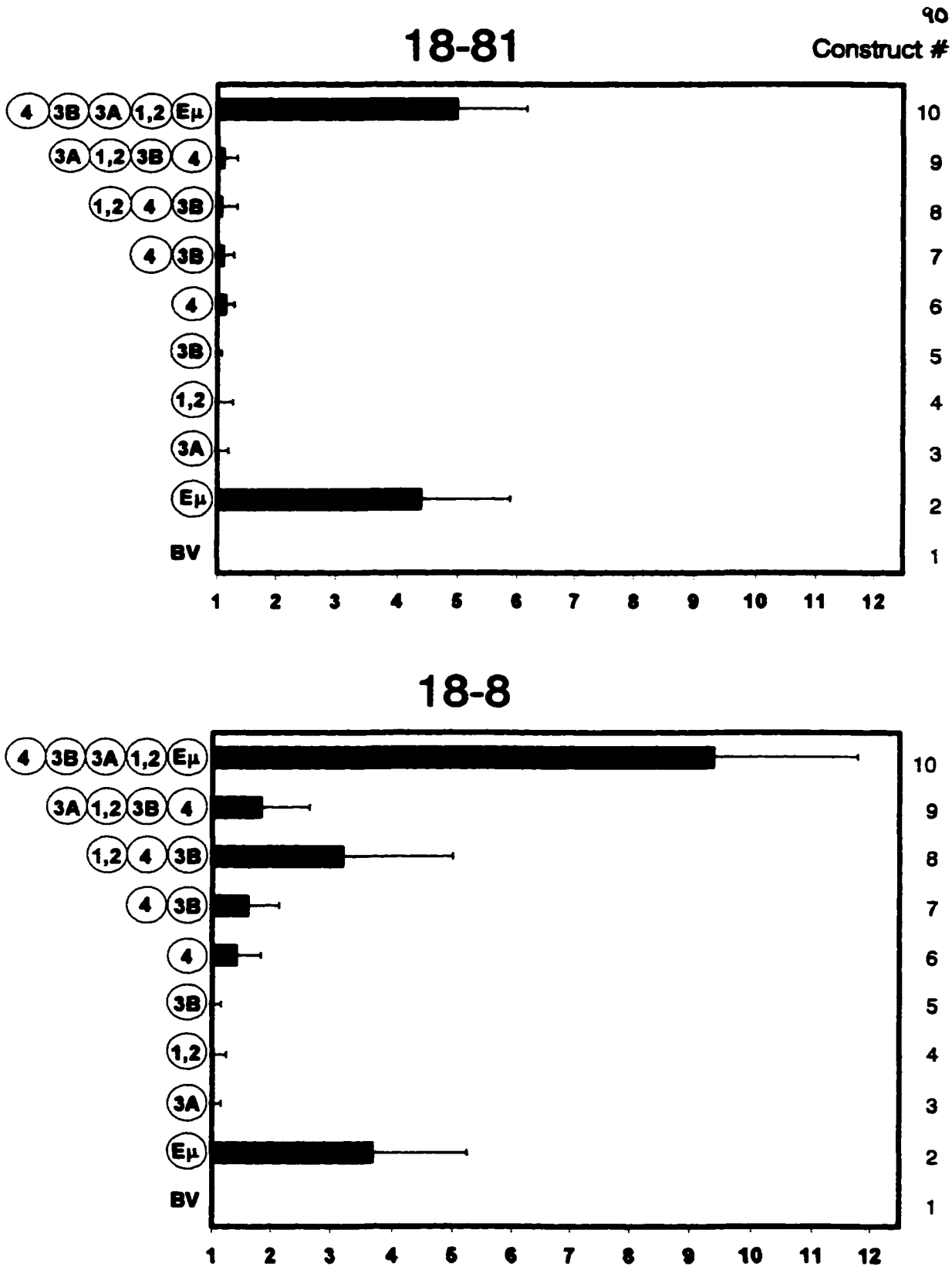


**Figure 13.**

**Enhancers activities as observed in cell lines M12.4.1 and A20, both representative of the murine surface $Ig^+$ B-cell stage. Enhancement levels were calculated as described in the** legend to Fig. 12.

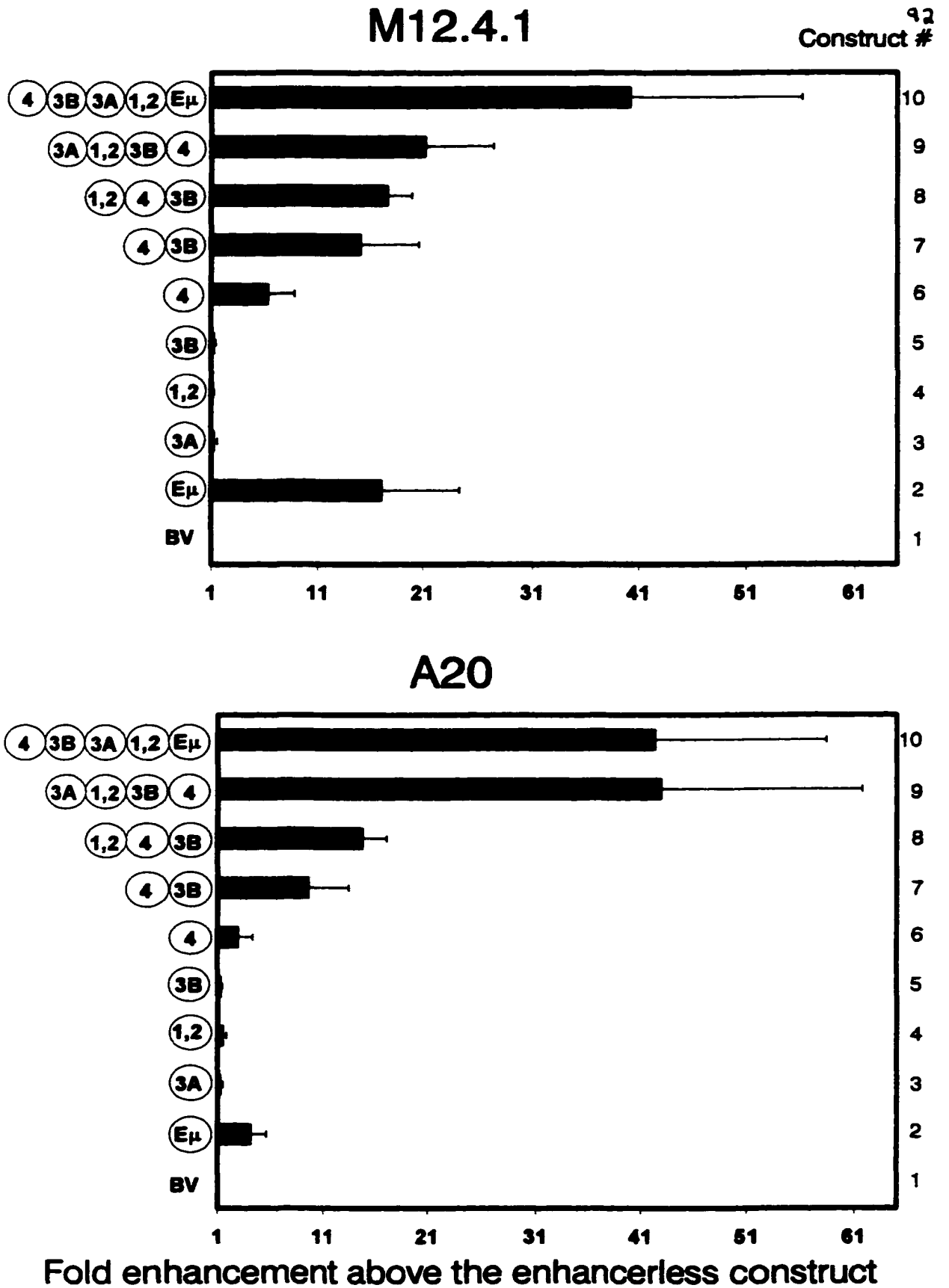


**Figure 14.**

Enhancer activities as observed in two human cell lines representatives of the surface $Ig^+$ B-cell stage (Raji and Namalwa). Enhancement levels were calculated as described in the legend to Fig. 12.

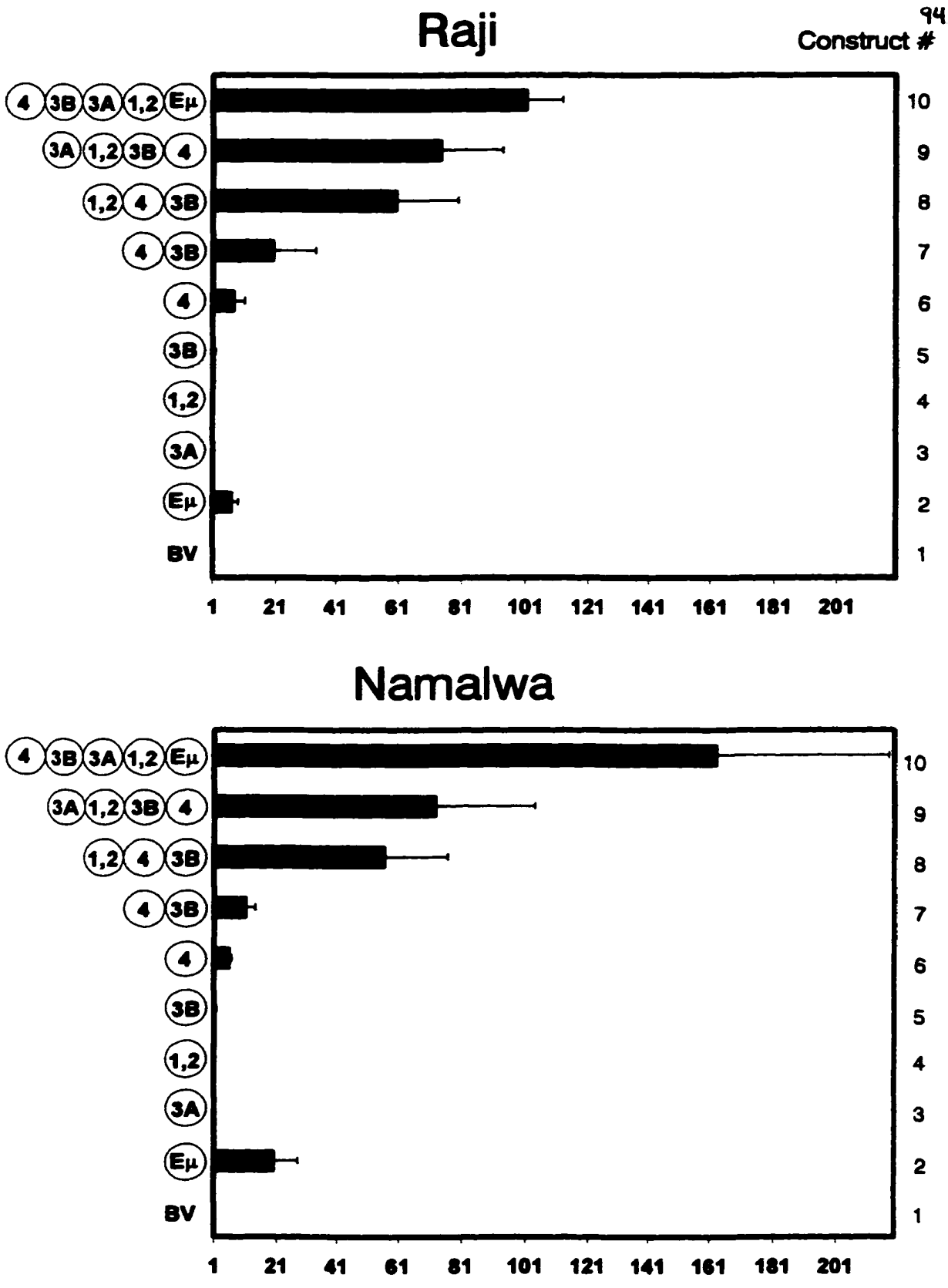


**Figure 15.**

Enhancer activities as assayed in S194 and P3X63Ag8 cell lines representative of the Ig-secreting stage in B cell development. Enhancement levels were calculated as described in the legend to Fig. 12. For these cell lines, 6-17 independent transfections were done with at least two different plasmid preparations of each construct.

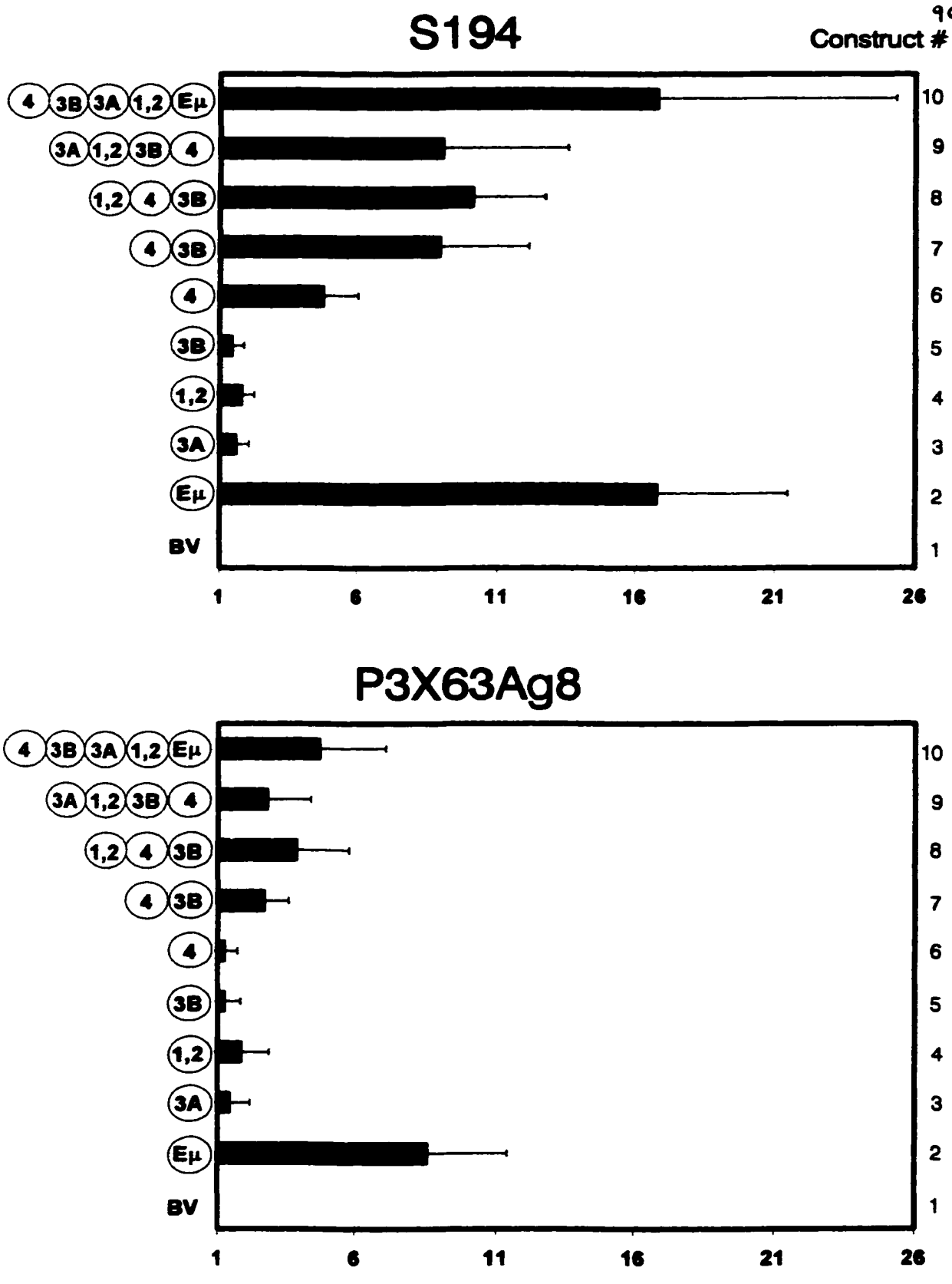


**Figure 16.**

Enhancer activities as assayed in non-lymphoid cells (Rat-1, fibroblast cell line).

Enhancement levels were calculated as described in the legend to Fig. 12.

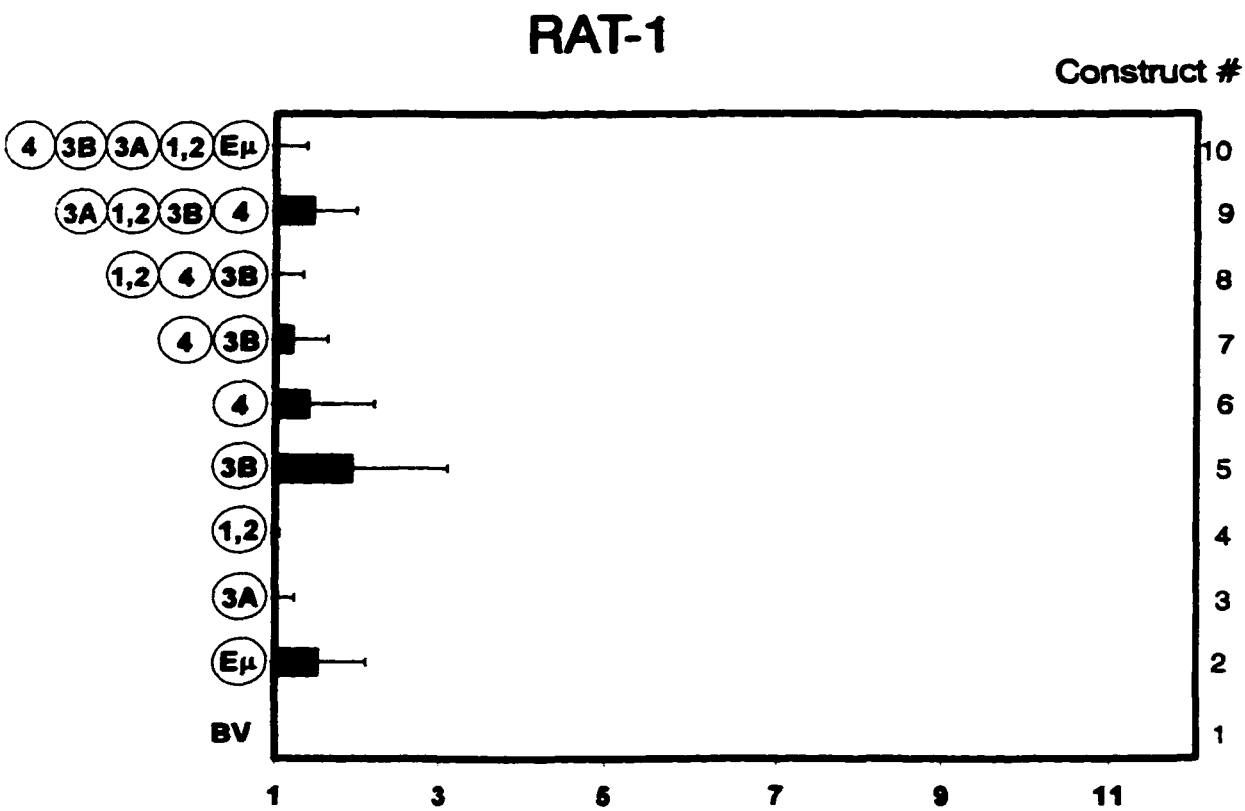


**Table I.**

**Relative contribution of Eμ and of the 3' enhancers, respectively, as a function of B cell development.**

[a] Eμ contribution was calculated as the ratio Eμ/(hs1,2,3A,3B,4,Eμ) x 100%.

[b] 3' enhancer contribution was calculated as the ratio [hs1,2,3A,3B,4]/[hs1,2,3A,3B,4,Eμ] x 100%.

| | Cell lines | Eμ contribution [a] | 3' element contribution [b] |
|---|---|---|---|
| Pre-B cells | 18-81 | 90% | 3% |
| | 18-8 | 30% | 10% |
| Surface Ig+ B cells | Raji | 6% | 70% |
| | Namalwa | 10% | 40% |
| | M12.4.1 | 40% | 50% |
| | A20 | 7% | 100% |
| Ig - secreting B cells | S194 | 100% | 50% |
| | P3X63Ag8 | 180*% | 60% |

the 3' IgH elements' contributions. However, at the surface $Ig^+$ cell stage, the relative contribution of Eμ is significantly lower while the predominant contributor being the 3' IgH elements. The lower relative contribution of Eμ was observed in both murine (surface $Ig^+$) B cells as well as human Burkitt's lymphomas [Figs. 14 (a & b); summarized in table I]. Importantly, as indicated by fold enhancement of Eμ (construct 2; Fig. 11) relative to the enhancerless construct (BV), Eμ's activity is itself not significantly altered in these cell lines. Thus the difference in relative contribution by Eμ actually reflects an increase in 3' IgH enhancer function.

### 4.4 - 3' Enhancer activities during B - cell differentiation

As described in the Introduction, the murine IgH locus houses enhancer elements both upstream (Eμ) and downstream (hs3A($C_\alpha$ 3'E), hs1,2(3'αE), hs3B(hs3), hs4) of the constant region exons (Fig. 5). Although activities of individual enhancer elements have been examined by others, we chose to examine the behavior of these enhancers when grouped together over the course of B cell development. To determine individual enhancer activities at each stage of B cell development, we transiently transfected test constructs 1- 6 (Fig. 11) into B cell lines representative of pre-B, surface $Ig^+$, and plasma cell stages of B cell differentiation. Transfections were performed in at least two cell lines for a given stage of B cell development to allow for cell line to cell line variations. The

fold activation by each element above the enhancerless construct is summarized in Figs. 12 (a & b), 13 (a & b), and 15 (a & b). Consistent with earlier reports, hs3A, hs1,2 and hs3B were inactive in pre-B cells and surface $Ig^+$ cells, while hs4 showed weak activity in the pre-B cell line, 18-8, was active in all B cell lines and in the plasma cells. Hs3A, hs1,2 and hs3B were weakly active in plasma cells. We tested our murine enhancer constructs in two human Burkitt's lymphoma lines, Raji and Namalwa, to examine whether their function was conserved since reports showed that the human IgH locus contained regions with homology to these 3' IgH enhancer elements (Chen and Birshtein, 1997; Mills et al., 1997). As presented in Figs. 14 (a & b), hs4 was the only element active in these cell lines; the rest of the enhancers (hs3A, hs1,2 and hs3B) were inactive in both human B cell lines.

### 4.5 - 3' Enhancer elements have strong synergistic activity

We next asked whether the enhancers could interact functionally with one another, and, if so, whether such interactions would change during B cell development. Constructs bearing single or multiple IgH enhancer elements (constructs 1- 9; Fig. 11) were transiently transfected into cell lines representative of each stage of B cell differentiation. Normalized luciferase values from 6-17 independent transfections were collected and fold activation above the enhancerless construct calculated. As shown in

Figs. 12 (a & b), 13 (a & b), 14 (a & b), 15 (a & b), there is very strong synergistic activity when the 3' elements are combined, and this synergy shows a clear developmental progression. In pre-B cells, all combinations of 3' elements were only weakly active [Figs. 12 (a & b)] . In surface $Ig^+$ cells however, there was a very large increase in activity in all combinations [note the change in scale between Figs. 12 (a & b) and 13 (a & b)]. Even combinations of just two elements (hs3B and hs4) yielded activities much larger than the sum of their individual activities, demonstrating functional synergy. Furthermore, this activity in each cell line was equal to or greater than that observed with Eμ alone [Figs. 13 (a & b) and 14 (a & b)]. As more 3' elements were added, the activation similarly increased, reaching ≈ 80 fold over the enhancerless construct, in the case of the Burkitt's lymphomas Raji and Namalwa [Figs. 14 (a & b)]. The activity found with all four 3' elements together vastly exceeded the sum of their activities separately and, in most cases, even surpassed the activity of Eμ [Figs. 13 (b), 14 (a & b)]. Interestingly, at the plasma cell stage, the dramatic synergy prevalent in the surface $Ig^+$ stage was not observed. Combinations of the 3' IgH elements were clearly stronger than individual elements, but the resulting activity appeared to be additive, not synergistic (Table II). In addition, Eμ's activity now was equal to or greater than all four 3' IgH elements combined together (Figs. 15 (a & b), (Table II)).

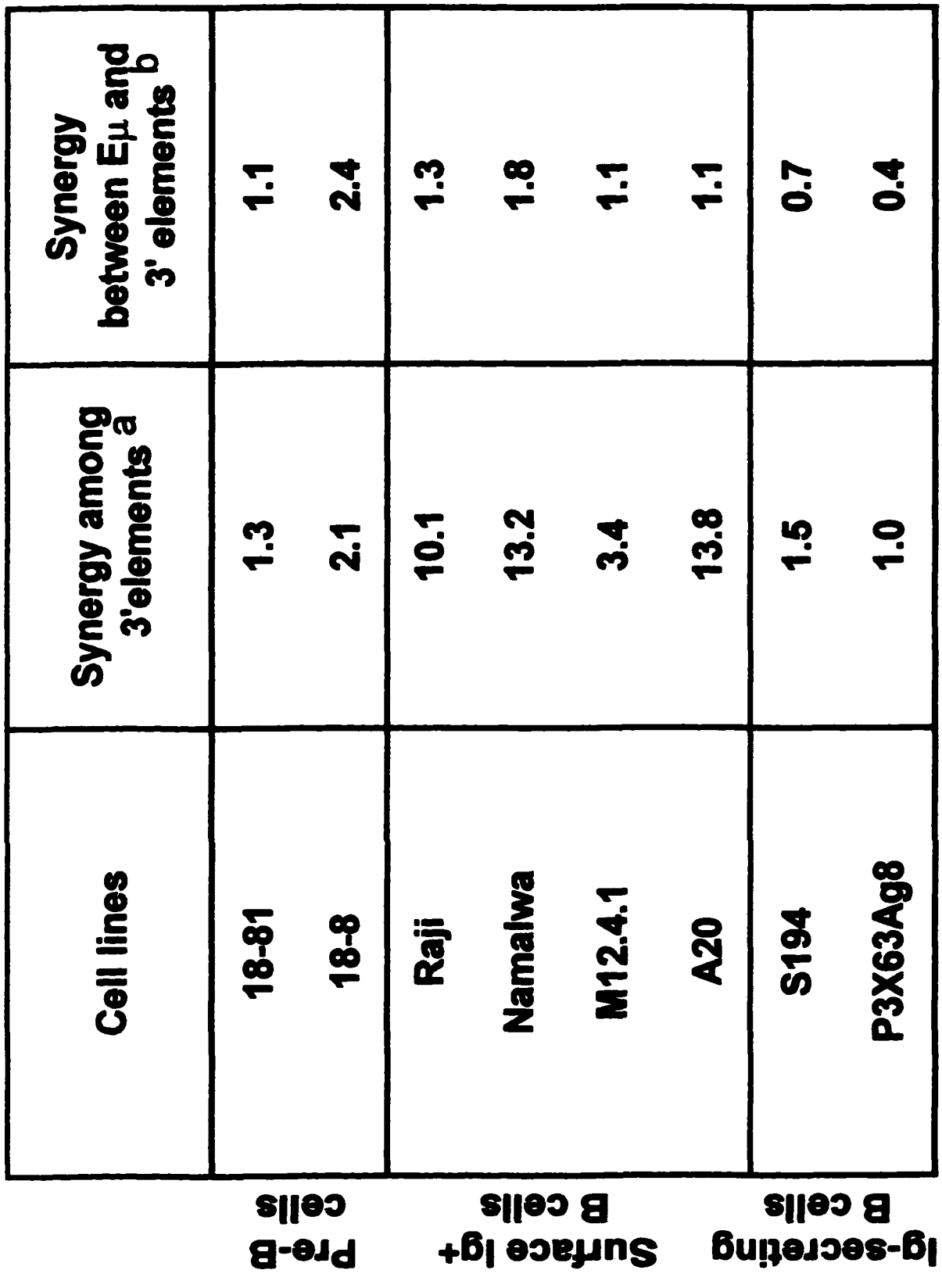
In conclusion, we found that activity of the 3' IgH elements varied both quantitatively and qualitatively over the course of B cell differentiation. The 3' IgH elements showed little or no function at the pre-B cell stage but were strongly synergistic at the B cell stage, with as few as two elements exhibiting an activity much greater than

**Table II.**

Enhancer synergy as a function of B cell development

[a] Synergy among the 3' enhancers was determined as fold enhancement with the combined 3' enhancers (Fig. 11, construct #9; [hs1,2,3A,3B,4]) divided by the sum of the enhancement values obtained with individual 3' enhancers (Fig. 11, constructs #3, #4, #5, #6; [hs1,2 + hs3A + hs3B + hs4]).

[b] Synergy between the upstream enhancer Eμ, and the 3' enhancers was determined as fold enhancement obtained with all enhancers combined (Fig. 11, construct #10; [hs1,2,3A,3B,4,Eμ]) divided by the sum of the enhancement values obtained with the combined 3' enhancers and Eμ, respectively (Fig. 11, constructs #2 and #9; [Eμ] + [hs1,2,3A,3B,4].



| | Cell lines | Synergy among 3'elements [a] | Synergy between Eμ and 3' elements [b] |
|---|---|---|---|
| Pre-B cells | 18-81 | 1.3 | 1.1 |
| | 18-8 | 2.1 | 2.4 |
| Surface Ig+ B cells | Raji | 10.1 | 1.3 |
| | Namalwa | 13.2 | 1.8 |
| | M12.4.1 | 3.4 | 1.1 |
| | A20 | 13.8 | 1.1 |
| Ig-secreting B cells | S194 | 1.5 | 0.7 |
| | P3X63Ag8 | 1.0 | 0.4 |

the sum of their individual functions. At the plasma cell stage, the activity of the 3' IgH elements remained strong. The dramatic synergy observed in B cells, however, was much less pronounced in the Ig-secreting cells. These changes in function of the 3' IgH elements contrast with the relatively stable intronic enhancer activity throughout B cell differentiation.

## 4.6 - The 3' enhancer elements do not synergize with the intronic enhancer element Eμ

While the above described analyses demonstrated strong functional interactions among the 3' IgH elements, we also were interested in potential interactions between Eμ and the 3' IgH elements. Therefore, we included a construct bearing all four 3' elements as well as Eμ (construct 10; Fig.11) and tested its function at the various B cell stages, as shown in Figs. 12 (a & b) - 15 (a & b).

In the pre-B cell stage, where we observe little or no 3' enhancer activity, addition of Eμ to the combined 3' elements results in an activity close to that of Eμ alone (Fig. 12 (a & b), Table I). In the murine and human surface $Ig^+$ cell lines, there may be a moderate increase in activity when Eμ is added to the 3' enhancers, however, the level of activity is additive, not synergistic [Figs. 13 (a & b), 14 (a & b), Table II]. In S194 plasma cells, combining Eμ with the 3' IgH enhancers appears to have neither additive nor synergistic

effects. The activities observed at this stage are equivalent to Eμ alone (activity generated from construct 10 was less than the sum of both Eμ (construct 2; Fig. 11) and 3' enhancer activities put together (construct 9; Fig. 11). In contrast, in P3X63Ag8 cells, combining Eμ and the 3' IgH enhancers together generates activity much less than Eμ alone [Fig 15 (b); Table II].

To ensure that the activities we observed with the various enhancer combinations were indeed B cell specific, we transiently transfected the fibroblastic cell line, Rat-1, with each of our test constructs (constructs 1-10). As shown in Fig. 16, none of the constructs exhibited strong activity in this cell line.

In summary, while the 3' IgH elements exhibit strong synergistic interactions with one another, there appears to be little functional interaction between the 3' IgH enhancers when considered as a unit and the intronic enhancer. In pre-B and B cells, addition of Eμ to the 3' IgH elements creates mostly additive effects, while in plasma cells, Eμ may actually repress 3' IgH element function.

## 4.7 - There is functional redundancy among IgH enhancer elements

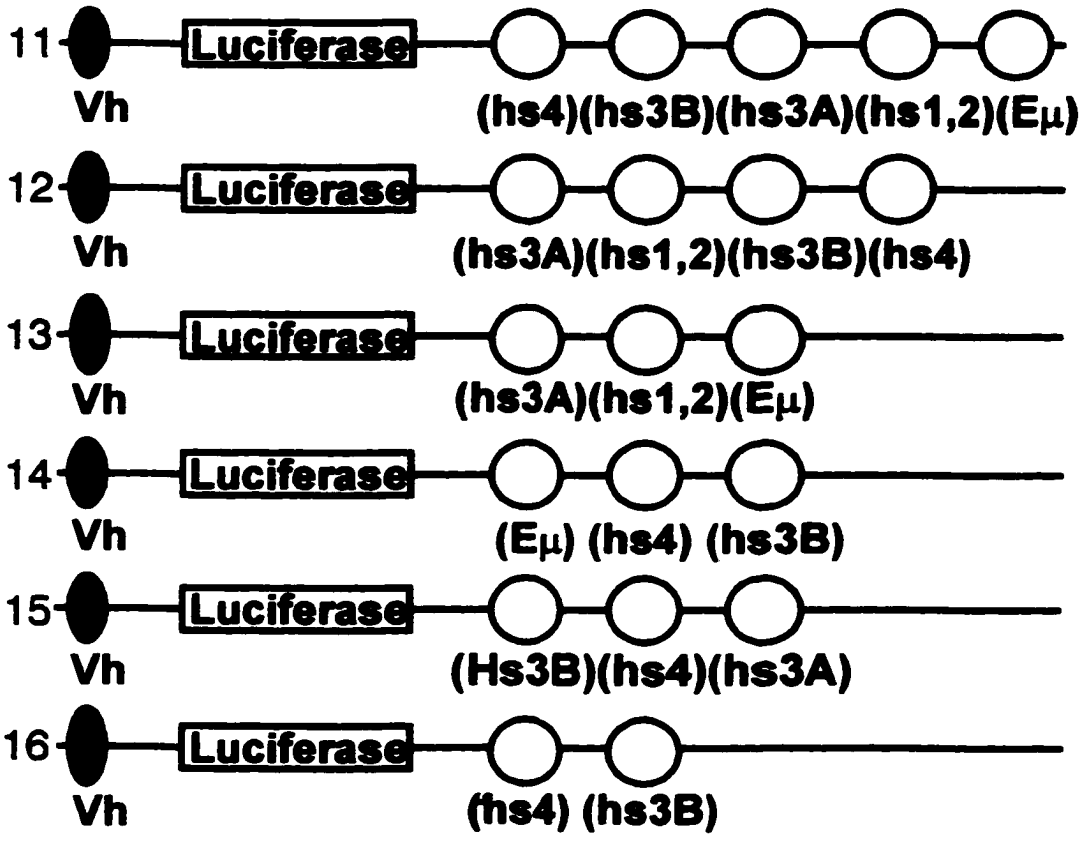
In previous sections, we observed that hs3A, hs1,2, hs3B, hs4 function more efficiently as a synergistic unit rather than as individual elements in surface $Ig^+$ B cells. We sought to determine which of the individual elements were contributing to this

synergy. One approach was through sequential deletion of hs3A and then hs1,2 from hs3A,12,3B,4 (construct 9; Fig. 11) where all the 3' IgH enhancer elements exist as a unit, generating constructs 8 (hs1,2,4,3B) & 7 (hs4,3B) (Fig. 11) respectively. These constructs were then transfected multiple times into B cells representing three different stages of development. The results are summarized in Figs. 12 (a & b), 13 (a & b), 14 (a & b) and 15 (a & b). Comparing constructs 8 & 9 in Figs. 12 (a & b), 13 (a), 14 (a & b) and 15 (a & b), the relative luciferase activities from both constructs were not significantly different from each other. Additionally, in Figs. 12 (a & b), 13 (a) and 14 (a & b) comparing constructs 7 & 9, we observed no significant differences in their relative luciferase acitivities suggesting there were functional redundancies among the 3' IgH enhancer elements - the loss of one element (hs3A), or two [(hs3A), (hs1,2)] elements, often resulted in little if any detriment to the overall activity of the unit as assayed in pre-B, Ig-secreting cells and in one of the surface $Ig^+$ cells. Thus, the remaining enhancer elements were sufficient to compensate for losses of one or a few elements.

To determine if the above observations were specific to loss of (hs3A) or (hs3A, 1,2), construct 15 (Fig. 17) that lacked the (hs1,2) enhancer element alone was generated. Multiple transfections were performed into Raji cells - a surface $Ig^+$ B lymphoma line where pronounced synergies among the 3' IgH enhancer elements were evident (see Table I). As indicated in Fig. 18 (a), the 3' IgH enhancer elements once again functioned more efficiently as a unit yielding a synergistic effect, albeit at lower overall levels (comparing construct 9, Fig. 14 (a) and construct 12, Fig. 18 (a)). Removal of hs1,2 from the full complement of IgH 3' enhancers (construct 15; Fig. 17), resulted in no detrimental effect

**Figure 17.**

Schematic diagrams of the test constructs used for functional redundancy analyses. Construct # 11 is the construct with all the IgH enhancer elements as a unit, construct #12-16 missing either one, two or three enhancer elements as indicated.

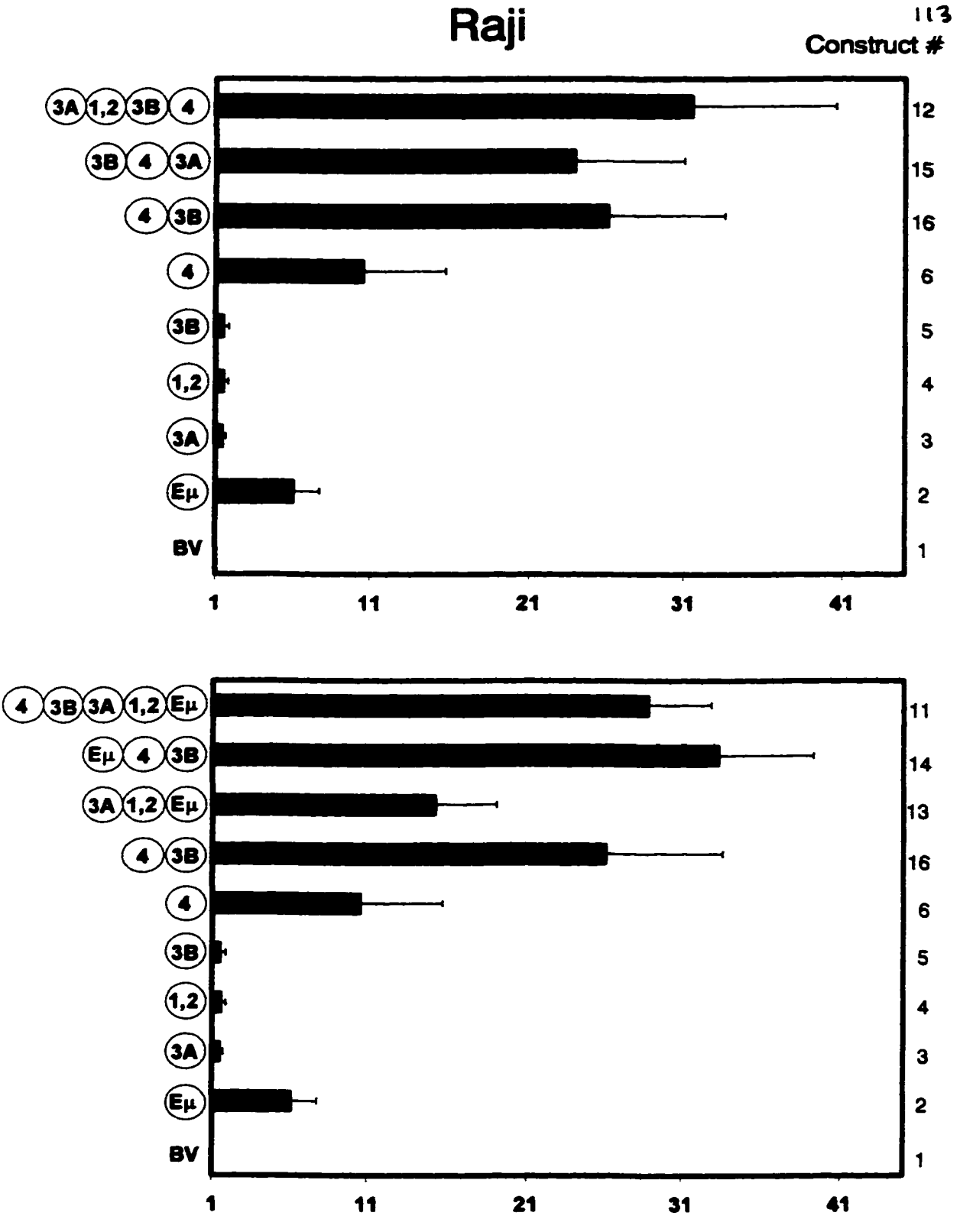


to the activity of the unit (Fig. 18 (a) comparing constructs 12 & 15), demonstrating that the redundancies amongst the 3' IgH enhancers observed previously were not specific to the loss of hs3A . Hs1,2 like hs3A, appeared not to contribute much at all to the overall activity of the unit since the removal of hs1,2 from construct 12 resulted in levels of luciferase activities that were not significantly different from those with all the 3' IgH enhancer elements intact (Fig. 18 (a), construct 12 & 15). Interestingly, the subsequent removal of hs3A from construct 15 (Fig. 17) also resulted in luciferase activity that was not significantly different from those with hs3A (Fig. 18 (a) comparing constructs 15 & 16), suggesting that the order of removal of hs3A and hs1,2 from the 3' IgH enhancer unit is irrelevant as the outcome remained the same in both cases [Figs. 14 (a) & 18 (a)]. Furthermore, from this particular transfection, (hs4,3B) was roughly equivalent to all 3' IgH enhancers together in activity (Fig. 18 (a) compare constructs 12 & 16), suggesting that pairing of hs4 and hs3B was sufficient to achieve maximal transcriptional activity. (It should be noted that in earlier transfections, pairing of hs4 and hs3B was significantly weaker than all 3' IgH enhancers together).

To test the significance of (hs4,3B) contributions in the context of all IgH enhancers (construct 11, Fig. 17), construct 13 consisting of only hs3A, hs1,2, and Eμ was generated and transfected into Raji cells. From Fig. 18 (b), the loss of hs3B and hs4 enhancer elements from the full complement of IgH enhancers (construct 11, Fig. 17) resulted in an approximately 50 % reduction in luciferase activity (comparing constructs 11 & 13 in Fig. 18 (b)] showing significant contribution of hs4 and 3B to the activity of the full complement of IgH enhancers. Interestingly, even though there were no

**Figure 18.**

Enhancer activities as observed in human cell line representative of the surface $Ig^+$ B-cell stage (Raji). Enhancement levels were calculated as described in the legend to Fig. 12.



significant differences in luciferase activities between (hs3A,1,2,Eμ) (construct 13, Fig. 17)(p value of 0.08) and (hs4,3B) (construct 16, Fig. 17) (Fig. 18 (b) comparing constructs 13 & 16), (hs3A,1,2,Eμ) still could not fully compensate for the loss of (hs4,3B) (Fig. 18 (b) comparing constructs 11 & 13) suggesting that there is something unique about the (hs4,3B) combination that could not be supplanted by the remaining IgH enhancer elements.

As noted above, constructs 16 and 12 were functionally equivalent, indicating that hs3A and hs1,2 were dispensable with respect to maximal 3' IgH enhancers activity in Raji cells. To test the notion this would also be true in the normal chromosomal context in which Eμ is present, construct 14 (Eμ, hs4, hs3B) (Fig. 17) was generated and transfected into Raji cells. In Fig. 18 (b), we see that the luciferase activities of constructs 11 & 14 were not significantly different (p value of 0.09), demonstrating that in Raji cells, (hs3A,1,2) was also dispensable in the context of the full complement of IgH enhancers. In summary, the loss of hs3A, hs1,2 or both (hs3A and hs1,2) did not affect the overall activity of the IgH enhancers as a unit in Raji cells. Functional redundancy among the IgH enhancer elements allowed for loss of individual elements without effects on phenotype.

Is the pairing of (hs4,3B) the only combination that can effectively substitute for full IgH enhancer activity? To approach that question, constructs bearing different enhancer element pairs were generated and transfected into surface $Ig^+$ B cell lines - Raji and M12.4.1 and also into an Ig-secreting line (S194). By analyzing the activities of each pair of elements, we hoped to be able to determine whether certain pairs functionally

interacted more efficiently than others, and if so, if they exhibited stage specificity.

## 4.8 - Hs4 synergistic interactions

Previous data presented here and elsewhere (Madisen and Groudine, 1994; Michaelson et al., 1995) showed that hs4 alone, unlike the other 3' IgH elements, acts as an enhancer in plasma cells, B cells and, in some cases, pre-B cells. We examined the ability of hs4 to synergize with other enhancers in pairwise combinations (constructs 17 - 21; Fig. 19) by transient transfection into Raji and M12.4.1. The results are shown in Figs. 20 (a) & 21(a).

As described previously (Sections 4.3 and 4.4), only hs4 and Eμ exhibited significant activity above background when transfected alone into Raji or M12.4.1 cells. None of the other 3' IgH elements functioned when transfected individually.

In Raji cells, hs4 was observed to functionally synergize with each of the other enhancer elements hs3A, hs1,2, hs3B, and Eμ and even with a second hs4 element. In this and subsequent sections, synergy was measured by using a paired t-test to ask whether "ab" was equal to (a+b) or alternatively, was significantly greater than (a+b). For enhancer pairs, involving hs4, $p < 0.05$ for $ab = a + b$, meaning that the combination (ab) was significantly more active than the sum of the individual activities (a + b). Table III summarizes the results of tests of functional synergy among the various enhancer element

**Figure 19.**

Schematic diagrams of the test constructs used for pairwise enhancer analyses. The $V_H$ promoter is represented by the filled ovals placed upstream of the luciferase reporter gene. Enhancers are represented by circles downstream of the luciferase gene, a distance away from the $V_H$ promoter. Constructs # 17-21, assess the effect of hs4 enhancer element when paired with the remaining IgH enhancers as indicated while constructs #22-26, measures the effect of Eμ pairing with the remaining IgH enhancers as indicated.

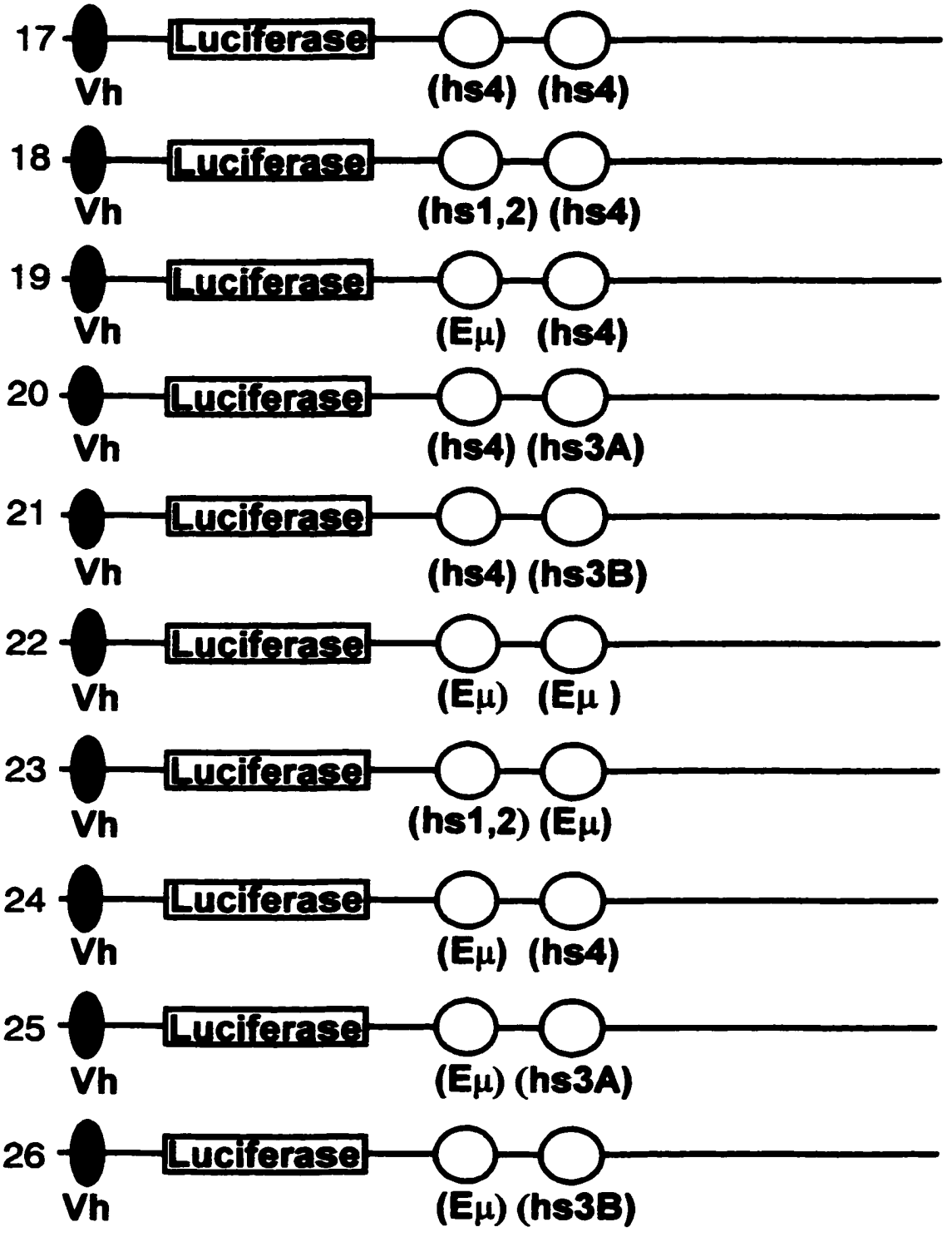


**Figure 20.**

Enhancer activities as observed in human cell line representative of the surface $Ig^+$ B-cell stage (Raji). Enhancement levels were calculated as described in the legend to Fig. 12. The top panel summarized the effect of hs4 pairs while the bottom panel, the results of Eμ pairs.

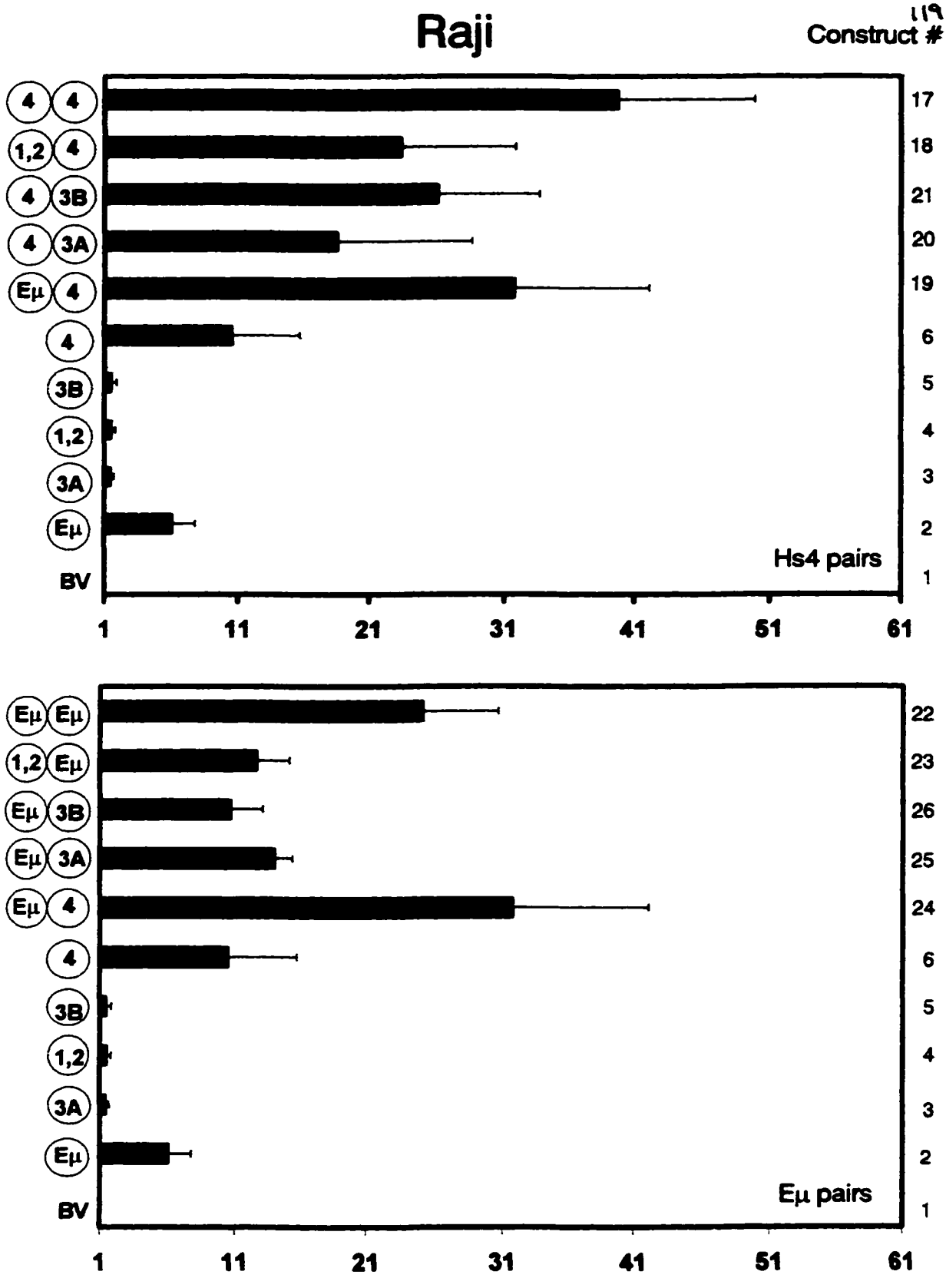


**Figure 21.**

Enhancer activities as observed in murine cell line representative of the surface $Ig^+$ B-cell stage (M12.4.1). Enhancement levels were calculated as described in the legend to Fig. 12. The top panel summarized the effect of hs4 pairs while the bottom panel, the results of Eμ pairs.

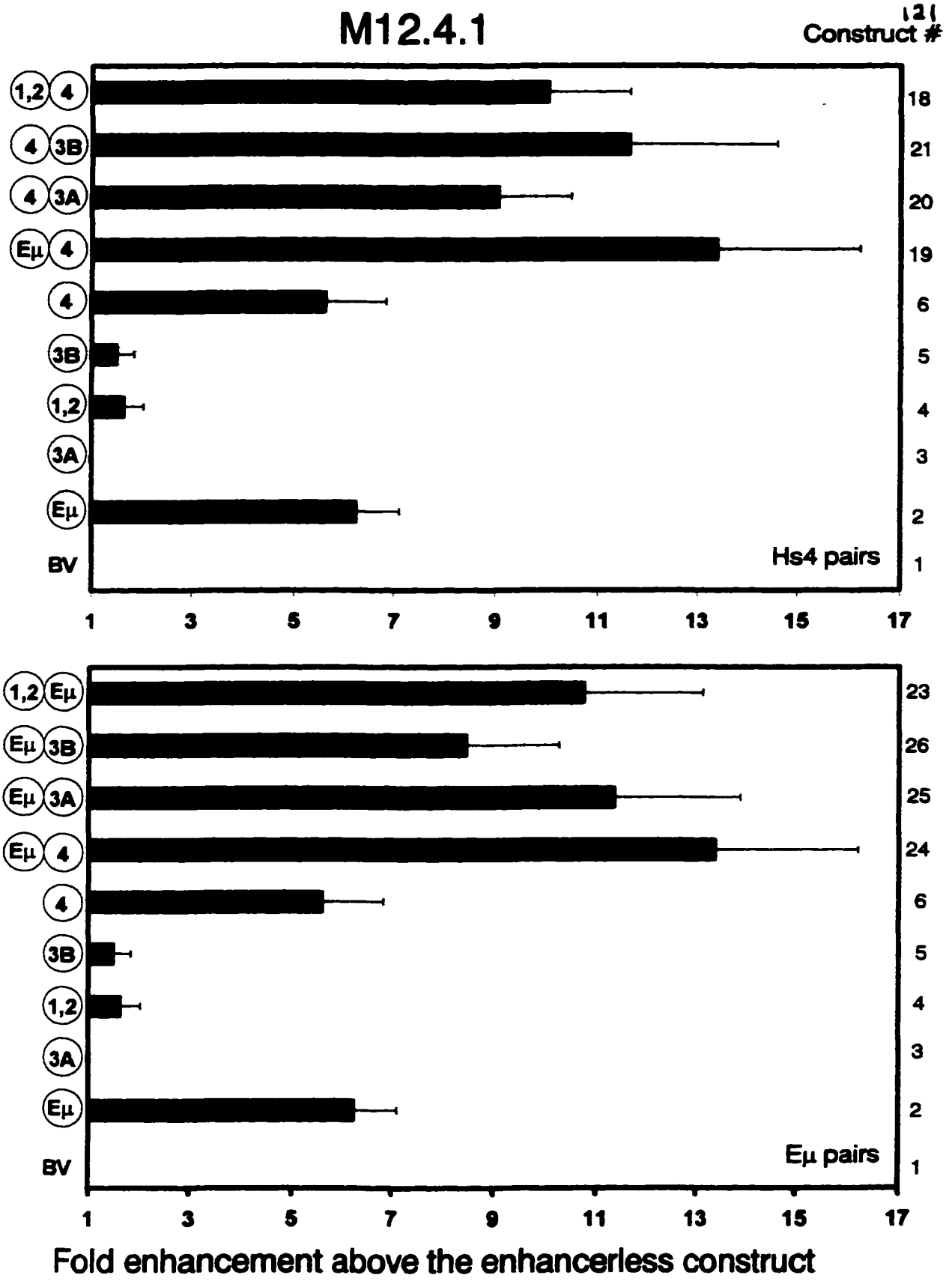
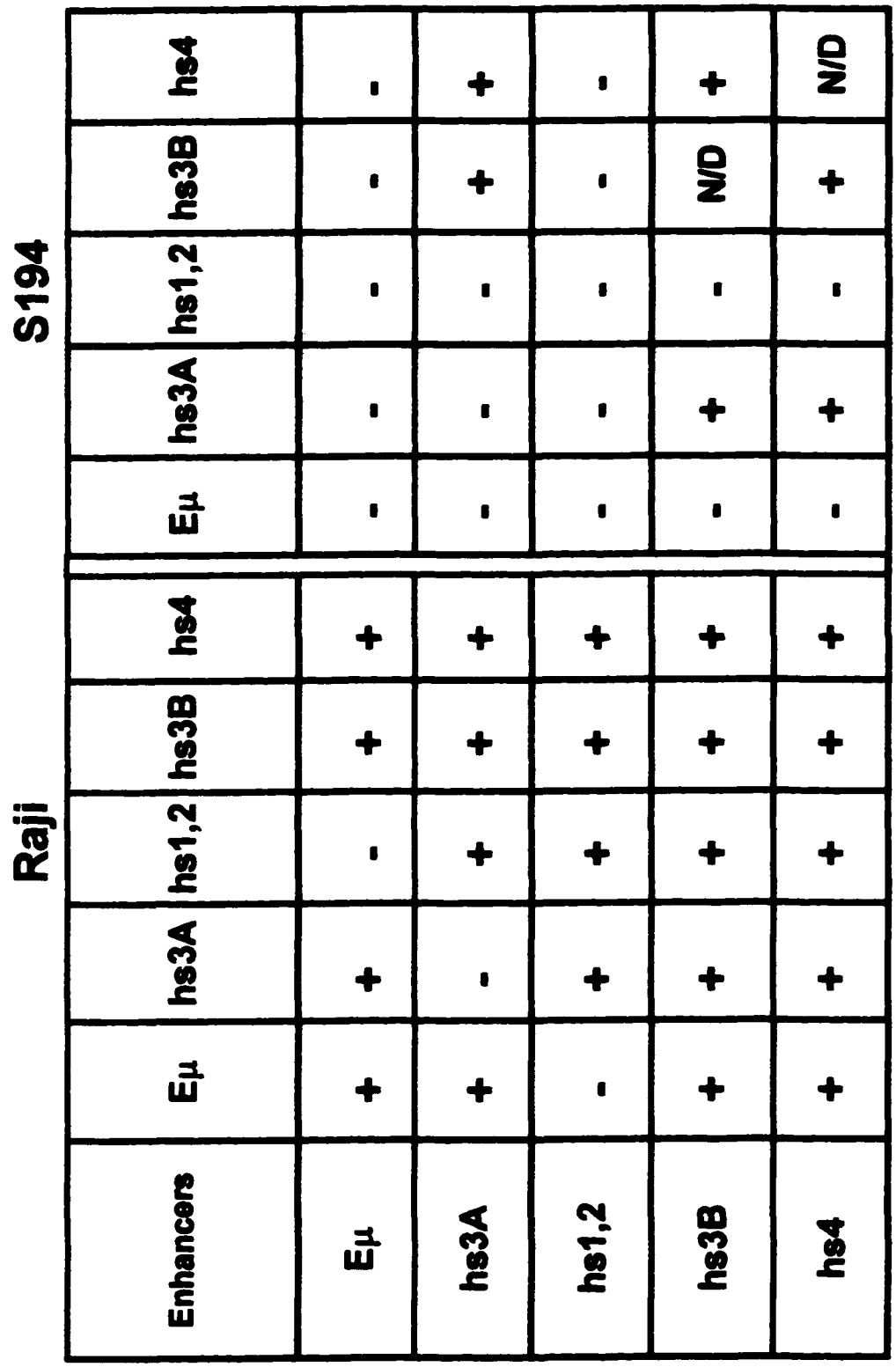


**Table III.**

Summary of pairwise enhancer synergy as analyzed in Raji and S194 cells. Synergy was measured by using a paired t-test. For example, synergy for (Eμ,Eμ) combination was determined by asking if the overall activity level of the combined (Eμ,Eμ) (Fig. 19, construct #22) is equal to the sum of the (Eμ) and (Eμ) (Fig. 11, construct #2). If the p value is $< 0.05$, then a "+" sign symbolizes synergy. If the p value $> 0.05$, a "-" sign symbolizes no synergy between the pairs. Data generated from Raji cells (six transfections) are on the left half of the table while the right half of the table are data obtained for S194 cells [eight transfections for heterologous pairs, e.g. (hs4,3B) and three transfections for homologous pairs, e.g. (Hs3B,3B)]. N/D indicates "not done".



| Raji | | | | | | S194 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Enhancers | Eμ | hs3A | hs1,2 | hs3B | hs4 | Eμ | hs3A | hs1,2 | hs3B | hs4 | |
| Eμ | + | + | - | + | + | - | - | - | - | - | + |
| hs3A | + | - | + | + | + | - | - | - | + | + | + |
| hs1,2 | - | + | + | + | + | - | - | - | - | - | + |
| hs3B | + | + | + | + | + | - | + | - | N/D | + | + |
| hs4 | + | + | + | + | + | - | + | - | + | + | N/D |

pairs. All of the hs4 pairs were significantly higher in activity than either hs4 or Eμ alone. The (hs4,Eμ) and (hs4,hs4) pairs were not significantly different from one another, but the others could be ordered in activity where (hs3A,4) < (hs1,2,4) < (hs3B,4) < (Eμ,4) (as determined in a paired t-test; note that the data shown in bar graphs, e.g. Fig. 20 are pooled means and variances). The most powerful pair (Eμ,4) yielded only between 1.4 to 2-fold greater activity (95% confidence level) than the least powerful pair. Given that in the same set of experiments, the activities of (hs3B,4) and (Eμ,4) were not significantly different from that of the full complement of enhancers (construct 11, Fig. 18), the implication is that loss of any one 3' IgH enhancer element, with the exception of hs4 or even loss of Eμ, would have little effect on overall transcriptional activity.

In M12.4.1, hs 4 again synergizes when paired with hs3B, hs3A, hs1,2, or Eμ (Table IV). The activity level generated from each pairing, has the general ranking as in Raji cells, with the most active pair (Eμ,4) yielding 1.3 - 1.6 fold greater activity tan the least active (hs3A,4). The same conclusions can be reached as were reached for Raji: loss of any element other than hs4 should not result in a significant reduction of gene activity, since any remaining enhancer when paired with hs4, should be able to compensate for the loss.

To determine if the redundancy of the IgH enhancer elements with respect to their interactions with hs4 was preserved in Ig-secreting cells, similar transfections were carried out in the plasma cell line S194. The results are presented in Fig. 22 (a).

Consistent with previous data, hs4 alone showed relatively strong activity when alone, while the other 3' IgH elements were weaker when assayed individually. Hs4,

**Table IV.**

**Summary of pairwise enhancer synergy as analyzed in M12.4.1 and S194 cells. Synergy value determined in the same manner as described in legend to table III.**

| Enhancers | M12.4.1 Eμ | M12.4.1 hs3A | M12.4.1 hs1,2 | M12.4.1 hs3B | M12.4.1 hs4 | S194 Eμ | S194 hs3A | S194 hs1,2 | S194 hs3B | S194 hs4 |
|---|---|---|---|---|---|---|---|---|---|---|
| Eμ | N/D | + | + | - | + | - | - | - | - | - |
| hs3A | + | N/D | - | - | + | - | - | - | + | + |
| hs1,2 | + | - | N/D | - | + | - | - | - | - | - |
| hs3B | - | - | - | N/D | + | - | + | - | N/D | + |
| hs4 | + | + | + | + | N/D | - | + | - | + | N/D |

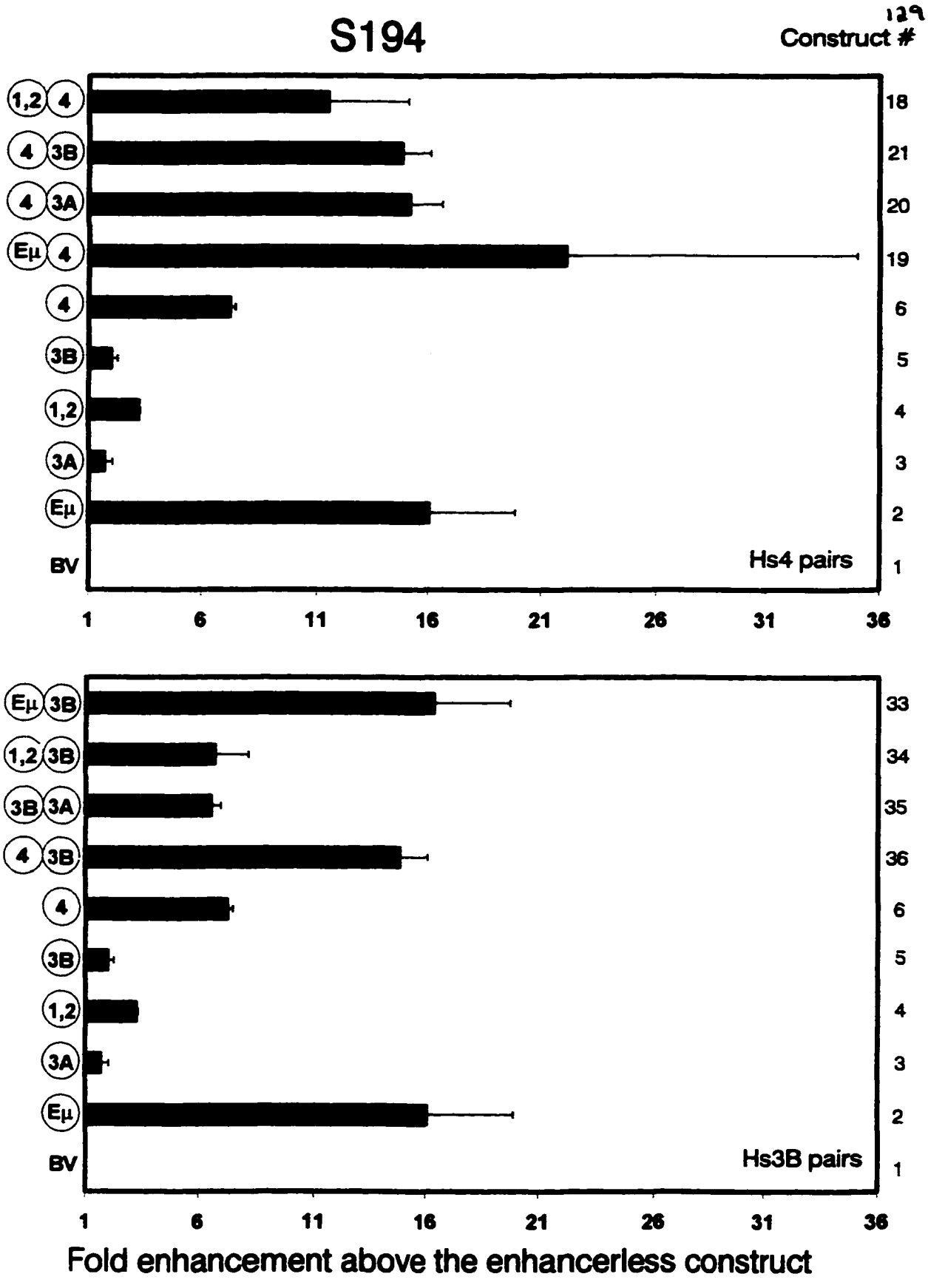
again, was able to functionally synergize with hs3A, hs3B ($ab = a + b$, $p < 0.05$). Interestingly, hs4 no longer synergized with Eμ or hs1,2. The overall activity level for (hs4,Eμ) and (hs4,1,2), was equal to the sum [see Fig. 22 (a) and Table III]. All of the hs4 pairings were roughly equivalent to one another ($ab = cb$, $p > 0.1$), none was significantly greater than Eμ alone suggesting redundancy amongst the IgH enhancers with respect to their interactions with hs4 and, in the presence of Eμ, the various hs4 pairings appeared dispensable. Loss of hs4 in this case would not result in deleterious effects upon activity, since Eμ alone is sufficient to compensate for the loss.

We conclude that the synergy generated by hs4 is fairly promiscuous at the B cell stage (Raji and M12.4.1), functionally interacting with every other element, including itself, stimulating transcription to levels greater than Eμ alone. Interestingly, the ability of hs4 to synergize becomes more restricted at the plasma cell stage (S194). Hs4 no longer synergizes with Eμ and hs1,2 while hs4 still synergizes with hs3A and hs3B, resulting in activity, roughly equivalent to Eμ alone. There do not appear to be any preferences amongst these synergistic interactions; combinations of either hs3A or hs3B with hs4 resulted in roughly similar levels of activation that were not significantly different from Eμ alone.

Finally, functional redundancy amongst hs3A, hs1,2, hs3B and Eμ with respect to their interactions with hs4, suggests that loss of any single enhancer element other than hs4 in surface $Ig^+$ and Ig - secreting B cells would not result in discernible changes in IgH expression levels, since there would be efficient compensation for the loss through interactions between hs4 and of any remaining enhancer element. In Ig-secreting cells but

**Figure 22.**

Enhancer activities as observed in murine cell line representative of the Ig-secreting cell stage (S194). Enhancement levels were calculated as described in the legend to Fig. 12. The top panel summarized the effect of hs4 pairs while the bottom panel, the results of hs3B pairs.



not in surface $Ig^+$ cells, hs4 also appears dispensable.

## 4.9 - Eμ synergistic interactions

Eμ, like hs4, is active throughout B cell development. We tested the ability of Eμ to interact functionally with other enhancers by creating a series of pairwise combinations (constructs 22-26, Fig. 19) and transiently transfecting the B cell lines Raji and M12.4.1. The data obtained are summarized in Figs. 20 (b) & 21 (b).

Like hs4 in Raji cells, Eμ functionally synergized with hs3A, hs3B, hs4 and a second copy of Eμ (Fig. 20 (b); Table III). Interestingly, only the (Eμ,hs4) and (Eμ,Eμ) pairs resulted in significant activities above the hs4 enhancer alone (see Fig. 20 (b) constructs 22 & 24 vs 6; p values $< 0.05$). While Eμ pairings with hs3A and hs3B were synergistic the overall levels of activity achieved were not significantly different from that of hs4 alone. Eμ when paired with hs1,2 resulted in only an additive effect. The activity level again was not significantly different from (Eμ,hs3A), (Eμ,hs3B) and hs4 alone (see Fig. 20 (b) constructs 23, 25, 26 vs 6); p values $> 0.05$). These data suggest that with respect to Eμ, the (Eμ,hs4) pairing is the most efficient in generating high activity. Loss of hs4 would most likely result in a strong negative effect in this case, as functional interactions among the remaining 3' IgH enhancers (hs3A, hs3B) with Eμ would result in activity levels no greater than that of hs4 alone.

In M12.4.1, Eμ is significantly stronger in activity than hs4 (Eμ ≥ hs4, p value < 0.05) [Figs. 21 (a & b) constructs 2 & 6]. Additionally, Eμ was able to functionally synergize with hs3A, hs1,2 and hs4 but not with hs3B (paired t-test as described earlier). Eμ paired with hs3B generates the sum of their individual activities. The activity levels for (hs3A), (hs3B), (hs1,2), (hs4) pairings with Eμ could be ordered (Eμ,hs4) > (Eμ,hs3A) > (Eμ,hs3B), but were not significantly different from one another the range of difference was only 1.3 to 1.8-fold (95% confidence limits) [Fig. 21(b), comparing constructs 23 - 26]. Loss of any one of the 3' IgH enhancers hs3A, hs1,2, hs3B or hs4), in this case, should not result in significant defects in the activity of the unit, as redundancy among the remaining enhancer elements with respect to its interaction with Eμ would be sufficient to compensate for such a loss. The difference in the results obtained with Raji and M12.4.1 therefore, was in the apparent essential role for hs4 in the former but not in the latter cell line.

A subset of the constructs was then transfected into the plasma cell line, S194 to see if the differences reported above were related to the developmental stages of the B-cells. The results are shown in Figs. 22, 29 and 30. Consistent with data just presented, Eμ failed to synergize with hs4 in plasma cells [Fig. 22 (a) & Table III, constructs 19 vs 2 & 6]. In addition, Eμ no longer synergized with hs3A [Fig. 30 (a), construct 25 vs 2 & 3], hs3B [Fig. 22 (b), constructs 33 vs 2 & 5], hs1,2 [Fig. 29, constructs 44 vs 2 & 4] or even with itself [Fig. 30 (b) constructs 22 vs 2][ab = (a + b), p values > 0.05; also see Table III]. The overall fold enhancement was less than additive for (Eμ,hs1,2), as well as (Eμ,Eμ) pairings, demonstrating inhibitory interactions among these elements. The

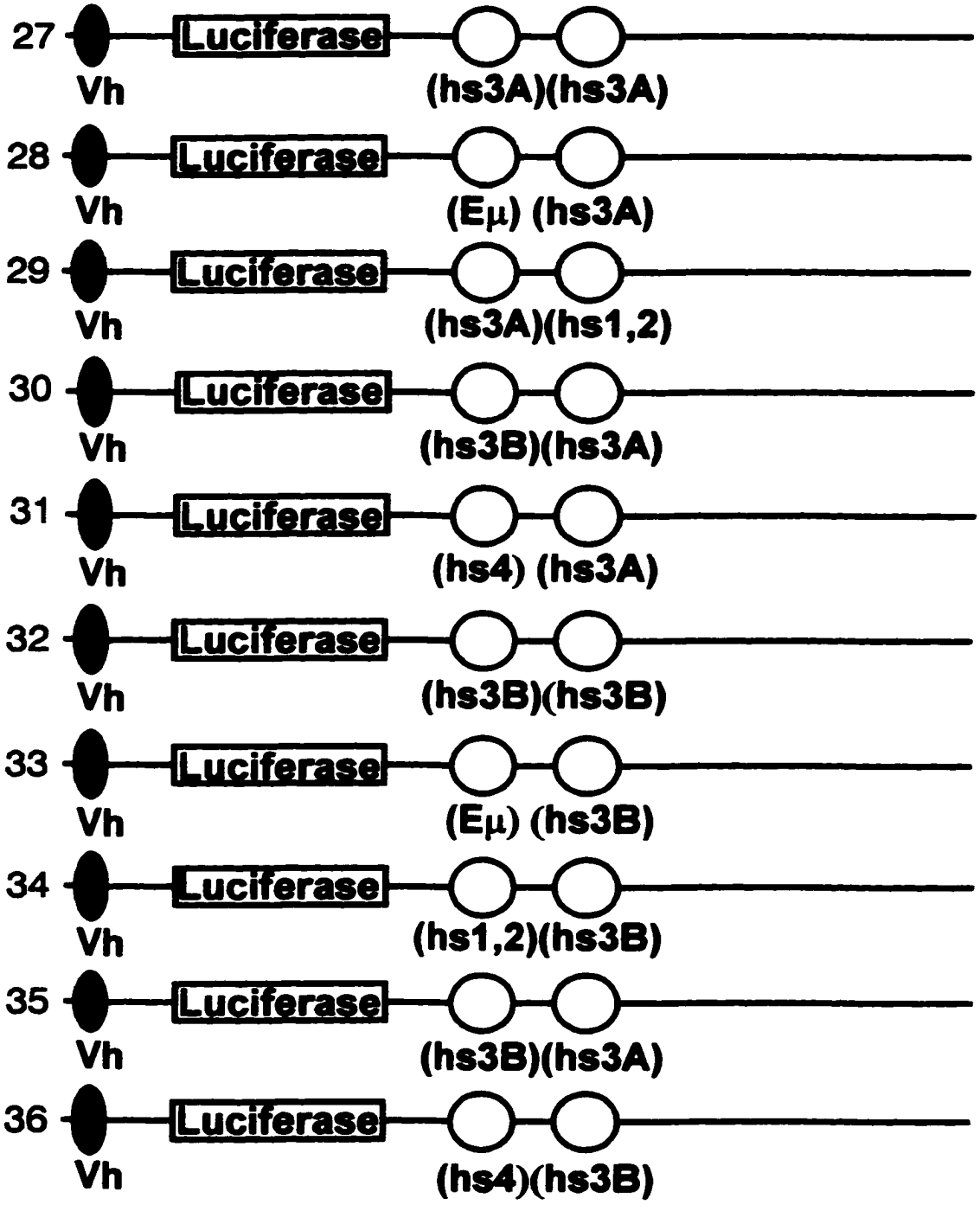
remaining pairs (Eμ,hs3A) and (Eμ,hs3B) exhibited only an additive effect, and since hs3A and hs3B are much weaker than Eμ in these cells, the pair activity levels were not significantly different from that of Eμ alone [Fig. 30 (a), construct 25; Fig. 22 (b) ,construct 33; p values for (Eμ,hs3A) and (Eμ,hs3B), > 0.05]. The (Eμ,hs4) pair was problematic in that it was compared to hs4 and Eμ alone in three transfection experiments. In one out of the three (Eμ,hs4) pair, gave uncharacteristic low activity (< hs4 and much < Eμ). The other two data points are consistent with Eμ and hs4 being additive. The overall implication from these data, as was the suggestion from the "hs4 pair" data, is that loss of any one of the 3' IgH enhancer elements would not affect the activity level of the remaining enhancer unit in Ig-secreting cells so long as Eμ was present.

## 4.10 - Hs3A and hs3B are related in sequence but show differences in functional synergy with other enhancer elements over B-cell differentiation

Hs3A and hs3B are highly related by sequence (Chaveau and Cogne, 1996; Saleque et al., 1997). They have not been studied functionally however, to determine whether they act similarly. We tested the functional interactions of these elements by making pairwise combinations with other IgH elements (constructs 27-36; Fig. 23) and transiently transfecting them into both Raji and M12.4.1 cell lines. The results are

**Figure 23.**

Schematic diagrams of the test constructs used for pairwise enhancer analyses. The $V_H$ promoter is represented by the filled ovals placed upstream of the luciferase reporter gene. Enhancers are represented by circles downstream of the luciferase gene, a distance away from the $V_H$ promoter. Constructs # 27-31, analysize the effect of hs3A enhancer element when paired with the remaining IgH enhancers as indicated while constructs #32-36, measures the effect of hs3B enhancer element pairing with the remaining IgH enhancers as indicated.



summarized in Figs. 24 (a & b) and 25 (a & b) and Table III & IV.

In Raji cells, we observed that hs3A and hs3B functionally are quite different. Hs3A does not synergize with itself while hs3B does. Interestingly, hs3A was observed to synergize with hs3B. There was a small but statistically significant difference in the activity of (hs4,3A) and (hs4,3B) pairs [(Hs4,3B) = (hs4,3A), $p = 0.01$] with (hs4,3B) being the more active pair. In (Eμ,hs3A) and (Eμ,hs3B) pairs, the (Eμ,hs3A) pair was the more active one. Only the hs3A or hs3B pairings with hs4, however gave an activity significantly greater than hs4 alone.

In M12.4.1, differences between hs3A and hs3B remained. Again the (hs4,3B) pair was more active than the (hs4,3A) pair. In addition, hs3A showed significant synergy with Eμ while hs3B did not. As in Raji, the (Eμ,hs3A) pair had greater overall activity than did (Eμ,hs3B).

To explore the possibility of stage-related effects among the hs3A and hs3B enhancer pairings, a subset of the constructs were also transfected in S194 cells, the results summarized in Figs. 22 (a & b), 30 (a & b) and Table III. We observed that in S194, both hs3A and hs3B again synergized with hs4 (p value $< 0.05$), but this time yielding an overall activity level not significantly different from each other. Neither element synergized with Eμ in this cell line. The (hs3A,3B) synergy seen in Raji but lost in M12.4.1, reappeared again in S194. Notably, hs3A did not synergize with itself, again suggesting a qualitative difference between hs3A and hs3B.

Taken together, these data demonstrate that hs3A and hs3B are functionally different despite similarities in DNA sequences. Additionally, their roles appeared to be

**Figure 24.**

Enhancer activities as observed in human cell line representative of the surface $Ig^+$ B-cell stage (Raji). Enhancement levels were calculated as described in the legend to Fig. 12. The top panel summarized the effect of hs3A pairs while the bottom panel, the results of hs3B pairs.

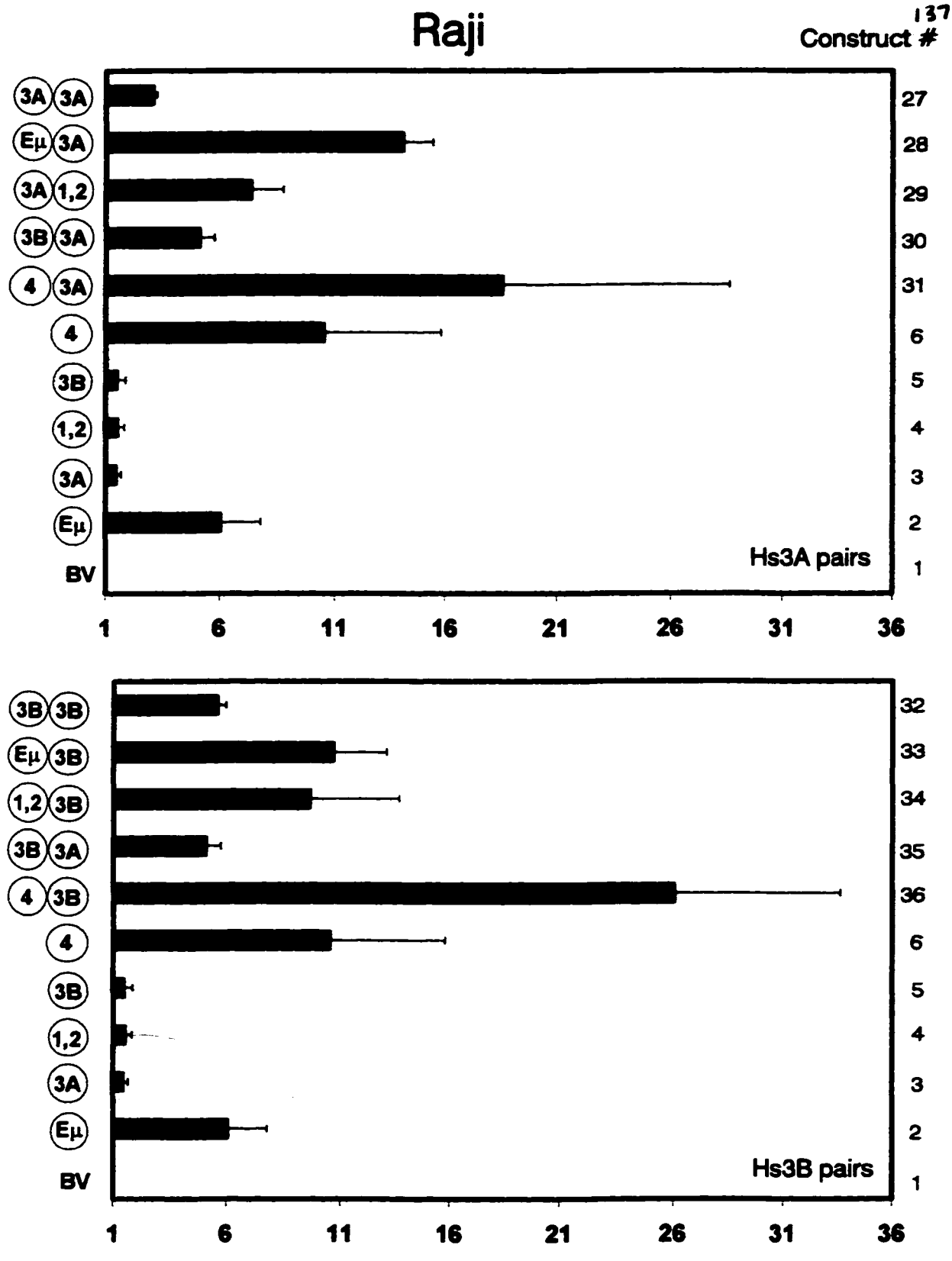
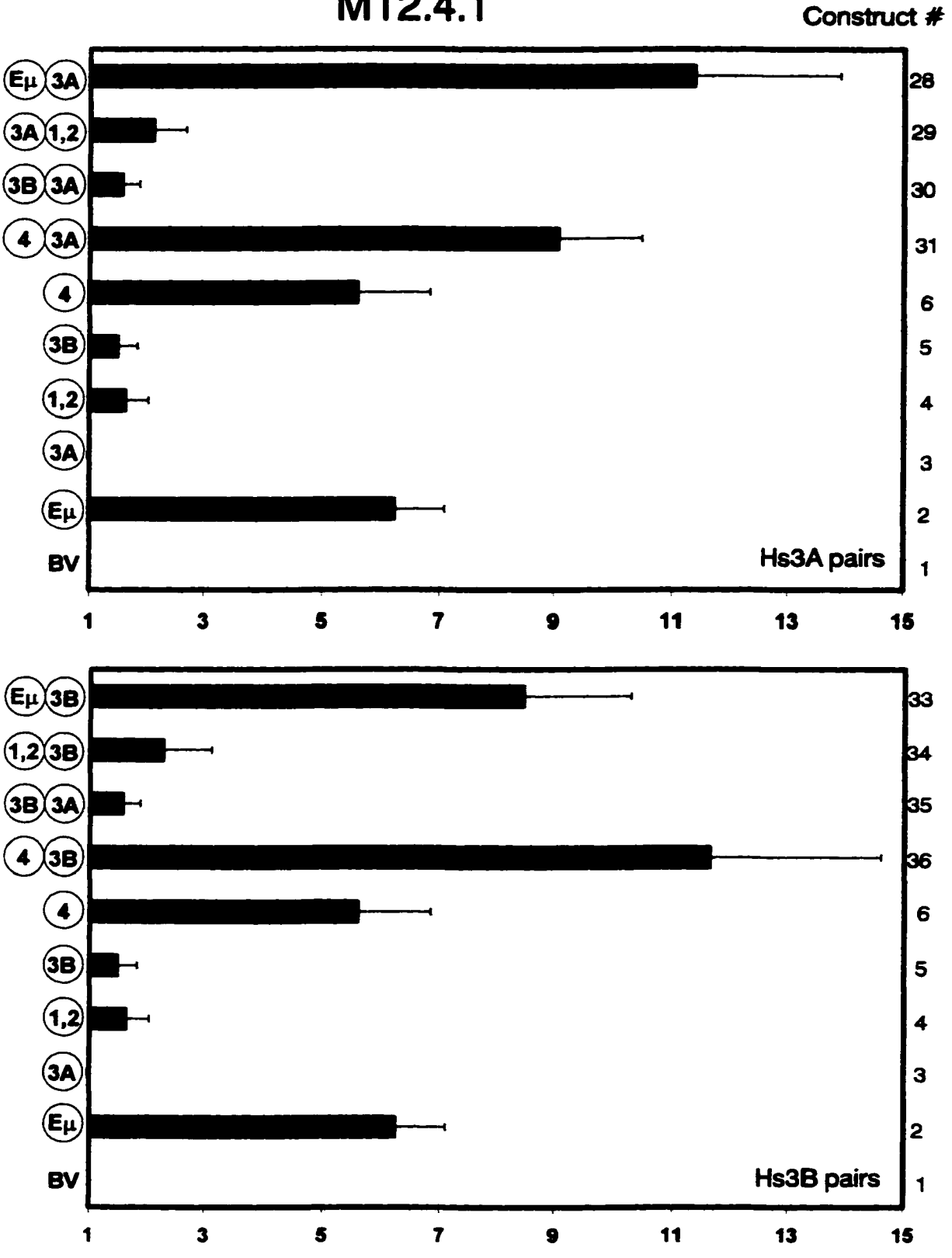


**Figure 25.**

Enhancer activities as observed in murine cell line representative of the surface $Ig^+$ B-cell stage (M12.4.1). Enhancement levels were calculated as described in the legend to Fig. 12. The top panel summarized the effect of hs3A pairs while the bottom panel, the results of hs3B pairs.

# M12.4.1



developmentally regulated over the course of B cell differentiation. For example; hs3A and hs3B both synergized with Eμ in Raji cells, only hs3A synergized with Eμ in M12.4.1 cells, and neither hs3A nor hs3B synergized with Eμ in S194 cells. Similarly, both hs3A and hs3B synergized with hs1,2 in Raji cells, but neither synergized with hs1,2 in either M12.4.1 or S194.

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## 4.11 - Hs1,2 synergistic interactions

From previous sections, we demonstrated the dispensability of hs1,2 in the context of the full complement of IgH enhancers, as well as within the context of the 3' IgH enhancer unit alone. To examine functional interactions between hs1,2 and other individual IgH enhancers, we made pairwise combinations of hs1,2 with each of the IgH enhancers (constructs 37-41; Fig. 26). These constructs were transiently transfected into Raji and M12.4.1 and the results summarized in Figs. 27 (a & b).

In Raji cells, hs1,2 synergized with hs3A, hs1,2,hs3B and hs4 but not with Eμ (Fig. 27 and Table III), Despite synergy in (hs1,2,3A) and (hs1,2,3B), their overall activities were roughly equivalent to one another [(Hs3A,1,2) = (Hs3B,12), p value 0.26] and not significantly different from Eμ (p values > 0.05). The most active pairing with hs1,2 was with hs4, overall activity level significantly above hs4 alone (constructs 38 and 41, Fig. 27).

**Figure 26.**

Schematic diagrams of the test constructs used for pairwise enhancer analyses. The $V_H$ promoter is represented by the filled ovals placed upstream of the luciferase reporter gene. Enhancers are represented by circles downstream of the luciferase gene, a distance away from the $V_H$ promoter. Constructs # 37-41, analyze the effect of hs1,2 enhancer element when paired with the remaining IgH enhancers as indicated.

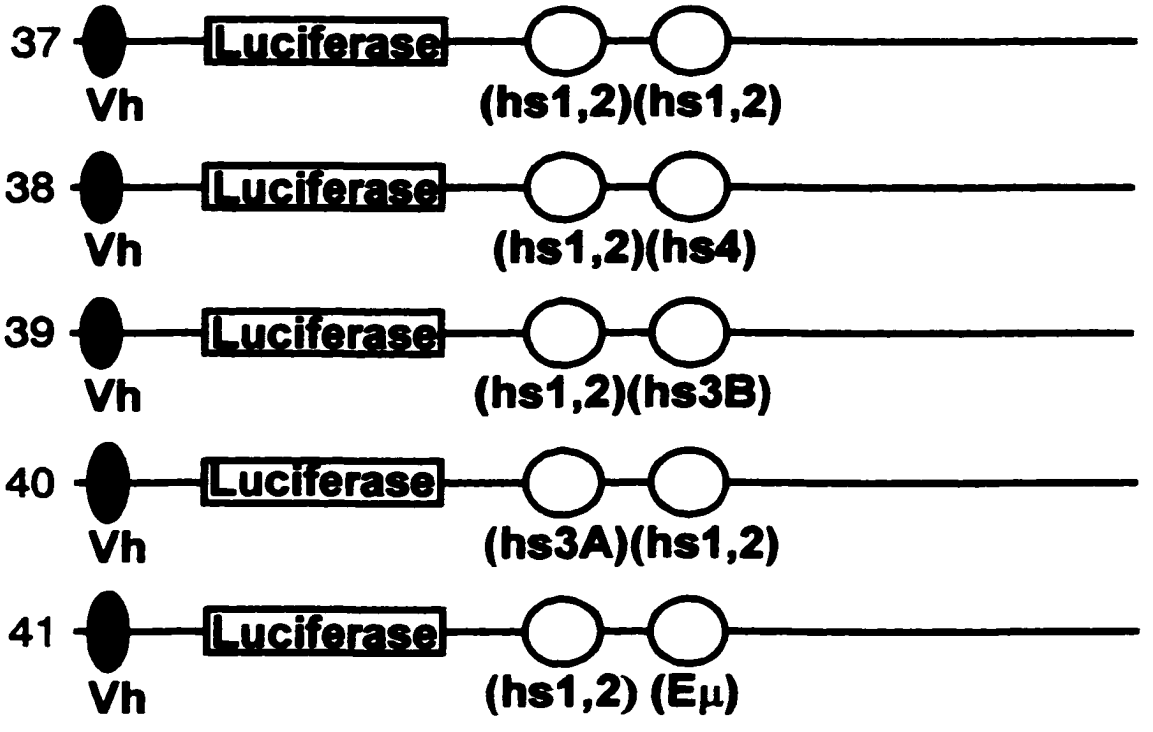
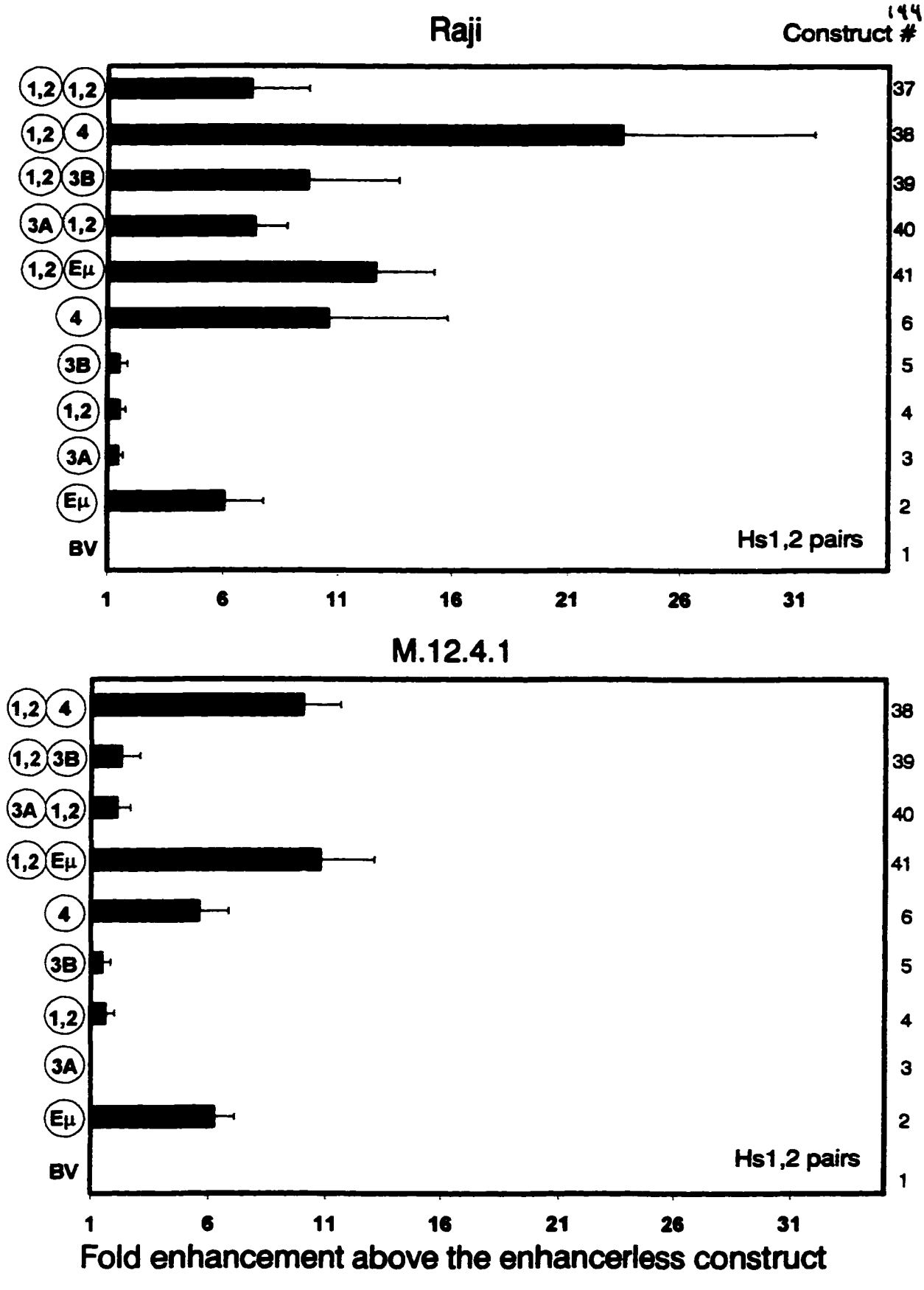


**Figure 27.**

Enhancer activities as observed in human cell line representative of the surface $Ig^+$ B-cell stage (Raji) (top panel); murine cell line representative of the surface $Ig^+$ B-cell stage (M12.4.1) (bottom panel). Enhancement levels were calculated as described in the legend to Fig. 12.



In M12.4.1, hs1,2 synergized only with Eμ and hs4, both pairs generating activity levels significantly above both Eμ and hs4 alone (p value < 0.05). The implication from the findings in both of these surface $Ig^+$ cell lines is that loss of both Eμ and hs4 at this cell stage would likely result in pronounced effects upon IgH transcription since the remaining pairs, namely (hs1,2,3A) and (hs1,2,3B) would not be sufficient to compensate for the loss (Fig. 27).

The most pronounced difference between Raji and M12.4.1 was the absence of synergy between hs1,2 and the two hs3 elements in the latter cell line. We asked how an Ig-secreting cell line compared in this respect. As shown in Figs. 22 and 30 (constructs 34 & 40) and summarized in Table III, hs1,2 did not synergize with either hs3A or hs3B. Additionally, synergy was also absent in pairings of hs1,2 with either hs4 or Eμ.

## 4.12 - Hs1,2 - Eμ inhibition is not distance dependent

Pairing of hs1,2 and Eμ resulted in synergistic activation in the B cell lines - Raji and M12.4.1, but repression of transcription in the plasma cell line S194 [Figs. 27 (a & b), 29, 30]. The repression results in plasma cells are consistent with some earlier published reports (Madisen and Groudine, 1994; Mocikat et al., 1995). Additionally, Mocikat et al, reported that the repressive interactions observed between hs1,2 and Eμ could be reversed upon increasing the distance separating the two enhancers. That is to

say, when the distance between Eμ and hs1,2 was increased from 3.7 kb to 14.9 kb, they observed an increase in activity, from repression when the separation was 3.7 kb, to a 2.4 fold increase in activity when the separation was 14.9 kb. To directly address this possibility, we made two constructs in which we inserted a 5 kb segment of DNA between hs1,2 and Eμ (constructs 42 & 43*; Fig. 28). The inserted DNA is a Hind III fragment obtained from the 5' end of the 12 kb Eco RI fragment of the murine IgH genome. It has been demonstrated by Kakkis et al (Kakkis et al., 1988), using transient assays as well as stable transfections into P3X63Ag8 cells, that this fragment harbors no enhancer-like activity. Constructs 42 and 43* are identical to one another except that in construct 43*, one of the Hind III restriction sites was regenerated during the ligation reactions.

The constructs with increased hs1,2 - Eμ spacing (construct 42 & 43*) were transiently transfected into S194 cells along with the original reporter (construct 44). As shown in Fig. 29, the repressive effect of hs1,2 observed for (hs1,2, Eμ) was maintained even when hs1,2 and Eμ were placed at least 6.5 kb apart. In fact, there was no significant difference in the activities from any of the constructs (construct 42 vs 44, $p > 0.05$; construct 43 vs 44, $p > 0.05$).

**Figure 28.**

Schematic diagrams of the test constructs used to study the effect of inserting a 5 kb of inert DNA fragment in between (hs1,2) and Eμ enhancer. The $V_H$ promoter is represented by the filled ovals placed upstream of the luciferase reporter gene. Enhancers are represented by circles downstream of the luciferase gene, a distance away from the $V_H$ promoter, the inert 5 kb fragment of DNA represented by unfilled rectangle in between (hs1,2) and Eμ. Constructs # 42 and 43*, differs in that in the latter construct, one of the Hind III restriction sites was regenerated during the blunt ended ligation. Construct #44, containing (hs1,2) adjacent to Eμ. Construct #45, contain (hs4,3B) pair for comparison purposes.

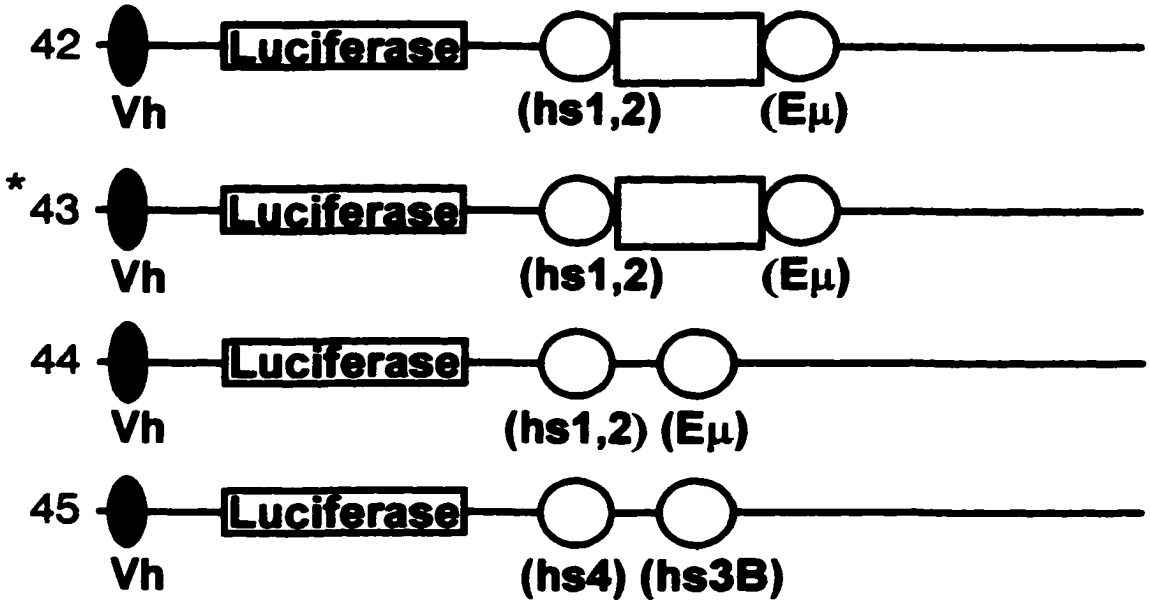


**Figure 29.**

Enhancer activities as observed in murine cell line representative of the Ig-secreting cell stage (S194). Enhancement levels were calculated as described in the legend to Fig. 12.

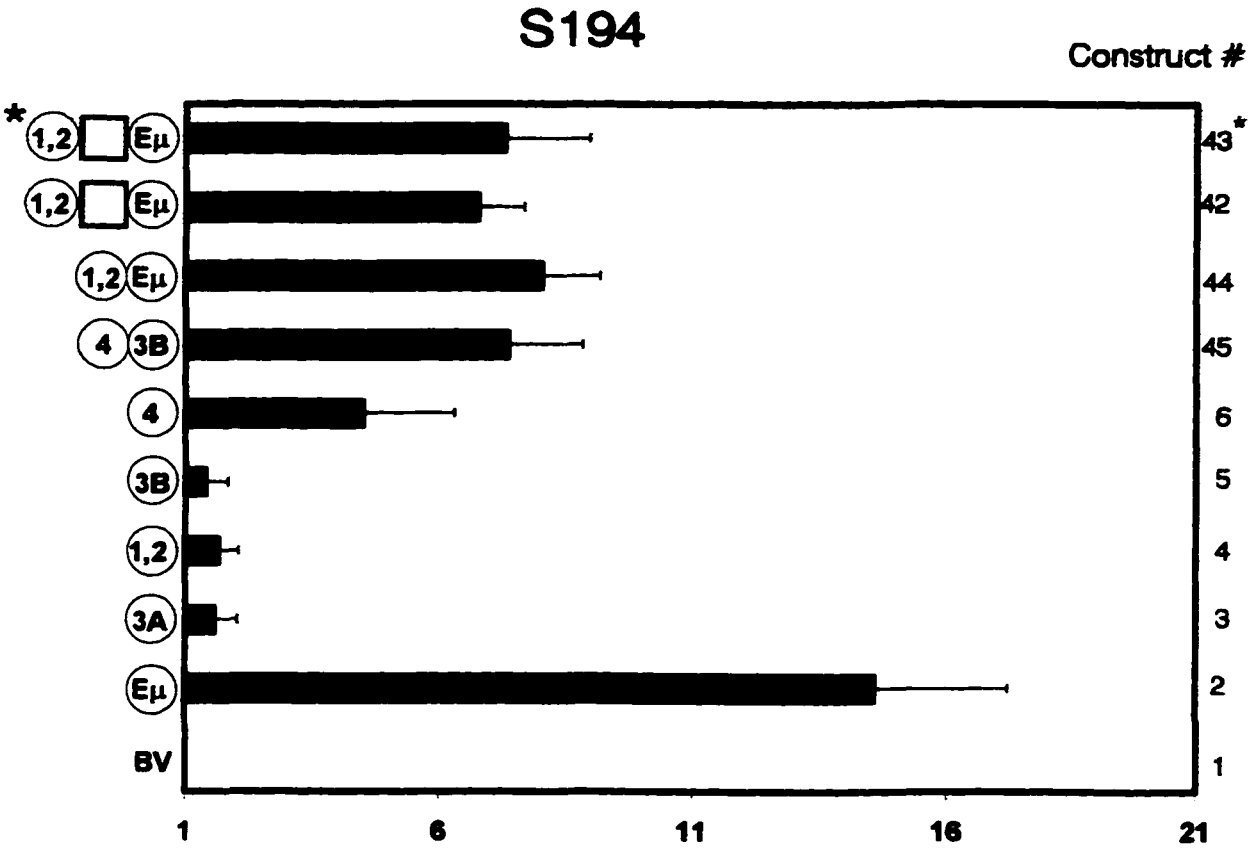
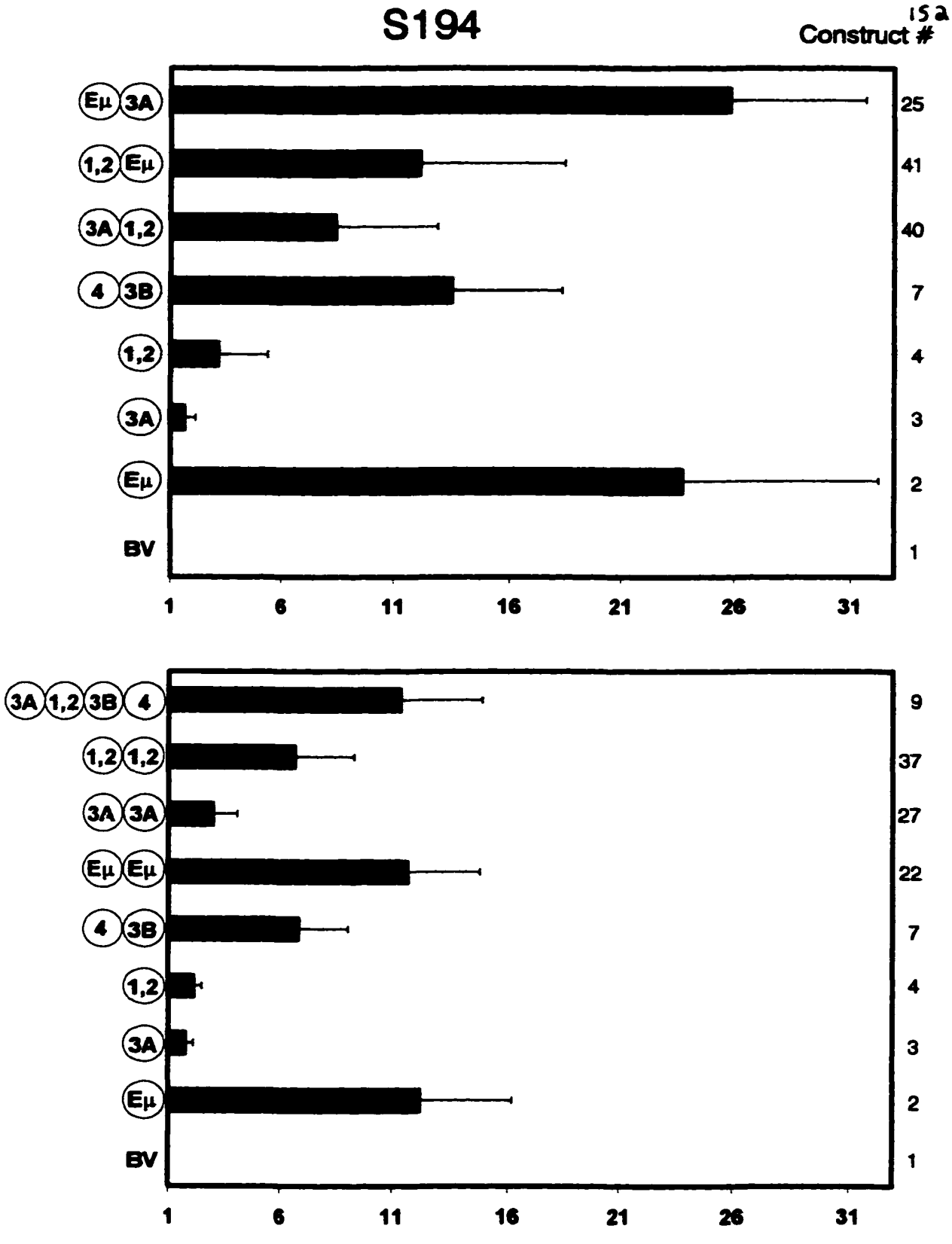


**Figure 30.**

Enhancer activities as observed in murine cell line representative of the Ig-secreting cell stage (S194). Enhancement levels were calculated as described in the legend to Fig. 12. Top panel, a collection of constructs # 25 (Fig.19), 40 &41 (Fig. 26); bottom panel, comparison of enhancer activities from various IgH enhancer dimers.




Fold enhancement above the enhancerless construct

## 4.13 - IgH enhancer synergy in reporter constructs does not depend upon relative position or orientation

Recent sequence analysis suggests that hs3A, hs1,2, and hs3B, are part of a larger palindromic structure of more than 25 kb of DNA (see Fig. 31). Because of the significant sequence similarity between hs3A and hs3B, as well as their relative positions within the palindromic structure, we were concerned that "proper" orientation and positioning, relative to their chromosomal context, would be necessary for optimal function. Arguing against this notion was the discovery that the 3' IgH enhancers downstream of the human immunoglobulin Cα genes lacked this palindromic structure (no equivalent for hs3B) (Chen and Birshtein, 1997; Mills et al., 1997). As a test of the possible functional importance of the palindromic structure in the murine locus, however, we created constructs 46-50* (Fig. 32). Construct 46 and 47 differ in the orientations of the enhancers with respect to the reporter gene. In construct 46, the enhancers (hs1,2, hs3B, hs4) are oriented such that hs1,2 is the farthest from the reporter gene promoter, the reverse of its normal positioning in the IgH locus. In construct 47, hs1,2 is closest to the reporter gene so that all these enhancers lie in the same order they are found in the chromosome, relative to IgH gene transcription. Construct 48 was made to see if placement of the enhancers relative one another was important. Here, hs4 and hs3B are switched with respect to hs1,2. Constructs 49 and 50* differ in that in construct 49, the orientation of hs3A and hs3B are in the same orientation with respect to one another

**Figure 31.**

Amplification of the 3' IgH enhancers with the proposed unit I containing the palindromic structure (hs3A, hs1,2, hs3B) and unit II, hs4 alone. Circles represent the various enhancers and the arrows above enhancers 3A, 1,2 and 3B refer to the orientation and positioning of homology in nucleotide sequences.

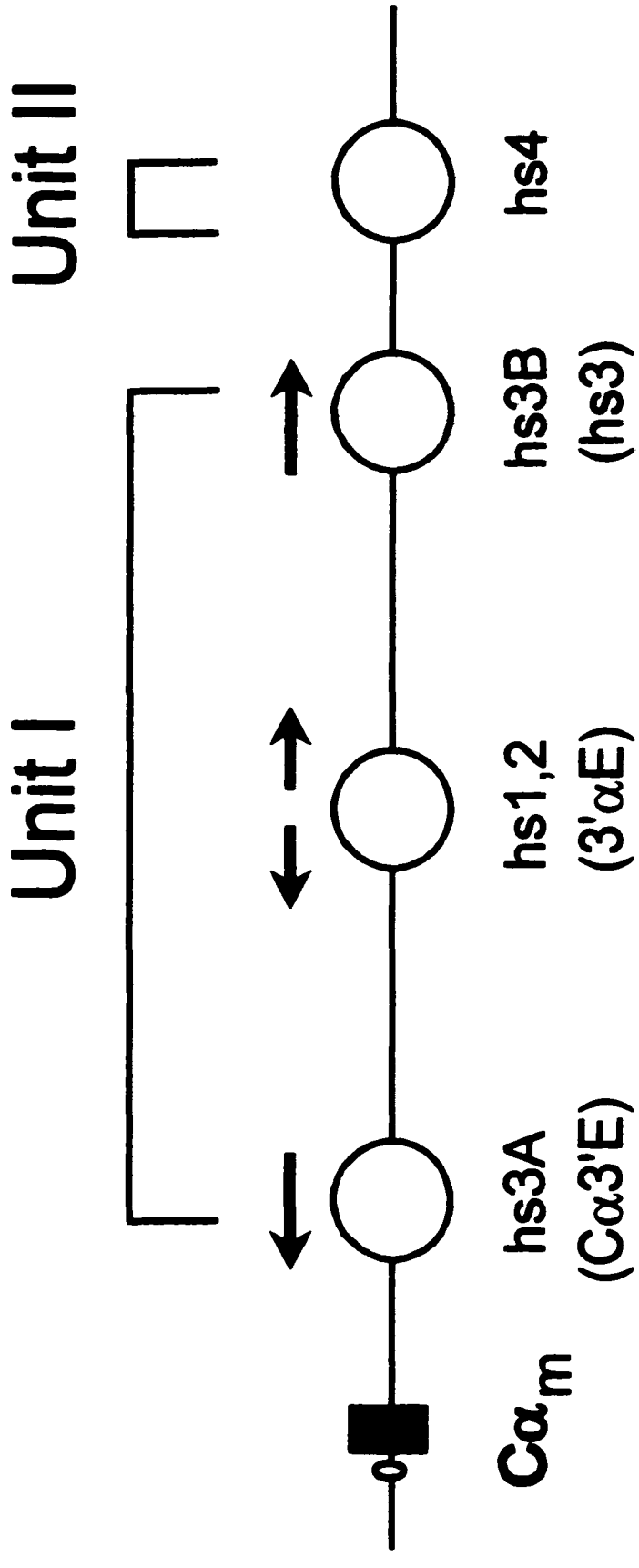
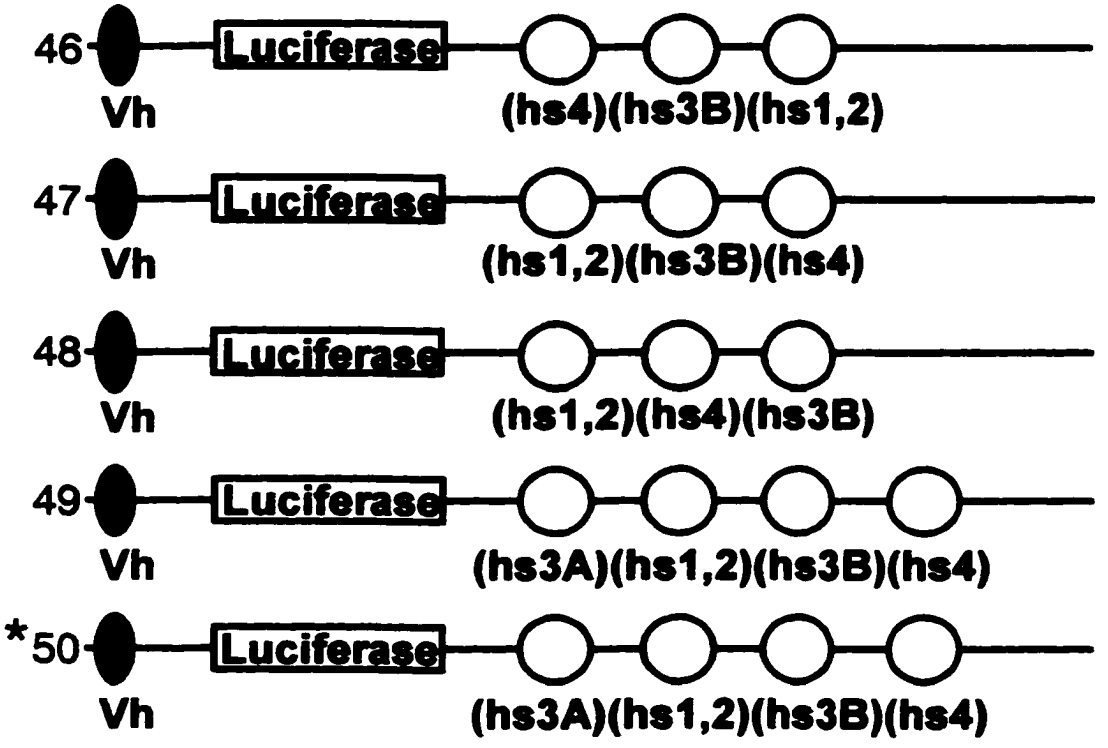


**Figure 32.**

Schematic diagrams of the test constructs used to study the effect of positioning and orientation of enhancers relative to adjacent enhancers . The $V_H$ promoter is represented by the filled ovals placed upstream of the luciferase reporter gene. Enhancers are represented by circles downstream of the luciferase gene, a distance away from the $V_H$ promoter. Constructs # 46 and 47 differs in that in the latter, (hs1,2) is closer to luciferase reporter gene (see material and method section). Construct #49 and 50* are the same except that in the latter, the enhancers are all oriented and positioned in the same fashion as would be in the IgH locus.

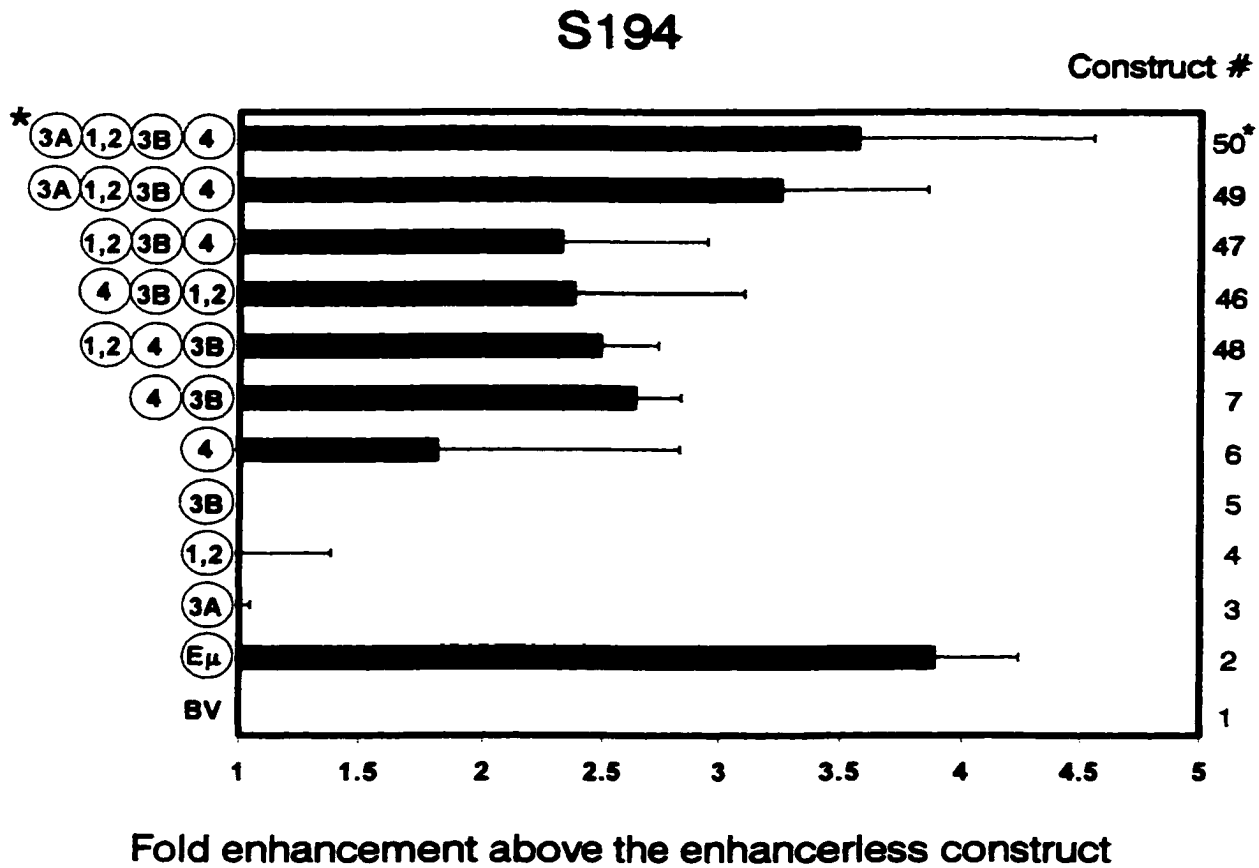


while in construct 50*, hs3A and hs3B are oriented away from each other as they are in normal IgH chromosomes. The latter would allow for formation of the palindromic structure while the former would not.

As shown in Fig. 33, the relative position and orientation of individual enhancer elements did not significantly affect their overall activity (constructs 49 = 50, $p > 0.5$; construct 46 = 47, $p > 0.5$). This suggests that the functional interactions of these elements are moderately promiscuous; they occur between different elements regardless of positioning as well as orientation and result in significant levels of activation.

**Figure 33.**

Enhancer activities as observed in murine cell line representative of the Ig-secreting cell stage (S194). Enhancement levels were calculated as described in the legend to Fig. 12. Construct #50* differs from construct #49 that only the former allows for the formation of a complete palindromic structure between hs3A, hs1,2 and hs3B.



# Chapter 4

## Discussion

## 4.15 - Discussion

The murine Ig heavy chain locus consists of multiple gene segments interspersed with an array of regulatory elements. Eμ, the first eukaryotic tissue specific enhancer identified, located approximately 4 kb downstream of the assembled $V_H$ gene's promoter region, has been demonstrated to be active throughout B cell development. In addition to Eμ, there are a series of 3' regulatory elements, spanning a distance of approximately 34 kb, downstream of the $C_\alpha$ exons. Four specific elements have been identified in this region: hs3A (formerly, $C_\alpha$3'E), hs1,2 (formerly, 3'αE), hs3B (formerly, hs3) and hs4. The "hs" designation indicates DNase I hypersensitive sites 1,2, 3A, 3B (apparent only in Ig-secreting B-cell lines) and hs4, (detected in pre-B as well as in plasmacytoma cell lines).

Initial studies indicated that each of these elements possessed activation functions, in one or more B - lineage cell lines. Much of the data were generated in studies which treated each element as a distinct enhancer. In vivo however, all of the 3' IgH elements are present together and thus, may function better as a unit.

Manipulations of the endogenous IgH locus of the mouse had already provided some information regarding IgH enhancer functions. Deletion of Eμ dramatically reduced VDJ joining in developing B lymphocytes. It did not result in complete abolition of VDJ joining however, indicating that other regulatory elements might be supporting low levels of VDJ joining (Chen et al., 1993; Serwe and Sablitzky, 1993); see Fig. 35). The

characterization of a number of plasmacytomas that were missing Eμ, yet maintained high level IgH expression for example cell line 9.9.2.1 (Fig. 34), suggested that other enhancers could fully compensate for the loss of Eμ (Aguilera et al., 1985; Eckhardt and Birshtein, 1985; Klein et al., 1984; Wabl and Burrows, 1984). Finally, the mutant cell line, LP1.2, was identified which lacked almost 34 kb of DNA downstream of the $C_\alpha$ exons, a deletion which included all of the subsequently identified 3' elements. The result was a significant decrease in IgA expression level (Gregor and Morrison, 1986; Michaelson et al., 1995) (see Fig. 34), suggesting that Eμ alone was insufficient to ensure high level IgA expression. In order to understand the functional interactions of the various enhancers within the heavy chain gene locus throughout B cell differentiation, we decided to assay their activities as a complex rather than as distinct units.

## Eμ's contributions shift to 3' IgH enhancer elements as B cells mature - a reflection of changes in cells' environment

While Eμ's activity was clearly very important early in B cell development (VDJ recombination process), and was dispensable in plasma cells (see Fig. 34), its contribution relative to the full complement of IgH enhancers over the course of development was not clear. Using test constructs 1, 2, 3, 4, 5 and 10 (Fig. 11), we transiently transfected cell lines representing pre-B, surface Ig $^+$ and Ig-secreting stages and assayed for their functions. We observed that Eμ alone was active throughout all stages of development consistent with published studies. When assayed with the 3' IgH

**Figure 34.**

Schematic of spontaneous and induced deletions of IgH regulatory regions in late stage B cells. IgH loci in several Ig-producing cells are diagrammed. Heavy chain variable ($V_H$) and constant ($C_H$) region coding sequences are represented as filled boxes. The relative positioning of the enhancers are described in the text. Enhancers are represented as circles. Missing enhancer element(s) are represented by [ ]. The names of the Ig-secreting cell lines are shown to the right of each map and the classes of heavy chain they produce are indicated to the right. Levels of heavy chain mRNA and protein in the cell lines are represented as +++ (for high level expression), +/- (low expression), - (no expression).

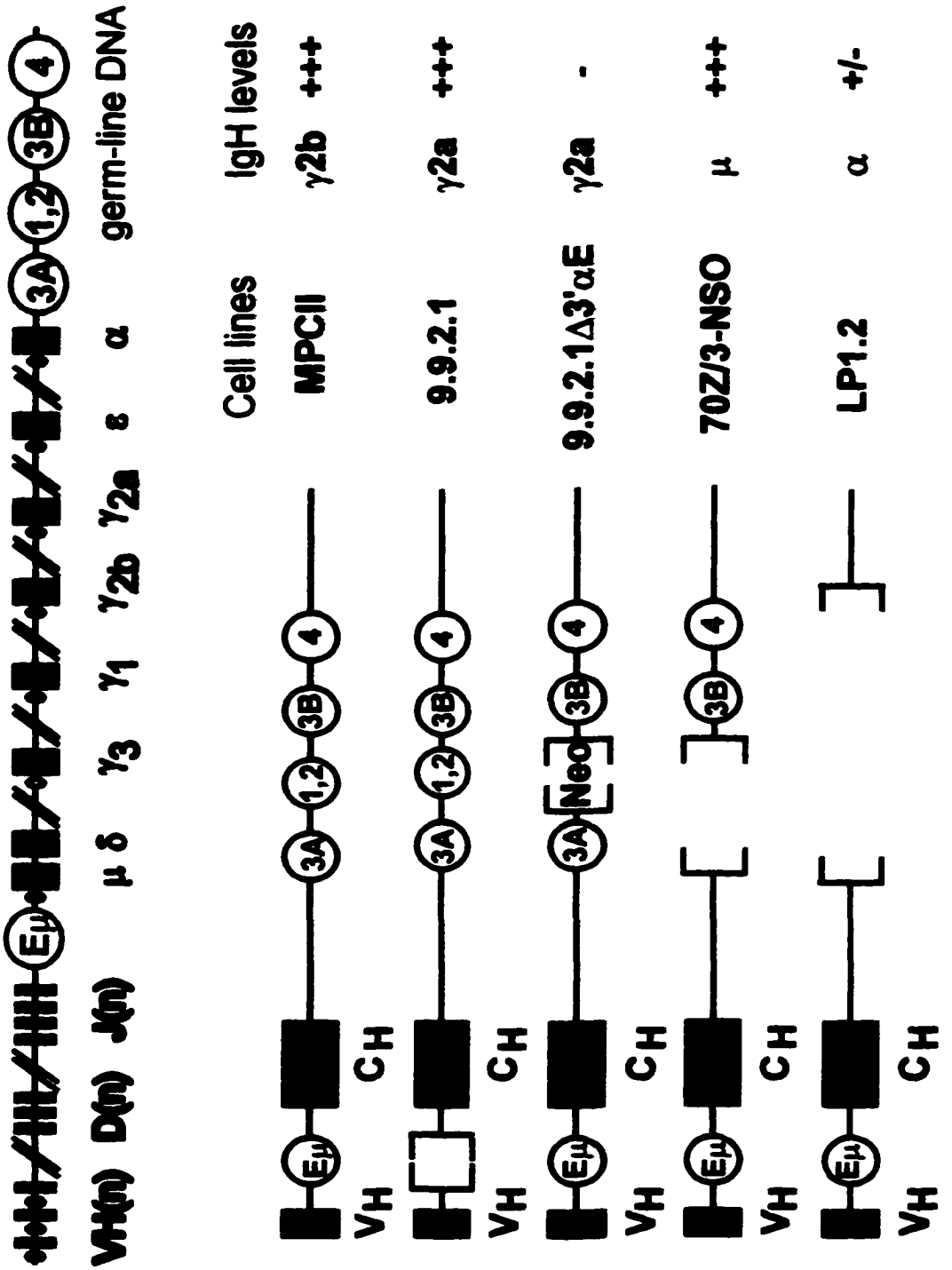
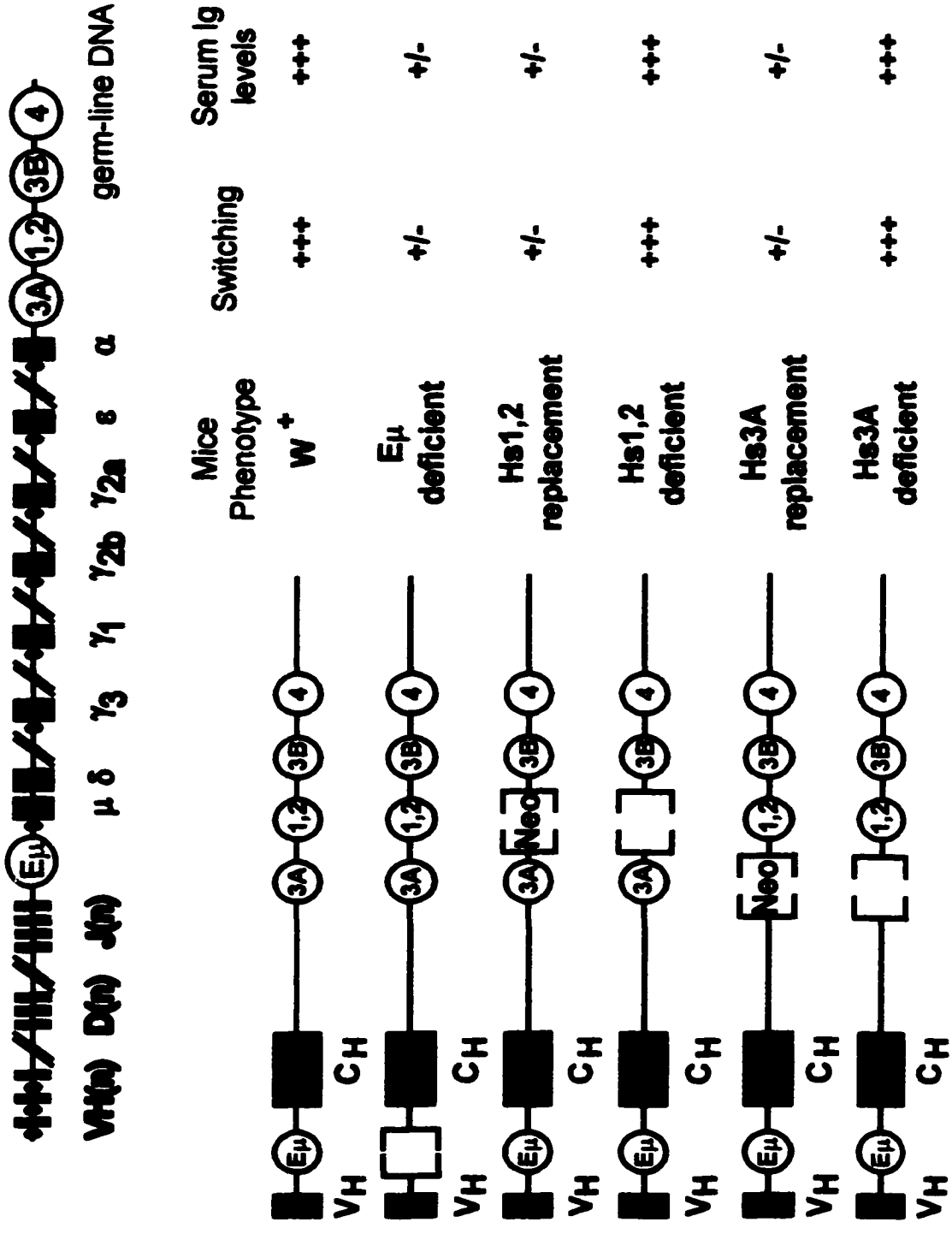


**Figure 35.**

Schematic of induced deletion of IgH regulatory regions in developing mice. Heavy chain variable ($V_H$) and constant ($C_H$) region coding sequences are represented as filled boxes. The relative positioning of the enhancers are described in the text. Enhancers are represented as circles. Missing enhancer element(s) are represented by [ ]. Replacement with $neo^R$ as indicated where applicable. The phenotype of the mice is on the right of the map, wild type mice represented by $W^+$. Effects of switching and serum Ig levels for each phenotype is on the far right of the map. +++ means normal levels of serum Ig as well as switching to all isotypes while +/- means impaired switching to some but not all isotype.



enhancers however, the overall activity level ranged from being not significantly different from Eμ alone to being significantly greater than Eμ alone as shown in Figs. 12 - 14, demonstrating differential functions for all IgH enhancers as B cells mature. We then analyzed the contribution by Eμ relative to all the IgH enhancers, accomplished by dividing the average activity value obtained for Eμ by the average activity value obtained for all IgH enhancers together. The resulting values, expressed in percentages were summarized in Table I. From Table I, Eμ's contribution relative to all the IgH enhancers showed a developmental progression from being the major contributor in pre-B cells (30 - 90 %), to a low in surface $Ig^-$ cells (7 - 40 %) and then, high in Ig-secreting cells (100 - 180 %). The data we obtained were consistent with findings from transgenic mice carrying the chloramphenicol acetyltransferase (CAT), regulated by the $V_H$ promoter and Eμ (Nato et al., 1998).

If Eμ's contribution showed a developmental progression, what about the 3' IgH enhancers? Dividing the average activity value obtained for 3' IgH enhancers as a unit by the average activity value for all IgH enhancers together (Eμ + 3' IgH), we found that over the course of B cell differentiation, the contribution by the 3' IgH enhancers also changed (Table I). In pre B cells where Eμ is dominant, the 3' IgH enhancers contributed very little to overall activity (3 - 10 %). In the surface $Ig^+$ stage, a dramatic increase in 3' IgH enhancers' contribution (40 - 100 %) was observed concurrent with a drop in Eμ's contribution. In the Ig-secreting stage, both Eμ and 3' IgH enhancers were found to contribute significantly to the overall activity.

We observed that within a given stage, for example at the surface $Ig^+$ stage, contribution by Eμ and 3' IgH enhancers showed some variation in percentages. Looking at the Tables I & II, M12.4.1 showed considerable differences from the other three surface $Ig^+$ cell lines assayed. We reasoned that these differences could reflect the different stages of arrest for these cell lines. For instance in M12.4.1, since both Eμ's and the 3' IgH enhancers' contributions were high, resembling the pattern obtained for Ig-secreting cells, we thought it possible that M12.4.1 represented a more mature surface $Ig^+$ B cell than either Raji, Namalwa or A20 cells. As discussed more below, M12.4.1 consistently displayed a phenotype intermediate to that of the other surface $Ig^+$ cells line and Ig-secreting cells.

From Table I, one can begin to understand why loss of Eμ in early B cell development would result in a more dramatic effect on IgH gene expression than in later B cell developmental stages. Eμ, at the pre B cell stage contributed approximately 90 % of the IgH expression in 18-81 cells, therefore, loss of Eμ would result in a dramatic reduction in IgH gene expression, in agreement with the findings of others (Serwe & Sablitsky, 1993 and Chen et al., 1993). The incomplete abrogation of IgH expression in their findings might be accounted for by the 3 % contribution by the 3' IgH enhancers observed in our experiments. While we observed a low level contribution by the 3' IgH enhancers in transient assays, it is unclear however, whether the 3' IgH enhancers actually are responsible for the maintenance of low level VDJ recombination observed in mice carrying loci lacking Eμ. If in fact the 3' IgH enhancer elements are responsible for the residual VDJ recombination in mice lacking Eμ, we would predict that further deletion of

hs4 would totally abolish recombination since amongst the 3' IgH elements only hs4 exhibited activity at the pre-B cell stage.

In the surface $Ig^+$ stage, pronounced synergy was observed for the 3' IgH enhancers, with the activity level surpassing that of Eμ alone (Table II). Addition of Eμ to the 3' IgH enhancer elements did not result in significant synergy. This suggested that loss of Eμ would result in less dramatic effects on IgH gene expression since the 3' IgH enhancers contributed anywhere from 50 - 100 % of total enhancer activity (Table I).

In the Ig-secreting stage however, both Eμ and the 3' IgH enhancers contributed significantly to transfected gene expression. At this stage, the activity of the combined 3' IgH enhancers was only equal to the sum of their individual activities (Table II). The addition of Eμ to the 3' IgH enhancers resulted in an activity that was lower than the sum of its parts [Fig. 15 (a)]. This suggested functional redundancy between these two regions and possible competition between them for available transcription factors. The implication is that only the 3' enhancer elements or Eμ must remain active in order to maintain high levels of gene expression. If so, then loss of either Eμ or the 3' IgH enhancers would not result in a significant defect in IgH gene expression. This would explain the existence of cell lines which lack Eμ, yet maintain high levels of IgH expression. From Table I however, in both Ig-secreting cells S194 and P3X63Ag8, contribution by Eμ was much greater than the 3' IgH enhancers although both were still considerably high. If S194 and P3X63Ag8 cells truly represent Ig-secreting stage B cells, loss of Eμ should result in 40 - 50 % reduction in IgH gene expression. In 9.9.2.1 cells, γ2a gene expression levels were found to be equivalent to γ2b gene expression levels in

MPC11. Since we did not transfect MPC11 with our test constructs directly, we don't know the initial % contribution by Eμ and the 3' IgH enhancers. This, along with cell line to cell line variations, means that it is possible that in 9.9.2.1, Eμ and the 3' IgH enhancers have equal activity. Thus, loss of Eμ would not result in significant changes in the level of IgH gene expression consistent with the phenotype of the cell line 9.9.2.1.

Presumably in 9.9.2.1, the expressed $\gamma_{2a}$ remains under the influence of the 3' IgH elements. Consistent with this hypothesis, $neo^R$ disruption of hs1,2 in 9.9.2.1 cells by $neo^R$ gene replacement through homologous recombination technique, resulted in mutant cell line 9.9.2.1Δ3'αE, that no longer expresses γ2a (Lieberson et al., 1995)(see Fig. 34) . It is possible that the insertion of $neo^R$ gene into the chromosome disrupted the γ2a gene expression, thereby redirecting the enhancing activities of hs3A, hs3B and hs4 to the expression of proximal $neo^R$ gene rather than the distal γ2a gene (Lieberson et al., 1995). It has been recently shown that the insertion of PGK-$neo^R$ cassettes into the immunoglobulin heavy chain constant region locus results in inhibition of class-switch recombination events upstream of the insertion site (Seidl et al., 1999). For example, insertion of PGK-$neo^R$ cassettes into the IgE constant region resulted in complete inhibition of class-switching to isotypes IgG3, IgG2b, IgG2a, IgE, partial inhibition of class-switching to IgG1, and no effect on switching to IgA (Seidl et al., 1999). These findings suggest the existence of a long-range 3' IgH regulatory region required for class-switching recombination to multiple $C_H$ genes. The PGK-$neo^R$ cassette insertion into the IgH locus disrupts the ability of the locus to undergo class-switching events to constant region genes upstream but not downstream of the insertion site. Additionally, these data

suggest that class-switching to γ1 might be regulated by mechanisms other than 3' IgH enhancers.

More direct evidence that insertion of the neo$^R$ gene into the IgH locus itself resulted in the disruption of IgH gene expression came from hs3A and hs1,2 knock-out studies done in mice (see Fig. 35). (Manis et al., 1998), using the Cre-loxP system, generated 'clean' knock-out mice of hs3A and hs1,2. Mice homozygous for the insertion of the neo$^R$ gene (both in hs3A and hs1,2 knock-out), showed defects in serum Ig levels as well as in class-switching to γ3, γ2b, γ2a, ε and, partially, to γ1. In both cases, IgA remained unaffected. Upon deletion of the neo$^R$ gene however, this phenotype was lost, demonstrating that disruption in class-switching events was due to insertion of the neomycin $^R$ gene into the locus. Additionally, their findings demonstrated that neither hs3A nor hs1,2 was crucial for class-switching to various isotypes (Manis et al., 1998).

The data summarized in Table I are not easily reconciled with the phenotype of the mutant cell line LP1.2. The mutant cell line LP1.2 lost approximately 34 kb of its genomic DNA containing all the 3' IgH enhancers and produced only 1/10 the wild type level of IgA protein (see Fig. 34). This suggested that Eμ alone was not sufficient to support high levels of IgA gene expression in Ig-secreting cells. Our findings predict that loss of the 3' IgH enhancers would still result in high levels of IgH gene expression, since contributions by Eμ were as great or greater than that of the 3' IgH enhancers (Table I). Perhaps in LP1.2, this is true but additionally, loss of approximately 34 kb of genomic DNA has brought a distant repressor(s) closer to both Eμ and the $V_H$ promoter which would then reduce Eμ activity. This repression, along with loss of all of the 3' IgH

enhancers would then explain the decreased IgA levels observed for LP1.2. An alternate possibility is that the reduction in IgA expression levels is due to loss of one or a set of essential factors required for Eμ's activation. A third possibility is that deletion of the 34 kb of DNA could potentially bring in a distant unknown enhancer in proximity to Eμ. From our data, we found that combination of Eμ with certain of the 3' IgH enhancers actually results in repression of each enhancers' activity. For instance, pairing Eμ with hs3A, hs3B or hs4 results in a lack of synergy, whereas pairing Eμ with either hs1,2 or another Eμ produced activities lower than the sum of their individual parts (Fig. 30, construct 41& 22). While the repression observed for (Eμ,Eμ) could be reasoned as competition for limiting amounts of transcription factors, this is not likely the case for the repression observed for (Eμ,hs1,2). In this particular pair, the overall activity level for (Eμ,hs1,2) is much lower than that of Eμ alone (Fig. 29, construct 44 vs 2); if competition for limiting amounts of transcription factors were the mechanism for repression in (Eμ,hs1,2) pairing, then, one might expect the overall activity level to be roughly equivalent to that of Eμ alone. The fact that the overall activity level was much lower than Eμ alone suggested additional repressive mechanisms other than simple competition for transcription factors. The repressor(s) if present, most likely acts specifically on the (Eμ,hs1,2) combination, rather than on Eμ and hs1,2 individually since (Eμ,hs3A), (Eμ,hs3B), (Eμ,hs4), (hs3A,3A) and (hs1,2,1,2) combinations did not result in repression. The distant enhancer in LP1.2 could behave like hs1,2 thereby repressing Eμ's activity. To test for repressor(s) binding to Eμ in Ig-secreting cells, simple deletions of Eμ could be linked to hs1,2 or full length Eμ and tested for their

ability to repress compared to wild type full length Eμ homodimers. Localization of the repressive sequences would allow identification of the responsible factor(s) since Eμ has been studied extensively.

Another possible explanation for the LP1.2 phenotype is that the 34 kb region deleted in this cell line contains additional activating elements that are as yet uncharacterized and function only in the context of the known 3' IgH enhancer elements. Recall that in our constructs, because of size constraints we did not assay the entire 34 kb of DNA fragment housing hs3A, hs1,2, hs3B and hs4 as a unit. It is possible that the genomic sequences found between these elements in the IgH locus could also participate in further synergistic activation when placed adjacent to the 3' IgH enhancer elements. The relative contribution of the 3' IgH enhancers to Ig transcription in Ig-secreting cells (Table I) might then be greatly underestimated in our studies.

To investigate if a distant repressor might account for the reduction in IgA gene expression when in proximity to Eμ and the $V_H$ promoter, one could start out by cloning the DNA sequences downstream of the hs4 enhancer element into a reporter gene construct with Eμ, driven by a $V_H$ promoter. Test constructs then transiently transfected into Ig-secreting cells could then be assayed for function. Constructs that resulted in repression of the reporter gene could then be identified, the DNA sequenced, and examined for possible binding by a repressor factor.

The shift in enhancer dependence from Eμ to the 3' IgH elements in going from pre-B to B cells is likely a reflection of changes within the cell's environment during the

course of development, resulting in the appearance of different groups of transcription factors that augment 3' IgH element function more significantly than Eμ's activity. There are a large number of B cell specific transcription factors which could potentially affect IgH enhancer function. Since our data showed that the factor(s) involved in the shift in enhancer dependence are acting upon the 3' IgH enhancers, two candiates would be the transcription factors BSAP and NF-κB. Both have been shown to interact with various 3' IgH enhancers but not Eμ; and have changing roles during B cell differentiation consistent with the changes in 3' IgH enhancer function.

B cell specific activator protein (BSAP), product of PAX-5 gene, is a DNA-binding transcription factor that is expressed in pro B, pre B and mature B cells but not in plasma cells reviewed in (Morrison et al., 1998; Nutt et al., 1997; Urbanek et al., 1992). BSAP binding sites have been identified in the regulatory sequence of a number of genes including those encoding Ig heavy chain genes, the Ig-α (mb-1) subunit of B cell antigen receptor, the B cell antigen coreceptor CD19, the κ-light chain gene, surrogate light chain genes VpreB1 and λ5, J-chain, B cell specific tyrosine kinase Blk, just to name a few. BSAP was thus speculated to play an important regulatory role early in B cell development; this hypothesis was confirmed when mice lacking PAX-5 exhibited an early block in B cell development, as manifested by absence of small pre B, B and plasma cells. Interestingly, the block occurred at two places - fetal liver and bone marrow revealing a dependency of both fetal and adult B lymphopoiesis on BSAP. BSAP is essential for B lineage commitment in fetal liver and for progression of B lymphoid development beyond an early pro B cell stage in adult BM. Additional BSAP binding

sites were observed upstream of or within, almost all IgH S regions including Sμ, Sγ1, Sγ2a, Sγ3, Sε and Sα as well as in two of the four 3' IgH enhancer elements namely hs1,2 and hs4. This suggests a role for BSAP in class-switching events and possibly regulation of hs1,2 and/or hs4 activity (Michaelson et al., 1995; Singh and Birshtein, 1993). Because mature B cells do not emerge from PAX-5 deficient mice, the functional implications of BSAP expression throughout the mature B cell stage and its disappearance at the plasma cell stage cannot be inferred from these studies. BSAP's possible role in class-switching or in the activity of hs1,2/hs4 cannot be inferred from these studies for the same reason.

Through transient transfection studies, however, BSAP was observed to repress the activity of hs1,2 in B cells (Singh and Birshtein, 1993). A mutation in the BSAP binding site of hs1,2 causes an increase in activity in pre-B cells, where BSAP is present, but this same mutation in hs1,2 has no effect in plasma cells, which lacked BSAP. Introduction of recombinant BSAP into a plasma cell line which does not express BSAP endogenously similarly caused a reduction in hs1,2 activity (Neurath et al., 1994). Thus, it was concluded that hs1,2 remained inactive from the pre-B cell stage to the mature B cell stage owing to the presence of BSAP protein.

The idea that hs1,2 remained inactive in mature B cells was further supported by studies of transgenic mice, housing an hs1,2 enhancer-dependent reporter gene. Splenic B lymphoctyes were isolated and separated into resting B cells and activated B cells populations. When analyzed for hs1,2 activity, reporter gene function was only detectable in activated B cells. The resting B cell population however, upon mitogenic stimulation, also resulted in detectable hs1,2 containing reporter gene activity, demonstrating that the

activity of this enhancer element was largely restricted to activated B lymphocytes (Andersson et al., 1999; Arulampalam et al., 1996; Arulampalam et al., 1994).

In our transient assays, hs1,2, as an isolated enhancer was inactive from pre B to surface $Ig^+$ B cells [Figs. 12 (a & b) - 14 (a & b)], the activity of hs1,2 possibly being repressed by the binding of BSAP to its binding site. Interestingly, hs4, which houses a number of BSAP binding sites within it, was active in surface $Ig^+$ B cells, even in the presence of BSAP, suggesting BSAP has different functions depending upon the location of its binding sites within the locus (Michaelson, 1995). When assayed along with the remaining 3' IgH enhancers however, we showed that the "inactive" enhancer element (hs1,2) becomes strongly active when combined with hs3A, hs3B and hs4 in surface $Ig^+$ cells. That is, repression of this element did not translate to repression of the entire 3' enhancer cluster. The union of all of the 3' IgH enhancers might allow for association with yet a new set of transcription factors(s), or the association could mask the BSAP binding sites within hs1,2 due to spatial orientation, thereby eliminating repression of hs1,2 enhancer activity. Alternatively, the BSAP binding sites within hs4 might have a much higher affinity for BSAP than those in hs1,2. As such, the majority of the BSAP could shift from binding hs1,2 to binding hs4, thereby freeing the hs1,2 binding site for the NF-αP activator and inducing hs1,2 function.

To test this hypothesis, a modified construct could be made containing a BSAP binding site-mutated (hs1,2) fragment along with the remaining 3' IgH enhancers. The activity of this test construct transfected into surface $Ig^+$ cells could then be compared with construct 9 (Fig.11), containing the $W^+$ hs1,2 fragment.

Another transcription factor that might contribute to the developmental shift in enhancer strength from Eμ to 3' IgH enhancer is NF-κB. In contrast with BSAP, NF-κB can be hypothesized to affect 3' IgH enhancer function in several different ways, such as i) the particular forms of NF-κB expressed early in B cell differentiation might not activate the 3' IgH enhancers, while NF-κB forms expressed later in B cell development might activate strongly, ii) the NF-κB may be retained in the cytoplasm by IκB early, but not late, in B cell development, or iii) NF-κB is alone insufficient to activate the 3' IgH enhancers and requires the presence of a separate factor(s) expressed only later in B cell development.

NF-κB, present in the cytoplasm of numerous cell types exists as dimers of a family of structurally related proteins reviewed by (Ghosh, 1998; Snapper, 1996). Five such proteins have been identified in the mammalian cells: p65, cRel, RelB, p50/p105, and p52/p100. All five of these proteins share a conserved N-terminal region - the Rel homology domain - within which there are DNA-binding, protein-dimerization, and nuclear localizing signal (nls) sequences. Pre-B and non-B cells express mainly p50 and p65, mature B cells express p50, Rel and plasmacytoma lines or LPS-activated B cells express p52, RelB,p50, p65 and Rel. In the cytoplasm, NF-κB homo or heterodimers exist as an inactive complex bound to the inhibitor protein, IκB. Upon cellular activation however, IκB becomes phosphorylated which leads to its degradation, allowing NF-κB to translocate to the nucleus where it binds to the κB binding site present in promoter/enhancer of a wide array of genes. For example, κB binding sites have been demonstrated in immunoglobulin κ light chain genes (κ3' enhancer) and heavy chain

genes (hs1,2, hs4) and in the germline promoter of constant heavy chain ε gene (Iε), Iγ3, Iγ1, and Iα just to mention a few. Because of the presence of κB binding sites in the germline promoters of a number of constant region genes, it was not surprising that mice deficient in p50 showed defects in serum Ig levels of γ1, ε and α. p50 is a subunit of heterodimeric transcription factor, NF-κB, that was originally identified as an inducible B cell-specific factor able to bind to the κB motif in intronic κ light chain enhancer. P50 is initally synthesized as a larger 105 kDa precursor protein which is proteolytically processed into the p50 subunit and IκBγ, thus, both are contained in a single translation unit. Similarly, p52 is synthesized as a 105 kDa precursor which is cleaved into p52 and IκBγ. The precursor forms are unable to translocate to the nucleus and are retained in the cytoplasm until processed. Once cleaved into their constituent subunits, the NF-κB homo or heterodimers associate with the IκB subunits and again are held in the cytoplasm. Only when cells receive the appropriate signals will the IκB be degraded and allow NF-κB to translocate to the nucleus and activate transcription. Splenic B cells from mice deficient in p50 showed a significant reduction in all isotypes except IgM, which was slightly elevated in $p50^{-/-}$ mice. The largest reduction in isotype level was IgE (approximately 40 fold less), serum IgG1 (approximately 10 fold less) and IgA (approximately 5 fold less) than normal mice. These findings suggested that one key cellular event dependent upon p50 was isotype switching. Moreover, in vitro activated B cells from these mice had reduced levels of germline transcripts from $\gamma_3$ and ε constant region genes (Snapper et al., 1996). The similarity of this phenotype to mice with $neo^R$ replacement of the hs1,2 enhancer, which similarly manifested reduced levels of $\gamma_3$ and ε germline transcription

suggested that p50 might be a regulator of hs1,2 activity or of other components of the 3' IgH enhancer with which hs1,2 might synergize. Hs1,2 has a κB binding site and mutational studies showed that this site contributed positively to hs1,2 activity in plasma cells (loss of this site resulted in approximately 50 % reduction in activity). In B cells, however, mutation of the κB site in hs1,2 resulted in significant activation of hs1,2, suggesting NF-κB binding at this stage functioned as a repressor rather than as an activator (Michaelson et al., 1995). This change in the outcome of NF-κB binding to hs1,2 is perhaps a reflection of changes in the components of the NF-κB dimer. This notion was demonstrated by gel shift analyses on mature B cells and plasma cells using the κB site of hs1,2 as a probe. Michaelson et al showed (1996) showed that while mature B cells exhibited a single complex, plasma cells possessed multiple complexes. Using a spectrum of antibodies against specific component of κB complexes, they concluded that the single complex observed in mature B cells was p50/c-Rel while the multiple complexes in plasma cells contained p50, cRel and RelB (Michaelson et al., 1995). These data were consistent with those of others in which it was shown in which they showed that the major κB binding complex in murine mature B cells was a p50/Rel heterodimer while the major inducible form in pre-B cells was a p50/p65 heterodimer (Miyamoto et al., 1994).

Hs4, like hs1,2, contains a single κB binding site (Michaelson et al., 1995). Unlike hs1,2 however, NF-κB binding resulted in activation of hs4 across all stages of B cells assayed despite changes in NF-κB components from pre B to Ig-secreting cell stage (Michaelson et al., 1996). The differences in NF-κB function at hs1,2 versus hs4 are due

to a requirement for an additional factor for example, NFE, which facilitates p50 and c-rel-dependent activation of hs1,2 (Linderson et al., 1997). Mutation of either the κB or NFE binding sites in hs1,2 reduces hs1,2 function in plasma cells. A single copy of the NF-κB/NFE composite element was sufficient to confer lymphoid-restricted activity upon a heterologous promoter, and mutation of either individual element abrograted all promoter activity, suggesting a functional interdependence of the two binding sites.

In summary, a model for the shift in IgH enhancer dependence can be proposed through the functions of the transcription factors BSAP and NF-κB. In this model, BSAP represses 3' IgH enhancer activity at early stages of B cell development (with the exception of hs4). BSAP-mediated repression is overcome by either the presence of activators or decreases in BSAP expression later in B cell differentiation. NF-κB, although expressed early in B cell development, is either unavailable to activate the 3' IgH enhancers by retention in the cytoplasm by IκB, or is able to enter the nucleus but is unable to activate 3' IgH enhancer function due to either a lack of an important secondary factor, such as NFE, or by having the incorrect combination of subunits necessary for full activity (p50/p50 vs p50/c-rel, for example). It is important to note that these possibilities are not mutually exclusive, and all could play a part in regulating 3' IgH enhancer activity.

**Functional redundancy in IgH enhancers at surface $Ig^+$ and Ig-secreting B cell stages**

In our subsequent studies of subsets of IgH enhancers [Fig. 19 (a & b)], we found

that in surface $Ig^+$ cells, loss of one (hs1,2) or two (hs1,2), (hs3A) enhancer elements from the full complement of IgH enhancers resulted in activities not significantly different from the full IgH enhancers unit. Our findings are consistent with the results obtained from (hs1,2) and hs3A knock-out mice (Manis et al., 1998). As described earlier, these mice had no measurable change in Ig gene expression. However, our data suggest further that loss of the combination of two elements would similarly have no measurable effect on gene expression.

Our data are also consistent with data obtained from pre-B cell line, 70Z/3 that lacks both hs3A and hs1,2. Not surprisingly (given lacked of activity of these two enhancers in pre-B cells), 70Z/3 produces normal levels of cytoplasmic μ-chain. When fused with an Ig-secreting stage cell line (NSO), that no longer produces Ig, the resulting hybrid, 70Z/3-NSO, produced μ from the defective pre-B cell allele (lacking hs3A and hs1,2) (see Fig. 34). The remaining IgH enhancers Eμ, hs3B and hs4 were apparently sufficient for maintaining high levels of IgH gene expression and for γ1 class-switching (Saleque et al., 1999).

From our findings, even a combination of two enhancers was sufficient to maintain high levels of IgH gene expression. We found that any pair containing hs4 generated activity significantly above Eμ in both representatives of the surface $Ig^+$ B cells - Raji and M12.4.1 (Figs. 20, 21, 24, 25 and 27). This suggests redundancy of Eμ, hs3A, hs3B and hs1,2 with respect to their interactions with hs4 at this stage. Loss of any one of the IgH enhancers, except for hs4, should not affect Ig gene expression subsequent to the pre-B cell stage (where Eμ is essential). If M12.4.1 truly represents surface $Ig^+$ cells,

then deletion of hs4 should also not pose a problem as long as Eμ remains intact since pairwise combinations with Eμ all resulted in activity that was not significantly different from that of the (hs4,3B) pair. If however, Raji cells better represent surface $Ig^+$ cells, an hs4 knock-out could reduce IgH expression by almost 50 % (see Fig. 20).

The pairwise combinations were also examined for stage specific activity by transfecting them into an Ig-secreting cell - S194. Pairwise combinations were not tested in pre B cells because the combination of all the 3' IgH elements were inactive (18-81), or significantly below Eμ alone (18-8). From Figs. 22 (a & b), 30 (a & b), we found that all pairings with hs4 resulted in activities significantly greater than hs4 but roughly equivalent to Eμ alone. Since Eμ alone was in turn, equivalent in activity to the full complement of IgH enhancers (Fig. 15, constructs 10 vs 2), this suggests that loss of Eμ from the IgH locus in these cells, as in surface $Ig^+$ cells, would be efficiently compensated for by hs4 -containing pairs.

Pairings among the hs3A, hs3B, hs1,2 enhancers, although resulting in overall activity levels that were not significantly different from one another and from Eμ in Raji, were significantly lower in activity than Eμ in both M12.4.1 and S194. This suggests redundancy among the three enhancer element such that loss of for example, hs3A in (hs1,2,3A) pair, will be compensated for by the (hs1,2,3B). Interestingly, the combinations (hs1,2,3A) (hs1,2,3B) and (hs3A,3B) also exhibit synergy that appeared to be dependent upon the stage of B cell development. For instance, both (hs1,2,3A) and (hs1,2,3B) are synergistic in Raji cells, but in M12.4.1 and S194 cells, this synergism was lost. (Hs3A,3B) pair on the other hand, was synergistic in Raji cells, disappeared in

M12.4.1 but reappeared again in S194 cells. These changes could be a reflection of either the availability of transcription factor(s) or the transcription factor binding affinities over the course of B cell development and hence, different functions attributed to each enhancer pair. It is possible that these pairs are involved in the regulation of processes other than transcription from an IgH promoter, perhaps in class-switching events. While it is clear that hs3A and hs1,2 individually were not required for the class-switching process as observed in knock-out mice studies, it is possible that pairings of hs1,2 with either hs3A or hs3B might. In the hybrid 70Z/3-NSO which lacked both hs3A and hs1,2, spontaneous class-switching in culture from IgM to IgG1 was still observed, suggesting that hs3A and hs1,2 were not required in class-switching to the γ1 isotype. It is possible that other enhancer pairs might regulate class-switching to specific isotypes. Perhaps, switching to IgG1 is regulated by (hs3B,4) instead since this pair show synergy in Ig-secreting stage. To investigate the possibility of (hs3A,1,2), (hs3B,1,2), (hs3A,3B) or (hs3B,4) regulating class-switching, one could delete the above pairs from existing surface $Ig^+$ B cells known to undergo class-switching upon mitogenic stimulations. Alternatively, transgenic mice using two different reporter genes covalently linked to one another but driven by two different promoters could be employed. One promoter would be driven by the universal $V_H$ promoter, while the other, a more specific I promoter for a given isotype, for example Iε. The activities for each promoter could then be determined in the presence of all the enhancers in resting splenocytes versus splenocytes induced with various B cell activators. By using different combinations of enhancers, one could potentially link a given enhancer(s) to a specific I promoter. Another alternative would be

to generate various enhancer pairs knock-out mice for example (hs3A,1,2), (hs3B,1,2) or (hs3A,3B), and then analyze them for defects in serum Ig levels as well as IgH gene expression.

**Hs3A, Hs3B although highly homologous to one another, display differences in function over B cell development**

Hs3A and hs3B, are highly homologus (97 % identical over 597 bp) (Chaveau and Cogne, 1996; Saleque et al., 1997) and are oriented in opposite directions (Fig. 31). Due to their homology, hs3A and hs3B were assumed to function interchangeably (Chauveau et al., 1998). We decided to explore this directly by using constructs 27-36 (Fig. 23). Transfections into Raji, M12.4.1 and S194 showed that hs3A and hs3B differ in their ability to synergize when paired with identical enhancers over the course of B cell development. It is possible that these synergies are reflections of their involvement in regulatory processes other than sustaining high level of gene expression, for instance, in class-switching as alluded to earlier. If the reasoning was correct then, we would expect more hs3A and hs3B pairs showing synergy in surface $Ig^+$ cell lines, rather than in Ig-secreting cell lines. Indeed, this was the case (see Tables III & IV). In M12.4.1 however, we noticed that there are fewer hs3A and hs3B enhancer pairs showing synergy, suggesting that M12.4.1 might be in an intermediate stage in development between Raji and S194. To test this hypothesis, in vivo and/or in vitro DNAse I foot-printing studies of Eμ, hs3A, hs1,2, h3B and hs4 would have to be performed in pre B (18-81), B

lymphomas (Raji and M12.4.1) and plasmactyoma (S194). The pattern generated for each element at each stage of B cell differentiation could be obtained and compared for similarities and differences. Assuming that there are differences at the various cell stages, reflecting the binding of stage-specific transcriptional factors to these elements, we could compare the various patterns generated in the cell lines and conclude within some degrees of confidence whether or not M12.4.1 has progressed further in B cell development than Raji.

Taken together, hs3A and hs3B are functionally different despite their high levels of homology at the DNA level. It is possible that the differences observed are due to the 100bp gap in hs3A.

**Enhancer positioning and palindromic structure appear irrelevant for efficient IgH expressions in our assay system**

One of the concerns in assaying the IgH enhancers was the differences in the distances separating these enhancer units. Perhaps what we observed for transient transfections might not truly mimic what goes on in the normal chromosomal context because of the restriction in the distance separating these enhancer elements. Because of size constraints in plasmid constructs, the enhancers we studied were placed adjacent to one another. Could the lack of synergy observed for certain pairings such as (Eμ,hs1,2) in Ig-secreting cells be due to spatial impairment rather than actual repression brought about by the enhancer element itself? As reported in results' section, we compared results when

these two elements were placed 5 kb apart, but found no change in overall activity. Others reported that increased spacing led to synergy between these elements (Mocikat et al., 1995). The differences in our observations could be due to the fact that we used an entirely different system for our analyses. We chose to use transient transfections while Mocikat et al, employed stable transfections. The other issue is the fact that Mocikat et al, used the mouse intronic enhancer (Eμ) with the rat (hs1,2) core sequences instead of the mouse intronic enhancer with the mouse core hs1,2. Perhaps, the two enhancer fragments needed to be from the same species to function properly. Our findings however, were consistent with data from (Furebring et al., 1997) which showed that increasing the distance separating Eμ and (hs1,2) did not result in a shift from repressive to synergistic effect as reported by (Mocikat et al., 1995). More extensive experiments have to be performed to determine whether or not confirm that spatial configuration indeed plays a role in determining enhancer synergy.

In line with these data, Chauveau, et al. (1998), examined the effect of orientation and positioning of enhancers with respect to their endogenous positions in the 3' IgH. As mentioned previously, others have postulated that the 3' IgH enhancers might function as two units : Unit I, comprised of hs3A, hs1,2, hs3B, and unit II, hs4 alone (Birshtein et al., 1997). Unit I, consisting of hs3A, hs1,2 and hs3B was speculated to function through the formation of a large palindromic structure - with hs1,2 in the center, hs3A and hs3B flanking (Fig. 31). Chaveau et al, assuming the high homologies in DNA sequence between hs3A and hs3B reflected functional homology, made a construct - hs3A, hs1,2 hs3A(rev) to simulate the hs3A, hs1,2, hs3B that normally occurs in the IgH locus. This

construct was transiently transfected into B cells of different stages of development. Comparing the activities obtained from construct hs3A, hs1,2, hs3A(rev), to that obtained from hs3A, hs1,2, hs3A (tandem repeat-same direction) in both surface $Ig^+$ and Ig-secreting cell stages, they reported that the construct which allowed for the formation of the palindromic structure functioned more efficiently than the one in which palindromic structure formation was interrupted through the improper orientations of the two hs3A fragment with respect to each other. Additionally, upon the inclusion of hs4, they observed significantly more activity than with just hs3A, hs1,2 and hs3A(rev).

Our studies with hs3A and hs3B showed that they are functionally different at the surface $Ig^+$ and Ig-secreting stages. Using these two elements in either the "natural" orientation or in a reverse orientation that could disrupt the palindromic structure, we observed no significant differences in the overall activity of these constructs (Fig. 33). This suggested that the palindrome might itself not play a key role in enhancer function. Moreover, our tests of removing one of the two hs3 elements (Fig. 33) and of changing enhancer position relative to the promoter (Fig. 33) also showed no discernible dependence upon particular arrangements of the 3' IgH enhancers. These data are consistent with the hs3A knock-out mice studies, in which loss of one "arm" of the palindromic structure had no apparent effect on IgH gene expression (Manis et al., 1998).

Another piece of evidence that efficient IgH gene expression could occur irrespective of the availability of "full" palindromic structure formation comes from analyses of the human IgH locus. Downstream of both human Ig Cα genes, are found enhancer complexes consisting of hs3A, hs1,2 and hs4. These complexes were very

similar to those found in the murine IgH locus. In both the Cα1 and Cα2 genes, hs3B was absent, thereby eliminating the possibility of forming a complete palindromic structure. Additionally, the hs1,2 found in each of the Cα genes was not oriented in the same direction with respect to hs3A and hs4. (Chen and Birshtein, 1997; Mills et al., 1997). Despite these differences between the human and murine enhancer complexes, the human IgH locus was still efficiently regulated, suggesting the irrelevance of complete palindromic formation for full IgH gene expression, consistent with our findings.

## Conclusion

In summary, our findings suggest functional redundancies amongst the IgH enhancers. It appears that not all IgH enhancers are required for high levels of IgH gene expression. If our transient assays are correct, then pairs of (Eμ,hs4) or (hs3B,4) are all that are necessary for maintenance of high IgH gene expression. Since Eμ is essential for the VDJ recombination process, it would not be possible to generate a mice that only has (hs4,3B). From our findings, it might be expected that mice with just Eμ, hs3B and hs4 would generate a normal population of B cells and Ig-secreting cells. Here, (hs3B) was included even though (Eμ,hs4) pair was sufficient for high level gene expression, because, potentially, (hs4,3B) pair might be involved in class-switching event to certain isotype. The effect on the IgH gene loci expression level would be minimal but class-switching to isotypes other than γ1 might be affected.

Each enhancer element has the ability to synergize when paired with another IgH

enhancer element as exemplified by hs3A pairing with hs1,2 and Eμ pairing with hs4 in surface $Ig^+$ cells, just to quote a few. The synergies observed for the various pairings were not static in nature but dynamic instead. Synergy was more pronounced in surface $Ig^+$ cells and tapered off in Ig-secreting cells. This could reflect changes in the cells' nuclear environment in terms of the availability of common transcription factors or perhaps the appearance of a new set of transcription factors. I would suggest that these synergy pairs that did not result in high levels of activity are involved in processes other than maintaining high levels of IgH gene expression, for instance in class-switching process.

To clarify the roles of the IgH enhancer elements in B cell ontogeny, more refined experiments need to be done. One potential approach is to delete the IgH enhancers from the mouse genome, first, individually, then, as pairs and, finally as a unit. For the deletion of all 3' IgH enhancer elements at once, it would be ideal to replace the approximately 34 kb genomic DNA encompassing hs3A, hs1,2, hs3B and hs4 deleted from the IgH locus with an 'inert' piece of DNA of equivalent size. That way, spatial configuration as well as distant repressor(s) effect would be accounted for. The problem arises when dealing with Eμ, and pairings involving Eμ. A different approach will have to be devised as the knockout of Eμ alone results in deleterious effects in VDJ recombination process. An alternative approach would be to introduce an already rearranged variable region in the place of Eμ. If Eμ were only needed for efficient VDJ rearrangement, then, this need would be fulfilled by an already rearranged variable region. This "altered" locus could be subsequently followed by deletion of any of the 3' IgH enhancers of interest.

The idea that enhancers might play different roles in the regulation of a locus over

the course of cell development as demonstrated in this thesis and in the work of other laboratories, is an idea that is not restricted to the immunoglobulin heavy chain locus. A somewhat analogous situation has been reported in the kappa (κ) light chain locus. Kappa light chain locus as described in chapter 1 (see Fig. 2), contained two enhancers - the intronic kappa enhancer (iEκ), located between the Jκ segments and the constant κ region; the 3' enhancer (3'Eκ), located approximately 9 kb downstream of the Cκ region. In the κ locus, the iEκ element becomes active during the pre B to B-cell transition (reviewed in Gorman and Alt, 1998). Studies of the function of iEκ in cell lines have suggested that iEκ is required for demethylation of the κ locus and expression of the κ germline transcripts (Klug et al., 1994; Lichtenstein et al., 1994). Mice homozygous for the deletion of iEκ and its adjacent MAR region (MiEκ), impaired VκJκ rearrangement, although not completely, and showed no effect on κ gene expression (Xu et al., 1996), suggesting contribution by other regulatory elements within the locus (possibly 3' kappa enhancer), for κ gene expression as well as efficient VκJκ rearrangement.

The 3' kappa enhancer (3' Eκ) in contrast to the intronic kappa enhancer was originally thought to be fully functional at later, mature B and plasma cell, stages of B cell development (Meyer and Neuberger, 1989). However, the 3' kappa enhancer deficient (3' $E\kappa^{-/-}$) mice studies, which resulted in a substantial decrease in the number of κ-expressing B cells and an increase in λ-expressing B cells accompanied by a decrease in κ versus λ gene rearrangement just like the $iE\kappa^{-/-}$ deficient mice, would suggest that the 3' Eκ is normally active during pre-B to B-cell transition (Gorman et al., 1996). Additionally, a reduction in κ expression was observed in resting B cells of the mutant

mice, but was normal in activated B cells, suggesting a critical role for 3' Eκ in establishing the normal κ/λ ratio, but not absolutely required for κ gene rearrangement or, surprisingly, for normal κ expression in activated B cells.

# Chapter 5

## Concluding Remarks

## 5.1 - Concluding remarks

The data presented in this thesis, address the roles of the various IgH enhancer elements over the course of B cell development.

Our findings show that Eμ alone is active throughout all stages of B cell development, while the 3' IgH enhancer elements are inactive individually, early B cell development, becoming active in later B cell stages, with the exception of hs4, which although active throughout all stages of B cell differentiation was weaker than Eμ except in Raji cells [see Figs. 12 (a &b) - 15 (a & b)].

When the IgH elements were assayed as a group, we found Eμ was dominant in pre-B cells where contributions to overall activity by the 3' IgH elements as a unit was negligible. At the surface $Ig^+$ stage, we observed a shift from Eμ to the 3' IgH elements as the dominant enhancers. The 3' IgH elements at this stage, displayed pronounced synergy in mouse and human B cell lines. At the Ig-secreting stage however, both Eμ and the 3' IgH enhancer elements contributed similarly to overall activity, demonstrating functional redundancy between this two regions which may account for the existence of a number of Ig-producing cell lines that expressed Ig heavy chain in the absence of Eμ (Aguilera et al., 1985; Klein et al., 1984; Wabl and Burrows, 1984; Zaller and Eckhardt, 1985)

We show functional redundancy among the IgH enhancers using various combinations of elements, suggesting that loss of any one element would be efficiently compensated for by the remaining IgH enhancer elements. Furthermore, even deletion of

two enhancers had little effect upon activity. The description of the hybridoma 70Z/3-NSO which lacks hs3A and hs1,2, and expresses high levels of IgM is consistent with our prediction (see 70Z/3-NSO, Fig. 34).

Although we observe a general functional synergy between IgH enhancer elements, closer examination has shown that while some pairs synergize poorly (e.g. hs3B and hs4), they still exhibit significant activity. Conversely, some pairs synergize greatly (e.g. hs3B and hs1,2) but show low overall activity. These results may reflect differences in functions associated with each enhancer pair. The (hs4,hs3B) pair, because of its high activity level, might be involved in ensuring high levels of IgH gene expression, while (hs3B,hs1,2) pair because of its low activity level and high synergy level, may play a role, in class-switching events to isotypes other than $\gamma 1$.

In light of these findings, I suggest that it will be more useful to examine double or even triple 'clean' knockouts of IgH elements in mice. In vivo, B cells normally are exposed to various lymphokines, signaling molecules, and antigen, all of which could potentially affect the activity of the IgH enhancer elements and their resulting roles in regulation of IgH gene expression.

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