The Role of Nuclear Position and Locus Conformation in Regulating V(D)J Recombination of the Tcrb Locus

by

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Dr. Tannishtha Reya

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Immunology in the Graduate School of Duke University

2008
ABSTRACT

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Abstract

Recombination of Tcrb gene segments in DN thymocytes is subject to allelic exclusion, such that only a single functional V\textsubscript{\beta} to DJ\textsubscript{\beta} rearrangement is generated per T-cell. For Tcrb to be allelically excluded the two alleles must initiate recombination asynchronously and once a \beta-protein is selected, feedback signals must suppress further recombination. Earlier studies of antigen-receptor loci implicated directed monoallelic association with pericentromeric heterochromatin in the initiation or maintenance of allelic exclusion. In this study we used three-dimensional fluorescent in-situ hybridization to directly visualize the nuclear localization of Tcra and Tcrb, pericentromeric heterochromatin, and the nuclear lamina. Here we provide evidence for a fundamentally different basis for Tcrb allelic exclusion. We demonstrate that Tcrb is highly associated with pericentromeric heterochromatin and the nuclear lamina in pro-B cells and in DN and DP thymocytes. We also find that Tcrb does not associate with peri-centromeric heterochromatin and the nuclear lamina in a strict monoallelic fashion. Rather, Tcrb alleles independently associate with the two compartments, leading to a stochastic distribution of nuclei containing both, one, or neither allele associated. In the subset of DN thymocyte nuclei with monoallelically associated Tcrb alleles, the non-rearranged allele is most often associated with repressive compartments. This suggests that association with these compartments inhibits
recombination prior to β-selection. This inhibition occurs without altering the
conformation of the locus. Moreover, the introduction of an ectopic enhancer into Tcrb,
led to both a repositioning of Tcrb away from these repressive compartments. This
repositioning was correlated with an increase in the frequency of recombination and a
break in allelic exclusion. These data lead us to propose that stochastic rather than
directed interactions of Tcrb alleles with repressive nuclear compartments bias initial
Tcrb recombination to be monoallelic in developing thymocytes and that such
interactions are essential for Tcrb allelic exclusion.
To my family and friends
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<tr>
<td>3D FISH</td>
<td>Three-dimensional fluorescence <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>12-RSS</td>
<td>12 base pair spacer recombination signal sequence</td>
</tr>
<tr>
<td>23-RSS</td>
<td>23 base pair spacer recombination signal sequence</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAF</td>
<td>barrier to autointegration factor</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BRG1</td>
<td>brahma-related gene 1</td>
</tr>
<tr>
<td>CE</td>
<td>coding end</td>
</tr>
<tr>
<td>CT</td>
<td>chromosomal territory</td>
</tr>
<tr>
<td>D</td>
<td>diversity</td>
</tr>
<tr>
<td>Dam</td>
<td>DNA adenine methyltransferase</td>
</tr>
<tr>
<td>DMR</td>
<td>differentially-methylated region</td>
</tr>
<tr>
<td>DN</td>
<td>CD4⁺CD8⁻ double negative</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>DP</td>
<td>CD4⁺CD8⁺ double positive</td>
</tr>
<tr>
<td>DSB</td>
<td>double strand break</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>Eα</td>
<td><em>Tcra</em> enhancer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Eβ</td>
<td>Tcrb enhancer</td>
</tr>
<tr>
<td>Eμ</td>
<td>IgH enhancer</td>
</tr>
<tr>
<td>EαKI</td>
<td>Eα knock-in allele</td>
</tr>
<tr>
<td>H3</td>
<td>histone 3</td>
</tr>
<tr>
<td>H4</td>
<td>histone 4</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>HP1</td>
<td>heterochromatin protein 1</td>
</tr>
<tr>
<td>HXK1</td>
<td>hexokinase isoenzyme 1</td>
</tr>
<tr>
<td>IC</td>
<td>interchromosomal compartment</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine based activation motif</td>
</tr>
<tr>
<td>J</td>
<td>joining</td>
</tr>
<tr>
<td>LacI</td>
<td>lactose repressor</td>
</tr>
<tr>
<td>lacO</td>
<td>operator sequence from the lactose operon</td>
</tr>
<tr>
<td>LAT</td>
<td>linker for activation of T cells</td>
</tr>
<tr>
<td>MMTV</td>
<td>mouse mammary tumor virus</td>
</tr>
<tr>
<td>Me</td>
<td>methylation</td>
</tr>
<tr>
<td>NHEJ</td>
<td>non-homologous end joining pathway</td>
</tr>
<tr>
<td>NPC</td>
<td>nuclear pore complex</td>
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<tr>
<td><strong>Abbreviation</strong></td>
<td><strong>Definition</strong></td>
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<tr>
<td>-----------------</td>
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<tr>
<td>PHD</td>
<td>plant homeodomain finger motif</td>
</tr>
<tr>
<td>PD$_\beta$I</td>
<td>promoter upstream of D$_\beta$I</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination activating genes</td>
</tr>
<tr>
<td>RNA pol II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>RSS</td>
<td>recombination signal sequence</td>
</tr>
<tr>
<td>SE</td>
<td>signal end</td>
</tr>
<tr>
<td>SP</td>
<td>CD4$^+$ or CD8$^+$ single positive</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>switch/sucrose non-fermentable</td>
</tr>
<tr>
<td>V</td>
<td>variable</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TEA</td>
<td>T early $\alpha$</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>Xa</td>
<td>active X chromosome</td>
</tr>
<tr>
<td>XCI</td>
<td>X chromosome inactivation</td>
</tr>
<tr>
<td>Xi</td>
<td>inactive X chromosome</td>
</tr>
<tr>
<td>YY1</td>
<td>yin yang 1</td>
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1. Introduction

Thymocyte Development

Self renewing hematopoietic stem cells (HSC) originating in the bone marrow differentiate into major immune system cell lineages (Bhandoola et al., 2007; Borowski et al., 2002). These are the myeloid lineage, which include monocytes, macrophages, eosinophils, basophils, neutrophils, mast cells, megakaryocytes, and dendritic cells; the erythroid lineage, which include erthyrocytes; and the lymphoid lineage, which include B-cells, T-cells, natural killer (NK) cells, and some dendritic cells (Cobaleda and Busslinger, 2008; Iwasaki and Akashi, 2007; Ney, 2006). As HSCs begin to differentiate, they lose their ability to self-renew and development occurs as these multiple potential precursor cells differentiate into the functional cells of the immune system (Lai and Kondo, 2008). The transition stages of the hematopoietic lineages are extremely plastic, in that ectopic expression of transcription factors in differentiated cells can often dedifferentiate them into precursor cells or transdifferentiate them into a different lineage (Cobaleda and Busslinger, 2008). Thus it is important to know both when a cell type takes on lineage characteristics and when they irreversibly committed to a lineage. The first split in hematopoietic lineages is between myeloid cells and lymphoid cells and is characterized by the loss of Flt3 (a tyrosine kinase receptor) (Adolfsson et al., 2005) (Figure 1). Cells that lose Flt3 become common myeloid progenitors (CMP) while
those that retain Flt3 become lymphoid primed multipotent progenitors (LMPP) and concurrent with the loss of VCAM1 surface expression LMPPs gradually lose the ability to become erythrocytes and then granulocyte/macrophages (Adolfsson et al., 2005; Lai and Kondo, 2006).

Figure 1: The developmental stages from HSC to CLP and their myeloid potential

In this model, CLP and CMP are generated asymmetrically from different MPPs. Prior to lymphoid lineage commitment at the CLP stage, MPPs loses myeloid lineage differentiation potential in a step-wise fashion (Lai and Kondo, 2008).

Conversely, CMPs are terminally differentiated towards the myeloid lineages and cannot give rise to lymphoid cells. Early transient expression of recombination activating genes 1 and 2 (Rag1 and Rag2) in LMPP marks the transition to early
lymphoid progenitors (ELP), which is followed by the upregulation of the IL-7 receptor as they progress to the common lymphoid progenitors (CLP) (Kondo et al., 1997).

LMPPs and ELPs can migrate to the thymus and are the primary cells responsible for differentiating into T-cells, while CLPs and the earliest B-cell progenitors (pre-pro B cells) can differentiate into T-cells but lack the ability to home to the thymus (Bhandoola et al., 2007). The different stages of thymocyte development are characterized by their expression of CD4 and CD8. Initially thymocytes express neither surface coreceptor and are termed double negative (DN) thymocytes. This stage is further subdivided by the surface expression of CD25 and CD44. DN1 (CD44+CD25-) thymocytes retain the ability to differentiate into B cells, macrophages, and dendritic cells (Godfrey et al., 1993). Notch1 signaling is critically important for the development and commitment to the T-cell lineage (Maillard et al., 2005; Radtke et al., 2004). Deletion of Notch1 or its downstream signaling protein, RBP-J, results in impaired T-cell development and differentiation of B-cells in the thymus (Radtke et al., 1999; Wilson et al., 2001). Ectopic expression of Notch in the bone-marrow results in the differentiation of T-cells in the Bone marrow (Pui et al., 1999). Delta-like4, a Notch1 receptor, is expressed in the thymus and is critical in activating Gata3 expression in DN2 (CD44+CD25+) thymocytes and the subsequent T-cell gene expression profile (Tanigaki and Honjo, 2007). DN2 thymocytes lose their potential to become B-cells but maintain their ability to become myeloid cells, dendritic cells, and natural killer cells.
Differentiation to DN3 (CD44-CD25+) thymocytes is the commitment step to the T-cell lineage as these cells are unable to dedifferentiate or transdifferentiate. It is at this stage that the antigen receptor loci Tcrb, Tcdr, and Tcrg recombine. The vast majority of DN3 thymocytes become αβ T-cells and progress to the DN4 (CD44-CD25-) stage of thymocyte development. DN4 thymocytes rapidly proliferate and become immature single positive (ISP) cells that express low levels of CD8 (Paterson and Williams, 1987). The ISP stage is transitory and the thymocytes quickly upregulate both CD4 and CD8 and become double positive (DP) thymocytes. In DP thymocytes Tcra recombines and upon the successful generation of an αβ TCR, these cells down regulate either CD4 or CD8 and become single positive (SP) thymocytes (Krangel et al., 2004). SP thymocytes then migrate out of the thymus to the periphery as mature T-cells.

DN3 thymocytes can differentiate into either αβ or γδ T-cells (Borowski et al., 2002; Pennington et al., 2005). There is evidence supporting that lineage fate is dictated prior to TCR gene rearrangement. Early data showed similar ratios of αβ to γδ in triple transgenic mice expressing rearranged Tcrb, Tcrg and Tcdr transgenes as compared to both Tcrb transgenic mice and Tcrg or Tcdr transgenic mice. These data suggest that γδ/αβ lineage commitment occurs independent of TCR recombination (Gerber et al., 2004). More recent data has demonstrated that cell fate is determined after TCR gene rearrangement. These data suggest the Tcrb, Tcdr, and Tcrg loci compete with each other, with the first functional receptor (pre-Tα/β or γδ) dictating the lineage
commitment of the cell. Hayes et. al. demonstrated that reducing the strength of γδ signaling in DN3 thymocytes could promote differentiation of γδ+ cells to the DP stage (considered αβ commitment) (Hayes et al., 2005). Supporting the idea that strong TCR signals promote γδ differentiation and weak TCR signals promote αβ differentiation, mice that express a transgenic γδ TCR without their cognate MHC differentiate into DP thymocytes (Haks et al., 2005). Thus, strong signals resulting from engagement of the transgenic γδ TCR complex by its ligand promote γδ lineage commitment, whereas weak signals resulting from impaired TCR signaling or elimination of its ligand promote αβ lineage commitment. Additionally it was shown that abrogating Notch signaling reduced the number of αβ T-cells and increased the number of γδ T-cells (Jiang et al., 1998; Washburn et al., 1997). This results from notch signaling being required for the survival of pre-TCR expressing DN3 thymocytes whereas Notch signaling is not required for the survival γδ expressing DN3 thymocytes (Garbe et al., 2006). These data suggests that differential synergy between TCR and Notch signaling determines lineage fate. Kreslavsky et. al. followed single T-cell precursors and demonstrated that commitment to either the αβ or γδ lineage occurred after TCR expression and that changing the TCR signal in single TCR-expressing cells altered its lineage commitment (Kreslavsky et al., 2008). Thus the decision between the αβ or γδ T-cell lineage is driven by the TCR expressed and is reinforced by Notch signaling.
There are two key checkpoints that are tightly regulated during αβ T-cell development. The first checkpoint is at the transition from DN3 to DN4 which ensures that developing thymocytes generate a functional β protein (Dudley et al., 1994; von Boehmer et al., 1999). At the DN3 stage RAG1 and RAG2 are expressed allowing Vβ to Dβ and Dβ to Jβ recombination (Krangel et al., 2004). There are two criteria that a Tcrb rearrangement must pass to be functional. First, the rearrangement must generate an in-frame coding sequence. Second, the tertiary structure of the encoded protein must be able to pair with pre-Tα. DN3 thymocytes express pre-Tα, a protein that mimics the structure of Tcra protein and forms a pre-T-cell receptor (pre-TCR) with a functional β protein (von Boehmer, 1997). The pre-TCR signals through CD3ε and linker of activated T-cell (LAT) to induce the differentiation of DN thymocytes to DP thymocytes (Falk et al., 2001; Zhang et al., 1999). This signal down regulates Rag1 and Rag2 gene expression and induces differentiation to the DN4 compartment and subsequent cell proliferation (Turka et al., 1991). In the absence of RAG, thymocyte development at DN3 is blocked and the Tcrb locus remains in its germ line configuration (Mombaerts et al., 1992; Shinkai et al., 1992). In the absence of LAT, the thymocyte is again stuck at the DN3 transition, but the Tcrb locus recombines (Zhang et al., 1999).

The second checkpoint is at the transition from DP to SP thymocytes. In DP thymocytes Rag2 and Rag2 are again expressed, but now Tcra undergoes VJ recombination (Starr et al., 2003). For DP thymocytes to progress past this checkpoint
they must express a functional TCR. Thus Tcra must recombine to form an in-frame coding sequence that generates a Tcra protein with tertiary structure capable of pairing with the β chain. There is an additional hurdle for DP thymocytes beyond the generation of a functional Tcra protein. The resulting TCR along with either the CD4 or CD8 coreceptor must be able to engage peptide loaded MHCI or MHCII and signal through the TCR (Scott et al., 1989). The strength of the signal is critically important to the survival of the DP thymocytes. An absence of a signal, due to failure of the TCR to recognize MHC, leads to death through neglect unless a secondary rearrangement of Tcra can save the DP thymocyte (Fehling et al., 1997). A weak signal due to recognition of MHC and self-peptide leads to survival through positive selection (Kisielow et al., 1988). However, a strong signal due to recognition of MHC and self peptide causes programmed cell death by negative selection to avoid autoimmunity (Groves et al., 1996; van Oers et al., 1996).

1.2 V(D)J Recombination

The hallmark of lymphocytes is the generation of a unique antigen receptor in each individual cell. This is accomplished by physically joining variable (V), diversity (D), and joining (J) gene segments of the antigen receptor loci through a process called V(D)J recombination. This involves the introduction of two double stranded breaks (DSB) into the genome with the intervening DNA sequence being either inverted or
excised from the genome of that cell. This process is mediated through a coupled cleavage reaction initiated by RAG1 and RAG2 at specific recombination signal sequences (RSSs).

1.2.1 Recombination Signal Sequences

Every V, D or J gene segment in an antigen receptor locus is flanked by an RSS. These RSSs direct the V(D)J recombination machinery to the gene segments and ensure that cleavage occurs at the appropriate base pair. There are three key sequences in an RSS. There is a well conserved heptamer (5’-CACAGTG), a less well conserved spacer sequence of either 12 bp or 23 bp, and a conserved nonamer (5’-ACAAAAACC). Efficient recombination only occurs between a 12-RSS and 23-RSS.

Mutational analysis of the heptamer has identified the first three nucleotides (5’-CAC) as absolutely critical to recombination. This is reflected in their 99% conservation across species (Hesse et al., 1989). The remaining four nucleotides are conserved in 81-91% of RSSs. However, despite being highly conserved these nucleotides are not required for recombination (Feeney et al., 2004). Mutating the fourth nucleotide varied the recombination efficiency while mutating the remaining three nucleotides had little effect on recombination efficiency. Additionally, mutations to the heptamer and nonamer only affected the efficiency of recombination, never the location of the cleavage site (Akamatsu et al., 1994). Functionally the heptamer has been shown to
interact with RAG2 and stabilize the recombination complex at the RSS (Swanson, 2004).

The nonamer shows less sequence conservation than the heptamer. There are no invariant positions in the nonamer, but the consensus nucleotide for any position occurs 68-90% of the time (Feeney et al., 2004). The most conserved nucleotides are a cytosine at the second position and a string of 3 or 5 adenines ending at position seven. An adenine in the sixth position greatly increases the efficiency of recombination and it has also been show that the stretch of three (or five) adenines needs to be flanked by a non-adenine nucleotide (Hesse et al., 1989). Functionally, the nonamer binds to RAG1, providing the initial binding site for the recombination complex (Difilippantonio et al., 1996).

The 12 bp spacer sequence allows for one helical turn of the DNA, while the 23bp spacer sequence allows for two full helical turns of the DNA. Although 12 and 23 are the most frequent sizes of spacer, recombination is permitted with an 11/13 or a 22/24 spacer. Despite the fact that no specific sequence is required for recombination, there is a remarkable amount of conservation of the spacer sequence, especially of the 12–RSS. 64-67% of 12 and 23 RSSs have an adenine at the fifth nucleotide. In fact the 12 bp spacer and the first half of the 23 bp spacer possess six positions where the most conserved nucleotide is the same (Hesse et al., 1989). Computational analysis of 356 murine RSS generated an ideal 23 spacer (5’-TTGCAACCACATCCTGAGTGTGT)
which differed from the consensus sequence (5’-TTGgAACCACATCggGAGccTGT) at 5 positions. An RSS with the ideal 23bp spacer recombined six fold more efficiently than the consensus sequence and four times more efficiently than Jκ1.1 (Cowell et al., 2002; Cowell et al., 2004). Thus the spacer sequence plays a strong role in regulating the efficiency of recombination.

Recombination preferentially occurs between gene segments with a 12-RSS and 23-RSS; this constraint is known as the 12/23 rule (Tonegawa, 1983). The 12/23 rule ensures that gene segments, such as V segments, do not rearrange with similar gene segments, e.g. other V segments, because they all have the same type of spacer. The pairing of a 12RSS and a 23RSS is determined by RAG binding and occurs prior to cleavage (Gellert, 2002).

Variation in sequence of the heptamer, nonamer and spacer influences VH, VK, and Jβ gene segment usage (Livak et al., 2000; Nadel et al., 1998). RSS variation also establishes specificity of gene segment usage in the Tcrb locus. The Vβ gene segments are flanked by 23-RSSs and Jβ segments are flanked by 12-RSSs. According the 12/23 rule these gene segments should be capable of recombining, but these rearrangements are not observed in vivo. This restriction, termed “beyond 12/23” (b12/23) was demonstrated by Bassing et. al. when they switched the positions of the Jβ1 12-RSS with the 5’ Dβ1 12-RSS. These mice only contained Vβ to Jβ rearrangements, indicating that Vβ 23-RSS only rearranged to the 5’Dβ1 12-RSS even though it was at the Jβ1 position
(Bassing et al., 2000). Similarly the 3’ Dβ 23RSS is more effective than Vβ 23 RSS at rearranging with the Jβ 12RSS (Wu et al., 2003). Finally, non-lymphoid cells transfected with RAG and extrachromosomal substrates recapitulate the endogenous prohibition of Vβ to Jβ rearrangement (Jung et al., 2003; Tillman et al., 2003). These data suggest that the mechanism responsible for b12/23 must be based on differences in the nucleotide sequence of the RSS. The spacer sequence has been shown to be critically important for enforcing the b12/23 restriction (Hughes et al., 2003). Thus RSS variation shapes the Ig and TCR repertoires and imposes restriction to the recombination reaction.

1.2.2 RAG

Murine RAG1 contains 1040 amino acids while RAG2 contains 527 amino acids. The enzymatic cleavage activity of the RAG proteins is largely concentrated in core regions; residues 384–1008 of RAG1 and residues 1–387 of RAG2. Core RAG1 contains a DDE motif composed of three acidic residues (D600, D708, and E962), that when mutated abolish DNA cleavage (Fugmann et al., 2000; Kim et al., 1999; Landree et al., 1999). These residues have been shown to be critical in the initial step of nicking the DNA strand. Residues 384–454 of RAG1 comprise a nonamer-binding region (NBR) that specifically binds the conserved nonamer of the RSS. The central domain (amino acids 528–760) of RAG2 has been shown to bind to the heptamer. RAG2 also has a key site of phosphorylation at T490 (outside of the core region) that has been implicated in its degradation. It is thought that this allows RAG2 levels to be tightly regulated.
throughout the cell cycle, such that recombination only occurs during G₀/G₁ (Lin and Desiderio, 1993). The core regions of RAG have been shown to be sufficient for recombination, particularly in vitro. The non-core regions of RAG are important for regulating recombination in vivo. Mice that have had their RAG genes replaced with core RAG, show a severe defect in V-DJ recombination with minimal effect on D-J recombination in both IgH and Tcrb (Dudley et al., 2003; Liang et al., 2002). One possible explanation for this difference in effects on recombination is that the less regulated recombination of D-J gene segments is “easier” and thus is less affected by reduction in RAG recombination efficiency compared to the more regulated V-DJ recombination. Thus a small reduction in RAG efficiency results is a more pronounced V to DJ phenotype. The non-core region of RAG2 contains a plant homeodomain (PHD) that is capable of binding to histone 3 trimethylated at lysine 4 (H3K4me3), a mark of active transcription. Thus core RAG2 may be unable to efficiently bind to V RSSs, leading to an unstable recombination complex and reduced V-DJ rearrangements (Liu et al., 2007; Matthews et al., 2007).

**1.2.3 V(D)J Cleavage and Repair**

In vitro recombination only requires a complex consisting of RAG1, RAG2, a 12-RSS, and a 23-RSS. (Figure 2) The addition of the high mobility group 1 and 2 (HMG1, HMG2) proteins greatly enhances the efficiency of in vitro recombination. HMG1 and HMG2 are non-specific DNA binding proteins that bend DNA and are thought to
deform the 23-RSS to allow better RAG binding (Gellert, 2002). Cleavage requires that both RSSs be bound to the RAG complex, but there is some debate over which RSS binds to the complex first. There is evidence for preferential nick formation (the first enzymatic step of recombination, which can occur at unpaired RSSs) occurring preferentially at V12RSS, suggesting that RAG binds to the 12-RSSs first (Curry et al., 2005). In vitro, RAG has also been shown to form more stable complexes with 12-RSSs. Thus RAG complexes may assemble on 12-RSSs and capture 23-RSSs to form a synaptic complex. However there is no published direct evidence for RAG binding in vivo.
Figure 2: The V(D)J recombination reaction.

RAG protines mediate RSS synapsis and cleavage to form blunt signal ends and hairpin coding ends. The DSB is then repaired through NHEJ. Coding segment (grey rectangle), 12 RSS (cyan triangle), 23 RSS (dark blue triangle). (Courtesy of Michael S Krangel).

Once the RAG complex forms a synaptic complex with two RSSs, RAG cleaves the 5’ end of the heptamer, creating a DNA nick with a free 3’ hydroxyl (-OH) group on the coding end (CE). This 3’ -OH group acts as a nucleophile and attacks the
phosphodiester bond between the nucleotides of the RSS and the gene segment on the other strand resulting in a DSB, with a closed hairpin loop at the CE and a blunt signal end (SE). The DSB in the post cleavage recombination complex are then repaired through the common non-homologous end joining (NHEJ) pathway. The Ku70/Ku80 complex binds to the DSB and recruits and activates DNA dependent protein kinases (DNA-PK) (Featherstone and Jackson, 1999; Ma and Lieber, 2002). Artemis is also recruited to the DSB, where DNA-PK phosphorylates it and activates its endonuclease activity (Goodarzi et al., 2006; Moshous et al., 2001). The CE hairpin is then imprecisely cleaved by Artemis resulting in an overhang of between 0 and 10 bp, though normally the overhang in successful rearrangements is between 1 and 4 nucleotides long. The nucleotides in the overhang can be removed by the inherent exonuclease activity of Artemis. Additionally, if terminal deoxynucleotidyl transferase (TdT) is present, up to 15 non-palindromic (N) nucleotides may be added to the CE in a template independent manner, but with a preference for guanine nucleotides. The DSB is resolved by DNA Ligase IV, in association with XRCC4, which ligates the CEs together and SEs together. If there was an overhang when the DSB was ligated, the resulting single stranded DNA will be filled with palindromic (P) nucleotides using the complementary strand as a template. Thus, imprecise opening of the hairpin, deletion of overhang nucleotides, and the addition of N and P nucleotides creates highly variable coding joints (CJ) which ensures a diverse repertoire of antigen receptors.
1.3 Nuclear Architecture

1.3.1 Chromosomal Territories and the Interchromatin Compartment

The twenty chromosomes of mice maintain structural domains during interphase, which are known as chromosomal territories (CT) (Cremer and Cremer, 2001). These CTs can be visualized simultaneously in a single cell by “painting” them with a combination of chromosome specific fluorochromes (van der Ploeg, 2000). These chromosomal territories are stochastically arranged within the nucleus, but there is evidence that their positioning is not entirely random. In cultured fibroblasts, small chromosomes tend to be located in the center of the nucleus while large chromosomes were located near the periphery (Bolzer et al., 2005). There is also evidence to suggest that chromosomes with low gene density more frequently adopt a peripheral location compared to gene-dense chromosomes which are located more centrally (Cremer et al., 2003; Croft et al., 1999). Thus, even though the exact position of a CT in any given nucleus is not predetermined, chromosomes are biased to nuclear locations depending upon the content of their DNA.

Electron-microscopic studies indicate that the three dimensional (3D) CT structure is highly perforated with many intruding non-chromatin spaces forming the interchromatin compartment (IC) (Visser et al., 2000). The IC provides a “work space” where transcription factors, chromatin proteins, and RNA-processing factors can be compartmentalized and accumulate in distinct nuclear domains (Misteli, 2007). The IC
starts at nuclear pores, expands between CTs and invaginates into their interior (Visser et al., 2000). The higher order chromatin structure of the CT creates a barrier between it and the IC and this barrier is hypothesized to be permeable to single proteins or small proteins aggregates, but not by larger protein complexes (Cremer et al., 2006). Thus, specific nuclear processes requiring macromolecular complexes such as transcription, replication, and repair occur at spatially defined locations outside of the CT (Misteli, 2007).

1.3.2 Large Scale Chromatin Changes

Chromatin exists in two states. Euchromatin, or “open” chromatin, is transcriptionally active and characterized by sensitivity to DNase digestion and restriction enzyme cleavage. Heterochromatin, or “closed” chromatin, is typically silent and refractory to DNase digestion and restriction enzyme cleavage (Arney and Fisher, 2004; Cremer et al., 2006; Richards and Elgin, 2002). Euchromatin domains are highly correlated with regions of high gene density while heterochromatin is generally gene poor (but about 450 genes are found in heterochromatin regions) (Gilbert et al., 2004; Yasuhara and Wakimoto, 2006). The transition between heterochromatin and euchromatin can be visualized as “chromosome puffs” of polytene chromosomes in Drosophila melanogaster. This “puffing” or decondensation of the chromatin occurs at the transcriptionally active regions of the polytene chromosomes (Andersson et al., 1984). In a mammalian cell line that contains an integrated two hundred copy tandem
array of a plasmid containing the mouse mammary tumor virus promoter, the chromatin of the array is decondensed from an approximately 0.5-micron spot to form a fiber 1-10 micron long upon promoter activation (Muller et al., 2001). Additionally in this cell line, the degree of decondensation was proportional to the amount of transcript produced by the array as detected by RNA FISH. Similarly, a lac repressor-ER fusion protein was capable of decondensing a region of chromatin containing lac operator sites in the presence of estradiol (Nye et al., 2002). Moreover, Chambeyron and Bickmore demonstrated that developmental activation of the endogenous Hoxb locus correlated with a ninefold increase in the spatial volume the locus occupied (Chambeyron and Bickmore, 2004a). Thus, there is a large scale chromatin decondensation in regions that are transcriptionally active (Chambeyron and Bickmore, 2004).

In addition to decondensation, multiple genes have been demonstrated to loop out of their CTs as they become transcriptionally active. Specifically, induction of Hoxb genes in embryonic stem cells induces the locus to loop up to 1 micron out of the territory of chromosome 11 (Chambeyron and Bickmore, 2004a; Chambeyron et al., 2005). Similarly in the MRC5 lung fibroblast cell line, the MHC locus moves away from its chromosomal territory upon stimulation with interferon-γ (Volpi et al., 2000). However, while transcription and decondensed chromatin are tightly correlated, they are not intrinsically linked to repositioning out of CTs. Both ubiquitously expressed genes and tissue-restricted genes, when transcriptionally active, can be found within
CTs (Mahy et al., 2002). The Hoxd locus, which contains genes important in regulating limb development, both loops out and decondenses in the tail-bud region, but only decondenses in the limb-bud region (Morey et al., 2007). Therefore transcription is not restricted to the IC but may occur more frequently and at higher levels when outside CTs.

1.3.3 Transcription Factories and Splicing Speckles

Clusters of multiple active RNA polymerases (Transcription Factories) have been visualized in the nucleus using antibodies against the transcriptionally active (serine2-phosphorylated) form of RNA pol II (Grande et al., 1997). Electron microscopy of transcription factories has shown that they are between 50 and 70 nm in diameter, irrespective of the cell type (Faro-Trindade and Cook, 2006; Jackson et al., 1998; Pombo et al., 1999). These factories contain between 6 and 8 RNA pol II molecules because there are approximately 65,000 RNA pol II molecules in a HeLa nucleus, but only about 10,000 sites of transcription (Cook, 1999). Transcription factories can be visualized in live cells with GFP tagged RNA pol II and these experiments have shown that RNA pol II complexes are mobile and can be found throughout the nucleus (Becker et al., 2002; Kimura et al., 2002). However, when RNA pol II is found in transcription factories it is immobile (Jackson et al., 1998; Martin and Pombo, 2003). Osborne et. al. analyzed the colocalization of several genes on mouse chromosome 7. They discovered that the genes Hbb and Eraf colocalized at transcription factories. Moreover, in nuclei with one
transcribed allele, it was the transcribed allele that colocalized with other genes at transcription factories. Thus, widely separated genes colocalize in shared RNA pol II factories in a transcription-dependent manner (Osborne et al., 2004). Nordormeer et. al. introduced the human β-globin LCR into a 3Mbp gene rich region on chromosome 8. They saw in fetal liver cells an LCR-dependent increase in gene expression, a relocation of the locus away from the CT, and an increased association with transcription factories (Noordermeer et al., 2008). These data suggest that transcription can be driven by positioning genes at transcription factories. Finally, in cells stably transfected with thousands of minichromosomes, active transcription of the minichromosomes only occurred in a subset of transcription factories (Xu and Cook, 2008). These data led the authors to hypothesize that transcription factories may “specialize” in the types of genes they transcribe. It is also hypothesized that transcription factories may coordinate the regulation of multiple genes by influencing a small local area within the nucleus (Misteli, 2007).

The spliceosome assembly factor SC35 also shows a punctate, or speckled, distribution within the nucleus. There are 15–40 large SC35 domains per nucleus that contain numerous splicing and RNA metabolic factors and are referred to as “splicing speckles” (Lamond and Spector, 2003). Gene rich euchromatic regions are frequently associated with splicing speckles (Shopland et al., 2003). Increased transcriptional activity of the \textit{Plp1} in differentiated oligodendrocytes correlated with its association
with splicing speckles (Neilson et al., 2002). Additionally, MyHC and MyoD became associated with splicing speckles when transcriptionally active in terminally differentiated muscle nuclei, but were not associated when transcriptionally inactive in proliferative myoblasts or in fibroblasts (Moen et al., 2004). Brown et. al. demonstrated that when Hbb and Hba were associated with splicing speckles their transcriptional activity increased (Brown et al., 2006). They also demonstrated that cotranscribed erythroid genes were enriched at splicing speckles, but did not see colocalization of these genes at transcription factories (Brown et al., 2008). They suggested, contrary to Osborne et.al. (Osborne et al., 2004), that associations between active genes may result from their location on decondensed chromatin that enables clustering around common nuclear speckles and any attributed colocalization with transcription results from the prevalence of transcription factories near splicing speckles. Thus, it is debatable if association with transcription factories regulates gene expression.

1.3.4 The Nuclear Envelope

The nuclear envelope defines the barrier between the nucleus and the cytoplasm and has four domains: the outer nuclear membrane, the nuclear pore, the inner nuclear membrane, and the nuclear lamina.
1.3.4.1 The Nuclear Lamina

The nuclear lamina is a network of filament-like lamin proteins that extends from the nuclear envelope into the nucleoplasm (Dechat et al., 2008). There are two lamin subtypes; LaminA/C and laminB1/B2. The B-type lamins are tightly associated with the inner nuclear membrane. While most of the A-type lamins are also found at the nuclear lamina, between 5-10% can be found in the nucleoplasm (Rusinol and Sinensky, 2006; Vlcek and Foisner, 2007). There are multiple proteins and complexes that interact with the inner nuclear membrane, the nuclear lamina, and chromatin (Figure 3).

![Figure 3: The interaction between the nuclear lamina and heterochromatin.](image)

Lamin B-associated LAP2β and Lamin B Receptor bind BAF and HP1, respectively and provide anchors for heterochromatin. LAP2β also binds histone deacetylase HDAC3, which contributes to heterochromatin formation (Schirmer and Foisner, 2007).
There are two nuclear lamina proteins responsible for generating a repressive nuclear compartment. First, the Lamin B receptor, which integrates into the inner nuclear membrane, binds to both laminB1 and Heterochromatin Protein 1 (HP1). HP1 binds to lysine 9 methylated histone 3 and binds histone methyl-transferases (HMT). Thus it can bind heterochromatin and propagate repressive marks to nearby histones. Secondly, LAP2β, also an integral inner nuclear membrane protein, can bind both barrier to autointegration factor (BAF) and histone deacetylase 3 (HDAC3) (Somech et al., 2005). BAF has been shown to nonspecifically bind chromatin while HDAC3 can deacetylate both histones H3 and H4. Thus the nuclear lamina has the potential to create a heterochromatic environment that is refractory to gene expression.

Several studies have addressed the biological significance of association of chromatin with the nuclear lamina. Pickersgill et. al. identified genes near the nuclear periphery by creating a chimeric protein between LaminB1 and DNA adenine methyltransferase (Dam). Adenine-methylated DNA fragments from a *drosophila melanogaster* cell line were then analyzed. It was found that these fragments were sparsely populated with genes and those genes were transcriptionally silent, replicated late in the cell cycle, and were depleted of active histone modifications (Pickersgill et al., 2006). This strongly suggested that the nuclear lamina is a repressive nuclear compartment. Additionally, regions of chromatin that were artificially tethered to emerin, a protein that interacts with laminB1, had reduced levels of transcription.
(Reddy et al., 2008). In a similar study, tethering genes to LAP2β resulted in a reduction of gene transcription (Finlan et al., 2008). However, a stably integrated plasmid targeted to the nuclear lamina through LaminB1 maintained high levels of transcription originating from a strong viral promoter (Kumaran and Spector, 2008). Thus, repositioning of genes to the nuclear lamina may be sufficient to suppress transcription from weak promoters, while strong promoters maybe capable of overcoming the repressive environment of the nuclear lamina.

1.3.4.2 The Nuclear Pore Complex

The nuclear pore complex (NPC) allows molecules to pass to and from the nucleus (Cook et al., 2007). Small molecules passively diffuse through the NPC, but macromolecular complexes greater than 40 kDa are actively transported across the nuclear membrane through the NPC (Mosammaparast and Pemberton, 2004). The NPC spans the nuclear envelope and fuses the inner and outer nuclear membranes together (D’Angelo and Hetzer, 2006). NPCs are composed of 30 different proteins, known as nucleoporins, and are about 60–125 MDa in mammals and about 40–60 MDa in yeast (Cronshaw et al., 2002; Rout et al., 2000; Yang et al., 1998). The NPC is an eightfold-symmetrical structure comprising a nuclear envelope embedded scaffold that surrounds a central transport channel and two rings (the cytoplasmic ring and the nuclear ring) above and below the channel. Stretching out into the nucleoplasm from
the nuclear ring are eight filaments that join a third ring to form the nuclear basket (Fahrenkrog et al., 1998; Suntharalingam and Wente, 2003).

In yeast, the NPC has been associated with active gene expression. A microarray of the yeast genome identified a subset of highly transcribed genes bound to NPC proteins (Casolari et al., 2004). Moreover, the induction of hexokinase isoenzyme 1 (HXK1) transcription correlated with its relocation to the NPC. The relocation required the 3’ untranslated region of the HXK1 primary transcripts. Additionally, when transcription of HXK1 was initiated by an alternate promoter, the gene did not relocate to the NPC, suggesting the mode of activation influences the nuclear position of the gene (Taddei et al., 2006). Induction of GAL gene expression in yeast results in active transcription in only a subset of cells. Those cells that actively transcribe GAL reposition their alleles to the NPC, while in the cells that are not transcribing GAL the alleles were randomly distributed in the nucleus (Cabal et al., 2006). Thus, the transcriptional state of GAL is directly correlated with the position within the nucleus. The increase in transcription levels was abrogated upon deletion of subunits of the SAGA histone acetyl transferase complex. SAGA was shown to directly bind to nucleoporins in the NPC, suggesting that SAGA could be a key element in recruiting genes to the NPC (Luthra et al., 2007).
1.3.5 Peri-centromeric Heterochromatin

Peri-centromeric heterochromatin is the sequence of the DNA near the centromere of the chromosome that is highly repetitive, gene poor, and heavily methylated. The repetitive sequences of the centromeric region of different chromosomes cluster together with other repetitive DNA sequences in the genome to form distinct foci in most mammalian nuclei. The major repetitive element of peri-centromeric heterochromatin is the $\gamma$-satellite tandem repeat. These heterochromatic regions contain high levels of heterochromatin protein 1 (HP1) and Ikaros. Heterochromatin protein 1 (HP1) is an evolutionarily conserved chromosomal protein with important roles in chromatin packaging, gene silencing, and the formation of centromeric clusters in the nucleus (Sadaie et al., 2008). HP1 association with peri-centromeric heterochromatin facilitates gene silencing through repressive chromatin modifications (Taddei et al., 2001). Peri-centromeric heterochromatin silencing in yeast is mediated through centromere-associated DNA repeats that are transcribed and give rise to small interfering RNAs (siRNA) (Reinhart and Bartel, 2002; Volpe et al., 2002). In yeast, the resulting dsRNA accumulates at the region of transcription and recruits the Clr4-Rik1-Cul4 complex, which mediates H3K9 methylation and initiates heterochromatin formation. This initial mark is then propagated and maintained by the recruitment of Swi6/HP1 (Iida et al., 2008). When transgenes are targeted in-cis to pericentromeric heterochromatin, nearby heterochromatin spreads into the transgene.
This results in the accumulation of HP1 and H3K9me2 and the repression of gene expression (Buhler et al., 2007; Grewal and Moazed, 2003).

Repositioning genes to centromeric heterochromatin is correlated with their reduced transcription. In B-cells it was shown that λ5 was not associated with centromeric heterochromatin in pre-B cell lines, but was associated in mature B-cell lines (Brown et al., 1997). Additionally, in that study cd2, cd4, and cd8a (which are not expressed in these cell lines) were associated with peri-centromeric heterochromatin in both pre-B cells and mature B cells, while cd19 (which is expressed in both cell lines) was rarely associated with peri-centromeric heterochromatin. Further study of cd8a demonstrated that it was associated with peri-centromeric heterochromatin in CD4SP cells when it is not expressed, but was not associated with peri-centromeric heterochromatin in DP thymocytes when it is expressed (Merkenschlager et al., 2004). Gata3 also associates with pericentromeric heterochromatin when its expression is repressed in Th1 cells (Hewitt et al., 2004). Repositioning of genes to peri-centromeric heterochromatin in developing T-cells is thought to be mediated through the transcription factor Ikaros. ImmunoFISH studies have revealed that the transcription factor Ikaros colocalizes to peri-centromeric foci with inactive, developmentally-regulated genes, suggesting that Ikaros may contribute to the peri-centromeric repositioning of these genes (Brown et al., 1999; Brown et al., 1997). When ectopically expressed in 3T3 cell lines, Ikaros binds directly to peri-centromeric foci (Cobb et al.,
Ikaros binds to the promoter of terminal transferase (TdT) gene and Ikaros binding is required for transcriptional inactivation during thymocyte development (Trinh et al., 2001). Therefore Ikaros binds to both peri-centromeric heterochromatin foci and to genes that are repressed when associated with peri-centromeric heterochromatin. Thus there is a strong correlation between peri-centromeric heterochromatin association and repressed transcription. However, it is not known if movement to peri-centromeric heterochromatin is sufficient to repress transcription in trans or if previously repressed genes are drawn to peri-centromeric heterochromatin. Tethering of genes to peri-centromeric heterochromatin is needed to prove or disprove a causative role for trans peri-centromeric heterochromatin repression.

1.4 Genomic Organization of Antigen Receptor Loci

There are seven murine antigen receptor loci that are capable of undergoing V(D)J recombination (Figure 4). The receptor loci share similar characteristics in organization. Numerous V segments are found dispersed at the 5’ end of each locus and each V segment usually has its own promoter that will drive transcription if that gene segment is incorporated into the final coding sequence. D gene segments, when present, are found in the middle of the locus. Finally multiple J gene segments are found at the 3’ end of the locus followed by the constant region exons.
Figure 4: Genomic organization of the antigen receptor loci.

A schematic of the seven murine antigen receptor loci that is not drawn to scale. V, D, and J gene segments are represented by square rectangles, promoters are represented by arrows, and enhancers are represented by ovals (Adapted from Cobb et al, 2006; Hesslein and Schatz, 2001; Krangel and Schlissel, 2004).

The IgH locus is the largest of the antigen receptor loci spanning 3.3 Mbp near the telomere on mouse chromosome 12. The locus is highly repetitive with 54% of the
locus being composed of interspersed repeats (Retter et al., 2007). There are 15 gene segment families that occupy the 5’ 3.0 Mbp of the locus. There are between 170 and 195 $V_H$ gene segments of which a little over half are functional (110 are genes and 85 are pseudogenes) (Johnston et al., 2006). $V_H$ gene segments are flanked on their 5’ end with a promoter and on their 3’ end by a 23-RSS. There are 15 $D_H$ gene segments in four families (DSP2, DFL16, DST4, and DQ52) spanning approximately 120Kbp. Each $D_H$ gene segment is flanked on each side by a 12-RSS (Retter et al., 2007). There are 4 tightly clustered $J_H$ gene segments flanked on their 5’ side with a 23-RSS. Finally there are multiple $C_H$ gene segments spanning about 190 Kbp which encode the constant region of $IgH$. Two germline transcripts originate in the 3’ end of the Igh locus: $\mu_0$ transcripts originate 5’ of DQ52 and $I\mu$ transcripts that originate immediately 3’ of the $E_\mu$ core (Alessandrini and Desiderio, 1991; Lennon and Perry, 1985). There are three known enhancer elements in the $Igh$ locus. The intronic enhancer ($E_\mu$) is located between the $J_H$ and $C_H$ gene segments. The 3’ regulatory region (3’RR) lies 3’ to the $C_H$ gene segments and is composed of 4 DNAse hypersensitivity sites. Finally the $E_\delta\gamma_3$ region found between the $C_\delta$ and $C_\gamma$ is also important for CSR (Mundt et al., 2001).

The $Igk$ locus consists of 140 $V_\kappa$ gene segments (95 are functional) and spans 3.2 Mbp on mouse chromosome 6 (Brekke and Garrard, 2004). $V_\kappa$ gene segment have a 3’ 12-RSS. Unlike $IgH$ there are no D segments in the $Igk$ locus. Downstream of the $V_\kappa$ region there is the $J_\kappa$ region consisting of 4 gene segments, each flanked on their 5’ end
with a 23-RSS. Finally there is a single constant region at the extreme 3′ end of the locus. Kappa locus transcription and recombination are regulated by three enhancers. One lies between the \( J_\kappa \) and \( C_\kappa \) gene segments (Ei) and the other two lie outside the transcription unit at the 3′ end of the locus (E3′ and Ed) (Liu et al., 2002; Meyer and Neuberger, 1989; Queen and Baltimore, 1983).

The \textit{Igl} locus is relatively small when compared to the other antigen receptor loci spanning only 240kbp of mouse chromosome 16. Two \( V_\lambda \) gene segments, each with a 3′ 23-RSS, are located at the 5′ end of the locus. These \( V_\lambda \) gene segments are followed by a single \( J_\lambda \) and \( C_\lambda \). There is a third \( V_\lambda \) in the middle of the locus followed by two \( J_\lambda \) \( C_\lambda \) clusters (each composed of a single \( J_\lambda \) and \( C_\lambda \)). There are two enhancers in the \textit{Igl} locus that are found downstream of the first \( C_\lambda \) and the third \( C_\lambda \).

The \textit{Tcra} locus spans 1.6 Mbp of mouse chromosome 14. The most notable feature of the \textit{Tcra} locus is that the \textit{Tcrd} locus is contained within it and there is a sharing of \( V \) gene segments. The 104 \( V \) gene segments in the \textit{Tcra/Tcrd} locus extend across 1.5 Mbp of the 5′ end of the locus and the gene segments are flanked by 23-RSSs. (krangell 2004) There are 61 tightly grouped \( J_\alpha \) gene segments (flanked 5′ by 12RSS) found at the 3′ end of the locus followed by a single constant gene segment. All \( V \) gene segments rearrange to \( J_\alpha \) gene segments with a bias for the 3′ \( V \) segments to rearrange to the 5′ \( J_\alpha \) and the 5′ \( V \) segments to rearrange to the mid and 3′ \( J_\alpha \) gene segments (Pasqual et al., 2002). There is a strong enhancer (E\( \alpha \)) located at the 3′ end of the locus that is
critical for transcription of both Tcrd and Tcra in mature T-cells and for recombination of Tcra (Sleckman et al., 1997). Ea binds transcription factors in DN thymocytes but Tcra remains closed and hypoacetylated and Ea is not required for Tcrd recombination. In DP thymocytes Ea promotes changes in chromatin structure across Vα and Jα gene segments and drives transcription of proximal Vα promoters as well as promoters found in the Jα array (Hawwari et al., 2005). There is a hierarchy of promoters in the Jα array with the strongest being the T-cell early alpha (TEA) found 5’ of the Jα gene segments. There exists a weaker promoter at Jα49 and several other very weak promoters in the middle of the Jα array. The numerous Vα and Jα gene segments allow for multiple rearrangements to occur in the Tcra locus with rearrangement excising the intervening DNA sequence. This has two important consequences. First, DP thymocytes have multiple attempts at forming a functional Tcra protein. And second, the thymocyte is committed to the αβ TCR lineage, because Tcrd is deleted upon activation of Tcra.

The Tcrd locus is composed of two Dδ gene segments, two Jδ gene segments, one Cδ region, and single inverted Vδ gene segment found 3’ of Cδ. Unlike IgH and Tcrb, which also recombine D gene segments, there is no ordering of recombination such that Vδ to Dγ, Dδ to Dδ, and Dδ to Jδ all occur in DN3 thymocytes. The V gene segments are flanked by a 23-RSS, the Dδ gene segments are flanked by a 3’ 12-RSS and a 5’ 23-RSS, and the Jδ gene segments have a 5’ 12-RSS. This configuration of 12 and 23 RSSs allows for both V to Dδ and Dδ to Jδ rearrangements, which are detected, and V to Jδ
rearrangements, which are not detected. There is one enhancer (Eδ) located between Cδ and Jδ2. Eδ increases the efficiency of Tcrd gene segment rearrangement but is not required for Tcra rearrangement (Monroe et al., 1999). In addition to the V gene segment promoters, both Dδ2 and Jδ1 have promoters (Carabana et al., 2005). Eδ has been shown to increase the transcript levels of the Dδ2 promoter, but there are residual transcripts found in Eδ deficient mice suggesting that Dδ2 has some function that is independent of Eδ.

The Tcrb locus (spanning about 700 Kbp) is arranged with 33 Vβ gene segments (19 are functional) in the 5’ region of the locus surrounded by two regions of trypsinogen genes. There are two outlier functional Vβ gene segments: Vβ2 is located on the extreme 5’ end of the first trypsinogen region and Vβ14 lies at the extreme 3’ end of the Tcrb locus in an inverted orientation. At the 3’ end of the locus, upstream of Vβ14, there are two DJCβ clusters, which each contain a single Dβ gene segment, 6 Jβ gene segments, and a single Cβ region. The Jβ gene segments have a 12-RSS and the Dβ gene segments have a 5’ 12-RSS and 3’ 23-RSS. Though Vβ to Jβ rearrangements are theoretically possible due to their RSS configuration, they are never seen. Upon rearrangement to any upstream Vβ gene segment the internal trypsinogen region is excised, while rearrangement to Vβ14 results in an inversion of the intervening DNA. There is a single enhancer (Eβ) located between Cβ2 and Vβ14 that is required for histone acetylation, transcription, and recombination of Tcrb (Bouvier et al, 1996).
Additionally there is a promoter located near D_{\beta}1 (PD_{\beta}1) that controls local accessibility of the DJC_{\beta}1 cluster (Spicuglia et al, 2002). The recombination of Tcrb is ordered in that D_{\beta} to J_{\beta} rearrangement occurs before V_{\beta} to DJ_{\beta} rearrangement.

1.5 Regulation of V(D)J Recombination

1.5.1 The Accessibility Hypothesis

V(D)J recombination is limited to antigen receptor loci by RSSs and to lymphocytes through RAG expression. Lineage specific restriction of recombination (e.g. antibody-encoding loci recombining in B-cells and TCR-encoding loci recombining in T-cells) as well as stage specificity (e.g. IgH recombination in pro-B cells and Tcra recombination in DP thymocytes) is explained via the “Accessibility Hypothesis”. This hypothesis was proposed based on the observation that germline transcription from V_{H} promoters occurred coincidently with recombination of these gene segments in pro-B cells and that the transcripts were not present in mature B-cells or mature T-cells (Yancopoulos and Alt, 1985). The accessibility necessary to induce transcription was hypothesized to also allow the recombination machinery access to the RSSs.

Several lines of evidence support this hypothesis. First, ectopic expression of RAG in non-lymphoid cells does not induce recombination at endogenous loci (Schatz and Baltimore, 1988). Second, when nuclear extracts from B and T cells (with RSSs in their native chromatin structure) were mixed with purified RAG1 core protein, DSBs were formed at endogenous antigen receptor in accord with the lineage and stage
specificity of the nuclear extract (Golding et al., 1999; Stanhope-Baker et al., 1996).

Third, H3 and H4 hyperacetylation of antigen receptor loci is tightly correlated with their recombination potential (McMurry and Krangel, 2000; Tripathi et al., 2002). Conversely the repressive histone mark, H3K9, correlates with decreased accessibility and impaired recombination and forced recruitment of a H3K9 specific methyltransferase suppress recombination (Morshead et al., 2003; Osipovich et al., 2004).

Finally, DNA methylation \textit{in vitro} and \textit{in vivo} is strongly associated with repressed chromatin and suppressed recombination (Cherry and Baltimore, 1999; Hsieh and Lieber, 1992; Mostoslavsky et al., 1998).

The foundation of the Accessibility Hypothesis was based upon the observation that transcription is correlated with recombination (Yancopoulos and Alt, 1985). Promoters and enhancers drive transcription and experiments that delete either enhancers or promoters have demonstrated a reduction in transcription and a corresponding reduction in recombination. Two germline transcripts originate in the 3’ end of the \textit{Igh} locus: \(\mu 0\) transcripts originate 5’ of D\(Q5\)2 and I\(\mu\) transcripts that originate immediately 3’ of the E\(\mu\) core (Alessandrini and Desiderio, 1991; Lennon and Perry, 1985). Deletion of E\(\mu\) leads to a 10 to 20 fold reduction in both \(\mu 0\) and I\(\mu\) transcripts and a corresponding decrease in D\(H\) to J\(H\) rearrangement (Afshar et al., 2006; Perlot et al., 2005). Deletion of E\(\mu\) did not affect V\(\mu\) transcription but did block V\(\mu\) to DJ\(\mu\) recombination. The inhibition of V\(\mu\) to DJ\(\mu\) recombination was attributed to a secondary
defect stemming from a reduction in rearranged DJH substrates (Afshar et al., 2006). A similar phenotype is seen in the Tcrb locus. Germline expression of the Vβ genes is, to a large extent, independent of Eβ while the germline transcripts traversing Jβ1 and Jβ2 are dependent upon Eβ. Eβ−/− mice have a striking Vβ to Dβ and Dβ to Jβ rearrangement defect (Mathieu et al., 2000). Deletion of both enhancer elements in the Igk locus results in a reduction of germline transcription and recombination (Inlay et al., 2002). In the Tcra locus, Ea is required for germline Jα expression and for normal Vα to Jα rearrangement (Sleckman et al., 1997). Promoter deletion studies showed a similar effect. Deletion of PDβ1 in Tcrb results in a loss of transcription and recombination of the Dβ1 and Jβ1 gene segments (Whitehurst et al., 1999). Deletion of TEA in the Tcra locus results in a loss of transcription as well as loss of recombination of the most 5′ Jα gene segments (Villey et al., 1996). Thus enhancers and promoters cooperatively create accessible regions that are permissive to both transcription and recombination.

1.5.2 Nucleosome Positioning

In vitro recombination substrates assembled onto nucleosomes are cleaved very poorly by RAG proteins but cleavage efficiency is increased by the addition of the ATP dependent nucleosome remodeling enzyme SWI/SNF (Golding et al., 1999; Kwon et al., 2000). BRG1 (the catalytic subunit of SWI/SNF) was found to associate with D and J gene segments in recombination-permissive IgH and Tcrb loci (Morshead et al., 2003). Moreover, in vitro, BRG1 was shown to increase the cleavage efficiency of
polynucleosomal constructs (Patenge et al., 2004). This suggested that nucleosomes positioned over RSSs are a barrier to recombination by preventing RAG access to the DNA. This idea was supported by the finding that a consensus nonamer sequence strongly positions nucleosome both in vivo and in vitro (Baumann et al., 2003). Thus, nucleosomes may be positioned over RSSs rendering them inaccessible to RAG and repositioning of nucleosomes may regulate recombination. Experimentally this was demonstrated by replacing PDβ1 with gal4 sites in a Tcrb “minilocus”. Binding of BRG1 to these gal4 sites, along with Eβ, was sufficient to induce recombination of Dβ and Jβ gene segments in the minilocus (Osipovich et al., 2007). This suggested that promoters facilitate recombination by repositioning nucleosomes and creating accessible RSSs, while enhancers facilitate recombination through activating promoters. These studies, while confirming the correlation between transcription and recombination, could not discern if promoters induced chromatin changes through transcription or through recruiting transcription factors necessary to create accessible DNA.

1.5.3 Transcription

Germline transcripts from the promoters of V gene segments have been found to correlate with recombination of those gene segments (Fondell and Marcu, 1992; Goldman et al., 1993; Schlissel et al., 1991; Yancopoulos and Alt, 1985). Experiments in which the transcription level was increased resulted in an increase in recombination. Inducing transcription from endogenous promoters in Igk by stimulating a pre-B cell
line with lipopolysaccharide (LPS) induced V\textsubscript{\kappa} to J\textsubscript{\kappa} recombination (Schlissel and Baltimore, 1989). Additionally, when the strong housekeeping phosphoglycerine kinase (PGK) promoter was introduced into the Ig\textsubscript{\iota} locus, both germline transcription and V\textsubscript{\kappa} to J\textsubscript{\kappa} recombination were dramatically increased (Sun and Storb, 2001). Thus, the rate of transcription closely mirrors the rate of recombination, suggesting that the correlation between germline transcription and recombination is not just an all or none phenomenon, as transcription efficiency is directly associated with the recombination efficiency.

To determine if promoters induce chromatin changes through transcription or through recruiting transcription factors, Abarrategui et. al. blocked transcription in the Tcra locus. By introducing a transcription terminator downstream of TEA, they showed a reduction in the H3 and H4 acetylation and H3K4 trimethylation of the downstream gene segments (Abarrategui and Krangel, 2006, 2007). More importantly, they showed that transcription elongation is required for recombination of 5’ J\textsubscript{\alpha} gene segments. Transcription may facilitate recombination in a number of ways. It is possible that H3K4Me3 recruits chromatin-remodeling enzymes leading to a repositioning of nucleosomes (Santos-Rosa et al., 2003). It is also possible that transcription mediated H3K4me3 directly targets RAG to transcribed RSSs through its PHD domain (Liu et al., 2007b; Matthews et al., 2007). Finally, it is possible that the movement of the polymerase through the gene segments may disrupt nucleosome structure sufficiently.
to create recombination-accessible RSSs (Belotserkovskaya et al., 2003; Carrozza et al., 2005; Schwabish and Struhl, 2006). These data strongly suggests that transcription makes a substantial contribution to RSS accessibility and recombination.

1.5.4 Nuclear Localization

Direct visualization of antigen receptor loci by three-dimensional fluorescent in-situ hybridization (3D-FISH) has demonstrated that these loci relocalize to and from the nuclear periphery during development. The Igh locus in embryonic stem (ES) cells, multipotent progenitors, and non B cells was preferentially located at the nuclear periphery. Then, as the Igh locus becomes active and recombinationally competent in pro-B cells, it is repositioned to a central nuclear location (Kosak et al., 2002). Interestingly, in immature B-cells and plasma cells more than 60% of Igh loci return to the nuclear periphery (Yang et al., 2005; Zhou et al., 2002). Thus, for Igh there is a correlation between recombination potential and a central nuclear position. Supporting this was the observation that the V\(^H\) region of the Igh locus is more frequently located at the nuclear periphery than the 3’ DJC\(^H\) region. Therefore, the 5’ peripheral orientation may reduce accessibility at the distal V\(^H\) gene segments while the D\(^H\) and J\(^H\) regions remain accessible, which is in accordance with D to J recombination being less regulated than V to DJ recombination (Kosak et al., 2002; Yang et al., 2005). However, repositioning to a central nuclear region is not necessarily sufficient for recombination. Igk moves from the nuclear periphery in ES cells and multipotent progenitors to a more
central position in pro-B cells prior to its recombination in pre-B cells and \( Igl \) is infrequently located at the periphery in progenitor cells, developing B cells, or T cells (Kosak et al., 2002). Conversely, repositioning to the nuclear periphery does not fully prevent recombination. \( Tcrb \) and \( Tcra/Tcra \) are frequently at the nuclear periphery throughout thymocyte development. \( Tcrb \) was found at the nuclear periphery in 50% of DN cells, 59% of DP and 76% of SP T cells while \( Tcra/Tcra \) was found at the periphery in 71% of DN cells, 46% in DP cells, and 55% of SP cells (Skok et al., 2007). These observations led to the hypothesis that recombination efficiency is regulated, in part, by positioning antigen receptor loci at the nuclear periphery by creating a closed chromatin environment that decreases the RSS accessibility to RAG.

1.5.5 Locus Contraction

Antigen receptor loci must adopt a conformation that brings the gene segments in close proximity for recombination to occur. 3D FISH analysis of developing B-cells using BAC clones as probes to visualize the 5’ and 3’ ends of the \( Igh \) locus demonstrated that the average distance between the probes is smaller in cultured \( \text{Rag}^{2-/-} \) pro-B cells than in either cultured wild-type B cells or CD8+ T cells (Kosak et al., 2002; Sayegh et al., 2005). Using a third BAC probe situated between the 5’ and 3’ probes, this contraction of the \( Igh \) locus was attributed to it adopting two conformations. 18.5% of \( Igh \) loci adopted a compacted conformation, where all the probes moved closer to each other but maintain a linear order, while 24.6% of the \( Igh \) loci adopted a looped conformation,
where the 5', central, and 3' BAC probes did not maintain the genomic linear alignment (Sayegh et al., 2005). Interestingly, in Rag2\(^{-/-}\) pro-B cells all \(V_H\) gene segments are on average about 0.4 \(\mu\)m away from the \(J_H\) gene cluster demonstrating that the spatial separation of these gene segments is independent of their genomic separation (Jhunjhunwala et al., 2008). In pre-pro-B cells the distance between the proximal \(V_H\) gene segments and the \(J_H\) gene cluster was similar to pro-B cells. However, in pre-pro-B cells the distal \(V\) gene segments were located farther away (about 0.6 \(\mu\)m) from the \(J_H\) gene cluster than in pro-B cells (Jhunjhunwala et al., 2008).

The rearrangements of distal \(V_H\) gene segments requires IL-7 signaling and \(Ezh2\) expression. IL-7 signaling, through the transcription factor STAT5, induces histone acetylation, germline transcription and recombination of the distal \(V_HJ558\) gene family, but is not required to contract \(Igh\). In comparison, the proximal \(V_H\) gene family 7183 had normal histone acetylation, germline transcription, and recombination in STAT5\(^{-/-}\) pro-B cells (Bertolino et al., 2005). Distal \(V_H\) segments have the active histone modification H3K36me2, while proximal \(V_H\) segments have the repressive histone modification H3K27me3 (Xu et al., 2008). \(V_H\) gene segments in pax5\(^{-/-}\) mice have reduced H3K27me3 in the proximal region and \(V_H\) gene segments in IL-7R\(^{-/-}\) pre-B cells have reduced H3K36me2 in the distal region (Xu et al., 2008). Thus the transcription factors STAT5 and Pax5 may influence distal and proximal \(V_H\) rearrangements by
differentially modulating their histone modifications during B lymphocyte development.

Contraction is required for recombination of the distal $V_H$ gene segments in pro-B cells and is so far regulated by three transcription factors: Pax5, YY1, and Ikaros. Pax5 has been shown to be required for recombination of the 5' $V_H$ gene segments, as Pax5 deficient pro-B cells have a 100 fold reduction in the recombination frequency of the distal $V_H$J558 gene family (Fuxa et al., 2004; Hesslein et al., 2003; Nutt et al., 1997). Importantly, the ends of the $IgH$ locus (indetified by probes for $V_H$J558 and $C_{\gamma}$) were separated by more than 0.5 $\mu$m in 84-94% of all Pax5/ pro-B cells, indicating that the $IgH$ locus is in an extended conformation (Fuxa et al., 2004). Thus, in pro-B cells Pax5 ensures that the distal $V_H$ regions are in close proximity to the $D_H$ and $J_H$ gene segments. However, Pax5 is insufficient by itself to contract the $IgH$ locus. Ectopic expression of Pax5 in DN thymocytes did not induce $IgH$ contraction or recombination to distal $V_H$ gene families, suggesting there is an additional B-cell specific factor necessary of $IgH$ contraction (Fuxa et al., 2004). YY1 is a zinc finger protein that functions as either a transcriptional activator or repressor depending on the promoter (Calame and Atchison, 2007). Loss of YY1 resulted in a significant increase in the number of $IgH$ alleles with an extended conformation in pro-B cells and led to a defect in $V_H$ to $D_H$ recombination (Liu et al., 2007a). Finally, the transcription factor Ikaros is required for $IgH$ contraction. In Ikaros deficient pro-B cells $IgH$ adopts an extended conformation,
with the intralocus distance significantly larger than in Ikaros expressing pro-B cells. Moreover, the distal V_H gene segments in Ikaros deficient pro-B cells were inaccessible, did not generate germline transcripts, and did not recombine (Reynaud et al., 2008). Thus Pax5, YY1, and Ikaros induce contraction of the Igh locus and are required for the recombination of distal V_H gene segments.

The Igk and Tcrb loci also alter their conformation in different developmental stages. 3D FISH shows that the Igk locus contracts in small pre-B cells and in immature B cells, because in 90% of small pre-B cells and in 78% of immature B-cells the 5’ and 3’ ends of the Igk locus were within 0.3 µm of each other (Roldan et al., 2005). In pro-B cells, the recombinationally inactive Igk adopts an extended conformation, since more than 85% of Igk alleles had their 5’ and 3’ ends between 1.0 and 1.5 µm apart in pro-B cells and in large cycling pre B-cells (Roldan et al., 2005). The Tcrb locus behaves correspondingly during thymocyte development. The 5’ and 3’ ends of the Tcrb locus are extended and separated by more than 0.3 µm 80% of the time in pre-B cells and αβ transgenic DP thymocytes. However, in DN thymocytes Tcrb is contracted, with the 5’ and 3’ ends within 0.3 µm of each other 80% of the time. The contraction of Tcrb is attributed to looping rather than compaction in DN thymocytes because the spatial orientation of three BAC probes did not reflect their linear genomic orientation (Skok et al., 2007). Thus developmental regulation of recombination can be achieved by altering the conformation of antigen receptor loci.
1.6 Allelic Exclusion

An important but poorly understood aspect of V(D)J recombination is the phenomenon of allelic exclusion, which limits Tcrb, IgH, and Igk loci to produce a functional protein from only a single allele (Corcoran, 2005; Khor and Sleckman, 2002; Krangel et al., 2004). Allelic exclusion of these loci is controlled at the level of rearrangement because mice that express pre-rearranged IgH and Tcrb transgenes do not recombine their endogenous loci and two pre-rearranged IgH transgenes can also be expressed in a single B cell (Sonoda et al., 1997; Uematsu et al., 1988). Both Tcrb and Igk recombine D to J on both alleles, while V to DJ rearrangement is subject to allelic exclusion. Allelic exclusion is thought to occur in two phases. First the alleles must asynchronously initiate recombination. Second upon a successful rearrangement there must be a feedback inhibition of recombination so that further recombination does not occur at the locus. Asynchronous recombination results from either 1) a mechanism that strictly orders the sequence that the alleles undergo recombination or 2) a mechanism that reduces the frequency that both alleles recombine such that synchronous recombination is highly unlikely. Feedback inhibition is mediated through pre-TCR or pre-BCR signaling, because mice deficient for pre-TCR and pre-BCR signaling molecules often contain nuclei with two in-frame alleles (Michie et al., 2001; Muljo and Schlissel, 2000).
If one-third of rearrangements are successful and both alleles are capable of rearranging, then an allelically excluded mature T-cell population should have a 60:40 ratio of cells with one rearrangement to cells with two rearrangements (figure 5). Theoretically, one third of all nuclei should successfully form an in-frame rearrangement on the first attempt. Thus one-third of cells (or 3/9 of the total population) will have the first allele VDJ rearranged and the second allele only DJ rearranged. The remaining two-thirds of the cells will attempt a second rearrangement, resulting in two-ninths (2/3 out-of-frame X 1/3 in-frame =2/9) of the total population that will have the first allele out-of-frame and the second allele in-frame, and four-ninths (2/3 out-of-frame X 2/3 out-of-frame =4/9) of the total population having both alleles out-of-frame. Cells that fail to successfully form an in-frame rearrangement on either allele will not progress to the DP compartment. This (3/9:2/9) 60:40 ratio is found experimentally. In mature B and T-cells, 50-60% of cells have only one allele with a VDJ rearrangement, while 40-50% of cells have one allele with an in-frame VDJ rearrangement and one allele with an out-of-frame VDJ rearrangement (Alt et al., 1984; Khor and Sleckman, 2005; Melchers et al., 1999).
A schematic representing Tcrb rearrangement in developing DN thymocytes. Initially both alleles have Db to Jb rearrangements (DJ). In-frame Vβ to DJβ rearrangement (VDJ+) and out-of-frame Vβ to DJβ rearrangements (VDJ-) lead to the following outcomes (Courtesy of Michael S. Krangel).

1.6.1 Asynchronous Rearrangement

Two types of mechanisms have been invoked to explain asynchronous recombination of Tcrb alleles in DN thymocytes and Igh alleles in pre-B cells (Jackson and Krangel, 2006b; Jung et al., 2006; Khor and Sleckman, 2002; Mostoslavsky et al., 2004). In deterministic models, the two alleles are nonequivalent substrates for recombination and the order in which the alleles recombine is predetermined. Mostoslavsky et. al. demonstrated via FISH that IgH, Igk, Igλ, and Tcrb alleles asynchronously replicate (Mostoslavsky et al., 2001). Between 38% and 56% of interphase nuclei were visualized with three copies of the different loci indicating that
one allele had replicated earlier than the other. The early replicating $Igk$ allele was established early in development and this choice was maintained in primary fibroblast cell lines. Asynchronous replication may reflect a differential marking of the alleles that renders one allele more accessible for rearrangement. In fact, for 83% of $Igk$ alleles and 90% of $IgH$ alleles, the late-replicating allele was in the germline configuration, indicating that rearrangement in B cells almost always occurs on the early-replicating allele (Mostoslavsky et al., 2001). Further studies showed that the late replicating $Igk$ allele carried this mark into the pre-B cell stage. In pre-B cells, $Igk$ was monoallelically associated with pericentromeric heterochromatin and 80% of the time it was the late replicating allele that was associated. These associations with pericentromeric heterochromatin correlated with $Igk$ becoming inaccessible to a trans-acting factor that demethylates DNA (Goldmit et al., 2005). The early replication of one $Igk$ allele persists further into mature B-cells. Genetically modified ‘double-expressing’ $Igk$ B cells underwent monoallelic demethylation, and the demethylated allele was the ‘preferred’ substrate for somatic hypermutation (Fraenkel et al., 2007). However, $Igk$ is biallelically expressed in immature B cells and in pre-B cells (Singh et al., 2003). Similarly, early replication of $Tcrb$ does not differentiate the transcription potential of the two alleles. Jia et. al. targeted a tailless human CD2 cDNA downstream of V$\beta$8.2, so that expression of hCD2 was driven by the V$\beta$8.2 promoter. These mice exhibited biallelic transcription of hCD2. Therefore the early replication mark does not reduce or inhibit germline
transcription of $V_\beta$ gene segments (Jia et al., 2007). Thus, early replication reflects an allelic difference for antigen receptor loci and this marking of alleles occurs early in development and is stably transmitted through cell division and differentiation. Early replication does not affect transcription, since both $Igk$ and $Tcrb$ are biallelically transcribed, but may increase the probability that $Igk$ alleles recombine.

A stochastic model postulates that reduced efficiency of recombination on both alleles diminishes the likelihood that the two alleles initiate recombination simultaneously (Schlissel, 2002). It was suggested that limiting regulators randomly and infrequently activate $Igk$ alleles in pre-B cells, thereby contributing to $Igk$ allelic exclusion (Liang et al., 2004). In addition, inefficient $V_\beta$ and $V_H$ recombination signal sequences were suggested to be rate limiting for recombination and to contribute to $Tcrb$ and $Igh$ allelic exclusion, respectively (Schlissel, 2002). There is evidence supporting the idea that changes in RSS sequence results in differential gene segment usage. A single nucleotide polymorphism in the heptamer of the $V_\kappa A2$ 12-RS results in a significant decrease in recombination efficiency when assayed in plasmid substrates (Nadel et al., 1998). Similarly reduced peripheral expression of $V_\beta 3$ in a family of individuals was found to correlate with an allelic polymorphism associated with a single nucleotide change in the $V_\beta 3$ 23-RS spacer (Posnett et al., 1994). Additionally switching the $V_\beta 14$ 23-RSS with the $D_\beta 1$ 23-RSS resulted in a nine-fold increase in $V_\beta 14$ usage, suggesting that RSS sequences are important in determining $V_\beta$ recombination.
frequencies *in vivo* (Wu et al., 2003). The observation that mice expressing only the core RAG proteins displayed a defect in V to DJ but not D to J recombination can be interpreted to mean that core RAG requires optimal RSS to recombine. This implies that V RSSs are less efficient substrates for recombination (Liang et al., 2002).

Monoallelic positioning of *Igk* and *Tcrb* to pericentromeric heterochromatin may regulate recombination. *Igk* monoallelically associates with peri-centromeric heterochromatin in 60% of pre-B cells (Goldmit et al., 2005). Skok et al. reported in 71-77% of DN, DP, and SP thymocytes *Tcrb* was monoallelically associated with peri-centromeric heterochromatin (Skok et al., 2007). They inferred that 82% of the monoallelically associated alleles were unrearranged in DP thymocytes. Therefore, monoallelic association with peri-centromeric heterochromatin of *Igk* and *Tcrb* in developmental stages during which they actively recombine suggests that this association may influence the initiation of *Igk* and *Tcrb* allelic exclusion. The deterministic model would argue that recombination does not occur on the associated allele, while the stochastic model would argue that recombination is less likely to occur on the associated allele. A thorough analysis that can positively identify the functional allele and its association with peri-centromeric heterochromatin is necessary to fully differentiate these two possibilities.
1.6.2 Feedback Inhibition

Feedback inhibition prevents recombination of \( Igh \) and \( Tcrb \) loci in pro-B cells and DN thymocytes after a functional rearrangement occurs on one allele, and prevents further recombination of these loci in pre-B cells and DP thymocytes, respectively.

Three potential mechanisms may enforce feedback inhibition. First, changes in V gene chromatin structure may limit access of the recombinase to chromosomal recombination signal sequences. Second, the loci may be repositioned to repressive nuclear compartments to prevent recombination. Finally, the loci may adopt extended conformations increasing the physical distance between V and DJ gene segments and preventing synapse formation.

Chromatin accessibility of antigen receptor loci can be measured by H3 and H4 acetylation, H3K4 methylation, DNase cleavage activity, restriction enzyme cleavage activity, RAG recombination activity, and germline transcription. As DN thymocytes transition to DP thymocytes, \( V_\beta \) gene segments show a reduction in germline transcription, a reduction in DNase accessibility, and a reduction of H3 and H4 acetylation (Chattopadhyay et al., 1998; Jackson et al., 2005; Senoo et al., 2003; Tripathi et al., 2002). Similarly V gene segments in the \( IgH \) locus showed a similar reduction in germline transcription, histone acetylation, and nuclease sensitivity in pre-B cells. Thus the conversion of V gene segments to inaccessible substrates may explain the lack of recombination of \( IgH \) and \( Tcrb \) in pre-B cells and DP thymocytes. Unfortunately,
changes in chromatin structure cannot fully explain feedback inhibition. $V_\beta$14 retains high levels of germline transcripts in DP thymocytes but does not rearrange. In addition, when $V_\beta$10 is brought into close proximity to $E_\beta$ it transcribes at high levels in DP thymocytes without an increase in recombination (Chattopadhyay et al., 1998; Senoo et al., 2003). Additionally, when Jackson et al. introduced $E_\alpha$ into the $Tcrb$ locus they were able to induce high levels of germline transcription, histone acetylation, and restriction enzyme sensitivity in DP thymocytes but no $V_\beta$ to DJ$\beta$ recombination (Jackson et al., 2005). Therefore chromatin accessibility may play a role but cannot fully explain feedback inhibition.

$Igh$ is infrequently associated with pericentromeric heterochromatin in pro-B cells. In pre-B cells after pre-BCR signaling, $Igh$ monoallelically associates with centromeric heterochromatin (Roldan et al., 2005). It was inferred that based upon the close correlation between centromeric recruitment and nonproductive recombination in the proximal Igh domains that the DJ$\alpha$-rearranged or nonfunctionally VDJ$\alpha$ rearranged Igh allele is recruited to the centromere at the onset of pre–B cell development (Roldan et al., 2005; ten Boekel et al., 1997). Therefore repositioning of antigen receptor alleles to pericentromeric heterochromatin may play a role feedback inhibition for $Igh$. Thus, $Igh$ in pre-B cells can maintain an accessible chromatin structure necessary to transcribe the functional allele while preventing further recombination of the locus by positioning the nonfunctional allele in pericentromeric heterochromatin. Finally, developmental
changes in antigen receptor locus conformation may enforce feedback inhibition. As recombination requires that rearranging gene segments be in close proximity, if antigen receptor loci adopt an extended conformation they would prevent recombination while maintaining transcriptional activity.

1.7 Monoallelic Gene Expression

Understanding the mechanisms that drive monoallelic gene expression may provide insights into the mechanism of allelic exclusion. There are three classes of monoallelically expressed genes. Genes of the X chromosome are monoallelically expressed in mammalian female nuclei (Payer and Lee, 2008). Imprinted genes are monoallelically expressed based upon epigenetic imprints founded in the parental germ cells (Reik and Walter, 2001; Sha, 2008). Finally, there is a class of autosomal genes, including odorant receptor genes, antigen receptor loci, interleukin genes, and natural killer cell receptors, and pheromone receptors that are subject to random monoallelic expression (Chess, 2005; Held et al., 1995; Hollander et al., 1998; Rodriguez et al., 1999).

1.7.1 X Chromosome Inactivation

In female nuclei, one X chromosome must be transcriptionally silenced to achieve dosage compensation between XX females and XY males (Lucchesi et al., 2005). In the mouse, the X-inactivation center orchestrates X chromosome inactivation (XCI) through three noncoding genes: \textit{Xist}, \textit{Tsix}, and \textit{Xite}. \textit{Xist} encodes a 17 Kbp noncoding RNA that accumulates on and “coats” the entire inactive X chromosome (Xi) (Borsani et
al., 1991; Brown et al., 1992). Upon initiation of X inactivation, Tsix transcription ceases, Xist transcription increases, and Xist transcripts spread to coat what will become the Xi (Lee et al., 1999). If one chromosome has an inactive Xist, it becomes the active X chromosome (Xa), while if Xist transcription is increased on one chromosome, that allele is most often inactivated (Marahrens et al., 1998; Nesterova et al., 2003; Newall et al., 2001). Conversely, in heterozygous Tsix deficient female mice, the wild type chromosome is preferentially selected as the Xa (Lee and Lu, 1999; Luikenhuis et al., 2001; Sado et al., 2001). Xite harbors intergenic transcription start sites and deletion of Xite downregulates Tsix on that X chromosome and prevents it from becoming the Xa (Ogawa and Lee, 2003). Therefore, the opposing effects of Xist and Tsix suggest that the relative levels of these two transcripts determine which X chromosome will be inactivated (Mlynarczyk and Panning, 2000). Thus, the noncoding RNAs Tsix and Xist are crucial in establishing an inactive X chromosome.

The allelic choice made by during XCI requires interchromosomal interactions between the two X chromosomes. Xu et. al. demonstrated that the X chromosomes paired prior to XCI (Xu et al., 2006). They demonstrated that this X-chromosome pairing occurs transiently at the onset of X inactivation and requires Tsix and Xite. Deleting Xite and Tsix perturbed X-chromosome pairing, while the autosomal insertion of Tsix and Xite induced X-autosome pairing and blocked the initiation of XCI. Tsix and Xite have binding sites for the transcription factor CTCF, and when CTCF expression
was reduced through RNAi knockdown experiments, X chromosome pairing was reduced to background levels. Thus pairing of the X chromosomes allows them to “communicate” with each other ensuring that only one is inactivated. Additionally when ES nuclei were treated with either actinomycin D or α-amanitin, Tsix transcription and X chromosome pairing was abolished. Therefore, X-chromosome pairing requires both the transcription factor CTCF and transcription (Xu et al., 2007). Thus the initiation of XCI is driven by Tsix and Xite transcription and mediated by CTCF dependent interchromosomal interactions.

Xist is believed to act as a scaffold for histone methyltransferases, histone deacetylases, and DNA methyltransferases (Beletskii et al., 2001; Ganesan et al., 2002; Zhao et al., 2008). The Xi has typical heterochromatic modifications compared to the euchromatic modifications of the Xa. There is a lack of H3 and H4 acetylation and an enrichment of both histone H3 dimethylated at lysine 9 (H3K9me2) and H3 trimethylated at lysine 27 (H3K27me3) on the Xi (Boggs et al., 2002; Heard et al., 2001; Jeppesen and Turner, 1993). DNA methylation also appears to play an important role in maintaining gene silencing on the Xi because it is hyper-methylated while the Xa is hypo-methylated (Bartlett et al., 1991; Pfeifer et al., 1990; Wolf et al., 1984). Additionally, treating cells with DNA-demethylating agents results in reactivation of several genes on the Xi (Mohandas et al., 1981). Thus the monoallelical expression of X chromosome
genes is achieved by creating a heterochromatic environment for one allele and a euchromatic environment for the other allele.

### 1.7.2 Autosomal Imprinting

Approximately 84 murine genes are monoallelically expressed based upon the parental origin of the allele. This biased expression of one parental allele over the other is called genomic imprinting (Sha, 2008). Monoallelic expression and silencing of imprinted genes has been shown to depend upon cis elements that are differentially methylated in a parent-of-origin dependent manner. These differentially methylated regions (DMRs) are rich in CpG dinucleotides and repetitive elements (Korf et al., 1998; Walter et al., 2006). The Igf2/H19 locus is a well characterized locus of reciprocally imprinted genes. Igf2 is expressed from the paternal allele and H19 is expressed from the maternal allele (Rachmilewitz et al., 1992). The DMR of the maternal allele is not methylated allowing CTCF to bind the DMR and prevent Igf2 promoter activation by a downstream enhancer. On the paternal allele, methylation of the DMR prevents CTCF binding, allowing the downstream enhancer to activate transcription of Igf2 (Bell and Felsenfeld, 2000; Hark et al., 2000; Kanduri et al., 2000) (Figure 6). The parental specific differential methylation of DMRs is initiated during gametogenesis by the methyltransferases Dnmt3A and Dnmt3L (Bourc’his et al., 2001; Kaneda et al., 2004). Methylation of DMRs is then maintained in somatic cells by methyltransferases such as Dnmt1 (Goll and Bestor, 2005; Howell et al., 2001).
Figure 6: DMR imprint control at the \textit{Igf2/H19} locus.

The differentially methylated region (DMR) controls allele-specific expression at the \textit{Igf2/H19} imprinted cluster (\textit{yellow bar}). When bound by CTCF, the DMR acts as an insulator and blocks activation of the maternal \textit{Igf2} promoter by the downstream enhancer. On the paternal chromosome, methylation at the DMR inhibits CTCF binding, allowing the activation of the paternal \textit{Igf2} promoter by the enhancer. Black lollipops represent methylated cytosine residues. Black arrows indicate direction of transcription. Enh, enhancer (Sha, 2008).

Monoallelic gene expression of imprinted genes is correlated with differential chromatin modifications. The DMRs of \textit{Igf2/H19} are sensitive to DNase I digestion in a parent-of-origin manner (Khosla et al., 1999). Activating histone modifications are found at hypomethylated DMRs on the active allele (i.e., H3 and H4 acetylation) whereas deactivating histone modifications (H3K9Me2 and H3K27Me2) are generally associated with highly methylated DMRs (Lewis and Reik, 2006). Differential histone
modification can also occur outside of DMRS. Allele-specific histone modification at the promoter of \textit{Igf2/H19} locus is thought to help regulate imprinting (Vu et al., 2004).

Similar to XCI, a characteristic feature of some imprinted clusters is the presence of one or more noncoding transcripts. These transcripts are often transcribed antisense to the protein-coding genes in the cluster (Royo et al., 2006). In mouse, the \textit{Air} noncoding RNA is transcribed from the paternal allele in the opposite direction to imprinted \textit{Igf2r} and its transcription has been shown to silence the paternal allele (Lyle et al., 2000; Sleutels et al., 2002). However unlike XCI, imprinted alleles are differentiated in germ cells. Therefore, unlike XCI, imprinting does not require the nucleus to initiate an allelic choice, but rather requires the nucleus to maintain the differential allelic expression. In this regard XCI is similar to the initiation of asynchronous recombination, while imprinting is similar to feedback inhibition.

\textbf{1.7.3 “Random” Monoallelic Gene Expression}

The most diverse group of monoallelically expressed genes are those genes that are “randomly” selected in somatic cells. These include odorant receptor genes, antigen receptor loci, interleukin genes, natural killer cell receptors, and pheromone receptors (Chess, 2005; Held et al., 1995; Hollander et al., 1998; Rodriguez et al., 1999). Moreover, the monoallelic expression of these genes is not absolute. Interleukin-3 (IL-3), IL-4, IL-5, and IL-13 were monoallelically expressed in a subset of CD4$^+$ T cell clones while other clones biallelically expressed these cytokines (Kelly and Locksley, 2000). These data
suggest that the decision to express a given allele is independent of the state of the other allele. Additionally, even though IL-4, IL-5, and IL-13 are clustered together on chromosome 11, in only 81% of IL-4 expressing clones were the other cytokine genes monoallelically expressed from the same chromosome.

There may be at least 1000 autosomal human genes that are subject to random monoallelic expression. Gimelbrant et. al. looked for single nucleotide polymorphisms in human RNA transcripts using a microarray (Gimelbrant et al., 2007). This approach allowed them to generate “transcriptome-derived genotypes” and identify monoallelically expressed genes. Moreover, these patterns of monoallelic gene expression were epigenetically stable. They showed that 5 to 10% of assessed autosomal genes display random monoallelic transcription in human cells (Gimelbrant et al., 2007). The mechanisms establishing random monoallelic gene expression are uncertain.
2. Thesis Prospectus

2.1 Thesis Proposal

At the onset of these studies, we wished to determine the role nuclear localization had on regulating V(D)J recombination of the Tcra and Tcrb loci. Earlier studies had shown that Igh and Igk changed their nuclear position during B-cell development (Goldmit et al., 2005; Roldan et al., 2005; Skok et al., 2001). Our original hypothesis was that Tcra and Tcrb would behave similarly to Igh and Igk. We believed that Tcra and Tcrb would associate with repressive compartments when recombinationally-inactive and reposition to “free” compartments when they recombined. To analyze the nuclear position of Tcra and Tcrb, we initiated a collaboration with Dr. Karen Reddy and Dr. Harinder Singh from the University of Chicago, whose lab had published the first three-dimensional fluorescent in-situ hybridization (3D FISH) studies of Igh and Igk (Kosak et al., 2002). In our collaboration, we prepared the slides, Dr. Reddy imaged the slides, and we analyzed the data. As our studies progressed, Skok et al. published their observations of the nuclear localization and locus conformation of Tcrb (Skok et al., 2007). Based upon their data they suggested that nuclear position of Tcrb was directed and that strict monoallelic associations with pericentromeric heterochromatin influenced allelic exclusion. Our initial results suggested that Tcra was rarely associated with repressive nuclear compartments and that Tcrb was frequently associated with these compartments. These observations led
us to design experiments that would determine if $Tcrb$ associations with repressive nuclear compartments 1) were stochastic, 2) occurred prior to $\beta$-selection, 3) influenced recombination, 4) could be disrupted by the introduction of an ectopic enhancer.

We were also interested in the role of locus conformation had on recombination of $Tcrb$. Earlier studies in B-cells suggested that recombination required a contracted $Igh$ locus and further studies of $Tcrb$ supported this conclusion (Kosak et al., 2002; Sayegh et al., 2005; Skok et al., 2007). Additionally, Jackson et al. suggested that decontraction played a role in maintaining feedback inhibition of $Tcrb$ in DP thymocytes (Jackson et al., 2005). Based upon this evidence we hypothesized that associations with repressive nuclear compartments influenced $Tcrb$ recombination by maintaining or inducing an extending conformation. We also hypothesized that EαKI mice which have accessible $V_\beta$ gene segments in DP thymocytes, enforce feedback inhibition by maintaining an extended locus conformation.

2.2 Specific Aim 1: To determine the role of nuclear positioning in regulating recombination of $Tcrb$.

By 3D FISH of pro-B cells, DN thymocytes, and DP thymocytes, we analyzed the nuclear compartmentalization of $Tcra$ and $Tcrb$. Specifically, we analyzed the developmental associations of these loci with pericentromeric heterochromatin and with the nuclear lamina. We wished to determine whether $Tcrb$ association with the pericentromeric heterochromatin or the nuclear lamina influenced recombination and if
these associations occurred prior to β-selection. This allowed us to distinguish whether these associations influence asynchronous recombination or enforce feedback inhibition. Additionally, by analyzing the distribution alleles on a per nucleus basis, we could infer whether associations with repressive nuclear compartments were stochastic or directed. We also analyzed the nuclear compartmentalization of Tcrb alleles carrying an ectopic enhancer. These EαKI alleles allowed us to determine whether an ectopic enhancer can perturb nuclear compartmentalization in DN thymocytes and whether suppressing these associations can break allelic exclusion. Finally, we observed the average distance between the two Tcrb alleles in DN thymocytes. This allowed us to determine if direct communication between the Tcrb alleles is required for allelic exclusion.

2.3 Specific Aim 2: To determine the role of locus conformation in regulating recombination of Tcrb.

Using 3D FISH, we analyzed the conformation of the Tcrb locus by measuring the distance between the 5’ and 3’ end of the locus. First, we replicated earlier studies, demonstrating that Tcrb is contracted in DN thymocytes and extended in DP thymocytes. Second, we determined whether Tcrb adopts an extended conformation when associated with repressive nuclear compartments. Finally, we analyzed locus conformation in EαKI mice, to determine if Eα can contract Tcrb in DP thymocytes.
Thus we could determine whether an extended Tcrb conformation could explain the maintenance of feedback inhibition for accessible Vβ gene segments on EαKI alleles.
3. Materials and Methods

3.1 Mice

129 and C57Bl/6 wild-type mouse strains were purchased from Jackson Labs. \( \text{Rag}^{2/-} \) mice (Shinkai et al., 1992), \( \text{Lat}^{2/-} \) mice (Zhang et al., 1999), \( \text{Ea}^{-/-}\text{Rag}^{2/-} \times \text{Tcrb} \) transgenic mice (Sleckman et al., 1997), \( \text{EaKI} \) mice (Jackson et al., 2005), and \( \text{EaKI} \text{Rag}^{2/-} \) mice (Jackson et al., 2005) were described previously. DP thymocytes from \( \text{Rag}^{2/-} \) mice without a \( \text{Tcrb} \) transgene were harvested from mice ten days after they were injected with 300 \( \mu \)g of anti-CD3 antibodies (2C11). All mice were used in accordance with protocols approved by the Duke University and University of Chicago Institutional Animal Care and Use Committees.

3.2 Cell isolation

\( \text{Rag}^{2/-} \) pro-B cells were isolated and cultured as described previously (Smithson et al., 1995). DP thymocytes were sorted to 98 % (wild-type) or 95% (EaKI) purity as described (Jackson and Krangel, 2006a).

3.3 FISH probes and antibodies

BAC clones RP23-75P5 (\( \text{C}_{\beta^{-}} \text{-distal end of the Tcrb locus} \)), RP23-203H5 (\( \text{C}_{\beta^{-}} \text{-proximal region of the Tcrb locus} \)), RP23-304L21 (\( \text{C}_{\alpha^{-}} \text{-distal end of the Tcra locus} \)), and RP23-97O1 (\( \text{Actb} \) locus) were used to prepare locus-specific DNA probes. The BAC clones were grown shaking at room temperature (RT) in LB media with 12.5 ug/ml chloramphenicol. Typically, 100 mls of LB media was inoculated with 100 ul of 3ml LB
culture grown from a single bacterial colony. The bacteria were pelleted and resuspended in 10mls of 10mM Tris, 50 mM EDTA. The cells were lysed for 5 mins at RT in 10mls of 200mM NaOH and 1% SDS (w/v). The reaction was stopped by adding 10ml of 3.0 M potassium acetate (pH=5.5) and incubated on ice for 30 min. The resulting solution was spun at 5,000 rpm for 30 min. The supernatant was passed through a nylon mesh and then extracted once with phenol, twice phenol:chloroform (1:1), and once with chloroform. The DNA was precipitated by adding 0.7X isopropanol, freezing at -80°C for 30 min, and spinning at 8,000 rpm for 30 min. The pellet was washed once with 70% EtOH and then resuspended in 10mM Tris. The BAC clones were labeled using a Biotin or Digoxigenin (DIG) nick-translation kit (Roche). Foci were then detected using either FITC-conjugated streptavidin (Jackson Immunoresearch Laboratories) to detect biotin-incorporated probes or Cy3- or Cy5-labeled anti-DIG antibody (Jackson Immunoresearch Laboratories) to detect DIG-incorporated probes. The probe used to detect peri-centromeric heterochromatin was composed of eight tandem copies of the major γ-satellite repeat sequence and was labeled by direct incorporation of dUTP-alexa488 or dUTP-alexa568 (Skok et al., 2001). The nuclear lamina was detected using a polyclonal goat anti-laminB1 (sc-6217, Santa Cruz Biotech) antibody followed by Cy5-labeled anti-goat (Jackson Immunoresearch Laboratories).
3.4 3D DNA Immuno-FISH

Methods for cell fixation and FISH that preserve three-dimensional structure were derived from Solovei et al. (Solovei et al., 2002). One million thymocytes in 300 µl PBS were attached to poly-L-lysine slides for 30 min at 4°C (each slide representing an independent cell isolation). Cells were then fixed in 4% paraformaldehyde, permeabilized with 0.5% saponin and 0.5% Triton-X, treated with 0.1N HCl, and freeze-thawed 4 to 5 times in 20% glycerol. Slides were stored at this point for up to 1 month at −80°C. Slides were denatured by submerging in 70% formamide 2X SSC at 76°C for 3 mins, followed by 1 min treatment in 50% formamide 2X SSC. Conjugated probes were precipitated in ethanol with blocking DNA (consisting of mouse C<sup>ot</sup> DNA, human placental DNA, and salmon sperm DNA), resuspended in 50% deionized formamide containing 2X SSC and 10% Dextran sulfate, boiled for 5 min, and pre-annealed at 37°C for at least 1 hour prior to slide hybridization. Probes were then added to the slide for hybridization under a rubber cement sealed coverslip in a humid chamber for 48-96h at 37°C. Following hybridization, the slides were washed 3 times in 50% formamide 2X SSC at 42°C followed by 3 washes in 0.2X SSC at 63°C. The slides were blocked with 4% BSA, 2X SSC and then incubated with the appropriate antibodies in 4% BSA, 2X SSC. Excess antibody was removed by washing 3 times in 0.1% Triton-X in 2X SSC. Secondary antibodies, if required, were incubated and washed in the same manner. Slides were then mounted in VectaShield (Vector Laboratories).
3.5 Confocal imaging

The slides were imaged on an Olympus confocal microscope or a Leica confocal microscope. Nuclei were magnified with an oil immersion 100X objective lens and a 2X digital zoom. Images from the confocal microscope were saved as stacks of TIFFs and processed with ImageJ software (Bertolino et al., 2005). The images were passed through a Kalman stack filter and then segmented such that foci of gene loci were of uniform size, that the nuclear lamina was the thinnest contiguous circle possible, and that centromeric heterochromatin regions had distinct edges. (Figure 7)

![Unsegmented image](image1.png) ![Segmented image](image2.png)

**Figure 7:** Example of image segmentation performed prior to scoring colocalization of alleles with γ-satellite or lamin B1.

Gene foci were then considered to be colocalized with γ-satellite or laminB1 if at least two adjacent pixels between the two signals were positive in both channels. A Tcrb allele was considered unrearranged if the distal and proximal foci overlapped or were within 2 pixels of each other and was considered rearranged if the distal focus was at least 5 pixels away from the nearest proximal focus. Foci separated by a distance of 2
to 5 pixels were considered non-informative and were dropped from the data set. Only nuclei with two detectable alleles were evaluated.

In pairing and conformational analysis, the distance between the centers of two foci was measured. The conformation was determined by measuring the distance between a Tcrb distal and proximal probe, while pairing analysis measured the distance between the two alleles. These calculations weighted the intensity of each pixel in probe focus based upon its intensity. Thus weighted center of the focus tended to be near the brightest pixels.

3.5 **Statistical Analysis**

Statistical differences between populations were determined by using Fisher’s exact two-tailed contingency table.
4. Characterization of Tcra and Tcrb nuclear localization and conformation

4.1 Introduction

An important but poorly understood aspect of V(D)J recombination is the phenomenon of allelic exclusion, which limits some antigen receptor loci to produce a functional protein from only a single allele (Jackson and Krangel, 2006b; Jung et al., 2006; Khor and Sleckman, 2002; Mostoslavsky et al., 2004). In developing αβ T lymphocytes, Tcrb gene rearrangement is subject to allelic exclusion, whereas Tcra gene rearrangement is not. Tcrb gene rearrangement occurs in two steps, first Dβ-to-Jβ, and then Vβ-to-DβJβ (Jackson and Krangel, 2006b; Khor and Sleckman, 2002). Tcrb allelic exclusion is manifested at the Vβ-to-DβJβ step, and is thought to be mediated in two phases (Jackson and Krangel, 2006b; Khor and Sleckman, 2002). First, it is highly unlikely that both Tcrb alleles will undergo Vβ-to-DβJβ recombination simultaneously. Second, productive recombination on one allele is thought to provoke a feedback signal that suppresses further Vβ-to-DβJβ recombination on the other allele. These two constraints permit Vβ-to-DβJβ recombination on the second allele only if nonproductive recombination occurs on the first allele.

Feedback inhibition of Tcrb gene recombination is initiated by pre-TCR signaling and is linked to DN to DP differentiation (Jackson and Krangel, 2006b; Khor and Sleckman, 2002; Michie and Zuniga-Pflucker, 2002). Previous studies have
indicated that suppression of further Tcrb gene recombination in DP thymocytes is associated with changes in Vβ chromatin structure that limit access of the recombinase to chromosomal recombination signal sequences, as well as with a locus conformational change (decontraction) that increases the physical distance between Vβ and DβJβ segments (Agata et al., 2007; Jackson et al., 2005; Skok et al., 2007; Tripathi et al., 2002).

The initiation of Tcrb allelic exclusion is less well understood. Two types of mechanisms have been invoked to explain asynchronous recombination of Tcrb alleles in DN thymocytes (Jackson and Krangel, 2006b; Jung et al., 2006; Khor and Sleckman, 2002; Mostoslavsky et al., 2004). In deterministic models, the two Tcrb alleles in each cell would represent nonequivalent substrates for the recombinase and this initial allelic choice would dictate the order of subsequent recombination events. In this regard, it was suggested, based on replication timing, that the two immunoglobulin Igk alleles are differentially marked and the early replicating allele undergoes DNA demethylation in pre-B cells and is predisposed to rearrange first (Mostoslavsky et al., 1998; Mostoslavsky et al., 2001). By contrast, stochastic models invoke mechanisms that reduce the efficiency of recombination on both alleles and thereby diminish the likelihood that the two alleles initiate recombination simultaneously (Schlissel, 2002). It was suggested that limiting regulators randomly and infrequently activate Igk alleles in pre-B cells, thereby contributing to Igk allelic exclusion. In addition, inefficient Vβ and VH recombination signal sequences were suggested to be rate limiting for recombination.
and to contribute to Tcrb and Igh allelic exclusion, respectively (Liang et al., 2002).

Whether asynchronous recombination of Tcrb alleles is established through a deterministic or a stochastic mechanism remains an important but unresolved question.

The association of antigen receptor loci with two distinct nuclear compartments has been suggested to regulate recombination. One such compartment is the nuclear periphery. This compartment is comprised of two sub-domains, the inner nuclear membrane-nuclear lamina and the nuclear pore complexes, that appear to divergently regulate gene activity (Brown and Silver, 2006). Positioning of genes at nuclear pore complexes is thought to promote gene activity, whereas association of genes at the inner nuclear membrane-nuclear lamina is considered to repress their activity (Kosak and Groudine, 2004; Misteli, 2007; Pickersgill et al., 2006; Schneider and Grosschedl, 2007).

Several genes have been documented to reposition away from the nuclear periphery in conjunction with their activation (Hewitt et al., 2004; Williams et al., 2006; Zink et al., 2004). Moreover, inducible tethering of active genes to the mammalian inner nuclear membrane was shown to inhibit their transcription (Reddy et al., 2008). Igh and Igk alleles were found to preferentially localize to the nuclear periphery in hematopoietic progenitors and T lineage cells. However, these loci reposition away from the nuclear periphery in pro-B cells, suggesting that repositioning is an important step in their activation (Fuxa et al., 2004; Kosak et al., 2002; Reddy et al., 2008; Yang et al., 2005). A second such compartment is peri-centromeric heterochromatin (Fisher and
Merkenschlager, 2002; Kosak and Groudine, 2004; Su et al., 2005). The silencing of developmentally regulated genes is often associated with their juxtaposition in trans to foci of peri-centromeric heterochromatin (Brown et al., 1999; Brown et al., 1997). Association of Igλ and Igκ alleles with peri-centromeric heterochromatin has been linked to allelic exclusion. Igλ alleles display minimal association with peri-centromeric foci at the pro-B cell stage, during which Igλ recombination occurs. However, the nonrearranged Igλ allele is recruited to peri-centromeric foci in pre-B cells, implicating this relocalization in feedback inhibition of further Igλ recombination (Roldan et al., 2005; Skok et al., 2001). In addition, the recruitment of one (late replicating) Igκ allele to peri-centromeric foci in pre-B cells is thought to bias initial Igκ recombination in these cells to the nonassociated allele (Goldmit et al., 2005).

A recent study provided evidence for predominantly monoallelic association of Tcrβ alleles with peri-centromeric heterochromatin throughout T cell development (Skok et al., 2007). Analysis of DP thymocytes indicated that the allele associated with peri-centromeric heterochromatin in a given thymocyte was decontracted, suggesting that it had not undergone rearrangement. Based on these observations the authors inferred that recruitment of one Tcrβ allele to peri-centromeric heterochromatin in DN thymocytes may inhibit its recombination, thus biasing the other allele to rearrange first. However, the recombination status of the two alleles was not directly determined but inferred from their states of contraction. Moreover, the relationship between
subnuclear localization and contraction was only addressed in thymocytes that already had received a signal for feedback inhibition. Thus, whether association of Tcrb alleles with peri-centromeric heterochromatin was relevant for asynchronous recombination in DN thymocytes or for feedback inhibition of recombination in DP thymocytes could not be resolved.

To address the aforementioned unresolved issues, here we examined the relationship between subnuclear localization and recombination of Tcrb alleles. We found that Tcrb alleles associated at high frequency with either the nuclear lamina or peri-centromeric heterochromatin. However, in contrast with previous work, we found these associations to be stochastic rather than directed, and not strictly monoallelic. By analyzing DN thymocytes deficient in feedback signaling, we established a direct role for subnuclear localization in the asynchronous rearrangement of Tcrb alleles. Moreover, we obtained evidence that reduced interaction of Tcrb alleles with the nuclear lamina or peri-centromeric heterochromatin is associated with a loss of allelic exclusion. We propose a stochastic rather than a deterministic model by which subnuclear localization contributes to the initiation of Tcrb allelic exclusion.

4.2 Results

4.2.1 Tcrb alleles frequently associate with repressive compartments

We studied association of Tcrb alleles with peri-centromeric heterochromatin and the nuclear lamina in nuclei of developing thymocytes. Locus behavior prior to β-
selection was assessed in DN thymocytes obtained from $\text{Rag2}^{-/-}$ mice, whereas locus behavior subsequent to $\beta$-selection was assessed in DP thymocytes of wild-type mice. As a control, we analyzed locus behavior in $\text{Rag2}^{-/-}$ pro-B cells. We identified the nuclear location of $\text{Tcrb}$ alleles independent of their rearrangement status by probing with BAC clone RP23-75P5, which identifies the $C_\beta$-distal end of the locus (Figure 8a,b). Peri-centromeric heterochromatin was identified using a probe that recognizes $\gamma$-satellite repeats, whereas immunostaining with an antibody specific for LaminB1 was used to identify the nuclear lamina (Figure 8b). We found that $\text{Tcrb}$ alleles frequently colocalized with $\gamma$-satellite repeats (47.2% of alleles) and laminB1 (54.5% of alleles) in pro-B cells (Figure 8b,c). $\text{Tcrb}$ alleles associated with peri-centromeric heterochromatin in DN thymocytes at a reduced frequency (37.1% of alleles) as compared to pro-B cells, but their association with the nuclear lamina (66.4% of alleles) was substantially higher than in pro-B cells. DP thymocytes displayed associations with peri-centromeric heterochromatin (48.4%) and the nuclear lamina (46.6%) that were similar to those in pro-B cells.
Figure 8: Subnuclear localization of Tcrb alleles in Rag2^{−/−} pro-B cells, Rag2^{−/−} DN thymocytes, and sorted wild-type DP thymocytes.

(a) Tcrb locus, including the relative positions of the BAC clones used to visualize its subnuclear localization. (b) 3D Immuno-FISH, showing both Tcrb alleles in representative nuclei of each of three cell types. Nuclei were hybridized with BAC RP23-75P5 to identify the distal end of the Tcrb locus (green) and a plasmid containing γ-satellite repeats to identify peri-centromeric heterochromatin (red), and were stained with an antibody specific for laminB1 to identify the nuclear lamina (blue). (c) Colocalization of Tcrb alleles with either γ-satellite repeats or laminB1. γ-satellite data, 604 pro-B, 432 DN and 862 DP alleles (two to three slides each); laminB1 data, 578 pro-B, 232 DN and 972 DP alleles (two to three slides each). *, P < 0.05.

For comparison, we analyzed subnuclear positioning of the constitutively active gene Actb (Figure 9). Only 10% of Actb alleles co-localized with γ-satellite repeats and 20% with LaminB1 in all cell types examined. Thus this highly transcribed gene only infrequently associates with peri-centromeric heterochromatin and the nuclear lamina.
Figure 9: Subnuclear localization of Actb alleles in Rag2−/−-pro-B cells, Rag2−/−DN thymocytes, and sorted wild-type DP thymocytes.

(a) 3D Immuno-FISH, showing both Actb alleles in representative nuclei of the three cell types. Actb (97O1, green), peri-centromeric heterochromatin (γ-satellite, red, upper panels), nuclear lamina (laminB1, blue, lower panels). (b) Colocalization of Actb alleles with either γ-satellite repeats or laminB1. Data are from 246 pro-B, 232 DN and 162 pre-B nuclei (one slide each).
We also analyzed subnuclear positioning of Tcra alleles (Figure 10a). Like Tcrb alleles, Tcra alleles frequently colocalized with γ-satellite (31.5%) or laminB1 (64.6%) in pro-B cells (Figure 10b,c).

Figure 10: Subnuclear localization of Tcra alleles in Rag2−/− pro-B cells, Rag2−/−

(a) Tcra locus, including the position of the BAC clone used to visualize its subnuclear localization. (b) 3D Immuno-FISH, showing Tcra alleles in representative nuclei of each of the three cell types. Nuclei were hybridized with BAC RP23-304L21 to identify the Tcra locus (green) and a plasmid containing γ-satellite repeats to identify peri-centromeric heterochromatin (red), and were stained with an antibody specific for laminB1 to identify the nuclear lamina (blue). (c) Colocalization of Tcra alleles with either γ-satellite repeats or laminB1. γ-satellite data, 302 pro-B, 52 DN and 178 DP alleles (one slide each); laminB1 data, 302 pro-B, 52 DN and 178 DP alleles (one slide each). *, P < 0.05.

In contrast to Tcrb alleles, Tcra alleles only infrequently colocalized with γ-satellite or laminB1 in DN (9.6% and 19.2% respectively) and DP (11.8% and 28.1% respectively) thymocytes. The Tcra locus is active in both DN and DP thymocytes due to
the activation of Tcrd gene segments in DN thymocytes and Tcra gene segments in DP thymocytes (Krangel et al., 2004). Thus, the Tcra locus is associated with peri-centromeric heterochromatin or the nuclear lamina when inactive (in pro-B cells) and dissociates from these compartments when active (in DN and DP thymocytes).

The nuclear compartments defined by γ-satellite hybridization and laminB1 staining partially overlap, as γ-satellite repeats were found both centrally in the nucleus and at the nuclear periphery. Colocalization of Tcrb alleles with γ-satellite, laminB1 or both was extremely high (77.8% in pro-B cells, 77.2% in DN thymocytes, and 72.6% in DP thymocytes) (Figure 11). By contrast, although 74.5% of Tcra alleles were associated with these compartments in pro-B cells, only 25.0% and 34.8% were associated in DN and DP thymocytes, respectively. Tcra association rates in DN and DP thymocytes were similar to those of Actb, which associated only 25% of the time in all cell types (Figure 11). It is striking that Tcrb alleles so frequently associate with peri-centromeric heterochromatin or the nuclear lamina in DN and DP thymocytes even though the Tcrb locus is expressed at both developmental stages.
Figure 11: Colocalization of Tcrb, Tcra, and Actb alleles with γ-satellite or laminB1.

Alleles colocalizing with γ-satellite alone, laminB1 alone, or simultaneously with γ-satellite and laminB1 were summed. Tcrb data, 424 pro-B, 232 DN and 390 DP alleles (one slide each); Tcra data, 302 pro-B, 52 DN and 178 DP alleles (one slide each); Actb data, 246 pro-B, 232 DN and 162 DP alleles (one slide each). *, P < 0.05.

4.2.1 Tcrb alleles independently associate with repressive compartments

Skok et al. reported monoallelic Tcrb association with peri-centromeric heterochromatin in about 75% of DN and DP thymocyte nuclei (Skok et al., 2007). Their data imply that the two alleles in any nucleus are intrinsically different and have distinct probabilities to associate with peri-centromeric heterochromatin. This difference might then bias Tcrb gene recombination to one allele and contribute to the initiation of allelic exclusion. However, our data do not support strict monoallelic association of the Tcrb gene with peri-centromeric heterochromatin or the nuclear lamina. In DN thymocytes, 37.1% of Tcrb alleles colocalized with γ-satellite (Figure 8c).

If the two Tcrb alleles in DN thymocytes were equivalent and associated independently
with peri-centromeric heterochromatin, 13.7% of DN nuclei should have two associated alleles (37.1% × 37.1%), 46.7% should have one associated allele (2 × 37.1% × 62.9%) and 39.6% should have neither allele associated (62.9% × 62.9%) (**Table 1**). This is very close to the observed allelic distribution in DN thymocytes (12.9%, 48.3%, and 38.8%, respectively) (**Table 1, Figure 12a**). Likewise, the observed distribution of DN thymocyte nuclei having both, one, or no alleles associated with the nuclear lamina was predicted almost exactly from calculations based on an overall association frequency of 66.4% (**Figure 8c, Table 1, and Figure 12b**). *Tcrb* alleles behaved similarly in recombinase-sufficient DN thymocytes (**Table 2**), and independently segregated to both nuclear compartments in nuclei of all cell types examined (**Table 1**). Similar observations were made for the *Actb* and *Tcra* genes in all three cell types (**Tables 3, 4**).
Figure 12: Distribution of nuclei with zero, one, or two Tcrb alleles colocalized with γ-satellite or laminB1.

(a) Colocalization with γ-satellite, in 289 pro-B, 116 DN and 486 DP nuclei. (b) Colocalization with laminB1, in 302 pro-B, 116 DN and 431 DP nuclei. (c) Colocalization with either γ-satellite or laminB1, in 289 pro-B, 116 DN and 431 DP nuclei. Data were accumulated from two to three independently prepared slides per cell type.
Table 1: Experimental and Theoretical Distribution

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<th>Nuclear compartment</th>
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<th>Number of nuclei</th>
<th>Allelic association rate (%)</th>
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Table 2: Independent segregation of Tcrb alleles with laminB1 or γ-satellite in recombinase-sufficient DN thymocytes.

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<th>Cell Type</th>
<th>Nuclear compartment</th>
<th>Number of nuclei</th>
<th>Allelic association rate (%)</th>
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<td>γ-satellite</td>
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Table 3: Independent segregation of Actb alleles with laminB1 or γ-satellite

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<th>Number of nuclei</th>
<th>Allelic association rate (%)</th>
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<td>5.7</td>
<td>82.3</td>
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Table 4: Independent segregation of Tcra alleles with laminB1 or γ-satellite

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<th>Cell type</th>
<th>Number of nuclei</th>
<th>Allelic association rate (%)</th>
<th>Neither allele</th>
<th>One allele</th>
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<th>Both alleles</th>
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<td></td>
<td>p(1-p)^2</td>
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<td>laminB1</td>
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<td>77.8</td>
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Notably, Immuno-FISH analysis with probes for the Tcrb locus, γ-satellite repeats and laminB1 revealed that only 5.3% of DN thymocyte nuclei had two Tcrb alleles “free” of both compartments (Figure 12c). In contrast, 35.5% had one allele colocalized with either γ-satellite or laminB1 and 59.5% had both alleles colocalized with either γ-satellite or laminB1. These experimentally determined values match closely the values calculated from rates of association with the individual compartments (Table 1).

We conclude that although the Tcrb locus is monoallelically associated with peri-centromeric heterochromatin and with the nuclear lamina in subsets of thymocyte
nuclei, these subsets arise stochastically as a result of two indistinguishable alleles independently associating with these nuclear compartments. Thus, there is no biological mechanism directing monoallelic association. We further conclude that fully 95% of DN thymocytes have at least one \( Tcrb \) allele associated with peri-centromeric heterochromatin or the nuclear lamina.

### 4.2.3 Biased localization of unrearranged \( Tcrb \) alleles

Given that \( Tcrb \) alleles associate at high frequency with pericentromeric heterochromatin and the nuclear lamina in DN and DP thymocytes, we hypothesized that these associations may inhibit \( V_\beta \)-to-\( D_\beta J_\beta \) recombination and promote either the initiation or the maintenance of \( Tcrb \) allelic exclusion. To address this hypothesis, we first asked whether rearranged and unrearranged \( Tcrb \) alleles were differentially associated with pericentromeric heterochromatin or the nuclear lamina in DP thymocytes. Unrearranged \( Tcrb \) alleles were identified by their hybridization with both the \( C_\beta \)-distal \( Tcrb \) probe (RP23-75P5) and a \( C_\beta \)-proximal probe (RP23-203H5) that identifies the region situated between \( V_\beta \) and \( D_\beta \) gene segments. Rearranged \( Tcrb \) alleles were identified by their hybridization to only the distal probe (RP23-75P5), as the region detected by the proximal probe (RP23-203H5) is deleted by \( V_\beta \)-to-\( D_\beta J_\beta \) recombination (Figure 8a).

We found that unrearranged \( Tcrb \) alleles more frequently colocalized with \( \gamma \)-satellite (66.9%) as compared to rearranged alleles (44.8%) (Figure 13a,b). Similarly,
unrearranged Tcrb alleles more frequently colocalized with laminB1 (57.0%) as compared to rearranged alleles (41.3%). Of note, this analysis includes data from two non-informative subsets of nuclei that may cause the relationship between localization and recombination to be underestimated. In one subset, both alleles are rearranged; in the other, both alleles are associated with a repressive compartment. For a more informative analysis, we focused on nuclei that had rearranged only one allele and in which the Tcrb gene monoallelically colocalized with either γ-satellite or laminB1. In this subset of nuclei the unrearranged allele colocalized with γ-satellite 70.5% of the time, whereas the rearranged allele colocalized only 29.5% of the time (Figure 13c). Similarly the unrearranged allele colocalized with laminB1 64.3% of the time and the rearranged allele colocalized 35.7% of the time. The strong bias for the unassociated allele to be the rearranged allele suggests that association with pericentromeric heterochromatin and association with the nuclear lamina may each inhibit V_{βr}-to-D_{βJ_{β}} recombination.
Figure 13: Subnuclear localization of rearranged and unrearranged Tcrb alleles.

(a) 3D Immuno-FISH, showing Tcrb alleles in DP nuclei using distal (75P5, green) and proximal (203H5, red) probes. Peri-centromeric heterochromatin (γ-satellite, blue, upper panels). Nuclear lamina (laminB1, blue, lower panels). (b) Colocalization of rearranged and unrearranged alleles with γ-satellite or laminB1 in DP nuclei. γ-satellite, 145 rearranged and 327 unrearranged alleles (one slide); laminB1, 179 rearranged and 403 unrearranged alleles (two slides). (c) Colocalization of rearranged and unrearranged alleles with γ-satellite or laminB1 in the subset of DP cells with nuclei having monoallelic Tcrb locus association and one rearranged Tcrb allele. γ-satellite, 145 nuclei (one slide); laminB1, 179 nuclei (two slides). (d) 3D Immuno-FISH, showing Tcrb alleles in nuclei of Lat−/− thymocytes. Probe strategy was identical to (a). (e) Colocalization of rearranged and unrearranged alleles with γ-satellite or laminB1 in the subset of Lat−/− cells with nuclei having monoallelic Tcrb locus association and one rearranged allele. γ-satellite, 161 nuclei (one slide); laminB1, 54 nuclei (one slide). (f) Colocalization of Tcrb alleles with γ-satellite or laminB1 in Rag2−/− DN nuclei and in Lat−/− DN nuclei containing two rearranged alleles. γ-satellite, 232 Rag2−/− and 36 Lat−/− alleles (two and one slides, respectively); laminB1, 232 Rag2−/− and 220 Lat−/− alleles (two slides each). *, P < 0.05.
4.2.4 Subnuclear localization biases initial Tcrb recombination

Data from DP thymocytes cannot distinguish whether a relationship between rearrangement status and subnuclear location reflects a post–β-selection sorting of alleles linked to feedback inhibition or a pre–β-selection relationship. The former possibility suggests that unrearranged alleles are moved to pericentromeric heterochromatin or the nuclear periphery as part of feedback inhibition of recombination in DP thymocytes. The latter suggests that association with these compartments influences which allele initially undergoes recombination in DN thymocytes. To distinguish these possibilities, we examined the relationship between rearrangement and subnuclear positioning in DN thymocytes that had yet to receive an allelic exclusion signal.

Thymocytes from Lat-deficient mice cannot signal through their pre-TCR and thus do not generate a feedback signal and do not differentiate to the DP stage (Jackson and Krangel, 2006a; Zhang et al., 1999). We identified rearranged and unrearranged Tcrb alleles in DN thymocytes of Lat−/− mice using the distal and proximal Tcrb probes (Figure 13d), as described above. We also identified Tcrb excision circles that selectively hybridized with the proximal probe (Figure 13d, lower right panel). Since Lat−/− DN thymocytes do not receive a feedback signal, it was possible that they all contain two rearranged Tcrb alleles. However, this was not observed. 40.5% of all Lat−/− nuclei had both alleles unrearranged, 24.8% of the nuclei had one rearranged allele, and 34.6% of
the nuclei had both alleles rearranged (Figure 14). Thymocytes with two rearranged alleles may not accumulate in these mice because the lifespan of a DN thymocyte may be limited and late stage DN thymocytes may die by apoptosis.

Figure 14: Rearrangement frequency in Lat−/− DN thymocytes.

Distribution of Lat−/− DN nuclei with neither Tcrb allele rearranged, one Tcrb allele rearranged, or both Tcrb alleles rearranged. The rearrangement status of the alleles was determined by 3D immuno-FISH. 370 Lat−/− nuclei (three slides).

In the informative subsets of Lat-deficient nuclei that contained only one rearranged allele and one allele colocalized with either γ-satellite or laminB1, we noted a strong bias for the rearranged allele to be the “free” allele (Figure 13d,e). Rearranged and unrearranged Tcrb alleles colocalized with γ-satellite in 34.8% and 65.2% of nuclei, respectively, and with laminB1 in 23.5% and 76.5% of nuclei, respectively. We conclude
that association with peri-centromeric heterochromatin or the nuclear lamina reduces the efficiency of recombination, such that in DN thymocytes with one associated and one non-associated allele, the non-associated allele is more likely to rearrange first.

An alternative interpretation of these data is that recombination reduces the probability that an allele remains associated with peri-centromeric heterochromatin or the nuclear lamina. To address this possibility we compared the positioning of unrearranged Tcβ alleles in Rag2−/− DN thymocyte nuclei to that of rearranged Tcβ alleles in the subset of Lat−/− DN thymocyte nuclei containing two rearranged alleles (Figure 13f). Notably, we detected no significant difference in colocalization of Tcβ alleles with γ-satellite repeats or laminB1 in the two populations. Thus recombination does not influence the association of Tcβ alleles with peri-centromeric heterochromatin or the nuclear lamina.

The two probe strategy for analysis of Tcβ alleles also allowed evaluation of the orientation of unrearranged Tcβ alleles relative to peri-centromeric heterochromatin and the nuclear lamina (Figure 13a,d). For those instances in which a clear orientation could be discerned, the Cβ-distal portion of the Tcβ locus was usually closest to peri-centromeric heterochromatin or the nuclear lamina (Figure 15). This orientation might provide the basis for preferential inhibition of Vβ-to-DβJβ recombination. Previous studies had similarly shown that peripheral Igh loci are oriented with V gene segments
nearer the nuclear periphery than C gene segments (Fuxa et al., 2004; Kosak et al., 2002; Reddy et al., 2008; Yang et al., 2005).

**Figure 15:** *Tcrb* locus orientation relative to pericentromeric heterochromatin and the nuclear lamina.

Colocalization of the distal (75P5) only, proximal (203H5) only or both probes with γ-satellite or laminB1 is indicated. γ-satellite data were from 54 nuclei (one slide); laminB1 data were from 53 nuclei (one slide).

4.2.5 Deletion of Eα does not affect *Tcra* nuclear localization.

Studies of the β-globin locus have shown that subnuclear positioning can be influenced by enhancers and locus control regions (Francastel et al., 1999; Ragoczy et al., 2006; Ragoczy et al., 2003). We asked whether TCR locus enhancers function similarly by comparing *Tcra* locus positioning in DP thymocytes of *Tcra* enhancer-deleted (Eα−/−) and wild-type mice. Surprisingly, we detected no effect of Eα-deletion on positioning of the *Tcra* locus relative to peri-centromeric heterochromatin or the nuclear lamina (Figure 16). Thus, either Eα plays no role in subnuclear positioning of the *Tcra* locus, or it is functionally redundant with other *Tcra* locus regulatory elements in this regard.
Figure 16: Deletion of Eα does not affect Tcra nuclear localization.

(a) 3D Immuno-FISH, showing Tcra alleles in nuclei of Eα<sup>−/−</sup> Rag2<sup>−/−</sup> Tcrb transgenic thymocytes. Tcra (304L21, green), peri-centromeric heterochromatin (γ-satellite, red), nuclear lamina (laminB1, blue). (b) Tcra colocalization with γ-satellite or laminB1 in wild-type DP and Eα<sup>−/−</sup> Rag2<sup>−/−</sup> x Tcrb transgenic thymocytes. WT DP, 178 alleles; Eα<sup>−/−</sup> Rag2<sup>−/−</sup> Tcrb tg DP, 210 alleles (one slide each).
4.2.6 An ectopic enhancer disrupts Tcrb localization and perturbs allelic exclusion.

To determine if Eα can alter the nuclear positioning of a genetic locus, we analyzed DP thymocytes of mice carrying an ectopic insertion of Eα into the Vβ portion of the Tcrb locus (EαKI mice) (Jackson et al., 2005). Because some but not all Vβ-to-DβJβ recombination events would delete the inserted Eα, we focused specifically on unrearranged alleles in EαKI DP thymocytes. We found that unrearranged EαKI alleles colocalized with γ-satellite at a frequency that was reduced as compared to unrearranged wild-type alleles (39.5% vs. 66.9%) (Figure 17). We also detected a reduction in colocalization of unrearranged EαKI alleles with laminB1 as compared to unrearranged wild-type alleles (47.9% vs. 57.0%), although this difference fell just short of achieving statistical significance (Figure 17b). Therefore Eα can inhibit associations with peri-centromeric heterochromatin and the nuclear lamina in DP thymocytes.
Figure 17: Influence of $E\alpha$ on nuclear localization and allelic exclusion.

(a) 3D Immuno-FISH, showing $Tcrb$ alleles in nuclei of $E\alpha$KI thymocytes. Distal $Tcrb$ (75P5, green), proximal $Tcrb$ (203H5, red), peri-centromeric heterochromatin ($\gamma$-satellite, blue, left panel), nuclear lamina (laminB1, blue, right panel). (b) Colocalization of unrearranged $Tcrb$ alleles with $\gamma$-satellite or laminB1 in wild-type and $E\alpha$KI DP thymocytes. $\gamma$-satellite, 145 WT and 114 $E\alpha$KI alleles (one slide each); laminB1, 179 WT and 48 $E\alpha$KI alleles (one slide each). (c) Colocalization of unrearranged $Tcrb$ alleles with $\gamma$-satellite or laminB1 in DN thymocytes of $Rag2^{-/-}$ and $E\alpha$KI $Rag2^{-/-}$ mice. $\gamma$-satellite, 145 $Rag2^{-/-}$ and 128 $E\alpha$KI $Rag2^{-/-}$ alleles (two slides each); laminB1, 316 $Rag2^{-/-}$ and 128 $E\alpha$KI $Rag2^{-/-}$ alleles (two slides each). (d) Quantification of $V\beta$-to-$D\beta$J$\beta$ recombination in wild-type and $E\alpha$KI DP nuclei. WT, 527 nuclei; $E\alpha$KI 129 nuclei (two slides each). *, P < 0.05.
We compared EαKI to wild-type alleles in DN thymocytes (both on a Rag2$^{-/-}$ background) to determine if the influence of E$_{a}$ on subnuclear positioning was developmentally regulated. Surprisingly, EαKI alleles colocalized with laminB1 at a frequency that was significantly reduced as compared to wild-type (41.4% vs. 66.4%) (Figure 17c). We also noted reduced colocalization with γ-satellite (30.5% vs. 37.1%), although the difference was not statistically significant. Therefore E$_{a}$ can influence locus compartmentalization in DN thymocytes, even though it cannot activate transcription in these cells.

The disruption of Tcrb nuclear localization in EαKI thymocytes prompted us to ask if relocalization was accompanied by a measurable perturbation in Tcrb gene recombination. Efficient allelic exclusion predicts that 60% of DP nuclei should have one allele rearranged while 40% should have both alleles rearranged (Khor and Sleckman, 2005; Mostoslavsky et al., 2004). Our data from wild-type DP thymocytes is consistent with these predictions, as 61.5% (324 of 527) of nuclei had one allele rearranged while 38.5% (203 of 527) had both alleles rearranged (Figure 17d). The latter likely represents a slight underestimate of nuclei with two rearranged alleles, as V$_{\beta}$14 rearrangements are undetectable by FISH. Notably, in EαKI DP thymocytes, 48.8% (63 of 129) of nuclei had one allele rearranged whereas 51.2% (66 of 129) had both alleles rearranged. Thus, addition of an ectopic enhancer into the Tcrb locus increased the number of nuclei with two rearranged alleles, implying a disruption of allelic exclusion.
4.2.7 *Tcrb* alleles do not pair in DN thymocytes

The inactivation of an X chromosome is associated with transient pairing of the two chromosomes. To determine if allelic exclusion of *Tcrb* also involves pairing of the loci, we examined the average distance between the two *Tcrb* alleles in *Lat<sup>−/−</sup>* DN thymocytes. Past analysis of X chromosomes, *IgH* and *Igk*, and the Th2 and Th1 loci scored pairing when two loci were within 0.5 or 0.6 µm of each other (Hewitt et al., 2008; Spilianakis et al., 2005; Xu et al., 2007). If pairing is occurring between *Tcrb* alleles, a subset of nuclei should reveal two alleles within 0.5 µm of each other. The distance between two alleles was calculated based upon the distance between the centers of the foci in three dimensions. We found that the median distance between two *Tcrb* alleles in 102 *Lat<sup>−/−</sup>* DN thymocyte nuclei was 4.41 µm (Figure 18). The distances between *Tcrb* alleles were normally distributed and there was no subset of nuclei that had *Tcrb* alleles within 0.5 µm of each other. The closest observed distance between two *Tcrb* alleles was 1.53 µm. This median distance between *Tcrb* alleles is, remarkably, significantly greater than that for *Actb* alleles. The two *Actb* alleles in *Rag<sup>−/−</sup>* DN thymocytes had a median distance separating them of 2.81 µm. Therefore the two *Tcrb* alleles do not pair and direct communication between the alleles in DN thymocytes does not contribute to the initiation of allelic exclusion.
Figure 18: Tcrb alleles do not pair in Lat⁻/⁻ DN thymocytes.

(a) Distances were measured between the centers of Tcrb foci in Lat⁻/⁻ DN thymocytes and between the centers of Actb in Rag2⁻/⁻ DN thymocytes. (b) A Box and Whisker plot of the distances plotted in a. The median (middle line), the 25th and 75th percentile (rectangular box), and the max and min (lines) are shown.
4.3 Discussion

Previous studies of the \textit{Igh}, \textit{Igk} and \textit{Tcrb} loci implicated regulated monoallelic association with peri-centromeric heterochromatin in the initiation or maintenance of allelic exclusion (Goldmit et al., 2005; Roldan et al., 2005; Skok et al., 2001; Skok et al., 2007). Here we have addressed in detail the nuclear localization of \textit{Tcrb} alleles during T lymphocyte development and provide a fundamentally different view based on the following observations. First, we found that \textit{Tcrb} alleles associate with peri-centromeric heterochromatin and with the nuclear lamina at high frequencies throughout T cell development. Second, we found that these associations occur stochastically rather than in a directed fashion, with the two \textit{Tcrb} alleles in a cell associating independently and with equal probability with the two compartments. Third, we found that associated \textit{Tcrb} alleles were less likely to have undergone \textit{V}_\beta\text{-to-}D_\beta J_\beta recombination, and that this bias was established prior to \(\beta\)-selection and independent of feedback inhibition. Fourth, we found that an ectopic enhancer inhibited \textit{Tcrb} locus association with peri-centromeric heterochromatin and the nuclear lamina and impaired allelic exclusion. We propose that stochastic, high frequency associations of \textit{Tcrb} alleles with peri-centromeric heterochromatin and with the nuclear lamina promote asynchronous \textit{V}_\beta\text{-to-}D_\beta J_\beta recombination and are critical for the initiation of \textit{Tcrb} allelic exclusion.

Our data suggest that the nuclear lamina and peri-centromeric heterochromatin are both repressive for \textit{V}_\beta\text{-to-}D_\beta J_\beta recombination and distinguish three subsets of DN
thymocytes based on the number of Tcrb alleles that are associated with these compartments. Approximately 35% of DN thymocytes have one allele associated with either the nuclear lamina or peri-centromeric heterochromatin. Asynchronous recombination in this subset can be readily understood based on the observed bias for the nonassociated allele to be the first to undergo V\(\beta\)-to-D\(\beta\)J\(\beta\) recombination. In approximately 60% of nuclei, both Tcrb alleles are associated with a repressive compartment. We propose that asynchronous recombination is promoted in this subset by inefficient recombination on both alleles. This will create an expanded time window for V\(\beta\)-to-D\(\beta\)J\(\beta\) recombination, thus diminishing the likelihood that such rearrangements will be initiated simultaneously on both alleles. Only 5% of nuclei display two alleles that are free of both the nuclear lamina and peri-centromeric heterochromatin. Thus, stochastic association of Tcrb alleles with repressive nuclear compartments may diminish the likelihood that V\(\beta\)-to-D\(\beta\)J\(\beta\) recombination is initiated biallelically in fully 95% of developing thymocytes. This asynchrony would allow thymocytes sufficient time to test the quality of recombination on the first allele and to initiate feedback inhibition of recombination on the second allele.

Our data suggest that V\(\beta\)-to-D\(\beta\)J\(\beta\) recombination can occur on both associated and nonassociated alleles but that it occurs more efficiently on nonassociated alleles. Nevertheless our analysis may underestimate the extent to which association with repressive nuclear compartments impairs recombination, because three color Immuno-
FISH does not allow concurrent analysis of rearrangement status and colocalization with both γ-satellite and laminB1. Thus, in the informative subset of cells with one free and one associated allele, the allele judged to be free of one compartment may have been associated with the undetected repressive compartment. Such biallelically associated cells are intrinsically uninformative in assessing a recombination bias and their inclusion would suppress any measured bias. Thus the preference to undergo recombination on a free as compared to an associated allele may be greater than the measured 65:35 (peri-centromeric) and 76:24 (nuclear lamina) ratios would suggest.

Regardless, because it is unclear whether associated Tcra alleles interact stably with the nuclear lamina and pericentromeric heterochromatin or transiently associate and dissociate from these compartments, we cannot determine the extent to which Vβ-to-DβJβ recombination is inhibited by molecular interactions with these compartments.

Several aspects of our observations differ markedly from those in a previous study (Skok et al., 2007). First, the authors of that study noted substantially higher frequencies of association of Tcra alleles with the nuclear periphery in all cell populations. Second, although they found Tcra alleles to associate with pericentromeric heterochromatin at frequencies similar to those reported here, they observed substantially higher frequencies of monoallelic association. A potential explanation for the difference in peripheral localization is that we scored colocalization by overlap between the TCR loci and laminB1 signals, whereas Skok et al. did not use a
specific marker to define the nuclear periphery. Differences in monoallelic Tcrb gene association with peri-centromeric heterochromatin are more difficult to explain (Skok et al., 2007). In addition, we provided direct measures of Tcrb allelic recombination status and assessed the significance of repressive compartment association for Tcrb gene recombination prior to β-selection. We conclude that Tcrb alleles associate stochastically and at high frequency with both the nuclear lamina and pericentromeric heterochromatin and that such associations inhibit Vβ-to-DβJβ recombination prior to β-selection. Our data suggests a fundamentally different basis for the initiation of Tcrb allelic exclusion in which asynchronous Vβ-to-DβJβ recombination is achieved stochastically rather than through strictly regulated allelic choice.

We found that the two Tcrb alleles do not pair with one another in DN thymocytes. In fact, they are on average located farther apart than the two Actb alleles. This strongly suggests that direct physical communication between the two alleles is not required to initiate allelic exclusion. A directed allelic choice, like X chromosome inactivation, might require the pairing of the Tcrb alleles. The lack of physical interaction between the Tcrb alleles is consistent with our hypothesis that asynchronous recombination is the result of stochastic associations with repressive compartments.

The difference between the average distance between two Actb alleles and two Tcrb alleles may reflect their different nuclear environments. There are two possibilities that may explain this observation. First, the Actb alleles are more centrally located than
Therefore, \( \textit{Actb} \) would have access to a smaller nuclear volume than \( \textit{Tcrb} \), which has a more peripheral location. Second, it is possible that the transcriptionally active \( \textit{Actb} \) alleles are more often found in the interchromatin compartment and this constraint limits \( \textit{Actb} \) to a smaller space than \( \textit{Tcrb} \).
5. The effect of nuclear localization and conformation on V(D)J Recombination

5.1 Introduction

We demonstrated that the initiation of allelic exclusion was influenced by the nuclear positioning of Tcrb. The Tcrb locus is documented to be in a contracted conformation in DN thymocytes and in an extended conformation in DP thymocytes (Skok et al., 2007). Since locus contraction is thought to be required for recombination, we hypothesized that nuclear compartmentalization may regulate recombination by altering the conformation of the Tcrb locus. Additionally, decontraction of the Tcrb locus in DP thymocytes is a probable mechanism for enforcing feedback inhibition. Along these lines, Jackson et al. showed that the introduction of an ectopic enhancer into Tcrb could increase germline transcription, histone acetylation, H3K4 methylation, and restriction enzyme accessibility, but could not induce recombination of Vβ gene segments in DP thymocyte nuclei (Jackson et al., 2005). One possible explanation is that the Tcrb locus remained extended in DP thymocytes, thus enforcing feedback inhibition of Vβ gene segments made accessible by Eα.

Thus we wished to ascertain whether locus contraction plays a role in initiating asynchronous recombination and to assess a role for locus decontraction in enforcing feedback inhibition. To address these issues, we first demonstrated that we could recapitulate earlier studies identifying a contracted conformation of Tcrb in DN
thymocytes and an extended conformation in DP thymocytes (Skok et al., 2007).

Second, we assessed the relationship between locus conformation and association with repressive nuclear compartments in DN thymocytes. Finally, we analyzed the conformation of Tcrb with or without an ectopic enhancer in DP thymocytes. These experiments led us to conclude that locus conformation regulates feedback inhibition but not asynchronous recombination.

5.2 Results

5.2.1 Tcrb Alleles have a contracted conformation in DN thymocytes and an extended conformation in DP thymocytes.

We examined the conformation of Tcrb in DN thymocytes from Rag2−/− and Lat−/− mice. This allowed us to analyze the conformation of the locus in both a recombination-competent and -incompetent background. This distance was calculated between the weighted centers of the foci defined by distal and proximal Tcrb probes. Our probing strategy only measured alleles in their germline configuration, because the proximal Tcrb probe is not visualized after recombination. This strategy prevents our observations from being biased by alleles with shortened distances due to Vβ to DJβ rearrangements. In both Lat−/− and Rag2−/− nuclei we documented a tear-drop like distribution of distances between distal and proximal foci (Figure 19). This distribution resembles a Rayleigh distribution and is typical when measuring the distance between two points in three dimensions (Bertolino et al., 2005; Reynaud et al., 2008; Sayegh et al., 2005). A rayleigh distribution arises when a two-dimensional or three-dimensional
vector has elements that are normally distributed, are uncorrelated, and have equal variance (personal communication with C. Murre). The median distance between the probes in Lat\(^{−/−}\) nuclei was 0.4570 \(\mu\)m and in Rag2\(^{−/−}\) nuclei was 0.3535 \(\mu\)m. If contraction is required for rearrangement, the extended median and larger variance seen in the Lat\(^{−/−}\) thymocytes may be explained by the selective loss of fully contracted alleles due to recombination. Nonetheless, these median distances were significantly shorter than the median distances seen in DP thymocytes (Table 5). Thus Tcrb exhibits a contracted conformation in DN thymocytes.
Figure 19: The Tcrb locus adopts a contracted conformation in DN and an extended conformation in DP thymocytes.

(a) The distance between the two BAC probes that stain the 5’ and 3’ ends of Tcrb locus in Lat−/− DN thymocytes (119 alleles), Rag2−/− DN thymocytes (136 alleles), sorted wild type DP thymocytes (102 alleles), Rag2−/− × βtg DP thymocytes (138 alleles), and DP thymocytes from Rag2−/− mice treated with an anti-CD3 treatment (48 alleles). (b) A Box and Whisker plot of the inter-probe distances in DN thymocytes. The median (middle line), the 25th and 75th percentile (rectangular box), and the max and min (lines) are shown.
Table 5: P values of the differences between cell populations of the inter-probe distances of Tcrb.

<table>
<thead>
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<th>sorted DP</th>
<th>Rag2(^{-/-}) + anti-CD3</th>
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To analyze DP thymocytes we examined sorted thymocytes from wild-type mice, thymocytes from Rag2\(^{-/-}\) mice with a Tcrb transgene, and thymocytes from Rag2\(^{-/-}\) mice that were injected with an anti-CD3 antibody. The median distances were 0.6929, 0.4115, and 0.6175 µm respectively (Figure 18). The sorted wild-type DP thymocytes and the Rag2\(^{-/-}\) anti-CD3 treated thymocytes displayed a fully decontracted conformation. Interestingly, the Rag2\(^{-/-}\) x Tcrb transgene thymocytes had an intermediate conformation that was more extended than Rag2\(^{-/-}\) thymocytes but almost indistinguishable from Lat\(^{-/-}\) thymocytes. Nonetheless, the more physiological wild type DP cell population is clearly decontracted as are the anti-CD3 treated Rag2\(^{-/-}\) mice. Therefore, consistent with previously published results (Skok et al., 2007), Tcrb adopts an extended conformation in DP thymocytes.

5.2.2 Nuclear Localization does not influence recombination by maintaining and extended Tcrb conformation.

We previously demonstrated that the Tcrb allele that is not associated with either the nuclear lamina or centromeric heterochromatin has a higher probability to be rearranged. We hypothesized that nuclear position may influence locus conformation,
such that peripheral or pericentromeric alleles may adopt an extended, or partially extended, conformation and thus be less likely to recombine. To investigate this possibility we analyzed both Lat$^{-/-}$ and Rag2$^{-/-}$ DN thymocytes. The median distance between proximal and distal foci for alleles colocalized with $\gamma$-satellite repeats was 0.4560 $\mu$m and for free alleles was 0.4420 $\mu$m (Figure 20a,b). The median distances for laminB1-colocalized and for free alleles was 0.3620 and 0.3645 $\mu$m, respectively (Figure 20c,d). Thus, the conformation of the Tcrb locus is independent of its nuclear position.
Figure 20: Association with either γ-satellite or laminB1 in DN thymocytes does not influence the conformation of Tcrb.

(a) The distance in microns between the distal Tcrb probe and the proximal Tcrb probe based upon the association with γ-satellite in Rag2−/− DN thymocytes (87 were associated and 164 alleles were free). (b) A Box and Whisker plot of the inter-probe distance, where the median (middle line) the 25th and 75th percentile (rectangular box) and the max and min (lines) are shown. (c) The distance between the two BAC probes based upon association with laminB1 in Lat−/− DN thymocytes (104 alleles were associated and 52 alleles were free). (d) A Box and Whisker plot of the inter-probe distances in DN thymocytes. The median (middle line), the 25th and 75th percentile (rectangular box), and the max and min (lines) are shown.
5.2.3 An ectopic enhancer does not perturb the conformation of \textit{Tcrb}

The introduction of the alpha enhancer into the \textit{Tcrb} locus breaks allelic exclusion in DN thymocytes, but does not allow further recombination of \textit{Tcrb} in DP thymocytes. The conformation of \textit{Tcrb} does not significantly change by the addition of E\textsubscript{a} in DN thymocytes (Figure 21 and Table 6). The median distance between the distal and proximal \textit{Tcrb} probes in wild-type alleles on a \textit{Rag2}\textsuperscript{−/−} background was 0.3535 \textmu m, while for \textit{Tcrb} alleles with E\textsubscript{a} on a \textit{Rag2}\textsuperscript{−/−} background the distance was 0.3325 \textmu m. There is also no significant difference when \textit{Rag2}\textsuperscript{−/−} and E\textsubscript{a}KI \textit{Rag2}\textsuperscript{−/−} mice are treated with an anti-CD3 antibody, which induces thymocyte differentiation to the DP stage (0.6175 \textmu m vs. 0.5905 \textmu m for wild-type and E\textsubscript{a}KI alleles respectively). There is a subtle, but significant, difference between the extent of decontraction when wild-type alleles are compared to E\textsubscript{a}KI alleles in sorted DP thymocytes (0.6232 \textmu m vs. 0.5016 \textmu m). Although, the E\textsubscript{a}KI allele is by this measure not fully decontracted, it is more extended than the contracted conformation found in DN thymocytes. The inability of E\textsubscript{a} to fully contract the \textit{Tcrb} locus in DP thymocytes may explain the lack of recombination on these alleles in spite of the fact the \textit{V}\textsubscript{\beta} gene segments are accessible and the locus is less associated with the nuclear lamina. Thus decontraction may prevent recombination of accessible E\textsubscript{a}KI alleles in DP thymocytes.
Figure 21: Eα does not contract the Tcrb locus in DP thymocytes.

(a) The distance in microns between the distal Tcrb probe (75P5) and the proximal Tcrb probe (203H5) in Rag2−/− (136 alleles) and EαKI Rag2−/− (76 alleles) DN thymocytes, sorted wild type (102 alleles) and sorted EαKI (134 alleles) DP thymocytes, and in Rag2−/− x β tg (48 alleles) and EαKI Rag2−/− x Tcrb tg (70 alleles) DP thymocytes.  (b) A Box and Whisker plot of the inter-probe distances. The median (middle line), the 25th and 75th percentile (rectangular box), and the max and min (lines) are shown.

Table 6: P values of the differences between cell populations of the inter-probe distances of Tcrb.

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<th>EαKI DP</th>
<th>Rag2−/− + anti-CD3</th>
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<tr>
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5.3 Discussion

Our data shows that Tcrb adopts a contracted conformation in DN thymocytes. Because Tcrb adopted a contracted conformation in both Lat−/− nuclei and Rag2−/− nuclei, we conclude that RAG is not required for contraction. Interestingly, the recombinationally-competent Lat−/− nuclei had a slightly larger median distance between probes than the recombinationally-incompetent Rag2−/− nuclei. One possible explanation for this observation is that selective recombination of the more contracted alleles. This would suggest that the closer Vβ and DJβ gene segments are to one another, the higher the probability those gene segments will recombine. Even though we conclude that the suppression of recombination of alleles associated with repressive nuclear compartments is not the result locus conformation, subtle variations in the conformation of a locus may influence recombination efficiency. A wide distribution of Tcrb conformations in DN thymocytes may bias recombination to a subset of alleles, thus reducing the probability of synchronous recombination.

Our data shows that Tcrb adopts an extended conformation in sorted wild-type DP thymocytes and Rag2−/− thymocytes treated with anti-CD3. Interestingly, Rag2−/− x Tcrb tg thymocytes had a more contracted Tcrb conformation. Earlier studies observed an extended Tcrb conformation in Rag2−/− x Tcrb tg DP thymocytes (Skok et al., 2007). This difference may be explained by the methods used to measure the conformation of the Tcrb locus. We directly plotted distances between the distal and proximal probes,
while Skok et al. grouped Tcrb alleles into two categories; contracted alleles (with distal and proximal probes closer than 0.3 µm) and decontracted alleles (with distal and proximal probes farther than 0.3 µm). If our data is analyzed using this criterion, we would have scored 20% of the alleles in a contracted state (14 of 64 alleles) which is not substantially different from their data. Thus we believe our methodology to be more informative in describing the conformation of the locus. The contracted state that we observe in Rag2−/− x Tcrb transgene DP thymocytes maybe the result of an accelerated transition to the DP compartment that prevents the full decontraction of Tcrb. Since a Tcrb transgene is capable of allelically excluding the endogenous loci, this would suggest that locus decontraction is not absolutely required to maintain feedback inhibition. Thus both accessible chromatin and a contracted locus conformation are required for recombination and the loss of either is sufficient to prevent recombination.

In conclusion, we attempted to further elucidate the potential role of locus contraction in regulating allelic exclusion. We demonstrated that we could recapitulate the results of earlier studies by showing a contracted conformation of Tcrb in DN thymocytes and an extended conformation of Tcrb in DP thymocytes. We extended these studies and found no relationship between locus conformation and nuclear compartmentalization in DN nuclei. Thus, recombination efficiency is not reduced at repressive nuclear compartments by maintaining Tcrb in an extended conformation. This supports our earlier suggestion that repressive nuclear compartments reduce the
availability of Tcrb to RAG leading to a reduction in recombination efficiency. We also demonstrated that the conformation of Tcrb does not change with the addition of an ectopic enhancer in DP nuclei. Therefore, allelic exclusion in DP thymocytes of EaKI mice may be maintained because Ea does not contract the Tcrb locus. Thus, our results are consistent with a role for locus conformation in enforcing feedback inhibition but not in initiating asynchronous recombination.
6. Discussion

Herein we have demonstrated that the nuclear compartmentalization of \( Tcrb \) alleles regulates recombination. We showed that \( Tcrb \) alleles are frequently associated with pericentromeric heterochromatin and the nuclear lamina in both DN and DP thymocytes. Each \( Tcrb \) allele independently associates with pericentromeric heterochromatin and the nuclear lamina, suggesting that compartmentalization is stochastic rather than directed. Moreover, \( Tcrb \) alleles that are associated with these repressive nuclear compartments were less likely to have undergone \( V_\beta \)-to-\( D_\beta J_\beta \) recombination. Nuclear compartmentalization biased recombination prior to \( \beta \)-selection and independent of feedback inhibition without altering the conformation of \( Tcrb \). We demonstrated that nuclear localization of \( Tcrb \) was disrupted by an ectopic enhancer. Furthermore, disrupting the nuclear localization in DN thymocytes correlated with an increase in recombination and impaired allelic exclusion. Additionally, we demonstrated that an ectopic enhancer did not alter the conformation of the \( Tcrb \) locus. These data led us to propose that stochastic, high frequency associations of \( Tcrb \) alleles with repressive nuclear compartments promote asynchronous \( V_\beta \)-to-\( D_\beta J_\beta \) recombination. We also propose that feedback inhibition is enforced by an extended conformation of \( Tcrb \). Thus, associations with pericentromeric heterochromatin and the nuclear lamina are critical for the initiation of \( Tcrb \) allelic
exclusion in DN thymocytes, while locus conformation is critical for feedback inhibition in DP thymocytes.

Our data suggest that association with repressive nuclear compartments may not play a substantial role in feedback inhibition, but are consistent with an important role for locus decontraction in feedback inhibition. Jackson et al. demonstrated that introducing Eα into Tcrb does not break allelic exclusion in DP thymocytes despite the fact that EαKI alleles have all the hallmarks of an active locus (Jackson et al., 2005). Decontraction of the Tcrb locus in DP thymocytes is a potential explanation for the lack of recombination in the presence of active histone marks and accessible DNA. Our data is consistent with this interpretation because there was a decontraction of EαKI alleles in DP thymocytes. These data strongly suggest that feedback inhibition in DP thymocytes is enforced in part by decontraction of the Tcrb locus. Therefore attempts to break allelic exclusion in DP thymocytes will have to, at a minimum, contract the Tcrb allele.

We observed rearranged alleles associated with both pericentromeric heterochromatin and the nuclear lamina. Thus we hypothesize that associations with repressive nuclear compartments do not prohibit recombination but rather reduce recombination efficiency. If an allele were stably associated with a repressive nuclear compartment and could not recombine, then there should be a deviation from the theoretical 60:40 distribution of nuclei with one or two rearranged alleles, because that
calculation assumes that both alleles are equally able to recombine. Visualizing DSBs at Tcrb alleles that are associated with these repressive compartments would prove that recombination occurs there. DSBs can be detected at loci that are actively recombining (Chen et al., 2000; Pilch et al., 2003). Therefore active recombination can be visualized with antibodies against γ-H2A.X, which detect histone variant H2A.X when it is phosphorylated in the vicinity of DSBs. Thus, if DN thymocyte nuclei were stained for γ-H2A.X, Tcrb, and either γ-satellite or laminB1 and all three signals colocalize, we would demonstrate that recombination was occurring at these repressive compartments. Additionally, it would be interesting to determine if recombination can occur within CT. 3D FISH experiments visualizing Tcrb or Tcra along with γ-H2A.X and their relevant chromosome paint would determine if recombination can occur within the CT. It may be that embedding Tcrb in its CT may reduce the efficiency of recombination by restricting access of the locus to RAG.

Unfortunately, the usefulness of 3D FISH is limited by the “snap shot” nature of the experimental protocol. This means that nuclear repositioning cannot be followed in real time and thus the dynamic motion of the alleles cannot be determined. If DSB are not found at repressive nuclear compartments, it is possible that the alleles are rapidly moving between nuclear compartments. Therefore, the rearranged alleles scored as associated with repressive compartments may have recombined when they were unassociated. We feel that this explanation, though possible, is unlikely for the
following reasons. First, our data would imply that associations with repressive compartments are fairly stably since we see a consistent high frequency of association with these compartments in pro-B cells, DN thymocytes, and DP thymocytes. Second, real-time analysis of induced $\gamma$-H2A.X foci indicate that they are stably positioned within the nucleus (Soutoglou et al., 2007). Finally, repositioning of loci to the nuclear periphery has been shown to require a round of cell division (Reddy et al., 2008). We therefore expect that $Tcrb$ alleles recombine, albeit inefficiently, while associated with repressive nuclear compartments.

How nuclear compartmentalization suppresses recombination efficiency remains unknown, but we can tentatively rule out several possible mechanisms. First, associations with pericentromeric heterochromatin and the nuclear lamina do not appear to regulate $Tcrb$ chromatin accessibility. We know that as DN thymocytes transition to DP thymocytes, $Tcrb$ becomes less acetylated and less accessible to DNase digestion, but high levels of association with repressive nuclear compartments occur at both stages (Jackson et al., 2005; Tripathi et al., 2002). Thus, accessibility seems to be regulated by factors that are independent of these associations. Histone modifications and DNase accessibility are measured on cell populations, so it is possible that the individual alleles that associate with repressive compartments are hypo-acetylated and less accessible to DNase. Moreover we can infer that associations with pericentromeric heterochromatin and the nuclear lamina do not prevent transcription of individual $Tcrb$
alleles. We showed that there are many DN nuclei with one allele and both alleles associated with repressive nuclear compartments, but a $V_\beta$ gene segment was shown to be biallelically transcribed in DN thymocytes (Jia et al., 2007). This would argue that alleles that are associated with repressive compartments are being transcribed. A 3D DNA-RNA FISH analysis that visualizes the colocalization of the $Tcrb$ locus, $V_\beta$ germline transcripts, and either pericentromeric heterochromatin or the nuclear lamina could prove that transcription occurs on associated alleles. In addition, we demonstrated that associations with pericentromeric heterochromatin and the nuclear lamina do not alter the conformation of the $Tcrb$ locus.

One intriguing hypothesis would be that recombination, like transcription, occurs more efficiently at areas of high concentration of recombination related proteins or “recombination factories”. Thus, regulating the movement of antigen receptor loci to areas within the nucleus with preformed recombination complexes may offer a simple way to ensure efficient recombination. If “recombination factories” do exist within the nucleus, antibodies that bind RAG should show a punctate staining pattern similar to RNA pol II or the splicing factor SC-35. Discrete foci of RAG may be a secondary effect of RAG accumulating at the accessible RSSs that result from transcription. It is known that the movement of RNA pol II through the V, D, J gene segments disrupts nucleosome structure and create accessible RSSs (Abarrategui and Krangel, 2006; Belotserkovskaya et al., 2003; Carrozza et al., 2005; Schwabish and Struhl,
It is provocative to think of RAG either co-opting transcription factories or creating recombination factories near transcription factories and taking advantage of the accessible environments that transcription factories provide. RAG foci colocalizing with active Pol II would suggest that recombination factories are expanded transcription factories, while RAG foci near Pol II foci, similar to splicing speckles, would suggest independent recombination factories. Also, if recombination is actively occurring at these RAG foci, they should co-stain with antibodies against γ-H2A.X. DNA FISH for Tcrb or Tcra with antibodies against RAG and γ-H2A.X would functionally demonstrate that recombination occurs at RAG foci.

We demonstrated a strong correlation between nuclear localization and recombination efficiency. Moreover we demonstrated that the introduction of an ectopic enhancer suppressed associations with repressive compartments and increased recombination efficiency and disrupted allelic exclusion. To formally prove that associations with repressive nuclear compartments suppress recombination, an experiment would have to demonstrate that forced repositioning of an actively recombining locus to a repressive compartment resulted in a reduction in recombination. Reddy et al. used a chromosomally integrated array of 256 Lac operators (lacO) and a Lac repressor (Lacl) fused to an inner nuclear membrane protein to tether the targeted region to the nuclear periphery (Reddy et al., 2008). They demonstrated an inhibition of transcription of nearby genes. We could integrate 256
lacO into the Tcra locus. Tcra locus in DP thymocytes offers an excellent test of this hypothesis, because this actively recombining locus is rarely found at the nuclear lamina. If a nuclear lamina tethered Tcra locus has reduced recombination in DP thymocytes, it would prove that recombination efficiency is regulated by nuclear positioning. Additionally, our conclusion that Eα disrupts allelic exclusion by repositioning Tcrb alleles could also be formally demonstrated by tethering EαKI alleles to the nuclear lamina. If tethering the EαKI Tcrb allele to the nuclear lamina resulted in a restoration of allelic exclusion then we can conclude that Eα influenced Tcrb allelic exclusion through nuclear localization. Conversely, if tethering cannot restore allelic exclusion then the process through which Eα perturbs allelic exclusion would remain unknown.

In conclusion, we found that Tcrb alleles associate stochastically with peri-centromeric heterochromatin and with the nuclear lamina at high frequencies throughout T cell development and that these associations are established prior to β-selection and independent of feedback inhibition. We found that these associations inhibited recombination, but that this inhibition did not occur by changes in the locus conformation. More importantly we found that an ectopic enhancer inhibited Tcrb locus association with peri-centromeric heterochromatin and the nuclear lamina in DN thymocytes and impaired asynchronous recombination. We propose that stochastic, high frequency associations of Tcrb alleles with peri-centromeric heterochromatin and
with the nuclear lamina promote asynchronous Vβ-to-DβJβ recombination and are critical for the initiation of Tcrb allelic exclusion. We also propose that feedback inhibition is enforced by an extended conformation of Tcrb which explains why EαKI Tcrb alleles do not break allelic exclusion in DP thymocytes.
7. References


involving four phenotypically and functionally distinct subsets of CD3−CD4−CD8−
triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J
Immunol 150, 4244-4252.


Biochem 74, 481-514.

(2000). CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2


fate. Immunity 22, 583-593.

Methylation of histone H3 at Lys-9 is an early mark on the X chromosome during X

encoding class I MHC-specific receptors on NK cells. Nature 376, 355-358.


(2003). Pax5 is required for recombination of transcribed, acetylated, 5' Igh V gene
segments. Genes Dev 17, 37-42.

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8. Biography

I was born in Sioux Falls, SD on June 25th, 1975. As the eldest of three children in a military family I attended many public schools for grades K-12. I lived in Sioux Falls for six years after which my family moved to Herzogenaurach Germany where we lived for three years. From there we moved to Lawton Oklahoma, Fairfax Virginia, and then Lansing Kansas where I attended Lansing High School for 9th and 10th grade. I completed high school and received my diploma from Robinson Secondary School in Fairfax Virginia. I attended the University of Virginia and graduated in 1997 with a BS in Chemistry and a specialization in biochemistry and a BA in Biology. My first undergraduate research mentor was Dr. James Garrison in the department of Pharmacology were I studied G protein signaling. I continued my undergraduate research studies in the department of biochemistry in the lab of Dr. Ronald P. Taylor, where we studied the clearance on immune complexes from the blood stream. I was then awarded an intramural research training grant for post baccalaureate students at the NIH. Under the supervision of Dr. Dimitros Boumpas in the NIAMS, we studied lupus, rheumatoid arthritis, and psoriatic arthritis. After two years at the NIH, I moved to the private sector and worked at Booz-Allen and Hamilton focusing on Scientific and Engineering Technical Advisor support for Defense Advanced Research Projects Agency and counter-proliferation and counter-terrorism tasks. In 2001 I matriculated to department of Immunology at Duke University. My doctoral research
was completed in the lab of Dr. Michael S. Krangel studying the role of nuclear localization and locus conformation played in allelic exclusion. Most of the initial data for my thesis was gathered in lab of Dr. Harinder Singh and the University of Chicago with the excellent mentoring of Dr. Karen Reddy.

Publications:


Abstracts:
