Chromosomal position of a V_H gene segment determines its activation and inactivation as a substrate for V(D)J recombination

Jamie Geier Bates, Dragana Cado, Hector Nolla, and Mark S. Schlissel

Division of Immunology, Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720

Complete *IgHC* gene rearrangement occurs only in B cells in a stage-specific and ordered manner. We used gene targeting to reposition a distal V_H gene segment to a region just 5′ of the D_H gene cluster and found its activation to be highly dependent on the chromosomal domain within which it resides. The targeted V_H gene segment rearranged at a higher frequency than its endogenous counterpart, its rearrangement was no longer ordered, and its ability to be silenced by allelic exclusion was lost. Additionally, the targeted V_H gene segment lost lineage specificity, as VDJ_H rearrangement was observed in thymocytes. These data suggest that locus contraction, mimicked by proximal targeting, can override any regulation imposed by DNA sequences immediately surrounding V_H gene segments.

CORRESPONDENCE
Mark S. Schlissel:
mss@berkeley.edu

Abbreviations used: BAC, bacterial artificial chromosome; cDNA, complementary DNA; ChIP, chromatin immunoprecipitation; DP, double positive; dsDNA, double-stranded DNA; hμ, human μ; icμ, intracellular μ; LM-PCR, ligation-mediated PCR; RSS, recombination signal sequence.

V(D)J recombination underlies the remarkable diversity of antigen receptors in the immune system (for review see reference 1). A common recombinase dependent on the lymphocyte-specific gene products RAG1 and RAG2 recognizes and cleaves pairs of conserved recombination signal sequences (RSSs), which flank all Ig and TCR *V*, *D*, and *J* gene segments. Components of the nonhomologous end-joining double-stranded DNA (dsDNA) break repair system then catalyze the formation of coding exons by ligating the pair of broken coding ends to one another. Because RSSs at each of the seven rearranging loci (*IgHC*; κ- and λ *LC*; and *TCRα*, *-β*, *-γ*, and *-δ* chains) are recognized by the same recombinase machinery, the cell-type and stage-specific regulation of rearrangement is thought to rely on the accessibility of the recombinase to specific rearranging loci within chromatin structure (2, 3).

V(D)J recombination is regulated in three types of ways. First, rearrangement occurs in a lineage-specific manner. *Ig* and *TCR* genes rearrange completely only in developing B and T cells, respectively. Second, rearrangement is ordered within a lineage with *IgHC* or *TCRβ* locus rearrangement preceding *IgLC* or *TCRα* locus rearrangement in B and T cells, respectively. In addition, *D*-to-*J* rearrangement precedes *V*-to-*DJ* rearrangement in both *IgHC* and *TCRβ* loci. Finally, recombination in the *IgHC* and *TCRβ* loci are subject to allelic exclusion: the observation that each developing lymphocyte assembles only one functional *IgHC* or *TCRβ* chain gene, contributing to the clonotypic specificity of antigen recognition.

The *IgHC* locus has been extensively studied in an attempt to decipher the molecular basis of regulated V(D)J recombination. As noted in the previous paragraph, V_H-to-DJ_H rearrangement invariably follows D_H-to-J_H rearrangement (4). Direct V_H-to-D_H rearrangement is not observed, even on alleles with a targeted deletion of the J_H cluster of gene segments (5). Perhaps more remarkably, a V_H gene segment will bypass intervening germline D_H segments to join to a partially rearranged DJ_H segment. The D_H-to-J_H step in *IgHC* gene assembly is not lineage specific. DJ_H alleles are found in up to 40% of T cells, but complete VDJ_H alleles are not seen in these cells (6). In addition, unlike V_H-to-DJ_H rearrangement, D_H-to-J_H rearrangement is not subject to allelic exclusion. *IgHC* transgenic mice contain endogenous DJ_H- but minimal VDJ_H-rearranged alleles (7, 8).

Several trans-acting factors and signaling pathways have been implicated in the regulation of V_H-to-DJ_H rearrangement. Mice deficient in the transcription factor Pax5, the histone

J.G. Bates's present address is Stanford University, Stanford, CA 94305.

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methyltransferase Ezh2, or the IL-7Rα chain show defects at this step of *IgHC* gene assembly (9–13). In each of these instances, the defect is greater for the more distal V_H gene segments, suggesting that long-range chromosomal interactions may play an important role in this regulation. This idea is consistent with the results of fluorescent in situ hybridization experiments showing developmentally regulated chromosomal compaction or looping of distal V_H genes into juxtaposition with the D_H-J_H end of the *IgHC* locus (14–17). STAT5 (activated by IL-7Rα signaling), Pax5, and Ezh2 have each been shown to localize to V_H region sequences in vivo (11, 18, 19). Remarkably, forced expression of Pax5 in developing T cells results in the activation of V_H-to-DJ_H rearrangement and partial locus compaction in the "wrong" lineage (14, 20).

Rearrangement within the *IgHC* locus is influenced in cis by the intronic heavy-chain enhancer. Targeted deletion of this element results in a moderate decrease in D_H-to-J_H rearrangement but a near-complete absence of V_H-to-DJ_H rearrangement (21–23). Perhaps surprisingly, deletion of the most J_H-proximal D_H gene, DQ52, along with a promoter 5′ of this gene segment that is responsible for germline transcription of the J_H cluster of gene segments, has little effect on *IgHC* rearrangement (23, 24).

In the present paper, we describe experiments aimed at distinguishing whether DNA sequences immediately surrounding V_H gene segments are sufficient for the proper regulation of V_H-to-DJ_H rearrangement or whether the regulation of rearrangement depends on the chromosomal position and the context of a V_H gene segment. We found that targeting a distal V_H gene segment to a region ∼1 kb 5′ of *DFL16.1* caused it to recruit activating chromatin modifications, to rearrange more frequently than its endogenous counterpart, to rearrange directly to unrearranged D_H gene segments, to violate allelic exclusion, and to lose lineage specificity. We conclude that chromosomal position profoundly affects the regulation of V_H gene segment rearrangement.

RESULTS

Targeted insertion of a distal V_H gene segment into the 5′ of D region of the IgHC locus

To test to what extent chromosomal proximity contributes to the regulation of V_H-to-D_H rearrangement, we used homologous recombination in embryonic stem cells to target a distal V_H*558* family gene segment along with ∼1.3 kb of upstream promoter sequence and ∼500 bp of downstream sequence to a region ∼500 bp 5′ of *DFL16.1* (Fig. 1). The distance from *DFL16.1* to the RSS of the targeted V_H gene (termed V_H-*KI*) is ∼1 kb. Both the conceptual translation of this V_H gene segment and its RSS closely match the consensus for the V_H*558* gene family (unpublished data). In addition, its promoter sequence contains the canonical octamer binding site. Presumably, an identical copy of this V_H gene, referred to as its endogenous counterpart, lies in the distal region of the *IgHC* locus, although the existence of a V_H gene with this exact sequence was not demonstrated in a paper on the sequence of the *IgHC* locus from another mouse strain (25).

V_H-*KI* is frequently rearranged and expressed in knock-in mice

To measure the frequency of V_H-*KI* rearrangement, we took advantage of the fact that the targeted V_H gene possesses an SspI restriction endonuclease site that only one other functional V_H gene is predicted to have (Fig. 2 A). We amplified complementary DNA (cDNA) synthesized from bone marrow, spleen, $CD4^+$ $CD8^+$ (double-positive [DP]) thymocyte, and IL-7–dependent pro–B cell culture RNA from wild-type and homozygous V_H-*KI* mice with a degenerate V_H gene primer that is complementary to the leader sequence of most V_H*558* family genes paired with a primer complementary to an *IgHC* constant region exon. RT-PCR products were subjected to digestion with SspI to assess what fraction of the products represent transcription of a rearranged V_H-*KI* gene segment. The wild-type samples had minimal cleavage from the contribution of endogenous V_H genes, whereas the fraction of cleavable product in the targeted animals was significant (Fig. 2 B), implying that the targeted V_H gene segment rearranges very frequently. In IL-7–dependent pro–B cell cultures, cells are not subject to selective pressure for pre-BCR assembly. In this setting, about half of the total V_H*558* family rearrangements involve the targeted V_H gene segment, and this does not appear to differ in primary cells from bone marrow and spleen of these animals. Thus, *IgHC* rearrangements involving the V_H-*KI* gene segment can apparently undergo positive selection during B cell development and contribute to the B cell repertoire in knock-in mice. As expected, the DP thymocytes do not produce spliced rearranged transcripts.

The targeted V_H gene segment recruits activating chromatin modifications in thymocytes

Because the targeted V_H-*KI* gene segment was inserted within a region containing developmentally regulated histone modifications in cell lines (Fig. 3 A) (26), we proceeded to compare the histone modifications surrounding the targeted V_H gene with those in the region 5′ of *DFL16.1* on the unaltered allele in heterozygous V_H-*KI* animals. We performed chromatin immunoprecipitations (ChIPs) on V_H-*KI* heterozygous RAG^{null} bone marrow cultured in IL-7 and on V_H-*KI* heterozygous RAG^{wt} thymocytes using antibodies that recognize H3 acetylation and H3K4 dimethylation. We used RAG^{null} bone marrow to ensure that sufficient germline sequence would be available for the PCR reaction. The primer set called 5′V amplifies a region ∼2 kb upstream of the V_H gene segment sequence, and 3′V amplifies a region ∼400 bp downstream of the targeted V_H gene segment RSS (Fig. 3 B). Predictably, we found that the targeted V_H gene segment is modestly enriched for H3 acetylation and significantly enriched for H3K4 dimethylation in pro–B cells. To our surprise, we found that the 3′ end of the V_H-*KI* insertion, but not the unperturbed allelic region 5′ of *DFL16.1*, was enriched for H3K4 dimethylation in thymocytes (Fig. 3 C). Thus, the V_H gene segment is capable of recruiting histone modifications not otherwise found 5′ of *DFL16.1* in unperturbed thymocytes.

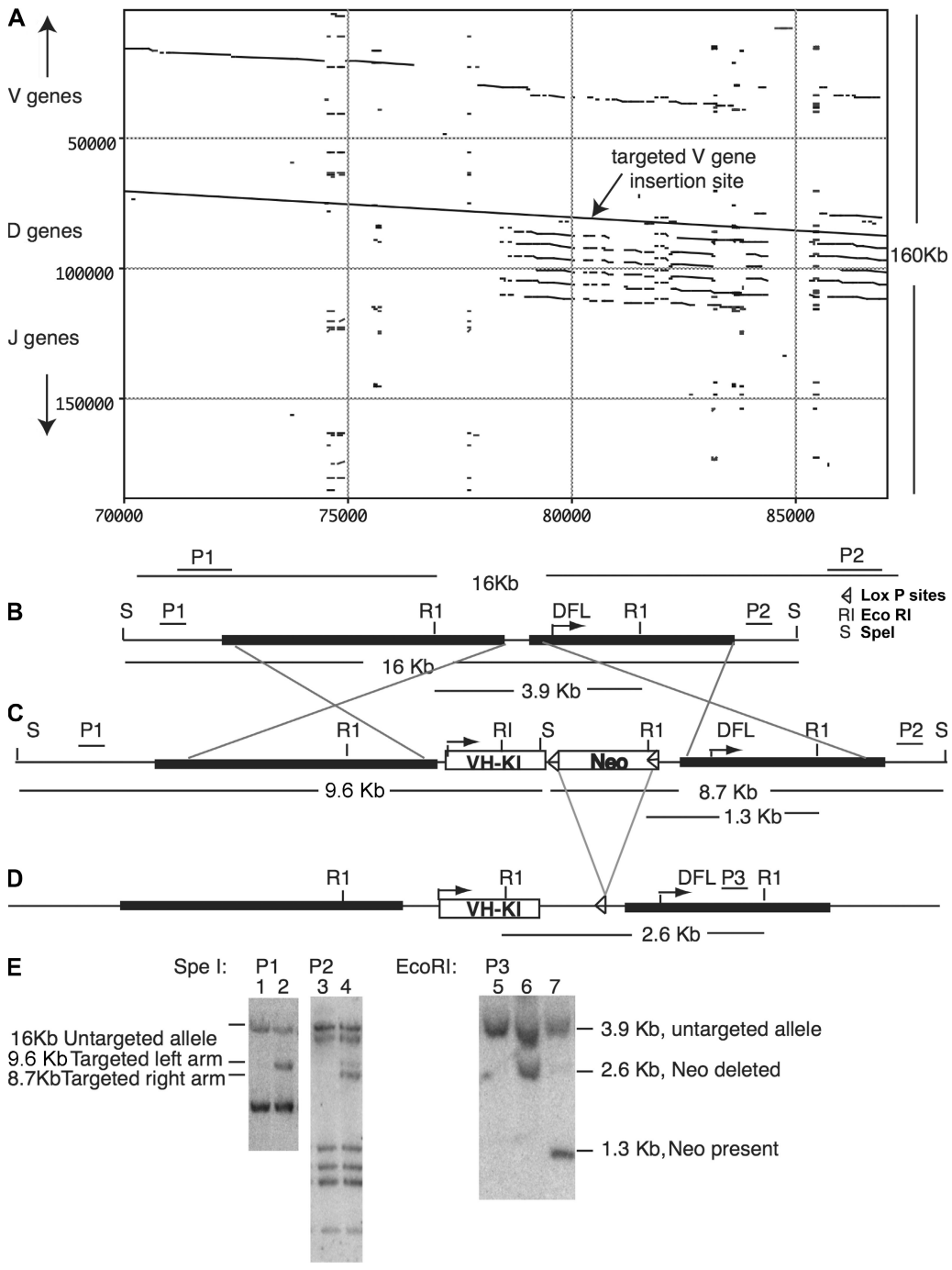

Figure 1. Targeted insertion of the V_H gene segment, *VH–KI*, into a region 5′ of *DFL16.1*. (A) A dot plot of a 160-kb nucleotide region of the *IgHC* locus spanning the V(D)J interval (y axis) against a 16-kb subregion surrounding *DFL16.1* (x axis) reveals the repetitive nature of the D_H cluster. The diagonal line across the entire map represents the exact match of the subfragment with the locus itself, and the other diagonal lines depict repeated regions. Probes for Southern blotting recognized multiple regions of DNA that were differentiated by size after digestion with the restriction enzyme SpeI. The Southern probes P1 and P2 are indicated below the sequences they recognize, and the position of the targeted V_H gene segment embedded in a region of repetitive 5′ of D gene sequence is indicated by the arrow. P1 recognizes only two regions, both within the *V*-to-*D* interval, and P2 recognizes multiple sequences in the D gene repeats. (B) The endogenous locus with the Southern probes P1 and P2, *DFL16.1* (DFL), and SpeI (S) and EcoRI (RI) sites. The SpeI sites at the endogenous locus give rise to the 16-kb segment, whereas targeting of the V_H gene segment introduces a third SpeI site between V_H*-KI* and *DFL16.1*. (C) The targeted locus depicts the addition of V_H*-KI*, the floxed Neo cassette, and the newly introduced SpeI site. (D) Upon Cre-mediated deletion of the floxed Neo, the EcoRI site within the Neo is removed, creating a 2.6-kb product, allowing the distinction of Neo-containing from Neo-deleted mice with P3. (E) Southern blot performed on SpeI-digested DNA and probed with P1 confirmed integration of the left arm (1, germline; 2, targeted). Digestion with SpeI and probing with P2 confirmed integration of the right arm (3, germline; 4, targeted). Mice positive for right- and left-arm targeting were mated onto Cre, and digestion with EcoRI and probing with P3 confirmed deletion of the Neo cassette.

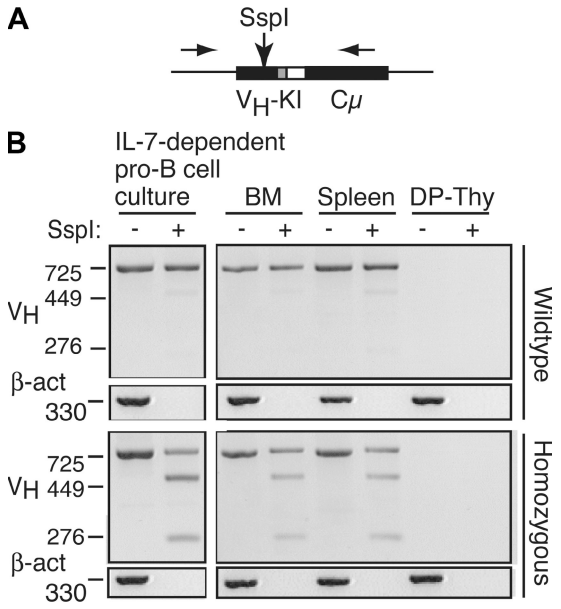

Figure 2. The frequency of rearrangement of the targeted V_H gene segment is substantially increased. A degenerate *V_H558* family leader sequence primer was paired with a constant region primer (Cμ) to amplify random-primed cDNA synthesized using RNA purified from wild-type and homozygous *V_H-KI* knock-in IL-7–dependent pro–B cell cultures, total bone marrow, total spleen, and CD4/CD8 DP thymocytes (DP-Thy). A restriction endonuclease, SspI, specific for the targeted V_H gene segment (as well as one endogenous V gene segment and four pseudogenes) was used to digest the PCR product. (A) A schematic of primers and restriction enzyme digest performed on cDNA. (B) Restriction enzyme–digested (+) and –undigested (−) amplified cDNAs from wild-type and *V_H-KI* knock-in cells were analyzed on agarose gels. As a control, β-actin was amplified, and the products analyzed in every other lane to correspond with the amplified cDNA shown above. Numbers indicate fragment lengths in nucleotides.

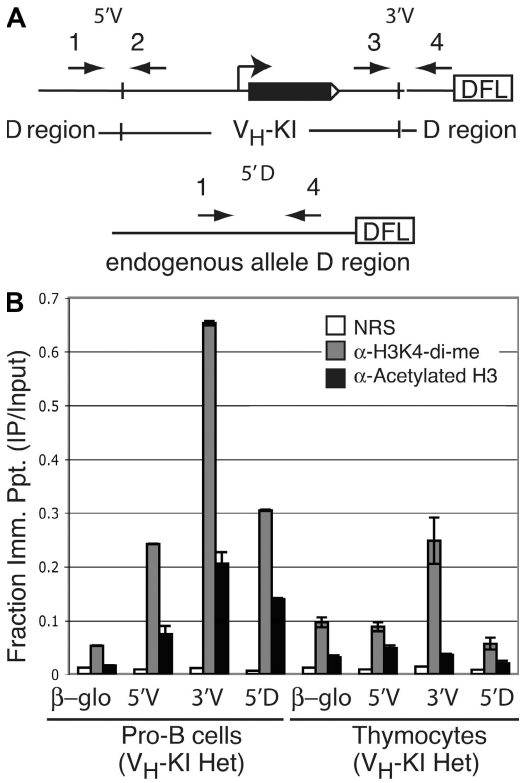

Figure 3. ChIP detects modified histone H3 surrounding the targeted V_H gene segment. (A) Schematic of the PCR primers used to analyze DNA recovered from ChIP using normal rabbit serum (NRS), antiacetylated H3, and antidimethylated H3K4. PCR assays using primers annealing 5′ of *V_H-KI* (1 and 2, 5′V), 3′of *V_H-KI* (3 and 4, 3′V), and on the endogenous allele 5′ of *DFL16.1* (1 and 4, 5′D) are depicted. (B) Enrichment of precipitated as compared with input DNA from IL-7–dependent pro–B cells from a *RAG^{null} V_H-KI* heterozygous (Het) mouse or thymocytes from a *V_H-KI* heterozygous *RAG^{wt}* mouse. Error bars represent one SD from an average of two experiments. β-globin (β-glo) was amplified as a negative control.

V_H-KI undergoes rearrangement in DP thymocytes

Given our observation that the targeted V_H gene segment can recruit H3K4 methylation in T cells, we went on to ask whether the lineage specificity of V_H-to-DJ_H rearrangement was likewise perturbed by repositioning this V_H gene segment. We purified wild-type, heterozygous, and homozygous *V_H-KI* genomic DNA from bone marrow, spleen, and sorted (>99% pure on reanalysis; unpublished data) DP thymocytes and used PCR to detect V_H-to-DJ_H and D_H-to-J_H rearrangements. As expected, we detected rearrangement of *DFL16.1*-to-J_H segments in both wild-type and mutant samples. In wild-type samples, *V_H558*-, *V_H7183*-, and *V_HQ52*-to-DJ_H rearrangement was limited to the bone marrow and spleen (Fig. 4). In contrast, abundant *V_H-KI*–to–DJ_H rearrangements were observed in all three tissues, including DP T cells from both *V_H-KI* heterozygous and homozygous animals. This demonstrates that T cells are capable of performing *V_H-KI*–to–DJ_H rearrangement in a context where endogenous V_H gene segment rearrangement is prohibited, and that such rearrangement does not require Pax5. Indeed, *V_H-KI* rearranges on a Pax5-null background in the bone marrow as well (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20071787/DC1).

Thus, position within the *IgHC* locus contributes to the lineage specificity of V_H-to-DJ_H rearrangement, and T cells possess all of the factors necessary to use V_H gene segments in V(D)J recombination.

The order of recombination is not tightly regulated in *V_H-KI* mice

In developing B cells, D_H gene segments almost invariably rearrange to J_H gene segments before V_H-to-DJ_H rearrangement (4). To test whether the *V_H-KI* gene segment undergoes normally ordered rearrangement, we assayed genomic DNA from wild-type, heterozygous, and homozygous *V_H-KI* mice for direct V_H-to-D_HQ52 rearrangement over a distance of ∼60,000 nucleotides (Fig. 5 A). As expected, we failed to detect V_H-to-D_H rearrangements of either *V_H558* or *V_H7183* family V_H gene segments in wild-type bone marrow. *V_H7183*-to-D_HQ52 rearrangements were detected sporadically and at low levels in wild-type spleen and thymus DNA samples. In contrast, we detected significant levels of such rearrangements in *V_H-KI* DNA samples from all three tissues in the

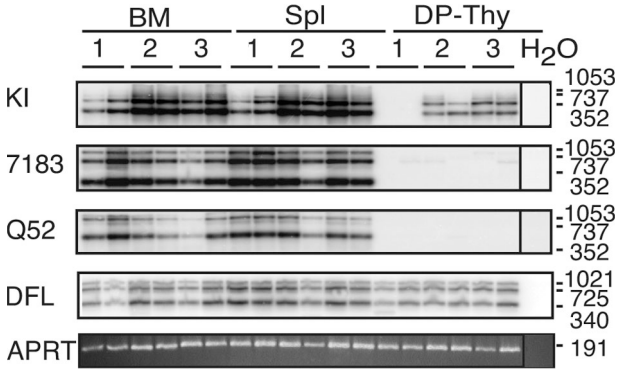

Figure 4. *V_H-KI* undergoes complete V(D)J rearrangement in CD4/CD8 DP thymocytes. Genomic DNA was purified from total bone marrow, total spleen (Spl), and sorted CD4/CD8 DP thymocytes (DP-Thy) from wild-type (1), heterozygous (2), and homozygous (3) *V_H-KI* mice. Two mice representing each genotype are shown. Products from a PCR rearrangement assay using degenerate primers for the *V_HJ558*, *V_H7183*, and *V_HQ52* gene families (as well as *DFL16.1* as a control) paired with a J_H3 reverse primer were analyzed on an agarose gel, Southern blotted, and probed with an internal J_H3 probe. The last lane in each set of assays (separated by the vertical black bar) is a PCR assay programmed with water in place of template. A phosphorimage is shown. APRT was amplified and run on an agarose gel as a loading control. Numbers indicate fragment lengths in nucleotides.

targeted mice (Fig. 5 B). These rearrangements occurred by deletion and not inversion, because the downstream primer was 3′ of the *DQ52* gene segment and not within the D_H gene segment itself. Thus, the ordered regulation of *IgHC* gene assembly is dependent on the position of V_H gene segments within the locus.

The targeted V_H gene segment violates allelic exclusion at the levels of rearrangement and protein expression

It has been suggested that ordered *IgHC* assembly might be necessary for effective allelic exclusion (3). Because we observed that positioning *V_H-KI* proximal to *DFL16.1* resulted in an increased frequency of direct V_H-to-D_H rearrangement, we went on to ask whether *IgHC* allelic exclusion was disrupted by this mutation as well. We addressed this possibility in two ways. First, we assayed genomic DNA purified from FACS-sorted wild-type and *V_H-KI* bone marrow pro– and pre–B cells and thymocytes for dsDNA breaks at various RSSs (Fig. 6 A). Such dsDNA breaks are reaction intermediates in V(D)J recombination and indicate active rearrangement of the gene segment under study at the time of DNA isolation (27). Pro–B cells were defined as $B220^+CD43^+$ intracellular μ^- ($ic\mu^-$) and pre–B cells as $B220^+CD43^-ic\mu^+$. Our analysis revealed that pro–B cells contain dsDNA breaks at RSSs 5′ of *DFL16.1* but not at those 5′ of *Jκ5*. In contrast, wild-type pre–B cells possess abundant breaks at RSSs 5′ of *Jκ5*, but not at those 5′ of *DFL16.1*. Remarkably, in the targeted animals, RSS breaks are easily detectable in pre–B cell DNA both 5′ of *DFL16.1* and 3′ of *V_H-KI*, indicating active *V_H-KI*–to–D_H rearrangement in violation of allelic exclusion (Fig. 6 A). The primers used to amplify 3′of *V_H-KI* signal end breaks were not degenerate and, therefore, did not amplify other members of the V_H gene family. Breaks at the *V_H-KI*

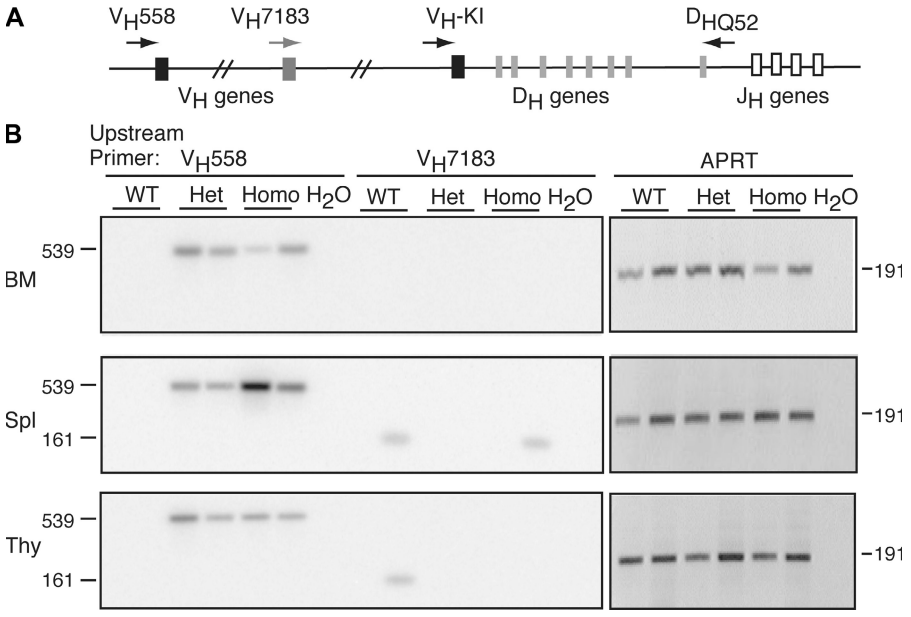

Figure 5. The order of *IgHC* gene assembly is perturbed in *V_H-KI* mice. DNA purified from wild-type, heterozygous (Het), or homozygous (Homo) *V_H-KI* bone marrow, spleen (Spl), or thymus (Thy) was analyzed for direct V_H-to-D_H rearrangement by pairing degenerate V_H gene segment–family primers (*V_HJ558* or *V_H7183*) with a reverse primer downstream of *D_HQ52* (A). The *V_HJ558* primer anneals to the V_H leader sequence, whereas the *V_H7183* primer anneals within FR3, resulting in PCR products of different sizes. (B) Each pair of lanes represents DNA from two different mice. The Southern blot analysis of PCR assays is shown (left), probed with an internal primer downstream of DQ52. The *APRT* gene was amplified from the same samples as a control and was visualized on an agarose gel (right). Numbers indicate fragment lengths in nucleotides.

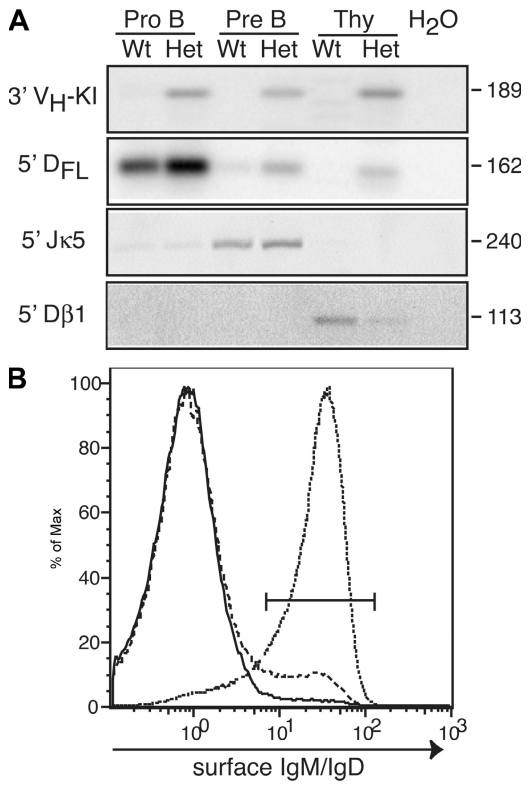

Figure 6. The targeted V_H gene segment violates allelic exclusion. (A) LM-PCR assay for RAG-induced dsDNA breaks at RSSs downstream of V_H-*KI* and upstream of *DFL16.1*, *Jκ5*, and *Dβ1* in DNA purified from sorted wild-type or heterozygous (Het) V_H-*KI* bone marrow from pro- ($CD43^+ic\mu^-$) and pre- ($CD43^-ic\mu^+$) B cells or thymocytes. Four to six littermate or age-matched mice of each genotype were pooled for staining and sorting. Southern blot analysis of the LM-PCR is shown probed with internal gene-specific primers. Numbers indicate fragment lengths in nucleotides. (B) V_H-*KI* is rearranged and expressed on splenic B cells from hμ-transgenic mice. Spleens were harvested from wild-type, wild-type × hμ-transgenic, and V_H-$KI^{+/-}$ × hμ-transgenic mice and stained with fluorochrome-labeled anti-B220 and anti–mouse IgM and IgD antibodies. Mouse IgM/IgD staining is displayed on a histogram after gating on $B220^+$ cells. Wild-type × hμ-transgenic mice (continuous line) have 2.51 ± 0.16% IgM/IgD^+ splenic B cells (horizontal bar indicates gate; $n = 4$), whereas V_H-*KI* heterozygous × hμ-transgenic mice (dashed line) have 9.98 ± 1.11% IgM/IgD^+ splenic B cells ($n = 4$). A wild-type mouse (dotted line) is shown as a reference for mouse IgM/IgD expression among $B220^+$ splenic B cells.

endogenous counterpart in wild-type pro–B cells were below the level of detection. However, we could not discern whether V_H-*KI* or 5′ of *DFL16.1* RSS breaks occur on unrearranged or DJ_H-rearranged knock-in alleles. Nonetheless, we conclude that the targeted V_H gene is able to undergo rearrangement into the pre–B cell stage, when endogenous V_H-to-DJ_H recombination is silenced by allelic exclusion.

To further examine *IgHC* allelic exclusion, we bred a well-characterized human μ (hμ) transgene onto either a wild-type or V_H-*KI* heterozygous genetic background. Expression of a transgenic hμ protein in developing B cells inhibits endogenous V_H-to-DJ_H rearrangement and surface expression of mouse *IgHC* on splenic B cells (Fig. 6 B, continuous line) (7). In contrast, the hμ transgene has far less of an effect on the expression of mouse *IgHC* protein in V_H-*KI* mice (Fig. 6 B, dashed line). Flow cytometric analysis of surface mouse IgM/IgD expression on $B220^+$ splenocytes in hμ-transgenic mice revealed a fourfold increase in mouse μ expression when one targeted V_H-*KI* allele is present (Fig. 6 B).

We went on to examine the frequency of V(D)J-rearranged alleles in bone marrow cDNA from wild-type and V_H-*KI* mice in the presence of the hμ transgene. This analysis confirmed that the targeted V_H gene was indeed abundantly rearranged in the bone marrow of targeted animals on the hμ transgenic background (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20071787/DC1). Collectively, these experiments show that repositioning a V_H558 gene segment to a location proximal to *DFL16.1* results in the disruption of *IgHC* locus allelic exclusion.

DISCUSSION

We have demonstrated that the chromosomal position of a V_H gene segment within the *IgHC* locus rather than V_H gene–associated sequences greatly influences the frequency, order, and cell-type specificity of its rearrangement. Eμ, the only known enhancer in the J_H region of the locus, is required to promote optimal accessibility of the *IgHC* locus for both D_H-to-J_H and V_H-to-DJ_H rearrangement, yet D_H-to-J_H rearrangement consistently precedes V_H-to-DJ_H rearrangement (4, 21–23). Moreover, several recent studies have demonstrated that locus contraction occurs in correlation with but independently of *V*-to-*DJ* rearrangement (15–17). We hypothesized that differential regulation of the clusters of D_H and V_H gene segments depends on the distance between these sequences within the nucleus. Through the repositioning of a normally distal V_H558 gene segment, this is precisely what we observed.

The frequency with which the V_H-*KI* gene segment rearranges is greatly enhanced by repositioning, which was quite unexpected considering similar experiments in T cells at the *TCRβ* locus (28). The *TCRβ* locus is much like the *IgHC* locus in that it has V, D, and J gene segments. It is the first locus to undergo rearrangement in T cells and, thus, like the *IgHC* locus, requires silencing to enforce allelic exclusion during *TCRα* rearrangement. *Vβ* gene segments are separated from *Dβ* and *Jβ* gene segments by ∼350 kb of DNA, with the exception of *Vβ14*, which lies ∼10 kb downstream of the *DJCβ* clusters on the far side of the only known enhancer in the locus, *Eβ* (29). The *TCRβ* locus undergoes ordered and cell type–specific rearrangement: *Dβ*-to-*Jβ* is followed by *Vβ*-to-*DJβ* rearrangement only in T cells and is strictly dependent on *Eβ*. *Eβ* deletion results in the absence of germline transcription that normally precedes any rearrangement, and homozygous *Eβ*-deleted animals lack αβ T cells altogether (30, 31). The domain of chromatin structure regulated by *Eβ*, as identified by a restriction enzyme accessibility assay, extends only 2 kb upstream of *Dβ1* (32).

Insertion of a targeted *Vβ* gene segment ∼7 kb upstream of *Dβ1* did not increase its frequency of rearrangement (26). However, deleting *Dβ1* along with the 350-kb *Vβ*-*Dβ* interval

resulted in a significant increase in the frequency of rearrangement of those *Vβ* gene segments now positioned much closer to *Eβ* (33). Thus, only when the entire *Vβ*-to-*Dβ* interval was deleted did the frequency of *Dβ* gene segment–proximal *Vβ* genes segments increase. This could be explained by the presence of a boundary element in the *Vβ*-to-*Dβ* interval that prevents the spreading of open chromatin from extending to the *Vβ* gene segments even when they are brought much closer to the *Dβ* and *Jβ* gene segments. Similarly, it is possible that the increase in rearrangement frequency of V_H*-KI* is caused by its position within the realm of accessibility potentially limited by an analogous chromosomal boundary.

Lineage specificity and the role of transcription factors and histone modifications in V_H-to-DJ_H rearrangement

We found that the chromosomal context of a V_H gene segment influences the lineage specificity of its rearrangement. Previous studies had shown that transgenic expression of Pax5 in thymocytes was sufficient to activate V_H-to-DJ_H rearrangement and cause compaction of the distal and proximal regions of the *IgHC* locus (14, 20). More recently, it was shown that Pax5 can bind directly to a subset of V_H gene segments and can recruit the recombinase to these V_H gene segments via a protein–protein interaction (19). Pax5 had also been shown to be necessary for removal of inhibitory histone methylation around the distal V_H gene segments (34). The 1.3 kb of 5′ of V_H promoter region sequence upstream of the targeted V_H gene does contain potential Pax5 binding sites, but V_H*KI*–to–DJ_H rearrangement was independent of Pax5 in thymocytes and in IL-7–dependent bone marrow culture, calling into question an obligatory role for this transcription factor in *IgHC* V(D)J recombination (Fig. S1). Our results are more consistent with Pax5 regulating V_H-to-DJ_H rearrangement by bringing distal V_H gene segments into proximity with D_H segments (compaction).

IL-7 signaling has been proposed to play a role in *IgHC* allelic exclusion by regulating STAT5 binding to V_H gene segment promoters (18, 35, 36). This idea was recently challenged by the observation that allelic exclusion is intact in the presence of a constitutively active STAT5 (37). Our data also argue against a role for V_H gene promoters in establishing allelic exclusion because the targeted V_H gene promoter is not sufficient to enforce allelic exclusion. It remains possible, however, that IL-7 signaling is required for V_H gene activation, because IL-7 signaling does play a role in T cell development (38, 39). Indeed, in $STAT5ab^{-/-}$ mice, Pax5 and Ezh2 expression are normal and chromosomal contraction occurs, but rearrangement is nonetheless impaired (36). Thus, STAT5 binding to V_H gene promoters and subsequent histone acetylation may be necessary to promote but not sufficient to properly regulate V_H gene segment activation. V_H gene segment promoters and RSSs from RAG^{null} IL-7–dependent pro–B cells are H3K4 methylated, but sorted double-negative thymocyte V_H gene segment promoters and RSSs remain unmethylated at H3K4, suggesting a role for this modification in the activation of V_H gene segment recombination (34). In agreement with this, we see recruitment of H3K4 methylation to the targeted V_H gene segment RSS in both IL-7–dependent bone marrow culture from RAG^{null} heterozygous V_H*-KI* mice and primary thymocytes from RAG^{wt} heterozygous V_H*-KI* mice.

Ordered rearrangement

Various mechanisms have been proposed to explain the ordered nature of *IgHC* gene assembly. One such mechanism involves the preferential binding of RAG complexes to 3′ of D_H RSSs, limiting the accessibility of the RAGs to the 5′ of D_H RSSs until after D_H-to-J_H rearrangement deletes the 3′ of D_H RSS. This, however, cannot be the case, because we see direct V_H to D_H joining on the targeted locus. It is worth noting that we only observed direct V_H*-KI*–to–D_H rearrangements involving the *DQ52* gene segment; no direct V_H*-KI*–to–*DSP2* family gene segment rearrangement was observed (unpublished data). Promoters upstream of the D_H gene segments become active upon D_H-to-J_H rearrangement (40, 41), and it may be that rearrangement-induced transcription attracts V_H genes to rearranged DJ_H gene segments. Although not all D_H gene segments were tested, the observation that *DQ52* is noticeably available for direct V_H*-KI*–to–D_H rearrangement could be a reflection of the promoter upstream of *DQ52* (driving the μ° germline transcript), which is active before D_H-to-J_H rearrangement (40, 42). The failure of V_H genes in their normal chromosomal positions to rearrange to the accessible *DQ52* might be caused by boundary element activity or simply distance within the nucleus.

Allelic exclusion and V_H gene segment position

Rearrangement of the targeted V_H*-KI* allele was not subject to allelic exclusion imposed by an hμ transgene. This observation is consistent with previously published results showing that endogenous D_H-proximal V_H gene segments continue to rearrange at a low but detectable frequency in *IgHC* transgenic mice (8). What is surprising is that the targeted V_H*-KI* gene segment was frequently expressed, increasing the fraction of cells concomitantly expressing both mouse and human *IgHC* by fourfold compared with wild-type hμ transgenic mice. This is in contrast to the inserted *Vβ* gene segment within the *TCRβ* locus, where in the presence of a transgenic *TCRβ* the targeted gene was not subject to allelic exclusion at the level of rearrangement, but protein expression was inhibited (28).

The targeted V_H*-KI* gene segment also escaped allelic exclusion in nontransgenic mice. We detected dsDNA RSS breaks 3′ of V_H*-KI* as well as 5′ of *DFL16.1* in sorted pre–B cell DNA from heterozygous knock-in but not wild-type animals. We detected a much stronger dsDNA RSS break signal from the targeted V_H*-KI* gene segment as compared with endogenous V_H gene segments in the pro–B cell samples, which can be explained by the increased frequency of rearrangement of the targeted gene segment (Fig. 2). The persistence of 5′ of *DFL16.1* breaks in pre–B cells and thymocytes from V_H*-KI* knock-in but not wild-type mice speaks more accurately to the contrast between the wild-type and

heterozygous animals. Thus, targeting a V_H gene segment to this chromosomal position affects the inactivation as well as the activation of the V_H gene segment rearrangement.

The chromosomal domain model

Changes in the proximity of V_H gene segments to $E\mu$ upon D_H-to-J_H rearrangement does not adequately explain the order, frequency, and cell-type specificity of V_H gene segment rearrangement in wild-type mice. *DQ52* rearrangement to J_H gene segments deletes as little as 700 bp of DNA, hardly enough to significantly alter the configuration of a 2-mb locus. Additionally, the targeted V_H*-KI* gene segment can rearrange directly to *DQ52*, which is over 80 kb away, roughly the same length of DNA that normally separates V_H*81X* and *DFL16.1* (~90 kb). This argues that distance alone might not account for such radical changes in the regulation of V_H gene segment recombination. We have demonstrated that D_H-to-J_H rearrangement itself does not account for the activation of the V_H*-KI* gene segment, because direct V_H*-KI*–to–D_H rearrangements are observed. It is possible that the region 5′ of *DFL16.1* into which we inserted *VH-KI* has enhancer or promoter activity or that the region of H3K4 methylation 5′ of *DFL16.1* in B cells (Fig. 3, A and B) (26) stimulates V(D)J recombination, but this cannot account for the activation of V_H*-KI* gene segment rearrangement in thymocytes. Of the two histone modifications we explored, the only modifications seen in thymocytes at this locus are those recruited by the targeted V_H gene, not by the endogenous locus (Fig. 3).

What might explain readily detectable V_H*-KI* rearrangement in thymocytes? We favor the possibility that V_H and D_H gene segments normally occupy distinct chromosomal domains, but the targeted V_H*-KI* gene segment now lies within the D_H domain. This D_H domain is normally accessible to the recombinase in both developing B and T cells. Our results lead us to two possible models. The V_H genes themselves can undergo activation in either B or T cells, but the timing, frequency, and lineage specificity of V_H-to-DJ_H rearrangement may be dependent on either (a) locus compaction or (b) a V_H domain control element that is unable to affect the repositioned V_H gene. Both of these models require that the V_H and D_H domains remain functionally separate, suggesting the existence of a boundary element. We are in the process of targeting the V_H*-KI* gene segment to a series of locations progressively further from *DFL16.1*.

MATERIALS AND METHODS

Cell lines. 63-12 (RAG2^{null} Abelson murine leukemia virus [AMuLV]–transformed pro–B cell [43]; provided by F. Alt, Harvard Medical School, Boston, MA), P5424 (RAG2^{null}, p53^{null} pro–T cell [44]; provided by P. Mombaerts, The Rockefeller University, New York, NY), and Pax5^{null} (AMuLV-transformed pro–B cell line derived from mice containing LacZ in place of exon 2 of Pax5 [45]; provided by M. Busslinger, Research Institute of Molecular Pathology, Vienna, Austria) cell lines were grown at 37°C/5% CO_2 in RPMI 1640 supplemented with 5–10% FCS, antibiotics, and 50 μM β-mercaptoethanol.

Primary pro–B cell culture. Bone marrow was harvested from the femurs of 4–8-wk-old mice and rid of red blood cells by ACK lysis. Cells were cultured on S17 stromal cells in the presence of 10% IL-7–containing culture supernatant in RPMI 1640 with 10% FCS, antibiotics and 50 μM β-mercaptoethanol. After culture, >95% of cells stained positively for surface CD19 or B220. Primary thymocytes were purified by centrifugation with HISTOPAQUE-1083 (Sigma-Aldrich), after which they were >90% CD4/CD8 positive. Animal experimentation procedures were approved by the University of California, Berkeley Animal Care and Use Committee.

ChIP. ChIP was performed as previously described (46). 10 μg of anti-dimethyl H3K4 antibody (Millipore), 15 μl of antisera against acetylated histone H3 (Millipore), or 10 μg of normal rabbit IgG (Santa Cruz Biotechnology, Inc.) were used for IP. The ratio of immunoprecipitated to input DNA for a given genomic region (IP/input) was defined as $2^{\wedge}(Ct_{input}-Ct_{IP})$, where Ct is the cycle threshold for real-time PCR with SYBR green technology. Chromatin from cell lines was precleared with protein A/G–sepharose (Millipore) blocked with sheared salmon sperm DNA and BSA, whereas chromatin from primary cells was precleared with unblocked sepharose before IP. Error bars represent one SD from an average of two experiments. 5×10^6 cell equivalents were used per IP for cell lines, and 2.5×10^6 cell equivalents were used for primary cells. PCR primers are listed in Supplemental materials and methods (available at http://www.jem.org/cgi/content/full/jem.20071787/DC1).

Targeting construct and V_H*-KI* mutant mice. The targeting vector consisted of a left arm of 5.6 kb (from 74623–80260 of the bacterial artificial chromosome [BAC] available from GenBank/EMBL/DDBJ under accession no. AC073553), the V_H gene segment in the sense orientation, a loxP-flanked neomycin resistance cassette in the opposite transcriptional direction, and a right arm of 2.5 kb (from 80260–82746 of the same BAC) of homologous sequence. The V_H gene was targeted to a position ~500 bp 5′ of *DFL16.1* (~80700 of the same BAC) in the same transcriptional orientation as *DFL16.1*. Cloning of the V_H gene segment and probes used for Southern blot analyses are described in Supplemental materials and methods.

Cell staining and sorting. Spleen and thymus were strained through a 40-μm cell strainer, whereas femurs and tibias were dissected from mice and crushed with a mortar and pestle or flushed with a syringe. Lymphocytes were isolated by density centrifugation using HISTOPAQUE-1083. Antibodies used for sorting DP thymocytes were anti-CD8α–FITC (BD Biosciences) and CD4-PE (BD Biosciences). For transgenic hμ analyses, splenocytes were stained with B220-PE (BD Biosciences), IgM-biotin (clone II/41; BD Biosciences), and IgD-biotin (clone 11–26; eBioscience) with streptavidin-Cy5 (BD Biosciences). Antibodies used for sorting cells for ligation-mediated PCR (LM-PCR) were rat anti–mouse IgM-biotin (clone 1B4B1; SouthernBiotech), IgD-biotin (for magnetic bead depletion; SouthernBiotech), CD43-biotin (clone S7; BD Biosciences) with streptavidin-cychrome (BD Biosciences), B220-PE (BD Biosciences), and anti–mouse IgM-FITC (clone II/41; BD Biosciences), for intracellular staining after fixation in 1% paraformaldehyde and permeabilization with 0.1% saponin.

LM-PCR. LM-PCR for detection of in vivo–generated broken signal ends was performed as previously described (47, 48). Primers are listed in Supplemental materials and methods.

Online supplemental material. Fig. S1 shows that *Pax5* is not necessary for V_H*-KI* rearrangement in developing B cells. Fig. S2 shows that V_H*-KI* is not subject to allelic exclusion. Supplemental materials and methods provides information about targeting construct cloning and probes for Southern screening, as well as primers used for PCR assays, including ChIP, frequency of V_H*-KI* gene rearrangement, recombination assays, and LM-PCR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20071787/DC1.

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REFERENCES

1. Hesslein, D.G., and D.G. Schatz. 2001. Factors and forces controlling V(D)J recombination. *Adv. Immunol.* 78:169–232.
2. Krangel, M.S. 2003. Gene segment selection in V(D)J recombination: accessibility and beyond. *Nat. Immunol.* 4:624–630.
3. Jung, D., C. Giallourakis, R. Mostoslavsky, and F.W. Alt. 2006. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annu. Rev. Immunol.* 24:541–570.
4. 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.
5. Chen, J., M. Trounstine, F.W. Alt, F. Young, C. Kurahara, J.F. Loring, and D. Huszar. 1993. Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the JH locus. *Int. Immunol.* 5:647–656.
6. Corcoran, L., I. Ferrero, D. Vremec, K. Lucas, J. Waithman, M. O'Keeffe, L. Wu, A. Wilson, and K. Shortman. 2003. The lymphoid past of mouse plasmacytoid cells and thymic dendritic cells. *J. Immunol.* 170:4926–4932.
7. Nussenzweig, M.C., A.C. Shaw, E. Sinn, D.B. Danner, K.L. Holmes, H.C. Morse III, and P. Leder. 1987. Allelic exclusion in transgenic mice that express the membrane form of immunoglobulin mu. *Science.* 236:816–819.
8. Costa, T.E., H. Suh, and M.C. Nussenzweig. 1992. Chromosomal position of rearranging gene segments influences allelic exclusion in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 89:2205–2208.
9. Nutt, S.L., P. Urbanek, A. Rolink, and M. Busslinger. 1997. Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev.* 11:476–491.
10. Hesslein, D.G., D.L. Pflugh, D. Chowdhury, A.L. Bothwell, R. Sen, and D.G. Schatz. 2003. Pax5 is required for recombination of transcribed, acetylated, 5′ IgH V gene segments. *Genes Dev.* 17:37–42.
11. Su, I.H., A. Basavaraj, A.N. Krutchinsky, O. Hobert, A. Ullrich, B.T. Chait, and A. Tarakhovsky. 2003. Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement. *Nat. Immunol.* 4:124–131.
12. Corcoran, A.E., A. Riddell, D. Krooshoop, and A.R. Venkitaraman. 1998. Impaired immunoglobulin gene rearrangement in mice lacking the IL-7 receptor. *Nature.* 391:904–907.
13. Chowdhury, D., and R. Sen. 2001. Stepwise activation of the immunoglobulin mu heavy chain gene locus. *EMBO J.* 20:6394–6403.
14. Fuxa, M., J. Skok, A. Souabni, G. Salvagiotto, E. Roldan, and M. Busslinger. 2004. Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. *Genes Dev.* 18: 411–422.
15. Sayegh, C., S. Jhunjhunwala, R. Riblet, and C. Murre. 2005. Visualization of looping involving the immunoglobulin heavy-chain locus in developing B cells. *Genes Dev.* 19:322–327.
16. Roldan, E., M. Fuxa, W. Chong, D. Martinez, M. Novatchkova, M. Busslinger, and J.A. Skok. 2005. Locus "decontraction" and centromeric recruitment contribute to allelic exclusion of the immunoglobulin heavy-chain gene. *Nat. Immunol.* 6:31–41.
17. Kosak, S.T., J.A. Skok, K.L. Medina, R. Riblet, M.M. Le Beau, A.G. Fisher, and H. Singh. 2002. Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. *Science.* 296:158–162.
18. Stanton, M.L., and P.H. Brodeur. 2005. Stat5 mediates the IL-7-induced accessibility of a representative D-Distal VH gene. *J. Immunol.* 174:3164–3168.
19. Zhang, Z., C.R. Espinoza, Z. Yu, R. Stephan, T. He, G.S. Williams, P.D. Burrows, J. Hagman, A.J. Feeney, and M.D. Cooper. 2006. Transcription factor Pax5 (BSAP) transactivates the RAG-mediated V(H)-to-DJ(H) rearrangement of immunoglobulin genes. *Nat. Immunol.* 7:616–624.
20. Hsu, L.Y., H.E. Liang, K. Johnson, C. Kang, and M.S. Schlissel. 2004. Pax5 activates immunoglobulin heavy chain V to DJ rearrangement in transgenic thymocytes. *J. Exp. Med.* 199:825–830.
21. Chen, J., F. Young, A. Bottaro, V. Stewart, R.K. Smith, and F.W. Alt. 1993. Mutations of the intronic IgH enhancer and its flanking sequences differentially affect accessibility of the JH locus. *EMBO J.* 12:4635–4645.
22. Serwe, M., and F.V. Sablitzky. 1993. (D)J recombination in B cells is impaired but not blocked by targeted deletion of the immunoglobulin heavy chain intron enhancer. *EMBO J.* 12:2321–2327.
23. Afshar, R., S. Pierce, D.J. Bolland, A. Corcoran, and E.M. Oltz. 2006. Regulation of IgH gene assembly: role of the intronic enhancer and 5′DQ52 region in targeting DHJH recombination. *J. Immunol.* 176:2439–2447.
24. Nitschke, L., J. Kestler, T. Tallone, S. Pelkonen, and J. Pelkonen. 2001. Deletion of the DQ52 element within the Ig heavy chain locus leads to a selective reduction in VDJ recombination and altered D gene usage. *J. Immunol.* 166:2540–2552.
25. Johnston, C.M., A.L. Wood, D.J. Bolland, and A.E. Corcoran. 2006. Complete sequence assembly and characterization of the C57BL/6 mouse Ig heavy chain v region. *J. Immunol.* 176:4221–4234.
26. Morshead, K.B., D.N. Ciccone, S.D. Taverna, C.D. Allis, and M.A. Oettinger. 2003. Antigen receptor loci poised for V(D)J rearrangement are broadly associated with BRG1 and flanked by peaks of histone H3 dimethylated at lysine 4. *Proc. Natl. Acad. Sci. USA.* 100:11577–11582.
27. 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.
28. Sieh, P., and J. Chen. 2001. Distinct control of the frequency and allelic exclusion of the V beta gene rearrangement at the TCR beta locus. *J. Immunol.* 167:2121–2129.
29. Glusman, G., L. Rowen, I. Lee, C. Boysen, J.C. Roach, A.F. Smit, K. Wang, B.F. Koop, and L. Hood. 2001. Comparative genomics of the human and mouse T cell receptor loci. *Immunity.* 15:337–349.
30. Bories, J.C., J. Demengeot, L. Davidson, and F.W. Alt. 1996. Gene-targeted deletion and replacement mutations of the T-cell receptor beta-chain enhancer: the role of enhancer elements in controlling V(D)J recombination accessibility. *Proc. Natl. Acad. Sci. USA.* 93:7871–7876.
31. Bouvier, G., F. Watrin, M. Naspetti, C. Verthuy, P. Naguet, and P. Ferrier. 1996. Deletion of the mouse T-cell receptor beta gene enhancer blocks alphabeta T-cell development. *Proc. Natl. Acad. Sci. USA.* 93:7877–7881.
32. Mathieu, N., W.M. Hempel, S. Spicuglia, C. Verthuy, and P. Ferrier. 2000. Chromatin remodeling by the T cell receptor (TCR)-beta gene enhancer during early T cell development: Implications for the control of TCR-beta locus recombination. *J. Exp. Med.* 192:625–636.
33. Senoo, M., L. Wang, D. Suzuki, N. Takeda, Y. Shinkai, and S. Habu. 2003. Increase of TCR V beta accessibility within E beta regulatory region influences its recombination frequency but not allelic exclusion. *J. Immunol.* 171:829–835.
34. Johnson, K., D.L. Pflugh, D. Yu, D.G. Hesslein, A.L. Bothwell, A. Thomas-Tikhonenko, D.G. Schatz, and K. Calame. 2004. B cell-specific loss of histone 3 lysine 9 methylation in the V(H) locus depends on Pax5. *Nat. Immunol.* 5:853–861.
35. Chowdhury, D., and R. Sen. 2003. Transient IL-7/IL-7R signaling provides a mechanism for feedback inhibition of immunoglobulin heavy chain gene rearrangements. *Immunity.* 18:229–241.
36. Bertolino, E., K. Reddy, K.L. Medina, E. Parganas, J. Ihle, and H. Singh. 2005. Regulation of interleukin 7-dependent immunoglobulin heavy-chain variable gene rearrangements by transcription factor STAT5. *Nat. Immunol.* 6:836–843.
37. Will, W.M., J.D. Aaker, M.A. Burchill, I.R. Harmon, J.J. O'Neil, C.A. Goetz, K.L. Hippen, and M.A. Farrar. 2006. Attenuation of IL-7 receptor signaling is not required for allelic exclusion. *J. Immunol.* 176:3350–3355.
38. Hoelbl, A., B. Kovacic, M.A. Kerenyi, O. Simma, W. Warsch, Y. Cui, H. Beug, L. Hennighausen, R. Moriggl, and V. Sexl. 2006. Clarifying the role of Stat5 in lymphoid development and Abelson-induced transformation. *Blood.* 107:4898–4906.
39. Yao, Z., Y. Cui, W.T. Watford, J.H. Bream, K. Yamaoka, B.D. Hissong, D. Li, S.K. Durum, Q. Jiang, A. Bhandoola, L. Hennighausen, and

J.J. O'Shea. 2006. Stat5a/b are essential for normal lymphoid development and differentiation. *Proc. Natl. Acad. Sci. USA*. 103:1000–1005.
40. Alessandrini, A., and S.V. Desiderio. 1991. Coordination of immunoglobulin DJH transcription and D-to-JH rearrangement by promoter-enhancer approximation. *Mol. Cell. Biol*. 11:2096–2107.
41. Yancopoulos, G.D., and F.W. Alt. 1985. Developmentally controlled and tissue-specific expression of unrearranged VH gene segments. *Cell*. 40:271–281.
42. Li, Y.S., R. Wasserman, K. Hayakawa, and R.R. Hardy. 1996. Identification of the earliest B lineage stage in mouse bone marrow. *Immunity*. 5:527–535.
43. Shinkai, Y., S. Koyasu, K. Nakayama, K.M. Murphy, D.Y. Loh, E.L. Reinherz, and F.W. Alt. 1993. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. *Science*. 259:822–825.
44. Mombaerts, P., C. Terhorst, T. Jacks, S. Tonegawa, and J. Sancho. 1995. Characterization of immature thymocyte lines derived from T-cell receptor or recombination activating gene 1 and p53 double mutant mice. *Proc. Natl. Acad. Sci. USA*. 92:7420–7424.
45. Urbanek, P., Z.Q. Wang, I. Fetka, E.F. Wagner, and M. Busslinger. 1994. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell*. 79:901–912.
46. Fernandez, L.A., M. Winkler, and R. Grosschedl. 2001. Matrix attachment region-dependent function of the immunoglobulin mu enhancer involves histone acetylation at a distance without changes in enhancer occupancy. *Mol. Cell. Biol*. 21:196–208.
47. Constantinescu, A., and M.S. Schlissel. 1997. Changes in locus-specific V(D)J recombinase activity induced by immunoglobulin gene products during B cell development. *J. Exp. Med*. 185:609–620.
48. Liang, H.E., L.Y. Hsu, D. Cado, L.G. Cowell, G. Kelsoe, and M.S. Schlissel. 2002. The "dispensable" portion of RAG2 is necessary for efficient V-to-DJ rearrangement during B and T cell development. *Immunity*. 17:639–651.