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A Dissection of Enhancer Function within the Immunoglobulin Heavy Chain Locus, using Transgenes and the Transcription Factor BSAP.

by Adrienne Alaie-Petrillo

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

2002

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ABSTRACT

A Dissection of Enhancer Function within the Immunoglobulin Heavy Chain Locus, using Transgenes and the Transcription Factor BSAP.

by Adrienne Alaie-Petrillo

Adviser: Laurel A. Eckhardt, Ph.D.

Five enhancers within the immunoglobulin heavy chain (IgH) locus serve to regulate transcription of the IgH genes in a stage-specific manner. In Ig-secreting cells, four 3' enhancers (hs3a, hs1,2, hs3b, hs4) can drive IgH gene expression in the absence of the intronic enhancer (Eμ). BSAP (B-cell-specific activating protein) is a transcription factor expressed in pro-B, pre-B, and surface Ig-positive cells but not in Ig-secreting cells. BSAP was shown to repress the 3' enhancer element hs1,2 in transient transfection assays. It was suggested, therefore, that BSAP suppresses 3' IgH enhancer function through much of B cell development, releasing this region of enhancers from suppression only when surface Ig+ cells are activated to differentiate into Ig-secreting cells. To test this hypothesis, we introduced a BSAP-expressing vector into an Eμ-deficient Ig-secreting plasmacytoma. Since Eμ is not available to compensate for loss of 3' enhancer function in this cell line, BSAP expression should result in a dramatic decrease in IgH gene expression if BSAP is indeed a repressor of 3'IgH enhancer function. We found, however, that ectopic expression of BSAP in this Ig-secreting cell line had very little effect on IgH gene expression. We conclude, therefore, that ectopically expressed BSAP,

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on its own, is not capable of repressing, in a global fashion, the 3' IgH enhancer region when produced within an established plasmacytoma.

Three of the four IgH 3′ enhancers (hs1,2, hs3b, hs4), were shown to function as a locus control region (LCR), in that they directed tissue-specific, insertion site-independent, and copy number-dependent expression of their linked transgene in stable transfections. However, recent studies failed to confirm these earlier findings, observing instead, only partial-LCR like activity. None of the research to date examined the IgH 3′ enhancers' ability to function as an LCR while in their native orientation and spacing as the enhancers span too large a distance to be mimicked in conventional cloning vectors. Using bacterial artificial chromosome technology (BAC), we tested whether the 3′ enhancers, in their native orientation and spacing, could direct insertion site-independent, copy-number dependent expression. We found that the IgH 3′ enhancers do not display classical LCR behavior.

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GENERAL INTRODUCTION

B cell ontogeny proceeds through several distinct stages, each stage clearly distinguishable from the preceding one by a discrete set of cell-surface markers. In order for a cell to progress from one stage to the next, it must satisfy specific developmental requirements. The function of the B cell is to produce antibodies that are used in immunological defense. It logically follows then, that the syntheses of different components of the antibody serve as the developmental checkpoints that the B cell must satisfy. If a cell fails to meet a requirement, it will be marked for destruction.

Antibodies are composed of four polypeptide chains: two identical heavy chains and two identical light chains. Each of these chains has a "variable" region that is unique to this protein and a "constant" region that is common to all antibodies of that particular class. The quaternary structure of an antibody defines its specificity as the binding site of an antibody is created by the combination of the variable region of the heavy chain with the variable region of the light chain. Therefore, each antibody possesses two identical regions to which a pathogen, in possession of a moiety of complementary shape, could bind. As each B cell produces antibodies of a single specificity, a large repertoire is generated which provides protection against a broad spectrum of pathogens.

Each B cell creates the unique variable region of their heavy chain by stitching together three distinct regions of DNA randomly selected from collections of segments known as the "variable" (V), "diversity"(D), and "joining"(J). To synthesize the variable region of a light chain, the B cell combines two segments: a "variable" segment and a "joining" segment. The formation of each of the chains allows the cell to progress to the

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next developmental stage. In order to create the heavy chain a murine Pro-B cell will select one from 20 "diversity" segments and link it to any one of the four "joining" segments. The DNA found between the two selected segments will be looped out of the genome, excised, and lost to the cell. This recombination event is known as the "D-J" join and it almost always occurs on both alleles. Additional nucleotides are often added to the ends of the selected segments (before the segments are ligated to one another), which greatly increase the diversity of the resulting polypeptide made by the combination of these two segments. The DJ join is followed by a second recombination event. The murine Pro-B cell will choose one from several hundred "variable" segments and combine it to the DJ, once again, looping out all intervening DNA to form the complete variable region or VDJ. Once VDJ rearrangement is finalized, the first heavy chain is transcribed, and through RNA splicing, the VDJ is placed next to the first "constant" gene of the heavy chain: the μ isotype. (see Figure 1). If the VDJ rearrangement results in a readable frame, the first heavy chain polypeptide is synthesized. This protein is combined with a precursor of the light chain known as the surrogate light chain, as the V-J of the light chain's variable region has not yet been stitched together. Two heavy chains in combination with two surrogate light chains go to the surface of the cell as the approximation of this cell's mature antibody.

Within the heavy chain gene, a 1 kb fragment located between the J gene segment cluster and the first constant region coding sequences (Cμ), was the first eukaryotic enhancer to be identified. Due to its location in the intronic region of the heavy chain, it was called the intronic enhancer or Eμ (Banerji et al. 1983; Gillies et al. 1983). (See figure 2). Eμ was found to be critical in early B-cell development as V to DJ

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rearrangement was severely impaired on mutated alleles (Chen et al. 1993; Serwe and Sablitzky 1993). Eμ is dispensable, however, when B cells reach their terminally differentiated plasma cell stage. This dispensability appears to be due to the fact that there are a group of four enhancers located 3′ of the last constant region exons (Cσ). The first of the 3′ enhancers, hs3a, is located ~ 4 kb downstream of Cα. Hs1,2 , hs3b, and hs4 are located 13.5 kb, 26.5 kb and 30 kb, respectively, downstream of hs3a (see figure 2). Thus, the 3′ enhancers span a distance of approximately 30 kb while the Ig promoter they regulate is located at a distance of ~ 200 kb. While both Eμ and the 3′ enhancers are active at the plasma cell stage (Ong et al. 1998), the 3′ enhancers appear to fully compensate for the activity of Eμ if, in the course of class switch recombination (CSR), it is deleted (Wabl and Burrows 1984; Eckhardt and Birshtein 1985). The determination that the 3′ enhancers were indeed responsible for driving transcription of the heavy chain gene in the absence of Eμ was made when insertion of a drug-resistance gene in the place of hs1,2 resulted in complete loss of transcription of the heavy chain (Lieberson et al. 1995).

While the 3′ enhancers appear to compensate for the loss of Eμ at the plasma cell stage, the reciprocal does not appear to be true: an intrachromosomal deletion of the four 3′ enhancers (that left transcription of the heavy chain under the control of Eμ), resulted in the dramatic reduction of transcription of this heavy chain (Gregor and Morrison 1986).

The 3′ enhancers cannot take the place of Eμ at earlier developmental stages, however, as they do not become active as a synergistic unit until the mature B cell stage (Ong et al. 1998). There are differences, also, in the level of activation of the individual enhancers.

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Whereas Eμ can greatly augment the expression of a linked transgene, each of the four 3′ enhancers when assayed individually, display very little enhancing activity at all stages of B cell development (Ong et al. 1998). In the endogenous locus, however, the replacement of a single 3′ enhancer (hs1,2) with a gene for drug resistance led to the abrogation of heavy chain transcription and the belief that hs1,2 was critical for transcription of the heavy chain at the plasma cell stage (Lieberson et al. 1995). Recent evidence has suggested that the strong promoter of the inserted drug resistance gene subverted the activity of the remaining 3′ enhancers (Fiering et al. 1995; Hug 1996). Therefore, the absolute contribution of hs1,2 towards overall transcriptional enhancement of the heavy chain remains to be elucidated. This study addresses this question.

The two distal 3′ enhancers, hs3b and hs4, have recently been shown to be critical for expression of a linked transgene in stable transfections in a plasmacytoma as deletion of this pair ablated expression of the reporter gene (Shi 2001). Further, transgenic mice with targeted deletions of hs3b and hs4 displayed aberrant class switch recombination (Manis et al. 1998). In contrast, the two proximal 3 ′enhancers, hs3a and hs1,2 were deleted with no apparent phenotype in both stable transfections in a plasmacytoma and in transgenic mice (Manis et al. 1998; Shi 2001).

Within each of the heavy chain's five enhancers are found recognition sites for a variety of both tissue-specific and ubiquitous transcription factors, with many of the factors known to bind to more than one of the enhancers. A few of the tissue-specific factors are critical for developmental progression beyond the Pre-B cell stage. Three such factors are B cell specific activating protein (BSAP) (Urbanek et al. 1994; Nutt et al. 1997), Pu.1 (Scott et al. 1994; McKercher et al. 1996), and early B cell factor (EBF) (Lan

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1995). Other factors such as Oct-2 (Corcoran et al. 1993) and Oca-B (Kim et al. 1996; Schubart et al. 1996) are critical for the proper functioning of later stage B cells.

At the time that the 3′ enhancers become active, they are located at ~ 200 kb away from the Ig promoter. If this B cell is selected for expansion and differentiation by its recognition of a pathogen, the cell may undergo a switch to expression of another constant gene. The type of antigen that provokes the response, determines the type of cytokines secreted by the Helper T cell, which ultimately determines which constant gene the B cell will switch to. The DNA between the constant genes is looped out, excised, and eventually lost. However, the distance between the 3′ enhancers and the promoter is still significant even in the event of a class switch to Cα, the constant gene most proximal to the 3′ enhancers.

Two models propose distinct mechanisms that allow the widely spaced regulatory elements to interact with one another. The "looping" model contends that the DNA located between the promoter and the enhancers is looped out during transcription thus allowing elements separated by large distances to come in physical contact with one another. Activation occurs via direct interactions between transcription factors bound to the enhancers and those bound near the promoter (Ptashne 1988; Ptashne 1997). The more recent "linking" model speculates that the promoter communicates with its enhancers via a protein bridge. In this model, protein candidates either bind directly to the hyperacetlylated chromatin found along the length of an active locus, or become anchored to the chromatin through their interaction with homeodomain transcription factors (as these have been shown to bind at low levels to a large proportion of active genes) (Morcillo 1997; Bulger 1999). This protein link allows elements that are not in

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physical contact with one another to work together. At this point, neither model can be definitively proven.

In addition to binding transcription factors that regulate the activity of gene loci, specific DNA sequences recognize and bind protein components of the nuclear scaffold. These 200-300 bp AT-rich sequences are known as matrix attachment regions (MARs)(Laemmli 1992). Within the heavy chain locus, MARs have been identified ~ 500 bp 5′ of the Ig promoter (Webb 1991), flanking the core enhancer element of Eμ (Cockerill et al. 1987), and 5′ of Cγ3 (Cockerill 1990). Although MARs occur on the average of every 30 kb in eukaryotic DNA (Garrard 1990), MARs have not been found within the 3′ enhancer region {Shi and Eckhardt, unpublished}. MARs appear to be involved in the transcriptional enhancement mediated by Eμ when assayed in transgenic mice as the addition of MARs to the Eμ core enhancer results in more efficient expression of linked transgenes. In addition, the linked transgenes are freed of the position effects they were subject to when linked to the core enhancer alone (Forrester et al. 1994; Jenuwein 1997). However, MARs do not have to be found within an enhancer region in order for it to function efficiently as neither the Ig's 3′ enhancers {Shi and Eckhardt, unpublished} nor the human growth hormone (hGH) locus control region (LCR) (Shewchuk 2001) contain MARs. The heavy chain locus also demonstrates that MARs do not serve as boundaries of a self-sufficient transcriptional region, as was once believed, for they are found in the middle of the heavy chain locus and are absent at the far 3′ end.

Three of the four 3′ enhancers (hs1,2, hs3b,hs4) have been labeled a locus control region (LCR) (Madisen and Groudine 1994). An LCR, originally identified in the β-

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globin locus, is functionally defined as a region of DNA capable of directing tissue-specific, copy-number dependent and integration site- independent expression of its linked gene (Grosveld et al. 1987). The original experiments involved linking hs1,2, hs3b,and hs4 to a *c-myc* gene and stably transfecting this construct into a human B cell line. The three 3′ enhancers drove copy-number dependent and position-independent expression of *c-myc* in the transformants (Madisen and Groudine 1994). However, recent experiments using all four of the 3′ enhancers have failed to duplicate these findings. Stable transfections of a construct driven by the 3′ enhancers into murine B cell lines have shown that the 3′ enhancers can overcome position effects to facilitate integration-site independent expression of their linked gene (Shi 2001). However, strict copy-number dependent expression was not observed. Similarly, recent studies in transgenic mice have demonstrated that the four 3′ enhancers can direct fully tissue-specific expression as well as integration site-independent expression of their linked transgene. Once again, though, copy-number dependent expression was not observed (Chauveau et al. 1999).

In this thesis, we employ two separate strategies to explore the function of the 3′ enhancers at the plasma cell stage. In our first study, we ectopically express a known negative regulator of hs1,2 (BSAP) in a plasmacytoma lacking Eμ, to examine hs1,2's contribution to overall transcriptional enhancement of the heavy chain gene at the plasma cell stage. In our second study, we investigate whether the 3′ enhancers, when assayed in their native orientation and spacing, function as an LCR at the plasma cell stage.

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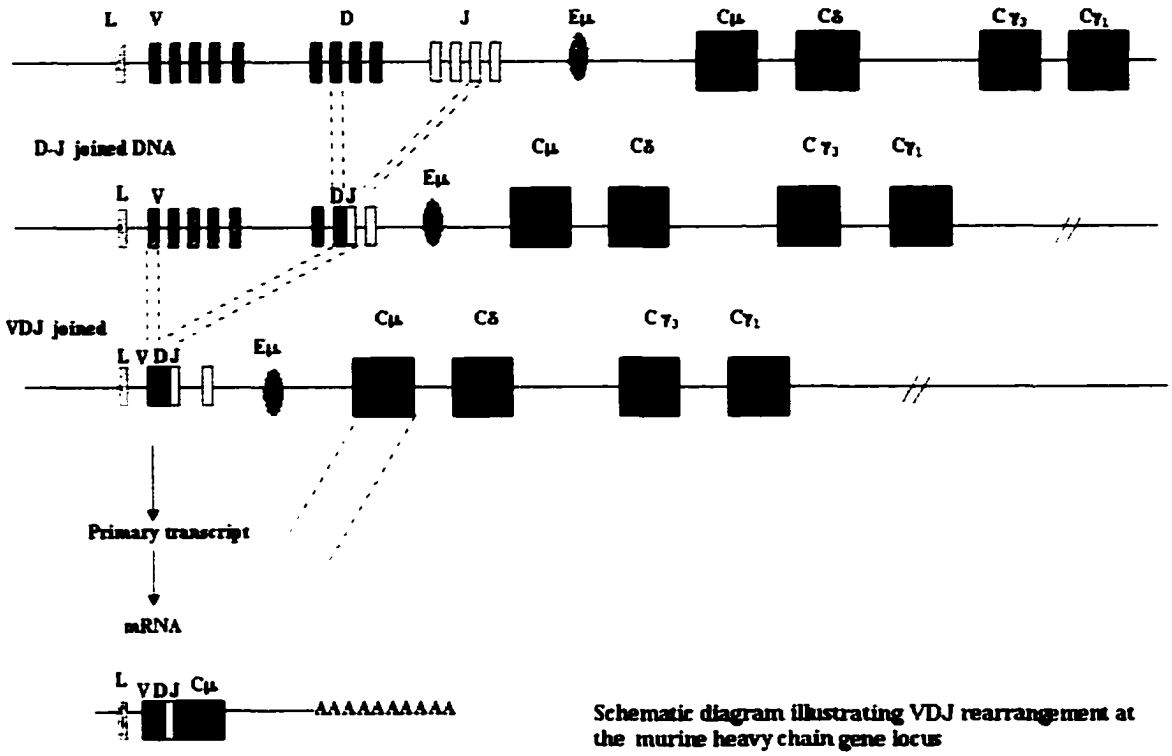
Figure 1



Schematic diagram illustrating VDJ rearrangement at the murine heavy chain gene locus

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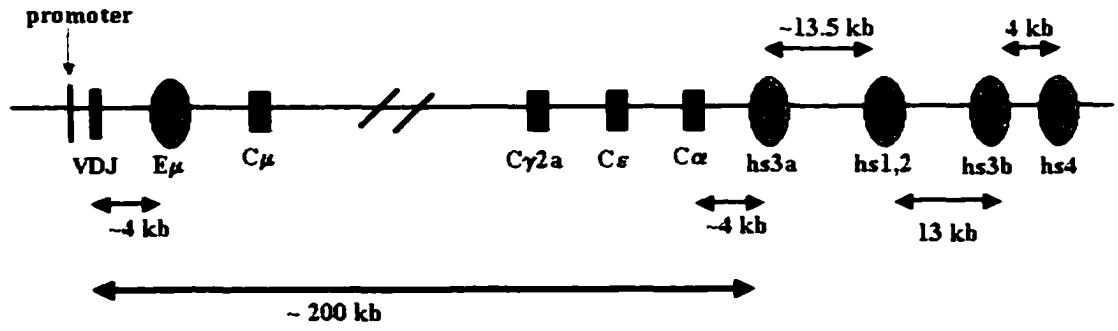
Figure 2



Schematic map of the Ig heavy chain locus illustrating the relative positions of the enhancers. Filled boxes represent constant region exons (Cμ, C γ2a, Cε, Cα) as well as assembled variable region exon (VDJ). Shaded ovals represent the various enhancers: Eμ, hs3a, hs1,2, hs3b, hs4.

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BSAP INTRODUCTION

Our first study of the heavy chain's 3′ enhancer region involves the stable, ectopic expression of B cell specific activating protein (BSAP)(also known as Pax 5) in a plasmacytoma. Pax 5 belongs to the Pax family of transcription factors that function in developmental regulation. The Pax proteins earn their familial designation by virtue of a 128 amino acid NH_2 – terminal paired domain through which they contact DNA. In mice, Pax 5 is expressed in the developing CNS, adult testis as well as throughout B cell ontogeny with the exception of the terminally differentiated plasma cell (Barberis et al. 1990; Adams et al. 1992). Pax 5 knock-out (-/-) mice exhibit midbrain defects, neurological abnormalities and the arrest of their B cell development at the pro-B cell stage (Urbanek et al. 1994). A close examination of the Pax 5 -/- pro-B cells reveals that they are not irreversibly committed to the B cell lineage, as they are capable of differentiating into many of the other haematopoietic lineages if deprived of IL-7 and provided with the proper cytokine stimulus (Nutt et al. 1999). Restoration of Pax 5 expression through retroviral transduction reinstates the B cell program proving that commitment to the B-lymphoid lineage is dependent upon Pax 5 (Nutt et al. 1999).

BSAP binding sites are non-palindromic and degenerative and have been found in the regulating elements, including promoters, enhancers, and switch regions of a vast array of B-cell specific genes such as CD19, Ig-α (mb-1), Blk, J chain and the Ig heavy chain 3′ enhancers hs1,2 and hs4 (Neurath et al. 1995; Michaelson et al. 1996; Rinkenberger et al. 1996). However, BSAP has not been found to be necessary for the expression of any gene with the exception of CD19, which is no longer transcribed in Pax 5 -/- mice(Nutt et

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al. 1997). Yet, BSAP contributes to the control of several genes as their expression was rapidly regulated upon addition of a hormone-inducible BSAP-estrogen receptor fusion protein in a BSAP-deficient pro-B cell line (Nutt et al. 1998). These genes included Ig-α, N-*myc*, LEF and PD.1. BSAP has also been implicated in the regulation of the germline ε promoter in *in vitro* cultured B cells (Liao et al. 1994; Qiu and Stavnezer 1998).

Once bound to one of its diverse recognition sequences, BSAP can act as a positive regulator, as is the case for CD19 and Ig-α, or as a negative regulator, as is the case for J chain and hs1,2 (Neurath et al. 1994; Rinkenberger et al. 1996; Nutt et al. 1998). However, it is not an intrinsic property of the particular BSAP recognition sequence that determines the behavior of BSAP at each of its sites, rather, it is the context in which each of these sites is located (Singh and Birshtein 1996; Nutt et al. 1998). For example, repression of the IgH 3′ enhancer hs1,2, as demonstrated by transient transfection, was found to be dependent upon an Oct-binding site and a G-rich motif binding site in addition to the two BSAP recognition sites (Singh and Birshtein 1996). When one of the two BSAP binding sites was removed from this context and placed in an expression vector, this BSAP binding site activated transcription, proving that the site itself was not inherently repressive (Singh and Birshtein 1996).

In the case of hs1,2 and the J chain promoter, it has been proposed that BSAP is mediating its repression of a particular regulatory sequence by blocking the activities and interactions of other activating proteins at this site (Neurath et al. 1995; Wallin et al. 1999). BSAP binding to its element in the J chain promoter prevents two other transcription factors, USF and MEF-2 from interacting with and activating the J chain promoter (Wallin et al. 1999). BSAP's binding at hs1,2 prevents the binding of the

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activating protein NF-αP (recently shown to be PU.1 (Linderson 2001)) until the plasma cell stage when BSAP is no longer expressed (Neurath et al. 1995).

Steric hindrance, however, is not the only proposed mode of BSAP's repression . In the case of the Ig kappa 3´ enhancers, BSAP has been shown to bind PU.1, and by that binding, repress the enhancement of transcription mediated by PU.1 and its accessory proteins (Maitra 2000).

Recently, a member of the Groucho family of corepressors, Grg4, has been shown to bind BSAP in its octapeptide motif (a conserved sequence motif found in most Pax proteins) (Noll 1993) and efficiently repress the transcriptional activity of BSAP (Eberhard 2000). As Grg4 does not contain any recognizable DNA-binding motifs, it is recruited to a particular site through its interaction with its co-repressor/ protein partner (Eberhard 2000).

Other proteins shown to bind BSAP are the TATA-box binding protein (TBP) and the Retinoblastoma protein (pRb) (Eberhard 1999) which both contact BSAP in its centrally located partial homeodomain. These interactions indicate that BSAP is able to contact the basal transcriptional machinery and that its activity can be regulated in a cell-cycle manner by pRb.

The development of conditional BSAP knock-out mice allowed investigators to define the role of BSAP in later B cell stages, as B lymphopoiesis in conventional BSAP knockout mice is arrested at the Pro-B cell stage (Urbanek et al. 1994; Nutt et al. 1997). Two different conditional knock-out mice were generated: one group deleted BSAP in response to drug induc ment, while another group deleted BSAP in developing B cells as they commenced expression of a tissue-specific signaling molecule (Horcher 2001).

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Together these transgenic mice showed that BSAP deletion in mature B cells leads to both the loss of expression and downregulation of many B cell specific genes (Horcher 2001). In addition, these mice displayed preferential loss of mature B cells, inefficient lymphoblast formation and reduced serum IgG levels (Horcher 2001). Therefore, BSAP is also necessary at the late stages of B cell development to help maintain a B cell's identity. Interestingly, loss of BSAP at this stage did not activate the terminal differentiation program: neither Blimp-1 nor J-chain was induced, suggesting that the repression of J-chain is more complex than BSAP's simply preventing interaction of the promoter with MEF-2 and USF.

We wished to use BSAP's role as a negative regulator of the IgH 3' enhancer, hs1,2, to determine the contribution of hs1,2 towards overall transcriptional enhancement of the heavy chain locus at the plasma cell stage. Earlier studies conducted by this laboratory involved replacement of hs1,2 in an Eμ-deficient Igγ2a-secreting plasmacytoma with a *neomycin* resistance (*neo*^{r}) gene. It was found that all transcription of the heavy chain gene ceased (Lieberson et al. 1995). However, evidence began to mount that insertion replacements in which a gene under the control of its own strong, constitutively active promoter constituted a "technical knock-out" (Kim et al. 1992; Fiering et al. 1995). This implied that the inserted gene and its promoter subverted the normal activities of the 3′ enhancers such that they worked in concert with the inserted promoter. The constitutively active promoter out-competed the endogenous promoter, which, in the case of the γ2a heavy chain, is located over 50 kb away. An alternative approach to assess the contribution of hs1,2, without perturbation of the chromosomal locus, is to repress the activities of this enhancer thereby orchestrating a "functional knock-out". BSAP has been

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shown to repress the activity of hs1.2 in transient transfection(Singh and Birshtein 1993; Neurath et al. 1995). We decided to ectopically-express BSAP in a plasma cell and note the effect on heavy chain transcript levels. Complicating the definitive assessment of hs1.2's role in enhancement, however, is data that shows the heavy chain intronic enhancer, Eμ, to be quite active at the plasma cell stage (Ong et al. 1998). We therefore chose to work with the 9921 cell line, an Igγ2a-secreting plasmacytoma that produces copious amounts of γ2a despite loss of Eμ due to a class-switching event (Eckhardt and Birshtein 1985). Working in an Eμ $^{-/-}$ background would allow us to assess the contribution of hs1.2 towards overall enhancement without the complication of enhancer activity redundancy.

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BSAP RESULTS

A BSAP-expression plasmid under the control of a CMV promoter and containing a *neo*^{R} gene was electroporated into 9921 cells and neomycin-resistant clones were screened by Western analysis for BSAP expression. Clones found to express BSAP at levels roughly equivalent to or greater than endogenously-expressed BSAP in a pre-B cell line (70Z/3) were chosen for analysis. As shown in Figure 3, clones 2, 4, 9, & 12 met these criteria and also expressed more BSAP than the surface-Ig+ cell line A20. Comparable results were obtained in three independent experiments, using extract prepared on three different occasions for each cell line.

As a test of the proper functioning of our ectopically expressed BSAP, we performed electrophoretic mobility shift assays. The four 9921/BSAP clones identified in Figure 3 expressed BSAP in a form that was able to bind an oligonucleotide containing a BSAP-recognition site [from the sea urchin histone promoter (H2a-2.2)] (Figure 4). Competitor DNA containing the BSAP-binding site eliminated the BSAP/DNA complex, while an irrelevant oligonucleotide did not (left panel, Figure 4). Anti-Pax-5 antibody confirmed that the DNA/protein complexes formed contained the BSAP protein (right panel, Figure 4). As shown, addition of this antibody either inhibited the BSAP/DNA complex or supershifted it to a size that barely entered the gel (compare +/- antibody lanes in right panel, Figure 4).

BSAP has been shown to negatively affect IgH enhancer hs1,2 function in transient transfection assays (Singh and Birshtein 1993; Neurath et al 1994). We analyzed the 9921/BSAP clones to determine whether BSAP would exert a similar effect on the endogenous IgH gene under IgH 3′ enhancer control. Western blots detecting Igγ2a and

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Igκ did not show any change in the ratio of these two Ig proteins as compared to the wild type cell line, suggesting that BSAP expression caused no appreciable change in the steady-state levels of Igγ2a (figure 5). This experiment was done four times with four different cell extract preps and always yielded comparable results.

We next employed Northern analysis to assess steady-state γ2a mRNA levels in the 9921/BSAP clones through the use of a phosphor imager and ImageQuant software. As established cell lines can change both their genotypic and their phenotypic characteristics over long periods of time in laboratory culture, we decided to continue our analysis of the 9921/BSAP clones comparing them to six representative parental 9921 subclones (labeled x2-x7 in figures 6 and 7). As shown in Figure 6, the five BSAP-producing clones, as a group, did not exhibit a decrease in γ2a heavy chain mRNA when compared to the six parental 9921 subclones. Instead, they expressed γ2a at levels that were both above and below the amount expressed by the six 9921subclones. A salient result of these Northern studies is that the steady-state transcript-level variation among both the 9921/BSAP clones and the parental subclones was found to be considerable: the BSAP clones exhibited an average variation of 37% while the parental subclones showed an average of 43% variation (Figure 6. Data obtained by ImageQuant analyses of phosphorimaging films from three independent experiments). However, the absence of an overall decrease in the steady-state level of γ2a mRNA in the 9921/BSAP clones is in accordance with the western analyses that failed to detect a decrease in γ2a protein levels.

As BSAP has been shown to negatively regulate the promoter of the B-cell specific J chain gene (Rinkenberger et al. 1996; Wallin et al. 1999), we measured the steady-state transcript levels of J chain in the 9921/BSAP and parental 9921 clones. As shown in

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Figures 6 and 7, any decrease found in individual 9921/BSAP clones (relative to GAPDH mRNA levels) was matched by individual parental 9921 subclones (e.g. compare BSAP clone 4 vs. parental clone x6, Figures 6 and 7). Once again, the variation in expressed level of J chain transcripts was significant: 50% among the 9921/BSAP clones and 34% among the parental clones (Figure 7. Data obtained by ImageQuant analyses of phosphorimaging films from three independent experiments).

As might be predicted from the fact that the variations were seen in both the gene-transfected and the parental clones: we did not observe a consistent correlation between higher levels of BSAP and lower levels of γ2a and J chain. For instance, 9921/BSAP clone 12 produced as much or more BSAP mRNA than clones 4 and 9, yet it produced much higher levels of γ2a and J chain mRNA. Also note in Figure 7 (far right panel) that the 9921/BSAP clones represented by BSAP clone 2 produces a large amount of BSAP relative to the Pre-B cell line 70Z/3, consistent with earlier Western blot and EMSA data (Figures 3 and 4).

Altogether these data suggests that ectopically-expressed BSAP, on its own, is not capable of repressing, in a global fashion, the entire 3′ enhancer region when produced within an established plasmacytoma. It is important to note that had we chosen only one 9921 subclone as our arbitrary reference point, we may have ascribed the variation observed among the BSAP clones to the transfected BSAP gene, rather than to epigenetic phenomena.

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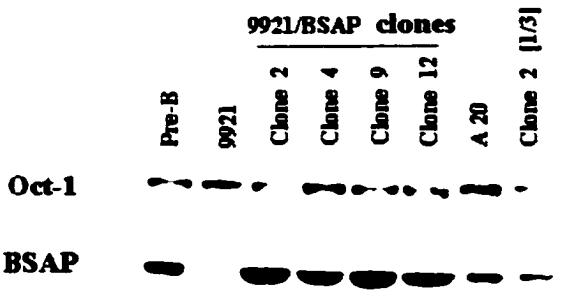
Figure 3 **Four 9921/BSAP clones produce as much BSAP as an endogenously-expressing Pre-B cell**

Western



50 μg nuclear extracts from indicated cell lines were probed with an anti-BSAP antibody to assess expression levels of BSAP. Pre-B cell line (70Z/3) produces BSAP endogenously and serves as a positive control. 9921 is an IgG2a-secreting plasmacytoma that does not produce BSAP. Clones 2,4,9,12 are 9921/BSAP clones generated from the stable transfection of 9921 with a BSAP-expression vector. A20 is a surface Ig+ B cell that produces BSAP endogenously. Oct-1 is shown for loading normalization. Addition of extract from Clone 2 demonstrates the semi-quantitative Nature of this assay.

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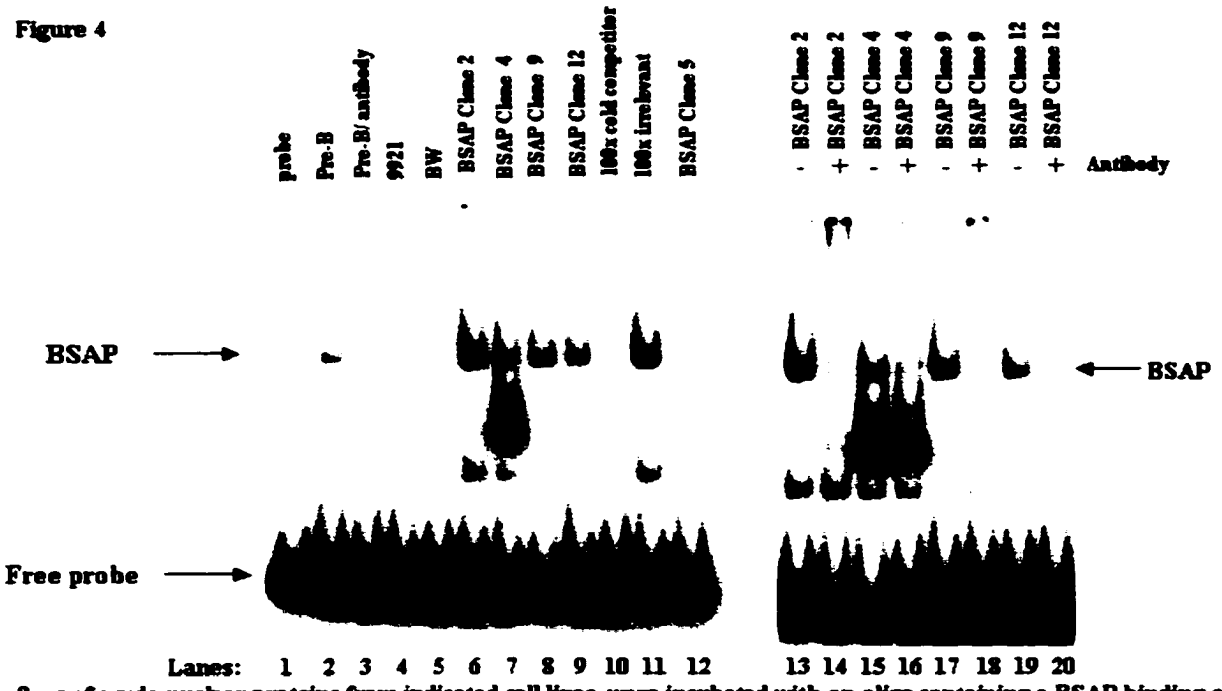
Figure 4



8 μg of crude nuclear proteins from indicated cell lines were incubated with an oligo containing a BSAP-binding site (H2a2.2) from the Sea Urchin histone promoter. The Pre-B cell line (70Z/3) produces BSAP endogenously and serves as a positive control. BW (BW5147) is a T cell lymphoma which does not produce BSAP and thus serves as the negative control. 9921 is an IgG2a-secreting plasmacytoma which also does not produce BSAP. BSAP Clones (2,4,5,9,12) were derived from the stable transfection of a BSAP expression vector into 9921 cells. The irrelevant competitor DNA is a 51 bp fragment of Eμ that contains a binding site for the Oct 1/Oct 2 proteins. In the expt. shown, the competitor DNAs were included in binding assays containing extracts from 9921/BSAP Clone 2.

BSAP binding sites ("a" and "b") in hs1,2 have been tested in cross-competition experiments with the H2a2.2 probe used in this EMSA. Both BSAP binding site in hs1,2 could effectively compete with the H2a2.2 site for binding to BSAP. Reciprocally, H2a2.2 could effectively compete away the binding of BSAP to either of its sites in hs1,2 (Neurath et al. 1994).

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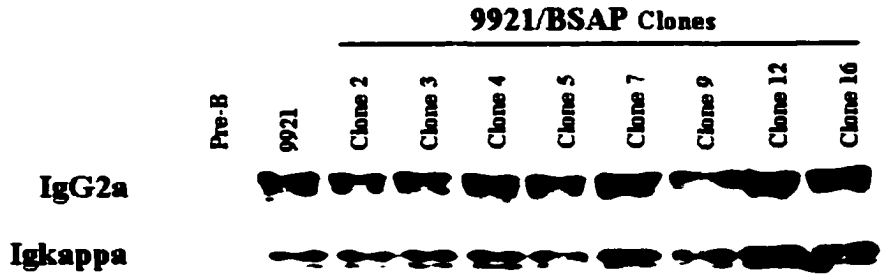
Figure 5 **9921/BSAP Clones do not exhibit a decrease in heavy chain expression as compared to parental line 9921.**

Western



Whole cell lysates (25 μg) from a Pre-B cell line (70Z/3) that produces IgM but no light chain; 9921, an IgG2a-secreting plasmacytoma, and eight 9921/BSAP clones generated from the stable transfection of 9921 with a BSAP expression vector, were probed with an anti-IgG2a antibody to assess steady-state expression levels of the IgG2a heavy chain. Ig kappa (light chain) is used for loading normalization. This is a representative experiment of four experiments performed.

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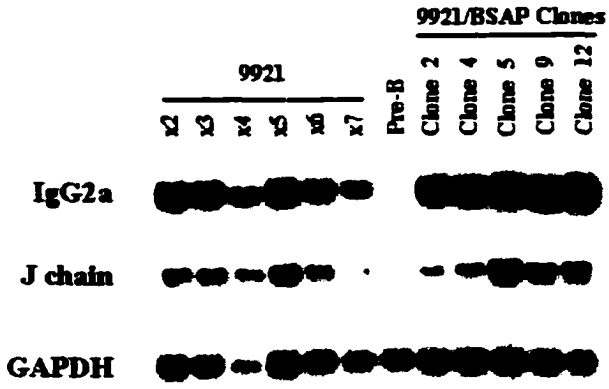
Figure 6
northern

9921/BSAP Clones do not exhibit a decrease in steady-state levels of heavy chain messages. Independent clones produce variable amounts of message.



Total cellular RNA (25 μg) from indicated cell lines were hybridized with probes to assess the steady-state levels of the IgG2a heavy chain and the J chain mRNA. Six independent subclones (labeled x2-x7) of 9921, an IgG2a-secreting plasmacytoma that does not produce BSAP were compared to five 9921/BSAP clones that were generated from the stable transfection of a BSAP-expression vector in 9921 cells. Pre-B cell line (70Z/3) does not produce either the γ2a heavy chain or the J chain and is used as a negative control.GAPDH was used for loading normalization. Experiment was performed in triplicate

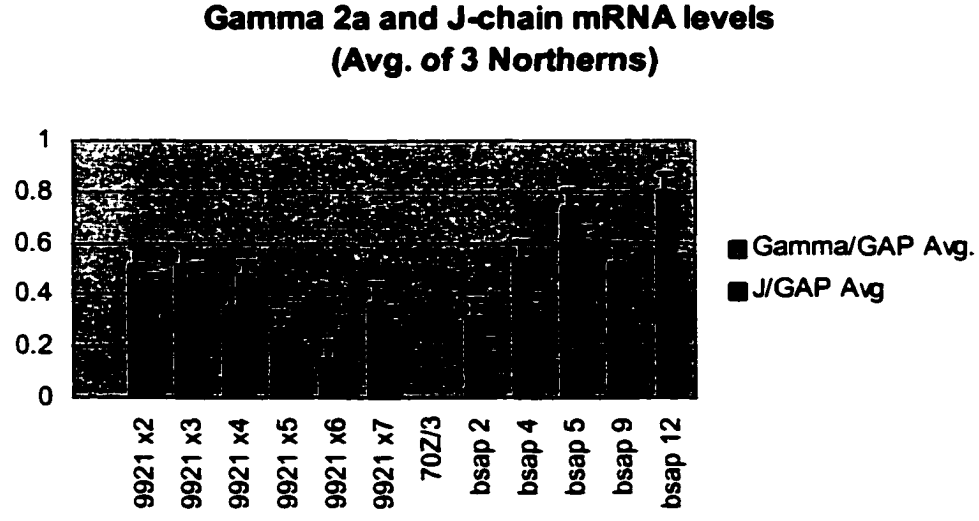


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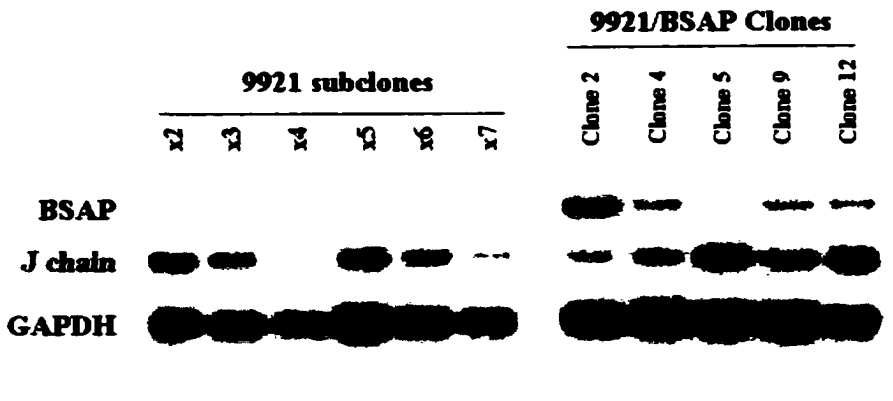
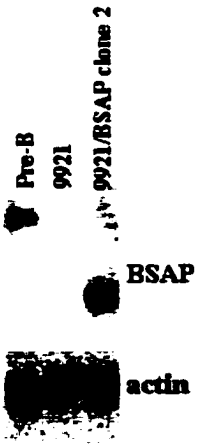
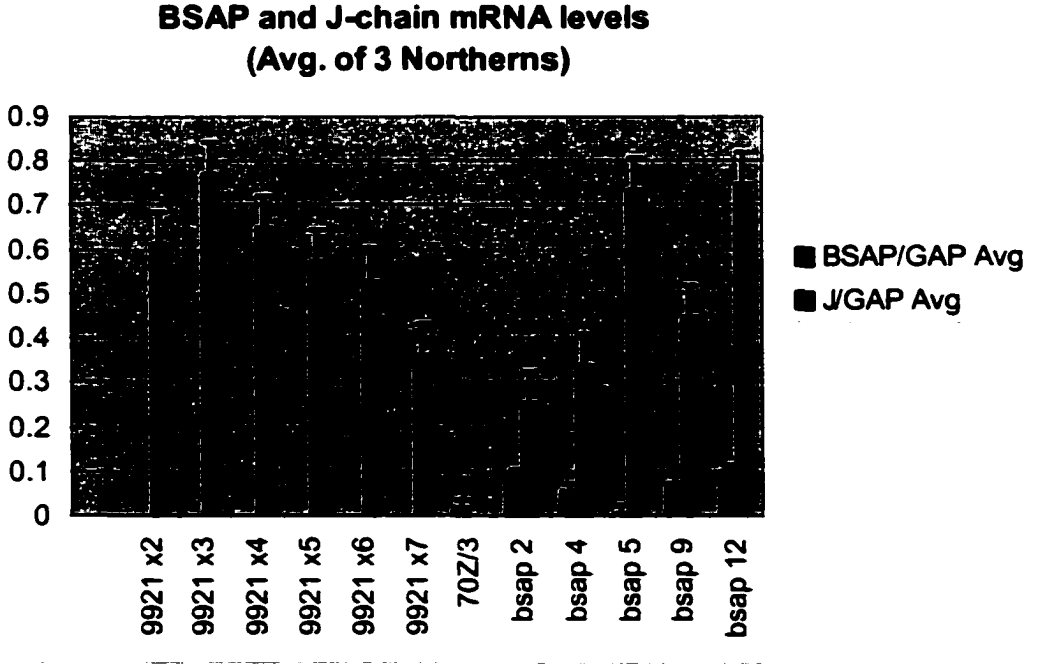
Figure 7 ***There is no correlation between levels of BSAP message and steady state levels of J chain message***

Northern





Total cellular RNA (25 μg) from indicated cell lines were hybridized with probes to assess the steady-state mRNA levels of BSAP and J chain. Six independent subclones (labeled x2-x7) of 9921, an IgG2a-secreting plasmacytoma that does not produce BSAP were compared to five 9921/BSAP clones that were generated from the stable transfection of a BSAP-expression vector in 9921 cells. In a separate experiment, mRNA from the Pre-B cell line 70Z/3 was compared to that from 9921 and the 9921/BSAP clone 2 (far right panel). Pre-B cell line (70Z/3) produces BSAP from the endogenous locus (hence the different size mRNA) and is used as a positive control. GAPDH (left and center panels) and actin (far right panel) were used for loading normalization.
Each experiment was done in triplicate



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BSAP DISCUSSION

hs1,2 has enhancing activity

Hs1,2 is one of the four enhancers located 3′ of the α constant gene region in the heavy chain locus. These enhancers become quite active as a synergistic unit at the surface Ig+ stage and remain active throughout the plasma cell stage, the stage at which transcription of the heavy chain is greatest (Ong et al. 1998). In addition to their activity as a unit, two of the four 3′ enhancers, hs1,2, and hs4, are capable, individually, of augmenting the expression of a linked reporter gene in transient transfection assays in plasmacytomas (Ong et al. 1998)

BSAP represses hs1,2 when hs1,2 is removed from its chromosomal context

BSAP is a transcription factor that is expressed at all stages of B cell development with the exception of the plasma cell. Once hs1,2 was found to contain two BSAP binding sites (Neurath et al. 1994), it was thought that BSAP might contribute to the regulation of this enhancer. It was relatively easy to test this hypothesis as the plasma cell provided an environment in which the hs1,2 is active and BSAP is absent. Cotransfection of BSAP and the hs1,2-linked reporter gene resulted in repression of the transcriptional enhancement mediated by hs1,2 (Neurath et al. 1994; Singh and Birshtein 1996). Thus, BSAP was labeled as a negative regulator of hs1,2.

The stable expression of BSAP in plasmacytoma line MPC11 resulted in a decrease in heavy chain γ2b expression, suggesting that BSAP's regulation of hs1,2 translated into a repression of the entire 3′ enhancer region (Wallin et al. 1998).

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Stable expression of BSAP in plasmacytoma 9921 does not result in a decrease in heavy chain expression

The plasmacytoma of this study, 9921, produces copious amounts of its endogenous heavy chain despite the loss of the intronic enhancer Eμ (Eckhardt and Birshtein 1985) which has been found to be quite active at this stage (Ong et al. 1998). Thus, transcription of the heavy chain is entirely dependent on other control elements, presumably the four 3′ enhancers. This may not be the case for other plasmacytomas in which Eμ is still within the heavy chain locus, including MPC11, the line chosen for the first BSAP transfection studies. Therefore, 9921 provided a less complicated environment in which to study the effects of the stable, ectopic expression of BSAP, since negative regulation of hs1,2, or of the entire 3′ region, could not be negated or camouflaged, in this context, by the compensatory enhancing activity of Eμ. We found, however, that the stable expression of physiologically relevant levels of BSAP in 9921 did not result in a decrease in heavy chain gene expression. We concluded this by comparing the levels of expression of the heavy chain in our five BSAP-expressing clones to the levels of expression in six independent parental subclones of 9921. Our BSAP-expressing clones produced variable amounts of heavy chain, with some clones producing more heavy chain than the 9921 parental subclones and other BSAP-expressing clones producing less than the parental 9921 cells. There was an overall variation in steady-state levels of heavy chain transcript among the BSAP-expressing clones of 37% while the variation observed among the 9921 parental subclones was 43%. We also did not observe a decrease in expression of the J chain, a plasma cell-specific gene previously shown by transient transfection (Wallin et al. 1999) and stable

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transfection (Rinkenberger et al. 1996; Wallin et al. 1998) to be negatively regulated by BSAP. The transient transfection data labeled BSAP as a negative regulator of J chain gene transcription by its ability to repress a J chain promoter motif linked to a CAT reporter gene (Wallin et al. 1999). This study also uses EMSA to assert that BSAP's binding to its motif, blocks the binding of two activating transcription factors (USF and B-MEF-2) to their respective motifs within the J chain promoter. Although USF and MEF-2 are present throughout all stages of B cell development, Wallin *et al.* conclude that these factors bind their respective promoter motifs (thus activating transcription) only when BSAP levels decrease at the plasma cell stage. However, the recent finding that J chain gene expression was not induced upon deletion of BSAP in a mature B cell (Horcher 2001), despite the fact that USF and MEF-2 are present, implies that BSAP's repression of the J chain gene is more complex than Wallin *et al.* suggest, and that protein binding motifs, when assayed in isolation, may not accurately mirror behavior at the endogenous locus.

The stable BSAP transfection studies contradict our findings in that they show decreases in J chain gene expression (Rinkenberger et al. 1996; Wallin et al. 1998). Wallin *et al.* (1998) (the same group published both stable studies in addition to the transient study discussed above) measured heavy chain gene expression (in addition to J chain gene expression) and observed a decrease in steady-state heavy chain transcription levels, also in contrast to our study. However, both of these studies lacked important controls. The determination that BSAP mediated repression of both the heavy chain gene and the J chain gene was based on analysis of only two MPC11-BSAP clones (Wallin et al. 1998). In addition, these two BSAP–expressing clones (and the clones generated in

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the Rinkenberger *et al.* study) were compared to a single parental clone. As our present study has shown, established cell lines can change over time in culture, with the result that gene expression levels can vary considerably. Thus, the "decreases" observed in the two MPC11- BSAP transformants may simply reflect natural variations in gene expression levels among clones of the same established cell line. Lastly, the levels of transfected BSAP in the BSAP-expressing clones were never compared to a cell line that produces BSAP endogenously. Thus, these studies may not reflect the behavior of BSAP at physiologically relevant levels.

Since 1998, additional work has been done that is more in accord with our present results (see below). However, there are several experiments that we could conduct, including a few that were tried unsuccessfully, that would allow us to definitively determine why we did not observe a phenotype when we stably transfected BSAP into our plasmacytoma. Were we unable to stifle the enhancing activity of hs1,2? Alternatively, did we stifle activity of hs1,2 but the loss of hs1,2's activity did not result in global repression of the entire 3' enhancer region?

I will propose future experiments to distinguish between these possibilities as I discuss our results further, as well as recent findings in the field.

Is our transfected BSAP able to repress hs1,2 in transient transfection?

Stable transfection of BSAP into plasmacytoma line MPC11 lead to decreases in expression of both the heavy chain and the J chain genes (Wallin et al. 1998). In the present study, stable transfection of BSAP into plasmacytoma 9921 did not result in decreased expression of either of these genes. As discussed above, it is possible that the

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difference is due to a difference both in quantitation and in the nature of the comparisons made between BSAP-expressing clones and parental clones. Alternatively, the lack of a decrease in gene expression in our studies might have been the result of an inactive BSAP protein in our cell lines. As shown in Figure 4, the BSAP produced in these lines could bind DNA appropriately (and specifically). But might this BSAP be unable to repress hs1,2 either because of an undetected mutation or because 9921 cells lacked a collaborating protein required for BSAP's repressing activity?

Unfortunately, I was unable to successfully perform transient transfections that might have confirmed that ectopically expressed BSAP in the 9921/BSAP clones could abrogate hs1,2 activity. The plan was to transfect an hs1,2-linked reporter gene into our 9921 plasmacytoma, and compare its activity to a control, enhancerless reporter gene. We should have detected an activity for the hs1,2-linked gene that significantly exceeded that of the enhancerless gene (as an hs1,2-linked reporter gene was shown to have activity in plasmacytoma lines S194 and P3X63Ag8) (Ong et al. 1998). In our next set of transient transfections, we would transfect the hs1,2-linked reporter gene into our 9921/BSAP transformants and again compare its activity to that of an enhancerless reporter gene. If we did not observe repression of the hs1,2-linked reporter gene, we could conclude that BSAP in these transformants lacks repressive activity. This, by itself, would explain our seeing no repression of the endogenous IgH gene in the 9921/BSAP transformants.

If, instead, we detected repression of the hs1,2-linked reporter (expression equivalent to the enhancerless control reporter gene), the activity of BSAP in these cells would be confirmed, and we could tentatively conclude that loss of hs1,2 activity is not sufficient

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to ablate the enhancer activity of the remaining 3′ IgH enhancers. Rather, hs1,2 may contribute to but not be critical for IgH gene expression. To test the latter notion further, we could transfect a reporter gene linked to the four 3′ enhancers (hs1-4) into one batch of 9921 cells and compare expression levels to those of an enhancerless construct transfected into another batch of 9921 cells. Once the level of enhancement over enhancerless construct was established for this hs1-4-linked reporter gene, we could transfect each of these constructs into our 9921/BSAP clones. If the expression level of the reporter gene linked to all four 3′ IgH enhancers was not diminished in the presence of the ectopic BSAP (whereas it was when the reporter gene carried only hs1,2), we could conclude that BSAP is incapable of repressing the 3′ enhancer region as a unit. This would provide an alternate explanation for our not seeing repression of the endogenous IgH gene in the 9921/BSAP clones, since, in this context, hs1,2 is naturally embedded within a chromosome carrying all of the other 3′ enhancers as well.

Finally, it is possible that we would have seen repression both of hs1,2 alone and of the full complement of 3′ IgH enhancers (hs1-4) in transient transfections into the 9921/BSAP clones. If this had been the experimental result, we could conclude that BSAP negatively regulates hs1,2 and that this downregulation can repress the four 3′ enhancers when they are linked in a mini-locus in transient transfection. We may have observed global 3′ repression (repression of the 3′ enhancers as a unit) in the transient transfections while not observing the same in our stable transfections, as this mini-locus is unlike the endogenous locus in two important aspects: the spacing between the individual enhancers is not maintained in the mini-locus nor is the spacing preserved between the promoter and four enhancers as a unit.

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While I began to conduct this set of experiments, I was unable to reliably transfect either the 9921cell line or the 9921/BSAP clones and measure reporter gene activity in transient assays. Therefore, I begun transient transfections of the hs1,2-linked reporter gene into plasmacytoma line S194 (as a previous graduate student in the laboratory, Jane Ong, showed hs1,2-reporter gene activity in this line). I also transiently transfected the four IgH 3′ enhancer-linked construct into S194 cells. While I observed significant enhancement of the four IgH 3′ enhancer linked- reporter gene (over enhancerless control construct) in the S194 cells, I was unable to reliably observe the 1.5-fold enhancement (over enhancerless construct) of the hs1,2-linked reporter gene previously reported by J. Ong. Therefore, I was unable to progress to the next set of transients in which I would cotransfect the BSAP expression plasmid (used in the present study) along with each of the enhancer-linked reporter plasmids.

Does ectopic expression of our BSAP in plasmacytoma line MPC11 result in downregulation of the heavy chain?

As only two BSAP-expressing MPC11 clones were generated (and important controls were lacking, as discussed above) in the Wallin *et al.* study (Wallin et al. 1998), it would be important to ascertain whether we would observe repression of heavy chain expression in our own set of stably-transfected MPC11/BSAP clones. Again, these experiments were begun but not completed. Once we had generated these MPC11/BSAP clones, we would have compared their heavy chain (and J chain) expression with that of six independent MPC11 parental clones (as was done for our 9921/BSAP study). If we observed statistically significant downregulation of the heavy chain in our MPC11/BSAP clones,

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we could conclude that the environment of MPC11 differs from 9921 enough to facilitate BSAP's repression of heavy chain expression in one plasmacytoma while not in the other. As it is known that established cell lines can differ markedly from one another even though they are of the same developmental stage, we would stably transfect BSAP into additional established plasmacytoma lines in an effort to clarify BSAP's behavior at this developmental stage. Each BSAP-transformed line displaying demonstrable reduction in heavy chain expression would be analyzed further, see below.

Does BSAP bind its sites in hs1,2 *in vivo* (in 9921 cells)?

A factor expressed at all stages in B cell development was found to have a binding site within hs1,2 at a distance of approximately 50bp downstream of BSAP binding site "a". However, this factor, NF-αP, was able to bind this site and enhance transcription only in the absence of BSAP, that is, at the plasma cell stage. This factor has recently been identified as the ets protein PU.1 (Linderson 2001). PU.1 is known to associate with NF-κB/Rel family members and activate transcription (Michaelson et al. 1996; Linderson 2001). As PU.1 would be present in 9921 (at its physiologic levels), our ectopic introduction of BSAP might result in competition for respective binding sites in hs1,2. To test whether our transfected BSAP was able to bind hs1,2 in vivo, (when PU.1 may have the competitive advantage) we could use DNA footprinting assays and Chromatin Immunoprecipitation (ChIP). Our 9921/BSAP clones should exhibit the same footprint over the hs1,2 binding sites as a pre-B or mature B cell which expresses BSAP endogenously. This pattern should differ from that observed in wildtype 9921 cells that do not express BSAP. A ChIP assay carried out in 9921/BSAP clones and 9921 wildtype

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cells would add information, as it would permit the definitive identification of BSAP as the protein bound to hs1,2 . In ChIP, the fixing of live cells simultaneously crosslinks proteins bound to DNA. DNA is then fragmented and the DNA/protein complex immunoprecipitated with an antibody to the protein of interest. Reversal of crosslinking followed by PCR of captured DNA would positively identify hs1,2. The finding that BSAP binds hs1,2 *in vivo*, however, would not preclude the possibility that PU.1 is interfering with the activities of BSAP. Recent findings demonstrating interactions between PU.1 and BSAP that result in either the repression of PU.1 by BSAP or the repression of BSAP by PU.1, depending on the context, (Maitra 2000) suggest that a more complex mode of action exists between these two transcription factors than simple steric hindrance. If we found that BSAP did not bind hs1,2 *in vivo* (and the pattern appeared as it did in wildtype 9921 cells), this would suggest, however, that steric hindrance is involved.

Once we found that BSAP was capable of binding hs1,2 *in vivo*, we could search for putative BSAP co-factors.

BSAP binds to two sites within hs1,2, designated "a" and "b". BSAP's repression of hs1,2 in transient transfection is dependent upon the integrity of both of its binding sites, as mutation of either of these sites greatly diminishes BSAP's ability to repress hs1,2 in surface Ig+ cells(Singh and Birshtein 1993). However, transient transfections showed that BSAP's repression of hs1,2-linked reporter genes was dependent on other protein binding sites as well. An octamer binding site located 5′ of the BSAP binding site "a" and a G-rich motif located 3′ of BSAP binding site "b" were found to be necessary for

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repression of hs1,2 in transient transfections in surface Ig+ cells (Singh and Birshtein 1996), as mutation of either one of these sites abolished repression. This suggests that BSAP works in concert with other transcription factors in its regulation of hs1,2. Though the Oct proteins that bind the octamer site are expressed in plasma cells, the protein or proteins that bind the G-rich motif, and their expression patterns, are as yet, unknown.

It is possible that we did not observe repression in our 9921/BSAP transformants because 9921 does not contain the full complement of BSAP's co-factors . If stable introduction of BSAP into plasmacytoma MPC11 resulted in repression of the endogenous heavy chain in our MPC11/ transformants (or other established plasmacytoma line transformants), we could employ ChIP assays in an attempt to reveal the cofactors that facilitate BSAP's repressive activity at hs1,2 (Wells and Farnham 2002). Westerns of proteins immunoprecipitated from the MPC11/BSAP clones and the 9921/BSAP clones would expose any differences in proteins bound to hs1,2. If differences were detectable, mass spectrometry could be employed to reveal the identity of the cooperating factors.

We could also search for BSAP cofactors in surface Ig+ cell lines as BSAP might repress hs1,2 and the 3′ enhancer region in surface Ig+ cells (though not in Ig-secreting plasma cells) due to the fact that surface Ig+ cell lines contain repressive co-factors while plasma cell, do not.

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BSAP exerts transcriptional repression through interaction with a corepressor, Grg4.

Though our studies have focused on BSAP's role as a negative regulator, BSAP also positively regulates the promoters of several target genes such as B cell surface proteins CD19 and Ig-α (mb-1), and transcription factors N-*myc* and LEF (Kozmik 1993; Nutt et al. 1998). Transient transfection data in plasmacytoma line SP2/0 showed that transfected BSAP was able to activate a CD19- promoter linked luciferase reporter gene (Eberhard 2000). However, a member of the Groucho family of corepressors known as Grg4 was shown to bind BSAP, and by that binding, repress BSAP's transactivation of the reporter gene (Eberhard 2000).

Although Grg4 is expressed at all stages of B cell development, and as such, should be present in plasmacytoma SP2/0, the cotransfection of increasing concentrations of Grg4 resulted in proportional decreases in BSAP's activation of the reporter gene (Eberhard 2000). The fact that cotransfection of BSAP along with the CD19-promoter-linked reporter gene resulted in activation of the reporter gene demonstrates that the plasmacytoma's endogenous Grg4 did not repress BSAP's transactivation function. It is possible that the endogenous levels of Grg4 in plasmacytoma SP2/0 are too low to work with transfected BSAP, or alternatively, that Grg4 is found in combination with other proteins when BSAP is no longer expressed. The plasmacytoma of this study, 9921, may also lack sufficient quantities of free Grg4 needed to work with BSAP in regulating hs1.2 *in vivo*.

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Would stable co-transfection of BSAP and Grg4 into plasmacytoma 9921 result in a decrease in heavy chain expression?

Perhaps our transfected BSAP was unable to effect repression of the heavy chain in 9921 cells due to an insufficient quantity of its cofactor Grg4. A western blot of 9921/BSAP extracts would be performed to reveal the presence of Grg4. However, the finding that Grg4 is expressed in 9921/BSAP cells would not tell us whether it was there in quantities sufficient to efficiently interact with our transfected BSAP, as repression of BSAP's transactivation function in Grg4-expressing plasmacytoma SP2/0 was not observed until exogenous Grg4 was cotransfected with BSAP (Eberhard 2000). Thus, we would proceed with stable transfection of both BSAP and Grg4 into 9921 cells to assess whether these two proteins are together sufficient to downregulate hs1,2's activity in its endogenous context of the four 3′ enhancers. If a decrease in heavy chain expression were manifest, we could conclude that BSAP, in combination with Grg4, is sufficient to regulate hs1,2, and that hs1,2 loss of function results in a loss of total enhancing activity of the 3′ region. If Grg4 were present in 9921/BSAP clones prior to Grg4 transfection, we could deduce that it was not present in quantities sufficient to cooperate with BSAP. If 9921/BSAP clones did not contain Grg4 prior to its transfection, we would have uncovered the reason why transfection of BSAP solely did not result in repression of the heavy chain.

If a decrease in heavy chain expression were not obvious in our 9921/BSAP/Grg4 clones, it might be due to the requirement of yet another cooperating factor. Grg4 has been found to lack a recognizable DNA-binding motif, so it is assumed that its recruitment to specific control regions is through its interaction with other proteins

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(Eberhard 2000). Investigators have hypothesized that Grg4 is stably recruited by BSAP to a specific site only in combination with another Grg4-binding protein (Eberhard 2000). To further pursue the notion that Grg4 is involved in BSAP's negative regulation of hs1,2, we might need to search for additional Grg4 binding factors.

Have we muted hs1,2's activity in the 9921/BSAP clones, but loss of hs1,2's enhancing power does not translate into global repression of the entire 3' enhancer region?

Thus far, we have explored the idea that BSAP is not downregulating the enhancing activity of hs1,2. However, as noted earlier, we must also consider the possibility that BSAP is suppressing the activity of hs1,2 but that the loss of hs1,2's enhancing power does not result in global repression of the entire 3′ enhancer region. If we do not have global repression of this control region, we should not expect to observe a decrease in heavy chain gene expression. As discussed below, work form our laboratory and others is consistent with this later possibility.

BSAP positively regulates the 3' enhancer, hs4

As measured in transient transfection assays, the four 3′ IgH enhancers display the greatest functional synergy in their activation at the surface Ig+ stage (Ong et al. 1998), a stage in which BSAP is endogenously expressed. That the 3′ enhancers as a whole are quite active at a time in which one of their repressors is in abundant supply is not altogether surprising when one views the diverse activities of BSAP. In addition to

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negatively regulating hs1,2, BSAP has also been shown to positively regulate the most 3′ of the four 3' enhancers, hs4, (Michaelson et al. 1996).

BSAP binding sites are different in sequence

BSAP binding sites have been shown to be quite degenerative, though they can be categorized into three general groups (Singh and Birshtein 1993). These disparate sites display a wide range of affinities for binding to BSAP (Czerny 1993). The Ig heavy chain locus alone contains many regions that have BSAP binding sites with various affinities. The sites located in the Sγ2a and Sε regions are of the highest affinity, those in hs1,2 are of the lowest affinity, while hs4 contains a site of intermediate affinity as well as several other sites of lower affinity. (Liao et al. 1992; Czerny 1993; Michaelson et al. 1996; Qiu and Stavnezer 1998). Therefore, hs4, an enhancer known to be positively regulated by BSAP, out-ranks hs1,2 both in the number of BSAP binding sites it contains and in the relative affinity of one of its sites.

Does BSAP in the 9921/BSAP clones prefer to bind to hs4 rather than to hs1,2?

To determine if the BSAP in the 9921/BSAP transformants displays a preference for a site in hs4 over either of its sites in hs1,2, we could use EMSA competition studies. The EMSA studies we have presented (Figure 4) used a BSAP binding site located in a Sea Urchin histone promoter (H2a2.2), as this is a site with particularly high affinity for BSAP. A comparison of the footprinting–protected region of the BSAP binding site in H2a2.2 shows an eight of eight base identity with additional flanking region sequence homology to the BSAP binding site "b" in hs1,2 (Singh and Birshtein 1993). (For

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sequence and BSAP contact points within the disparate oligos, see Materials and Methods). Further, cross-competition experiments using the BSAP binding sites in H2a2.2 and in hs1,2 have shown that each site effectively competes with the other for binding to BSAP (Neurath et al. 1994). We will use the site in hs4 as well as each of the two sites in hs1,2 as cold competitors in the binding reactions. By titrating the amount of molar excess used as the cold competitor, we can quantify the ability of each site to attract BSAP. In addition, we can use each site as the labeled probe and determine whether we can directly detect a quantitative difference in BSAP's ability to bind its respective sites. If we find that our transfected BSAP more readily binds hs4 than hs1,2, we can propose that hs4 would better compete for the quantities of ectopic BSAP. This finding would suggest that we are not supressing the activity of hs1,2 in our 9921/BSAP clones because BSAP is not binding hs1,2 in vivo. If BSAP binds each site with equal affinity, however, we can speculate that the lack of an observable decrease in heavy chain expression in our transformants is based on the fact that hs4 is a stronger enhancer than hs1,2 (Ong et al. 1998). Thus, BSAP's activation of hs4 would eclipse BSAP's muting of hs1,2, and the 3′ region, as a whole, would remain active.

BSAP mutant that binds hs1,2 but not hs4

As the BSAP binding sites in hs1,2 are of a different sequence from the BSAP binding site in hs4, it may be possible to generate a BSAP mutant that is capable of binding to hs1,2 but not to hs4. Stable transfection of this mutant would allow us to assess the consequence of BSAP binding to hs1,2 only . If we then observed a decrease in heavy chain expression, (global 3' repression), we could postulate that the muting of hs1,2's

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activity in our original 9921/BSAP clones was outmatched by BSAP's activation at hs4. If we did not observe a decrease in heavy chain expression, we would consider two possibilities: 1) BSAP is not suppressing hs1,2's activity (perhaps because the plasmacytoma lacks critical cooperating factors, see earlier discussion), or, 2) BSAP's muting of hs1,2 does not lead to global repression of the 3' region.

Hs1,2 is dispensable for high-level transcription of the heavy chain gene

Lieberson et al. from this laboratory successfully replaced hs1,2 *in situ* with a neomycin-resistance (*neo*^{r}) gene in the Eμ-lacking plasmacytoma of this study, and found that transcription of the endogenous heavy chain ceased (Lieberson et al. 1995). Hs1,2 was, therefore, believed to play a pivotal role in transcription of the heavy chain in a plasma cell that lacked Eμ. Evidence began to mount, however, that the replacement of hs1,2 with a gene driven by a strong promoter might have resulted in the subversion of this enhancer region's long-range interactions with its own promoter (Fiering et al. 1995; Hug 1996). If this were indeed the case, then replacement of hs1,2 with a *neo*^{r} cassette did not constitute a "clean" deletion of hs1,2 but rather a "technical" knock-out of the entire 3' enhancer region (discussed in Lieberson *et al.*, 1995) Experiments in mice using the Cre/loxP system confirmed the "technical knock-out" theory. The bacterial recombinase Cre efficiently loops out DNA located between two of its 32 bp "loxP" recognition sites, leaving just one loxP site at the place of recombination. Hence, this system allows drug resistance genes to be used in the initial selection of an insertion event and then deleted to prevent their further interference in the workings of the locus under examination. While a homozygous replacement of hs1,2 with a *pgk*-promoter

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driven *neo*^{r} gene flanked by loxP sites resulted in severe defects in Class Switch Recombination (CSR), clean deletion of the *neo*^{r} cassette (by Cre-mediated loxP site recombination) returned the CSR phenotype to normal (Manis et al. 1998). Therefore, the remaining 3′ enhancers were able to compensate for the loss of hs1,2, implying that there is some functional redundancy among these enhancers. Recent results from this laboratory confirm this redundancy. When an IgH transgene under the control of the four 3′ enhancers was stably transfected into the 9921 cell line, clean deletion of both hs1,2 and hs3a did not result in reduced transcription of the linked reporter gene(Shi 2001). However, clean deletion of the other two 3′ enhancers, hs3b and hs4, led to a dramatic decrease in (or loss of) IgH reporter gene expression (Shi 2001). This suggests that the contribution of hs1,2 towards enhancement at the plasma cell stage is not necessary for high-level transcription of the heavy chain locus. In that vein, if BSAP were able to negate the activity of hs1,2, the above data would suggest that the activity of the remainder of the 3' locus would be enough to compensate for whatever transcriptional enhancement was lost at hs1,2. In other words, BSAP's ectopic expression in a Ig-secreting line does not translate into a global repression of the entire 3′ region.

As the transition between the surface Ig+ B cells and Ig-secreting plasma cells results in a large increase in the expression of the Ig heavy chain gene, the factors governing regulation of transcription of this gene have been the subject of considerable interest. A myriad of transcription factors involved in the positive regulation of heavy chain gene transcription have been identified. However, factors involved in the suppression of heavy chain gene transcription have remained elusive. BSAP's ability to negatively regulate hs1,2 in transient transfection resulted in BSAP's label as the Ig heavy chain gene's

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premier negative regulating factor, though research to date, has not proven this notion. Therefore, a definitive determination of BSAP's ability to function as a negative regulator of the Ig 3′ enhancer region would be of some importance.

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BAC INTRODUCTION

Much attention has been devoted to the study of the Ig heavy chain's four 3' enhancers although several aspects of this enhancer region have complicated this examination. First, the four 3' enhancers span ~ 30 kb of DNA. Consequently, the 3' region could not be excised out of the genome en bloc and cloned in a conventional vector as these vectors cannot stably maintain large fragments of DNA. Second, the interaction of these enhancers with their Ig promoter takes place over a distance that can be as long as 200 kb in length (depending on whether class switching had deleted intervening DNA). Thus, the faithful duplication of the endogenous spacing between the promoter and its enhancers is also unachievable in a conventional cloning vector. Thirdly, the second 3' enhancer downstream of the α constant gene, hs1,2, is flanked by an inverted repeat (Chauveau and Cogne 1996). In addition, hs1,2 is situated equidistantly between two of its cooperating 3' enhancers, hs3a, and hs3b, which share 97% identity in sequence but are found in an inverted position relative to one another (Chauveau and Cogne 1996). Thus, hs1,2 is positioned at the center of a large palindromic region. This structure has made homologous recombination within this enhancer region, an infrequent event.

However, Bacterial Artificial Chromosome (BAC) technology allowed us to overcome the limitations of the cloning vectors and the difficulties of the region's architecture. The backbone of a Bacterial Artificial Chromosome is a ~ 7 kb vector that can stably hold several hundred kilobases of exogenous DNA (Shizuya 1992; Yang et al. 1997). The BAC is propagated in *E coli* as a supercoiled plasmid at a copy-number of one per cell (Shizuya 1992). As the BACs are propagated in recombination-deficient *E. coli*, they are not prone to chimerism, though modification of the BAC can be easily achieved by the

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addition of the RecA recombinase gene (Yang et al. 1997). Thus, a BAC could accommodate the entire heavy chain locus in its natural orientation. Further, modification of the 3' enhancer region would take place while the DNA was in *E. coli*, thereby dramatically increasing the speed at which recombinants could be identified and selected. Once the BAC's Ig locus contained the desired alteration, it would then be introduced into the mammalian cell where it would integrate into the genome at random.

A BAC containing the Ig locus would enable us to investigate several aspects of 3' enhancer function. Of primary interest would be the determination of whether the 3' enhancer region, in its native orientation and spacing, behaves as an LCR: directing tissue-specific, copy number-dependent, and integration site-independent expression of its linked gene (Grosveld et al. 1987). Earlier studies indicated that the 3' enhancers were functioning as an LCR (Madisen and Groudine 1994), whereas more recent data suggests otherwise (Chauveau et al. 1999; Shi 2001). However, in each of the previous studies, a mini-locus was constructed by excising the enhancers from their chromosomal location and placing them directly next to one another and the promoter they would regulate. Thus, the spacing of these mini-loci was not representative of the endogenous locus while our BAC's Ig locus would contain the physiologically relevant spacing. If our 3' enhancers are capable of insulating their linked gene from the disparate chromatin environments encountered at each site of integration, we should observe equivalent expression at each of these sites.

In order for our 3′ enhancers to be maximally active, and thus capable of directing high-level expression of their linked gene, we had to select a host cell of the appropriate

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developmental stage. We knew that BAC-propagated DNA could function normally as recent experiments involving the β-globin gene cluster showed that the BAC-derived locus displayed proper developmental stage-specific expression (Huang 2000). Therefore, we chose to transform our BAC-derived Ig locus into the Eμ-deficient plasmacytoma, 9921, as the four 3′ enhancers are quite active at this stage (Ong et al. 1998) and recent experiments proved that this cell could support production of two distinct heavy chains (Shi 2001).

The BAC we obtained did not include any of the Ig locus 5′ of the ε gene though it terminated 45 kb downstream of the most distal 3′ enhancer, hs4. (See Figure 8). This meant that the 3′ enhancer region was intact. However, as our BAC did not include an Ig promoter, variable region, or intronic enhancer (Eμ), our first modification of the BAC was to insert an Ig promoter and rearranged variable region upstream of the α gene (Figure 8). We did not insert an intronic enhancer (Eμ) into our BAC construct as this enhancer is active at the plasma cell stage (Ong et al. 1998) and could thus contribute supplemental enhancing activity that would complicate our study. In addition, a direct comparison of the expression levels of the two heavy chains produced by the BAC-transformed 9921 clone (endogenous γ2a chain vs. BAC-derived α chain) could be assessed, as neither Ig-locus contained the intronic enhancer, Eμ. Comparing the expression levels would allow us to determine whether our relevantly spaced 3′ enhancers could direct expression levels of their linked gene equivalent to those produced endogenously by a plasmacytoma.

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However, as different heavy chain isotypes may be produced in dissimilar amounts, this study also includes an expression level comparison between our 9921/BAC clones and an Igα-producing plasmacytoma (J558).

Finally, the determination of the basal level of expression of our BAC's α chain lays the foundation for future experiments. Though the contribution of pairs of the 3′ enhancers (hs3a + hs1.2 and hs3b+ hs4) has been determined in transgenic mice (Manis et al. 1998), and in a stably-transfected plasmacytoma (Shi 2001), our BAC could be used to establish the contribution a single enhancer has towards overall transcriptional augmentation. An additional round of modification of our BAC in which loxP sites are placed around one of the four 3′ enhancers would allow us to selectively delete this enhancer and examine the effects on transcription of the heavy chain.

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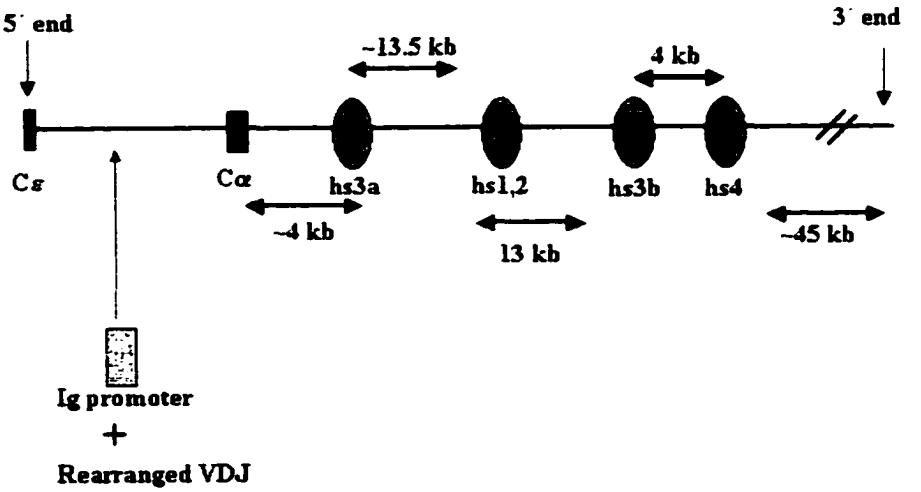
Figure 8 **Portion of heavy chain locus included in BAC (94 kb)**



Schematic map of the 94 kb portion of the heavy chain locus included in the BAC. The Ig promoter and rearranged VDJ were knocked-in upstream of Cα before the BAC was transfected into 9921 cells.

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BAC RESULTS

The four Ig heavy chain 3′ enhancers have been tested for their ability to act as a Locus Control Region (LCR) (Madisen and Groudine 1994; Shi 2001). Recent studies in this laboratory have shown that these IgH enhancers behave in an LCR-like fashion: though expression of the linked genes was found to be position-independent there was not a strict correlation between gene copy number and level of expression (Shi 2001). Each construct included the full complement of 3′ enhancers. However the enhancers were not positioned as they are in the endogenous locus; namely, the spacing that exists between each of the four enhancers was not conserved in these mini-loci, as the backbone of the vector could not support such a large piece of DNA. Further, the gene regulated by these enhancers was not located at a distance nearly as vast as that found endogenously. These mini-loci were constructed as such due to the limitations of the conventional cloning vectors in wide usage.

To test whether a gene driven by these enhancers (with their natural spacing) could exhibit expression that was insertion site-independent and copy-number dependent, we decided to employ the Bacterial Artificial Chromosome (BAC) technology as a BAC can hold pieces of DNA as large as 300 kb. The BAC we acquired, designated141e18, contained ~ 94 kb of the Ig heavy chain locus. The 5′ end of the BAC interrupted the ε constant region while the 3′ end extended 45 kb downstream of hs4- the most 3′ of the four 3′ enhancers (see Figure 8). As our BAC did not include either an IgH promoter or variable region, our first task was to knock-in a pre-assembled variable region with its concomitant promoter. All of the following experiments were undertaken as collaboration between myself and another graduate student in the laboratory, Buyi Zhang.

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A variable region with specificity for NP [(4-hydroxyl-3-nitrophenyl)acetyl] (Sonoda et al. 1997)was inserted upstream of the α constant region gene by homologous recombination. The BAC would direct transcription of a α heavy chain with a variable region specific for NP.

Our BAC was co-transfected along with a neomycin resistance gene into our γ2a-producing plasmacytoma line 9921 and approximately 40 G418-resistant clones were chosen for analysis.

Southern blots were performed to determine whether our G418-resistant clones contained any copies of the BAC. Confirmation of the presence of the BAC was a 4 kb band that would be generated upon digestion of the BAC with *Bam HI* and hybridization of the digested DNA with a 500bp probe derived from upstream of the α constant gene. As the endogenous locus gives a 14kb band when subjected to the same digestion and hybridization our, 9921/BAC clones would have two bands (see Figure 9). These Southern blots demonstrated the presence of the 5′ end of the BAC in these cell clones, (see Figure 10) but our expression analysis required that each transformant carried an intact BAC containing all four 3′ enhancers – the most 3′ of which, hs4, is located – 48 kb downstream of the probe used for these Southern analyses.

To ascertain that the entire 3′ enhancer region had integrated into the genome, we performed PCR using primers to amplify the unique 3′ end of the BAC (located 45 kb downstream of hs4). This "end" contains both DNA form the murine IgH locus and BAC vector sequences. Clone 21 did not contain this 3′ end though clones 1, 6, 7, 18, 33 and 40 were found to contain the absolute 3′ end of the BAC (see Figure 11).

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All expression analyses were conducted on the six 9921/BAC clones that contained the entire length of the BAC. Western blots to assess whether the clones could support expression and translation of the α heavy chain gene were performed first. All expression of the α heavy chain would be BAC-derived as the plasmacytoma line 9921 produces γ2a heavy chain endogenously. As shown in figure 12, five of the six 9921/BAC clones were permissive for expression of the BAC's heavy chain gene. Clone 18 did not produce α heavy chain and therefore was not included in further expression analyses (discussed again below).

As one of our goals was to determine if the heavy chain locus, when driven by the 3′ enhancers in an endogenous configuration, could exhibit copy-number dependent expression it was important to insure that we were not managing a mixed population of "clones". Therefore, each of the five cell clones was subcloned and each subclone was checked by ELISA to ascertain that they retained α heavy chain expression before further analysis ensued (data not shown).

In order to discriminate between our subclones that contained one copy of the BAC and those that contained multiple copies, we performed Southern blots on our subclones and analyzed our autoradiographs by densitometry using the band derived from endogenous IgH locus for normalization. As shown in Figure 13, clone 6.4 contains 4 copies of the BAC; clearly distinguishable from single-copy clones such as 7.11, 33.2, and 40.2 by the darker 4 kb band. The endogenous 14 kb band appears darker than the recombinant 4 kb band in single-copy clones (see Figures 10 and 13) due to the fact that 9921 cells contain one copy of the expressed heavy chain locus and three copies of the c-

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myc/ heavy chain translocated chromosome. Thus, four copies of endogenous loci would hybridize to the 500 bp probe used in these Southern analyses.

To address the question of position-independent expression, we analyzed the clones for the steady-state levels of transcripts (α mRNA) and Igα protein. If the 3′ enhancers, with their endogenous spacing, are enough to fully insulate the Igα transcription unit from the effects of the chromatin surrounding the BAC's site of integration, we should see expression in every clone with a full-length BAC and equivalent levels of expression in single-copy clones. Our clone 18, which contained a full-length BAC, did not express an α heavy chain (see western Figure 12). This finding could lead us to the conclusion that the presence of a copy of the full length BAC was not sufficient to insure expression of that BAC. However, the caveat to this typical criteria for determining LCR behavior is that the BAC is too large to state with certainty that it was not perturbed somewhere along its length or within the transcription unit itself. Therefore, we looked at levels of expression of those clones that contained the same numbers of copies of the BAC, namely single copies, to further address this question. As shown in Figure 14 extracts from most of the clones contained as much or more α-heavy chain then the Igα-producer, J558. There were detectable differences in the level of α protein present, however, when comparing single-copy clones to one another. For instance, extracts from clone 40.2 contained a significantly greater amount of α heavy chain protein than extracts from clone 7.11 (4-fold difference, see Figure 14). This casts doubt on the idea that the 3′ enhancers in their natural spacing, constitute an LCR. Our copy-number dependence expression analysis was hindered by the fact that the single-copy clones varied in Igα expression level. Therefore, there was no way to determine what constituted one "dose"

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of the BAC. There was, however, a significantly greater level of steady state α protein in 6.4, our multiple-copy clone, than in any of our single-copy clones (clone 6.4 produced 14x more α chain than clone 1.1; 4x more than clone 7.11; 6x more than clone 33.2, and 2x more α chain than clone 40.2).

Ig heavy chains are long-lived (half-lives are in the order of days) so that proteins accumulated in cells may not sensitively reflect differences in transcription rate levels. In order to look at the BAC closer to the level of transcription, we performed Northern analyses. Each one of our blots was placed on a phosphor imaging screen and quantitated by Imagequant software. The transcript data correlated nicely with what was observed at the protein level. For instance, clone 40.2, the single-copy clone that contained the greatest amount of α protein also contained the greatest amount of α transcript; (see Figure 15). Once again, it is hard to assign a single "dose" quantity that our multiple-copy clone can be compared to. Clone 6.4 makes four times more transcript than single-copy clone 7.11; but seven times more than single-copy clone 33.2 (Figure 15, quantitation data obtained by ImageQuant analyses of phosphor films from three independent experiments).

Consistent with previous work in this laboratory (Shi 2001), we found that the 9921/ BAC clones were able to support expression of both the endogenous Igγ2a locus and the Igα locus residing on the BAC (Figure 15). Our multiple copy subclone, 6.4, had the highest amount of α transcript while simultaneously expressing endogenous γ2a transcript at levels comparable to the other clones. There was not, however, a correlation between high levels of the endogenous γ2a transcript and high levels of the BAC's α transcript (compare clone 1.1's low level of α transcript to its high level of γ2a transcript

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in Figure 15). Our Northern data showed that we had not universally achieved heavy chain expression from our BAC equivalent to that of the endogenous locus. This is another indication that the – 94 kb BAC lacks a classical LCR.

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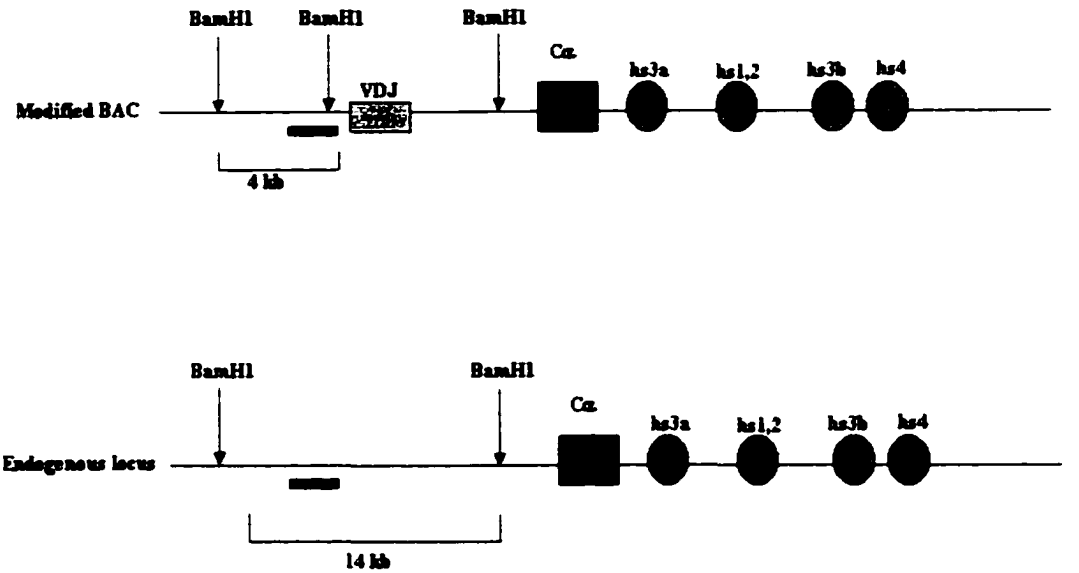
Figure 9



probe used for Southern analysis

Schematic map showing restriction region in the the endogenous locus and the BAC that has been modified to include a VDJ region upstream of Cα.

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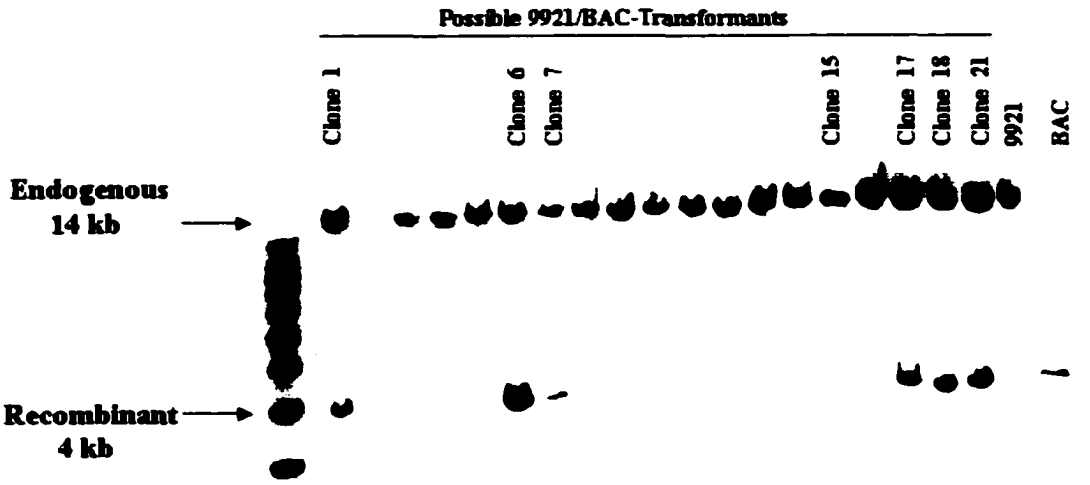
Figure 10
Southern



Southern blot using ~25 μg BamHI-digested genomic DNA from 9921, an IgG2a-secreting plasmacytoma and 19 possible BAC-transformed 9921 clones. Hybridization with a 500 bp probe selected from upstream of the IgA constant gene results in a 14 kb band from the endogenous loci and a 4 kb band from the BAC DNA. Clones 1, 6, 7, 15, 17, 18, and 21 reveal the presence of the BAC DNA.

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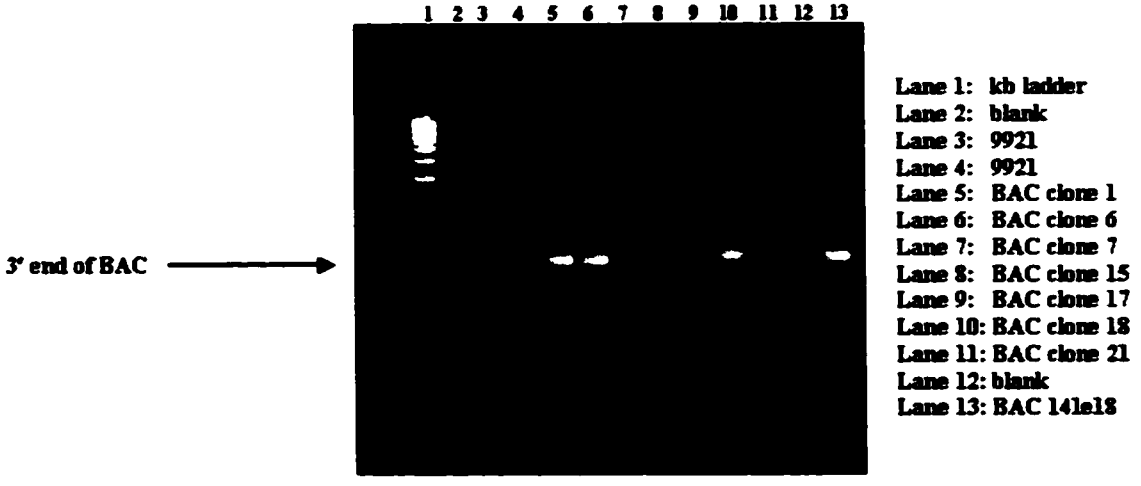
Figure 11 **9921/BAC clones 1, 6, 7, and 18 contain a full-length BAC**



EtBr-stained agarose gel showing PCR products. PCR was performed to identify 9921/BAC clones that contain a full-length BAC. PCR primers amplified the unique 3' end of the BAC. This "end" contains both DNA from the IgH locus and BAC vector sequences. Wild-type 9921 serves as a negative control (lanes 3 &4), while BAC 141e18 (lane 13) serves as our positive control. BAC clones 1, 6, 7, & 18 (lanes 5, 6, 7, &10, respectively) contain the absolute 3' of the BAC while BAC clones 15, 17, & 21 (lanes 8, 9,& 11, respectively), do not.

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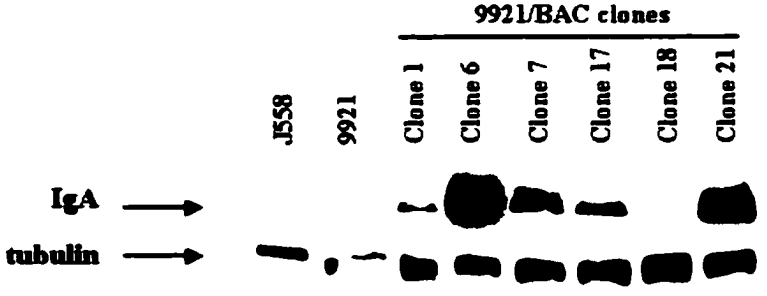
Figure 12

Western **Five BAC-transformed clones express the BAC-derived IgA protein**



Whole cell lysates from J558, an IgA-secreting plasmacytoma; 9921, an IgG2a-secreting plasmacytoma; and 6 BAC-transformed 9921 clones that secrete IgG2a from the endogenous locus were incubated with an anti-IgA antibody to establish which clones expressed IgA from the BAC (Bacterial Artificial Chromosome). Tubulin is shown for loading normalization.

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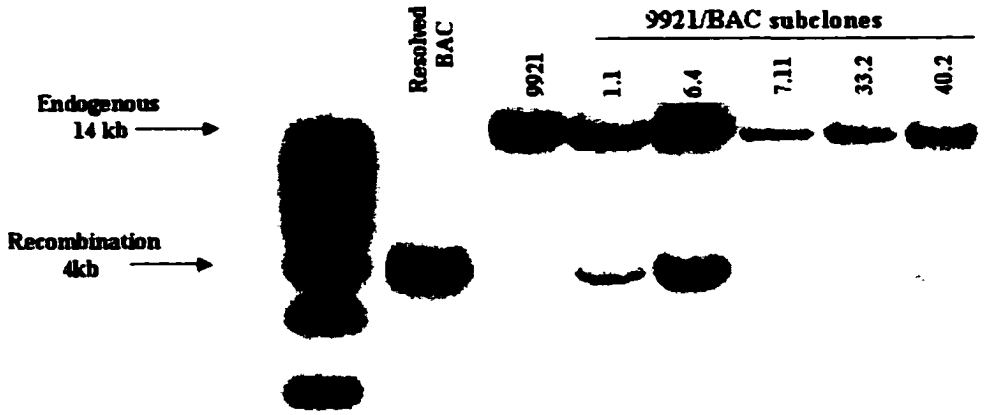
Figure 13

Southern **Four 9921/BAC clones contain a single-copy of the BAC, one contains multiple copies**



Southern blot using ~25 µg BamHI-digested genomic DNA from 9921, an IgG2a-secreting plasmacytoma and 5 BAC-transformed 9921 subclones. Hybridization with a 500 bp probe selected from upstream of the IgA constant gene results in a 14 kb band from the endogenous loci and a 4 kb band from the BAC DNA. Several exposures of this autoradiograph were scanned by densitometry and band intensities were determined by ImageQuant software to determine BAC copy number.

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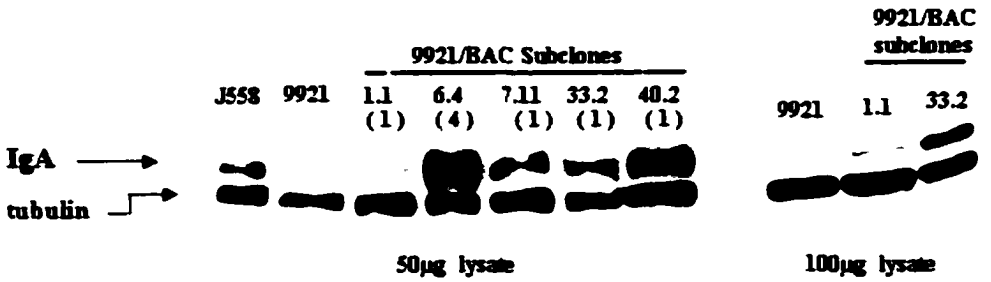
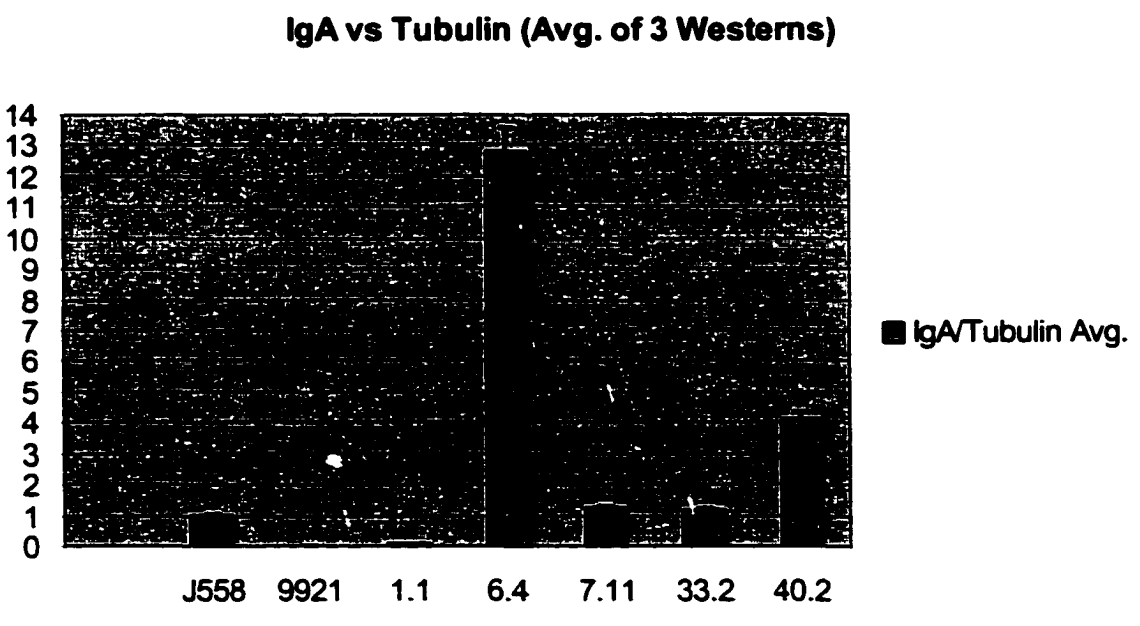
Figure 14
Western

9921/BAC subclones containing a single copy of the BAC produce variable amounts of BAC-derived IgA protein



Whole cell lysates from J558, an IgA-secreting plasmacytoma; 9921, an IgG2a-secreting plasmacytoma; and 5 BAC-transformed 9921 subclones that secrete IgG2a from the endogenous locus and IgA from the BAC (Bacterial Artificial Chromosome) were incubated with an anti-IgA antibody to establish IgA expression levels among the 5 subclones. Cell lysates were prepared in each case from identical numbers of cells. Expression levels from the two subclones (1.1 & 33.2) producing the least amount of IgA are shown when double the amount (100 µg) of extracts are used (right panel). Note that the Westerns in the left and right panels were developed with substrates for different times and cannot be directly compared. Tubulin is shown for loading normalization. Numbers in parentheses are the estimated BAC copy-number for each clone.



J558 IgA set to 1.

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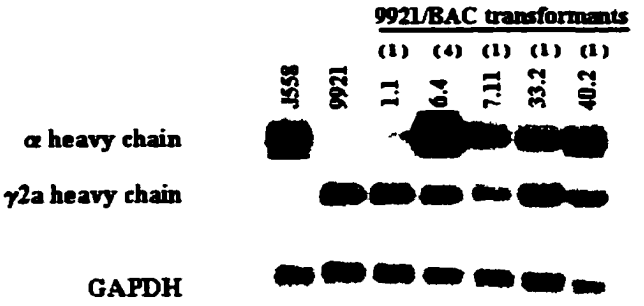
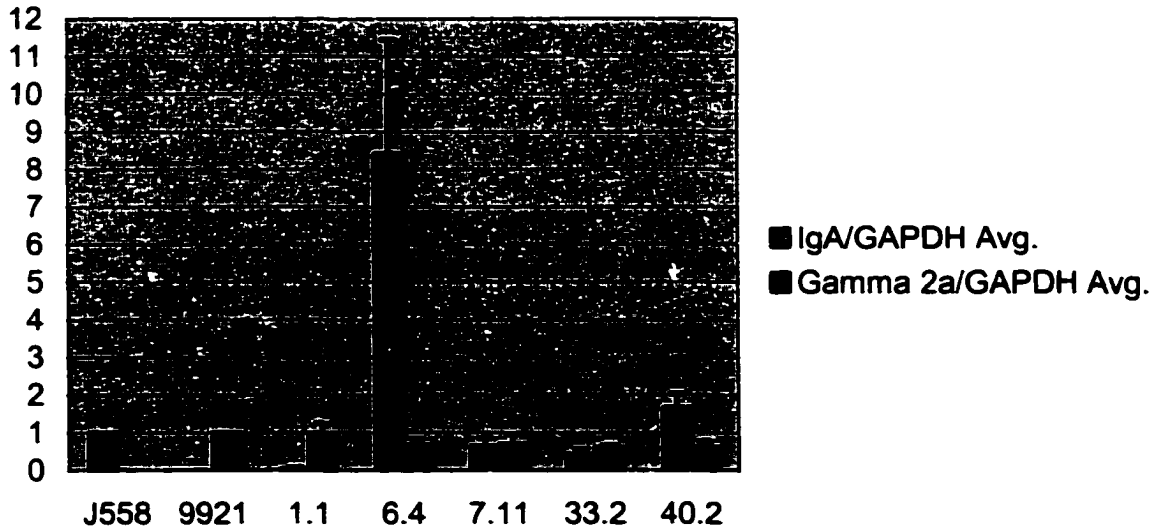
Figure 15
Northern

Single-copy BAC transformants produce a variable amount of α heavy chain mRNA



Northern blot using 25μg total cellular RNA from J558, an Igα-secreting plasmacytoma; 9921, an Igγ2a-secreting plasmacytoma; and five 9921/BAC subclones which secrete Igγ2a from the endogenous locus and Igα from the transfected BAC. A 4.4 kb fragment containing exons 2 and 3 of the α heavy chain was used to detect α message. A 4.1 kb fragment containing exons 1,2 and 3 of γ2a was used to detect γ2a message. GAPDH was used for loading normalization. Each blot was stripped between successive hybridizations. Each experiment was done in triplicate. The level of Igα transcript in each cell, relative to the Igα-producer J558 was .804 (clone 1.1), .28 (clone 7.11), .15 (clone 33.2), 1.4 (clone 40.2), 3.3 (multicopy clone 6.4).

Gamma 2a and IgA mRNA levels in BAC transformants (Avg. of 3 Northerns)



Comparison of γ 2a bars shows relative γ 2a expression in each cell line.
Comparison of α bars shows relative α expression in each cell line.
J558 α set to 1. 9921 γ 2a set to 1.

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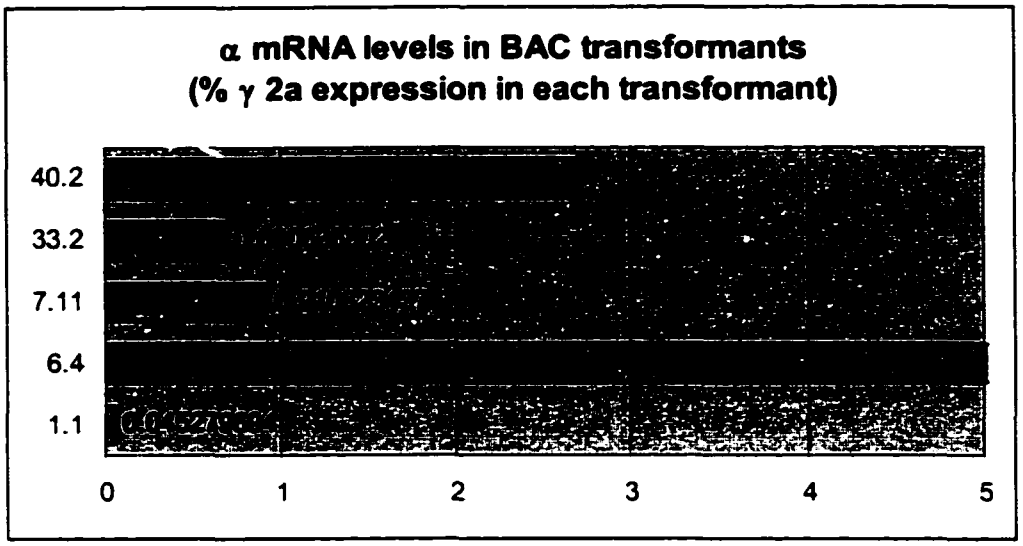

α mRNA expression levels in multicopy transformant 6.4 exceed the scale of the graph. (see values on graph)

The probes for γ 2a and α are of similar size and specific activity to allow for this comparison.

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BAC DISCUSSION

Discovery and initial characterization of hs1,2

The discovery that the Ig heavy chain locus was capable of supporting copious production of the heavy chain in the absence of the intronic enhancer Eu, (Eckhardt and Birshtein 1985) led researchers to speculate that there were additional, as yet undiscovered, enhancers located within the locus. When this laboratory identified hs1,2, another heavy chain enhancer located 3′ of the α constant gene(Lieberson et al. 1991), work was immediately begun to delete this enhancer in the Eμ- deficient cell line of this study, 9921. As this work was in progress, three additional 3′ Ig heavy chain enhancers (hs3a, hs3b, and hs4) were discovered (Giannini et al. 1993; Matthias and Baltimore 1993; Michaelson et al. 1995). When this laboratory successfully replaced hs1,2 with a neomycin-resistance (*neo*^{r}) gene, transcription of the endogenous heavy chain ceased (Lieberson et al. 1995). Hs1,2 was, therefore, believed to play a pivotal role in transcription of the heavy chain gene in a plasma cell that lacks Eμ.

Selectable markers interfere with locus

Evidence began to mount, however, that the replacement of hs1,2 with a gene driven by a strong promoter may have resulted in the subversion of this enhancer region's long-range interaction with its own promoter (Fiering et al. 1995; Hug 1996). If this were indeed the case, then replacement of hs1,2 with a *neo*^{r} cassette would not allow us to assess the contribution of hs1,2 towards transcriptional enhancement as this replacement did not constitute a "clean" deletion of hs1,2 but rather a "technical" knock-out of the entire enhancer region.

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Modification of hs1,2 deletion construct to include loxP sites

A former graduate student in this laboratory (Xuerong Shi) modified the construct used to knock-out hs1,2 by positioning loxP sites around the *neo*^{r} gene. Once homologous recombination into the 3' locus of the heavy chain was verified, Cre would be transiently expressed, and the *neo*^{r} gene would be excised from its place in the chromosome (the former site of hs,12). However, the 3' locus proved highly refractory to modification: the screening of 892 neomycin-resistant clones did not yield a single homologous recombinant. One of the reasons posited for the difficulty in manipulating this region was that hs1,2 is flanked by palindromic sequences and is situated in the middle of two enhancers (hs3a and hs3b) that share 97% sequence identity though they are in an inverted position relative to one another. Thus, hs1,2 is thought to be situated at the midpoint of a large inverted repeat (Chauveau and Cogne 1996).

Further modification of the hs1,2 deletion construct to extend 5' flank

My goal when I joined this project was to increase the efficiency of homologous recombination. To achieve that end, I decided to extend the length of the 5′ flanking region. The addition of 4.4 kb to the 5' end of this construct brought the total length of the 5' flanking region to 11.4 kb. This new construct was transfected into 9921 cells and the abovementioned graduate student screened 374 neomycin-resistant clones. Unfortunately, a homologous recombinant remained elusive.

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An alternative approach to manipulating the 3' locus of the heavy chain using Bacterial Artificial Chromosomes (BAC)

As *in situ* manipulation of the heavy chain's 3' region proved exceedingly difficult, we searched for other methods that would allow us to delete regions of the 3' locus in an efficient manner. The Bacterial Artificial Chromosome (BAC) offered us this promise. A BAC is an F-based plasmid that can stably hold up to 300 kb of DNA if it is propagated by recombination-deficient bacteria. Homologous recombination of any region of DNA contained within the BAC can be accomplished by transfection of a drug-resistant shuttle vector that carries the modification of interest (flanked by arms of homology) as well as the RecA recombinase gene into the host bacteria (O'Connor 1989; Yang et al. 1997). The bacteria that harbor the BAC that has undergone the desired recombination grow on selective plates while the unwanted bacteria, do not. Once the screen identifies the BAC that has been customized appropriately, the BAC is purified from the bacteria, linearized, and stably transfected into the mammalian cell of interest. The results of the homologous recombination can thus be studied directly as no further modification of the BAC is required.

Modification of the BAC containing a portion of the heavy chain locus

Our laboratory received a BAC that contained a 94 kb piece of the heavy chain locus. Work on the BAC was begun by me and Buyi Zhang, another graduate student in the lab. Mapping of the BAC revealed that the 5' end terminated in exon 2 of the ε constant gene while the 3' end terminated 45 kb downstream of hs4. As our BAC contained an intact α

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constant gene in addition to the four 3' enhancers, we could study the enhancers' regulation of this gene once we inserted a preassembled variable region and an Ig heavy chain promoter upstream of the α gene (Sonoda et al. 1997). Once this modification was complete, we stably transfected our BAC into 9921 cells.

Expression of the BAC's α heavy chain

The basal level of expression of the BAC's α heavy chain would need to be established before deletions allowed us to assess the individual contributions of the 3' enhancers. However, a close inspection of the BAC's expression level would provide us with data in the ongoing debate as to whether the heavy chain's 3' enhancers could act as an LCR and direct copy-number dependent and insertion-independent expression of their linked gene (Grosveld et al. 1987). Madisen and Groudine's (Madisen and Groudine 1994) experiments suggested that hs12, hs3b, and hs4 could act as an LCR, while other experiments, including recent data from this lab, suggested that the 3' enhancers, did not behave as a true LCR (Shi 2001). However, each of these earlier studies removed the enhancers from the endogenous locus and placed them directly next to one another. Therefore, neither the distance between the 3' enhancers and the promoter (which could reach 150 kb) nor the distance spanned by the 3' enhancer region itself (~25 kb) was intact in the earlier constructs. Hence, expression from the BAC should most closely mimic the endogenous activity of the 3' enhancers as the enhancers were in their native chromosomal conformation, and the gene they were regulating (α heavy chain,) was located at the physiological distance.

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The majority of BAC clones express BAC's α heavy chain

With the aid of our Southern analyses that revealed the presence of the 5' end of the BAC and our PCR analyses that established the presence of the 3' end of the BAC, we identified six clones that contained a full-length BAC. Of these six clones, five expressed the BAC-derived α heavy chain. We also obtained three clones that expressed the heavy chain without presence of the absolute 3' end of the BAC. However, the PCR that detected the presence of the 3' end of the BAC amplified a region of DNA 45 kb downstream from hs4, the most distal of the 3' enhancers. Thus, the BAC in those clones most likely contained the complete 3' enhancer region. Nevertheless, as we did not have an assay that differentiated between the BAC's 3' enhancers and the endogenous 3' enhancers, we could not verify the presence of the BAC's 3' enhancers hence we chose not to analyze these clones. We also obtained a single clone (clone 18) that contained a full-length BAC but did not express the BAC's α heavy chain. As our BAC is 94 kb long, however, we reasoned that the integrity of the DNA may be compromised somewhere along its length though we did not expend time to confirm this hypothesis. The clones that expressed their full-length BAC were subcloned before quantitative expression analysis began.

3' enhancers do not exhibit classical LCR behavior

For a region to be labeled as a classical LCR, it must provide copy-number dependent expression of its linked gene in addition to integration site-independent expression (Grosveld et al. 1987). Thus, a clone that contains five copies of an LCR-linked

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transgene should transcribe five times more RNA than a clone containing one copy of the LCR-linked transgene, as every site of integration is made permissible to expression by the LCR. This implies that the LCR is able to insulate its linked genes from the surrounding chromatin environment. As five out of six of the clones that contained the full-length BAC expressed the BAC's α heavy chain, we suggest that the 3′ enhancers facilitate integration site-independent expression. As mentioned above, we posit that the single clone that did not express its full-length BAC, sustained damage within the BAC or had its BAC undergo rearrangement upon integration into the genome.

The 3′ region, however, may not fully shield its linked heavy chain from non-permissive chromatin neighborhoods, as the expression level of α heavy chain varied among the single-copy clones. This variation made the determination of copy-number dependent expression difficult as well, as there was no clonal consensus on what constituted one "dose" of BAC expression. Our multiple-copy clone, however, did express far greater levels of α heavy chain (message and protein) than any of our single-copy clones. Relative to the normal single-copy Igα locus of the IgA-producer J558, our multicopy clone produced 3x as much α heavy chain message (4x as much α protein). However, we were not able to establish a copy number versus expression level trend, as we only possessed one multiple-copy clone. This absence of numerous multiple copy clones may reflect the plasmacytoma's difficulty in integrating the 94 kb locus.

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The β-globin LCR (the original LCR) does not always exhibit classical LCR behavior

The conglomeration of DNAseI hypersensitive sites located 5' of the β- globin gene cluster span a 19 kb region. When removed from their normal chromosomal context and tested in transgenic mouse assays, these hypersensitive sites behaved as an LCR (Grosveld et al. 1987). In these assays, however, the hs sites were placed proximal to one another due to the size limitations of conventional cloning vectors. As in this study, the new BAC and YAC (Yeast Artificial Chromosome) technology, made it possible to test the endogenous locus, with its native spacing, in transgenic mouse assays. Surprisingly, in several different studies, the endogenously-spaced β-globin LCR was not consistently able to shield its linked gene from the silencing effects of some chromosomal locations nor could the LCR establish consistent expression levels of the linked genes (Milot 1996; Kaufman 1999; Alami 2000). Though theβ –globin LCR does not strictly conform to the behavior of a classical LCR, it has not been stripped of its designation, nor have the parameters for classifying regions as a LCR been modified. By the accepted standards, though, the original LCR should no longer be considered an LCR.

Expression levels of the BAC's α heavy chain in 9921 are equivalent to α heavy chain expression levels produced by our control plasmacytoma, J558

Though we did not observe uniform levels of the BAC's α heavy chain among our 9921/BAC clones, we were able to achieve α heavy chain protein levels that were equivalent to or greater than endogenous α heavy chain produced by our control

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plasmacytoma, J558, in all but one of the BAC clones. This suggests that the BAC's 3' enhancers were behaving in a physiologically relevant manner directing appropriate high-level expression of their linked gene.

No correlation between expression levels of endogenous γ2a heavy chain and the BAC's α heavy chain in the 9921/BAC clones

However, in contrast to J558, our 9921/BAC clones produced both the BAC's α heavy chain and the cell's endogenous γ2a heavy chain. Though earlier experiments in this laboratory demonstrated that 9921 could support production of two heavy chains (Shi 2001), my BSAP studies demonstrated that each 9921 subclone exhibited a variation in the amount of heavy chain produced. Thus, we wondered whether a clone producing copious amounts of endogenous heavy chain was also producing copious amounts of the BAC's heavy chain. Alternatively, would abundant production of the endogenous heavy chain preclude abundant production of the BAC's heavy chain? Quantitative northern blot analysis of our 9921/BAC clones established that there was no relationship between the amounts of the two different heavy chains produced within a single clone. A clone was just as likely to produce high levels of both heavy chains, as it was to produce a high level of endogenous heavy chain and lower level of BAC heavy chain.

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Expression of BAC heavy chain is less than the expression of endogenous heavy chain

The 9921/BAC clones did not, on average, produce as great an amount of the BAC's α heavy chain as they did their endogenous γ2a heavy chain. This difference is not due to a disparity in the number of enhancers regulating the respective heavy chain promoters, as the four 3' enhancers without the assistance of the intronic enhancer, Eμ, drive both of these heavy chains. However, if additional, as yet undiscovered, enhancers exist within the heavy chain locus upstream of the ε constant gene region (where our BAC terminates), we could propose this as a reason for the difference in expression levels. What may be a more likely explanation for the difference in expression levels, however, is that the 94 kb length of our BAC does not contain any of the matrix attachment regions (MARs) known to exist within the heavy chain locus. In the endogenous locus, a MAR is located 500 bp upstream of the Ig promoter, 5′ and 3′ of Eμ, and 5′ of Cγ3 (Cockerill et al. 1987; Cockerill 1990; Webb 1991). As the 5′ end of our BAC is downstream of Cγ3, none of the known MARs are found on our BAC. In addition, studies of the 3′ enhancers have failed to detect the presence of additional MARs (Shi and Eckhardt, unpublished). Thus, the endogenous heavy chain locus would be attached to the scaffold, while our BAC, which integrated into the genome in a random fashion, may not be. This fact may account for the difference in expression levels of the endogenous and BAC-derived heavy chains.

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Future modifications of our BAC: add a Matrix attachment region

Future modifications to the BAC might include the addition of a matrix attachment region. An analysis of clones that contained the MARs-inclusive BAC would reveal whether the addition of a matrix attachment region resulted in an augmented expression of the BAC's heavy chain. Also, we could determine whether addition of a proximal MAR allows our 3' enhancer region to function as a classical LCR complete with copy number-dependent expression and insulation from the surrounding chromatin. However, as recent studies of the human growth hormone LCR showed that the region did not contain any MARs (Shewchuk, 2001), we might find that the addition of a MARs does not transform our 3' region into an LCR.

Future modification of our BAC: delete hs4

Hs4 was shown to be the strongest of the 3' enhancers when assayed by transient transfection in plasmacytomas (Ong et al. 1998). Recent stable transfection studies have shown that the clean deletion of both hs3 and hs4 resulted in a loss of transcription of the linked gene (Shi 2001), and a thoroughly abnormal phenotype of B cells in mice (Manis et al. 1998). Thus, hs4 has become the natural first choice for elimination from the BAC. As this study has determined expression levels of a BAC with an intact 3' region, analysis of the expression levels of the hs4-deficient BAC would allow us to ascertain the contribution of hs4 towards overall enhancement of the heavy chain at the plasma cell stage.

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MATERIALS AND METHODS

Cell lines

9921 is a γ2a/κ -producing Ig class switch variant that arose spontaneously from the cell line 971. 971 was, in turn, isolated from mutagenized 45.6.2.4 cells. This lineage is described in Eckhardt and Birshtein (Eckhardt and Birshtein 1985). 45.6.2.4 is a tissue culture-adapted subline of the γ2b/κ producing BALB/c mouse tumor MPC11 (Laskov and Scharff 1970). 70Z/3, a μ-producing pre-B cell line, was a gift from Dr. B.K. Birshtein, Albert Einstein College of Medicine (Bronx, NY). J558 is a Igα/λ secreting plasmacytoma that was obtained from ATCC (catalogue no. TIB-6). S194 plasmacytoma (IgA-secreting plasmacytoma derived from Balb/c) was a gift from Dr. B.K. Birshtein, Albert Einstein College of Medicine, Bronx, NY.

J558, 9921 and BSAP-transformed 9921 clones were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD; catalogue no. 12100-061) with 10% bovine calf serum (BCS) (Hyclone Laboratories, Logan, UT; catalogue no. SH30072.03). 70Z/3 and S194 were maintained in RPMI 1640 Medium (Life Technologies; catalogue no. 31800-089) with 10% BCS and 50μM β-mercaptoethanol. All media contained 1% penicillin-streptomycin (Life Technologies; catalogue no.15140-122) and 2mM L-glutamine (Life Technologies; catalogue no. 21051-016). All cells were maintained at 37□C in an atmosphere of 7 to 8% CO_2.

Construction of BSAP expression plasmid

A 3.3 kb fragment encoding the cDNA of the human BSAP gene was liberated from phBSAP-1s (kindly provided by Dr. M. Busslinger, Research Institute of Molecular

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Pathology, Vienna, Austria) by *Sal* I digestion and ligated into the corresponding Sal I site of expression vector pVR1012 -*neo* (a gift from Dr. H. Yu, Moffitt Cancer Center and Research Center, Tampa, FLA). The transcription unit of pVR1012-*neo* consisted of the cytomegalovirus (CMV) enhancer/promoter region, a leader sequence followed by an intron, a multiple-cloning site, and a polyadenylation signal. This vector also carries the bacterial *neo*^{r} gene under control of an SV40 enhancer/promoter. The pVR1012-*neo* plasmid containing BSAP cDNA was designated pVR-BSAP.

Modification of BAC:

A 500 bp PCR product (IPAU) generated from the intronic promoter (IP) region of the α heavy chain gene (to serve as the "upstream" homologous arm) was engineered to have *Sal I* and *Xho I* ends. Once cloned into the *Sal I* + *Xho I* sites of pBluescript® II SK (+) this plasmid was designated pBS IPAU.

A rearranged VDJ segment specific for NP ((4-hydroxyl-3-nitrophenyl) acetyl) was liberated from pIV$_H$B1-8L2neo^r (Sonoda et al. 1997) by *Cla I* digestion and inserted into the *Cla I* site of pBSII SK (+). This plasmid was designated pBS VDJ (+).

Another ~ 500 bp PCR product generated from a more downstream position in the IP region of the α heavy chain gene (to serve as the "downstream" arm) was also engineered to have *Sal I* and *Xho I* ends. This fragment was cloned into the *Sal I* site of pBS VDJ (+). This plasmid was designated pBSVDJIPAD.

IPAU was released from pBSIPAU by a *Xho I* and *Xba I* digestion. VDJIPAD was released from pBSVDJIPAD by an *Xho I* and *Xba I* digestion. These two fragments were combined with an *Xho I*-digested pBSII SK(+). The resulting plasmid was designated pBSUVD.

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pBSUVD was cut with *Xho I* to release the IPAUVDJIPAD which was cloned into the *Sal I* site of pSV1RecA. This final shuttle vector was designated pSVUVD.

Construction of RVA+4.4plox*neo*

RVAΔ*neo*Δtk (Lieberson et al. 1995) was digested with *EcoRI* and ligated to a 4.4 kb *EcoRI* fragment liberated from pIgA. This 3-way ligation yielded an intermediate plasmid designated RVA+4.4Δ*neo*Δtk. RVA+4.4Δ*neo*Δtk was digested with *Sal I* and flush-ended. A ~ 2 kb *neo*^{r} gene surrounded by loxP sites was taken from a *Not I* digest of p*lox*P2neo (a gift from Dr. F.W. Alt, the Children's Hospital, the Center for Blood Research, Harvard Medical School, Boston, MA), flush-ended, and inserted into the *Sal I* site of RVA+ 4.4Δ*neo*Δtk. This construct was designated RVA+4.4plox*neo*.

Cell Transfections:

Stable:

DNA was introduced into 9921 cells by electroporation. For BSAP studies: aliquots of 12 μg of *Dra* I-linearized pVR-BSAP plasmid DNA were combined with a 1ml suspension of 10^7 9921 cells in DMEM (without serum). For BAC studies: a 1 ml suspension of 10^7 9921 (Xuerong's 9921 subclone #2) cells in DMEM (without serum) were combined with 10 μg resolved BAC and 500 ηg *Xho*-I linearized psk-2loxPneo (Shi 2001). The mixtures were then dispensed into a 0.4 cm (width) electroporation cuvette (Bio-Rad, Hercules, CA; catalogue no.165-2088). An electric pulse was delivered at 960 μF and 250V by a Bio-Rad Gene Pulser□ electroporator and Capacitance Extender□ (Bio-Rad). The cells were then diluted in non-selective media

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and plated at 500cells/well in 96-well culture plates. After 48 h, culture medium supplemented with 1.5 mg/ml G418 (GibcoBRL, Grand Island, NY; catalogue no. 11811) to select for stable transformants expressing the *neo*^{r} gene. Colonies were visible 10-14 days after transfection.

Northern Analyses

Total cellular RNA was isolated using the TRIZOL reagent (GibcoBRL; catalogue no.15596) as per the manufacturer's instructions. Approximately 25μg total RNA/ sample were analyzed. RNA samples were denatured with formamide, size-fractionated on 1% formaldehyde-agarose gels and transferred to nylon membranes (Osmonics/MSI, Inc., Westborough, MA, Cat. NJOHY00010), essentially as described by others (Maniatis et al. 1989). Blots were hybridized with random-primed, ^{32}P-labeled DNA probes at 37□C for ~48 h in a hybridization solution of 50% formamide, 2X Denhardt's solution, 5X SSC, 50mM $NaPO_4$, ph 7.4, 50μg/ml sonicated salmon sperm DNA, 0.1% SDS and 50μg/ml polyA. To remove non-specifically bound probe, blots were washed 4X (30 min per wash) in 2X SSC, 0.2% SDS at 37□ or 42□ C for low stringency; or 4X (30 min each wash) 0.1X SSC, 1% SDS at 42□ or 68□ C for high stringency.

To strip the blots of specifically-bound radioactive probe, blots were washed 4X (15 min per wash in 0.01X SSC, 0.01% SDS at 100□C). **For BSAP studies:** γ2a-mRNA levels were assessed using a 312 bp fragment of the CH3 region of γ2a obtained by *Sst* I-digestion of pγ2a-CH3 (Tilley and Birshtein 1985). BSAP mRNA levels were assessed using a 3.3 kb *Sca* I- fragment containing the hBSAP gene liberated from phBSAP. A 1.2 kb cDNA clone (Jc21) was used to identify J chain mRNA (Cann et al. 1982). c-*myc*

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mRNA was detected with a 6 kb genomic DNA fragment containing exons 2 and 3 of the c-*myc* gene, liberated from the pc-myc Bam/Bam plasmid (Calza et al. 1984) by *BamHI* digestion. To ensure uniform levels and integrity of RNA samples, blots were stripped and rehybridized to mouse β-actin probe (3.2 kb pTRI-β-Actin mouse plasmid linearized by *Xba*I and *Hind*III, Ambion, Austin, Texas). Alternatively, a murine glyceraldehyde phosphate dehydrogenase (GAPDH) probe was used for normalization (GAPDH-mouse DECAprobe template, Ambion). **For BAC studies:** To detect α heavy chain mRNA, a 4.4 kb *EcoRI* fragment encompassing half of exon 2 and all of exon 3 of the α heavy chain gene was liberated from the plasmid RVA+ 4.4, DT.

To detect γ2a heavy chain messages a 4.1 kb fragment containing exons 1, 2 and 3 of the γ2a gene was isolated by an *EcoRI* and *BglII* digest of pγ2a-RI. GAPDH was used for normalization.

Western Analyses

Western blot analyses were performed by standard methods (Maniatis et al. 1989) with nuclear extracts prepared as described (Dignam et al. 1983). Whole cell extracts were prepared for analyses of Ig proteins. In brief, 5 x 10^6 cells were resuspended in 50μl extraction buffer (20mM HEPES, pH 7.9, 20% glycerol, 400mM Kcl, 0.5mM EDTA, 0.5mM EGTA, 0.025% NP40, 0.5mM DTT) and lysed by 5 freeze-thaw cycles.

The nuclear extracts and whole cell extracts were size-fractionated by electrophoresis through 8% SDS-PAGE gels. After electrophoresis, protein was transferred to nitrocellulose membrane (Osmonics/MSI, Inc., Westborough, MA, Cat. #WP2HYA0010). For BSAP detection, 50 μg nuclear extract were applied to the gel and blots were incubated with goat polyclonal IgG anti-Pax5 (BSAP) (Santa Cruz

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Biotechnology, Inc. Santa Cruz, CA; catalogue no. sc1974x) at a 1/3000 dilution followed by incubation with horseradish peroxidase (HRP)- conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, catalogue no. sc2020) at a 1/3000 dilution.

To normalize protein loading, Oct-1 was detected using rabbit polyclonal IgG anti-Oct-1 at a 1/200 dilution (Santa Cruz Biotechnology, catalogue no. sc232) followed by an HRP-conjugated donkey anti-rabbit Ig (Amersham, Arlington Heights, IL; catalogue no. NA934) at a 1/3000 dilution. Each blot was subsequently developed for 5 minutes in SuperSignal chemiluminescent substrate (Pierce, Rockford, IL; catalogue no. 34080)

For γ2a and κ western blots, 25μg whole cell extract were applied to the gels and γ2a was detected using an alkaline phosphatase-conjugated rabbit anti-mouse IgG2a (Zymed, San Franscisco, CA; catalogue no. 61-0222). Igκ was detected with biotin-conjugated, goat anti-mouse Ig kappa light chain (Amersham Pharmacia Biotech. Piscataway , NJ: catalogue no. rpn-1179) followed by incubation with alkaline phosphatase-conjugated avidin (Zymed; catalogue no. 43-4422). Each blot was subsequently developed with the BCIP/NCT phosphatase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD; catalogue no. 50-81-00). **For BAC studies:** For IgA western blots whole cell extracts were run on 6.5% polyacrylamide gels and IgA was detected using rabbit anti-mouse IgA at a 1/500 dilution(Zymed; catalogue no. 61-6700) followed by incubation with HRP-conjugated donkey anti-rabbit IgG at a 1/5000 dilution (Amersham; catalogue no. NA934). Tubulin was detected with rat IgG2a anti-α tubulin at a 1/300 dilution (Serotec; catalogue no. MCAP77) followed by incubation with HRP-conjugated mouse anti-rat IgG2a at a 1/5000 dilution (Zymed; catalogue no.03-9620).

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Dot Blots:

As a quick screen for identifying BAC-transfected clones expressing IgA whole cell lysates were applied directly onto the nitrocellulose. After allowing the lysate to dry for 15 minutes the nitrocellulose was blocked for 30 minutes in 1X PBS with 6% Carnation Dry milk. IgA was detected using Rat anti-mouse IgA at a 1/500 dilution (Pharmingen; catalogue no. 556960) in 1X PBS, 6 % Carnation Dry milk, 0.1% Tween 20 for 30 minutes. Three 10minute washes in 1X PBS, 0.1% Tween 20were followed by a 30 minute incubation with HRP-conjugated goat anti-rat IgG used at a 1/5000 dilution (Serotec; catalogue no. STAR 72). Three 10 minute washes were followed by a 5 minute development in SuperSignal chemiluminescent substrate (as described for Western blots).

Electrophoretic mobility shift assay

Nuclear extracts were prepared by the mini-extract method of Schreiber *et al.* (Schreiber et al. 1989) with slight modification. Typically, cells were grown to a density of approximately 1 X 10^6/ml and 10^7 cells were taken to prepare the extract. The protein concentrations of the extracts were determined by the Bradford assay with bovine serum albumin as the standard (Bio-Rad, Hercules, CA, Cat.#500-0006). The following oligonucleotides were synthesized, annealed, and purified by electroelution in a 20 % polyacrylamide gel before use in EMSAs for direct binding or competition studies: oligo containing H2A-2.2 site [site shown in italics] (Barberis et al. 1989). Underlined bases indicated contact point G residues, as identified by methylation interference footprinting (Singh and Birshtein, 1993).

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5′-AATTCAGGGTT*GTGACGCAGCGGTGGGTGACG*ACTGTCG-3′

3′-TTAAGTCCCAA*CACTGCGTCGCCACCCACTGC*TGACAGC-5′

51 bp fragment of Eμ containing octamer site [51bp sequence in italics, octamer site underlined] (Yu et al. 1989).

5′GATCC*TGAGCAAAACACCACCTGGGTAATTTGCATTTCTAAAATAAGTTGAGGATTG*

3′GACTCGTTTTGTGGTGGACCCATTAAACGTAAAGATTTTATTCAACTCCTAACTTAA

BSAP binding site "a" in hs1.2 (underlined bases indicate contact point G residues, as determined by methylation interference footprinting) (Singh and Birshtein, 1993).

5′- CATCATCAATAGGGGTCATGGACCCCAGTCCC-3′

3′- GTAGTAGTTATCCCCAGTACCTGGGGTCAGGG-5′

BSAP binding site "b" in hs1.2 (underlined bases indicate contact point G residues, as determined by methylation interference footprinting) (Singh and Birshtein, 1993).

5′-CCCTGGGGTTGAGCCACCCATCCTTGCCCATCTCCTGTCATGTCC-3′

3′-GGGACCCCACAACTCGGTGGGTAGGAACGGGTAGAGGACAGTACAGG-5′

Annealed oligonucleotides were labeled with α-(^{32}P)ATP (NEN, Boston, MA) to a specific activity of ~3 X 10^8 cpm/μg by the Klenow fill-in reaction. 8 μg crude nuclear protein were incubated with 7.5μg poly (dI-dC) (Roche Molecular Biochemicals, Indianapolis, IN) in 10mM Hepes, pH 7.9, 100mM NaCl, 10% glycerol, 0.5% $MgCl_2$, and 1mM dithiothreitol in a total volume of 15μl for 15 minutes at 4° C. ~ 1.75 ng (8 x 10^4 cpm) of DNA was then added to the reaction mixture and incubated for an additional 30 minutes at 4° C.

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For super-shift analyses, anti-Pax5 (BSAP) antibody (4 μg, Santa Cruz Biotechnology) was added to the reaction mixture along with the protein. The protein-DNA complexes were separated on a 4% polyacrylamide gel in Tris-borate/EDTA (TBE) buffer.

Southern Analyses

Agarose gel electrophoresis, transfer to membranes and DNA hybridizations were performed essentially as described previously in Radomska *et al.*(Radomska et al. 1994). For BAC 189a22 and 141e18 mapping: 1 μg of restriction enzyme-digested DNA was size fractionated on 1% agarose gels and transferred to nylon membranes. For genomic Southerns: ~25 μg of restriction enzyme-digested DNA was size-fractionated on 0.8% agarose gels and transferred to nylon membranes. Blots were pre-hybridized and hybridized at 65° C in buffer containing 7.5X Denhardt's solution, 3X SSC, 0.1 M sonicated sperm DNA and 0.5% SDS. Probes were labeled by the random primed method (Megaprime DNA labeling system, Amersham Pharmacia Biotech; rpn 1605) . To remove non-specifically bound probe, blots were washed in 0.1% SSC and 0.1% SDS at 65° C for 30 minutes to 2 hours as needed. Probes for BAC mapping included:

hs1,2: a 4 kb *Xba I* fragment containing hs1.2 was liberated from pλm 2-3.5

Eμ: a 1 kb *Xba I* fragment containing Eμ from pUC 18-enh

γ2b: a 310 bp fragment containing the CH_3 region of γ2b was liberated by *Sac I* digestion from pγ2b-CH3

α: pIgA3 cut with *EcoRI* and *PvuII* yields a 600 bp fragment which contains a portion of both CH_2 and CH_3 of α heavy chain DNA

hs3a: an *Xba I* digestion of pBSCα releases hs3a as a 1 kb fragment

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γ2a: 312 bp fragment of the CH_3 region of γ2a obtained by *SacI* digestion of pγ2a-CH3

ε: pCε cut with *XbaI* and *HindIII* yields a 900 bp fragment containing a portion of both CH_3 and CH_4 ε heavy chain DNA.

hs4: a 2.1 kb fragment (originally contained within pUNPST) that spans hs4, was released from pBK-CMV 3′ Pst/Bgl II by a SalI + EcoRI digestion.

IPAD/IPAU: 500 bp fragments from the IP region of the α heavy chain gene generated by PCR

PCR:

PCR was performed as follows to obtain the "homologous arms" used to construct the shuttle vector. Primers IPAUF and IPAUR amplify the ~500 bp directly upstream ("U") of the IP region of the Igα gene. Bases underlined are engineered restriction enzyme recognition (RE) sites.

IPAUF: 5'- ACGCGTCGACCAGTAGGATGTGTAGAGGAT-3' (RE site = Sal I)

IPAUR: 5'- CCGCTCGAGCCAGGACTCCACATGCAT-3' (RE site = Xho I)

Primers IPADF and IPADR were used to amplify the ~500 bp immediately downstream ("D") of the IP region of the Igα gene.

IPADF: 5'- CCGCTCGAGCTCAGTCTGACCCATCCACA-3' (RE = Xho I)

IPADR: 5'- ACGCGTCGACAGCCACAACAGCCTGAGT-3' (RE = Sal I)

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100 ng of the BAC DNA was used as a template with each primer at a concentration of 0.25 μM in a reaction mixture that contained 100mM Tris-HCl, 50mM KCl, 1.5 mM $MgCl_2$, 0.2mM each dNTP and 1.25 U Pfu. The total reaction volume was 40 μl. PCR reactions took place in a GeneAmp 9600 (Perkin Elmer, Norwalk, CT). Thirty cycles of 94°C for 1minute, 55°C for 1minute, 72°C for 1minute were followed by a final extension at 72°C for 7 minutes. PCR products were visualized by gel electrophoresis and then isolated by electroelution and used in the construction of the shuttle vector (see above).

ELISA:

Microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with a 1/500 dilution of Rabbit polyclonal anti-mouse IgA (Zymed; catalogue no. 61-6700) overnight at 4°C. Non-specific binding of proteins was then blocked by Blocking solution (3% BSA, 1X PBS, 0.1 % Na-Azide). The plates were then incubated with culture supernatant (50μl/well).

Igα heavy chains were assayed with a 1/2000 dilution of HRP-conjugated goat anti-mouse IgA (Zymed, catalogue no.62-6720) using ABTS [2,2'-Azino-bis (3 ethylbenzothiazoline-6-sulfonic acid)] (Zymed, catalogue no. 00-2024) as the chromogenic substrate. The absorbance at 405 nm was measured in an Elisa plate reader (Bio-Rad).

Transient transfection assays:

4×10^6 S194 cells were resuspended in 800μl RPMI (with 10% BCS) and added to 0.4cm electroporation cuvettes containing 10 μg DEAE and reporter plasmids [100 ng

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Renilla luciferase vector (pRL, Promega; catalogue # E 1960) with .48 pmol of either $V_H$luc, $V_H$luc1,2 or $V_H$luc3A 1,2 3B 4 (Stevens et al. 2000)]. After a 10 minute incubation at room temperature an electric pulse was delivered at 960 μF and 300V. The cuvette was then placed at 37° C for an additional 10 minutes. Cells were plated into 60 x 30 mm plates containing 4.2 mls of RPMI + 10% BCS. After 48 hours in culture, cells were harvested as follows: cells were spun down and washed once in 1 ml of 1X PBS. Cells were resuspended in 400μl Passive Lysis Buffer (Dual Luciferase® Reporter Assay kit, Promega, Madison, WI, catalogue no. E1960) and vortexed for 20 minutes after which a 5-minute 14K spin at 4° C was performed. Supernatant was collected and aliquoted as 20μl volumes into 96 well plates. 100μl of Promega's LAR II reagent was added by automation to each well followed by 100 μl of Promega's Stop and Glo reagent (added by automation)to each well in a Luminoskan RT 1.3-0 (Labsystems). The luciferase values were analyzed by Ascent software (Research Edition 2.1, Labsystems).

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