

Restrictions Limiting the Generation of DNA Double Strand Breaks during Chromosomal V(D)J Recombination

Robert E. Tillman, Andrea L. Wooley, Maureen M. Hughes,
Tara D. Wehrly, Wojciech Swat, and Barry P. Sleckman

Washington University School of Medicine, Department of Pathology and Immunology, St. Louis,
MO 63110

Abstract

Antigen receptor loci are composed of numerous variable (V), diversity (D), and joining (J) gene segments, each flanked by recombination signal sequences (RSSs). The V(D)J recombination reaction proceeds through RSS recognition and DNA cleavage steps making it possible for multiple DNA double strand breaks (DSBs) to be introduced at a single locus. Here we use ligation-mediated PCR to analyze DNA cleavage intermediates in thymocytes from mice with targeted RSS mutations at the endogenous TCR β locus. We show that DNA cleavage does not occur at individual RSSs but rather must be coordinated between RSS pairs flanking gene segments that ultimately form coding joins. Coordination of the DNA cleavage step occurs over great distances in the chromosome and favors intra- over interchromosomal recombination. Furthermore, through several restrictions imposed on the generation of both nonpaired and paired DNA DSBs, this requirement promotes antigen receptor gene integrity and genomic stability in developing lymphocytes undergoing V(D)J recombination.

Key words: lymphocyte • antigen receptor • RAG • DNA cleavage • DNA repair

Introduction

The precise specificity and vast diversity of the adaptive immune response is achieved through the expression of lymphocyte antigen receptors with polymorphic variable regions. Genes encoding variable regions are assembled during lymphocyte development from V, D, and J gene segments by a site specific DNA recombination process, termed V(D)J recombination (1). Variable gene segments (V, D, and J) are bordered by recombination signal sequences (RSSs)* composed of conserved heptamers and nonamers flanking nonconserved 12 or 23 base pair spacers (hereafter referred to as 12-RSSs and 23-RSSs, respectively) (1). Recombination only occurs between variable gene segments flanked by 12- and 23- RSSs, a restriction known as the 12/23 rule (1). The V(D)J recombination reaction proceeds through DNA cleavage and joining steps and is performed by an enzymatic complex collectively referred to as the V(D)J recombinase.

This complex includes the lymphoid specific recombinase activating gene (RAG)-1 and -2 proteins which introduce DNA double strand breaks (DSBs) precisely at the RSS/coding segment border (2-4). The resulting hairpin closed coding ends and blunt phosphorylated signal ends are then joined by generally expressed proteins involved in the nonhomologous end joining pathway of DNA DSB repair to form coding and signal joins, respectively (5-8).

There are six antigen receptor loci that encode the TCR α , β , γ , and δ chains and the Ig H and L (κ and λ) chains. These loci are composed of many variable gene segments with flanking RSSs. DNA cleavage by RAG-1/2 at isolated RSSs could result in numerous DNA DSBs at multiple loci in developing lymphocytes. Although some of these DSBs would be resolved through standard coding and signal join formation, many would require resolution by other means such as open-and-shut or hybrid join formation before cell cycle progression is permitted (9). Furthermore, these DSBs could be resolved through interchromosomal V(D)J recombination or serve as substrates for RAG-1/2 mediated transposition resulting in chromosomal translocations (10, 11). Accordingly, mechanisms that limit DNA DSB generation to gene segments that ultimately participate in coding join formation

Address correspondence to Barry P. Sleckman, Washington University School of Medicine, Department of Pathology and Immunology, 660 S. Euclid Ave., Campus Box 8118, St. Louis, MO 63110-1093. Phone: 314-747-8235; Fax: 314-362-4096; E-mail: Sleckman@Immunology.WUSTL.edu

*Abbreviations used in this paper: DSB, double strand break; LMPCR, ligation-mediated PCR; RAG, recombinase activating gene; RSS, recombination signal sequence.

would promote efficient variable region gene assembly and genomic stability.

The V(D)J recombination reaction is regulated in tissue, lineage, and developmental stage specific manners (12, 13). Although in some cases this regulation may occur at postcleavage steps, it is primarily effected at the DNA cleavage step of the reaction by limiting accessibility of antigen receptor genes to the V(D)J recombinase (12–14). This occurs through, among other things, changes in chromatin structure, methylation, and/or transcription that restricts DNA cleavage by the V(D)J recombinase (12, 13). For example, DNA DSB generation in Ig genes is restricted to developing B cells with further restriction of IgH and IgL chain gene DSBs to pro-B and pre-B cells, respectively (15). Similar considerations apply to DSBs generated in TCR genes during T cell development (15). However, the extent of DNA DSB generation in an accessible locus will be determined primarily by regulatory features of the V(D)J recombination reaction. In this regard, accumulation of DSBs can be limited by restrictions placed on the DNA cleavage step and/or by promoting rapid resolution of DSBs (i.e., as open-and-shut joins) introduced at gene segments that do not ultimately participate in coding join formation.

Analysis of the DNA cleavage step in vitro and on extra-chromosomal and transgenic recombination substrates has demonstrated that it is most efficient when 12/23 RSS pairing is permitted (16–19). This restriction, which provides the basis for the 12/23 rule, reflects a requirement for 12/23 RSS synapsis before DNA cleavage (20, 21). However, cleavage at isolated RSSs is observed in these analyses, and the extent to which this restriction limits DNA DSB generation at the numerous variable gene segments in endogenous antigen receptor loci is not known. Furthermore, RSSs impose significant constraints on the V(D)J recombination reaction beyond enforcing 12/23 compatibility (22, 23). Whether these restrictions occur at the DNA cleavage step, further limiting DNA DSB generation, is also unknown.

TCR β variable region genes are assembled from V β , D β , and J β gene segments with D β to J β rearrangement preceding V β to D β rearrangement. We have analyzed the DNA cleavage step of the V(D)J recombination reaction in the context of endogenous TCR β gene rearrangements during normal thymocyte development. These experiments used mice with targeted mutations of RSSs in the endogenous TCR β locus and, importantly, were designed to evaluate the effects of these mutations on DNA cleavage not only at mutant RSSs but also at their normal wild-type RSS partners. Our findings provide important insights into the regulation of the DNA cleavage step of the V(D)J recombination reaction in vivo within the context of the chromosome. In addition, they have implications for the manner in which DNA DSBs are limited during chromosomal V(D)J recombination and are discussed in the context of maintaining genomic stability during antigen receptor variable region gene assembly in developing lymphocytes.

Materials and Methods

Flow Cytometric Analyses. Thymocytes were stained with FITC-conjugated anti-CD8, PE-conjugated anti-CD4, or PE-conjugated as described previously (22). Stained samples were analyzed on a Becton Dickinson FACScan™.

Isolation of Thymocyte DNA. Whole thymocytes were treated with anti-CD4 (RL172.4), anti-CD8 (3.155), and complement as described previously (22). The resulting cells were lysed and DNA isolated as described previously (24).

Oligonucleotide Primers. The BW-1, BW-2, and BW-1H oligonucleotide primers have been described previously (25). The sequences of other primers used are as follows. 5A: 5'-TGACG-CACAGCCTTAGGG-3'; 5B: 5'-GGGCTGTTACTTCTTC-ATAGGGTGG-3' P2: 5'-GCTTATCTGGTGGTTTC-3'; 3A: 5'-TTGACGATAGACTTGTGGC-3'; 3B: 5'-CAGTGGAACTTACATCCCTTTTGTG-3'; P1: 5'-CCTCTCTCAAGGTCCATCAA-3'; BWJ: 5'-CCGGAGATCTGAATCCACA-TCA-3'; JA: 5'-CTGTCTGTCCCAAGGC-3'; JB: 5'-TCTC-TGGGTCGCAAGCC-3'; V2: 5'-TCCTGGGGACAAAGAGGTCAAATC-3'. VA2: 5'-TTCAGTCAGTGTATATAG-AGC-3'; VB2: 5'-GATGGTTTCAATGGTGTCTCTG-3'; VP2: 5'-GAACCAAAGTCTATGCTCTGAG-3'; VA11: 5'-TAGC-CTGGATCTGTTCTG-3'; VB11: 5'-AGGTCTCTGGGC-TATCTA-3'; VP11: 5'-TCCCAGCCTATTTAGTCTTCAG-3'; VA8.3: 5'-GATGACACCAATGAGTGAAG-3'; VB8.3: 5'-CTGAACTATGGCTGTGAGA-3'; VP8.3: 5'-TTTCAGATGACCAGGACTG-3'. V15: 5'-CCCATCAGTCATCCC-AACTTATCC-3'. VA15: 5'-GACTCTTGAGACCTGACT-ATC-3'. VB15: 5'-GCAATGAACCTGTCTGAGTTG-3'. VP15: 5'-GGAGTGAGTAGACTTGTGTTG-3'. VA12: 5'-GGAC-CATTAGTAGAGGACAAAC-3'. VB12: 5'-CCTGATATTC-CCGTACTIONGACTGGC-3'; VP12: 5'-GAAATCTTGGGTGAAAAATGAC-3'; R2U: 5'-TCACTCAAATCCTC-TTCAGA-3' R2D: 5'-CCCAGAGAACCACAGAAA-3'; R2P: 5'-GCTGACTGCCTACCCCAT-3'.

Ligation-mediated PCR and PCR. The BW linker was generated by annealing the BW-1 and BW-2 oligonucleotides. Purified thymocyte genomic DNA (2–3 μ g) was ligated to 100 pmoles of the BW linker in a volume of 50–60 μ l with 1–3 units T4 DNA Ligase (Promega) at 16°C for 12–14 h. Ligated samples were extracted with phenol and chloroform before PCR analysis. Heminested PCRs were performed using the BW-1H or BWJ primers and A series primers (i.e., VA12, VA11, JA) in the primary reaction and BW-1H or BWJ primers and B series primers (i.e., VB12, VB11, JB) in the secondary reaction. Primary ligation-mediated PCRs (LMPCRs) were performed on 30–40 ng of ligated genomic DNA with 10 pmoles of each primer, 50 mM KCl, 10 mM Tris (pH 8.0), 1–2 mM MgCl₂ and Taq in 50 μ l reaction volume. The conditions for primary LMPCR reactions were 92°C, 1 min; 50–60°C, 1 min; and 72°C, 1 min for 18 cycles. Secondary LMPCRs were performed on 10 μ l of the primary LMPCR using primary LMPCR conditions. Cycling conditions for secondary LMPCR reactions were 92°C, 1 min; 55–65°C, 1 min; and 72°C, 1 min for 28 cycles. The RAG-2 gene was amplified using the R2U and R2D primers using the primary LMPCR reaction conditions and the secondary LMPCR cycling conditions. Signal joins were amplified by a heminested PCR of nonligated genomic DNA using primary and secondary LMPCRs conditions with V primers and the 5A (primary PCR) and 5B (secondary PCR) primers.

Southern Blotting. PCR products were size fractionated on 1–1.5% agarose gels and transferred to Zeta-probe membranes

(Bio-Rad Laboratories) followed by hybridization with oligonucleotide probes as described previously (22).

Results

Experimental Approach. The mouse TCR β locus consists of 21 functional V β gene segments spanning ~650 kb upstream of two DJ β gene clusters (DJ β 1 and DJ β 2), each with a single D β gene segment, six functional J β gene segments, and associated constant region genes (C β 1 and C β 2; Fig. 1 A). The J β 1^ω allele has a targeted deletion of the DJ β 2 gene cluster limiting rearrangements on this allele to the DJ β 1 gene cluster (22). V β and J β gene segments are flanked by 23- and 12-RSSs, respectively, and the D β 1 gene segment by a 5' 12-RSS and a 3' 23-RSS. Because direct V β to J β rearrangement is prohibited, despite 12/23 compatibility, all rearrangements (D β to J β and V β to D β) on the J β 1^ω allele involve the D β 1 gene segment (22, 23).

DNA cleavage intermediates were assayed in thymocytes from mice heterozygous for 5' or 3' D β 1 RSS mutations on the J β 1^ω allele (J β 1^{M4/ω} and J β 1^{M2/ω}, respectively, see below; Fig. 1 A). Importantly, as all rearrangements must

use the D β 1 gene segment, the effects of these mutations could be assayed not only at the mutant D β 1 RSSs but also at their native V β or J β 1 RSS partners. To enrich for cells actively undergoing TCR β gene rearrangements, thymocytes were treated with anti-CD4, anti-CD8, and complement with the resulting cells being >90% CD4⁻CD8⁻ (double negative, DN; Fig. 1 B). Signal end DNA cleavage intermediates were assayed in genomic DNA isolated from DN thymocytes by LMPCR using a nested pair of TCR β locus-specific primers and a linker-specific primer. Polymorphisms on the mutant (J β 1^{M4} and J β 1^{M2}) and wild-type (J β 1^ω) alleles enabled direct quantitative comparison of signal end DNA cleavage intermediates derived from each allele in samples from thymocytes heterozygous for RSS mutations (J β 1^{M4/ω} and J β 1^{M2/ω}).

DNA Cleavage Intermediates from D β to J β Rearrangement. The J β 1^{M4} allele was generated by precisely replacing the 5' D β 1 12-RSS on the J β 1^ω allele with the J β 1.2 12-RSS without alterations in the nucleotides flanking the RSS (22; Fig. 1 A). D β to J β rearrangement occurs on both alleles in J β 1^{M4/ω} thymocytes (22). Accordingly, equivalent levels of DNA cleavage intermediates would be expected from D β to J β rearrangement at each allele in J β 1^{M4/ω} DN thymocytes. Assay of linker ligated genomic DNA from J β 1^{M4/ω} DN thymocytes revealed equivalent levels of 3' D β 1 signal ends derived from the J β 1^{M4} and J β 1^ω alleles (Fig. 2, A and B). Similarly, equivalent levels of J β 1.1 and J β 1.2 signal ends were detected from the J β 1^{M4} and J β 1^ω alleles (Fig. 2, A and C, and data not shown). Products from linker ligated J β 1.2 signal ends could be detected from as little as 90 cell equivalents of ligated genomic DNA demonstrating the assay is sensitive at low template levels (Fig. 2 C). LMPCR products derived from J β 1^{M4} and J β 1^ω alleles can be distinguished due to size differences generated by the introduction of a *loxP* site between D β 1 and J β 1.1 on the J β 1^{M4} allele (Fig. 2, A-C). All products were ligase dependent, and sequence analysis of these and subsequent products revealed that they were generated by blunt end ligation of the linker to precisely cut signal ends (data not shown). These findings demonstrate that the experimental approach used here permits the quantitative assessment of DNA cleavage intermediates generated during rearrangement at the TCR β locus in DN thymocytes. This is evidenced by the detection of equivalent levels of DNA cleavage intermediates from D β to J β rearrangement at the J β 1^{M4} and J β 1^ω alleles in J β 1^{M4/ω} thymocytes.

DNA Cleavage Is Coordinated at D β and J β RSSs. The J β 1^{M2} allele exhibits a block in D β to J β rearrangement due to a mutation of the 3' D β 1 RSS heptamer (CA-CAGTG to ATTTTAA; reference 23; Fig. 1 A). As expected, 3' D β 1 signal ends from the J β 1^ω, but not the J β 1^{M2}, allele were readily detected by LMPCR of genomic DNA from J β 1^{M2/ω} DN thymocytes (Fig. 2, A and B). The J β 1 RSSs on the J β 1^{M2} allele are unaltered and as such are identical to those on the J β 1^ω allele. Strikingly, although J β 1.1 and J β 1.2 signal ends can be readily detected from the J β 1^ω allele, these signal ends are not detected from the

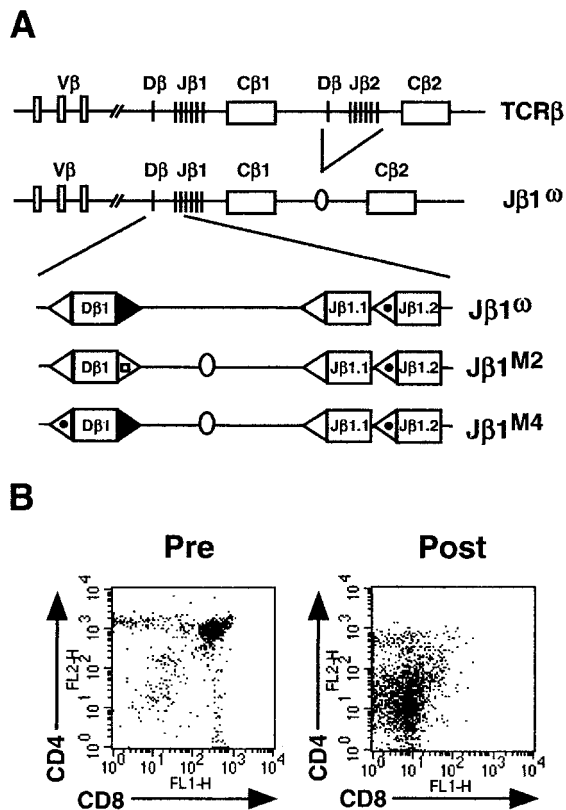


Figure 1. Schematic of the wild-type and mutant TCR β alleles. (A) Wild-type and modified J β 1^ω, J β 1^{M2}, J β 1^{M4} TCR β alleles. Shown are the 5' D β 1 and J β 1.1 12-RSSs (open triangles), J β 1.2 12-RSS (dotted open triangle), the 3' D β 1 23-RSS (closed triangle), and the 3' D β 1 23-RSS with the heptamer mutation (open triangle with open square). The 70 base pair fragment containing a *loxP* site is also shown (oval). (B) Flow cytometric analysis of thymocytes before (Pre) and after (Post) treatment with anti-CD4, anti-CD8, and complement.

J $\beta 1^{M2}$ allele in J $\beta 1^{M2/\omega}$ DN thymocytes (Fig. 2 C, and data not shown). Low levels of cleavage at the J $\beta 1.2$ RSS on the J $\beta 1^{M2}$ allele should be detected, if present, as cleavage at the J $\beta 1.2$ RSS the J $\beta 1^\omega$ allele can be detected from as little as 45 cell equivalents of J $\beta 1^{M2/\omega}$ DN thymocyte DNA (Fig. 2 C). Together, these findings demonstrate that DNA DSB generation at D β and J β RSSs is coordinated during D β to J β rearrangement, as mutation of the 3'D β 23-RSS prevents cleavage at native J β 12-RSSs on the same allele. This occurs within the context of the 12/23 rule as DNA

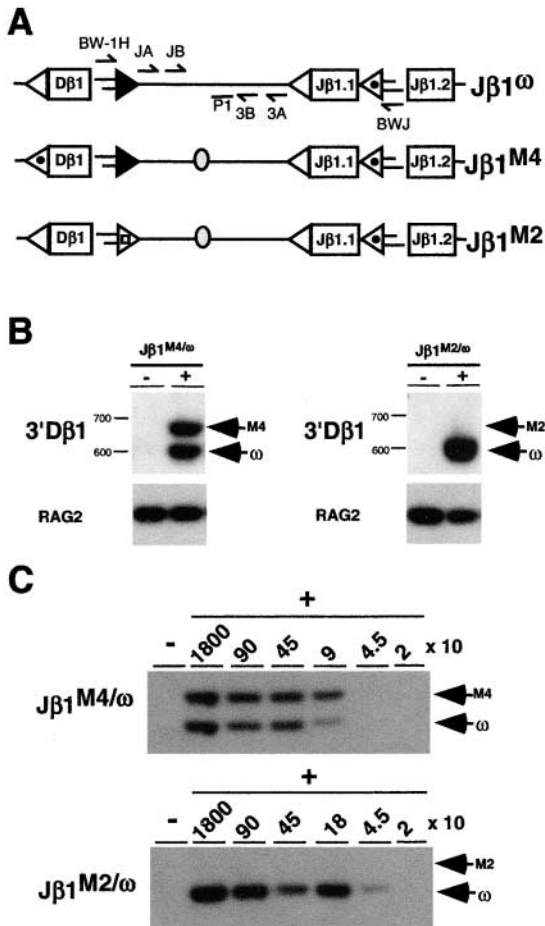


Figure 2. DNA cleavage is coordinated at 3'D β and J β RSSs. (A) Schematic of the J $\beta 1^\omega$, J $\beta 1^{M2}$, and J $\beta 1^{M4}$ alleles as described in the legend to Fig. 1 with the BW linker ligated to cleaved 3' D $\beta 1$ and J $\beta 1.2$ signal ends. The position of the oligonucleotide primers used to amplify linker ligated to the 3' D $\beta 1$ (BW-1H, 3A, and 3B) and J $\beta 1.2$ (BWJ, JA, and JB) signal ends are shown as is the position of the oligonucleotide (P1) used to probe these PCR products. Genomic DNA from J $\beta 1^{M4/\omega}$ or J $\beta 1^{M2/\omega}$ DN thymocytes was incubated with the BW linker in the presence (+) or absence (-) of T4 DNA ligase and heminested LMPCRs performed to detect 3' D $\beta 1$ (B) and J $\beta 1.2$ (C) signal ends as described in the Materials and Methods section. Analyses of J $\beta 1.2$ signal ends was performed on ligated thymocyte DNA that was serially diluted into nonligated DNA keeping the total amount of DNA constant. Cell equivalents of ligated template DNA are indicated. Products from ligation of the BW linker to signal ends from the J $\beta 1^\omega$ (ω), J $\beta 1^{M4}$ (M4), and J $\beta 1^{M2}$ (M2) alleles are indicated. Molecular weight markers are shown. Also shown is a RAG-2 gene PCR performed on ligated and nonligated template DNA and probed with the R2P oligonucleotide.

cleavage is not coordinated between J $\beta 1$ 12-RSSs on the J $\beta 1^{M2}$ allele. However, it is notable that cleavage at J $\beta 1$ 12-RSSs is not promoted by V β 23-RSSs suggesting that coordinated cleavage is regulated by constraints beyond 12/23 compatibility (see below).

Requirements for DNA Cleavage at the 5' D β RSS. Replacing the 5'D $\beta 1$ 12-RSS with the J $\beta 1.2$ 12-RSS on the J $\beta 1^{M4}$ allele causes a block in V β to D β rearrangement due to a requirement for the 5'D $\beta 1$ 12-RSS to target V β rearrangement beyond simply enforcing the 12/23 rule (22; Fig. 1 A). To determine whether this restriction occurs at the DNA cleavage step of the V(D)J recombination reaction, 5'D $\beta 1$ signal end cleavage intermediates were assayed in J $\beta 1^{M4/\omega}$ DN thymocytes (Fig. 3 A). These analyses yielded a 350 base pair LMPCR product expected from ligation of the linker to 5'D $\beta 1$ signal ends from either the J $\beta 1^{M4}$ or J $\beta 1^\omega$ allele (Fig. 3 B). This product digested completely with Eco0109 (present only in the 5'D $\beta 1$ RSS) and not Taq I (present only in the J $\beta 1.2$ 12-RSS), demonstrating that it is derived entirely from J $\beta 1^\omega$ 5'D $\beta 1$ signal ends (Fig. 3, A and B). Signal ends from cleavage at the J $\beta 1^\omega$ 5'D $\beta 1$ RSS can be detected from less than 600 cell equivalents of J $\beta 1^{M4/\omega}$ DN thymocyte DNA (Fig. 3 C).

The inability to detect J $\beta 1^{M4}$ 5'D $\beta 1$ signal ends could result from efficient cleavage at this RSS followed by rapid resolution of signal ends through signal join formation or through hybrid and open-and-shut join formation which

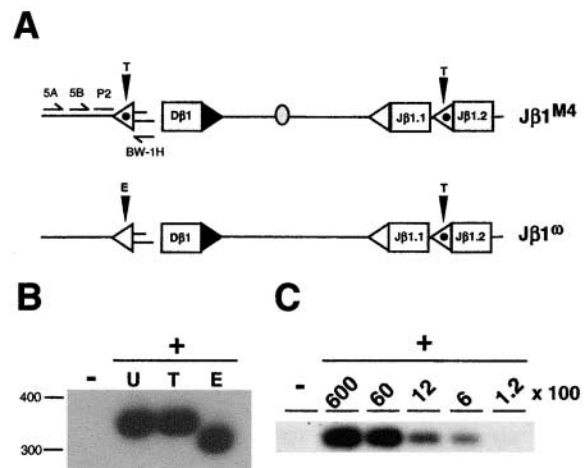


Figure 3. Cleavage at the 5'D β RSS. (A) Schematic of the J $\beta 1^\omega$ and J $\beta 1^{M4}$ alleles as described in the legend to Fig. 1 and the position of the oligonucleotide primers (5A, 5B, and BW-1H) used to amplify linker ligated to 5' D $\beta 1$ signal ends and the oligonucleotide probe (P2) for these products. The TaqI (T) and Eco0109 (E) sites present in the native J $\beta 1.2$ and 5' D $\beta 1$ RSSs are indicated. (B) Linker ligated (+) and nonligated (-) J $\beta 1^{M4/\omega}$ DN thymocyte DNA assayed for 5'D β RSS signal ends by heminested LMPCR using the above primer sets as described in Materials and Methods section. Undigested (U) LMPCR products or products digested with TaqI (T) or Eco0109 (E) are indicated. (C) 5'D β signal ends were assayed in ligated J $\beta 1^{M4/\omega}$ DN thymocyte DNA that was serially diluted into nonligated DNA keeping the total amount of DNA constant. The cell equivalents of ligated DNA are indicated. Molecular weight markers are shown.

may occur in settings where standard joining of RAG-1/2 cleavage products is prohibited (26–29). Analysis of $J\beta 1^{M4/\omega}$ DN thymocyte DNA by PCR revealed that $V\beta/D\beta$ signal and hybrid joins were derived entirely from the $J\beta 1^\omega$ allele as evidenced by the digestion of PCR products with Eco0109 and not Taq I (Fig. 4). PCR analysis of $J\beta 1^{M4}$ DJ β rearrangements in 20 previously described $J\beta 1^{M4/\omega}$ T cell hybridomas demonstrated that none had undergone $V\beta/D\beta$ hybrid join formation (22; data not shown). Furthermore, sequence analyses of these rearrangements revealed only one with sequence heterogeneity at the 5'D β 1 heptamer-coding junction consistent with possible open-

and-shut joining (data not shown). Together these data demonstrate that the absence of detectable $J\beta 1^{M4}$ 5'D β 1 signal ends in $J\beta 1^{M4/\omega}$ DN thymocytes does not result from efficient cleavage at this RSS with rapid resolution of signal ends as signal, open-and-shut or hybrid joins. Rather the inability to detect these ends reflects a primary defect at the DNA cleavage step due to constraints imposed by the 5'D β 1 RSS beyond simply having an appropriate spacer length.

Unique Restrictions Coordinate DNA Cleavage during $V\beta$ to $D\beta$ Rearrangement. To determine whether the novel restriction on DNA cleavage at the 5' D β 1 RSS is coordinated with $V\beta$ RSSs, $J\beta 1^{M4/M4}$, and $J\beta 1^{\omega/\omega}$ mice were bred to SJL mice to generate SJL $\beta/J\beta 1^{M4}$ and SJL $\beta/J\beta 1^\omega$ mice, respectively. The SJL TCR β locus has a 100 kb deletion that deletes nine functional $V\beta$ gene segments, including $V\beta 8.3$, $V\beta 11$, and $V\beta 12$ (Fig. 5 A). Thus, DNA DSBs at RSSs flanking these $V\beta$ gene segments in SJL $\beta/J\beta 1^{M4}$ and SJL $\beta/J\beta 1^\omega$ mice must be derived from the $J\beta 1^{M4}$ and $J\beta 1^\omega$ alleles, respectively. Signal ends from DNA cleavage at $V\beta 8.3$, $V\beta 11$, and $V\beta 12$ RSSs on the $J\beta 1^\omega$ allele were detected over a 100-fold dilution of linker ligated DNA from SJL $\beta/J\beta 1^\omega$ DN thymocytes (Fig. 5 B). Strikingly, $V\beta 8.3$, $V\beta 11$, and $V\beta 12$ RSS signal ends from the $J\beta 1^{M4}$ allele were not detected over similar dilutions of linker ligated DNA from SJL $\beta/J\beta 1^{M4}$ DN thymocytes (Fig. 5 B). The observed differences were not due to differential LMPCR efficiencies between the two samples as signal ends from cleavage at $V\beta 2$, $V\beta 15$, and 5'D β 1 RSSs (present on the $J\beta 1^{M4}$, $J\beta 1^\omega$, and SJL β alleles) were detected at similar levels in SJL $\beta/J\beta 1^{M4}$ and SJL $\beta/J\beta 1^\omega$ thymocyte DNA (Fig. 5 B). As expected, 5'D β 1 RSS cleavage intermediates in SJL $\beta/J\beta 1^{M4}$ thymocytes were derived entirely from the SJL β allele (data not shown). Together these findings demonstrate that $V\beta$ to $D\beta$ rearrangement proceeds through a coordinate DNA cleavage step that is constrained by RSSs beyond simple 12/23 compatibility, as replacing the 5'D β 1 12-RSS with the $J\beta 1.2$ 12-RSS prevents cleavage at native $V\beta$ 23-RSSs on the $J\beta 1^{M4}$ allele.

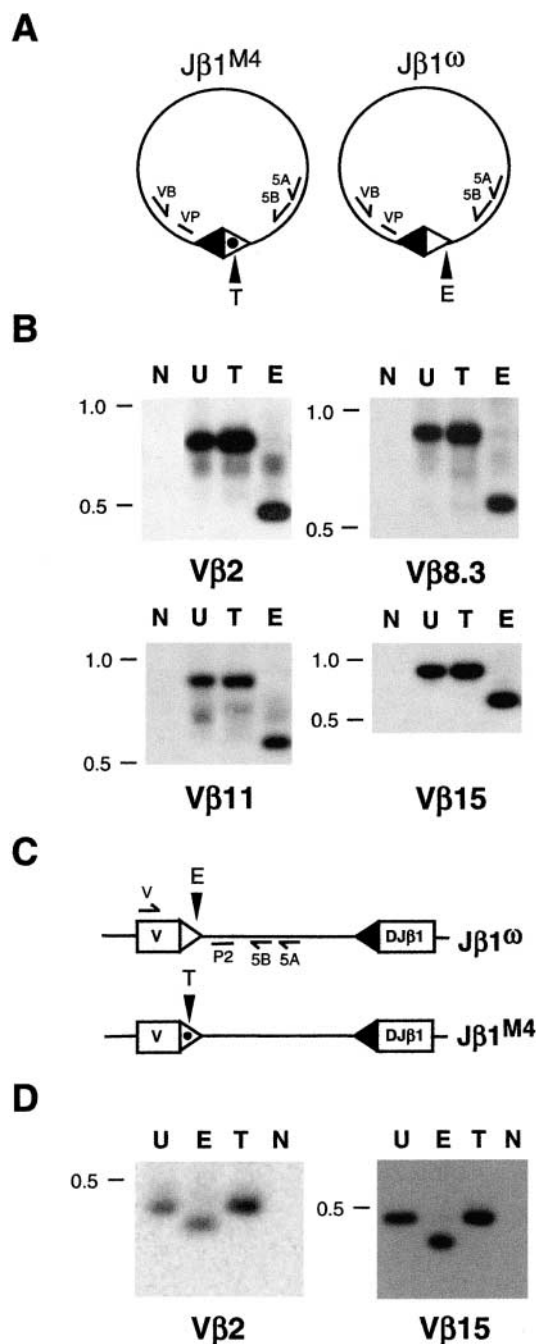


Figure 4. $V\beta/D\beta$ signal and hybrid joins. Shown are schematics of $V\beta/D\beta$ signal joins (A) and $V\beta/D\beta$ hybrid joins (C) on the $J\beta 1^{M4}$ and $J\beta 1^\omega$ alleles. The $V\beta$ 23-RSS (filled triangle) and the $J\beta 1^{M4}$ and $J\beta 1^\omega$ 5'D β 1 12-RSSs (open and open dotted triangles, respectively), the Eco0109 (E) and Taq I (T) sites in the 5'D β and $J\beta 1.2$ RSSs are indicated. (B) $V\beta/D\beta$ signal joins were amplified from $J\beta 1^{M4/\omega}$ DN thymocyte DNA by heminested PCR using the 5A/5B oligonucleotide primers and $V\beta 2$ ($V\beta 2$), $V\beta 8.3$ ($V\beta 8.3$), $V\beta 11$ ($V\beta 11$), or $V\beta 15$ ($V\beta 15$) oligonucleotide primers. PCR products were probed with $VP 2$, $VP 8.3$, $VP 11$, and $VP 15$ oligonucleotides, respectively. (D) $V\beta/D\beta$ hybrid joins were amplified by heminested PCR using the 5A/5B oligonucleotide primers and $V 2$ ($V\beta 2$) or $V 15$ ($V\beta 15$) oligonucleotide primers. PCR products were probed with the $P 2$ oligonucleotide. $VP 8.3$, $VP 11$, and $VP 15$, respectively. Shown are PCR products from reactions without template DNA (N) or those from reactions with template DNA after the PCR products were undigested (U) or TaqI (T) or Eco0109 (E) digested. Molecular weight markers are shown.

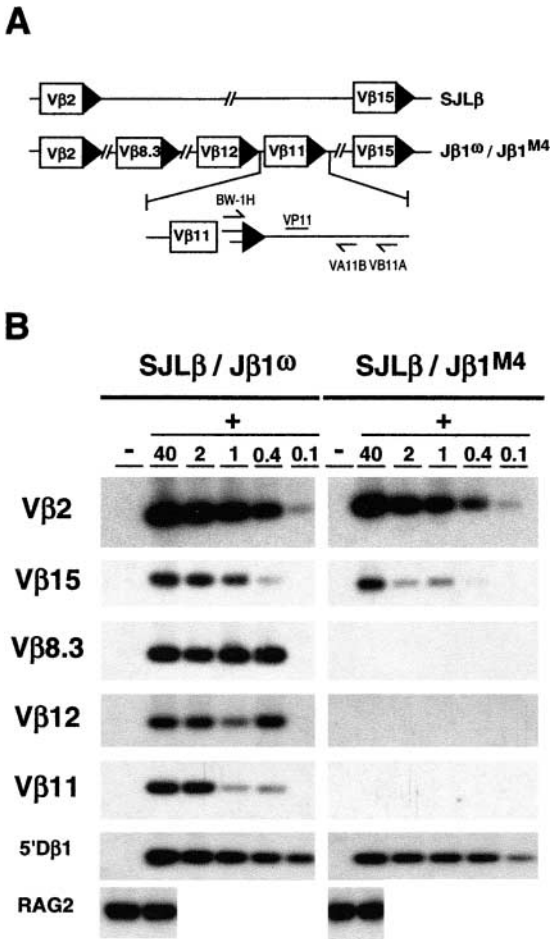


Figure 5. Coordination of DNA cleavage during Vβ to Dβ rearrangement. (A) Schematic of Vβ 2, 8.3, 12, 11, and 15 gene segments on the SJLβ, Jβ1^{M2}, and Jβ1^{M4} alleles. Also shown are the oligonucleotides used to amplify BW linker ligated Vβ11 signal ends (BW-1H, VA11, and VB11). (B) Ligated (+) DNA from SJLβ:Jβ1^{M2} and SJLβ:Jβ1^{M4} DN thymocytes was diluted into nonligated (-) DNA (relative dilutions are indicated) and assayed by a hemi-nested LMPCR for cleavage at the Vβ 2, 15, 8.3, 12, and 11 RSSs. PCR products were probed with the VP2, VP15, VP8.3, VP12, and VP11 oligonucleotides, respectively. Assay of cleavage at the 5'Dβ RSS was performed as described in the legend to Fig. 3. RAG-2 gene PCR products on ligated and nonligated DNA samples are also shown.

Discussion

By analyzing mice with RSS mutations in the endogenous TCRβ locus, we demonstrate that DNA cleavage is coordinated between appropriate RSS pairs in the context of chromosomal V(D)J recombination. Importantly, this requirement restricts the introduction of DNA DSBs primarily to pairs of RSSs flanking gene segments that subsequently participate in coding join formation. Cleavage at Jβ RSSs fails to occur in the absence of a functional 3' Dβ RSS (Jβ1^{M2} allele). Similarly, cleavage at Vβ RSSs fails to occur in the absence of a functional 5' Dβ RSS (Jβ1^{M4} allele) demonstrating that coordination of DNA cleavage occurs over chromosomal distances in excess of 400 kb.

These findings reveal several important constraints imposed on the generation of paired DNA DSBs during chromosomal V(D)J recombination. As was observed from in vitro analyses, we find that DNA cleavage in vivo is also regulated within the context of the 12/23 rule (20, 21, 30). However, pairing and cleavage between the 5'Dβ1 12-RSS and Vβ 23-RSSs on the Jβ1^{M4} allele did not occur, demonstrating the existence of restrictions on RSS synapsis beyond 12/23 compatibility. This may be due to the functional synapsis of Vβ/Dβ and Dβ/Jβ, but not Vβ/Jβ, 12/23 RSS pairs. Alternatively, expression of developmental stage and/or lineage specific factors may alter the ability of the V(D)J recombinase to use specific RSSs or RSS pairs to generate a synaptic complex. Such factors may function, for example, by promoting RSS synapsis over great distances in the chromosome. Importantly, these findings suggest that some aspects of the developmental regulation of variable region gene assembly may occur at the level of the V(D)J recombination reaction itself.

Cleavage at Vβ or Jβ RSSs lacking appropriate RSS partners (Jβ1^{M4} or Jβ1^{M2} alleles, respectively) was not coordinated through pairing with cryptic RSSs which are predicted to occur at significant frequencies within the genome. This suggests that mechanisms are in place that disfavor synapsis involving cryptic RSSs. Such mechanisms would limit rearrangements involving cryptic RSS within the TCRβ locus which would be nonfunctional and possibly preclude subsequent rearrangements. Furthermore, rearrangements involving cryptic RSSs outside of the locus could result in chromosomal deletions, inversions, or translocations (see below).

Coordination of the DNA cleavage step also promotes efficient assembly of variable region genes by limiting the generation of nonpaired DNA DSBs. For example, cleavage at isolated RSSs during Dβ to Jβ rearrangement could lead to the introduction of as many as 15 DSBs at a single TCRβ allele (all 3'Dβ and Jβ RSSs). DNA DSBs not ultimately resolved as coding and signal joins would have to be resolved as open-and-shut or hybrid joins. Open-and-shut joins accompanied by signal end nucleotide loss would preclude subsequent rearrangement of the gene segment. Hybrid join formation would limit further rearrangements by changing the RSS spacer lengths of the involved gene segments. In addition, hybrid join formation may prevent expression of fully assembled variable region genes. For example, hybrid join resolution of DNA DSBs at Vβ and Jβ gene segments flanking a fully assembled (VD)Jβ variable region gene would invert the orientation of the variable region gene with respect to the constant region gene. In this regard the requirement for cleavage at Vβ 23-RSSs to be coordinated with 5'Dβ 12-RSSs, and not Jβ 12-RSSs, prevents both nonpaired and paired introduction of DNA DSBs at Vβ 23-RSSs and Jβ 12-RSSs.

The generation of DNA DSB intermediates during V(D)J recombination poses a risk for genomic instability through their resolution as chromosomal translocations. This potential risk is underscored by the high incidence of chromosomal translocations and lymphoid tumors in mice deficient

in proteins involved in the response to and repair of DNA DSBs (31–34). The requirement for coordinated DNA cleavage reduces the risk of chromosomal translocations by limiting the generation of nonpaired signal ends which could be joined to DNA DSBs generated elsewhere in the genome or serve as substrates for RAG-1/2-mediated transposition. This requirement also promotes chromosomal stability within the context of paired DNA DSB generation. Variable region genes generated through interchromosomal V(D)J recombination, resulting in chromosomal translocations, are found at very low frequencies in mature T cells (35). Analyses of extrachromosomal recombination substrates have suggested that interchromosomal V(D)J recombination may be prohibited at the joining step with persisting DNA DSBs signaling apoptosis (36). These findings, although controversial (37), suggest that developing lymphocytes may attempt interchromosomal recombination at a higher frequency than would be predicted from analyses of completed rearrangements in mature lymphocytes.

Our findings demonstrate that coordination of the DNA cleavage step occurs preferentially in an intrachromosomal manner. In this regard the 3'D β 1 RSS on the J β 1 $^{\omega}$ allele coordinates cleavage with J β RSSs on the J β 1 $^{\omega}$ allele (intrachromosomal) and not the J β 1 M2 allele (interchromosomal) in J β 1 $^{M2/\omega}$ thymocytes. Similarly, the 5'D β 1 RSS on the SJL β allele coordinates cleavage only with the V β RSSs on the SJL β allele in SJL β /J β 1 M4 thymocytes. These findings demonstrate that interchromosomal V(D)J recombination is prohibited primarily at the DNA cleavage step but do not exclude the possibility that mechanisms may also exist at the joining step of the reaction. V(D)J recombination between two alleles of an antigen receptor locus would result in a chromosomal translocation that would not likely promote genomic instability. Importantly, though, this restriction would also be expected to limit recombination between different loci and to cryptic RSSs in nonreceptor loci which could lead to pathologic chromosomal translocations. The bias for coordinating cleavage between RSSs on the same allele may be due to the probability of synapsis between RSSs linked on the same versus separate chromosomes or to features of synaptic complex assembly. Alternatively, it may be inherent to the manner in which the V(D)J recombination reaction is regulated if, for example, mechanisms are in place to ensure that a single allele rearranges at a time. Such mechanisms may be important to allow for allelic exclusion, which requires that a newly assembled variable region gene be "tested" to determine whether it encodes a functional antigen receptor chain before rearrangement proceeds on the other allele (38).

We thank O. Kanagawa for helpful advice and reagents and E. Unanue, K. Murphy, M. Krangel, and B. Khor for critical review of the manuscript.

This work is supported in part by the National Institutes of Health grant 1 R01 AI47829-01 (B.P. Sleckman). B.P. Sleckman is a recipient of an Investigator Award in General Immunology and Cancer Immunology from the Cancer Research Institute and a Career Award in the Biomedical Sciences from the Burroughs

Wellcome Fund. R.E. Tillman is supported by a predoctoral training grant in tumor immunology from the Cancer Research Institute. M.M. Hughes is supported by a National Institutes of Health graduate training grant.

Submitted: 26 October 2001

Revised: 4 December 2001

Accepted: 12 December 2001

References

1. Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature*. 302:575–581.
2. Schatz, D.G., M.A. Oettinger, and M.S. Schlissel. 1992. V(D)J recombination: molecular biology and regulation. *Annu. Rev. Immunol.* 10:359–383.
3. van Gent, D.C., J.F. McBlane, D.A. Ramsden, M.J. Sadofsky, J.E. Hesse, and M. Gellert. 1995. Initiation of V(D)J recombination in a cell-free system. *Cell*. 81:925–934.
4. McBlane, J.F., D.C. van Gent, D.A. Ramsden, C. Romeo, C.A. Cuomo, M. Gellert, and M.A. Oettinger. 1995. Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. *Cell*. 83:387–395.
5. Gellert, M. 1997. Recent advances in understanding V(D)J recombination. *Adv. Immunol.* 64:39–64.
6. Weaver, D.T. 1995. V(D)J recombination and double-strand break repair. *Adv. Immunol.* 58:29–85.
7. Moshous, D., I. Callebaut, R. de Chasseval, B. Corneo, M. Cavazzana-Calvo, F. Le Deist, I. Tezcan, O. Sanal, Y. Bertrand, N. Philippe, et al. 2001. Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell*. 105:177–186.
8. Jeggo, P.A. 1998. Identification of genes involved in repair of DNA double-strand breaks in mammalian cells. *Radiat. Res.* 150:S80–91.
9. Lewis, S.M., J.E. Hesse, K. Mizuuchi, and M. Gellert. 1988. Novel strand exchanges in V(D)J recombination. *Cell*. 55:1099–1107.
10. Agrawal, A., Q.M. Eastman, and D.G. Schatz. 1998. Transposition mediated by RAG1 and RAG2 and its implications for the evolution of the immune system. *Nature*. 394:744–751.
11. Hiom, K., M. Melek, and M. Gellert. 1998. DNA transposition by the RAG1 and RAG2 proteins: a possible source of oncogenic translocations. *Cell*. 94:463–470.
12. Sleckman, B.P., J.R. Gorman, and F.W. Alt. 1996. Accessibility control of antigen-receptor variable-region gene assembly: role of cis-acting elements. *Annu. Rev. Immunol.* 14:459–481.
13. Hesselein, D.G., and D.G. Schatz. 2001. Factors and forces controlling V(D)J recombination. *Adv. Immunol.* 78:169–232.
14. Hempel, W.M., P. Stanhope-Baker, N. Mathieu, F. Huang, M.S. Schlissel, and P. Ferrier. 1998. Enhancer control of V(D)J recombination at the TCRbeta locus: differential effects on DNA cleavage and joining. *Genes Dev.* 12:2305–2317.
15. Stanhope-Baker, P., K.M. Hudson, A.L. Shaffer, A. Constantinescu, and M.S. Schlissel. 1996. Cell type-specific chromatin structure determines the targeting of V(D)J recombinase activity in vitro. *Cell*. 85:887–897.
16. McMurry, M.T., C. Hernandez-Munain, P. Lauzurica, and

- M.S. Krangel. 1997. Enhancer control of local accessibility to V(D)J recombinase. *Mol. Cell. Biol.* 17:4553–4561.
17. Steen, S.B., L. Gomelsky, and D.B. Roth. 1996. The 12/23 rule is enforced at the cleavage step of V(D)J recombination in vivo. *Genes Cells.* 1:543–553.
 18. Eastman, Q.M., T.M. Leu, and D.G. Schatz. 1996. Initiation of V(D)J recombination in vitro obeying the 12/23 rule. *Nature.* 380:85–88.
 19. van Gent, D.C., D.A. Ramsden, and M. Gellert. 1996. The RAG1 and RAG2 proteins establish the 12/23 rule in V(D)J recombination. *Cell.* 85:107–113.
 20. Hiom, K., and M. Gellert. 1998. Assembly of a 12/23 paired signal complex: a critical control point in V(D)J recombination. *Mol. Cell.* 1:1011–1019.
 21. Agrawal, A., and D.G. Schatz. 1997. RAG1 and RAG2 form a stable postcleavage synaptic complex with DNA containing signal ends in V(D)J recombination. *Cell.* 89:43–53.
 22. Bassing, C.H., F.W. Alt, M.M. Hughes, M. D’Auteuil, T.D. Wehrly, B.B. Woodman, F. Gartner, J.M. White, L. Davidson, and B.P. Sleckman. 2000. Recombination signal sequences restrict chromosomal V(D)J recombination beyond the 12/23 rule. *Nature.* 405:583–586.
 23. Sleckman, B.P., C.H. Bassing, M.M. Hughes, A. Okada, M. D’Auteuil, T.D. Wehrly, B.B. Woodman, L. Davidson, J. Chen, and F.W. Alt. 2000. Mechanisms that direct ordered assembly of T cell receptor beta locus V, D, and J gene segments. *Proc. Natl. Acad. Sci. USA.* 97:7975–7980.
 24. Sleckman, B.P., C.G. Bardon, R. Ferrini, L. Davidson, and F.W. Alt. 1997. Function of the TCR α enhancer in $\alpha\beta$ and $\gamma\delta$ T cells. *Immunity.* 7:505–515.
 25. Schlissel, M., A. Constantinescu, T. Morrow, M. Baxter, and A. Peng. 1993. Double-strand signal sequence breaks in V(D)J recombination are blunt, 5'-phosphorylated, RAG-dependent, and cell cycle regulated. *Genes Dev.* 7:2520–2532.
 26. Melek, M., M. Gellert, and D.C. van Gent. 1998. Rejoining of DNA by the RAG1 and RAG2 proteins. *Science.* 280:301–303.
 27. Bogue, M.A., C. Wang, C. Zhu, and D.B. Roth. 1997. V(D)J recombination in Ku86-deficient mice: distinct effects on coding, signal, and hybrid joint formation. *Immunity.* 7:37–47.
 28. Han, J.O., S.B. Steen, and D.B. Roth. 1997. Ku86 is not required for protection of signal ends or for formation of non-standard V(D)J recombination products. *Mol. Cell. Biol.* 17:2226–2234.
 29. Lewis, S.M., and J.E. Hesse. 1991. Cutting and closing without recombination in V(D)J joining. *EMBO J.* 10:3631–3639.
 30. Hiom, K., and M. Gellert. 1997. A stable RAG1-RAG2-DNA complex that is active in V(D)J cleavage. *Cell.* 88:65–72.
 31. Liao, M.J., and T. Van Dyke. 1999. Critical role for Atm in suppressing V(D)J recombination-driven thymic lymphoma. *Genes Dev.* 13:1246–1250.
 32. Gao, Y., D.O. Ferguson, W. Xie, J.P. Manis, J. Sekiguchi, K.M. Frank, J. Chaudhuri, J. Horner, R.A. DePinho, and F.W. Alt. 2000. Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. *Nature.* 404:897–900.
 33. Xu, Y., T. Ashley, E.E. Brainerd, R.T. Bronson, M.S. Meyn, and D. Baltimore. 1996. Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma. *Genes Dev.* 10:2411–2422.
 34. Elson, A., Y. Wang, C.J. Daugherty, C.C. Morton, F. Zhou, J. Campos-Torres, and P. Leder. 1996. Pleiotropic defects in ataxia-telangiectasia protein-deficient mice. *Proc. Natl. Acad. Sci. USA.* 93:13084–13089.
 35. Aster, J.C., and J. Sklar. 1992. Interallelic V(D)J trans-rearrangement within the beta T cell receptor gene is infrequent and occurs preferentially during attempted D beta to J beta joining. *J. Exp. Med.* 175:1773–1782.
 36. Han, J.O., S.B. Steen, and D.B. Roth. 1999. Intermolecular V(D)J recombination is prohibited specifically at the joining step. *Mol. Cell.* 3:331–338.
 37. Tevelev, A., and D.G. Schatz. 2000. Intermolecular V(D)J recombination. *J. Biol. Chem.* 275:8341–8348.
 38. Alt, F.W., G.D. Yancopoulos, T.K. Blackwell, C. Wood, E. Thomas, M. Boss, R. Coffman, N. Rosenberg, S. Tonegawa, and D. Baltimore. 1984. Ordered rearrangement of immunoglobulin heavy chain variable region segments. *EMBO J.* 3:1209–1219.