ABSTRACT

ORCUTT, TIMOTHY M. Dissecting the Epigenetic Regulation of Vβ Recombination.(Under the direction of Michael Sikes.)

V(D)J recombination in developing lymphocytes is essential for producing a diverse repertoire of antigen receptors (TCR and Ig). During recombination, the proteins encoded by the recombinase activating genes (RAG1 and RAG2) bind specific DNA sequences flanking individual V, D, and J coding segments within each antigen receptor gene, and introduce double strand DNA breaks at the coding sequence/targeting sequence boundaries. These double strand breaks are then repaired by ubiquitous DNA repair machinery to generate novel coding segment joints. The ability of each developing lymphocyte to independently assemble unique V(D)J joints results in the enormous diversity of antigen receptors expressed by our immune system. Despite a conserved enzymatic activity in both B and T lymphocytes, the assembly of T cell receptors (TCRs) and Immunoglobulins (Igs) in T and B cells respectively follows a highly orchestrated program in which the accessibility of individual targeting sequences varies during lymphocyte development. For example, when the TCRβ locus is rearranged, it initially assembles joints between D and J elements. Only after DJ joining do upstream V sequences become accessible and rearrange with the preassembled DJ's. We have previously shown that DJ rearrangement requires modification of the chromatin structure surrounding individual D and J segments via the coordinated actions of D-associated promoters and a single downstream enhancer. Like the D elements, each V element in TCRβ is associated with a transcriptional promoter. But the role these V promoters play in V-to-DJ

recombination remains unknown. Similarly, because enhancer deletion ablates D-to-J assembly, the potential role of enhancer activity in V recombination has not been directly tested. We hypothesize that V-to-DJ rearrangement requires both enhancer and promoter-dependent changes in the chromatin surrounding the Vβ RAG binding site, as well as that surrounding the Dβ 5' binding site. To test this hypothesis, I have constructed a panel of recombination substrates which harbor unrearranged or prerearranged DJ elements downstream from a single V element. These "miniloci" were stably transfected into the chromatin of a recombinase-inducible T cell line, and the chromatin status, expression and recombination potential of each was assessed.

Dissecting the Epigenetic Regulation of Vβ Recombination

by
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DEDICATION

I dedicate this thesis to my wife Amber, our first child Emily, and our future children.

BIOGRAPHY

I was born in Syracuse, NY but grew up in Orlando, FL. I moved to High Point, NC in 1995 and graduated from Southwest Guilford High School in 1998. I received my B.S. in Biology from High Point University in 2002 and married the same year. My wife and I moved to Raleigh, NC so that I could pursue an advanced degree in Microbiology. Our first child was born in 2006.

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I would like to thank Dr. Michael Sikes for everything he has taught me. I would not have been able to accomplish what I have as a graduate student without his guidance and understanding. I would also like to thank my committee members, Dr. Scott Laster and Dr. Eric Miller, for all of their input. I would like to thank my family and friends for their encouragement. I would like to thank my parents for everything they have done for me. I wouldn't be the person I am today without them. Most of all I would like to thank my wife Amber for her understanding and support. She is an excellent wife and mother. Her love has helped me through some challenging times.

TABLE OF CONTENTS

LIST OF FIGURES

LITERATURE REVIEW

Introduction

The body comes in contact with a wide variety of foreign objects on a daily basis. A number of these are potentially dangerous pathogens. Protection against such pathogens is the role of the immune system. The human immune system has developed two main response systems, known as the innate immune response and the adaptive immune response, to aide in these efforts. The adaptive and innate immune responses use different methods to fight off infection yet still interact with each other.

The cells of the immune system are derived in the bone marrow and most cells including B lymphocytes mature there as well. A second lymphocyte, the T cell, matures in the thymus (1). Mature lymphocytes, which are the primary cells that direct the adaptive immune response, move to the secondary organs of the immune system via the blood stream. These organs include the lymph nodes, the spleen, and lymphoid tissues. Pathogens can invade the body in many different ways. Lymphocytes screen antigens derived from invading pathogens with the help of specialized antigen presenting cells (APC), including macrophages and dendritic cells, within secondary lymphoid organs like the lymph node, spleen, peyer's patch, or other specialized tissues within the mucosa (1). Subsets of naïve lymphocytes continually pass through these organs. APCs present antigen on their cell surface for the lymphocytes to recognize. Once the lymphocytes recognize the antigen, they

are activated, proliferate, and then direct a highly orchestrated response targeted to the specific pathogen they recognized (1).

Organization of the Antigen Receptors

B and T lymphocytes each recognize discreet antigens through unique antigen receptors expressed on each cell's surface. These receptors, the T cell receptor (TCR) and the immunoglobulin (Ig) expressed by T and B cells respectively, are structurally and functionally very similar. The Ig is a heterotetrameric protein that consists of two identical heavy chains covalently linked by multiple disulfide bonds. Each heavy chain is also linked via disulfide bonding to a single light chain molecule. The antigen binding domain is formed by the three-dimensional structure formed between the N-termini of the heavy and light chain pairs. Consequently, each Ig contains two identical antigen binding domains (1). There are two types of light chains, lambda and kappa, and five classes of heavy chains, M, D, G, A, and E. Whereas the kappa and lambda light chains are derived from separate genes, the five classes of heavy chain proteins are encoded by a single gene, and are selectively expressed as the result of somatic rearrangement within the portion of the gene that encodes the receptor's effector function. The class of immunoglobulin is determined by the heavy chain. This means there are five classes of immunoglobulins, IgM, IgD, IgG, IgA, and IgE (1). Once a naïve B cell is activated, the Ig transcript undergoes alternative splicing, and results in the expression of a secreted protein called an antibody.

T cell receptors are also composed of two distinct proteins, assembled as a heterodimer. There are four TCR proteins, alpha, beta, delta, and gamma. However, developmental and structural restrictions limit TCR assembly to alpha/beta or gamma/delta heterodimers. TCRs remain on the surface of the T cell, and are never expressed in a secreted form. As with Ig's, the antigen binding domain of the TCR is formed by the three-dimensional structure of the paired N-termini of the alpha and beta or gamma and delta chains (1). Although T cells bearing the gamma/delta TCR play critical roles in shaping the immune response to select antigens, particularly in the gut (2), the majority of adaptive immune responses are mediated by T cells that express an alpha/beta TCR. Unless specifically stated otherwise, the T cells and TCRs described in the rest of this document will be of the alpha/beta lineage.

The Development of T cells

T cells are derived from the same pluripotent hematopoietic stem cells as all immune cells (3). Cells fated for T cell development first migrate from the bone marrow to the thymus. At this point, they are not "hard-wired" to become T cells. Rather, the unique cytokine microenvironment of the thymus directs their progression towards the T lineage, and precludes their development into either B cells or myelopoietic cells (3, 4). The stages of T cell development can be distinguished by variations in the expression of key components of the TCR signaling complex including CD3, CD4, and CD8. The lymphoid progenitors that first enter the thymus lack all three molecules, and are identified as triple negative (TN)

pro-T cells. More commonly, CD3 status is ignored, and the cells are simply identified as CD4/8 double negative (DN) cells.

While in the double negative stage, thymocytes differentially express a variety of other surface molecules. In particular, expression of the cell adhesion molecule CD44, and eventually the alpha subunit of the IL-2 receptor, termed CD25, are used to further subdivide DN development into DN1 ($44^+/25^-$), DN2 ($44^+/25^+$), DN3 ($44^-/25^+$), and DN4 ($44^-/25^-$) stages (3). Developmental progression beyond the DN stage is contingent on the cell's ability to signal through a preTCR molecule composed of a TCRβ chain protein paired with a surrogate α chain termed preTα (5). This test of TCRβ expression occurs in the DN4 stage, which has consequently been dubbed "β selection" (6). Cells that fail to express the preTCR are deleted by apoptosis.

The majority of cells survive β selection, after which they undergo a brief period of proliferation and upregulate the expression of both CD4 and CD8. Since these cells express both CD4 and CD8, they are termed double positive (DP) thymocytes. During DP development, cells gain expression of the TCRα locus, which replaces the preTα in a mature TCR complex (7). To complete maturation, each cell undergoes a period of positive selection in which the cell's newly formed TCR attempts to engage with major histocompatibility complexes (MHC) expressed on the surface of thymic stromal epithelial cells. At this point, any DP cell that cannot recognize MHC bound to "self" derived peptides undergoes apoptosis. Only those cells that bind to the peptide:MHC on thymic stromal cells are permitted to proceed through T cell maturation (8, 9).

There are two classes of MHC molecules, each geared to present antigenic peptides of a specific origin. Peptides derived from intracellular proteins are presented by MHC class I molecules (MHC I), while peptides from extracellular proteins are expressed by class II (MHC II) molecules. Though most cells in the body express either MHC I or MHC II molecules, thymic stromal epithelial cells express both classes of MHC. The CD4 and CD8 molecules expressed by DP cells are required co-receptors for a TCR:MHC interaction. If the T cell recognizes self MHC on the thymic stromal cell, the class of MHC recognized determines whether the T cell will mature into a CD4 or a CD8 single positive (SP) T cell. Cells that recognize MHC class I become CD8 T cells, and specifically downregulate expression of CD4; while cells that recognize MHC class II become CD4 T cells, downregulating CD8 expression (8).

Once a T cell undergoes positive selection, negative selection occurs. Antigen presenting cells (APCs) again present self peptides bound in their MHC molecules to the newly immature SP thymocytes. Whereas the ability to form TCR:MHC interactions was required for survival of the DP cell during positive selection, any immature SP cell that recognizes the self peptide:MHC complexes on the surface of APC cells during negative selection undergoes apoptosis (10). These cells would eventually mount a response against the host organism that could result in severe autoimmune disease. Those cells that survive negative selection are termed mature naïve T cells. These cells then leave the thymus and circulate throughout the body's blood system and secondary lymph tissues in search of their cognate antigens (1). CD8 T cells become cytotoxic T cells once they are activated.

Almost any cell can present antigen on its surface via MHC I if it is infected with an intracellular pathogen such as a virus. The main function of cytotoxic T cells is to eliminate these infected cells to prevent the proliferation of the infecting pathogen. Most CD4 T cells become helper cells once they are activated. There are two subsets of T helper cells, T_H1 and T_H2. T_H1 cells stimulate macrophages, while T_H2 cells stimulate B cells (1).

V(D)J Recombination

Proteins necessary for human life are encoded by as few as 25,000 inherited genes (11). Paradoxically, B and T lymphocytes fashion a repertoire of more than 10^9 different receptors capable of recognizing an enormous array of different pathogens from this limited amount of genetic material (12, 13). To circumvent the genome's size limitation and achieve such a wide breadth of receptor expression, developing lymphocytes undergo a process of somatic DNA rearrangement, termed V(D)J recombination, whereby the genes for individual antigen-specific receptors are assembled from large pools of coding segments (14). Consequentially, each mature B or T cell expresses a receptor (immunoglobulin or T cell receptor, respectively) specific to select antigens (15).

Despite cell-specific segregation of Ig and TCR rearrangements, both types of antigen receptor genes are assembled via a conserved mechanism. For each locus, the sequences that encode the antigen recognition domain are fragmented into segments termed variable (V), joining (J), and in some cases, diversity (D). V, J, and D gene segments are further duplicated into arrays of related sequences such that

the germline, or inherited form of each locus contains multiple copies of each element (15). In the case of TCR alpha and gamma loci and the Ig kappa and lambda loci, an antigen recognition domain is assembled when one V gene segment is joined to one J gene segment. The antigen recognition domains of TCR beta and delta loci and the Ig heavy chain locus contain D segments between the V and J segments, and are assembled in two steps: first, D-to-J, then V-to-DJ (16).

Rearrangement of all loci is mediated by a single enzymatic complex, which targets recombination signal sequences (RSs) that flank each V, D, and J gene segment (17, 18). RSs are composed of conserved AT-rich nonamer (5'ACAAAAACC3') and palindromic heptamer (5'CACAGTG3') sequences separated by nonconserved spacer sequences of either 12 or 23 basepairs (17). During rearrangement, select RSs are bound by the lymphocyte-specific components of recombinase, encoded by the Recombination Activating Genes (Rag-1 and –2) (19, 20), which introduce double-stranded DNA breaks precisely at the boundaries between the RSs and their coding sequences (21). Significantly, for two gene segments to recombine, the targeted RS of one must have a 12-bp spacer (12 RS) and the other a 23-bp spacer (23 RS). This restriction, known as the 12/23 rule, prevents V-V or J-J rearrangements in most loci (18, 22, 23). The intermediate products of RAG cleavage are termed signal ends (SEs) and coding ends (CEs), respectively. These intermediates are ligated by DNA repair enzymes to form extrachromosomal signal joints (SJs) and chromosomal coding joints (CJs) (21).

Defects in V(D)J recombination can have severe consequences. For example, the inability to complete recombination impairs lymphocyte development

leading to immunodeficiencies of varying severity (24-27). Conversely, inappropriate targeting of recombination can result in chromosomal translocations that lead to lymphoid malignancies (28, 29). To safeguard against inappropriate rearrangements, V(D)J recombination is tightly regulated during lymphocyte development such that genes encoding the T cell receptor (TCR) are only rearranged in developing T cells, while developing B cells only rearrange genes that encode the immunoglobulin (Ig) molecules (16, 30). This regulation is predominantly imposed *in cis* by epigenetic control of the accessibility or openness of individual chromatin regions within each locus to engagement by recombinase.

Differential Recombinational Accessibility

Despite sharing recombinase and its RS targets, each antigen receptor gene is assembled along a distinct developmental pattern. For example, TCR assembly during $\alpha\beta$ T cell development initiates with joining of D and J segments within the TCRβ locus of early pro-T cells (DN1/DN2). V-to-DJ recombination then completes TCRβ assembly in later pro-T cells (DN3) (31). If the $\tilde{V}\square\tilde{\square}DJ\beta$ joint is in-frame, the resulting expression of TCRβ protein during β selection in DN4 cells blocks further TCRβ recombination via the process of allelic exclusion (16). Analogous patterns are observed for Ig heavy and then light chain loci during B cell development (32). The mechanisms that regulate patterning of V(D)J recombination play an essential developmental role, providing a means for lymphocytes to test the function of CJs. For example, developing $\alpha\beta$ T cells that fail to generate in-frame joints of either

TCRβ allele, and would never be able to engage antigen, are deleted without further investment (16).

Early experiments demonstrated that the regulation of antigen receptor gene assembly cannot be accounted for simply by differential expression of RAG-1 and RAG-2. Indeed, while ectopic expression of the RAG proteins in murine fibroblasts directs efficient recombination of transiently transfected substrates, endogenous antigen receptor genes remain refractory to recombinase unless transcriptionally activated (33, 34). Likewise, D-J joining at TCRβ and IgH occurs at a developmental stage when TCRβ and IgH V gene segments and V and J gene segments of the TCRα and Ig light chains are not rearranged (16). The developmental patterning of rearrangement has been proposed to reflect programmed decondensation of the chromatin surrounding individual gene segments, making them more accessible to recombinase (30, 35-38). In support of this accessibility model, recombination has been strongly correlated with indicators of chromatin "opening" including hyperacetylation of histones H3 and H4 (39-43) and germline transcription (44-47).

At individual TCR and Ig loci, where one or a small number of enhancers must regulate the activity of multiple V-, D-, or J-associated promoters, targeted enhancer deletion impairs germline transcription (48-55) and leads to a reduction in the levels of histone acetylation over large portions of the targeted locus (56). Significantly, enhancerless loci also show marked decreases in V(D)J recombination (48-55), suggesting that enhancers help coordinate the epigenetic modifications that permit recombinase recruitment. For example, deletion of the TCRβ enhancer, Eβ, leads to a 2-3-fold drop in histone acetylation levels throughout a 30 kb domain that contains

both the D1-J and D2-J cassettes of mouse TCRβ (56), and completely blocks Dβ germline transcription and rearrangement (48, 49). While working with Eugene Oltz, Dr. Sikes previously showed that enhancer activity mediates recombinational accessibility on at least two fronts. Specifically, he found that enhancer activity is required to recruit histone acetyltransferase (HAT) activity to Dβ and Jβ (57), and is also required to activate a germline promoter positioned immediately 5' of the Dß1 gene segment (58). This promoter, termed PDβ1 is also required for Dβ1-toJβ recombination, but is not required for histone acetylation (57, 59), suggesting a stepwise model of enhancer-mediated TCRβ accessibility in which the enhancer first recruits HAT activity to the two Dβ-Jβ domains, and then activates Dβ-associated promoters that relieve other unidentified barriers to Dβ-to-Jβ recombination. Deletion of PDβ1 in knockout mice confirmed Dr. Sikes's *in vitro* results, and further showed that recombination of the second Dβ element, Dβ2, was unaffected by loss of the Dβ1 promoter (60, 61).

A growing list of germline promoters have now been implicated in targeting V(D)J recombination (30, 32, 62, 63). Despite their importance in controlling recombination, exactly how promoters contribute to recombinational accessibility is unclear. Findings in chromatinized templates and the endogenous TCRβ locus have suggested a role for the chromatin remodeling activities of hSWI/SNF (64-66). Since hSWI/SNF and related complexes are recruited to individual promoter sites, one attractive model of promoter-mediated accessibility would have the enhancer working in concert with a select promoter to recruit chromatin remodeling complexes to the promoter-associated gene segment. Indeed, one study has now shown a

direct interaction between the Dβ1 promoter and the TCRβ enhancer, Eß (67). Alternatively, germline transcription activated by each promoter may drive recombinational accessibility by extending initial chromatin opening at the promoter to downstream gene segment targets. While multiple studies have suggested that recombination and transcription do not always correlate (57, 68-71), the Krangel laboratory used targeted knock-in of a transcriptional terminator to show that transcription does indeed provide a significant increase in downstream gene segment usage (72).

TCRβ V elements are each associated with promoters immediately 5' of the leader exon. These promoters are responsible for germline and rearranged expression of their associated V elements, and share a common c-AMP response element-like decamer motif (73). In the mouse, most Vβs are positioned >250kb upstream of the lone identified enhancer, Eβ. Eβ deletion leads to a profound reduction in histone acetylation levels of upstream Vβ elements and blocks D-J recombination (74). Does the enhancer directly promote V-DJβ recombination by modulating chromatin opening at V and D regions?

Studies aimed at addressing the potential role of promoter activity in V-DJ recombination have yielded conflicting results. Early transgenic studies suggested that PVβ activity is dispensible for V rearrangement (75). In contrast, knockout of PVβ13.1 showed a roughly 10-fold decline in Vβ13.1 rearrangements (though Vβ13.1 germline expression was unaffected) (70). In each of these cases, analyses were complicated by the proximity of neighboring Vβ promoters and the developmental pressures and dynamic composition of the thymus. As such, the role

of PVβ in recombination, and its mode of action are still unresolved. Additionally, whereas D-to-J recombination involves a single promoter acting over a short distance, V-to-DJ joining spans a much greater distance and could require the activity of two separate promoters, PVβ and PDβ. The research described in this thesis investigated the role of Eβ, PVβ, and PDβ in directing the V-to-DJ recombination of chromatinized substrates in a RAG-inducible T cell model.

MATERIALS AND METHODS

Tissue Culture

The mature B cell line, M12 (76), the mature T cell line, BW5147 (77), were cultured in RPMI 1640 growth medium supplemented with 10% heat-inactivated FBS, 2mM L-Glutamine, 0.01% penicillin/streptomycin, and 50μM β-mercaptoethanol. In addition, growth medium for the M12-5B3 (57) and BW5147-TRT2.5 RAG-inducible sublcones was supplemented with 3mM Histidinol and 1.5μM tetracycline. Finally, G418 (1.5mg/ml final concentration) was added to 5B3 and TRT2.5 subclones transfected with TCRβ minilocus substrates. For recombinase induction, cells were collected, washed with PBS, and cultured in growth medium that lacked tetracycline for 48 hours.

Building the Vβ-DJβ Recombination Miniloci

The P^+E^+, P^+E^-, P^-E^+, Tet wt, and TET ΔTATA constructs have been previously described (57, 59). To dissect the individual components of recombinational accessibility, a rearrangement substrate was developed which contained unique insertion sites for Vβ14 promoter (EcoR1), Dβ1 promoter (Not I) and enhancer (Xho I) elements. The recombination substrate backbone was prepared by insertion of a 4.8 kb HindIII fragment spanning the Vβ14 gene segment into the HindIII site of pGEM11Z (Promega). The HindIII site downstream of the Vβ14 sequence was destroyed, and either a 900 bp PCR fragment containing the

PDβ1 promoter, a Dβ1Jβ1.1 coding joint, and downstream Jβ1.2 fragment or a 600 bp fragment lacking the PDβ1 amplified from DP C57Bl/6 thymocyte DNA was inserted into the EcoRI site, creating pVβ14-DJ-J. Oligonucleotide primers for DJ fragment amplification included: 5'Dβ1-Kpn (including the PDβ1) (5'-GACGCACAGCCTTAGGG-3') or 3'Dβ1TATA (lacking the promoter) (5'-AAGCTGTAACATTGTGGGGACAGG-3'), each paired with the downstream primer 3'Jβ2A (5'-GGATCCAGCCTCATTAGA-3'). Amplification reaction mixtures (50 μl) each contained thymocyte genomic DNA (500 ng), as well as 10 mM Tris-Cl, pH 8.3; 50 mM KCl; 2 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, and 50 ng of the indicated primers and 2 U of Taq polymerase. Multiple reaction mixtures for each fragment were amplified (94°C, 1 min.; 60°C, 1 min.; 72°C, 1.5 min.) for 15 cycles. PCR product replicates were pooled, blunted, and phosphorylated, precipitated, and purified on a 1% agarose gel prior to ligation. A separate vector harboring deletion the PVβ14 promoter was generated from pVβ14-DJ-J by QuickChange site-directed mutagenesis (Stratagene) as per the manufacturer's instructions using the following oligonucleotide primers: ΔPVβ14: sense (5'-CTCTACCGAATTCGCCTAGACAAAG-3'), antisense (5'-TCTAGGCGAATTCGGTAGAGCTTC-3'). Promoter deletion introduced a unique EcoRI restriction site (underscored in the primer sequences) used to screen clones. Each construct was verified by sequencing.

At the same time, a 9 kb XhoI / EcoRI fragment spanning the Ig_H μ constant region was inserted into the corresponding sites of a separate pGEM11Z plasmid. To this pGEM-Cμ, either an 690 bp StuI/NcoI fragment of the Eβ enhancer or a 475

bp AluI/AluI fragment of the minimal iEκ enhancer was added by blunt-end ligation into the unique Xho I site upstream of the Cμ element. Each final minilocus was prepared by ligating a blunt HindIII/XhoI Vβ14-DJ-J fragment from the 3 pGEM-Vβ-DJ-J variants into the unique NotI site of either pCμE$^-$, pCμEβ, or pCμEκ.

Stable Transfections

The TRT2.5 RAG-inducible cell line was generated by stably transfecting BW5147 cells with linearized pTET-RAG1 (8μg), pTET-RAG2 (7μg), and pTET-Tak (5.5μg) vectors (78), along with linearized pSV-HIS (0.5μg), which encodes resistance to histidinol. For transfection, the indicated amounts of each linearized plasmid were mixed, precipitated, and resuspended in 20μl sterile water. Ten million BW5147 cells in 300μl serum free RPMI 1640 were mixed with the DNAs, and electroporated at 250V, 960μF, ∞ resistance in a 0.4 cm cuvette. Following a 10 min. incubation on ice, the cells were diluted in 100 ml RPMI 1640 supplemented with 1.5μM tetracycline, and plated 1ml/well in 4 24-well plates. After 48 hours of recovery, histidinol was added to a final concentration of 3mM. After 2 weeks, individual clones were isolated by aspiration into separate culture plates. Each clone was separately induced for 48 hours in the absence of tetracycline, and RNA from each was screened for the expression of RAG-1 and RAG-2 by RT-PCR as described below. The TRT2.5 clone was selected based on high-level RAG expression and used for subsequent analyses.

Each TCRβ minilocus was stably transfected into TRT2.5 cells. For the transfection, 10μg minilocus plasmid DNA was linearized with PvuI, precipitated, and

resuspended in 20 μl sterile H_2O along with 1μg of linearized pKJ-1 which encodes resistance to the drug, G418. A total of 10^7 TRT2.5 cells were resuspended in 300ul serum free RPMI 1640, mixed with the DNAs, and added to a 0.4 cm gap electroporation cuvette. The mixture was electroporated (250V, 960μF, ∞ resistance), and then incubated on ice for ten minutes. The cells were then plated in TRT2.5 culture medium. The cells were allowed to recover for 2 days before G418 was added to a final concentration of 1.5mg/ml. After 2 weeks, the resultant heterogenous pool of transfectants was mixed and used for subsequent rearrangement analyses. Individual transfections produced a minimum of 50-100 colonies per plate, with minilocus copy number in each clone varying from 1 to >20.

Chromatin Immunoprecipitaion Assay

Monoclonal antibodies used for ChIP included anti-Histone 3 acetylated K9 (Upstate, 06-942), anti-RAG2 (Santa Cruz, sc-7623), and anti-BrgI (Santa Cruz, sc-10768). Cells were cultured in the presence or absence of tetracycline for 48 hours, and $2x10^8$ were harvested and resuspended in 20 ml of culture media. Protein:DNA complexes were cross-linked by the addition of 550 μl of 37% Formaldehyde (final concentration of 1%) for 10 minutes at room temperature. One milliliter of 2.5M glycine was then added, and the mixture was allowed to incubate at room temperature for 5 minutes. Cells were centrifuged at 1500 rpm for 4 minutes at 4^oC. The supernatant was discarded and the cell pellet was washed 2 times with 10 ml PBS. The pellet was then washed 3 times with 10 ml Run-on Lysis Buffer (10mM Tris-Cl pH7.5, 10mM NaCl, 3mM $MgCl_2$, 0.5% NP-40). The cells were centrifuged at

1000 rpm for 5 minutes in between each wash. The pellet was resuspended in 3 ml of MNase reaction buffer (10mM Tris-Cl pH7.5, 10mM NaCl, 3mM $MgCl_2$, 1mM $CaCl_2$, 4% NP-40) and 60 μl of PMSF. Fifty units of MNase (Sigma) which introduces double strand DNA breaks in the linker region between nucleosomes was added, and the mixture was allowed to incubate at 37°C for 30 minutes. To stop the reaction, 45 μl of 200mM EGTA was added. The cell mixture was then supplemented with 60 μl of PMSF, 300 μl 10% SDS, and 120 μl 5M NaCl. The sample was sonicated at 50% power for 5 30 second intervals separated by chilling on ice. The sonication further sheared chromatin fragments into 300-600 bp sizes. IP dilution buffer (20mM Tris-Cl pH 8.0, 2mM EDTA, 1% TritonX-100, 150mM NaCl, PMSF) was added to bring the final volume to 15 ml.

The chromatin sample was pre-cleared with Protein A-coated agarose beads by adding 100 μl of beads and rocking at 4°C for 15 minutes. The beads were pelleted at 2000 rpm at 4°C for 2 minutes. The supernatant was transferred to a new tube containing 50 μl protein A beads and 10-50 μg of the indicated antibody were added. The sample then rocked overnight at room temperature. Reactions underwent a series of washes (ChIP wash 1, ChIP wash 2, ChIP wash 3, and finally in TE) for 5 minutes each at room temperature. ChIP wash 1 contained 20mM Tris-Cl pH8.0, 2mM EDTA, 1% TritonX-100, 0.1% SDS, 150mM NaCl, 1mM PMSF. For ChIP was 2, the 150mM NaCl in ChIP wash 1 was replaced with 500mM NaCl. ChIP wash 3 contained 10mM Tris-Cl pH 8.0, 1mM EDTA, 0.25M LiCl, 1% NP-40, 1% deoxycholate. After all washes, 200 μl of elution buffer (25mM Tris-Cl pH8.0, 10mM EDTA, 0.5% SDS) was added and samples were incubated at 65°C for 15

minutes. Pronase was added to the supernatant to a final concentration of 1.5 μg/μl and the reaction was incubated at 42°C for 1 hour. The NaCl concentration was adjusted to 200mM and the reaction was incubated overnight at 65°C to reverse the protein:DNA crosslinks. The samples then underwent extraction with phenol:chloroform, and then chloroform alone, before the DNA was precipitated with ethanol. DNA pellets were then resuspended in water for real-time PCR amplification, and quantitated by PicoGreen fluorescence (Molecular Probes) according the manufacturer's directions.

PCR and RT-PCR analyses

The recombination potential and germline transcription of pooled transfectants for each construct was evaluated following induction of RAG-1/2 gene expression. Genomic DNAs were harvested from transfectant cells 48 hours after recombinase induction. For analysis of Vβ-DJβ coding joins, genomic DNAs were isolated following incubation of cells with proteinase K (100 μg/ml, 12 hr at 56°C) by precipitation with an equal volume of isopropanol. Amplification reaction mixtures (50 μl) each contained transfectant DNA (500 ng), as well as 10 mM Tris-Cl, pH 8.3; 50 mM KCl; 2 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, and 50 ng of the Vβ14 REC primer (5'-CCTAAGCACGGAGAAGCTGC-3') and 50 ng of the 3'Jβ2-B primer (5'-ATGTAGTCCCAGACATGAGAGAGCC-3'). Reaction mixtures were incubated at 72°C (3 min) prior to the addition of Taq polymerase (1.1 U), then amplified (94°C, 1 min.; 60°C, 1 min.; 72°C, 1.5 min.) for 32 cycles PCR products were separated on 2% agarose gels.

For analysis of RAG1/2 and germine transcription, total cellular mRNA was harvested from each transfectant using TriReagent (Sigma). Total mRNA (3 μg) from each transfectant was reverse transcribed (42^{O}C, 1 hr) in reaction mixtures (20 μl) containing dNTP mixtures (250 μM), random hexamer (5 pmol), dithiothreitol (8.75 mM), and RNAsin (20 U, Promega), as well as MuLV Reverse Transcriptase (100 U, New England Biolabs), followed by heat inactivation (75^{O}C, 15 min). To examine germline expression, the cDNAs (300 ng) were amplified for 27 cycles. Amplification products were separated on 1% agarose gels, and examined by Southern blotting. Primers for amplification and probes included: Vβ14 RNA (5'-TCCTGGGCATGTTCTTGGGTG-3') and Vβ14 RS (5'-GAAGGACTAAAGAGAGGGTGTGGT-3'). PCR products were detected separated on a 1% agarose gel, blotted to Zeta-Probe (BioRAD), and hybridized to radiolabeled probes positioned within the amplicon. Products were visualized by autoradiography.

Real-time PCR

For quantitative amplification of RAG1/2 expression in TRT subclones of BW5147, RNA was harvested from M12 control cells or the indicated TRT subclones 48 hours after RAG induction using TriReagent (Sigma), and reverse transcribed as described above. Separate 25 μl PCR reactions for expression of RAG1, RAG2, and the constitutively expressed β-actin message were run in triplicate for each of two RNA harvests using 300 ng cDNA mixed with the appropriate oligonucleotide primers (1 μM) and Sybr Green Master Mix (BioRAD). Each amplification reaction

was cycled 50 times (94^{o}C, 30 sec.; 55^{o}C, 30 sec.; 72^{o}C, 30 sec.) on a MyiQ cycler (BioRAD), along with a 6 log dilution curve of wildtype C57Bl6 thymocyte cDNA. Relative concentrations from each C_T value were generated, and fold RAG induction was calculated in each TRT sample by adjusting RAG values to β-actin, and then normalizing adjusted RAG levels from induced cells to those in matched uninduced cells. Oligonucleotide primers used to assess RAG transgene expression in TRT subclones included: RAG1 (5'-CCGCTAAAGAGTGTCCAGAGTCCC-3'); RAG2 (5'-AACCTCCAATGAAATCCCTCCAC-3'); each paired with the downstream primer TETRAG3' (5'-GCCCTGCCACTCATCGCAG-3'); 5'β-actin (5'-AGAGCTATGAGCTGCCTGACGGCC-3'); and 3'β-actin (5'-AGTAATCTCCTTCTGCATCCTGTC-3').

To assess changes in chromatin organization or availability following promoter or enhancer deletion, 2 ng each of the DNAs isolated by ChIP, was amplified in triplicate 25 μl reactions containing 1 μM concentrations of the appropriate primers (listed below), Syber Green Master Mix (BioRAD), along with a 5 log dilution curve of DNA isolated from each transfectant cell harvest prior to antibody addition. Each amplification reaction was cycled 50 times (94^{o}C, 30 sec.; 53^{o}C, 30 sec.; 72^{o}C, 30 sec.) on a MyiQ cycler (BioRAD). Absolute concentrations from each C_T value were generated, and fold enrichment was calculated for each DNA target in the immunoprecipitated samples over the matched input sample. Values were then normalized to values obtained for separate amplifications of the TEA or Cμ untranslated region negative control target sequences as indicated. Forward and reverse primers for each of the DNA regions assessed by ChIP

included respectively: Vβ14 sense (5'-CACTGAGTAGGGTGGGGC-3'); Vβ14 antisense (5'-CTTGAATCATGTTGTTTTCCAGAC-3'); Dβ1 sense (5'-TCCTTATCTTCAACTCCC-3'); Dβ1 antisense (5'-GGATCTAAACACATCTAGGC-3'); Jβ1.1 sense (5'-CAGCTCTTGATGAATATCATCATAGG-3'); Jβ1.1 antisense (5'-AAAGCATGTCCTCCGTGTCC-3'); CμUT sense (5'-TTGCTTGCTCTGCACACACCCTGC-3'); CμUT antisense (5'-CCTGAATGCTGCCTGCACCAGG-3'); TEA sense (5'-AGTCGATGCTGCTTGAATTCTCCC-3'); TEA antisense (5'-TTTCAAGTTGGCATCTGGACGTGG-3').

RESULTS

Chromatin structure and recombinational accessibility

Recombination is essentially a two-step process involving RAG-dependent cleavage and the subsequent ligation of liberated ends by ubiquitous double-strand break repair enzymes (17). Dr. Sikes' previous finding that signal end (SE) intermediates generated by RAG cleavage prior to ligation is promoter- and enhancer-dependent (59) suggests that recombination is imposed at the level of RAG accessibility to RS targets. To measure the impact promoter and enhancer activity has on RAG access to substrate targets, we used chromatin immunoprecipitation (Figure 1) coupled with real-time PCR to quantify the accessibility of RS sequences in 5B3 transfectants of a wildtype minilocus (P^+E^+), or miniloci from which either the Dβ1 promoter (P^-E^+) or the enhancer (P^+E^-) had been deleted (Figure 2).

Eurkaryotic DNA is wrapped around a protein core of histone molecules to form chromatin. The degree to which chromatin is condensed has long been correlated with gene regulation in general, and V(D)J recombination specifically. When the N-terminal tails of histones 3 and 4 gain covalent acetyl additions at select residues, their ability to form condensed or hetero-chromatin is greatly diminished (79, 80). Consequently, acetylation of lysine 9 on histone 3 has long served as a marker of open or euchromatin, and is found in recombinationally active loci.

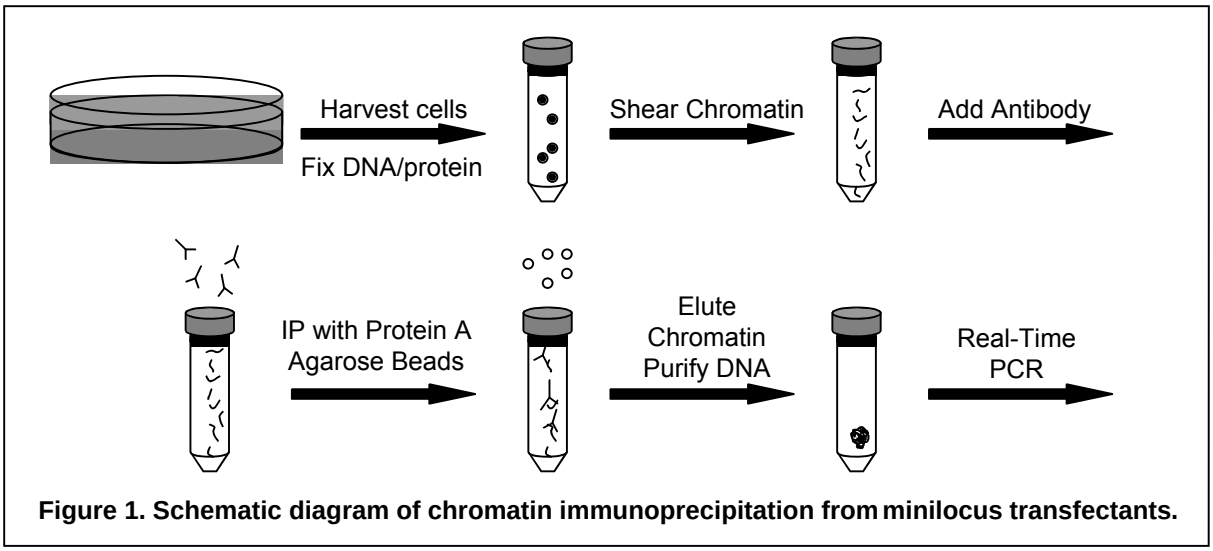
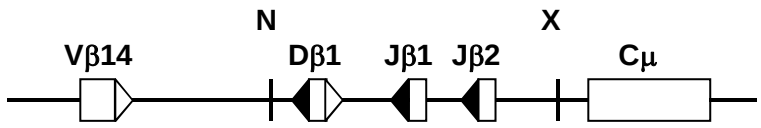


Figure 1. Schematic diagram of chromatin immunoprecipitation from minilocus transfectants.



Minilocus		
P^+E^+	PDβ1	Eκ
P^+E^-	PDβ1	ϕ
P^-E^+	ϕ	Eκ

Figure 2. Miniloci to test the role of promoter activity in RS accessibility. Miniloci with or without PDβ1 or the B cell-specific enhancer, Eκ, were stably transfected into the RAG inducible B cell line, M12 5B3. Unique cloning sites including NotI (N), and Xho I (X) are indicated.

Histone 3 acetylation was measured using real-time PCR to test the extent to which enhancer and/or the promoter activity impact the chromatin organization at the minilocus Vβ, Dβ, and Jβ RS sequences. The DNA fragments used in the PCR were the result of the chromatin immunoprecipitation assay (Figure 1), in which genomic DNA within each transfectant cell was covalently linked to its associated histones (or any bound protein) in the presence of formaldehyde, and then the cellular chromatin was mechanically and enzymatically sheared into 200-600 bp DNA lengths. Antibodies to the acetylated histone 3 tail bound only the DNA fragments associated with open chromatin, and antibody:chromatin complexes were purified from the total chromatin population in the presence of Protein-A coated agarose beads.

Purified DNA from each IP was amplified at three different locations along the germline TCR gene segments. The PCR primers were designed to amplify across the RS sequences of the V, D, and J regions. Because the IPed DNA is always contaminated with unbound sequences, amplification values for each IPed sample were expressed as fold above those obtained from input chromatin (ie, before antibody addition). Adjusted values for each IP were then normalized to those obtained from PCR of the T early alpha (TEA), a germline TCRα promoter that has previously been shown to exist in a heterochromatic (ie, hypoacetylated) form in 5B3 cells (59). As can be seen in Figure 3A, elevated levels of histone 3 acetylation were detected across each of the gene segments in both P^+E^+ and P^-E^+. The relative differences between the levels of Dβ and Jβ histone acetylation have been shown before (57).

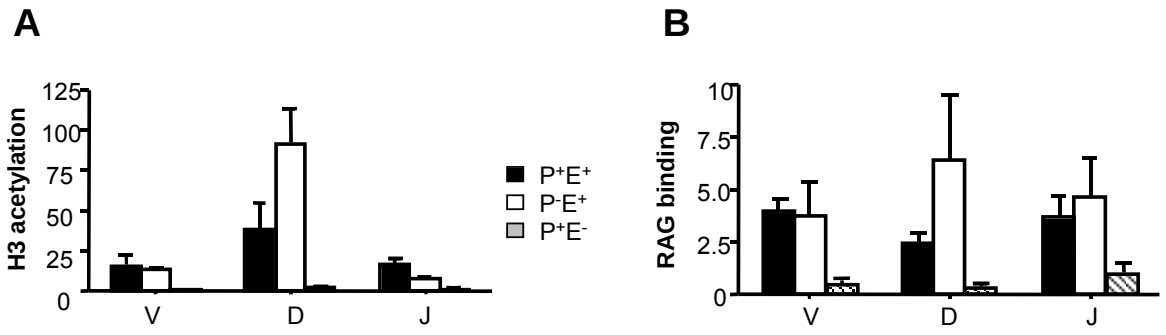


Figure 3. Chromatin accessibility at Vβ14 is enhancer-dependent. (**A)** ChIP assays for acetylation of histone H3 associated with P^+E^+, P^+E^-, and P^-E^+ substrates. Real-time PCR was used to obtain quantitative data for histone acetylation at the Vβ14, Dβ1, and Jβ1 RS sequences within each substrate. Values were normalized to the hypoacetylated TEA region of TCRalpha. **(B)** ChIP assays for RAG binding at the Vβ14, Dβ1, and Jβ1 RS sequences within P^+E^+, P^+E^-, and P^-E^+ substrates. Real-time PCR was used to obtain quantitative data for enrichment of each RS sequence in protein:DNA complexesimmunoprecipitated with anti-Rag2 Ab. Values were normalized to the Rag-refractory μ constant region of IgH. Bars represent mean + SEM for 3 independent amplifications from each of 3 immunoprecipitations.

More recently, a study of chromatin accessibility in a RAG-deficient DN3 cell line also showed that a small subset of Vβ elements assayed showed less histone acetylation than Dβ1 (65). Our results therefore appear consistent with the literature. There seems to be no difference in acetylation in any of the gene segments with or without the promoter. Again, this is consistent with Dr. Sikes's previous findings for histone acetylation between D and J segments (57). However, the deletion of the enhancer dramatically reduced histone 3 acetylation across all three gene segments, suggesting that the enhancer plays a major role in RS accessibility throughout the TCRβ locus.

To correlate the histone acetylation findings with the availability of each RS for recombination, I used ChIP assays to directly measure RAG protein binding at the minilocus V, D, and J RS targets (Fig. 3B). Because RAG2 is only recruited to the RS as part of a multimeric complex with RAG1, and does not directly contact DNA itself, I performed the ChIP assays on cells grown in the absence of tetracycline for 48 hours using anti-RAG2 Abs to detect only recombinationally "competent" protein:DNA assemblies. To control for variations in the efficiency of immunoprecipitation or DNA loading, each realtime PCR signal was first normalized to the antibody untreated controls, and then expressed as fold above that detected within the 3' untranslated region of Cμ (an RS-free portion of the minilocus over 9 kb downstsream of the Jβ1.1 RS). The use of the Cμ UTR for normalization was preferable to a sequence outside the minilocus since it would be subject to the same variations in transgene copy number as the RS sequences.

As with histone acetylation, RAG binding was essentially dependent on the presence of an enhancer within the minilocus. The P^+E^- transfectants showed little RAG binding to either the V, D, or J1.1 RS. The slightly enriched binding of RAG complexes at the Jβ1.1 RS relative to the V and D RSs likely reflects the higher affinity of this RS for RAG (81). Unexpectedly, RAG binding to each minilocus RS was not effected by deletion of PDβ1, despite the fact that P^-E^+ shows a dramatic (>30-fold) reduction in D-to-J recombination (59). Likewise, RAG was readily detected binding to the Vβ14 RS in both the wildtype and promoterless transfectants despite the absence of Vβ recombination. Together, these findings suggest that promoter-mediated recombinational accessibility could be regulated at a point other than the ability of RAG proteins to bind their RS targets.

The transcriptional activity of PDβ1 is dependent on a TATA box positioned within the 5'RS of Dβ1. Because of its unique location, the TATA box was not removed when Dr. Sikes originally constructed the P^-E^+ minilocus. Therefore, even though the promoterless minilocus is recombinationally defective, I wanted to determine if the TATA box was occupied. Attempts to use ChIP to detect the presence of the TATA binding protein (TBP) failed. However, it is well documented that TBP binding leads to the recruitment of the SWI/SNF chromatin remodeling complex, which forces histones away from the site of transcriptosome formation during transcription initiation (82). Therefore, to indirectly assess the occupancy of the PDβ1 TATA box in promoterless transfectants, I used ChIP assays to measure binding of the ATPase subunit of SWI/SNF, BrgI (Figure 4). A recent study of the chromatin in a RAG-deficient pro-T cell line found that Dβ1 chromatin showed higher

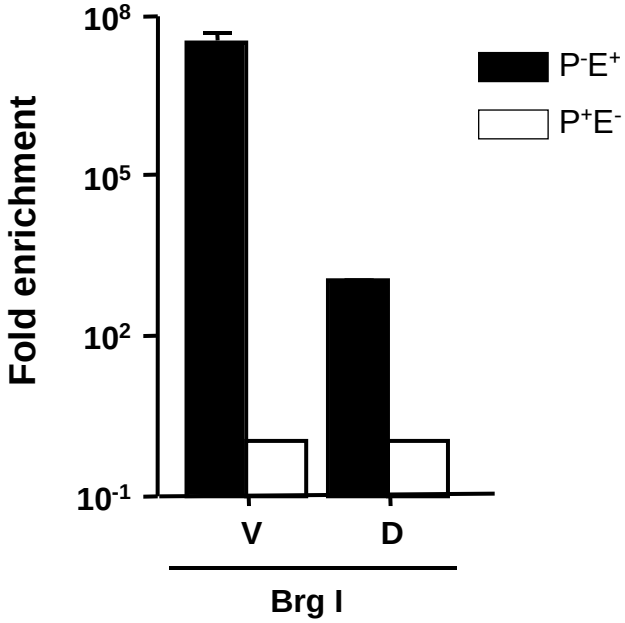


Figure 4. Promoter activity recruits SWI/SNF chromatin remodeling complexes. ChIP assays were used to measure binding of the Brg I subunit of SWI/SNF within P^+E^- and P^-E^+ substrates. Real-time PCR was used to quantitate enrichment of Vβ14 and Dβ1 RS sequences within the immunoprecipitated protein:DNA complexes of each sample. Values were first normalized to the transcriptionally inactive TEA region of TCRalpha. Additionally, values obtained for the promoterless transfectants were expressed as fold enrichment over those obtained for paired amplifications of the inert enhancerless transfectants. Bars represent mean + SEM for 3 independent amplifications from each of 2 immunoprecipitations.

levels of histone 3 acetylation and BrgI binding than any of the upstream Vβ elements and roughly equivalent levels of BrgI binding as Vβ14 (65). While my histone acetylation analysis of P^+E^+ transfectants mirrors those results, and while histone acetylation and RAG binding in the minilocus were unaffected by PDβ1 deletion (Figure 3), loss of the Sp1 and GATA-3 binding sites that drive PDβ1-dependent transcription (58) clearly reduced the ability of the TATA box to recruit BrgI below that seen for Vβ14. When compared to the findings of Morshead et al., this represents a >5-log drop in BrgI recruitment. Because RAG binding is normal despite this dropoff in BrgI binding, I suggest that chromatin remodeling induced by BrgI might be necessary to allow the bound RAG to synapse with RAG complexes bound at the Jβ elements. Indeed, the Oltz laboratory has recently shown that artificial targeting of the BRG1 component of hSWI/SNF 5' of Dβ1 is sufficient to restore D-to-J recombination in promoterless miniloci (83).

Because the TATA box serves as a nucleation site for an entire array of proteins other than BrgI, I separately assessed the ability of RAG to bind the D and J RS elements in constructs in which PDβ1 and the enhancer were replaced with a simplified Ptet promoter (Figure 5). Ptet links heptamerized binding sites for the chimeric tTAk transcription factor (containing the Tet Repressor DNA binding domain and the VP16 transactivation domain (78)) with the PDβ1 TATA box found inside the Dβ 5'RS. When he compared transcription and Dβ-to-Jβ recombination of wildtype miniloci (TET wt) with that of miniloci harboring a mutated TATA box (TET ΔTATA) Dr. Sikes had previously found that TATA mutation dramatically reduced Dβ germline transcription, but also correlated with a >10-fold reduction in Dβ-to-Jβ

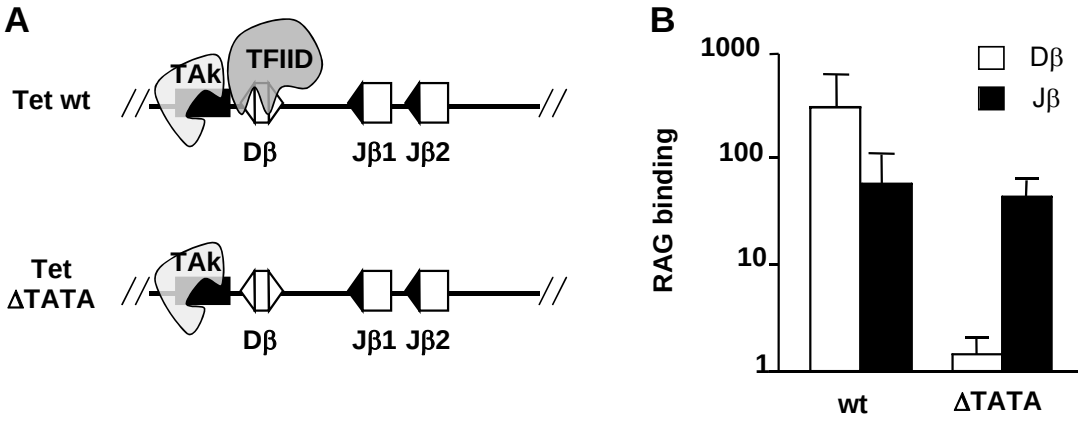


Figure 5. RAG binding requires TATA-dependent activities. (**A)** Schematic diagram of the DJβ regions of two miniloci containing the enhancer-independent Ptet promoter coupled to either the wildtype Dβ1 5′RS (Tet wt) or to a Dβ1 5′RS from which the resident TATA box has been removed by site-directed mutagenesis.**(B)** ChIP assays for RAG binding at the Dβ1 and Jβ1 RS sequences within Tet wt and Tet ΔTATA substrates. Real-time PCR was used to obtain quantitative data for enrichment of each RS sequence in protein:DNA complexesimmunoprecipitated with anti-Rag2 Ab. Values were normalized to the Rag-refractory constant region of Ig lambda. Bars represent mean + SEM for 3 independent amplifications from each of 2 immunoprecipitations.

recombination (Sikes and Oltz, unpublished observations). When I assessed RAG binding at the D and J RSs of these transfectants 48 hours after RAG induction, I found that RAG binding to the J RS was unaffected by TATA mutation. By contrast, mutation of the Dβ1 TATA mutation dramatically impaired RAG binding at the Dβ1 RSs despite the ability of the TET promoter to recruit its chimeric TAk transcription factor. The inefficiency with which promoterless miniloci recruit BrgI to the Dβ TATA box, coupled with the sensitivity of RAG binding to a TATA mutation in the Ptet-containing substrates, strongly suggest that promoter activation induces multiple changes in the Dβ RS chromatin that are separately necessary for RAG binding and then rearrangement.

Promoter regulation of Vβ-to-DJβ recombination

Although multiple studies have identified roles for promoter and enhancer activities in initially directing recombinase to the Dβ 3'RSS and Jβ RSS (48, 49, 57, 59-61, 67, 84), it remains unclear how recombinase is subsequently redirected to more distal Vβ elements to complete assembly of the TCRβ coding exons. TCRβ V elements are each associated with promoters immediately 5' of the leader exon. These promoters are responsible for germline and rearranged expression of their associated V elements, and share a common c-AMP response element-like decamer motif (73). In the mouse, most Vβs are positioned 250kb to 1000kb upstream of the lone identified enhancer, Eβ. It is somewhat contentious whether Vβ promoter activation is Eβ-dependent (most recently, studies showed that Vβ transcription of a TCRβ transgene in DN2/DN3 thymocytes was Eβ-independent

(71)). However, Eβ deletion leads to a profound reduction in the histone acetylation levels of upstream Vβ elements and blocks TCRβ recombination (56, 85), suggesting that Eβ might promote V-DJβ recombination by modulating the chromatin opening at V and D regions.

Studies aimed at addressing the potential role of PVβ in V-to-DJ recombination have also yielded conflicting results. Early transgenic studies suggested that PVβ activity is dispensable for V rearrangement (75). In contrast, recent knockout of PVβ13.1 showed a roughly 10-fold decline in Vβ13.1 rearrangements, despite no effect on Vβ13.1 germline expression (70). In each of these cases, analyses were complicated by the proximity of neighboring Vβ promoters (as evidenced by the above-mentioned transcription following PVβ13.1 deletion) and the developmental pressures and dynamic composition of the thymic environment. Consequently, the role of PVβ in recombination, and its mode of action remain unresolved. Additionally, whereas D-to-J recombination involves a single promoter acting over a short distance, V-to-DJ joining spans a much greater distance and could require the activity of two separate promoters, PVβ and PDβ.

In investigating the regulation of Vβ recombination, I wished to address multiple outstanding questions. First, once the enhancer has opened the locus and driven joining of the DJ substrate used in V-DJ assembly, does it play a similar role in driving V recombination? Secondly, does PDβ control V-to-DJ rearrangement after D-to-J joining has occurred, or does PDβ become dispensable? Third, does promoter activity 5' of Vβ contribute to rearrangement?

Whereas the M12 5B3 recombinase-inducible B cell line was effectively used to test the roles of promoter and enhancer activity in driving D-to-J recombination, previous studies using TCRβ miniloci in transgenic mice showed that Vβ recombination only occurs in T cells (86). Therefore, to investigate Vβ recombination control, Dr. Sikes and I first worked to generate a recombinase-inducible T cell model by cotransfecting tet-responsive RAG-1 and RAG-2 expression vectors (78) and a plasmid encoding resistance to histidinol into the murine BW5147 mature T cell line. Importantly, BW5147 has rearranged both TCRβ and TCRα alleles, but does not express a functional TCR (77). As such, endogenous TCRβ rearrangements would not contribute to Vβ recombination signals I would be measuring in subsequent experiments.

After screening multiple homogeneous stable transfectants by Southern hybridization for genomic incorporation of the TETRAG constructs, we selected two clones containing >4 copies each of TET-Rag1 and TET-RAG-2. These clones, termed TRT2.5 and TRT4.6, were grown under RAG-inducing conditions (tetracycline-free media) along with the 5B3 recombinase-inducible B cell line for 48 hours. RNA from induced and matched uninduced cells was collected and reversed transcribed. Real-time PCR was then performed to measure the levels of RAG1 and RAG2 that were produced by each cell line. Figure 6 shows the fold induction of RAG1 and RAG2 in each cell above that in the uninduced controls. Both T cell lines showed high levels of RAG1 induction (>100-fold above basal levels), similar to that expressed in the B cell line, with TRT2.5 inducing TETRAG1 as much 100,000 fold over basal levels. However, the TRT clones expressed substantially less RAG2

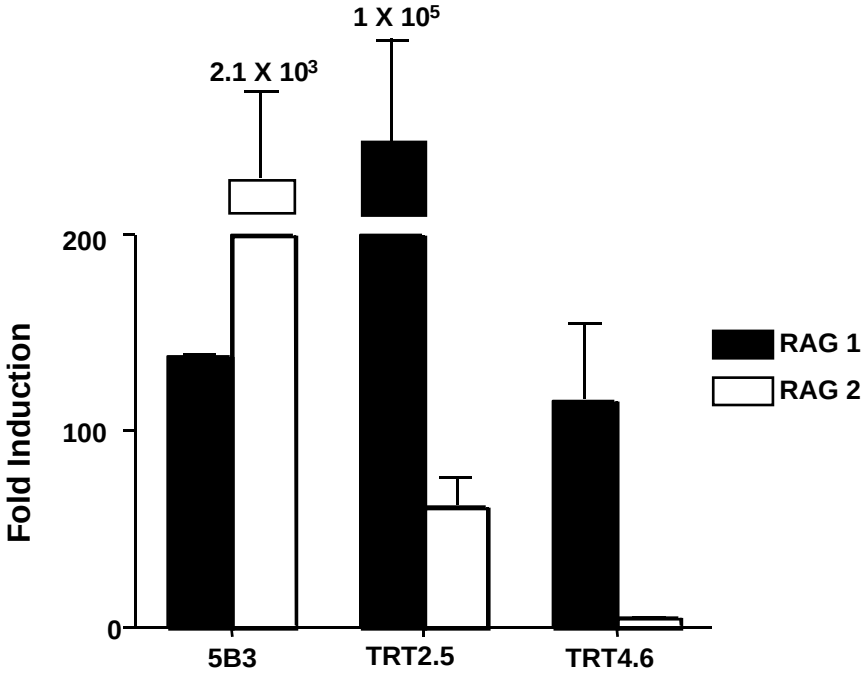
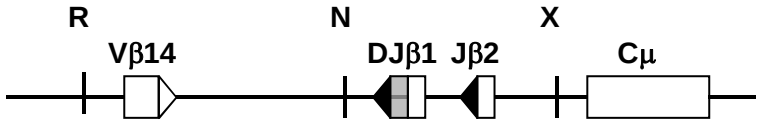


Figure 6. RAG expression in inducible T cell lines. Expression of TET-RAG1 (black bars) and TET-RAG2 (white bars) in 2 different T cell lines (TRT2.5 and TRT4.6) was measured 48 hours after tetracycline withdrawal by reverse transcription-coupled real-time PCR. Values for each transcript were controlled for loading variability by normalizing to co-amplified β-actin transcripts, and then expressed as fold above those obtained from matched cultures grown in the presence of tetracycline. Bars represent mean + SEM for 3 separate amplifications of 2 independent inductions per clone.

after induction than did 5B3 (TRT2.5 = 48-fold, and TRT4.6 = 8-fold over background). These results were also verified by gel electrophoresis of traditional semi-quantitative PCRs. Importantly, no RAG RNAs were detected in any of the clones assayed when PCRs were cycled as many as 35 times (data not shown). Despite reduced levels of RAG2, TRT2.5 was chosen for subsequent analyses of Vβ recombination in transfected miniloci.

In addition to developing TRT2.5, testing the role of promoter activity in Vβ recombination necessitated that I modify the TCRβ minilocus by replacing the Igκ enhancer with Eβ, and replace germline Dβ1 and Jβ1.1 with a preformed DJβ1 joint (Fig. 7, PVβEβ). Four additional constructs were also made to separately test the contributions of enhancer and promoter activity to Vβ recombination. These included an enhancerless substrate (PVβE$^-$), a substrate containing the Igκ enhancer (PVβEκ), a substrate lacking the Vβ14 promoter (PV$^-$Eβ), and finally a substrate that contained both the PVβ and Eβ elements, but lacked the Dβ promoter (PVβEβPD$^-$),

Each construct was linearized and stably transfected into TRT2.5 along with a plasmid encoding resistance to the drug, G418. After two weeks, two heterogeneous pools of each transfectant were assessed for Vβ transcription (Fig. 8). Importantly, Dr. Sikes has previously shown that such bulk transfectant pools contain between 10 and several hundred individual clones that contain from 1 to >20 copies of the inserted TCRβ minilocus (87). Despite the presence of a strong DNA contaminant in each sample, RT-PCR analyses of Vβ germline transcription quantitatively detected a transcript that initiated upstream of the leader exon, spliced to the second exon,



Minilocus			
PVβEβ	PVβ14	PDβ1	Eβ
PVβE$^-$	PVβ14	PDβ1	ϕ
PVβEκ	PVβ14	PDβ1	Eκ
PV$^-$Eβ	ϕ	PDβ1	Eβ
PVβEβPD$^-$	PVβ14	ϕ	Eβ

Figure 7. Miniloci to test the role of promoter activity in V-to-DJ recombination. Miniloci with or without PVβ14, with or without PDβ1, and with Eβ, Eκ, or no enhancer were stably transfected into the RAG inducible T cell line, TRT2.5. Unique cloning sites including EcoRI (R), NotI (N), and Xho I (X) are indicated.

and continued through the Vβ14 RS of PVβEβ (Fig. 8A, lanes 1,2 and 7-9). Because the downstream primer was positioned within the Vβ14 RS, and V-to-DJ rearrangement would have deleted the primer binding site, allowing me to identify the transcript as germline. Consistent with the negative impact Eβ deletion has on Vβ chromatin structure in the endogenous TCRβ locus (84), the enhancerless minilocus showed no Vβ transcriptional activity (lanes 3, 4), while the less active Eκ enhancer was able to drive wildtype levels of Vβ transcription (lanes 5, and 6). Dr. Sikes had previously shown that deletion of PDβ from the TCRβ minilocus abolishes Dβ germline transcription. To confirm that Vβ germline transcription detected in Fig. 8A was being directed by the presumed Vβ14 promoter, I tested the ability of a Vβ promoterless minilocus to drive Vβ germline transcription (Fig. 8B). As predicted, PVβ deletion was as effective as Eβ deletion in blocking transcription through the germline Vβ sequences (lanes 3-6), while transcription in wildtype and PDβ deficient controls was essentially normal (lanes 1-2 and 7-8, respectively).

My analyses of transcriptional control confirmed that both promoter and enhancer activities are required to direct Vβ transcription prior to recombination. To determine if each element also contributed to V-to-DJ recombination, I induced RAG expression in each bulk clone for 48 hours, and then assessed rearrangement status in crude PCR extracts made from each induced sample and matched uninduced controls (Fig. 9). As with transcription, I found that Vβ14 recombination was absolutely dependent on the inclusion of an enhancer element. While two

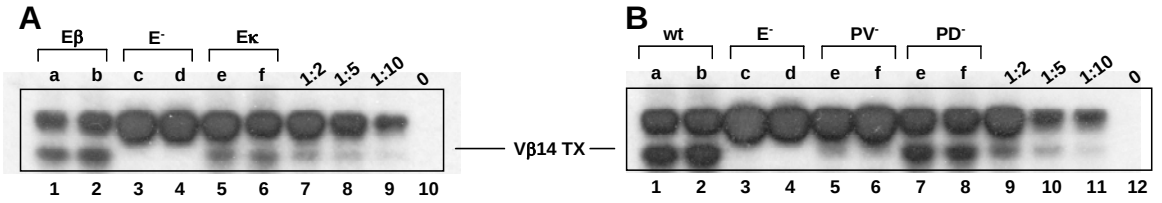


Figure 8. Vβ transcription is enhancer and promoter-dependent. (A) PCR assay for Vβ14 germline transcription in two independent TRT2.5 transfectants each of PVβEβ (lanes 1 and 2), PVβE- (lanes 3 and 4), or PVβEκ (lanes 5 and 6). The linearity of amplification was determined by serial dilution of the PVβEβ clone a (lanes 7-10). **(B)** PCR assay for Vβ14 germline transcription in two independent TRT2.5 transfectants each of wildtype PVβEβ (lanes 1 and 2), or PVβEβ substrates lacking either the enhancer (lanes 3 and 4), the Vβ14 promoter (lanes 5 and 6), or the Dβ1 promoter (lanes 7 and 8). The linearity of amplification was determined by serial dilution of the wildtype PVβEβ clone a (lanes 9-12). All samples were from cells cultured in the absence of RAG induction.

independent transfectant pools of the wildtype PVβEβ exhibited V-to-DJ recombination 48 hours after RAG induction (9A, lanes 1-4), neither of two enhancerless transfectants exhibited any detectable Vβ assembly (lanes 5-6). However, the level of minilocus Vβ recombination in TRT2.5 transfected with PVβEβ was substantially below that seen in the wildtype thymus (9B, compare lanes 1 and 3), despite there only being a single V present in the minilocus vs 20 V's in the endogenous locus. Such inefficiency appears to be a limitation inherent in all recombinase-inducible cell systems, having been previously show (87, 88).

Despite the low levels of V-to-DJ recombination I observed in the transfectants, the sensitivity of PCR allowed me to test the impact of promoter deletion on PVβEβ assembly. Interestingly, deletion of either PVβ or PDβ severely impaired Vβ recombination (lanes 6 and 7), despite the fact that Vβ transcription was normal in PDβ-deficient constructs. Similarly, replacement of Eβ with the Eκ enhancer blocked Vβ recombination (lane 4) despite the fact that it had had only a very modest impact on germline transcription. These findings show that Vβ recombination is regulated very similar to Dβ recombination, requiring both promoter and enhancer activity. However, while Dβ recombination can be driven by any active enhancer, Vβ rearrangement may specifically require Eβ. Also, V-to-DJ joining requires promoter activity proximal to both RS elements, likely because of the distance between the V and D.

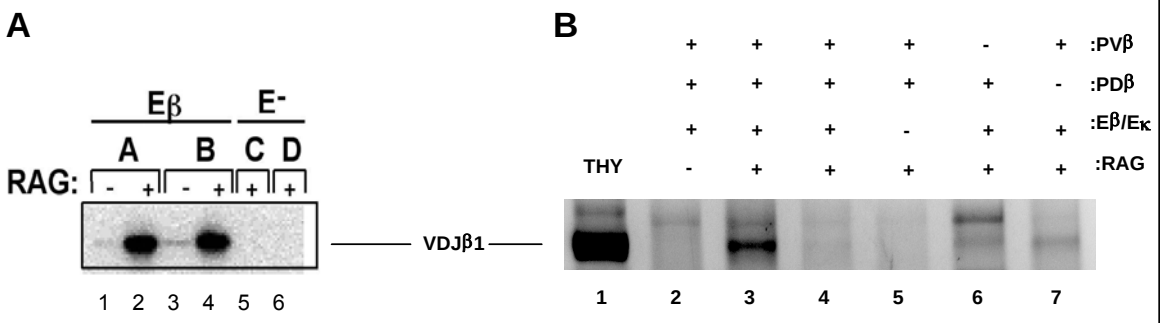


Figure 9. The Role of enhancer and promoter activity in V to DJ Recombination. (A) PCR assays for V-DJ rearrangement in TCRβ miniloci harboring a preformed DJβ joint and either the Eβ enhancer (lanes 1-4) or no enhancer (lanes 5, 6). **(B)** PCR assay for Vβ14 recombination in TRT2.5 transfectants harboring a preformed DJβ1 joint together with the indicated combinations of Vβ14 promoter (PVβ), Dβ1 promoter (PDβ), and either Eβ (lanes 2, 3, and 6, 7), Eκ (lane 4), or no enhancer (lane 5). Lane 2 represents a construct harboring promoters in front of both V and D, together with Eβ, but in the absence of RAG induction. Lane 3 represents the same substrate 48 hours after RAG induction. All other samples are shown post RAG induction.

CONCLUSIONS

Antigen recognition by the adaptive immune system depends on the generation of a diverse and dynamic repertoire of antigen receptor molecules. The genes for receptors are generated *de novo* during lymphocyte development by the somatic DNA rearrangement process, V(D)J recombination (89). How V(D)J recombination is differentially used to assemble the various Ig and TCR genes during B and T cell development appears rooted in the sequence and conformation of the antigen receptor genes themselves, and not imposed at the level of the recombinase enzymes. To date, limited insight has been gained regarding the *cis* elements that control V(D)J recombinational accessibility. Clearly, enhancers positioned within each locus are critical for rearrangement of their associated gene segments, playing dual roles to both target histone acetylation events that relax heterochromatin condensation, and separately driving activation of promoters that flank individual gene segments (83). A growing body of evidence also suggests that these germline promoters contribute to recombinational accessibility (83); though their mechanism of action remains unclear.

To begin dissecting the roles of promoter activity in V(D)J recombination, I made use of a novel recombinase-inducible cell system, the components of which include progenitor lymphocyte cell lines engineered to express high levels of RAG1 and RAG2 upon withdrawal of tetracycline from the culture medium, and a series of modified TCRβ rearrangement substrates stably transfected into the inducible cell lines. Importantly, previous work by Dr. Sikes and his former mentor had shown that

recombinase-inducible cell systems faithfully recapitulated the enhancer- and promoter-dependence of the endogenous mouse TCRβ gene locus (59, 87, 88). Using this model system, I addressed two overarching questions: (i) how does promoter deletion lead to a block in Dβ-to-Jβ recombination? and (ii) what promoter and enhancer activities are necessary for subsequent Vβ-toDJβ recombination?

Dr. Sikes had previously shown that deletion of either the enhancer or the Dβ1 promoter from TCRβ miniloci stably transfected into the recombinase-inducible B cell line, 5B3, lead to a profound block in Dβ-to-Jβ recombination. Using chromatin immunoprecipitation and realtime PCR, I examined each of these transfectants to determine what impact promoter or enhancer deletion had on the accessibility of individual gene segments to either the RAG proteins or the BrgI component of the SWI/SNF chromatin remodeling complex.

In his previous laboratory, Dr. Sikes had shown that while PDβ1 is essential for D-to-J recombination in TCRβ miniloci, it does not contribute to histone acetylation of DJβ1 chromatin (57). My findings (Fig. 3A) corroborate these earlier findings. So how might PDβ1 be regulating recombination? When a promoter is activated, it has a profound impact on the structure/organization of associated sequences. Most eukaryotic promoters act only when in physical communication with enhancer elements. In the case of the antigen receptor loci, this communication involves the bending/looping of often hundreds of kilobases of sequence to bring the promoter and enhancer elements together (90). Additionally, transcription initiation involves recruiting ATP-dependent chromatin remodeling complexes to the promoter site. These chromatin remodelers act to push histones off of the core promoter

elements such as TATA boxes, initiator sequences, and downstream promoter elements (DPEs) (82), necessary to allow formation of the transcription preinitiation complex (PIC). Once the PIC is assembled, ATP is cleaved again to provide the energy necessary to transiently melt apart the double-stranded DNA at the transcription fork. Finally, transcription is propagated down the gene as the RNA polymerase tracks along the sense strand in a complex that retains chromatin remodeling and DNA melting activities (91). In truth, any of these activities could conceivably be essential for providing RAG protein access to the RS sequences that flank the transcribed V, D, and J gene segments.

Unexpectedly, when I assessed RAG binding at the D and J gene segments of wildtype and promoterless TCRβ miniloci, I found that RAG binding at the Dβ1 RS was NOT impaired by promoter deletion (Fig. 3B). How can this be explained given the promoterless substrate's profound defect in D-to-J rearrangement? First, when the TATA box was destroyed in a simplified construct RAG binding was drastically impaired at the Dβ1, but not at the downstream Jβ RS (Fig. 5), suggesting that assembly of the transcriptosome PIC components at the TATA box is essential for the Dβ RS to gain access to RAG. In fact, when the PDβ1 was deleted in a knockout mouse model, a lone CpG dinucleotide uniquely positioned inside the Dβ1 3'RS remained hypermethylated, whereas it was hypomethylated in wildtype alleles (61). Perhaps assembly of the PIC is responsible for directing demethylation of this CpG, which in turn facilitates RAG binding to the RS. Since the Jβ RS elements do not have similar CpG dinucleotides, their ability to bind RAG might not be similarly effected by PDβ1 deletion.

Recent studies suggest that functional synaptic complexes are formed only when RAG-1/2 binds first to a 12-RS, and then recruits a compatible 23-RS, while separate binding of RAG-1/2 to independent 12- or 23-RSs will not give rise to rearrangement (92). Because the 5B3 cell system induces super-physiological levels of RAG expression (87), it is possible that the bulk of RAG binding detected in the promoterless transfectants represents recombination dead-ends. Two controls argue against this possibility as a stand-alone explanation for the observed binding. First, Dr. Sikes never saw any differences in D-to-J recombination in the wildtype, promoterless, or enhancerless substrates when he titrated the amount of RAG-1 and –2 induced in each transfectant (Sikes and Oltz, unpublished data). Second, the RAG-2 in the bound complexes is phosphorylated and degraded as the cell enters S phase (16). Since 5B3 cells are rapidly cycling cells, RAG:DNA complexes in these cells would be continually recycling. More likely, the RAG binding detected at Dβ in the promoterless miniloci reflects RAG binding to both the Dβ 5' and 3' RSs. Given their close proximity to one another, it would be impossible to distinguish RAG binding at one D RS relative to the other by ChIP unless separate constructs were generated in which each RS was individually destroyed.

Regardless of whether some or all of the RAG-bound complexes detected by ChIP represent nonfunctional associations, they clearly demonstrate that the V, D, and J sequences in the promoterless miniloci are fully accessible to RAG binding. Consequently, the uncoupling of RAG binding and recombination in promoterless miniloci might suggest that additional steps after RAG binding are necessary for the formation of a recombination complex. In fact, I found that the promoter-proximal

Dβ1 TATA box is significantly less effective at recruiting the BrgI component of SWI/SNF in promoterless miniloci than is the upstream Vβ core promoter (Fig. 4). Given that the SWI/SNF complex has been shown to play an essential role in the rearrangement of chromatinized oligonucleotide models *in vitro* (93), loss of BrgI in the promoterless miniloci could well explain the mechanism by which PDβ1 confers recombinational accessibility to the Dβ1 RS. We suggest that histone acetylation by enhancer-recruited histone acetyltransferase proteins provides an initial "opening" or loosening of the chromatin structure throughout the locus. This allows the promoter to recruit chromatin remodeling complexes to the Dβ elements, which relieve structural inhibitions such as methyl groups on the CpG dinucleotide within the Dβ1 3'RS. After removal of this final block, synaptic RAG:DNA complexes are formed and D-to-J recombination occurs.

In testing the contributions of individual promoter elements to Vβ recombination, I found that both the PDβ and the PVβ promoters are required for V-to-DJ joining (Fig. 9), though each was also absolutely dependent on enhancer activity. The continued need for PDβ after D-to-J joining is consistent with findings that DJ joined segments in vivo are maintained in a state of accessible chromatin through the DN and DP stages of development. The need for the Vβ promoter as well would suggest that each stage of V(D)J recombination follows a conserved program of enhancer- and promoter-mediated changes in the accessibility of promoter-proximal RS elements. In developing thymocytes, Vβ transcription is not detected at appreciable levels until cells reach the DN3 stage where Vβ-to-DJβ recombination is also detected (Lamb and Sikes, unpublished data). Consequently,

the delay in Vβ recombination *in vivo* could stem from a delayed activation of Vβ promoter activity.

Taken together, the data I present in my thesis suggest that all stages of TCRβ recombination are dependent on promoter and enhancer elements scattered through the locus to make their associated RS elements available for RAG binding. Specifically, my findings suggest a model in which Eβ acts first to provide a general state of openness to the V, D, and J chromatin through histone acetylation. The enhancer then plays a second role, helping to recruit transcription factors to the germline promoters flanking Dβ gene segments. These transcription factors include chromatin remodeling complexes and transcriptosome components. One or more of these elements confers accessibility of the Dβ 3'RS elements to RAG-1 and RAG-2, possibly through the SWI/SNF-dependent removal of histone proteins from the RS, or PIC-dependent removal of CpG methylation within the 3'RS. Once, D-to-J assembly has been completed, an undefined repression is relieved, allowing the promoter-dependent formation of a synaptic complex between Vβ and the newly formed DJ joint. Future work in the laboratory will focus on dissecting the promoter control of Vβ recombination, and why V and D RSs can readily bind RAG proteins prior to DJ recombination, but do not drive V-to-D joining.

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