Localized epigenetic changes induced by D<sub>H</sub> recombination restricts recombinase to DJ<sub>H</sub> junctions

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Genes encoding immunoglobulin heavy chains (Igh) are assembled by rearrangement of variable (V<sub>H</sub>), diversity (D<sub>H</sub>) and joining (J<sub>H</sub>) gene segments. Three critical constraints govern V<sub>H</sub> recombination. These include timing (V<sub>H</sub> recombination follows D<sub>H</sub> recombination), precision (V<sub>H</sub> gene segments recombine only to DJ<sub>H</sub> junctions) and allele specificity (V<sub>H</sub> recombination is restricted to DJ<sub>H</sub>-recombined alleles). Here we provide a model for these universal features of V<sub>H</sub> recombination. Analyses of DJ<sub>H</sub>-recombined alleles showed that DJ<sub>H</sub> junctions were selectively epigenetically marked, became nuclease sensitive and bound localized 5′(JH) gene segments. Three critical constraints govern VH recombination. These include timing (VH recombination follows D<sub>H</sub> recombination), precision (V<sub>H</sub> gene segments recombine only to DJ<sub>H</sub> junctions) and allele specificity (V<sub>H</sub> recombination is restricted to DJ<sub>H</sub>-recombined alleles). Here we provide a model for these universal features of VH recombination. Analyses of DJ<sub>H</sub>-recombined alleles showed that DJ<sub>H</sub> junctions were selectively epigenetically marked, became nuclease sensitive and bound recombinase proteins, which thereby permitted D<sub>H</sub>-associated recombination signal sequences to initiate the second step of Igh gene assembly. We propose that VH recombination is precise, because these changes did not extend to germline D<sub>H</sub> segments located 5′ of the DJ<sub>H</sub> junction.

Genes that encode antigen receptors of lymphocytes are assembled via DNA-recombination events that juxtapose gene segments spread over several megabases of the genome. V(D)J recombination, as this process is known, is precisely coordinated with the lineage and developmental stage of lymphocytes<sup>1,2</sup>. Thus, genes that encode immunoglobulins rearrange in the B lymphocyte lineage, whereas genes that encode T cell antigen receptors rearrange in the T lymphocyte lineage. In the B lineage, genes that encode immunoglobulin heavy chains (Igh) rearrange first, followed by rearrangement of genes that encode immunoglobulin light chains (IgL and Igk). Similarly, in the T lineage, genes that encode the T cell antigen receptor β-chain (Tcrb) rearrange first, followed by rearrangement of Tcrα genes. The loci that rearrange first in each lineage (Igh and Tcrb) consist of variable (V), diversity (D) and joining (J) gene segments and require two recombination events to generate fully recombined alleles. In each case, D-to-J recombination precedes recombination of the V region to the preformed DJ junction to produce VDJ-recombined alleles. Thus, understanding the assembly of genes that encode antigen receptors involves the delineation of mechanisms that select a locus for rearrangement and impose the order of V(D)J recombination at the Igh and Tcrb loci.

V(D)J recombination requires recruitment of the recombinase components RAG-1 and RAG-2 to loci destined for rearrangement. Thereafter, RAG-1 and RAG-2 introduce double-strand breaks at special recombination signal sequences (RSSs) that flank gene segments to initiate recombination. The accessibility of a locus to the RAG recombinase determines the ‘choice’ of the antigen-receptor gene that will recombine. This is called the ‘accessibility hypothesis’<sup>3</sup>. Accessibility, in turn, is regulated by cis-acting accessibility-control elements (ACEs), which coincide with promoters and enhancers in antigen-receptor loci<sup>4</sup>. At one level, therefore, the order of rearrangements of the gene encoding the B cell antigen receptor can be viewed as follows: Igh accessibility precedes Igk accessibility, and in the Igh locus, D<sub>H</sub> segments become accessible before the V<sub>H</sub> segments do.

Since the earliest formulation of the accessibility hypothesis, chromatin structure has been considered a key determinant of locus accessibility<sup>5,6</sup>, however, the molecular features that distinguish accessible loci from inaccessible loci are just beginning to be understood<sup>7–9</sup>. All antigen-receptor loci contain acetylated histones before the initiation of recombination in the appropriate lymphocyte lineage and at the appropriate developmental stage<sup>1,4,10</sup>. Where examined, rearrangeable loci are also marked with activation-associated histone methylation, such as di- or trimethylation of histone H3 Lys4 (H3K4me2 or H3K4me3, respectively). Conversely, the repressive histone modification dimethylation of histone H3 Lys9 (H3K9me2) is diminished before recombination<sup>11,12</sup>. Moreover, recruitment of the H3K9 methyl transferase G9a to a Tcrb minilocus recombination substrate attenuates recombination, which thereby provides direct evidence of the inhibitory effects of this modification<sup>13</sup>. The function of specific positive modifications in V(D)J recombination remains unclear, however, because it is difficult to modulate these marks independently and to assess the effects on recombination. The recognition that the plant homeodomain...
of RAG-2 binds H3K4me3 has led to a model in which epigenetic histone modifications mark a locus for the recruitment of RAG-1 and RAG-2 (refs. 14–16).

The Igh locus comprises approximately 150 VH gene segments, 10–13 D1H gene segments and 4 J1H gene segments17. The initial activation of D1H recombination (rather than V1H recombination) and the ‘preferential’ use of certain D1H gene segments are explained by several observations. First, analysis of RAG-deficient pro-B cells has shown that only the 5′- and 3′-most D1H gene segments that flank the D1H region (DFL16.1 and DSQ2, respectively) and the region encompassing the IJH gene segments extending to exons of the μ-chain constant region (Cμ) have hallmarks of active chromatin11,18. These include the presence of activating histone modifications, nucleosome sensitivity and pockets of DNA demethylation (R. Selimyan, I.L., R.Subrahmanyam, F.W.A., R. Sen et al., data not shown). Unlike flanking D1H gene segments, the intervening D1H gene segments (DSP2 segments) are marked by H3K9me2, a repressive chromatin modification, which may result in less use of these gene segments in the mature B cell repertoire9,10. The absence of activating histone marks in the V1H part of the Igh locus has led to a model in which V1H gene segments are relatively inaccessible to recombine at this stage15. Second, the IJH region has the greatest density of RAG proteins in the Igh locus26; in contrast, RAG proteins are undetectable at V1H gene segments in pro-B cells. Thus, recombina

Localized activation of DJH junctions

To analyze the state of DJH-rearranged loci, we generated a panel of cell lines with specific Igh rearrangements. For this, we transiently transfected RAG-2-deficient mouse fetal liver–derived 6312 cells with a RAG-2 expression vector and isolated single-cell clones with recombinant Igh alleles. Because RAG-2 was expressed transiently, these clones were genetically stable thereafter. We assayed DJH recombination by PCR (Supplementary Fig. 1) and used representative clones (2B9, 2F1, 2C10 and 1E3) in chromatin assays. We first examined the changes that accompany rearrangement of a DSP2 gene segment located in the middle of the DJH cluster. One allele in 2B9 cells had a DSP2.2b-JH1 rearrangement, and the second allele had undergone V1H recombination, which resulted in the deletion all unrearranged D1H gene segments (Fig. 1a). We designed primers specific to the 5′ region of the rearranged DSP2.2b gene segment and compared the histone-modification state of the DJH-rearranged allele with that of germline alleles in the parent cell line.

Chromatin-immunoprecipitation (ChIP) assays showed that the 3-kilobase region 5′ of the DJH junction had a greater abundance of activation-related modifications, such as acetylation of histone H3 and feedback inhibition of V1H recombination24,25. Before these features of V1H gene segment selectivity come into play, however, three general aspects of V1H recombination must be addressed. First, why does V1H recombination always follow DJH recombination? Second, why does V1H recombination occur selectively on DJH-recombined alleles? Third, what is the mechanism that directs V1H gene segments

Figure 1 Chromatin accessibility at a DSP2.2b-JH1-rearranged allele. (a) Germline Igh locus in 6312 cells and the DJH-rearranged Igh locus in 6312-derivative 2B9 cells, which have a DSP2.2b-JH1 junction in one allele and a V1H rearrangement in the other allele (not drawn to scale); light green (block) arrows represent DJH-associated repeat sequences10,11. Black horizontal lines (bottom) indicate positions of amplicons analyzed by real-time PCR (throughout). (b) ChIP assay of 6312 and 2B9 cells with antibodies to modified histones (vertical axes), followed by real-time PCR and calculation of the abundance of each amplicon as described11. Numbers in the parentheses indicate position (in kb) 5′ of the DSP2.2b segment. The promoter of the gene encoding γ-actin, the Jµ4 gene segment and Eµ serve as controls for active chromatin, and constant region Cµ3 serves as a control for inactive chromatin (throughout). (c) Dnase I sensitivity of nuclei (2 × 10⁶) from cells with DSP2.2b-JH1 Igh alleles (2B9) or germline Igh alleles (6312), assessed by quantitative real-time PCR. DSP2, unrearranged DSP2 gene segments; Eµ, known Dnase I–hypersensitive site in the IγC-G intron; Cµ3, Dnase I–insensitive site. Data are from two independent experiments (b; average and s.d. of duplicates) or one experiment representative of two independent experiments (c; second experiment, Supplementary Fig. 3b).
H3K9me2 modification but a lower abundance of H3K4me3 modification 3 kb 5′ of the DJI junction. These changes provided plausible explanations for earlier analyses of DJH transcription showing that DFL16.1 and DSP2 rearrangements activate promoters that were dormant in the germline configuration, which results in more recruitment of RNA polymerase II and more sense- and antisense-oriented transcription from the DJH-rearranged alleles. However, both the density of RNA polymerase II and antisense transcripts are much lower in abundance within 2 kb 5′ of the DJH junction than at the peak near the DJH junction. We concluded that DJH recombination led to chromatin activation and transcription that was highly restricted near the DJH junction.

As the most prominent ACE at the Igh locus is the intronic enhancer Eγ, we determined whether the changes in the abundance of histone modifications at the DJH junction were dependent on Eγ by analyzing the status of DJH junctions in pro-B cell lines from Eγ-deficient mice transformed with the v-Abl oncogene of Abelson mouse leukemia virus. We did ChIP assays, with antibody to H3K9ac (anti-H3K9ac) and anti-H3K4me3, of three Eγ-deficient cell lines: FA3, which has two Igh alleles in the germline configuration; FA8, which has a DSP2.8-JH1 rearrangement in one allele and a DQ52-JH2 rearrangement in the other allele; and FA10, which has a DSP2.7-JH2 rearranged allele and a germline allele. We used the Eγ-sufficient 6312 cell line as control for those positive modifications at the Igh locus. We found that in the absence of Eγ, both the germline and the DJH-rearranged loci were completely devoid of those active histone marks (Supplementary Fig. 2), which indicated that activating histone modifications associated with DJH junctions required the intronic enhancer Eγ.

To further confirm the idea that DJH junctions were locally activated, we examined the histone-modification status of unrearranged DJH gene segments in cells that contained DJH-recombined alleles. Unrearranged DSP2 gene segments in 2B9 cells were inactive by several criteria; they lacked H3K9ac and H3K4me3 and retained H3K9me2 (Fig. 2a,b). The same was true in a different cell line, 2F1, that had undergone DQ52-JH1 rearrangement in one allele and DSP2.2a-JH2 rearrangement in the second allele (Fig. 2a,c). We also investigated sequences around DFL16.1 in these cells to determine whether DJH rearrangements affected the peak of activation at the 5′ end of the DJH-Cγ domain. We found that neither DSP2.2 nor DQ52 rearrangement affected the activation state of DJH16.1 positively or negatively (Fig. 2b,c). Consistent with that observation, the DNase I sensitivity of the unrearranged DFL16.1 segment was not altered for DSP2.2b-rearranged alleles (Fig. 1c and Supplementary Fig. 3b). We concluded that DJH recombination led to highly localized histone modification and accessibility changes at DJH junctions that did not extend to germline DJH gene segments upstream.

### Chromatin changes at uniquely located DJH gene segments

Unlike the intervening DSP2 gene segments, the 5′-most and 3′-most DJH gene segments are associated with active chromatin marks in the germline configuration. To determine whether rearrangements of these gene segments also led to additional local chromatin activation, we investigated the state of a DFL16.1-JH1−recombined allele in 2C10 cells and a DQ52-JH1−recombined allele in 2F1 cells (Fig. 3a). The sequences upstream of DFL16.1 were lost.

Figure 2: Histone modifications at unrearranged upstream DJH gene segments in DJH-rearranged Igh alleles. (a) Germline Igh locus in 6312 cells and the rearranged loci in the 6312 derivatives 2B9 and 2F1 cells (not drawn to scale): 2B9 cells have a DSP2.2b-JH1 rearrangement in one allele and a JH3 rearrangement in the second allele; 2F1 cells have DQ52-JH1 and DSP2.2a-JH2 rearrangements. These configurations leave seven germline DJH gene segments in 2B9 cells (one DFL16.1, one DFL16.2 and five DSP2); 2F1 cells have ten germline DJH gene segments (one DFL16.1, one DFL16.2 and eight DSP2) in the DQ52-JH1−rearranged allele and five germline DJH gene segments (one DFL16.1, one DFL16.2 and three DSP2) in the DSP2.2a-JH2−rearranged allele. (b,c) ChIP assay of 6312 cells (b,c), 2B9 cells (b) and 2F1 cells (c) with antibodies to modified histones (vertical axes), followed by real-time PCR analysis of various amplicons (horizontal axes) and calculation of the abundance of each as described. Numbers in parentheses indicate position of amplicon (in kb) 5′ of DFL16.1 (DFL). Data are from two independent experiments (average and s.d. of duplicates).
in the second allele of 2C10 cells, which had undergone V\textsubscript{H}-to-DJ\textsubscript{H} recombination, and the sequences immediately upstream of DQ52 were lost in the second allele of 2F1 cells, which had rearranged an upstream DJ\textsubscript{H} gene segment (DSP2.2a-J\textsubscript{H}2). This configuration of rearrangements in the two cell lines allowed us to unequivocally probe the chromatin state surrounding rearranged DFL16.1 and DQ52 gene segments.

The abundance of H3K9ac was much greater near the recombined DFL16.1-J\textsubscript{H1} junction than in the same location in the germline configuration (Fig. 3b). A greater abundance of H3K9ac also was evident at DFL16.1 (~1.7), the approximate position of the H3K9ac peak in the unrearranged state, but dropped off rapidly further upstream. The same trend was evident for H3K4me3 modification (Fig. 3b). We observed no major changes in H3K4me2 or H3K9me2 modifications around the rearranged DFL16.1 relative to those in the germline segments analyzed in this study and that DSP2.9 rearrangement did not substantially alter the chromatin state around DFL16.1.

Figure 3: Histone modifications at Igh alleles with DFL16.1 and DQ52 rearrangements. (a) Germline Igh locus in 6312 cells and the DJ\textsubscript{H}-rearranged loci in 6312-derived 2C10 and 2F1 cells (not drawn to scale), along with the positions of amplicons analyzed by real-time PCR: 2C10 cells have a DFL16.1-J\textsubscript{H1} rearrangement in one allele and a V\textsubscript{H} rearrangement in the second allele (DFL16.1 primers used here do not bind to the VDJ-recombined allele); 2F1 cells have a DQ52-J\textsubscript{H1} rearrangement in one allele and a DSP2.2a-J\textsubscript{H2} rearrangement in the second allele (DQ52 primers do not bind to the DSP2.2a-J\textsubscript{H2} rearranged allele). (b, c) ChIP assay of 6312 cells (b, c), 2C10 cells (b) and 2F1 cells (c) with antibodies to modified histones (vertical axes), followed by real-time PCR analysis of various amplicons (horizontal axes) and calculation of the abundance of each as described\textsuperscript{11}. Numbers in parentheses indicate position (in kb) 5’ of the rearranged DJ\textsubscript{H} gene segment. DFL, DFL16.1; DQ, DQ52. Data are from two independent experiments (average and s.d.).

Changes in the abundance of histone modifications at DJ\textsubscript{H} junctions involving DFL16.1 and DQ52, we did not observe any changes in DNase I sensitivity at or near these junctions (Supplementary Figs. 4 and 5) relative to that of the corresponding locations on germline alleles. These observations demonstrated that chromatin alterations in response to DJ\textsubscript{H} recombination were highly localized regardless of DJ\textsubscript{H} gene use.

We hypothesized that localized changes that distinguish DJ\textsubscript{H} junctions from upstream unrearranged DJ\textsubscript{H} gene segments would provide a plausible mechanism for the targeting of V\textsubscript{H} recombination to the DJ\textsubscript{H} junction. In this context, DSP2.9 occupies a special position in the DJ\textsubscript{H} cluster. As the first DJ\textsubscript{H} gene segment 3’ of DFL16.1, it is nearest the pocket of active chromatin at the 5’ end of the germline DJ\textsubscript{H} cluster other than DFL16.1 itself. It is therefore possible that chromatin changes associated with DSP2.9 recombination could lead to a large domain of activated chromatin that encompasses both DSP2.9 (rearranged) and DFL16.1 (unrearranged) gene segments. Alternatively, a DSP2.9-rearranged allele may contain two distinct minidomains of active chromatin. To distinguish between those alternatives, we examined the structure of a DSP2.9-J\textsubscript{H2}–rearranged allele in 1E3 cells, which had a DFL16.1-J\textsubscript{H1} rearrangement in the other allele (Fig. 4a). Notably, we again found a highly localized peak of H3K9ac and transcription-associated H3K4me3 modification at the DJ\textsubscript{H} junction. In the presence of J\textsubscript{H} cluster other than DFL16.1 itself. It is therefore possible that chromatin changes associated with DSP2.9 recombination could lead to a large domain of activated chromatin that encompasses both DSP2.9 (rearranged) and DFL16.1 (unrearranged) gene segments. Alternatively, a DSP2.9-rearranged allele may contain two distinct minidomains of active chromatin. To distinguish between those alternatives, we examined the structure of a DSP2.9-J\textsubscript{H2}–rearranged allele in 1E3 cells, which had a DFL16.1-J\textsubscript{H1} rearrangement in the other allele (Fig. 4a). Notably, we again found a highly localized peak of H3K9ac and transcription-associated H3K4me3 modification at the DJ\textsubscript{H} junction. In the presence of J\textsubscript{H}
modified for small numbers of cells (microChIP) to analyze bone marrow pro-B cells isolated by flow cytometry. To account for the heterogeneity of bone marrow pro-B cells, we used a ‘pan-DSP’ primer that hybridized to all six DSP gene segments and one that was unique to DFL16.1, together with a JH1 primer (Fig. 5a), to assess the histone-modification state of DJH1 junctions by real-time

Figure 4 Chromatin accessibility at DSP2.9-JH2-rearranged alleles. (a) Germline IgH locus in 6312 cells and the DJH-rearranged loci in 1E3 cells are shown (not drawn to scale), along with the positions of amplicons analyzed by real-time PCR: 1E3 cells have a DSP2.9-JH2 junction in one allele and a DFL16.1-JH1 junction in the second allele that would not bind the DSP2.9-specific primers. (b) ChIP assay of 6312 and 1E3 cells with antibodies specific for modified histones (vertical axes), followed by real-time PCR analysis of various amplicons (horizontal axes), and calculation of the abundance of each as described. Numbers in bars indicate amount of template DNA (in ng) used for PCR. Data are from two independent experiments (Supplementary Fig. 3c). (c) DNase I sensitivity of DSP2.9-JH2 (1E3) and the germline IgH allele (6312; assessed as in Fig. 1c). Data are from two independent experiments (b; average and s.d. of duplicates) or one experiment representative of two independent experiments (c; second experiment, Supplementary Fig. 3c).

Figure 5 Chromatin accessibility at DJH junctions in primary pro-B cells. (a) C57BL/6 IgH locus and location of PCR primers (arrows below). (b) MicroChIP assay of pro-B cells (2 x 10^5 to 2.5 x 10^5 cells isolated by flow cytometry from the bone marrow of C57BL/6 mice) with antibodies specific for modified histones, followed by real-time PCR analysis (primer colors, a) of DJH junctions (DFL-JH and DSP-JH) with forward primers that anneal 5′ of the DFL16.1 segment (orange) or all DSP2 segments (brown) and a reverse primer that anneals 3′ of the DSP2.9 segment. ND, not determined. (c) MicroChIP assay as in b, followed by PCR amplification (primer colors, a) of serially diluted input DNA (wedges: 2, 1 and 0.5 ng, top two blots; 800, 200 and 50 pg, bottom two blots (left and right)), or 200 pg ChIP DNA (DJH1 junctions) or 100 pg ChIP DNA (germline DJH, γ-actin and β-globin), with the same DJH forward primers as in b and a reverse primer that anneals 3′ of JH4 (green), and then Southern blot analysis (oligonucleotides, Supplementary Table 1). TE, Tris-EDTA; DFL-JH (1~4) and DSP-JH (1~4), amplicons corresponding to rearrangement of DFL16.1 and DSP2, respectively, to the four JH gene segments; H3K4me3-1 and H3K4me3-2, two analyses with anti-H3K4me3. Second blot from top (left and right), lower exposure of the Southern blot, as signal from the DJH4 bands was saturated with the exposure time above. Bottom, controls as in b. (d) Quantification of cumulative signal intensity in c (lane numbers correspond to those in c). Numbers in bars indicate amount of template DNA (in ng) used for PCR. Data are from two independent experiments (b; average and s.d.) or are representative of three independent experiments (c,d).
Fig. 5c H-rearranged abundance as described20 (IP/Inputcorr = ((IPSP − IPrIg) / input) × 1,000, where IPSP and IPrIg are the amount of DNA recovered by ChIP assay with RAG-specific antibody or rabbit immunoglobulin G, respectively). Numbers in parentheses indicate positions (in kb) 5′ (−) or 3′ (+) of the gene segment. Amplicons for JH4 and β-globin serve as the positive control and negative control, respectively. Data are from two or three independent experiments (average and s.d. of duplicates).

Fig. 6 Association of RAG-1 with DJH-rearranged Igh alleles. (a) Germline Igh locus in D345 pro-B cells25 and the DJH junctions in the D345 derivatives 3E and 1C6 cells (not drawn to scale), along with the positions of amplicons analyzed by real-time PCR. 3E cells have a DSP2.2-JhJ2 junction, and 1C6 cells have a DFL16.1-JhJ1 junction; the second allele in these cells is in germline configuration. (b,c) ChIP assay of D345 cells (b,c), 3E cells (b) and 1C6 cells (c) with a RAG-1-specific antibody, followed by quantitative real-time PCR and calculation of abundance as described20 (IP/Inputcorr = ((IPSP − IPrIg) / input) × 1,000, where IPSP and IPrIg are the amount of DNA recovered by ChIP assay with RAG-specific antibody or rabbit immunoglobulin G, respectively). Numbers in parentheses indicate positions (in kb) 5′ (−) or 3′ (+) of the gene segment. Amplicons for JH4 and β-globin serve as the positive control and negative control, respectively. Data are from two or three independent experiments (average and s.d. of duplicates).

PCR. We found a much greater abundance of H3K9ac marks as well as H3K4me3 marks at DFL16.1-JH1 and DSP2-JH2 junctions than at the corresponding germline DJH segments (Fig. 5b). To examine the chromatin state of DJH junctions that used other JH gene segments, we used a reverse primer located 3′ of JH4; for these assays, the PCR was followed by Southern blot analysis (Fig. 5c) and quantification of the signals in the Southern blots (Fig. 5d). We compared PCR products from 0.1–0.2 ng of microChIP material with those of serial dilutions of input material (2.0, 1.0 and 0.5 ng for DJH junctions; 0.8, 0.2 and 0.05 ng for germline fragments). We found greater enrichment for DJH junctions, but not for germline DJH gene segments, in DNA obtained by microChIP than in input DNA (Fig. 5c,d). Together these assays demonstrated that DJH junctions were selectively targeted for epigenetic modifications linked to the recruitment of RAG-1–RAG-2.

Restricted recruitment of recombinase to DJH junctions

To determine whether changes in histone modifications located at the DJH junction correlated with the recruitment of recombinase, we used ChIP to locate RAG-1 on DJH recombined alleles. We began with a pro-B cell line that lacks endogenous RAG-1 and has transgenic expression of the catalytically inactive RAG-1 (D708A) protein in the germline

Using a RAG-1-specific antibody for ChIP, we found considerable enrichment for RAG-1 in the JH region and complete depletion of RAG-1 over most DJH gene segments in D345 cells (Fig. 6a, b,c), as shown before25. Notably, we detected a small peak of RAG-1 coincident with small amounts of activating histone modifications just 5′ of DFL16.1 (Fig. 6b,c). The density of RAG-1 peaked approximately 1.7 kb upstream of DFL16.1, as noted before for H3K9ac and H3K4me3 marks11. This peak may have represented ‘spillover’ of RAG proteins from the JH-associated recombination center because of the spatial proximity of DFL16.1 to the JH domain21.

We found more RAG-1 at the recombined DSP2.2-JH2 junction in 3E cells (Fig. 6b). However, RAG-1 binding was close to background (represented by an amplicon from the locus encoding β-globin) at upstream unrearranged DSP2.2 and DFL16.1 gene segments. The pattern of recruitment of RAG-2 in these cells was similar to that of RAG-1 (Supplementary Fig. 6). Thus, there was considerable enrichment for both RAG-1 and RAG-2 at a recombined DSP2.2 gene segment. Similarly, the density of RAG-1 was much greater at the recombined DFL16.1-JH1 junction in 1C6 cells than at the unrearranged DFL16.1 gene segment in the same cells (Fig. 6c). We noted the same pattern of RAG-1 binding in 2C11 cells (Supplementary Fig. 7). We confirmed that the histone-modification pattern of the D345 derivatives closely resembled that of the 6312-derived clones reported above (Figs. 1–3 and Supplementary Fig. 8). We concluded that DJH recombination led to accumulation of RAG proteins at DJH junctions.

The high density of RAG-1 at DFL16.1-JH1 in 1C6 cells indicated that the peak of RAG-1 centered 5′ of DFL16.1 on unrearranged alleles shifted to the DFL16.1-JH junction after recombination (Fig. 6c). This result was directly analogous to the shift in histone modification peaks on DFL16.1-JH1–recombined alleles (Fig. 3b). Moreover, we noted higher RAG-1 density at DJH junctions than at the germline JH4 region in both 3E cells and 1C6 cells. Because the density of RAG protein in the germline Igh locus was concentrated over the JH gene segments, these observations suggested that RAG-1 and RAG-2 redistributed toward the DJH junctions on rearranged alleles. We propose that refocusing RAG-1–RAG-2 not only maximizes use of the DJH RSS for V H recombination but also serves to limit the low but detectable occurrence of direct V H-to-JH rearrangements27.

DISCUSSION

Three regulatory features are shared by all V H recombinations. First, V H recombination always follows DJH recombination (timing). This timing could be mediated by a late-acting ACE associated with V H gene segments, such that DJH recombination would have always occurred before this ACE was activated. However, such an ACE has not been identified. Second, V H recombination is selectively activated on alleles that have undergone DJH recombination (allele specificity). This underappreciated facet of V H recombination can be inferred from the state of Igh alleles in mice with a knock-in mutation that express Igh in germline configuration, in contrast to normal B cells, in which both Igh alleles...
VH–to–germline D H recombination was observed in mice in which chromatin state for RAG proteins to recognize the VH RSS and induce recruitment of RAG proteins to V H gene segments. Indeed, it is be conferred in part by interleukin 7–dependent histone modification, the permissive chromatin state required for access to VH may gene segment, which would thereby lead to V H recombination. This 'preference' for JH-DH synapsis would be dictated by the nature of the associated RSSs, which contain 23 nucleotides or 12 nucleotides of spacer DNA, respectively, between conserved nonamer and heptamer sequences. After DH recombination, RAG proteins are 'preferentially' recruited to DJH junctions, which permits them to initiate the reaction at the 5′ DJH RSS; now the complementary RSS would be that of a VH gene segment, which would thereby lead to VH recombination. This model extends the recombination-center model proposed for germ-line antigen receptor loci to DH-combined Igh loci. Essentially, DHH recombination brings DH RSSs into the recombination center, which allows them to participate in the second step of Igh gene assembly. The idea that DH RSSs become available to initiate recombination only after DJH recombination also provides an explanation for the allele specificity of VHH recombination.

Our model circumvents the need to invoke independent activation and recruitment of RAG proteins to VHH gene segments. Indeed, it is easy to imagine that recruitment of RAG all along the 2.5-megabase VH region would result in many more RAG-induced DNA breaks and translocations. We hypothesize that restriction of the presence of RAG to a discrete part of the Igh locus with sequential bringing in of the appropriate gene segments provides the correct recombination order with minimal genomic instability. We do not suggest that recruitment of RAG to the DJH RSS is sufficient to initiate VHH recombination. Instead, RAG proteins bound to the DJH-associated RSS must find and gain access to a VH RSS for hairpin formation to occur. It is likely that locus conformation, mediated by looping and/or compaction, has a role in the spatial positioning of VH gene segments in the vicinity of DHH-associated RAG proteins. Lack of such positioning is the likely explanation for less distal VHH recombination in pro-B cells deficient in the transcription factor Pax5 or transcriptional regulator YY1 (refs. 30,31). Additionally, correctly positioned VH gene segments must also be in the appropriate chromatin state for RAG proteins to recognize the VH RSS and induce nicking. The permissive chromatin state required for access to VHH may be conferred in part by inter leukin 7–dependent histone modifications and Pax5-dependent loss of H3K9me2 (ref. 12).

One caveat to the model is that DJQ52-associated RSSs, which are rich in RAG before rearrangement, should be able to recombine with germline VHH gene segments to produce VH-DHQ52 junctions. Indeed, such rearrangements are in fact observed, but only when the spatial configuration of the Igh locus is altered. The first instance of VHH-to–germline DHH recombination was observed in mice in which a VH gene segment was knocked in very close to DFLI6.1 (ref. 36). The knocked-in VHH segment rearranged 'preferentially' to DQ52 located 50 kb away rather than to DFLI6.1 located only 1.0 kb away. That product was probably generated by binding of RAG at an unarranged DQ52, followed by capture of the RSS associated with the knocked-in VHH segment. We propose that a synthesis between germline DQ52 and the knocked-in VH gene segment was possible in this situation because both gene segments were in the same chromatin domain demarcated by CTCF- and YY1-binding sites 5′ of DFLI6.1 (refs. 21,22). In the normal configuration of the Igh locus, RAG-bound complexes at DQ52 RSSs would be more effectively captured by JH RSSs because VH gene segments are located outside the D HJ domain.

A second instance of VHH to germline DQ52 rearrangement has been reported on Igh alleles mutated at the two CTCF-binding elements upstream of DFLI6.1 (ref. 23). These modifications remove the newly identified looping or barrier sites 5′ of DFLI6.1 that sequester all DH gene segments in one chromatin domain.22,23 In the absence of the normal looping or barrier sites, perhaps the D HJ domain extends into the proximal VHJ region, thereby incorporating one or more VH gene segments into the D HJ domain. Functionally, this would be analogous to the proposed structure described above, generated on the allele with the knocked-in VH gene segment. Therefore, VHH RSS(s) would be available for synthesis with the DQ52 RSS, which would lead to proximal VHH-to–germline DQ52 rearrangements.

Finally, our observations provide a plausible mechanism for the precision of recombination of VHH to DJHJ junctions but not to germ-line DJH RSS 4 kb upstream. Specifically, we found that activating histone modifications and nucleosome sensitivity of DJHJ junctions did not extend even 4 kb to the nearest unarranged DJH gene segment. Because these changes occurred in recombinase-deficient cells, our working hypothesis is that these changes direct the recruitment of RAG to DJHJ junctions while avoiding upstream germ-line DH gene segments. Consistent with our proposal, direct analysis of the binding of RAG also showed the greatest amount of RAG-1–RAG-2 at DJHJ junctions and very little at germ-line DHJ segments. Notably, the peak of RAG binding seemed to shift from its pre-rearrangement position over JH gene segments to focused accumulation at DJHJ junctions, which thereby further accentuated the use of the DHJ RSS in the next rearrangement step. We propose that the exquisite specificity of the recombination of VHH to DJHJ junctions is imposed by the localized changes in chromatin structure and consequent restriction of RAG proteins to DJHJ junctions.

Several mechanisms can be considered by which chromatin changes are restricted to DJHJ junctions. First, as large portions of the DJHJ region are actively maintained in silent (H3K9me2-marked) chromatin, it is possible that some of these heterochromatin-associated enzymes are brought along with the recombining DJHJ segment. After rearrangement, the DJHJ promoter is activated by proximity to Eμ and results in activation- and/or transcription-associated histone modifications near the DJHJJ junction. However, such modifications cannot spread further in the 5′ direction because of silencing activities there. Second, DJHJ promoters activate bidirectional transcription after rearrangement. It is possible that the greater abundance of antisense transcripts generated from the recombined DJHJ promoter may have a role in maintaining the heterochromatic state of upstream germ-line DH gene segments. Third, DJH promoters may function as boundary elements. In this scenario, although the DJH gene segment recombines into the highly active JHJ region, the positive effect of this part of the locus is prevented from spreading into the upstream germ-line DJ gene segments by the newly active, rearranged DJHJ promoter. Further studies are needed to determine the factors that restrict chromatin structural changes to DJHJ junctions.

**METHODS**

Methods and any associated references are available in the online version of the paper.
Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R. Subrahmanyan designed and did all the experiments; R. Subrahmanyan and R. Sen analyzed and interpreted the data; H.D. assisted with Southern blot analysis; I.I. did the ChiP analysis of H3K4me2; T.C. assisted in the initial characterization of DJ12-rearranged cell lines; Y.J. and D.G.S. provided D345 cells and discussions of the results; R. Sen analyzed and interpreted the data; H.D. assisted with Southern blot analysis; R. Subrahmanyan and R. Sen wrote the manuscript; and D.G.S. and F.W.A. read and critiqued the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Bergman, Y. & Cedar, H. Epigenetic control of recombination in the immune system. Semin. Immunol. 22, 323–329 (2010).
2. Schatz, D.G. & Ji, Y. Recombination centres and the orchestration of V(DJ) recombination. Nat. Rev. Immunol. 11, 251–263 (2011).
3. Perlot, T. & Alt, F.W. Cis-regulatory elements and epigenetic changes control genomic rearrangements of the IgH locus. Adv. Immunol. 99, 1–32 (2007).
4. Thomas, L.R., Cobb, R.M. & Oltz, E.M. Dynamic regulation of antigen receptor gene assembly. Adv. Exp. Med. Biol. 650, 103–115 (2009).
5. Blackwell, T.K. et al. Recombination between immunoglobulin variable region gene segments is enhanced by transcription. Nature 324, 585–589 (1986).
6. Yancopoulos, G.D. & Alt, F.W. Developmentally controlled and tissue-specific expression of unrearranged VH gene segments. Cell 40, 271–281 (1985).
7. Osipovich, O. & Oltz, E.M. Regulation of antigen receptor assembly by genetic-epigenetic crosstalk. Semin. Immunol. 22, 313–322 (2010).
8. Spicuglia, S., Pekowska, A., Zacarias-Cabeza, J. & Ferrier, P. Epigenetic control of Tcrb gene rearrangement. Semin. Immunol. 22, 330–336 (2010).
9. Subrahmanyan, R. & Sen, R. Epigenetic features that regulate IgH locus recombination and expression. Curr. Top. Microbiol. Immunol. 356, 39–63 (2011).
10. Subrahmanyan, R. & Sen, R. RAGs’ eye view of the immunoglobulin heavy chain gene locus. Semin. Immunol. 22, 337–345 (2010).
11. Chakraborty, T. & Ji, Y. Repeat organization and epigenetic regulation of the DH-Cmu domain of the immunoglobulin heavy-chain gene locus. Mol. Cell 27, 842–850 (2007).
12. Johnson, K. et al. Cell-specific loss of histone 3 lysine 9 methylation in the VH locus depends on Pax5. Nat. Immunol. 5, 853–861 (2004).
13. Osipovich, O. et al. Targeted inhibition of V(DJ) recombination by a histone methyltransferase. Nat. Immunol. 5, 309–316 (2004).
14. Liu, Y. Subrahmanyan, R., Chakraborty, T., Sen, R. & Desiderio, S. A plant homeodomain in RAG-2 that binds hypermethylated lysine 4 of histone H3 is necessary for efficient antigen-receptor-gene rearrangement. Immunity 27, 561–571 (2007).
15. Matthews, A.G. et al. RAG2 PHD finger couples histone H3 lysine 4 trimethylation with V(DJ) recombination. Nature 450, 1106–1110 (2007).
16. Ramón-Maqués, S. et al. The plant homeodomain finger of RAG2 recognizes histone H3 methylated at both lysine-4 and arginine-2. Proc. Natl. Acad. Sci. USA 104, 18993–18998 (2007).
17. Johnston, C.M., Wood, A.L., Bolland, D.J. & Corcoran, A.E. Complete sequence assembly and characterization of the C57BL/6 mouse Ig heavy chain V region. J. Immunol. 176, 4221–4234 (2006).
18. Chakraborty, T. et al. A 220-nucleotide deletion of the intronic enhancer reveals an epigenetic hierarchy in immunoglobulin heavy chain locus activation. J. Exp. Med. 206, 1019–1027 (2009).
19. Chowdhury, D. & Sen, R. Stepwise activation of the immunoglobulin mu heavy chain gene locus. EMBO J. 20, 6394–6403 (2001).
20. Ji, Y. et al. The in vivo pattern of binding of RAG1 and RAG2 to antigen receptor loci. Cell 141, 419–431 (2010).
21. Guo, C. et al. Two forms of loops generate the chromatin conformation of the immunoglobulin heavy-chain gene locus. Cell 147, 332–343 (2011).
22. Guo, C. et al. CTCF-binding elements mediate control of V(DJ) recombination. Nature 477, 424–430 (2011).
23. Featherstone, K., Wood, A.L., Bowen, A.J. & Corcoran, A.E. The mouse immunoglobulin heavy chain V-D-J intergenic sequence contains insulators that may regulate ordered V(DJ) recombination. J. Biol. Chem. 285, 9327–9338 (2010).
24. Degner-Leiss, S.C. & Feeney, A.J. Epigenetic and 3-dimensional regulation of V(DJ) rearrangement of immunoglobulin genes. Semin. Immunol. 22, 346–352 (2010).
25. Hewitt, S.L., Chaumeil, J. & Skok, J.A. Chromosome dynamics and the regulation of V(DJ) recombination. Immunol. Rev. 237, 43–54 (2010).
26. Dahl, J.A. & Collas, P. A rapid micro chromatin immunoprecipitation assay (microChIP). Nat. Protoc. 3, 1032–1045 (2008).
27. Korolov, S.B., Novobrantseva, T.I., Hochedlinger, K., Jaenisch, R. & Rajewsky, K. Direct in vivo VH to JH rearrangement violating the 12/23 rule. J. Exp. Med. 201, 341–348 (2005).
28. Akamatsu, Y. et al. Deletion of the RAG2 C terminus leads to impaired lymphoid development in mice. Proc. Natl. Acad. Sci. USA 100, 1209–1214 (2003).
29. Dudley, D.D. et al. Impaired VDJ recombination and lymphocyte development in core RAG1-expressing mice. J. Exp. Med. 198, 1439–1450 (2003).
30. Hesselink, D.G. et al. Pax5 is required for recombination of transcribed, acetylated, 5′ IgH V gene segments. Genes Dev. 17, 37–42 (2003).
31. Liu, H. et al. Yin Yang 1 is a critical regulator of B-cell development. Genes Dev. 21, 1179–1189 (2007).
32. Bertolino, E. et al. Regulation of interleukin 7-dependent immunoglobulin heavy-chain variable gene rearrangements by transcription factor STAT5. Nat. Immunol. 6, 836–843 (2005).
33. Chowdhury, D. & Sen, R. Transient IL-7/IL-7R signaling provides a mechanism for feedback inhibition of immunoglobulin heavy chain gene rearrangements. Immunity 18, 229–231 (2003).
34. Stanton, M.L. & Brodeur, P.H. Stat5 mediates the IL-7-induced accessibility of a representative D-Distal VH gene. J. Immunol. 174, 3164–3168 (2005).
35. Xu, C.R., Schaffer, L., Head, S.R. & Feeney, A.J. Reciprocal patterns of methylation of H3K36 and H3K27 on proximal vs. distal IgVH genes are modulated by IL-7 and Pax5. Proc. Natl. Acad. Sci. USA 105, 8685–8690 (2008).
36. Bates, J.G., Cado, D., Nolla, H. & Schissel, M.S. Chromosomal position of a VH gene segment determines its activation and inactivation as a substrate for V(DJ) recombination. J. Exp. Med. 204, 3247–3256 (2007).
ONLINE METHODS

Cell culture. Rag2\(^{-/-}\) 6312 cells and their derivative cell lines 2B9, 2C10, 2F1 and 1E3, as well as D345 cells and their derivatives 3E, 1C6 and 2C11, were grown in RPMI medium supplemented with FBS, antibiotics and 2-mercaptoethanol. The derivative cells with DJ\(_H\) rearrangement were generated by transient transfection of a RAG-2 expression vector into 6312 cells or a RAG-1 expression vector into D345 cells, followed by single-cell cloning and characterization of DJ\(_H\) junctions by PCR. A bicistronic retroviral vector that coexpressed RAG-2 and green fluorescent protein (GFP) was transfected into 6312 cells or retroviral vectors expressing RAG-1 and RFP were transfected together into D345 cells with an Amacor nucleofector (Cell Line Nucleofector Kit V, Program W-01). At 24 h after transfection, GFP\(^+\) 6312 cells and RFP\(^+\) D345 cells were sorted and grown in RPMI culture medium. After 4–7 d of culture, GFP\(^+\) 6312 and RFP\(^+\) D345 cells were sorted as single cells into 96-well dishes for population expansion. Clones were screened by PCR for the presence of alleles with DJ\(_H\) recombinaiton and were chosen to represent a range of DH segment use. Generation of the v-Abl-transformed 6312 and D345 cells has been described 20,37.

ChIP. ChIP analysis of modified histones and RAG-1 was done as described 11,20. Antibodies for ChIP were as follows: anti-H3K9ac (06-942) and anti-H3K9me2 (07-441) were from Millipore; and anti-H3K4me2 (39141) and anti-H3K4me3 (39159) were from Active Motif. Hybridoma cell lines producing monoclonal antibody to RAG-1 (23) or to RAG-2 (11) were generated by Epitomics (39159) were from Active Motif. Hybridoma cell lines producing monoclonal antibody to RAG-1 (23) or to RAG-2 (11) were generated by Epitomics (39159) or of 32 cycles (for germline fragments; primers and probes, Supplementary Table 1). The abundance of RAG-1 at specific genomic loci was analyzed by real-time PCR (IP/Inputcorr) was calculated as described 20.

Statistical analysis. Results were calculated with Microsoft Office Excel 2007. Graphs were generated with GraphPad Prism 5 or Microsoft Office Excel 2007.

DNase I sensitivity. DNase I sensitivity was assayed as described 15. Nuclei from 2 × 10\(^5\) cells were treated with increasing amounts of DNase I (0–20 U/ml), followed by purification of the genomic DNA, then by real-time PCR in duplicate (primers, Supplementary Table 1). Sensitivity was determined for two independent DNase I–treated samples for each cell line.

Micro-ChIP of primary pro-B cells. Bone marrow from 16 C57BL/6 mice was labeled with biotinylated anti-Mac-1 (553309), anti-Gr-1 (553125), anti-Ter119 (553672), anti-CD3\(\varepsilon\) (553060), anti-IgM (553406), anti-Ly-6C (557359) and anti-DX-5 (553856; all from BD Biosciences). Labeled cells were then bound to streptavidin microbeads (130-048-102; Miltenyi Biotec), and samples were depleted of those cells by passage through LD columns (130-042-901; Miltenyi Biotec). The flow-through fraction was then stained with fluorescein isothiocyanate–anti-B220 (553088; BD Biosciences), phycoerythrin–indotricarbocyanine–anti-CD19 (552854; BD Biosciences), phycoerythrin–anti-CD43 (553271; BD Biosciences) and allophycocyanin–anti- AA4.1 (17-5892; eBioscience), and cells expressing all four markers were sorted on a FACSAria (BD). The total yield of 1.3 × 10\(^6\) pro-B cells was divided into five tubes; one was used as input material, and the other four were used for micro-ChIP in duplicate with anti-H3K9ac and anti-H3K4me3. A published micro-ChIP protocol 26 was adopted with minor modifications. ChIP samples (100–200 pg) were analyzed by real-time PCR (Fig. 5b) or by Southern blot analysis of PCR products (Fig. 5c) to assess enrichment for specific targets. Southern blot analysis followed one round of PCR of 35 cycles (for DJ\(_H\) rearrangement) or of 32 cycles (for germline fragments; primers and probes, Supplementary Table 1). Animal experiments were reviewed and approved by the NIA/IRP Animal Care and Use Committee (Animal Studies Protocol 338-LMBI-2013).

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