

REGULATION OF T CELL RECEPTOR GENE ASSEMBLY BY LOCAL AND
LONG-RANGE CHANGES IN CHROMATIN ACCESSIBILITY

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To my parents, Barb and Greg, your love sustains me

and

To my wife, Jen, your love inspires me

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LIST OF ABBREVIATIONS

Ac	acetylation
ACE	accessibility control element
ACH	active control hub
ATP	adenosone triphosphate
BAC	bacterial artificial chromosome
BCR	B cell receptor
bp	base pair
BRG1	brahma-related gene 1
BRM	brahma
BM	bone marrow
CE	coding end
ChIP	chromatin immunoprecipitation
CJ	coding join
CLP	common lymphoid progenitor
D	diversity
DN	CD4-CD8- double negative
DNA-PKc	DNA-dependent protein kinase
Dnmt	DNA methyl transferase
DP	CD4+CD8+ double positive
DSB	double strand break

E α	TCR α enhancer
E β	TCR β enhancer
FCS	fetal calf serum
FISH	fluorescent in situ hybridization
GFP	green fluorescent protein
H	heavy chain
H	Hinf I
H3-K9	histone 3-lysine 9
HAT	histone acetyltransferase
HDAC	histone deacetylase
HMG	high mobility group
HMT	histone methyltransferase
HP1	heterochromatin protein 1
Ig	immunoglobulin
IL-7	interleukin-7
ISWI	imitation switch
J	joining
L	light
LCR	locus control region
LM-PCR	ligation mediated polymerase chain reaction
MAR	matrix attachment region
Me	methylation

MeCP	methyl-CpG-binding protein
MHC	major histocompatibility complex
MNase	micrococcal nuclease
NHEJ	non-homologous end joining pathway
P	promoter
P	Pvu II
PD β 1	promoter upstream of D β 1
PD β 2	promoter of D β 2
PKC	protein kinase C
RAG	recombination activating genes
RES	restriction enzyme sensitivity
RNA pol II	RNA polymerase II
RSS	recombination signal sequence
SCID	severe combined immunodeficiency
SE	signal end
SEC	signal end complex
SJ	signal join
SP	CD4+ or CD8+ single positive thymocyte
SWI/SNF	mating type switching/sucrose non-fermenting
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TEA	T early α

TF	transcription factor
V	variable
3C	chromosome conformation capture

CHAPTER I

INTRODUCTION

As mammals, our major defense against an ever-changing constellation of pathogens is provided by B and T lymphocytes, which express clonally distributed antigen receptors. An enormous diversity of B and T cell receptors (BCR and TCR) are generated during lymphocyte development in an antigen-independent manner. The large repertoire of lymphocytes, each bearing a signature antigen-binding specificity, is poised to recognize pathogens and signal for their elimination by host effector functions.

The ability of lymphocytes to generate such an enormous diversity of antigen receptors ($> 10^8$ in healthy individuals), coupled with known restrictions on our genomic complexity, confounded explanation for decades. In the mid-1970s, Susumu Tonegawa and colleagues discovered that, unlike other known genes, those encoding for immunoglobulin (Ig) proteins were inherited in a non-functional form. Indeed, the variable region exons of Ig and TCR genes must be assembled from arrays of variable (V), diversity (D), and joining (J) gene segments via somatic recombination (Brack, Hiramata et al. 1978; Weigert, Gattmaitan et al. 1978). This genetic reorganization occurs only in precursor, receptor-negative lymphocytes and is an integral component of their program for ordered development. The assembly of all antigen receptor genes is mediated by a single V(D)J recombinase consisting of the recombination activating genes-

1 and -2 (RAG-1 and RAG-2) proteins, which serve as its key enzymatic components (Schatz, Oettinger et al. 1989; Oettinger, Schatz et al. 1990). The RAG complex targets conserved recombination signal sequences (RSSs) flanking all Ig and TCR gene segments (Sakano, Huppi et al. 1979).

Although the generation of receptor diversity by V(D)J recombination is beneficial, it is also an inherently dangerous process. Defects in V(D)J recombination can cause immunodeficiencies or chromosomal translocations that lead to lethal lymphoid malignancies (Kuppers and Dalla-Favera 2001; Bassing, Swat et al. 2002). With regards to the latter aberration, cryptic RSSs and unusual DNA structures can serve as RAG targets leading, in some cases, to the translocation of protooncogenes into highly expressed antigen receptor loci (Raghavan, Swanson et al. 2005). Thus, normal immune development requires the stringent regulation of recombinase targeting, which is controlled at several levels, including: (i) tissue-specificity (e.g., precursor B cells rearrange only Ig, not TCR loci), (ii) locus-specificity (e.g., TCR β rearrangements occur prior to TCR α rearrangements), and (iii) allelic exclusion (only one functional allele is produced for each Ig and TCR gene).

Early insights into the molecular mechanisms controlling antigen receptor gene assembly came from the discovery that unrearranged (germline) gene segments are transcribed coincident with their recombination (Van Ness, Weigert et al. 1981; Yancopoulos and Alt 1985). These observations led to the hypothesis that V(D)J recombination is regulated by changes in chromatin that permit or deny access of nuclear factors to gene segments. In non-lymphoid

cells, Ig and TCR loci reside in closed chromatin, which is inaccessible to the transcription and recombinase machinery. However, at the appropriate stage of lymphocyte development, chromatin associated with specific clusters of gene segments opens and becomes a target for transcription/recombination. The links between gene expression and recombination suggested that transcriptional control elements within antigen receptor loci might also serve to regulate chromatin accessibility at neighboring gene segments. Consistent with this model, targeted deletion of promoters or enhancers from antigen receptor loci severely impairs their recombination in cis (Oltz 2001; Krangel 2003; Schlissel 2003; Dudley, Chaudhuri et al. 2005). Thus, the biologic action of V(D)J recombinase is tightly regulated by promoters/enhancers, which serve as accessibility control elements (ACEs) to guide antigen receptor gene assembly and lymphocyte development.

V(D)J Recombination: A Mechanistic Perspective

V(D)J recombination is mediated by RSSs that directly flank all Ig and TCR gene segments. Each RSS contains a conserved palindromic heptamer and an AT-rich nonamer, which are separated by a non-conserved spacer of 12 or 23 bp in length. Under physiologic conditions, recombination requires two gene segments flanked by a 12 and a 23 bp RSS (Sakano, Huppi et al. 1979). Experiments conducted with artificial substrates have demonstrated that: (i) V(D)J recombinase is restricted to precursor lymphocytes (Lieber, Hesse et al. 1987), (ii) all Ig and TCR genes are assembled by a single recombinase activity

(Yancopoulos, Blackwell et al. 1986), and (iii) the tissue-specific components of V(D)J recombinase are encoded by a pair of linked genes, termed Recombination Activating Genes 1 and 2 (RAG-1 and -2) (Schatz, Oettinger et al. 1989; Oettinger, Schatz et al. 1990). Early functional experiments with RAG expression vectors showed that RAG-1/2 are sufficient to confer recombinase activity to any cell type tested (Oettinger, Schatz et al. 1990; Oltz, Alt et al. 1993). Accordingly, loss of RAG function by targeted deletions in mice or natural mutations in humans produce a severe combined immunodeficiency (SCID) due to an inability to initiate V(D)J recombination (Mombaerts, Iacomini et al. 1992; Shinkai, Rathbun et al. 1992; Schwarz, Gauss et al. 1996).

The advent of in vitro V(D)J recombination systems produced a bounty of data that support the following model for recombination by RAG proteins (Fig. 1) (McBlane, van Gent et al. 1995; Eastman, Leu et al. 1996). First, the RAG-1/2 complex binds to an RSS, with initial contact between RAG-1 and the nonamer sequence (Swanson and Desiderio 1998). Association of RAG-1 with RAG-2 enhances contact between recombinase and the heptamer (Swanson and Desiderio 1999). The stoichiometry of active RAG complexes in vivo remains unclear; however, current evidence suggests that RAG first binds to a 12 bp-RSS and introduces a single-strand nick precisely at heptamer/coding border (Eastman, Leu et al. 1996; van Gent, Ramsden et al. 1996). The RAG complex then searches for a 23 bp-RSS, forming a synapse, and introduces a similar nick at the second RSS (Jones and Gellert 2002; Mundy, Patenge et al. 2002).

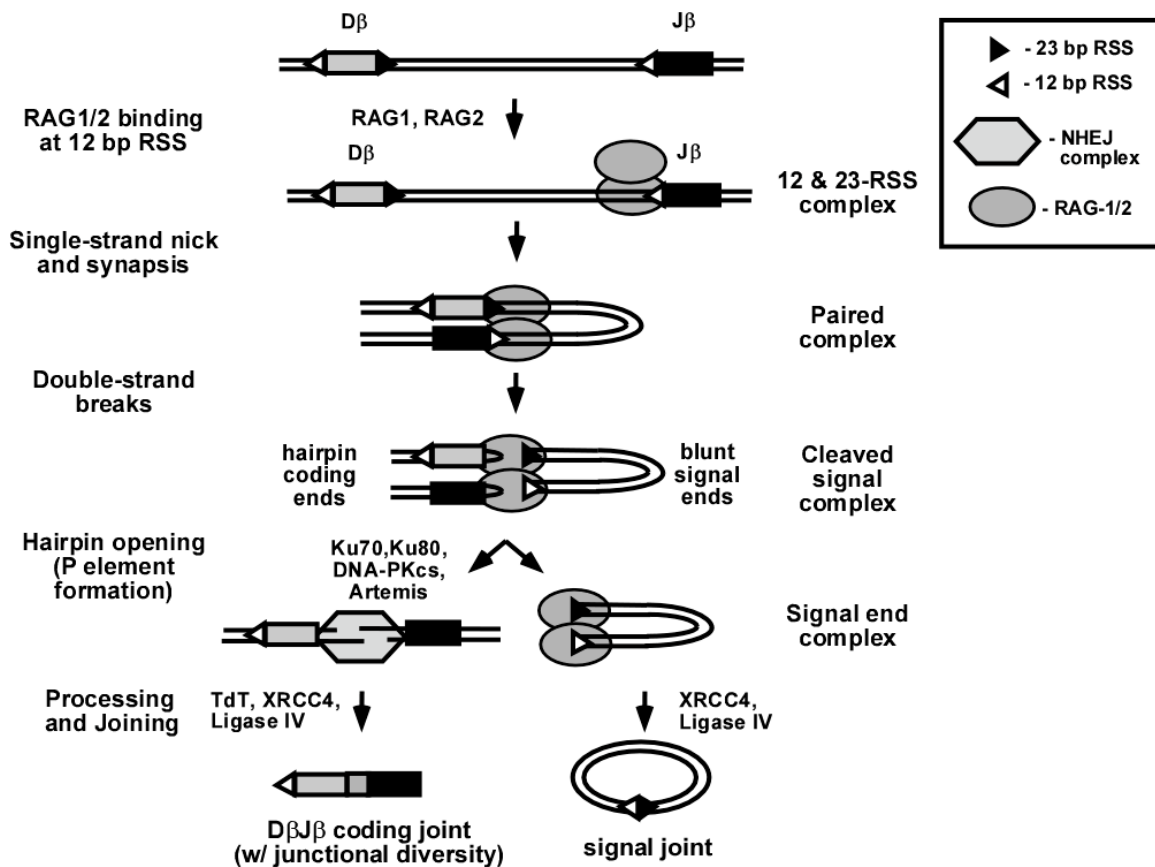


Figure 1. General V(D)J recombination mechanism. The mechanism is exemplified for a portion of the TCR β locus and shows rearrangement of a single D β /J β pair. RSSs are represented by black and white triangles and coding segments are depicted as black or gray rectangles. In brief, the RAG-1/2 recombinase complex (gray ovals) forms a synapse with two compatible RSSs, introduces double-strand breaks at the RSS/coding border, and the breaks are resolved by the NHEJ machinery as imprecise coding joins and precisely fused signal joins. Refer to text for a detailed description of the process.

The liberated hydroxyl groups then attack the opposing phosphate backbones at each RSS to generate a pair of blunt signal ends (SE) and sealed hairpins at the coding ends (CE) (Roth, Menetski et al. 1992). In vitro studies indicate the existence of a post-cleavage complex, which contains the RAG proteins as well as the CEs and SEs (Agrawal and Schatz 1997; Hiom and Gellert 1998). This complex is transient in nature and dissolves rapidly to generate a SE complex (SEC) that retains bound RAG proteins and CEs as free DNA hairpins.

Formation of the SEC and CEs represents the endpoint of RAG-dependent events in vitro. Completion of V(D)J recombination requires resolution of DNA ends to rescue the chromosome and generate coding joins (CJs). Studies of V(D)J recombination in CHO mutant cells engineered to express RAG proteins revealed an important role for the ubiquitous double-strand break repair machinery in the resolution of both SEs and CEs (Pergola, Zdzienicka et al. 1993; Taccioli, Rathbun et al. 1993). Together with subsequent studies, the following model has emerged for the resolution of V(D)J breaks by the non-homologous end-joining (NHEJ) repair pathway (Dudley, Chaudhuri et al. 2005). Free ends are first recognized by a heteromeric complex of KU proteins, which in turn recruit the catalytic component of DNA-dependent protein kinase (DNA-PK_{cs}). Activated DNA-PK_{cs} phosphorylate numerous targets that control cell cycle progression (e.g., p53) and subsequent DNA repair. These include the Artemis protein and the variant histone, H2AX, which is phosphorylated over a broad region surrounding the break (Chen, Bhandoola et al. 2000). Phosphorylation of Artemis activates its endonuclease activity, which

is critical for opening hairpins at CEs (Ma, Pannicke et al. 2002) and creating palindromic sequences (P elements) at many V(D)J junctions (Lafaille, DeCloux et al. 1989). Moreover, endonuclease activity associated with Artemis generates further diversity at CJs via the random deletion of nucleotides from exposed ends (Ma, Pannicke et al. 2002). The precursor lymphocyte-specific protein, terminal deoxynucleotidyl transferase (TdT), enhances junctional diversity through the random addition of nucleotides at CEs (Komori, Okada et al. 1993).

Final resolution of both CEs and SEs is achieved following the recruitment of XRCC4, which binds to and activates DNA ligase IV (Li, Otevrel et al. 1995; Grawunder, Wilm et al. 1997). More recent studies suggest that an additional, yet unidentified repair factor may facilitate V(D)J recombination in vivo (Dai, Kysela et al. 2003). Notwithstanding, the end result of the repair process is a highly modified CJ, which enhances sequence diversity at the CDR3 region of Ig and TCR proteins. The exposed CJs are resolved rapidly by the NHEJ machinery, whereas SEs are resolved slowly and the resultant SJs are usually deleted from the genome as episomal circles (Hesslein and Schatz 2001).

Mouse knockouts confirmed the in vivo relevance of these cell model studies on NHEJ repair. In addition to radiosensitivity, mice harboring null mutations of KU, DNA-PK_{cs}, Artemis, XRCC4, or DNA-ligase IV all exhibited a SCID phenotype due to defects in the formation of coding joins or opening of hairpins (reviewed in (Dudley, Chaudhuri et al. 2005). Dual deletion of most NHEJ components and p53 produced mice with aggressive lymphocytic tumors exhibiting chromosomal translocations that are hallmarks of defective V(D)J

recombination (Gao, Ferguson et al. 2000; Dudley, Chaudhuri et al. 2005). Recombinase activity and NHEJ are also coupled via changes in RAG protein stability during the cell cycle. Specifically, RAG-2 is phosphorylated, ubiquitinated, and rapidly degraded in dividing cells (Lin and Desiderio 1993; Jiang, Chang et al. 2005). This cell cycle-dependent control restricts recombinase activity to resting G_0/G_1 cells, where the NHEJ mechanism of DNA repair predominates (Lee and Desiderio 1999). The importance of this regulatory mechanism was recently confirmed in mice that express a phosphodeficient mutant of RAG-2 in thymocytes. These mutant animals possessed high levels TCR signal ends in cycling pre-T cells and exhibited defective TCR joins that were reminiscent of those from NHEJ-deficient mice (Jiang, Ross et al. 2004). Together, these in vivo studies underscore the importance of proper targeting, regulation, and constraint of V(D)J recombination during the stepwise process of lymphocyte development.

Genomic Architecture of Ig and TCR Loci

The TCR and Ig components of antigen receptors are encoded by seven distinct genetic loci. Two distinct classes of T cells exist, which express either a $TCR\beta/TCR\alpha$ or $TCR\gamma/TCR\delta$ heterodimers. The B cell antigen receptor is a tetrameric structure composed of two identical Ig heavy chains (IgH) covalently linked to their partner light chains (IgL, either $Ig\kappa$ or $Ig\lambda$). In contrast to the split nature of gene segments that comprise variable exons, the constant regions of antigen receptor genes exhibit a normal exon/intron structure. Each of the exons

encode for a single Ig-fold domain, a β -barrel structure that is commonly found in many surface receptors.

The TCR β locus spans approximately one Mb on mouse chromosome 6 (Glusman, Rowen et al. 2001). The 5' region of the locus is composed of 35 V β segments, 14 of which are nonfunctional pseudogenes. The 3' region of the locus harbors two D β J β clusters, each containing one D β and six functional J β segments (Fig. 2). Coding exons for the TCR β constant region reside downstream of each D β J β cluster (C β 1 or C β 2). Finally, a single V β element, called V β 14, lies downstream of C β 2 and rearranges by an inversional mechanism. All V β gene segments are flanked on their 3' sides by a 23 bp RSS, while the J β elements are bordered by 12 bp RSSs. The two D β elements are flanked by a 12 and a 23 bp RSS, on their 5' and 3' sides, respectively. In theory, this RSS arrangement should permit direct V β →J β recombination. However, these joins are rarely observed in vivo due to undefined constraints of the recombination process, termed “beyond 12/23 restriction” (Bassing, Alt et al. 2000; Jung, Bassing et al. 2003; Tillman, Wooley et al. 2003).

The gene segments encoding mouse TCR α and TCR δ are intermingled in a single locus spanning 1.5 Mb on chromosome 14 (Glusman, Rowen et al. 2001; Bosc and Lefranc 2003). In total, the locus contains over 100 V segments, some of which rearrange only with J α gene segments, some with only D δ J δ joins, and some contribute to both the TCR α and TCR δ repertoires (Krangel, Carabana

et al. 2004). A pair of D δ and J δ segments lies between the V cluster and the C δ coding region. Further downstream of C δ lie 60 J α gene segments followed by the C α coding region.

The TCR γ locus is distributed across a short region of DNA (~200 kb) on mouse chromosome 13 (Glusman, Rowen et al. 2001). This locus consists of seven V γ gene segments and one V γ pseudogene interspersed among three functional J γ -C γ units and one nonfunctional J γ -C γ unit. All of the TCR γ gene segments are positioned in the same transcriptional orientation, with V γ segments flanked by 23-bp RSSs and J γ gene segments flanked by 12-bp RSSs.

The mouse IgH locus spans a region on chromosome 12 of approximately three megabases (Chevillard, Ozaki et al. 2002). The constant region coding exons, ordered C μ , C δ , C γ 3, C γ 1, C γ 2b, C γ 2a, C ϵ , and C α are spread over a region of approximately 200 kb at the 3' end of the locus. Four J H gene segments are positioned in a small cluster located 7.5 kb upstream of C μ coding exons and the thirteen D H segments are located in a linear array further upstream. Approximately 150 V H segments are dispersed over a 1 Mb region upstream from the D H cluster. In mouse, these V H segments are arranged in families that share a high level of sequence similarity. The D-proximal family, termed 7183, is preferentially used in IgH rearrangements by pro-B cells (Malynn, Yancopoulos et al. 1990). The most distal V H family (J558) is the largest and predominates the peripheral B cell repertoire (Chevillard, Ozaki et al. 2002). The D H gene segments are flanked by 12 bp-RSSs on both sides, while the V H and J H segments each have 23 bp RSSs. In keeping with the 12/23 rule,

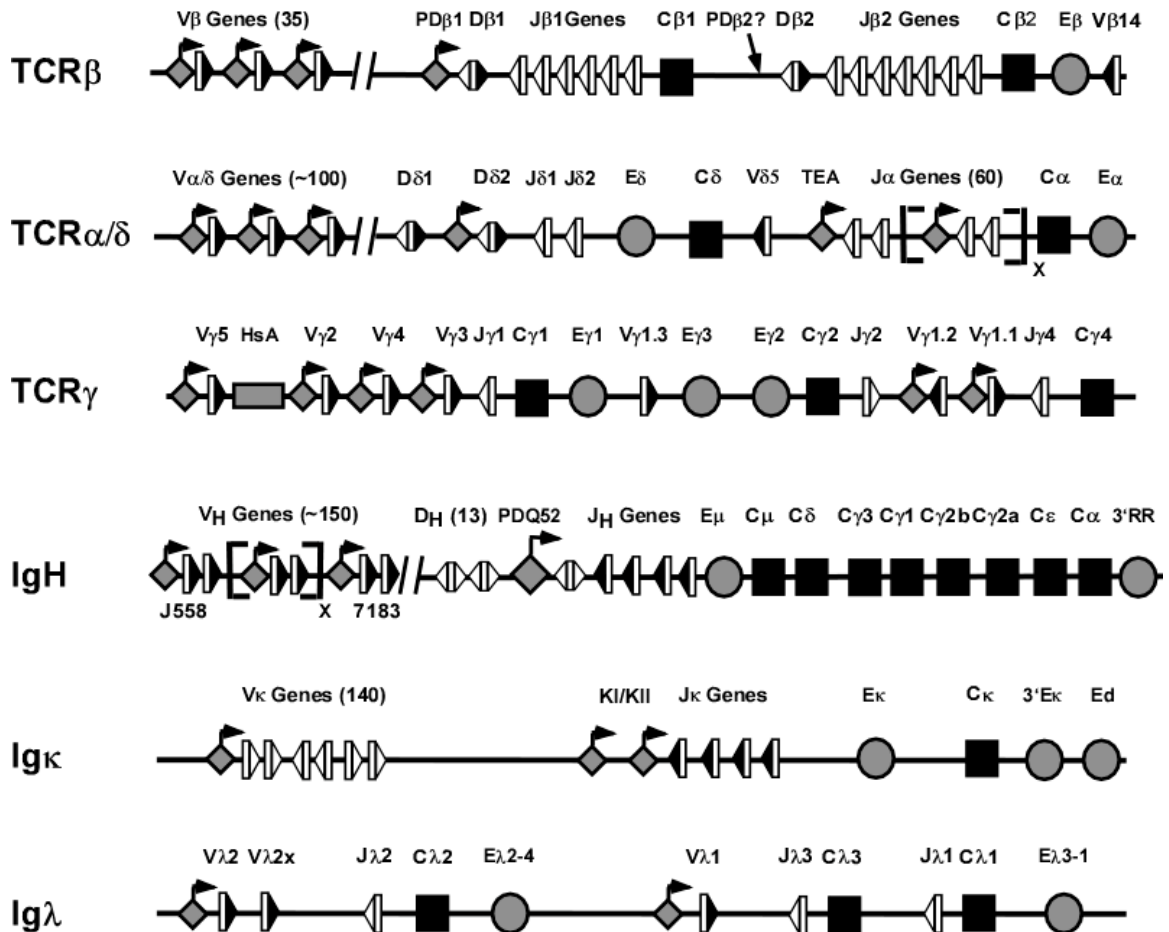


Figure 2. Schematic depiction of mouse Ig and TCR loci (not to scale). Gene segments are represented by rectangles and RSSs are depicted as triangles (23 bp, black and 12 bp, white). Transcriptional promoters and enhancers are shown as gray diamonds and circles, respectively, and constant regions as black squares. Estimated numbers of gene segments are displayed above the represented V, D, and J regions. For the IgH locus, the most proximal (7183) and distal V_H families (J558) are shown.

this RSS composition precludes $V_H \rightarrow J_H$ joining and ensures the inclusion of a D_H element in all IgH joins.

The $Ig\kappa$ locus is composed of approximately 140 $V\kappa$ and 4 functional $J\kappa$ gene segments, which are spread over 3 Mb on mouse chromosome 6 (Kirschbaum, Pourrajabi et al. 1998; Thiebe, Schable et al. 1999). A single $C\kappa$ exon lies 2.5 kb downstream of the $J\kappa$ cluster. A subset of $V\kappa$ gene segments are in a reverse transcriptional orientation relative to the $J\kappa$ segments. As such, rearrangements involving these segments occur via large-scale inversion of DNA between the selected $V\kappa/J\kappa$ segments rather than the usual deletion mechanism of joining (Gorman and Alt 1998). In addition to the RSSs associated with $V\kappa$ and $J\kappa$ gene segments, consensus RSSs are positioned downstream of the $J\kappa$ cluster (Muller, Stappert et al. 1990). These RSSs can recombine with $V\kappa$ gene segments to inactivate the targeted $Ig\kappa$ allele during the process of receptor editing.

The mouse $Ig\lambda$ locus spans about 200 kb on chromosome 16 and harbors three distinct cassettes of $V\lambda/J\lambda$ gene segments and $C\lambda$ exons (Gorman and Alt 1998). Only two of the three $V\lambda$ gene segments ($V\lambda 1$ and $V\lambda 2$) are used predominantly in developing B cells. In cells that fail to express functional $Ig\kappa$ genes, these $V\lambda$ segments rearrange preferentially to their most proximal $J\lambda$ - $C\lambda$ clusters ($V\lambda 2$ with $J\lambda 2$ and $V\lambda 1$ with $J\lambda 1/J\lambda 3$) (Reilly, Blomberg et al. 1984). As a result, the repertoire of mouse $Ig\lambda$ rearrangements is far more restricted than that observed for the $Ig\kappa$ locus.

Control of Antigen Receptor Gene Assembly in Lymphocyte Development

The generation of functional T and B lymphocytes requires the precise orchestration of antigen receptor gene assembly and a highly ordered program of cellular differentiation (Busslinger 2004; Rothenberg and Taghon 2005). Both lineages derive from pluripotent stem cells in adult bone marrow, which differentiate into common lymphoid progenitor (CLPs) cells. T cell progenitors migrate from the bone marrow and complete their development in the thymus. In contrast, B lymphopoiesis occurs in the liver during fetal development but continues in the bone marrow of adults. T and B cell precursors initially lack surface antigen receptors but, upon their commitment, they rapidly initiate the program of V(D)J recombination at either TCR or Ig loci. This ordered process is an integral component of developmental pathways, with the protein products from each step guiding cellular differentiation and subsequent steps of gene assembly. The end result of this genetic program is the acquisition of TCR or Ig expression and a signature antigen binding specificity on each lymphocyte clone.

To initiate V(D)J recombination, precursor lymphocytes must first express the tissue-specific components of recombinase – the RAG genes. The RAG-1/2 genes are located approximately 15 kb apart on chromosome 2 in mouse, and are under the transcriptional control of multiple cis-acting elements. These elements work in concert to repress RAG expression in non-lymphoid cells and activate expression in precursor lymphocytes (Yu, Misulovin et al. 1999; Hsu, Lauring et al. 2003). More recent studies have shown that RAG-1/2 expression initiates in CLPs and a significant portion of these cells target the IgH locus for

D_H→J_H recombination (Borghesi, Hsu et al. 2004). This expression pattern likely explains the presence of D_HJ_H joins in thymocytes (Born, White et al. 1988). However, neither the ordered assembly nor the cell-type specificity of V(D)J recombination can be explained simply by RAG expression patterns because both genes are expressed at varying levels throughout all stages of precursor lymphocyte development.

One of two classes of TCRs can arise during T cell lymphopoiesis. The majority of precursors become α/β rather than γ/δ T cells, and lineage commitment appears to hinge on which set of genes first undergo productive rearrangements (Robey 2005). Upon T lineage commitment, thymocytes lack expression of the CD4/CD8 co-receptors and are termed double negative (DN) pro-T cells. The DN population can be further categorized into the DNI-DNIV subsets based on CD44/CD25 expression (Rothenberg and Taghon 2005). The DNII-DNIII subsets first target recombinase to the D β J β clusters, followed by V β →D β J β rearrangement. Assembly of a functional TCR β gene leads to expression of a pre-TCR in DNIV cells, which consists of the TCR β chain, the surrogate TCR α chain (pT α), and the CD3 co-receptor complex (von Boehmer 2005). Expression of the pre-TCR inhibits further V β →D β J β recombination but stimulates several other processes (collectively called β -selection), including (i) clonal expansion of TCR β ⁺ pro-T cells, (ii) differentiation into CD4⁺/CD8⁺ double positive (DP) pre-T cell stage, and (iii) activation of V α →J α recombination (Shinkai, Koyasu et al. 1993; Aifantis, Buer et al. 1997). T cell clones that express a functional TCR α gene undergo positive selection and differentiate into

the CD4 helper or CD8 cytotoxic T cell lineage. Autoreactive clones are removed from the T cell repertoire by apoptosis during negative selection in the thymus. Additionally, precursor T cells can undergo multiple rounds of $V\alpha \rightarrow J\alpha$ recombination until these cells express a TCR that progresses through both the positive and negative selection checkpoints (Hawwari, Bock et al. 2005; Huang, Sleckman et al. 2005).

Should a CLP commit to the B cell lineage, its subsequent development can be tracked using a combination of surface marker expression and the rearrangement status of Ig loci. The first developmental stage, termed a pro-B cell, is identified by expression of the lineage marker B220 and the CD43 surface protein. Pro-B cells can be categorized further into fractions A-C (Li, Wasserman et al. 1996). Fraction A/B cells first target the IgH locus for $D_H \rightarrow J_H$ recombination, which almost always occurs on both alleles. Fraction B cells then initiate $V_H \rightarrow D_H J_H$ recombination, which appears to be a less efficient process and targets each allele sequentially (Hardy, Carmack et al. 1991). Formation of a functional $V_H D_H J_H$ exon permits expression of IgH protein (IgM isotype) in the cytoplasmic compartment (Ig μ protein). In turn, Ig μ associates with two surrogate light chains ($\lambda 5$ and V_{pre-B}) and the signaling molecules Ig α and Ig β to generate the pre-B cell receptor (pre-BCR) (Hombach, Tsubata et al. 1990; Melchers 2005).

The pre-BCR triggers a proliferative burst to expand the numbers of pro-B cells expressing IgH protein, which can then couple with distinct IgL chains (Young, Ardman et al. 1994). The vast majority of pre-B cells first target the Ig κ

locus for $V_{\kappa} \rightarrow J_{\kappa}$ recombination (Ehlich, Schaal et al. 1993). However, if both Ig κ alleles are assembled out-of-frame, the pre-B cell clone retargets recombinase activity to the Ig λ locus. Functional rearrangement at either IgL locus permits expression of a complete BCR. The emerging B cell terminates RAG expression and migrates to the spleen where it undergoes further differentiation to become a mature B lymphocyte (Hardy and Hayakawa 2001).

Regulation of V(D)J Recombination by the Accessibility Hypothesis and Beyond

The stepwise, ordered assembly of antigen receptor genes requires targeting, then retargeting, of V(D)J recombinase to distinct regions within TCR and Ig loci at different stages of lymphocyte development. The numerous levels of regulation include: (i) tissue-specificity, (ii) ordered assembly within each locus (D \rightarrow J then V \rightarrow DJ), (iii) stage-specificity (e.g., TCR β in pro-T and TCR α in pre-T cells), and (iv) allelic exclusion. The selectivity of these genomic rearrangements occurs despite the use of a common recombinase that is expressed at all stages of precursor B and T cell development (Yancopoulos, Blackwell et al. 1986). Moreover, the RSS substrates for V(D)J recombinase are virtually indistinguishable when comparing TCR and Ig loci.

A first clue to the mechanisms by which a common enzyme/substrate system differentially targets gene segments for recombination came from the discovery of “germline transcripts” by the Alt and Perry laboratories. Their studies revealed that transcription of germline gene segments is initiated in the cell types that target these segments for recombination (Van Ness, Weigert et al.

1981; Yancopoulos and Alt 1985). For example, unrearranged V β segments are transcribed in pro-T cells but not in pre-T or B lineage cells (Senoo and Shinkai 1998). Since these initial observations, the general correlation between germline transcription and V(D)J recombination has been extended to all TCR and Ig gene segments and even artificial substrates (reviewed in (Sleckman, Gorman et al. 1996; Oltz 2001).

The link between transcription and recombination led to the hypothesis that each step in antigen receptor gene assembly is controlled by alterations in chromatin accessibility to the common recombinase complex. Specifically, recombinationally inert gene segments would be packaged into a chromatin configuration that is refractory to RAG binding and cleavage; whereas targeted gene segments would be packaged into an “open” chromatin configuration that is accessible to both RAG and transcriptional complexes. Further support for the accessibility hypothesis derived from studies showing that fibroblasts engineered to express RAG-1/2 could target actively expressed chromosomal substrates (“open”) for recombination while endogenous TCR and Ig loci remained both transcriptionally and recombinationally silent (Schatz, Oettinger et al. 1992). Subsequently, the accessibility hypothesis has been validated by numerous experimental approaches that directly or indirectly measure levels of chromatin accessibility at gene segments.

Although changes in chromatin accessibility can account for the majority of regulatory processes governing V(D)J recombination, the RAG complex and its substrate RSSs can also influence rearrangement efficiencies. Mouse

knockouts and cell model studies show that a truncated form of RAG-2 (lacking its C-terminus) encodes for its “core” enzymatic activity and can efficiently perform D→J but not V→DJ recombination (Kirch, Rathbun et al. 1998; Liang, Hsu et al. 2002; Akamatsu, Monroe et al. 2003). In a recent development, Cortes and colleagues reported that the C-terminus of RAG-2 binds directly to all four core histones (West, Singha et al. 2005). Specific mutations in the C-terminus that abolish its binding to histones also impair $V_H \rightarrow D_H J_H$ but not $D_H \rightarrow J_H$ recombination in pro-B cell lines. One exciting possibility is that the C-terminus of RAG-2 may serve as bridge between chromatin and recombinase to facilitate the long-range synapsis of RSSs.

The precise sequence of RSSs also contributes to restrictions in the order and type of gene segments used at the TCR β locus. Although the 12/23 rule permits direct joining between $V\beta$ and $J\beta$ gene segments, these recombination products are almost never observed in vivo (Bassing, Alt et al. 2000). Using an elegant series of knockout and substrate models, Alt and Sleckman have shown that an intrinsic property of the $J\beta$ -RSS restricts its efficient usage to recombination with $D\beta$ - but not with $V\beta$ -RSSs. In contrast, the $V\beta$ -RSSs are more compatible for recombination with the 5'D β -RSS (Bassing, Alt et al. 2000; Sleckman, Bassing et al. 2000). The precise mechanisms involved in this “beyond 12/23” control remain unknown. However, the specificity of gene segment selection at TCR β does not rely on thymocyte-specific factors and likely reflects a more general feature of the recombinase itself, which may preferentially pair certain RSSs for coupled cleavage (Jung, Bassing et al. 2003;

Tillman, Wooley et al. 2003). Consistent with this possibility, ordered $D\beta \rightarrow J\beta$ then $V\beta \rightarrow D\beta J\beta$ recombination is not controlled by simple proximity of the $D\beta$ and $J\beta$ gene segments. This order is recapitulated at engineered TCR β loci in which the $V\beta$ cluster is positioned proximal to $D\beta 1$ (Ferrier, Krippel et al. 1990; Senoo, Wang et al. 2003).

Notwithstanding these important but more specialized restrictions, it has become clear that chromatin accessibility is the primary determinant for establishing the recombination potential of gene segment clusters.

Chromatin Structure and Accessibility Control Mechanisms

A significant hurdle for the evolution of eukaryotes from prokaryotes was the packaging of approximately two meters of chromosomal DNA into nuclei that are several microns in size. Eukaryotes solved this problem by packaging genomic DNA into nucleosomes, the basic building block of chromatin. A single nucleosome consists of ~146 bp of DNA wrapped around an octamer of four histone pairs (H2A, H2B, H3 and H4) (Wolffe and Guschin 2000; Khorasanizadeh 2004). In most chromatin, nucleosomes are separated by ~20 to 60 bp of spacer DNA, which gives rise to a simple structure resembling “beads on a string”. The histone protein, termed H1, can bind to linker DNA and is essential for the condensation of “open” chromatin into more compact forms (e.g., the 30 nm fiber) (Wolffe and Guschin 2000). The mechanisms that give rise to even higher degrees of chromatin compaction remain vague.

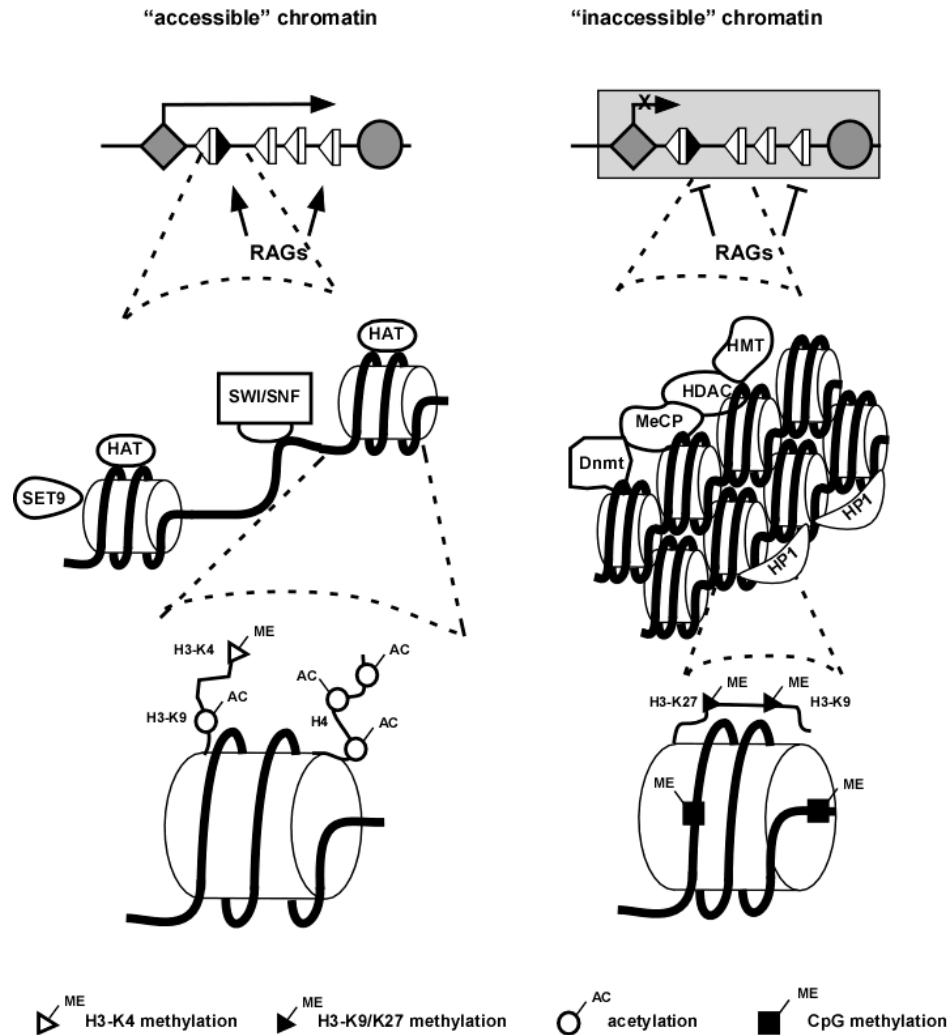


Figure 3. Top: schematic representation of recombinase accessible (left) and inaccessible chromatin (right). Germline promoters and enhancers are depicted as diamonds and circles, respectively. The two types of chromatin are shown at increasing levels of resolution (top to bottom). Middle: Nucleosomal DNA (dark spirals) wrapped around an octamer of 4 histones (H2A, H2B, H3, H4), which is represented as a cylinder. Nucleosomes are loosely packed in accessible chromatin (left) and usually associate with activating TFs, HATs, and nucleosome remodeling complexes (SWI/SNF). Inaccessible chromatin has more densely packed nucleosomal arrays (right) and associates with an interacting cascade of chromatin modifiers that usually includes DNA methyltransferases (Dnmt), methyl-CpG binding proteins (MeCP), histone deacetylases (HDAC), histone methyltransferases, and the heterochromatin protein HP-1. Bottom: The general patterns of chromatin modifications at accessible (left) and inaccessible chromatin (right) are shown. A key for symbols representing each modification is given at the bottom.

Eukaryotes harbor three general types of chromatin in their nuclei (Fig. 3). The most highly compacted form, constitutive heterochromatin, is heavily stained by DNA-specific dyes and represents the most inaccessible state. Accordingly, very few expressed genes are found in heterochromatic regions, which include pericentric repeats and the inactive X chromosome (Fahrner and Baylin 2003). A second form of chromatin, termed euchromatin, is not highly stained by DNA dyes and represents an open state that contains most of the cell's expressed genes. Regions of euchromatin are generally more accessible to nuclear factors and susceptible to attack by nucleases. A third configuration of chromatin, called facultative heterochromatin, is an intermediate form that exhibits many hallmarks of inactive chromatin but is not constitutively closed. Regions of facultative heterochromatin contain genetic loci that are silent but can be induced for expression given the proper cues and chromatin remodeling (Fahrner and Baylin 2003). In addition to standard histones, eukaryotes express a panel of variants that perform specialized functions. These include: (i) macroH2A, which is a major component of constitutive heterochromatin (Chadwick, Valley et al. 2001), (ii) H3.3, which replaces H3 at expressed genes and marks the locus for continued expression (McKittrick, Gafken et al. 2004), and (iii) H2AX, which is found in approximately 10% of nucleosomes and becomes phosphorylated at sites of DNA damage (Chen, Bhandoola et al. 2000).

Although the nucleosomal structure of cellular DNA solves the basic packaging problem, it generally impedes interactions between DNA and most non-histone proteins, including transcription factors (TFs) and the basal

transcription machinery (Geiman and Robertson 2002). In this regard, numerous lines of evidence indicate that V(D)J recombinase can engage its target RSSs in nucleosomal DNA only after substrates become accessible. First, RAG cleavage of RSSs is blocked *in vitro* when substrates are packaged into mononucleosomes (Kwon, Imbalzano et al. 1998). Second, antigen receptor loci undergoing rearrangement exhibit many hallmarks of accessible euchromatin, including hypersensitivity to nucleases; whereas recombinationally silent loci are largely refractory to nucleases (Chattopadhyay, Whitehurst et al. 1998; Chowdhury and Sen 2003). Third, Schlissel and colleagues have shown that recombinant RAG proteins cleave RSSs in nuclei from primary lymphocytes with the appropriate tissue-, stage-, and allele-specificity (Stanhope-Baker, Hudson et al. 1996). Together, these studies suggest that most antigen receptor loci begin as facultative heterochromatin in CLPs. Upon lineage commitment, developmental cues signal for an opening of specific chromatin domains to render the appropriate gene segments accessible to recombinase.

Eukaryotes have developed a complex set of mechanisms to alter chromatin accessibility at both the local and long-range levels. Many of these mechanisms involve the recruitment of protein complexes that covalently modify either the histone or DNA components of chromatin (Berger 2002; Richards and Elgin 2002). A broad panel of transcription factors recruits protein complexes that acetylate, methylate, phosphorylate, or ubiquitinate histones. These modifications enable nucleoprotein modules to recruit other co-activators, including components of the core transcription machinery. These observations

have led to the “histone code” hypothesis. According to this hypothesis, modifications in N-terminal tails of histones generate binding sites for additional chromatin remodeling complexes, which in turn control the transcriptional status of flanking genes (Fig. 3) (Jenuwein and Allis 2001).

A well-recognized example of the histone code hypothesis is the modification of lysine-9 on histone H3 (H3-K9). This amino acid is targeted by a broad spectrum of histone acetyltransferases (HATs) and histone deacetylases (HDACs), which do not bind DNA directly but are recruited by TFs or repressor complexes (Nakayama, Rice et al. 2001; Emerson 2002; Narlikar, Fan et al. 2002).

Acetylation of H3-K9 leads to high-affinity interactions with bromodomains in other HAT or nucleosome remodeling complexes, which further augment chromatin accessibility (Peterson and Workman 2000). Accordingly, expressed loci normally associate with nucleosomes bearing H3-K9 acetylation, whereas silent loci are characterized by hypoacetylated H3-K9 residues (Litt, Simpson et al. 2001). Recent studies have extended these links to the process of V(D)J recombination. Acetylation of nucleosomes can partially relieve the inhibition of RAG-mediated cleavage at RSSs in vitro (Kwon, Morshead et al. 2000). Moreover, antigen receptor loci that undergo active rearrangement are associated with hyperacetylated histones in vivo, whereas inert gene clusters remain hypoacetylated (McMurry, Hernandez-Munain et al. 1997; Chowdhury and Sen 2003; Morshead, Ciccone et al. 2003).

In contrast to acetylation, methylation at H3-K9 leads to the reduced expression of associated transcription units (Lachner, O'Carroll et al. 2001). The degree of methylation at H3-K9 also influences the magnitude of gene repression and the formation of distinct chromatin configurations. Di-methylation at H3-K9 is found predominantly at repressed genes in euchromatin or facultative heterochromatin. This epigenetic mark is imprinted by two histone methyltransferases (HMTs) in mammals, called G9a and GLP (Tachibana, Sugimoto et al. 2002; Peters, Kubicek et al. 2003; Tachibana, Ueda et al. 2005). Tri-methylation of H3-K9 is observed predominantly at constitutive or pericentric heterochromatin and is the enzymatic product of two redundant HMTs called Suv39h1 and Suv39h2 (Peters, O'Carroll et al. 2001; Peters, Kubicek et al. 2003).

Consistent with the histone code hypothesis, methylated H3-K9 recruits an entirely different set of remodeling complexes relative to its acetylated counterpart. This set of complexes feature the presence of a chromodomain and function to impair chromatin accessibility (Bannister, Zegerman et al. 2001; Lachner, O'Carroll et al. 2001). Indeed, H3-K9 methylation marks antigen receptor gene segments that are recombinationally inert and this modification can dominantly repress accessibility to V(D)J recombinase at chromosomal substrates (Morshead, Ciccone et al. 2003; Johnson, Pflugh et al. 2004; Osipovich, Milley et al. 2004).

In addition to acetylation/methylation of H3-K9, mammalian histones are marked by a constellation of covalent modifications (Jenuwein and Allis 2001;

Cosgrove, Boeke et al. 2004). Several of these epigenetic marks have been studied extensively in the context of gene expression, and to some extent, for correlations with V(D)J recombination. These include: (i) histone H4 acetylation, which correlates with transcriptional activation at open chromatin (Jenuwein and Allis 2001), (ii) H3-K4 methylation, which is accomplished by SET1 and is characteristic of expressed genes (Santos-Rosa, Schneider et al. 2002), (iii) H3-K27 methylation, which is targeted by the Ezh2 component of polycomb complexes and serves as a long-term memory mark for silent chromatin (Cao, Wang et al. 2002; Kuzmichev, Nishioka et al. 2002), and (iv) H3-K79 methylation by the DOT1 methyltransferase, which identifies active chromatin and prevents silencing in yeast (Ng, Ciccone et al. 2003). Collectively, these and other histone modifications comprise a flexible, yet highly complex code, which specifies numerous cellular processes, including gene activation and V(D)J recombination.

The exquisite specificity of the histone code is underscored by recent studies of the IFN β regulatory region, which forms an enhanceosome structure upon binding its cognate TFs. Acetylation of the enhanceosome-proximal nucleosome at H3-K9/K14 is required for the recruitment of TFIID via a pair of bromodomains (Agalioti, Chen et al. 2002). In contrast, the SWI/SNF nucleosome remodeling complex is recruited via interactions between its bromodomain and an acetylated lysine at H4-K8.

In addition to histone tail modifications, the DNA component of chromatin can be covalently marked by methylation at CpG dinucleotides (Bird 2002). This reversible modification is mediated by a family of DNA methyltransferases

(Dnmt) that exhibit distinct functions. In mammals, the Dnmt1 enzyme maintains CpG methylation following cellular replication, while Dnmt3 isoforms perform de novo methylation (Bestor 2000; Chen, Ueda et al. 2003). In general, CpG methylation is indicative of transcriptional repression, whereas actively expressed genes are hypomethylated (Bird 2002). Mounting evidence suggests a functional interplay between the H3-K9 and DNA methylation machineries. Nucleosomes methylated at H3-K9 present a docking site for heterochromatin-associated proteins, including isoforms of HP1 (Bannister, Zegerman et al. 2001; Lachner, O'Carroll et al. 2001). The HP1 α isoform interacts with Dnmt3, which may then target local CpG sites for methylation (Fuks, Hurd et al. 2003). Modified CpG sites interact with a specialized set of DNA binding proteins (e.g., MeCP2) that form complexes with HDACs and HMTs (Jones, Veenstra et al. 1998; Fuks, Hurd et al. 2003). Thus, recruitment of G9a or Suv39h1/2 suppresses gene expression through a self-reinforcing mechanism that relies on extensive cross-talk between the histone and DNA methylation machineries. In the context of antigen receptor gene assembly, the vast majority of recombinationally active loci are hypomethylated on CpG dinucleotides, whereas recombinationally inert loci exhibit CpG hypermethylation (Bergman, Fisher et al. 2003). Moreover, CpG methylation has been shown to directly suppress V(D)J recombination of ectopic or transgenic substrates (Engler, Haasch et al. 1991; Hsieh, Gauss et al. 1992; Demengeot, Oltz et al. 1995).

Chromatin modifications and transcription factors serve as binding platforms for ATP-dependent complexes that remodel neighboring nucleosomes

and expose associated DNA (Kingston and Narlikar 1999). Three major families of remodeling complexes have been characterized to date. Two of these families, termed ISWI and Mi-2, function mainly as transcriptional repressors (Emerson 2002; Narlikar, Fan et al. 2002). In contrast, members of the SWI/SNF family facilitate transcription of nucleosomal substrates and can interact with the activation domains of TFs (Peterson and Workman 2000). In keeping with the histone code, components of the SWI/SNF complex possess bromodomains to enhance binding at acetylated regions within the chromatin of expressed loci (Hassan, Neely et al. 2001). Although the composition of SWI/SNF can vary, two general classes have been identified. These classes are functionally distinct and contain either Brg1 or Brm as their critical ATPase subunit (Kadam and Emerson 2003). The precise mechanism of SWI/SNF action on nucleosome arrays has not been established. However, the functional outcome of SWI/SNF action is three-fold: it alters the translational position of nucleosomes on DNA, it modifies histone octamers to increase DNA accessibility, and it loops out intervening DNA between nucleosome entry and exit sites (Kingston and Narlikar 1999; Kassabov, Zhang et al. 2003). A link between nucleosome remodeling and V(D)J recombination is suggested by recent *in vitro* studies. Pretreatment of mononucleosome substrates with SWI/SNF partially rescues RSS cleavage by RAG proteins (Kwon, Morshead et al. 2000). Moreover, chromatin immunoprecipitation (ChIP) analyses revealed that Brg1 occupies regions within Ig and TCR loci that are recombinase-accessible (Morshead, Ciccone et al. 2003).

Regulation of Chromatin Accessibility and V(D)J Recombination by Cis-Acting Elements

Gene expression programs are specified in large part by a collection of cis-acting elements that include transcriptional promoters, enhancers, locus control regions (LCRs), silencers, and boundary elements. A primary function of these regulatory motifs is to dock TFs that indirectly modulate the accessibility of neighboring chromatin. The observed link between germline transcription and recombination of gene segments suggests these two processes share common regulatory elements. Consistent with this possibility, transcriptional control elements are scattered throughout TCR and Ig loci. Promoters and enhancers within these loci are mostly arranged in a split configuration, which may afford modular control of transcription and/or recombination at distinct clusters of gene segments.

This regulatory model was confirmed in early studies using TCR β or TCR δ transgenic substrates, which demonstrated a direct role for transcriptional enhancers in targeting their efficient recombination (Ferrier, Covey et al. 1990; Lauzurica and Krangel 1994). Likewise, recombination of chromosomal substrates in cell models requires the inclusion of any active enhancer/promoter combination, even those of viral origin (Oltz, Alt et al. 1993; Sikes, Suarez et al. 1999). Germline deletion of enhancers or promoters within antigen receptor loci consistently impairs rearrangement of linked gene segments. Together, these studies demonstrate that the biologic action of V(D)J recombinase is tightly

regulated by promoters and enhancers, which serve as accessibility control elements to guide antigen receptor gene assembly and lymphocyte development.

Regulation of TCR β gene assembly

The TCR β locus provides an excellent model for cis-acting regulation because the D β J β region contains only a single enhancer (E β) and one germline promoter in each D β J β cluster (Fig. 2). E β function is T lineage-specific and is activated at the earliest stage of thymocyte development (McDougall, Peterson et al. 1988). Accordingly, inclusion of E β in a transgenic TCR β minilocus activates its recombination in DN thymocytes (Ferrier, Covey et al. 1990). The ACE function of E β was confirmed by its targeted deletion in mice, which cripples recombination at both D β J β clusters (Bories, Demengeot et al. 1996; Bouvier, Watrin et al. 1996). The E β knockout also ablates germline transcription of both D β J β clusters and converts their associated chromatin modifications into a heterochromatic pattern (e.g., H3/H4 hypoacetylation and CpG hypermethylation) (Mathieu, Hempel et al. 2000; Spicuglia, Kumar et al. 2002). Interestingly, the ACE function of E β can be replaced with a heterologous enhancer. In transgenic miniloci, the IgH enhancer, E μ , can target D β →J β recombination in both B and T lineage cells (Ferrier, Covey et al. 1990). However, targeted replacement of the endogenous E β element by E μ permits TCR β recombination in thymocytes but not in B lineage cells, where E μ normally functions (Bories, Demengeot et al. 1996). These findings suggest that a negative regulatory element, which is

missing from the transgenic substrate, may repress E_{μ} function in precursor B cells if the enhancer is positioned within the TCR β locus.

A second reason that many studies of accessibility control have focused on the TCR β locus is the extensive characterization of a germline promoter associated with the D β 1 gene segment. This promoter, called PD β 1, is positioned directly 5' of D β 1, includes a consensus TATA sequence within the 5'D β -RSS, and directs germline transcription through the D β 1J β cluster in pro-T cells (Sikes, Gomez et al. 1998; Doty, Xia et al. 1999). Germline transcription analyses indicate the presence of an analogous promoter near D β 2; however, the putative PD β 2 element remains to be characterized (Whitehurst, Chattopadhyay et al. 1999).

The essential ACE function of PD β 1 in TCR β gene assembly has been demonstrated at both the endogenous locus and in model substrates (Sikes, Suarez et al. 1999; Whitehurst, Schlissel et al. 2000). Deletion of PD β 1 cripples transcription and rearrangement of the D β 1J β but not the D β 2J β cluster, suggesting that the promoter may influence chromatin accessibility over a limited range (Whitehurst, Chattopadhyay et al. 1999). In this regard, D β →J β recombination in minilocus substrates is severely impaired by moving PD β 1 only 400 bp from its native location, even though the promoter remains transcriptionally active (Sikes, Meade et al. 2002).

In contrast to D β →J β rearrangement, much less is known about the cis-acting elements that regulate the second step of TCR β gene assembly, V β →D β J β recombination. V β gene segments are clearly active for germline

transcription and exhibit hallmarks of active chromatin in DN cells (Senoo and Shinkai 1998; Jackson and Krangel 2005). However, neither of these features are altered in E β knockout thymocytes, suggesting this element does not control chromatin accessibility at V β segments (Mathieu, Hempel et al. 2000). The additional element(s) that controls V β accessibility likely is not located between the V β cluster and D β 1 because germline deletion of this region has no effect on TCR β gene assembly (Senoo, Wang et al. 2003). Recent studies have demonstrated that V β promoters, which drive transcription of rearranged V β D β J β exons, also contribute an ACE function for their recombination. Deletion of the V β 13 promoter significantly inhibits its rearrangement in cis; however, allelic exclusion of the gene segment remained intact (Ryu, Haines et al. 2004). Accessible chromatin is restricted to regions surrounding V β segments in DN cells rather than spread throughout the entire V β cluster (Jackson and Krangel 2005). Together, these findings suggest that V β promoters may function as enhancer-independent ACEs to induce highly localized changes in chromatin and target V β gene segments for recombination. However, validation of this model awaits additional V β promoter knockouts and a more extensive characterization of chromatin in thymocytes from these animals.

Regulation of additional TCR and Ig genes

In addition to insights gained by examination of the TCR β locus, much has been learned by studying genetic loci that encode the five other antigen

receptors. This section describes several unique aspects of cis-acting regulation in these additional TCR and Ig loci.

Transcription within the TCR α/δ locus is controlled by distinct enhancers. The E α element is positioned downstream of C α , while E δ is situated between the V α and J α clusters (Fig. 2). Targeted deletion of the E α results in a severe reduction of germline J α transcription and V α →J α rearrangement in developing thymocytes (Sleckman, Bardon et al. 1997). More recent studies have shown that E α controls not only the J α cluster but also affects germline transcription and chromatin modifications at the subset of proximal V α gene segments that are used preferentially in DP cells (Hawwari and Krangel 2005). Thus, the ACE function of E α extends over an astounding range of at least 400 kb.

Elimination of E α did not significantly alter the level of TCR δ rearrangement but attenuated transcription of rearranged TCR δ genes (Sleckman, Bardon et al. 1997). In contrast, germline deletion of E δ severely impairs recombination of TCR δ gene segments, but spares V α →J α rearrangement (Monroe, Sleckman et al. 1999). Interestingly, regional control within the TCR α/δ locus by the two separate enhancers cannot be explained by enhancer location because replacement of E α with E δ fails to restore TCR α recombination (Bassing, Tillman et al. 2003).

The promoter elements that control transcription at the TCR α/δ locus have been studied in considerable detail. A germline promoter, termed T early alpha (TEA) is positioned upstream of the most 5' J α (J α 61) gene segment. A localized ACE function for TEA was confirmed by its germline deletion, which

abrogates both transcription and recombination specifically of 5' J α segments (Villey, Caillol et al. 1996). Furthermore, recent studies indicate that a series of at least four germline promoters control the accessibility of specific regions within the J α cluster (Hawwari, Bock et al. 2005).

Gene expression at the Ig κ locus is controlled by a collection of cis-acting elements that includes three enhancers: one in the J κ /C κ intron (iE κ), a second located 9 kb downstream of C κ (3'E κ), and a recently defined element, called Ed, positioned downstream of 3'E κ (Fig. 2) (Gorman and Alt 1998; Liu, George-Raizen et al. 2002). To date, the ACE functions of only iE κ and 3'E κ have been tested by germline deletions. Single deletions of either enhancer significantly impair V κ →J κ rearrangement (5-10X each), while a dual E κ /3'E κ deletion completely cripples J κ transcription and recombination in cis. These results suggest that a collaborative effort between cis-acting enhancers is required for efficient transcription and rearrangement to occur at the Ig κ locus. Pre-B cells that fail to generate an alloreactive Ig κ allele proceed to rearrange their Ig λ locus. Consistent with the emerging theme that promoters regulate accessibility to recombinase in a highly localized manner, targeted insertion of a neo expression cassette upstream of J λ 1 dramatically increases its germline transcription and rearrangement (Sun and Storb 2001). Thus, the strength of a promoter driving J λ germline transcription, rather than its specific architecture, may determine the efficiency of recombination at Ig λ .

Role of Transcription in Accessibility Control of V(D)J Recombination

Cis-acting elements regulate chromatin accessibility and recruit factors that facilitate efficient transcription of linked genes. Numerous studies established tight spatial and temporal correlations between transcription and changes in chromatin accessibility that render gene segments accessible to V(D)J recombinase. These studies suggest that transcription itself may regulate the recombination potential of gene segments. Alternatively, chromatin alterations that generate recombinase accessibility may coincidentally permit transcription as a byproduct of chromatin opening at promoters.

Many studies support a role for transcription in accessibility control mechanisms. Expression of transfected recombination substrates almost invariably correlates with their recombination efficiencies (Blackwell, Moore et al. 1986; Oltz, Alt et al. 1993). Targeted deletion of germline promoters that drive transcription through linked gene segments block their efficient rearrangement (Villey, Caillol et al. 1996; Whitehurst, Schlissel et al. 2000; Sikes, Meade et al. 2002; Hawwari, Bock et al. 2005). Mice defective for IL-7 signaling exhibit a dramatic reduction in both transcription and recombination of distal V_H gene segments (Corcoran, Riddell et al. 1998).

Despite these findings, mounting evidence suggests that promoters and enhancers function as ACEs via mechanisms that are independent of transcription. Numerous examples have been reported where transcription of gene segments is insufficient for their recombination (Okada, Mendelsohn et al. 1994; Tripathi, Mathieu et al. 2000). Certain V_H segments are transcribed in

wild-type or PAX-5-deficient pro-B cells but are not rearranged efficiently (Angelin-Duclos and Calame 1998; Hesslein, Pflugh et al. 2003). Moreover, targeted insertion of E α adjacent to the V β 12 segment drives its transcription in DP thymocytes but fails to target it for rearrangement (Jackson, Kondilis et al. 2005).

Conversely, several examples of transcription-independent recombination have been reported. Tethering of the glucocorticoid receptor to episomal substrates disrupts nucleosomal arrays at neighboring gene segments and leads to their recombination in the absence of detectable transcription (Cherry and Baltimore 1999). Likewise, inversion of the PD β 1 promoter in chromosomal substrates cripples transcription through D β J β gene segments but D β →J β rearrangement is unaffected (Sikes, Meade et al. 2002). Thus, a regulatory model has emerged in which transcriptional read through of gene segments is neither necessary nor sufficient for their recombination. Instead, the ACE function of promoters is necessary to induce localized changes in chromatin accessibility that facilitates recognition by the RAG complex. It remains likely, however, that transcription can serve to either augment or to propagate recombinase accessibility beyond promoter-proximal regions.

Classic sterile transcripts initiate from either germline or V segment promoters and proceed in a sense direction through target gene segments and RSSs. However, recent studies have shown that a second form of germline transcription exists within the V_H cluster. Corcoran and colleagues detected both genic and intergenic transcripts through the V_H region in pro-B cells (Bolland,

Wood et al. 2004). These newly identified RNAs were expressed in an anti-sense orientation relative to the V_H promoters and coding regions. Importantly, anti-sense transcription is developmentally regulated and correlates with the targeting of V_H gene segments for recombination (i.e., activated subsequent to D_H→J_H recombination and extinguished following V_H→D_HJ_H rearrangement). Analogous to its function at the β-globin locus (Gribnau, Diderich et al. 2000), anti-sense transcription may play an important role in the initiation and/or propagation of remodeling events that extend chromatin accessibility over the broad V_H region.

Control of Recombinase Accessibility by Chromatin Modifications and Remodeling

Although it is clear that accessibility to V(D)J recombinase requires chromatin remodeling, the epigenetic and biochemical mechanisms involved in this process are just beginning to emerge. Similar to studies of gene expression, numerous correlations now exist between chromatin modifications, nuclease sensitivity, and V(D)J recombination. Despite these links, causal relationships between many of these processes have not been established. Moreover, tantalizing new data suggest that RAG proteins play a more direct role in bridging chromatin and recombination because the C-terminus of RAG-2 binds directly to histones in vitro (West, Singha et al. 2005). This may translate in vivo to a regulatory scheme in which the RAG complex associates with higher affinity to histones bearing specific modifications, increasing the local concentration of recombinase at specific RSSs.

CpG methylation

Methylation of CpG dinucleotides is an important component of many mechanisms that enforce heritable silencing of genetic loci. Accordingly, hypermethylated regions within the genome generally adopt inaccessible chromatin configurations (Vermaak, Ahmad et al. 2003). The repressive nature of this DNA modification is likely due to the recruitment of methyl-CpG binding proteins, such as MeCP2, which interact with HDAC activities and can recruit nucleosome remodeling complexes that establish a repressive chromatin environment (Nan, Ng et al. 1998; Fuks, Hurd et al. 2003). Consistent with this model, gene segments located in regions of CpG hypermethylation are usually silent with respect to V(D)J recombination (Bergman, Fisher et al. 2003). Thus, erasure of CpG methylation is thought to be a prerequisite for the establishment of a recombinase accessible locus.

A primary function of ACEs may be to target demethylation or to protect gene segments from de novo methylation (Demengeot, Oltz et al. 1995; Mostoslavsky, Singh et al. 1998). Deletion of either PD β 1 or E β from the endogenous TCR β locus produces a dramatic increase in CpG methylation and a corresponding decrease in nuclease sensitivity within the D β J β cluster (Mathieu, Hempel et al. 2000; Whitehurst, Schlissel et al. 2000). In one reported instance, CpG methylation was shown to directly suppress V(D)J recombination. The 3'D β 1-RSS contains a CpG dinucleotide. Analysis of joins at TCR β loci lacking PD β 1 suggested that methylation at this dinucleotide is incompatible with RAG-

mediated cleavage (Whitehurst, Schlissel et al. 2000). Subsequent in vitro studies confirmed this interpretation (Nakase, Takahama et al. 2003). However, because the vast majority of RSSs lack a CpG motif, the primary effect of CpG methylation at antigen receptor loci likely is to inhibit chromatin accessibility of gene segments to recombinase.

Changes in DNA methylation play a dual regulatory role at the $Ig\kappa$ locus, ensuring its stage-specific activation while restricting functional rearrangement to a single $Ig\kappa$ allele (Bergman and Cedar 2004). During the pro-B \rightarrow pre-B cell transition, a single, randomly selected $Ig\kappa$ allele undergoes demethylation within the $J\kappa C\kappa$ region (Mostoslavsky, Singh et al. 1998). The demethylated allele exhibits numerous hallmarks of an accessible locus, including early replication, germline $J\kappa$ transcription, and histone hyperacetylation (Goldmit, Ji et al. 2005). In contrast, the remaining hypermethylated allele associates with repressive chromatin and is decorated with the heterochromatin protein, HP1. Monoallelic demethylation is enhancer-dependent ($iE\kappa$ and $3'E\kappa$ are required) and the hypomethylated allele is indeed targeted for the vast majority of $V\kappa\rightarrow J\kappa$ recombination (Mostoslavsky, Singh et al. 1998). Thus, ACE-mediated demethylation may be a primary mechanism for maintaining allelic exclusion at $Ig\kappa$.

Histone modifications

In general, gene segments within recombinationally active loci exhibit the same pattern of histone modifications that characterize expressed genes. For

example, recombinogenic D β J β clusters in pro-T cells possess high levels of H3-K9 and H4 acetylation, high levels of H3-K4 methylation, but low levels of H3-K9 methylation (Tripathi, Jackson et al. 2002; Morshead, Ciccone et al. 2003). The opposite pattern of histone modifications is seen at the D β J β region in pro-B cells (Morshead, Ciccone et al. 2003). These correlative data strongly suggest that the histone code is a primary determinant in controlling tissue-, stage-, and allele-specific changes in chromatin accessibility to the RAG complex.

Histone acetylation

Numerous correlations have emerged between H3/H4 acetylation and the recombination potential of antigen receptor gene segments (McMurry and Krangel 2000; Chowdhury and Sen 2003; Morshead, Ciccone et al. 2003; Espinoza and Feeney 2005). These data suggest that histone hyperacetylation is a necessary component of recombinase accessibility; however, definitive cause/effect relationships between these two processes have not been established. A growing body of evidence indicates that histone hyperacetylation clearly is not sufficient for targeting rearrangements. Deletion of a germline promoter left histones hyperacetylated over most of the D β 1J β cluster in an artificial TCR β minilocus, but these gene segments failed to rearrange (Whitehurst, Schlissel et al. 2000). A similar disconnect between hyperacetylation and recombination was reported for distal V_H gene segments in pro-B cells from PAX-5-deficient mice (Hesslein, Pflugh et al. 2003).

Hypoacetylation of H3/H4 is clearly a feature of recombinationally inert gene segments. The conversion from a hyper- to a hypoacetylated status at H3-K9 appears to be an important component of allelic exclusion. During the pro-B→pre-B cell transition, the loss of IL-7R signaling leads to a simultaneous reduction in acetylation levels and chromatin accessibility at V_H gene segments (Chowdhury and Sen 2003). A similar reduction in acetylation is observed at V_β segments during the DN→DP transition (Tripathi, Jackson et al. 2002). Little is known about the HATs and HDACs that mediate changes in the acetylation status of antigen receptor loci. However, deletion of E_β perturbs the ratio of HAT complexes at the germline promoter region, leading to an increased occupancy by P300 at expense of CBP and PCAF (Spicuglia, Kumar et al. 2002). The shifting balance of HAT complexes may alter the precise array of H3 and H4 lysine residues that are targeted for acetylation and thereby fail to present the proper docking platform for requisite chromatin remodeling complexes (Agalioti, Chen et al. 2002).

H3-K9 methylation

Chromatin at recombinationally inert loci is invariably hypoacetylated at H3-K9 but is enriched for methylation on this histone residue (Morshead, Ciccone et al. 2003; Johnson, Pflugh et al. 2004). For example, V_H segments display a tissue-specific difference in di-methyl H3-K9, with a hypermethylated status in thymocytes and non-lymphoid cells versus a hypomethylated/hyperacetylated status in pro-B cells (Johnson, Pflugh et al. 2004). This tissue-specific erasure of

H3-K9 methylation at V_H segments requires the expression of PAX-5 in pro-B cells. Thus, PAX-5 potentially regulates $V_H \rightarrow D_H J_H$ recombination at two distinct levels – IgH locus contraction and revision of chromatin modifications at the V_H cluster (Fuxa, Skok et al. 2004; Johnson, Pflugh et al. 2004). In contrast to its tissue-specific regulation, H3-K9 di-methylation apparently is not involved in stage-specific control of $V_H \rightarrow D_H J_H$ recombination because the V_H cluster remains hypomethylated in pre-B cells after allelic exclusion inhibits their recombination (Johnson, Pflugh et al. 2004). However, the status of tri-methyl H3-K9, a modification that has been implicated in more stable forms of gene repression, has not been examined at the V_H or any other gene segment cluster.

Unlike other histone modifications, a direct cause/effect relationship between H3-K9 methylation and recombinase accessibility has been established using a TCR β minilocus. Recruitment of the G9a histone methyltransferase (HMT) to active chromosomal substrates cripples both germline transcription and D $\beta \rightarrow$ J β recombination even when functional ACEs are present (Osipovich, Milley et al. 2004). The repressive effects of G9a recruitment on histone modifications and substrate accessibility are highly localized and reversible in nature. These features are reminiscent of the transient silencing induced at the TdT and RAG loci in DP thymocytes, where only small regions proximal to their promoters are reversibly methylated at H3-K9 (Su, Brown et al. 2004). In contrast, persistent and widespread H3-K9 methylation occurs upon the heritable silencing of these genes during DP \rightarrow SP differentiation. It remains possible that pro-B cells employ a similar strategy to rapidly establish inaccessible chromatin at V_H segments for

allelic exclusion (and perhaps pro-T cells for V β segments). This may occur by recruitment of an HMT to establish highly localized regions of H3-K9 dimethylation at V_H segments, which would rapidly extinguish their accessibility to recombinase. A more stable form of repression may develop upon differentiation to the pre-B cell stage via widespread distribution of tri-methyl H3-K9 and CpG methylation throughout the entire V_H cluster.

H3-K27 methylation

Methylation of H3-K27 normally associates with the stable repression of transcription units (Peters, Kubicek et al. 2003). The methyl-H3-K27 mark is imprinted by Polycomb group proteins, such as the Ezh2 methyltransferase, which is a critical component of the PRC2 repressor complex (Cao, Wang et al. 2002; Kuzmichev, Jenuwein et al. 2004). To date, there have been no reports of H3-K27 methylation status at recombinase accessible versus inaccessible antigen receptor loci. This may be due to the limited utility of available antibodies for CHIP assays. However, the Ezh2 gene has been deleted specifically in B lineage cells using a conditional knockout approach. Surprisingly, the Ezh2 deletion inhibits rearrangement of distal V_H gene segments but has no effect on their germline transcription or histone acetylation (Su, Basavaraj et al. 2003). Ablation of Ezh2 reduces the overall levels of histone methylation at distal V_H segments but it remains unclear whether this decrease corresponds to methylation at the H3-K27 residue. Because Ezh2 and H3-K27 methylation are

normally repressive, this unexpected finding may reflect an indirect rather than a direct effect of the HMT on recombinational control at the distal V_H cluster.

Nucleosome remodeling

In vitro studies have clearly established that positioned nucleosomes form potent barriers for RAG-mediated cleavage of substrates (Kwon, Imbalzano et al. 1998). This reductionist approach also revealed that the precise phasing of a nucleosome relative to an RSS profoundly influences the efficiency of RAG cleavage. Importantly, treatment of nucleosomal substrates with Brg1, the ATPase component of many SWI/SNF remodeling complexes, rescues RAG cleavage (Kwon, Morshead et al. 2000). These studies are even more exciting given the recent finding that many RSSs have an intrinsic nucleosome positioning function, which may provide an inherent protection from inappropriate recombination until the associated nucleosome is remodeled (Baumann, Mamais et al. 2003). Thus, recombinase accessibility at compatible RSSs almost certainly relies on the reorganization of resident nucleosomes via the action of ACEs.

In this regard, a subset of histone modifications (e.g., acetylation), as well as the basal transcription machinery itself, can recruit SWI/SNF complexes to sites of active transcription (Hassan, Neely et al. 2001). Recent ChIP studies have revealed that the catalytic component of this remodeling complex, Brg1, is broadly associated with clusters of gene segments that are poised for recombination (Morshead, Ciccone et al. 2003). Importantly, this association is enhancer-dependent for the D β J β cluster in pro-T cells (Spicuglia, Kumar et al.

2002). A medium resolution map of nucleosomes at the D β J β cluster suggests that deletion of E β may increase nucleosome density at the recombinase-inaccessible D β segment (Spicuglia, Kumar et al. 2002). Despite these advances, large gaps exist in our knowledge of the genetic and epigenetic requirements for recruitment of remodeling complexes to antigen receptor loci. Likewise, the ACE-dependent features of nucleosome organization and reorganization that occurs at targeted gene segments needs to be addressed.

Statement of the Problem

Although precursor T and B lymphocytes share a common enzyme and a common substrate, V(D)J recombination is strictly regulated at several levels including tissue and stage-specificity. Previous experiments have shown that this is not due to differential expression of the RAG recombinase since this enzyme complex is expressed throughout T and B cell lymphopoiesis. Therefore, it has been proposed that substrate specificity is regulated by changes in the chromatin accessibility at V, D, and J gene segments. In keeping with this hypothesis, it has been shown that germline transcripts of antigen receptor genes originate from rearranging loci (Van Ness, Weigert et al. 1981; Yancopoulos and Alt 1985). Due to this discovery, the role of transcriptional control elements in the regulation of V(D)J recombination quickly came under scrutiny.

The TCR β locus, like all antigen receptor loci, contains multiple cis-acting elements including a single enhancer (E β) and two germline promoters in its

D β J β clusters. In mice, deletion of either transcriptional control element, leads to a significant loss in levels of transcription as well as D β →J β rearrangement. While deletion of E β cripples rearrangement across the entire D β J β cluster, removal of the germline promoter, PD β 1, specifically inhibits recombination and transcription of the D β 1J β gene family (Bories, Demengeot et al. 1996; Bouvier, Watrin et al. 1996; Whitehurst, Chattopadhyay et al. 1999). Interestingly, studies using an artificial substrate, the TCR β minilocus, have demonstrated that promoter positioning is critical in directing efficient rearrangement (Sikes, Meade et al. 2002). Relocation of the promoter to a position less than 500 bp from its native position results in a considerable decrease in recombination. Furthermore, rearrangement of D β and J β gene segments can occur despite mutations that abrogate transcription through the D β 1J β cluster. Together, these data suggest that in addition to directing transcription an alternate role exists for the germline promoter, PD β 1, in directing D β →J β rearrangement.

The primary goal of the research presented in this dissertation is to define the molecular and epigenetic mechanisms by which cis-acting elements in the TCR β locus regulate V(D)J recombination. Specifically, my studies focus on the individual and cooperative impact of the germline promoter, PD β 1, and the enhancer, E β , on chromatin structure during D β →J β rearrangement in thymocytes.

CHAPTER II

DIFFERENTIAL REGULATION OF CHROMATIN ACCESSIBILITY BY CIS-ACTING ELEMENTS

Introduction

In lymphocytes, the regulation of antigen receptor loci involves the concerted action of promoters and enhancers that are separated by large distances. A collection of these cis-acting elements controls not only the transcription of functional immunoglobulin (Ig) and T-cell receptor (TCR) genes, but also regulates a unique genetic process required for their assembly (Oltz 2001; Krangel 2003; Jung and Alt 2004). The assembly process, termed V(D)J recombination, creates a diverse repertoire of Ig and TCR variable region genes from large arrays of V (variable), D (diversity), and J (joining) gene segments. Rearrangement of the gene segments in precursor lymphocytes is mediated by an enzymatic complex (V(D)J recombinase) containing RAG-1 and RAG-2 proteins (Schatz, Oettinger et al. 1989; Oettinger, Schatz et al. 1990; Hesslein and Schatz 2001). The RAG complex targets Recombination Signal Sequences (RSSs) that directly flank the coding region of each gene segment. Individual RSSs include a conserved heptamer/nonamer pair separated by a non-conserved spacer that is either 12 or 23 base pairs (bp) in length. For two gene segments to undergo recombination, one segment must be flanked by a 12 bp RSS and the other segment by a 23 bp RSS (12/23 rule). Following recognition of two compatible RSSs, the RAG complex introduces double-stranded breaks

precisely at the RSS/coding region borders. The resultant DNA ends are resolved by ubiquitous repair factors to form signal joints and chromosomal coding joints (Roth, Lindahl et al. 1995; Hesslein and Schatz 2001; Bassing, Swat et al. 2002). Thus, all Ig and TCR rearrangements share a common enzyme/substrate system (RAG-1/2 and RSSs).

Despite this shared recombination system, the assembly of antigen receptor genes is controlled at numerous levels, including tissue- and stage-specificity. For example, TCR gene rearrangements are restricted to developing thymocytes, whereas complete Ig gene assembly occurs only in precursor B cells (Oltz 2001; Krangel 2003; Mostoslavsky, Alt et al. 2003). In the context of stage-specificity, thymocytes initiate rearrangement of the TCR β locus in pro-T cells (D β →J β then V β →D β J β). Assembly of an in-frame TCR β gene leads to the expression of a pre-T cell receptor, differentiation of the pro-T cell clone to the pre-T cell stage, and the initiation of TCR α rearrangement (Shinkai, Koyasu et al. 1993). Prior studies have shown that the tissue- and stage-specific aspects of V(D)J recombination are governed by changes in the accessibility of gene segment clusters to the RAG1/2 complex (Stanhope-Baker, Hudson et al. 1996). Chromatin associated with rearranging gene segments adopts a more “open” configuration than chromatin at recombinationally inert regions within a locus (Mathieu, Hempel et al. 2000; Chowdhury and Sen 2003). Recombinase accessibility has also been correlated with the transcription of unrearranged gene segments, suggesting that the two processes share common regulatory elements (Yancopoulos and Alt 1985). Indeed, deletion of either transcriptional promoters

or enhancers from antigen receptor loci dramatically impairs V(D)J recombination in cis (Bories, Demengeot et al. 1996; Bouvier, Watrin et al. 1996; Whitehurst, Chattopadhyay et al. 1999; Krangel 2003; Mostoslavsky, Alt et al. 2003). These findings indicate that enhancers and germline promoters function as accessibility control elements (ACEs) to regulate the tissue- and stage-specific assembly of antigen receptor genes.

The TCR β locus has served as a tractable model to study the mechanisms by which ACEs regulate V(D)J recombination. The mouse TCR β locus consists of approximately 30 V β genes located upstream of two distinct D β J β clusters (refer to Figs. 2 and 4). In pro-T cells, transcription of each D β J β cluster is initiated at germline promoters that neighbor the D β gene segments (PD β 1 and PD β 2) and require the activity of a single enhancer located at the 3' end of the locus (E β) (Bories, Demengeot et al. 1996; Bouvier, Watrin et al. 1996; Sikes, Gomez et al. 1998; Whitehurst, Chattopadhyay et al. 1999). Consistent with this regulatory architecture, deletion of E β abrogates rearrangement of both D β J β clusters, while deletion of PD β 1 specifically impairs recombination at the D β 1J β cluster. The ACE function of PD β 1 is position-dependent in model recombination substrates and must reside proximal to the D β 1 gene segment to direct its efficient rearrangement (Sikes, Meade et al. 2002). Together, these findings suggest several possible models by which germline promoters and E β coordinately regulate recombinase accessibility at the TCR β locus. For example, E β may function solely to activate the D β germline promoters. These promoters would then serve as position-dependent ACEs to open chromatin

throughout the D β J β clusters. In this model, E β would exhibit no inherent ACE function in opening TCR β chromatin. Alternatively, E β may possess an independent ACE function that directs a generalized opening of chromatin throughout the D β J β clusters. However, activation of the germline promoters may be required to direct a highly localized form of chromatin remodeling that fully unmasks the neighboring D β -RSS for recombination.

To determine how ACEs control TCR β gene assembly, I measured chromatin accessibility in thymocytes from mice lacking either the D β 1 germline promoter (Δ PD β 1) or the E β enhancer element (Δ E β). I found that deletion of E β dramatically reduces chromatin accessibility throughout the D β J β cluster, including D β - and J β -RSSs. In contrast, deletion of PD β 1 significantly reduces the accessibility of chromatin associated with the proximally located D β 1-RSS but spares accessibility at neighboring J β -RSSs. These data indicate that distinct aspects of chromatin remodeling are orchestrated by each transcriptional control element to trigger TCR β gene assembly.

Methods

Cell Lines and Mice

The RAG-deficient pro-B (63-12) and pro-T cell lines (P5424) have been described previously (Shinkai, Rathbun et al. 1992; Chattopadhyay, Whitehurst et al. 1998). These cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% Penicillin-Streptomycin, and 0.05 mM β -mercaptoethanol. Analyses of wild-type TCR β loci were performed using thymocytes from RAG1-deficient mice. Mice harboring deletions of either E β or PD β 1 were bred onto a RAG-deficient background and have been described previously (Mathieu, Hempel et al. 2000; Whitehurst, Schlissel et al. 2000).

Restriction Endonuclease Sensitivity Assays

Nuclei were prepared from either cultured cells or primary lymphocytes (1×10^7) by resuspension in NP-40 lysis buffer on ice for 5 minutes (10 mM Tris [pH 7.4], 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 0.15 mM spermine, and 0.5 mM spermidine) (Weinmann, Mitchell et al. 2001). Cell nuclei were centrifuged (1000 rpm, 5 min., 4°C), washed with 100 μ l of chilled RE digestion buffer (10 mM Tris [pH 7.4], 50 mM NaCl, 10 mM MgCl₂, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM β -Mercaptoethanol, 0.15 mM spermine, and 0.5 mM spermidine) (Weinmann, Mitchell et al. 2001), centrifuged, and resuspended in the recommended buffer for restriction enzyme digestion (50 μ l, New England Biolabs). The nuclei were

incubated on ice for 1 hour with increasing amounts of REs (refer to Fig. 5 legend for concentrations of each enzyme). The RE digestions were terminated by the addition of 2X proteinase K buffer (50 μ l, 100 mM Tris [pH 7.4], 200 mM NaCl, 2 mM EDTA, and 1% SDS) and incubated at 56°C for 1 hour. Each DNA sample was then supplemented with 50 μ l of 1X proteinase K buffer, 50 μ l of RE digestion buffer, and 5 μ l of proteinase K (10 mg/ml) and allowed to incubate overnight at 37°C (Weinmann, Mitchell et al. 2001). The genomic DNA was extracted with phenol/chloroform, treated with RNase A (20 μ g, 4 hours, 37°C), precipitated with ethanol, and resuspended in 200 μ l TE buffer.

Ligation-Mediated PCR

Genomic DNA (5 μ g) isolated from RE-treated nuclei was ligated with linkers specific for overhangs resulting from each enzyme digestion in a 100 μ l reaction (see Table 1 for linker sequences). The linker-ligated DNAs (3 μ l) were amplified using a nested PCR strategy with primers specific for the following regions within the endogenous TCR β locus: the 3'D β 1-RSS (D β 1-RSS (HinfI)), D β 1/J β 1.1 intervening sequences (D β 1(Hinf)J β 1), the J β 1.6-RSS (J β 1.6-RSS(PvuII)), and the 3'D β 2-RSS (D β 2-RSS(HinfI)) (see Table 2 for PCR primer sequences and reaction profiles). The initial amplification was performed for 12 cycles using a linker-specific primer and a TCR β sequence-specific primer. A 3 μ l aliquot of this reaction was used as template for a second, 25-cycle amplification with nested primers (see Table 2). A control PCR assay for total DNA content (C λ) has been described previously (Sikes and Oltz 1999). A

separate PCR assay for enzyme cleavage efficiency in the constitutively expressed c-myc gene was performed using conditions shown in Table 2. Quantification of RES data was accomplished by PhosphorImager analysis (Fuji) and all values were normalized for total DNA content (Cλ).

Table 1. RE accessibility assay linker sequences.

Site of RE Digestion	Linkers [BW1 and BW2(variable)]
D β 1-RSS (Hinfl)	BW1: 5'GCGGTGACCCGGGAGATCTGAATTC3' BW2(Hinfl-1): 5'AATGAATTCAGATCTCCCGGGTCACCGC3'
D β 1(Hinfl)J β 1	BW1: 5'GCGGTGACCCGGGAGATCTGAATTC3' BW2(Hinfl-2): 5'ATTGAATTCAGATCTCCCGGGTCACCGC3'
J β 1.6-RSS (Pvull)	BW1: 5'GCGGTGACCCGGGAGATCTGAATTC3' BW2 (Pvull): 5'GAATTCAGATC3'
D β 2-RSS (Hinfl)	BW1: 5'GCGGTGACCCGGGAGATCTGAATTC3' BW2 (Hinfl-2): 5'ATTGAATTCAGATCTCCCGGGTCACCGC3'
C-myc (Hinfl)	BW1: 5'GCGGTGACCCGGGAGATCTGAATTC3' BW2 (Hinf-3): 5'AGTGAATTCAGATCTCCCGGGTCACCGC3'
C-myc (Pvull)	BW1: 5'GCGGTGACCCGGGAGATCTGAATTC3' BW2 (Pvull): 5'GAATTCAGATC3'

Table 2. RE accessibility assay nested PCR primer sequences and reaction profiles.

PCR amplicon	PCR primers and probes	PCR conditions ¹	Comment
D β 1-RSS	PCR1: 5'GCGGTGACCCGGGAGATCTGAATTC3' 5'TTATGGACGTTGGCAGAAGAGGAT3' PCR2: 5'CCGGGAGATCTGAATTCATTC3' 5'TGCATCCTTTGCTGCTAGGGCC3' 5'GATCTAAACACATCTAGGCTTG3' (probe)	PCR1: 60°C, 12 PCR2: 60°C, 25	Fig. 5,6,7,9
D β 1(Hinf)J β 1	PCR1: 5'GCGGTGACCCGGGAGATCTGAATTC3' 5'GGTTCCTTTACCAAAGAAGACTTCTG3' PCR2: 5'CCGGGAGATCTGAATTCATTC3' 5'GCACAGTGCCATAGGATGAGGAG3' 5'GAGTAATCGCTTTGTG3' (probe)	PCR1: 62°C, 12 PCR2: 62°C, 25	Fig. 9
J β 1.6-RSS	PCR1: 5'GCGGTGACCCGGGAGATCTGAATTC3' 5'GGCATTCTGTGTCCAGAACACG3' PCR2: 5'CCGGGAGATCTGAATTCCTGT3' 5'CCCAGAGAAGAGCAAGCGACCAGGC3' 5'GGTCATCCAACACAGGCACAACCC3' (probe)	PCR1: 62°C, 12 PCR2: 62°C, 25	Fig. 5, 6, 7
D β 2-RSS	PCR1: 5'GCGGTGACCCGGGAGATCTGAATTC3' 5'GGTAGGGACCTTATCACTTCACTCC3' PCR2: 5'CCGGGAGATCTGAATTCATTC3' 5'CCTTATCACTTCACTCTCCACCC3' 5'GGTGCTACCCAGAGCCTG3'(probe)	PCR1: 62°C, 12 PCR2: 62°C, 25	Fig. 6, 7
C-myc for D β 1-RSS D β 1(H)J β 1, D β 2-RSS	PCR1: 5'GCGGTGACCCGGGAGATCTGAATTC3' 5'CCCTTGGGGAGAAGGACG3' PCR2: 5'CCGGGAGATCTGAATTCATTC3' 5'CCGCAACATAGGATGGAG3' 5'GCTGCTTCCCACCCGCCCC3' (probe)	PCR1: 58°C, 12 PCR2: 58°C, 25	Fig. 5, 6, 7, 9
C-myc for J β 1.6-RSS	PCR1: 5'GCGGTGACCCGGGAGATCTGAATTC3' 5'GGGGAAGACCACTGAGGGGTC3' PCR2: 5'CCGGGAGATCTGAATTCCTGC3' 5'CACTGAGGGGTCAATGCACTCGG3' 5'CGGAGAAGCTGGCCTCCTACC3' (probe)	PCR1: 62°C, 12 PCR2: 62°C, 25	Fig. 5, 6, 7

¹ Annealing temperature and cycle number

Results

Chromatin Accessibility of D β - and J β -RSSs

The nucleosomal structure of cellular DNA impedes its interaction with most non-histone proteins, including V(D)J recombinase (Kwon, Imbalzano et al. 1998). Germline promoters and enhancers within antigen receptor loci function to reorganize chromatin structure and generate accessibility to the RAG complex (Whitehurst, Chattopadhyay et al. 1999; Mathieu, Hempel et al. 2000). However, the precise role of each ACE in the control of chromatin accessibility at individual RSSs is unknown. For this purpose, we designed restriction endonuclease sensitivity (RES) assays that independently probe chromatin accessibility at specific D β - and J β -RSSs. This experimental approach offers a distinct advantage over assays that monitor cleavage by the RAG complex because such assays require simultaneous accessibility at two compatible RSSs. Thus, if an ACE deletion impairs accessibility at the D β - but not at J β -RSSs, RAG cleavage would be blocked at both gene segments. However, the appropriate RES assays would reveal differential accessibility of chromatin at the D β - versus J β -RSS.

A potential complication of RES experiments is that certain restriction sites may lie in a DNA linker between two positioned nucleosomes. In this case, the RE site would be accessible in all cells, independent of the ACE functions that regulate D β →J β rearrangement. Indeed, preliminary experiments identified a small set of RE sites within the D β J β cluster that were equally accessible in

nuclear chromatin from pro-T and non-T cells (data not shown). Thus, an important criterion applied to each RES assay used in our studies is that a specific site must be accessible in cells poised for TCR β assembly (pro-T cells), but inaccessible in other cell types. We first probed chromatin accessibility at restriction sites that lie within three distinct RSSs: Hinf I sites at the 3' D β 1- and D β 2-RSSs (Fig. 4, H#1 and H#2, respectively) and a Pvu II site in the J β 1.6-RSS (Fig. 4, P#1). Nuclei from two RAG-deficient cell lines that represent the pro-T (P5424) and pro-B stages (63-12) were treated with escalating concentrations of Hinf I or Pvu II. In both cell types, the TCR β locus is frozen in its germline configuration due to the lack of RAG proteins. This feature of the cell model circumvents complications of ongoing D β \rightarrow J β recombination that would delete RE targets in wild-type pro-T cells. Genomic DNAs from the treated nuclei were analyzed by ligation mediated-PCR reactions (LM-PCR), which were designed to detect specific products of RE cleavage. As shown in Figure 5, RE sites within the D β 1- and J β 1.6-RSSs were cleaved efficiently in the pro-T cell line but were largely resistant to cutting in the pro-B cell line (top panel). These results cannot be attributed to global differences in the efficiency of RE digestion between the two cell lines because similar levels of Hinf I and Pvu II cleavage products were detected for sites in the ubiquitously expressed c-myc gene (Fig. 5, middle panel).

To verify the utility of these assays *in vivo*, we tested whether the accessibility of chromatin at the D β - and J β -RSSs exhibit tissue-specific differences in primary mouse lymphocytes. Accordingly, we compared RE

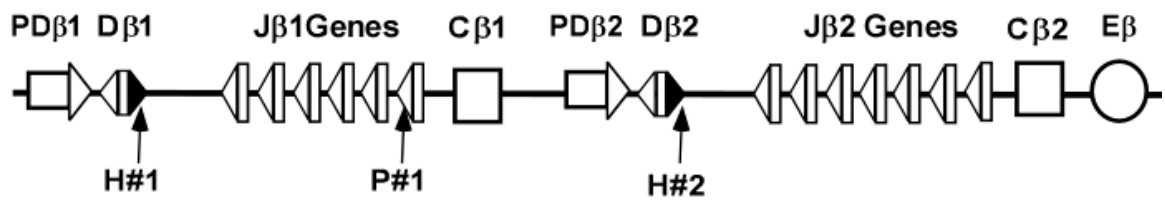


Figure 4. Schematic map depicting the DβJβ region of the mouse TCRβ locus (~25 kb). The locations of Hinf I (H) and Pvu II (P) sites used in the RE sensitivity assays are indicated. Constant region exons (Cβ1 and Cβ2) are shown as a single box and the map is not drawn to scale.

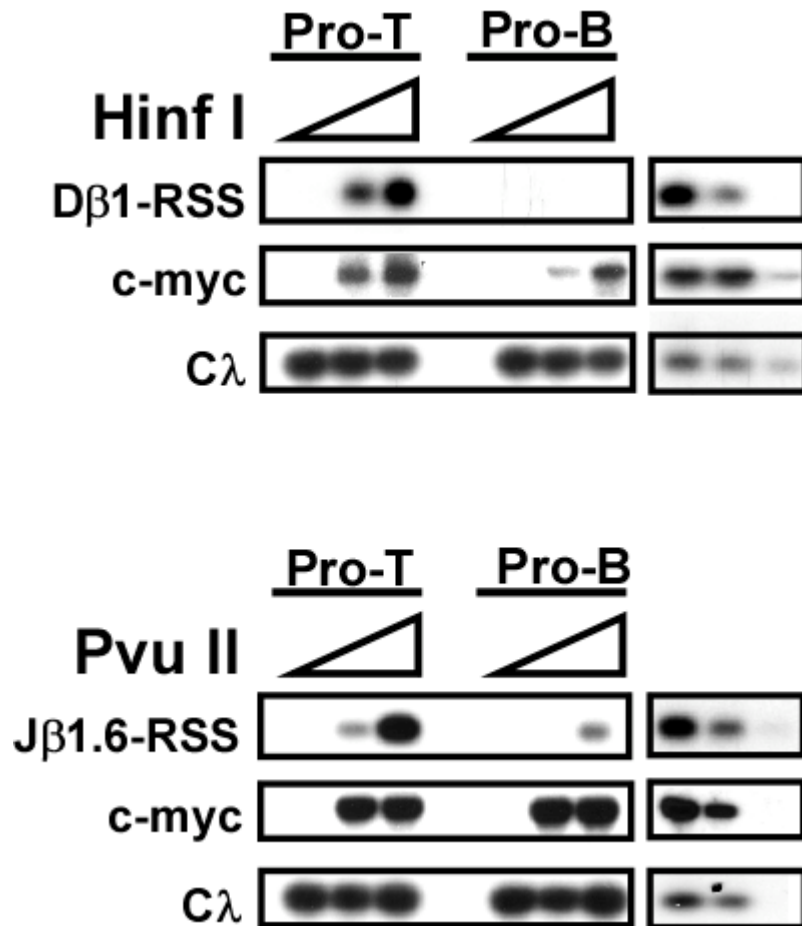


Figure 5. Nuclei from pro-T (P5424) and pro-B (63-12) cells were treated with increasing amounts of the indicated enzyme (0.1 and 1 U of Hinf I, 0.01 or 0.1 U of Pvu II). Enzyme cleavage at the indicated site(s) was analyzed using LM-PCR and Southern blotting as described in the Methods section. Control PCR assays for DNA content (C λ) and enzyme cutting efficiency (c-myc) were performed using the same samples of Hinf I- or Pvu II-digested linker-ligated DNA. The linearity of each assay was confirmed by serial dilutions of the maximally digested pro-T sample.

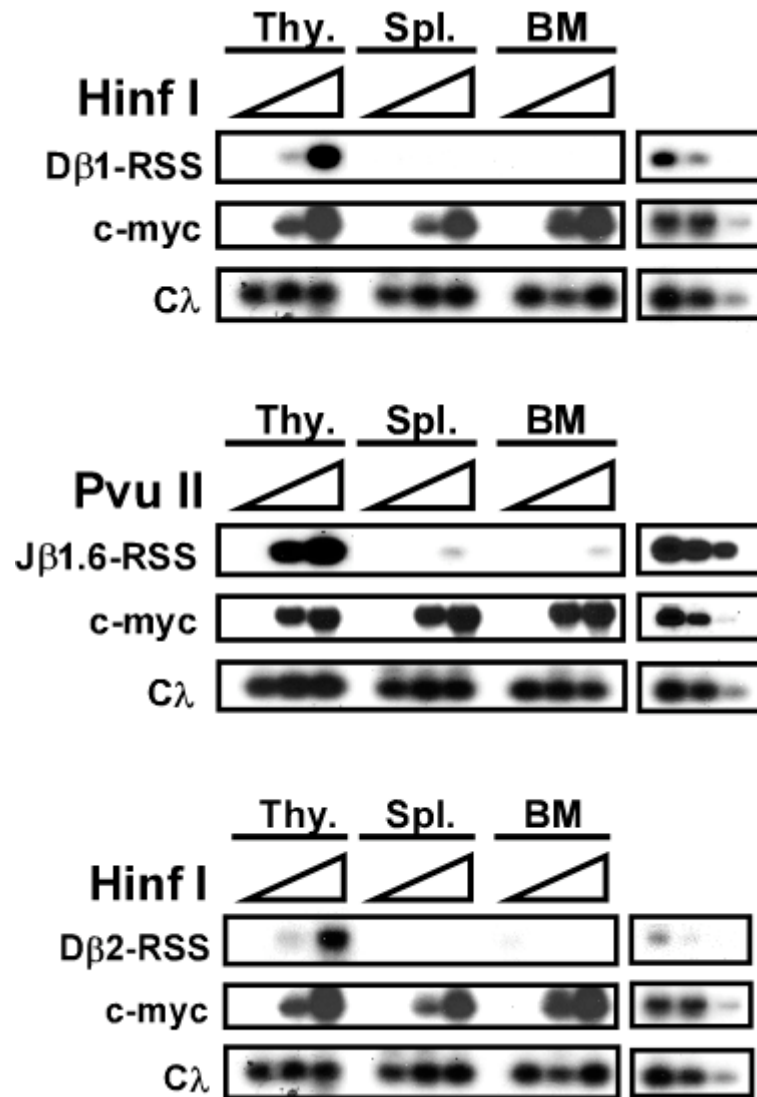


Figure 6. Nuclei from RAG-deficient thymocytes, splenocytes, and bone marrow cells were analyzed using RES assays as described in Figure 5.

cleavage in nuclei obtained from RAG-deficient thymocytes (pro-T cells) versus nuclei from tissues in these mice that lack T-lineage cells (spleen and bone marrow). Consistent with data from transformed cell lines, the D β 1-, D β 2-, and J β 1.6-RSSs were cleaved efficiently in RAG-deficient thymocytes, whereas RE cleavage products were nearly undetectable in nuclei from spleen and bone marrow (Fig. 6). Thus, the RES assays serve as bona fide readouts for changes in chromatin accessibility at these D β - and J β -RSSs.

Differential regulation of RSS accessibility by PD β and E β

The critical first step of TCR β gene assembly is D β →J β rearrangement, which requires the function of both known ACEs -- the E β enhancer and the D β 1 germline promoter (at the D β 1J β cluster) (Bories, Demengeot et al. 1996; Bouvier, Watrin et al. 1996; Whitehurst, Chattopadhyay et al. 1999). However, the overall function of each ACE in the regulation of long-range versus local chromatin accessibility at D β - and J β -RSSs is unknown. Indeed, it remains unclear whether E β possesses an inherent ACE function or completely depends on its communication with PD β to influence recombinase accessibility. To address these fundamental issues, we monitored changes in chromatin accessibility at TCR β loci in mice that lack either E β or the PD β 1 germline promoter. The first mutation removes the core E β element (Δ E β) and completely blocks D β →J β recombination in cis (Bouvier, Watrin et al. 1996). The germline promoter knockout eliminates approximately 3 kb upstream of the D β 1 gene segment, including the functional PD β 1 element (Δ PD β 1). Recombination at the

D β 1J β cluster is profoundly reduced in mice harboring the Δ PD β 1 mutation, whereas D β 2 \rightarrow J β rearrangement is unaffected (Whitehurst, Schlissel et al. 2000).

To monitor the effects of ACE function on D β J β accessibility, we obtained thymocytes from mice containing either wild-type (WT), Δ PD β 1, or Δ E β alleles bred onto a RAG-deficient background. As shown in Fig. 7, RES assays revealed substantial levels of chromatin accessibility at all three RSSs in RAG^{-/-} thymocytes harboring wild-type TCR β alleles (D β 1-, J β 1.6-, and D β 2-RSSs). In sharp contrast, RSSs throughout both D β J β clusters were largely inaccessible to RE digestion in Δ E β mice, even at the highest enzyme concentrations. Quantitative analysis of these data indicates that cleavage at the D β 1-, J β 1.6-, and D β 2-RSS was inhibited five to ten-fold in the Δ E β thymocytes (Fig. 8). These data demonstrate that the deletion of E β impairs chromatin accessibility throughout both D β J β clusters.

The dramatic decrease in D β 1 \rightarrow J β rearrangement observed at Δ PD β 1 alleles may result from a loss of chromatin accessibility at either the D β 1-RSS, the J β -RSSs, or at all RSSs within the gene segment cluster. To distinguish between these possibilities, I performed RES assays on thymocytes from RAG-deficient mice containing Δ PD β 1 alleles. Consistent with its overall effect on D β \rightarrow J β rearrangement, deletion of PD β 1 severely impaired accessibility to Hinf I at the 3'D β 1-RSS but spared chromatin accessibility at an analogous site in the 3'D β 2-RSS. When compared with WT TCR β alleles, Hinf I cleavage at the

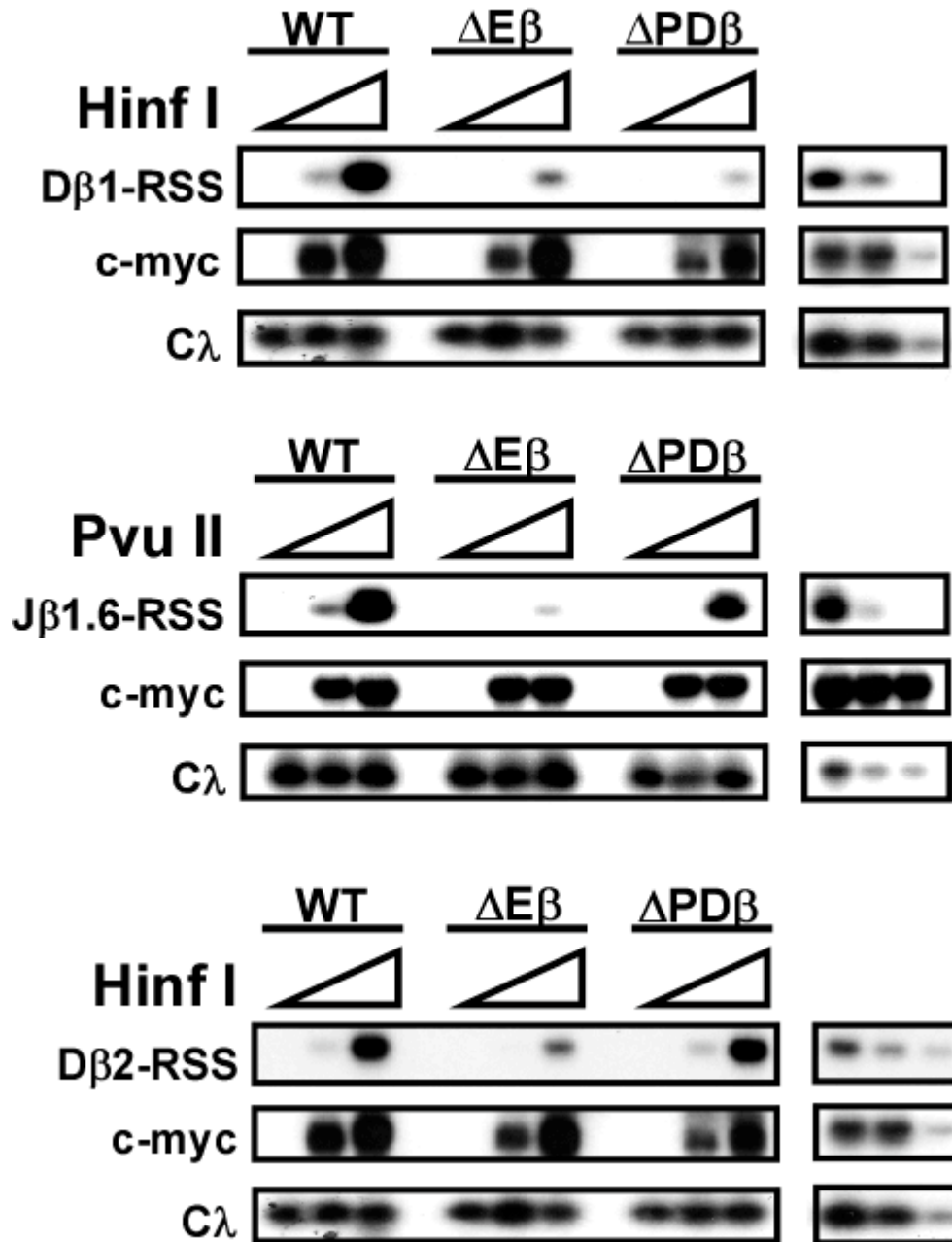


Figure 7. Thymocyte nuclei from RAG-deficient mice containing either wild-type (WT), $\Delta E\beta$, or $\Delta PDB1$ alleles were treated with increasing concentrations of the indicated enzyme and analyzed as described in Figure 5.

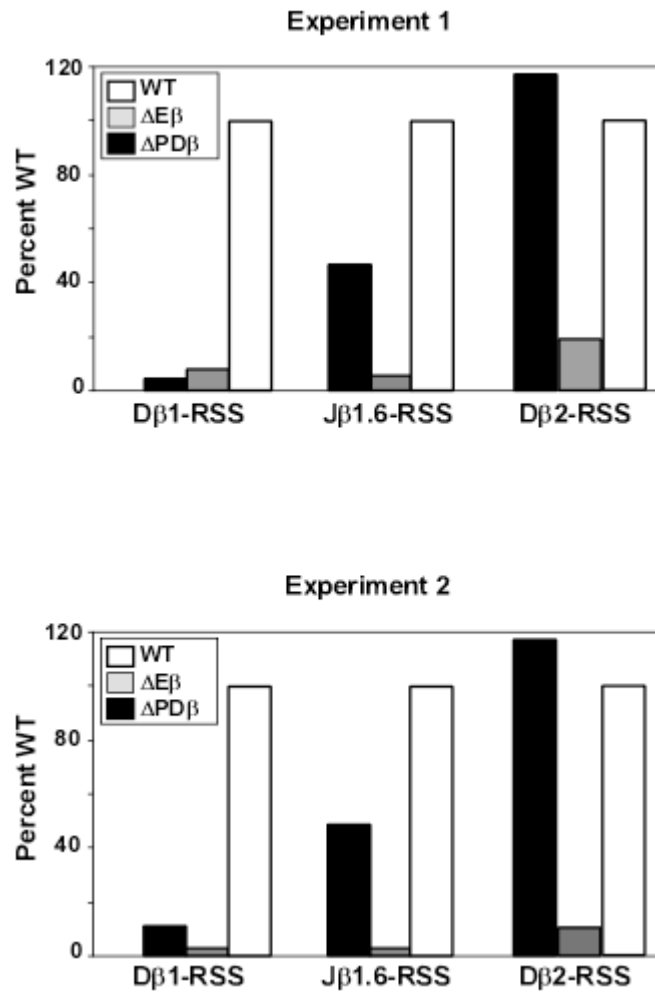


Figure 8. Quantification of LM-PCR assays shown in Figure 7. Signal intensities for the RE cleavage products were measured using a Fuji PhosphorImager and normalized for DNA content ($C\lambda$). Results for $RAG^{-/-}\Delta E\beta$ and $RAG^{-/-}\Delta PD\beta$ thymocytes are displayed as a percentage of the signals obtained from $RAG^{-/-}$ thymocytes (WT).

3'D β 1-RSS was reduced more than eight-fold in Δ PD β 1 thymocytes (Fig. 8). In contrast, the PD β 1 deletion had only modest effects on chromatin accessibility at the J β 1.6-RSS (approximately two-fold relative to the WT TCR β allele) (Fig. 8). These data suggest that the ACE function of this germline promoter is largely restricted to the D β 1 gene segment, while E β exhibits a much broader influence on chromatin accessibility that spreads throughout both D β J β clusters.

To define the precise chromatin domains under the influence of PD β 1 and E β , I examined cleavage at additional Hinf I sites within the D β 1J β cluster (Fig. 9). Consistent with chromatin accessibility at the RSSs, numerous Hinf I sites distributed throughout the J β 1.4 to J β 1.6 region were refractory to enzyme cleavage in Δ E β thymocytes (~8-fold average reduction relative to WT). Accessibility at these same sites was only modestly inhibited upon deletion of the D β 1 germline promoter. Although the exact magnitude of changes in chromatin accessibility varied between Hinf I sites upon PD β 1 deletion, all of these sites were significantly less accessible upon deletion of E β . Importantly, RE cleavage at the Hinf I site located most proximal to the D β 1-RSS was relatively unaffected in Δ PD β 1 thymocytes (H#3, 450 bp 3' to D β 1, Fig. 9). These data demonstrate that the PD β 1 germline promoter has a minimal range of influence on chromatin accessibility within the D β 1J β cluster (< 450 bp). Together, these findings indicate that E β serves as a promoter-independent ACE to regulate long-range chromatin accessibility at RSSs throughout both D β J β clusters, with the exception of the D β 1-RSS. In contrast, the ACE function of PD β 1, which

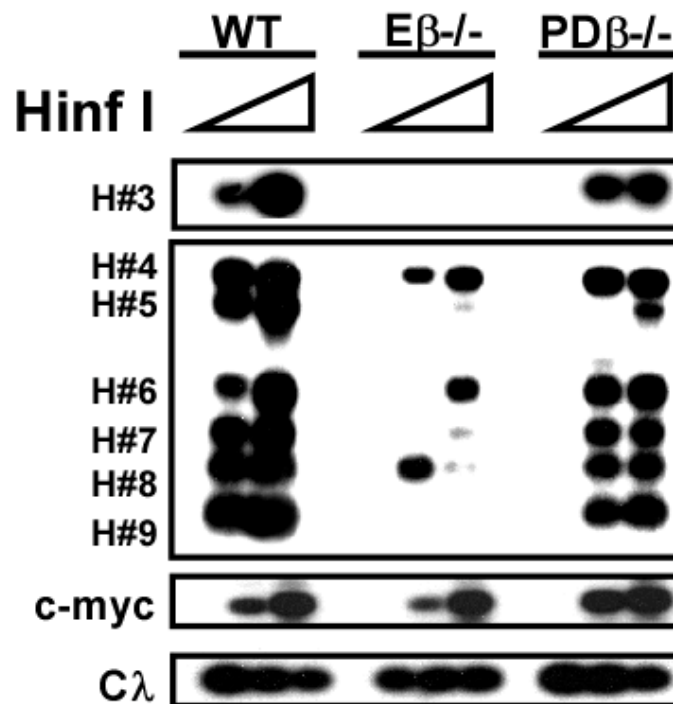
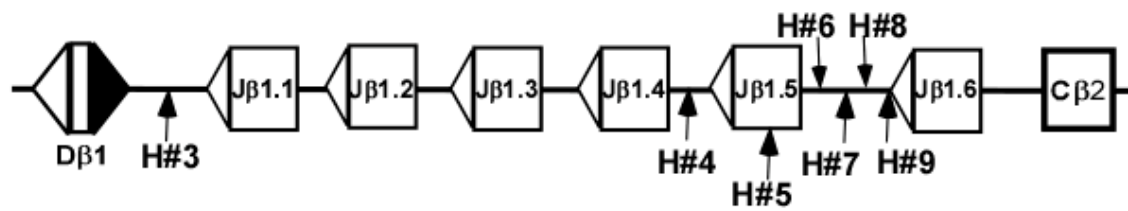


Figure 9. Thymocyte nuclei from the indicated TCRβ genotypes on a RAG-deficient background were treated with Hinf I or Pvu II and analyzed as described in Figure 5. The relative positions of RE sites are displayed in a schematic of the Dβ1Jβ cluster (top).

depends on $E\beta$ directs a highly localized opening of chromatin that is required to render only the most proximal RSS accessible.

Discussion

During Ig and TCR gene assembly, germline promoters and enhancers serve as accessibility control elements that direct chromatin remodeling at each locus and regulate the availability of RSSs to V(D)J recombinase (Oltz 2001; Krangel 2003; Jung and Alt 2004). Enhancers serve as location-independent regulatory elements, while the ACE function of germline promoters is highly dependent on their location relative to target gene segments (Sikes, Suarez et al. 1999; Sikes, Meade et al. 2002). However, little was known regarding the specific role of each ACE in controlling local versus long-range accessibility to RSSs.

Our studies indicate unique functions for the germline promoters and enhancer in opening $D\beta J\beta$ chromatin. Loci lacking $PD\beta 1$ undergo $E\beta$ -dependent remodeling that renders $J\beta 1$ -RSSs accessible for RE cleavage, while $D\beta 1$ -proximal sequences remain inaccessible. These data demonstrate that $E\beta$ possesses an inherent ACE function independent of any interaction with the $D\beta 1$ germline promoter. Notwithstanding, the generality of our finding that $E\beta$ possesses an inherent ACE function is supported by previous studies showing that the Ig heavy chain enhancer $E\mu$ can regulate chromatin accessibility at a prokaryotic promoter in the absence of any collaboration between these two

elements (Jenuwein, Forrester et al. 1997). The inherent ACE function of E β is exerted throughout both J β clusters at distances of at least 15 kb. Interestingly, proximal sequences downstream of E β remain hypoacetylated at histone 3 in thymocytes (Mathieu, Hempel et al. 2000), indicating a directionality for its ACE function.

Enhancer-mediated accessibility at the TCR β locus also activates the D β germline promoters, which are uniquely required for highly localized remodeling of chromatin at the D β 1-RSS. The spatially restricted ACE function of germline promoters provides a unifying explanation for a series of observations relevant to the control of antigen receptor gene assembly. First, PD β 1 functions as a position-dependent ACE in model recombination substrates. Efficient substrate rearrangement requires a promoter position of less than 500 bp from the D β 1 gene segment (Sikes, Meade et al. 2002). This range of ACE function in model substrates is consistent with our new finding that deletion of PD β 1 inhibits chromatin accessibility at distances of less than 450 bp downstream of D β 1. Second, efficient rearrangement of J α gene segments requires a series of germline promoters, which exert only a limited range of ACE function (Hawwari, Bock et al. 2005). Third, promoters (germline or conventional) are consistently located proximal to target gene segments at all antigen receptor loci. Fourth, the ACE function of germline promoters is independent of transcription through target gene segments (Sikes, Meade et al. 2002). The latter finding suggests that the most important role for promoters in V(D)J recombination is to recruit factors that direct highly localized remodeling of chromatin associated with proximal RSSs,

which leads to V(D)J recombinase accessibility and ensuing gene rearrangement.

CHAPTER III

FORMATION OF A PD β /E β HOLOCOMPLEX

Introduction

The transcriptional regulation of multigenic loci is controlled by a dynamic cross-talk between cis-acting elements, including promoters, enhancers, and locus control regions (LCRs). In many cases, these complex loci contain multiple promoters that compete for activation by a common enhancer or LCR to achieve cell type- or stage-specific expression of a given gene (Bulger and Groudine 1999; Smale and Fisher 2002; Tolhuis, Palstra et al. 2002). This regulatory strategy requires communication between promoters and enhancers that are separated in the genome by distances ranging from one to hundreds of kilobases. Two basic models have been proposed for promoter/enhancer cross-talk (Bulger and Groudine 1999). In one model, enhancer activation opens a limited area of chromatin, which ultimately spreads throughout the locus and permits access of transcription factors to a distal promoter (Bulger and Groudine 1999; Dorsett 1999; Engel and Tanimoto 2000). An important feature of this model is that no direct contact between the promoter and enhancer elements is required.

A second long-standing paradigm for transcriptional regulation by promoters and their distal enhancers is the looping model. This model states that regulatory elements communicate via through-space interactions between

proteins bound to the DNA elements (Tolhuis et al). Emerging studies have verified this model for the β -globin and HNF-4 α loci (Tolhuis et al, Hatzis et al). Moreover, studies of the HNF-4 α locus have provided insight into the temporal molecular interactions that lead to promoter activation by a distal enhancer. Upon enterocyte differentiation, histone modifiers are recruited to the HNF-4 α enhancer element, while the transcriptional machinery assembles proximal to the promoter region. The activated enhancer then tracks along the intervening DNA in a unidirectional fashion, altering nucleosomes and acetylating H3 and H4 proteins as it proceeds, until reaching the HNF-4 α promoter. Assembly of a stable promoter/enhancer complex triggers a new wave of chromatin modifications within the promoter region, which generates an environment permissible for transcription. Despite these insights into the control of other genetic loci, the mechanisms of crosstalk between transcriptional promoters and enhancers in the regulation of chromatin accessibility for V(D)J recombination remains unclear.

To explore the mechanisms by which the germline promoter, PD β 1, and the distal enhancer, E β , control recombination within the D β J β cluster, I used mice lacking each of these regulatory elements to perform Chromosome Conformation Capture assays at the TCR β locus. Importantly, I find that PD β and E β are in direct physical contact, forming a stable holocomplex in thymocytes. These results suggest a new paradigm for TCR β gene assembly in which PD β 1 and E β form a holocomplex which is required to control RSS accessibility during T cell development.

Methods

Cell Lines and Mice

The RAG-deficient pro-B (63-12) and pro-T cell lines (P5424) have been described previously (Shinkai, Rathbun et al. 1992; Chattopadhyay, Whitehurst et al. 1998). These cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% Penicillin-Streptomycin, and 0.05 mM β -mercaptoethanol. Analyses of wild-type TCR β loci were performed using thymocytes from RAG1-deficient mice. Mice harboring deletions of either E β or PD β 1 were bred onto a RAG-deficient background and have been described previously (Mathieu, Hempel et al. 2000; Whitehurst, Schlissel et al. 2000).

3C analyses

We employed a modified version of 3C methods that were described previously (Tolhuis, Palstra et al. 2002; Spilianakis and Flavell 2004). In brief, formaldehyde (2% final concentration) was added to 1×10^7 cells in RPMI/10% FCS and cross-linked 10 minutes on ice. The reaction was quenched with glycine (0.125 M final concentration). Nuclei were isolated using an ice-cold cell lysis buffer containing 10 mM Tris (pH 8.0), 10 mM NaCl, 0.2% NP-40, and protease inhibitors. Nuclei were resuspended in restriction enzyme buffer containing 0.3% SDS followed by 2% TX-100, each of which were incubated with shaking at 37°C for 1 hour. These samples were digested with Xba I (400 U

+BSA) overnight at 37°C followed by 400 additional units of enzyme and four hours of incubation. Xba I digestion was terminated by addition of 1.6% SDS and incubation at 68°C for 25 minutes. Samples were diluted in 1X ligation buffer (30 mM Tris, 10 mM MgCl₂, and 1% TX-100, 10 mM DTT, and 1 mM DTT) and incubated under conditions that favor intramolecular ligation (500 U of T4 DNA ligase in a total reaction volume of 7 ml). Ligations proceeded overnight at 16°C, an additional 500 U of ligase were then added and incubated at 16°C (4 hours), followed by a 30 minute incubation at room temperature. Samples were treated with Proteinase K overnight at 68°C and RNase A (37°C, 1 hour) prior to standard DNA purification.

Control templates for the PCR reactions were prepared from a BAC that spans 204 kb of the murine TCR β locus (BAC clone #RP23-421M9). The BAC (30 μ g) was digested with Xba I overnight at 37°C. Equimolar amounts of the resultant Xba I fragments were ligated at a high concentration using T4 DNA ligase to form all possible ligation products (Spilianakis and Flavell 2004). The control templates for IKK β were prepared from a PCR-generated genomic fragment spanning two Xba I sites. The purified PCR product was digested to completion with Xba I, and the three resultant fragments were ligated to generate all possible products. Touchdown PCR assays were developed for each set of primers and optimized to ensure linearity. Each TCR β PCR assay utilized an anchor primer situated downstream of E β and another primer located within either the V β 14, C β 2, D β 2, D β 1, or 30 kb 5' of D β 1 (see Table 3 for primer sequences and reaction profiles). IKK β primers reside in two Xba I fragments

separated by a single restriction fragment and serve as a control for cross-linking efficiencies. PCR products were analyzed on 2% agarose gels, blotted, and hybridized to a radiolabeled internal DNA probe. All PCR reactions were performed in triplicate and provided consistent results.

Table 3. Chromosome Conformation Assay PCR primer sequences and reaction profiles.

PCR amplicon	PCR primers and probe sequences	PCR conditions ¹	Comment
E β Anchor V β 14 C β 2 D β 2 D β 1 5' D β 1 probe	5'GCCAATCCTGCTCTATCCATC3' 5'GATGGTACCAGGCAAAGCTAC3' 5'CTGTACCTAACATCCTCAACCC3' 5'GGATGATGACTACACCTCCATG3' 5'GCTTGTTTCAGAGAGGCCAG3' 5'GCCTGTGGTCACTGTGCTTTG3' 5'GTAGCCCAGCCTTCCTAAG3'	66°C, 5 cycles 64°C, 5 cycles 62°C, 5 cycles 57°C, 18-20 cycles	Fig. 11, 12
5' IKK β 3'IKK β probe	5'CGTGTCCCTTCTCTAGCCTG3' 5'GCATGCCCTCTGGTCCCTAG3' 5'GGCATCAAACCTTGCTCTGTGGC3'	66°C, 5 cycles 64°C, 5 cycles 62°C, 5 cycles 60°C, 20 cycles	Fig. 11, 12

¹ Annealing temperature and cycle number for touchdown PCR

An initial 3 minute 94°C denaturation step was added to the beginning of each PCR followed by cycles of 94°C for 30 seconds, annealing temp for 30 seconds, and 72°C for 1 minute.

Results

Direct interactions between distal E β and D β regions in vivo

To directly probe the spatial organization of D β J β clusters in vivo, I utilized Chromosome Conformation Capture (3C) using cells that contain either accessible or inaccessible TCR β loci (Dekker, Rippe et al. 2002). In the 3C technique, nuclear chromatin is chemically cross-linked and then subjected to restriction enzyme digestion. Following digestion, only the distal regions that form stable interactions will remain covalently attached in the cross-linked chromatin. The digested chromatin is then ligated at concentrations that favor intramolecular reactions between cross-linked restriction fragments rather than between random pieces of DNA. The ligated chromatin is then stripped of protein and specific ligation products are detected by PCR analysis. Using this method, PCR primers specific for distal regions of DNA will generate amplification products only if the two regions associate in a stable conformation that brings them into spatial proximity (Dekker, Rippe et al. 2002; Tolhuis, Palstra et al. 2002; Spilianakis and Flavell 2004).

I began the 3C analyses by using stable cell lines that harbor either an accessible (pro-T, P5424) or inaccessible TCR β locus (pro-B, 63-12). Cross-linked chromatin from these cells was digested with Xba I (refer to Fig. 11 for details), which generates separate restriction fragments encompassing E β , PD β 1, PD β 2, or several regions lacking known transcriptional control elements (e.g., C β 2 and 5'D β 1, Fig. 10). Ligated DNA was analyzed by PCR using an

invariant anchor primer located directly downstream of E β (primer E) and a panel of primers derived from several relevant Xba I restriction fragments (Fig. 10). The efficiency of each PCR assay was monitored using a sample that contained all possible ligation products from the D β J β clusters. This sample was prepared from a bacterial artificial chromosome (BAC) following complete Xba I digestion and ligation of the fragments at high DNA concentration to force intermolecular reactions.

As shown in Fig. 11, chromatin from pro-T cells yields amplification products for the E β -specific primer when it is coupled with either the D β 1 or D β 2 primers (primers B or C). These 3C data indicate a stable interaction between the enhancer and D β regions in this accessible TCR β locus. Intrachromosomal association of these regions is cell type-specific because little or no amplification is observed in pro-B cells, which contain inaccessible TCR β loci. Consistent results were obtained from triplicate PCR amplifications with two independent DNA preparations (data not shown). Proximal restriction fragments within the TCR β locus (E β /V β 14) or the constitutively active IKK β locus provide equivalent levels of amplification in both cell types. These control assays exclude the possibility that cell type specificity observed for E β /D β interactions is solely due to differences in chromatin cross-linking between the two cell lines. Furthermore, the E β /D β interactions are restricted spatially because a primer specific for C β 2 (primer D), which lies between the D β 2 region and E β , does not afford PCR products with the E β anchor. Likewise, long-range interactions are not observed between E β and a region upstream of the D β 1 germline promoter(5'D β primer A).

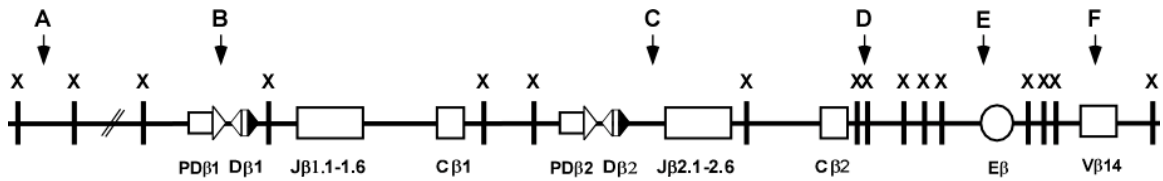


Figure 10. Chromosome Conformation Capture (3C) assay of D β J β clusters. Schematic depiction of a 60 kb region spanning the murine D β J β clusters. Xba I restriction enzyme sites are denoted by “X” and specific Xba I fragments assayed by the 3C technique are highlighted below the schematic (e.g., 5’D β 1). The relative locations of each Xba I fragment are drawn to scale with the exception of 5’D β 1, which resides approximately 30 kb upstream of D β 1. Primers used in the 3C assays are represented by bold lines (A-F). Primer E, located directly downstream of E β , is the invariant anchor primer used in all PCR assays.

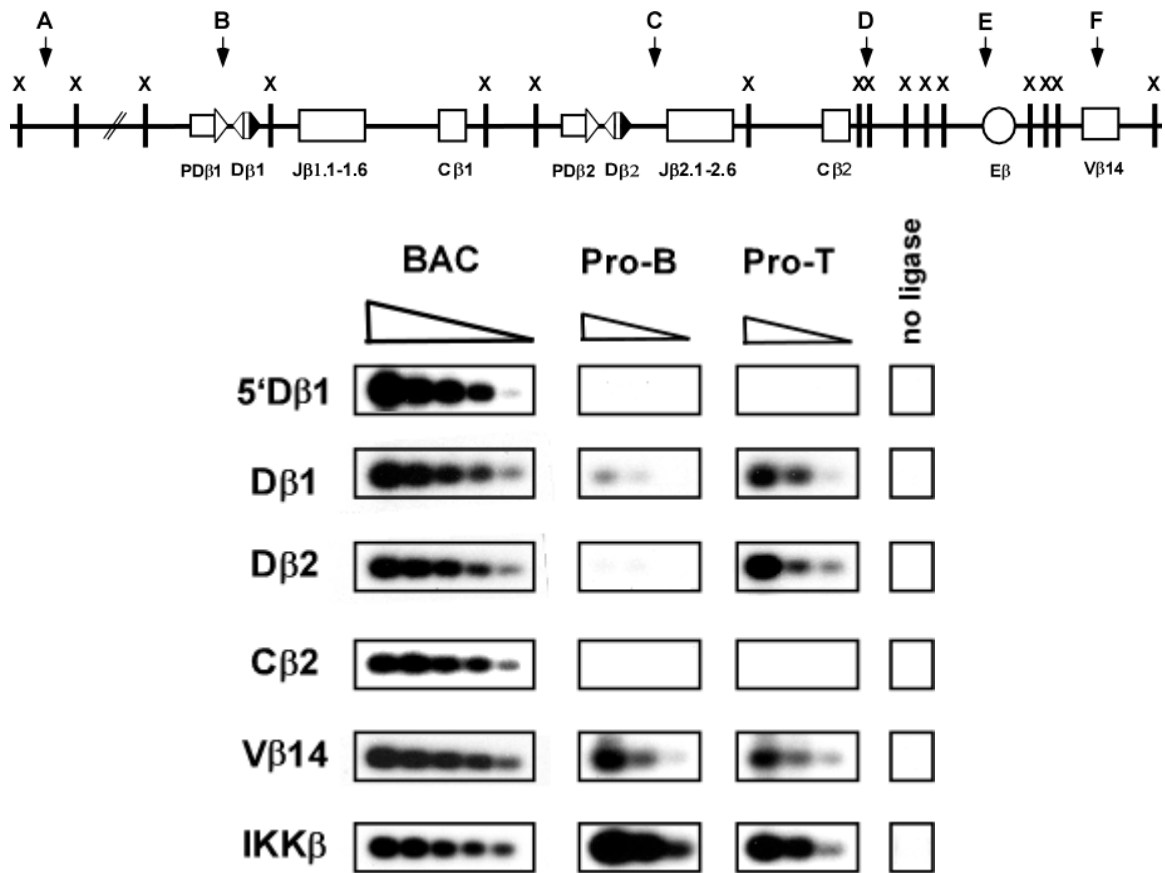


Figure 11. 3C analyses of the D β J β clusters in stable cell lines. Cross-linked chromatin from the P5425 (pro-T) and 63-12 (pro-B) cell lines were subjected to 3C analyses using the anchor primer E located within the E β fragment. This anchor was paired in PCR assays with a series of primers that detect potential interactions between E β and other regions within the TCR β locus (primer A: 5'D β 1, primer B: D β 1, primer C: D β 2, primer D: C β 2, and primer F: V β 14). Each analysis contained a titration of template DNAs corresponding to 600, 200, and 50 ng to confirm assay linearity. The V β 14 and IKK β assays provide controls for cross-linking efficiencies. PCR products from these two assays derive from proximal Xba I fragments within their respective chromosomes, which should cross-link with similar efficiencies in both cell types. The relative efficiencies of each PCR assay were monitored using control templates containing all possible ligation products from these regions of the TCR β and IKK β loci (see Methods). Background signals in each assay were controlled using Xba I digested chromatin that was not subjected to ligation conditions (no ligase). Representative data are shown for experiments that were performed in triplicate on two separate preparations of cross-linked DNA.

An identical pattern of association between E β and the two D β regions is observed in primary thymocytes from RAG-deficient mice (Fig. 12). The interaction is spatially restricted to the D β regions (i.e., C β 2 and 5'D β 1 regions are excluded) and is tissue-specific because only low levels of cross-linking are observed in T cell-deficient splenocytes from these animals. Importantly, the in vivo association between these distal regions is ACE-dependent. Amplification products from the E β /D β 1 assay are dramatically reduced (>10-fold) upon deletion of either the enhancer or the D β 1 germline promoter (Δ E β or Δ PD β 1, respectively). As expected, interactions between E β and the D β 2 region are disrupted by the enhancer deletion but are unaffected by removal of the D β 1 promoter. Taken together, results from 3C analysis directly demonstrate that regions containing the D β germline promoters stably associate with the distal E β element to form a stable holocomplex in DN thymocytes poised for D β →J β recombination.

Discussion

The tissue- and stage-specific expression of multigenic loci typically relies on the regulated cross-talk between multiple promoters and distally located enhancers. This regulatory strategy is particularly important for the ordered assembly and expression of antigen receptor loci in precursor lymphocytes. During Ig and TCR gene assembly, germline promoters and enhancers serve as accessibility control element that direct chromatin remodeling at each locus and regulate the availability of RSSs to V(D)J recombinase (Oltz 2001; Krangel 2003;

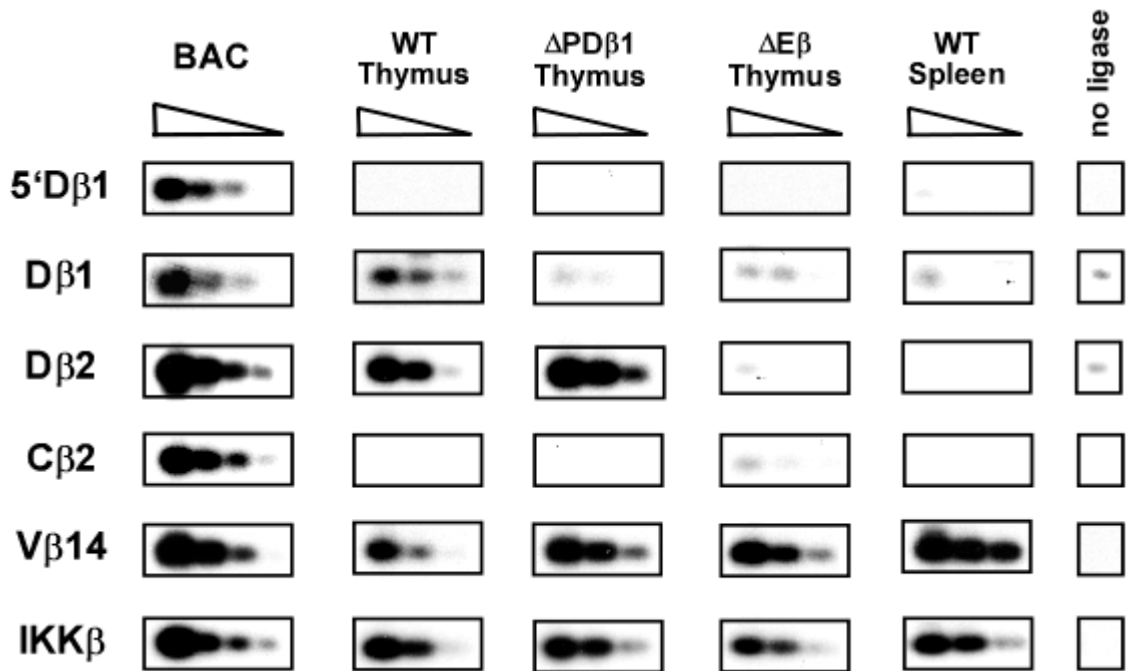
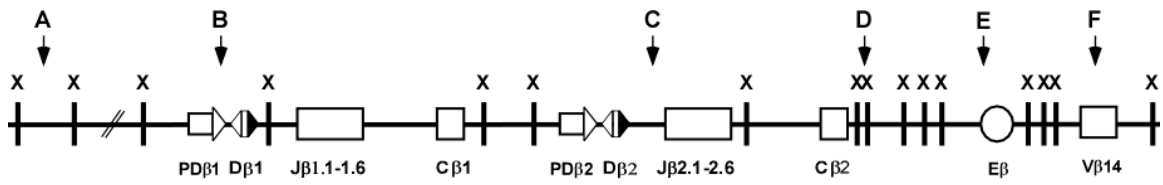


Figure 12. In vivo 3C analysis of the D β J β region in DN thymocytes from RAG-deficient mice harboring either WT, Δ PD β 1, or Δ E β alleles of the TCR β locus. Samples were analyzed as described in Fig. 11. Splenocytes from the RAG-deficient mouse were included as a control for the cell type specificity of observed interactions.

Jung and Alt 2004). Although a cooperative interaction between promoter and enhancer elements is required for rearrangement of linked gene segments, the mode of action for each ACE is distinct. Enhancers serve as location-independent regulatory elements, while the ACE function of germline promoters is highly dependent on their location relative to target gene segments (Sikes, Suarez et al. 1999; Sikes, Meade et al. 2002). Despite these advances, little was known regarding the mechanisms of ACE cross-talk in vivo.

I have used mice lacking either the TCR β enhancer, E β , or the D β 1 germline promoter, PD β 1, to establish a mechanistic framework for ACE communication during the earliest stages of thymocyte development. These findings demonstrate that D β →J β recombination requires a functional interplay between ACEs, which includes their stepwise activation, formation of a stable promoter/enhancer holocomplex, and local versus long-range opening of D β J β chromatin (Chapters II and III). Initially, E β is activated by tissue-specific transcription factors (Capone, Watrin et al. 1993). Subsequently, enhancer function is sufficient to direct H3-K9 acetylation through most of the D β J β clusters and opens J β -associated chromatin. At this early stage of TCR β activation, the D β 1-RSS is largely protected from H3-K9 acetylation and remains in a recombinase-inaccessible state. The enhancer-mediated reorganization of TCR β chromatin then permits the binding of additional transcription factors to the D β 1 germline promoter (Spicuglia, Kumar et al. 2002).

Despite their independent roles in opening D β J β chromatin, cooperation between germline promoters and E β is required to initiate TCR β gene assembly.

Previous models for cross-talk between ACEs at antigen receptor loci invoked either direct contact between promoters and enhancers (looping) or contact-independent communication (linking model) (Bulger and Groudine 1999; Dorsett 1999; Engel and Tanimoto 2000). ChIP data reveal a reciprocal association between promoter- and enhancer-specific factors (SP1 and RUNX1, respectively) that cannot be explained by coincidental binding of each factor to a cryptic site in the non-cognate element (e.g., a cryptic SP1 site in E β) (Oestreich et al. 2006). These in vivo data, coupled with 3C analyses, firmly establish that the distal E β and PD β elements directly contact one another to form a stable holocomplex. Importantly, this holocomplex forms precisely at the developmental stage that D β J β clusters are targeted for rearrangement (pro-T cells). In contrast to SP1, a subset of promoter factors associate with E β even after PD β 1 deletion (e.g., TBP), strongly suggesting that the undefined D β 2 promoter continues to associate with the enhancer in Δ PD β 1 thymocytes. Alternatively, E β may directly recruit these factors for delivery to germline promoters during formation of the stable holocomplex (Spicuglia, Kumar et al. 2002). Resolution of these issues awaits a functional definition of PD β 2 and the generation of appropriate knockout mice.

Interactions between E β and germline promoters could occur as either a tripartite complex containing all three elements or exclusive bipartite complexes containing E β and either of the promoters (Tolhuis, Palstra et al. 2002; Spilianakis and Flavell 2004; Liu and Garrard 2005). ChIP assays for SP1 support the latter mode of interaction. In RAG-deficient thymocytes, SP1 binds

specifically to PD β 1 and associates with E β but not with D β 2 (Oestreich et al. 2006). In a tripartite complex, it is likely that SP1 immunocomplexes would also contain D β 2 sequences. Thus, ACE interactions at the TCR β locus are reminiscent of those observed at the multigenic globin and Ig κ loci, in which multiple enhancer/LCR elements are permitted to associate with only a single promoter at any given time (Tolhuis, Palstra et al. 2002; Spilianakis and Flavell 2004; Liu and Garrard 2005). The mechanisms that restrict interactions between multiple promoters with E β and the identity of factors that facilitate formation of holocomplexes remain to be determined. Notwithstanding, these studies clearly establish a contact mechanism for ACE communication at the TCR β locus and strongly suggest that PD β /E β holocomplexes direct highly localized changes in chromatin accessibility to trigger TCR β gene assembly.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

The research in this dissertation has focused on understanding the regulation of V(D)J recombination at the TCR β locus. V(D)J recombination is the primary mechanism for generation of immunologic diversity and is one of two known site-specific processes of DNA rearrangement in mammals. Although the generation of receptor diversity by V(D)J recombination is beneficial, it is also an inherently dangerous process. Defects in V(D)J recombination can cause immunodeficiencies or chromosomal translocations that lead to lethal lymphoid malignancies. The rearrangement of antigen receptor genes is regulated by distal transcriptional control elements, which modulate chromatin accessibility of Recombination Signal Sequences (RSSs) to V(D)J recombinase. However, the manner in which these elements regulate chromatin structure and their functional interplay during lymphocyte development remained unclear.

To define the mechanisms by which cis-acting elements control TCR β gene assembly, I used RE sensitivity to demonstrate that, in the absence of PD β 1, the E β enhancer has an intrinsic ACE function. This function generates a nearly full level of chromatin accessibility throughout both D β J β clusters, most notably at the J β -RSSs. However, the striking exception is the D β 1 gene segment, which remains inaccessible in promoterless loci. This “privileged” D β 1 gene segment becomes accessible only after formation of the promoter/enhancer

holocomplex identified by the 3C studies. These findings inspire two questions that will be important to address in future studies: (i) why is formation of a holocomplex required rather than relying exclusively on the ACE function of E β , and (ii) how are the additional restrictions placed on D β 1 accessibility?

Clearly, the requirement for ACE cross-talk affords more stringent control over D β →J β recombination by precluding inappropriate TCR β rearrangements in lymphoid progenitors that coincidentally activate either ACE. Another possibility is that formation of the PD β /E β holocomplex facilitates subsequent V β →D β J β recombination. Perhaps, the holocomplex interacts with other cis-elements in the V β cluster to draw these distal regions into spatial proximity. The holocomplex may also serve as a temporary “glue” that holds D β and J β coding ends together until the double-strand break repair machinery can rescue the chromosome via coding join formation (Bogue et al., 1998). The targeted deletion and subsequent relocation of E β to a location upstream of the J β 1 gene cluster would in part test this hypothesis. Following double-stranded break formation at the D β - and J β -RSSs, the now untethered J β gene element would lead to a functional coding joint if the holocomplex is not essential. Alternatively, it is possible that chromosomal translocations and/or immunodeficiencies could be seen if indeed holocomplex formation is required for efficient repair.

The mechanisms that protect D β 1-associated chromatin from the intrinsic ACE function of E β remain unknown. Interestingly, unlike J β -RSSs, both of the D β -RSSs contain a consensus sequence for nucleosome positioning (Baumann, Mamais et al. 2003). Accessibility of a fixed nucleosome over the D β 1-RSS may

require additional aspects of chromatin remodeling by factors specifically recruited to the PD β 1/E β holocomplex. Experiments to test this hypothesis are outlined below. Alternatively, a transcriptional repressor may associate with D β 1, but not with the J β region, prior to promoter activation. Expulsion of this putative repressor may occur only after holocomplex formation. In what may be a related finding, the D β 1-RSS contains a CpG sequence that remains hypermethylated in Δ PD β 1 thymocytes (Whitehurst, Schlissel et al. 2000). This chromatin modification recruits repressor complexes via interactions with methyl-CpG binding proteins (Jones, Veenstra et al. 1998; Nan, Ng et al. 1998). Identification of such inhibitory complexes by ChIP assay and mutational analysis of this site would be useful in determining if this is indeed the case. Thus, formation of the PD β /E β holocomplex may generate a high local concentration of chromatin modifiers and remodeling complexes that, in turn, counteract D β -associated repressors, remodel a positioned nucleosome at the D β 1-RSS, and trigger TCR β gene assembly (Fig. 13).

Therefore, a primary goal of future studies will be to examine the role of nucleosomal organization in the regulation of V(D)J recombination at the TCR β locus. To address this issue, it will be necessary to conduct nucleosome mapping studies of the TCR β locus. Low resolution, micrococcal nuclease (MNase) Southern blotting analyses would be invaluable by showing whether or not nucleosomes are positioned in an ordered array at the locus. Subsequent, higher resolution assays, such as nucleosome scanning or high resolution LM-

PCR assays could further define nucleosome positioning at the D β 1-RSS and other locations of interest (e.g. PD β 1, J β -RSSs, and D β 2-RSS).

An additional finding of these studies is that the germline promoter, PD β 1, and the distally located E β interact, presumably via factors bound to each ACE, generating a stable PD β /E β holocomplex. As such, experiments identifying the transcription factors involved in PD β /E β holocomplex formation would prove to be extremely helpful. Mutational analysis of transcription factor binding sites in both PD β 1 and E β coupled with 3C analysis would be critical in identifying such complexes. Continued ChIP analysis will also provide candidate protein complexes for mutational and 3C analysis. Furthermore, precise definition of the promoter associated with the D β 2 gene segment will lead to a new wave of experiments examining transcription factor binding at “PD β 2” as well as possible interactions with other cis-acting elements (e.g. PD β 1 or E β) within the TCR β locus.

It is quite possible that the formation of the PD β 1/E β holocomplex creates a unique binding surface to recruit additional remodeling factors (e.g., SWI/SNF) to direct highly localized chromatin modifications critical for unmasking the TATA box and D β 1-RSS to trigger both germline transcription and D β →J β recombination. To this end, I have taken advantage of a system in which the ATPase subunit, Brg1, a critical component of the SWI/SNF complex, can be targeted to artificial TCR β miniloci. These studies show that targeted recruitment of Brg1 can rescue chromatin accessibility at the D β 1-RSS in a promoterless

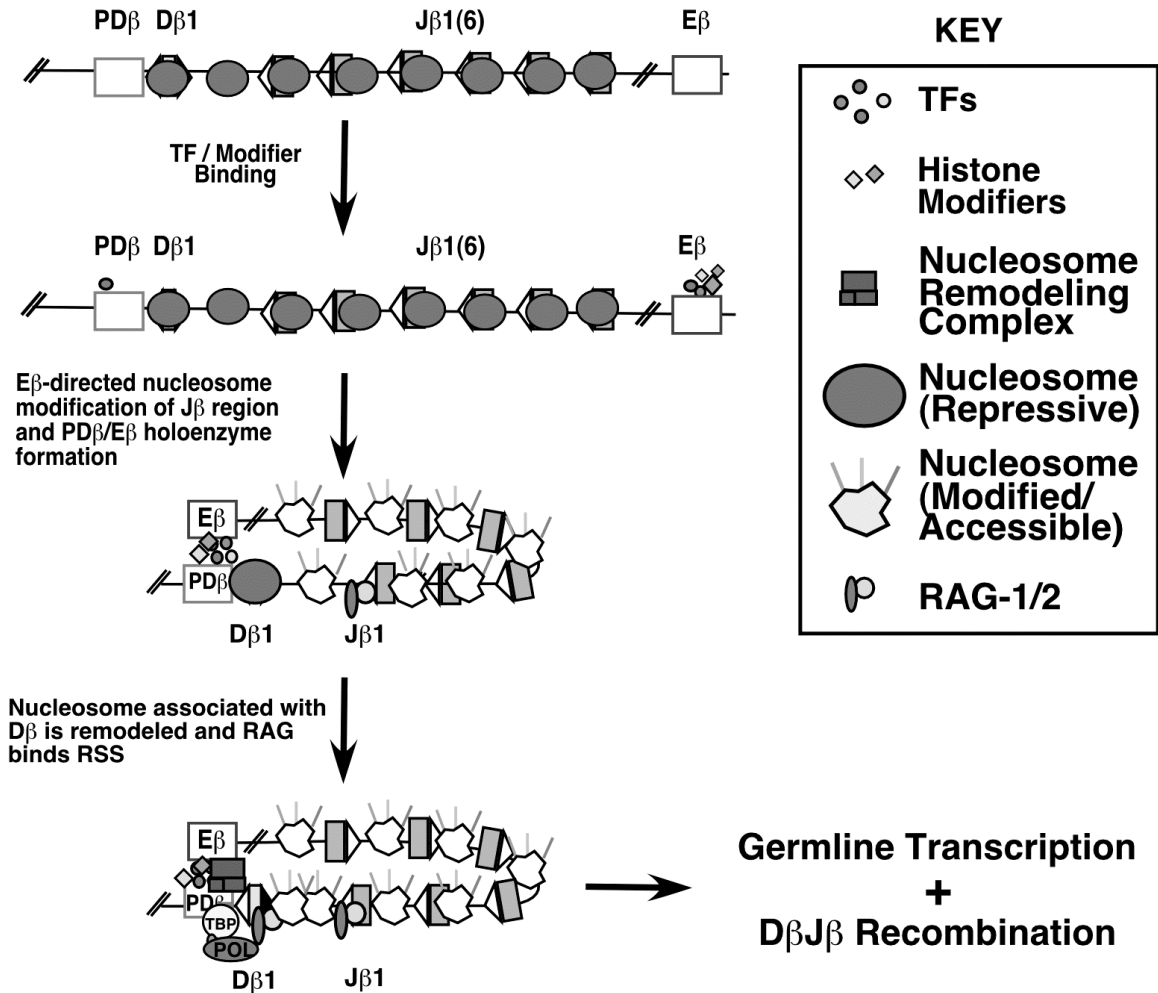


Figure 13. Proposed model for ACE function in the regulation of DβJβ recombination. Refer to “Conclusions and Future Directions” for a complete description. For simplicity, only the Dβ1Jβ cluster of gene segments is shown.

TCR β minilocus substrate (Fig. 14). It is likely that multiple chromatin modifiers are involved in the regulation of substrate accessibility. In addition to Brg1, targeted recruitment of the histone methyltransferase (HMT), G9a, is able to repress D β →J β rearrangement (Osipovich, Milley et al. 2004). However, the particular effect of additional modifiers on rearrangement is unknown. Similar studies examining additional proteins and protein complexes will help to establish the functional hierarchy of chromatin modifiers in the regulation of substrate accessibility.

Despite the utilization of a unique recombinatorial process for functional assembly, antigen receptor loci share many hallmarks of transcriptional regulation with numerous other genes. The data provided in this dissertation offer exciting revelations about the mechanisms of gene regulation not only at antigen receptor loci but, undoubtedly, at other genes as well. Indeed, it has already been shown that the β -globin and HNF-4 α genes share common mechanisms of transcriptional regulation with those seen at the Ig κ and TCR β loci (Hatzis and Talianidis 2002; Tolhuis, Palstra et al. 2002; Liu and Garrard 2005; Oestreich, Cobb et al. 2006). Therefore, it is likely that the lessons garnered from continued exploration into the regulation of V(D)J recombination will enhance our knowledge of this inherently dangerous but vital process, while providing new insight into the global mechanisms of gene regulation.

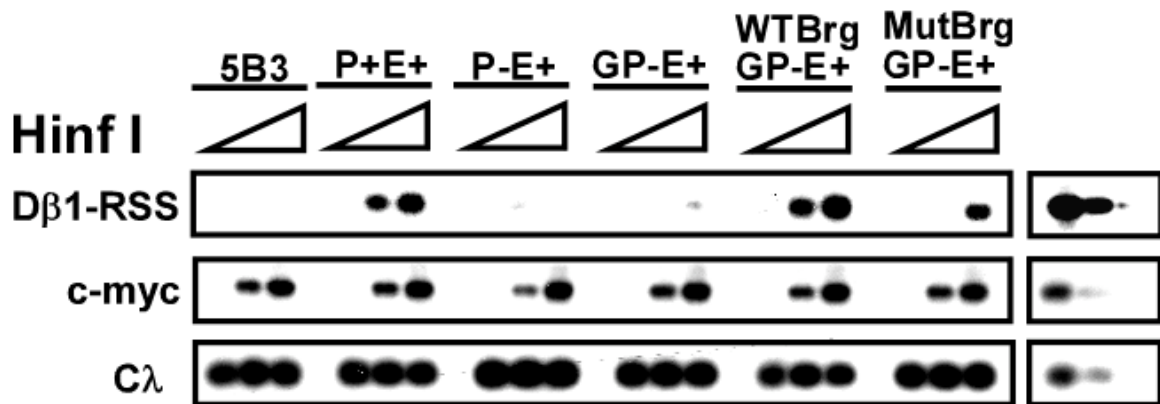


Figure 14. Targeted recruitment of Brg1 leads to enhanced chromatin accessibility in the D β 1-RSS. Nuclei from 5B3 cells were treated with increasing amounts of the indicated enzyme (0.1, 0.5, and 2.5 U of Hinf I). Enzyme cleavage at the indicated site(s) was analyzed using LM-PCR and Southern blotting as described in the Methods section (Chapter II). Control PCR assays for DNA content (C λ) and enzyme cutting efficiency (c-myc) were performed using the same samples of Hinf I-digested, linker-ligated DNA. The linearity of each assay was confirmed by serial dilutions of the maximally digested 5B3, P+E+ sample.

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