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# Regulation of immunoglobulin heavy-chain gene rearrangements

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**Summary:** Regulated assembly of antigen receptor gene segments to produce functional genes is a hallmark of B- and T-lymphocyte development. The immunoglobulin heavy-chain (IgH) and T-cell receptor β-chain genes rearrange first in B and T lineages, respectively. Both loci require two recombination events to assemble functional genes; D-to-J recombination occurs first followed by V-to-DJ recombination. Despite similarities in overall rearrangement patterns, each locus has unique regulatory features. Here, we review the characteristics of IgH gene rearrangements such as developmental timing, deletion versus inversion, $D_H$ gene segment utilization, ordered recombination of $V_H$ gene segments, and feedback inhibition of rearrangement in pre-B cells. We summarize chromatin structural features of the locus before and during recombination and, wherever possible, incorporate these into working hypotheses for understanding regulation of IgH gene recombination. The picture emerges that the IgH locus is activated in discrete, independently regulated domains. A domain encompassing $D_H$ and $J_H$ gene segments is activated first, within which recombination is initiated. $V_H$ genes are activated subsequently and, in part, by interleukin-7. These observations lead to a model for feedback inhibition of IgH rearrangements.

## Overview of the locus

Immunoglobulin heavy-chain (IgH) genes are assembled by somatic recombination of $V_H$, $D_H$, and $J_H$ gene segments. $V_H$ gene segments number in the hundreds in the mouse (1, 2) and lie at the 5′ end of the IgH locus (*Fig. 1*) close to the telomere of the short arm of chromosome 12. Similar numbers of $V_H$ genes are located close to the telomere of chromosome 14 in humans (3). Close to half the murine $V_H$ genes belong to the J558 family. The majority of these are located at the 5′ end of the $V_H$ cluster, though several are also found interspersed amongst other gene families throughout the locus. The extreme 3′ end of the $V_H$ locus comprises the $V_H$ 7183 gene family, with $V_H$81X being the most 3′ $V_H$ gene segment. The length of the $V_H$ locus has been estimated to be approximately 1.5 megabases (Mb) (1). The $D_H$ gene segment

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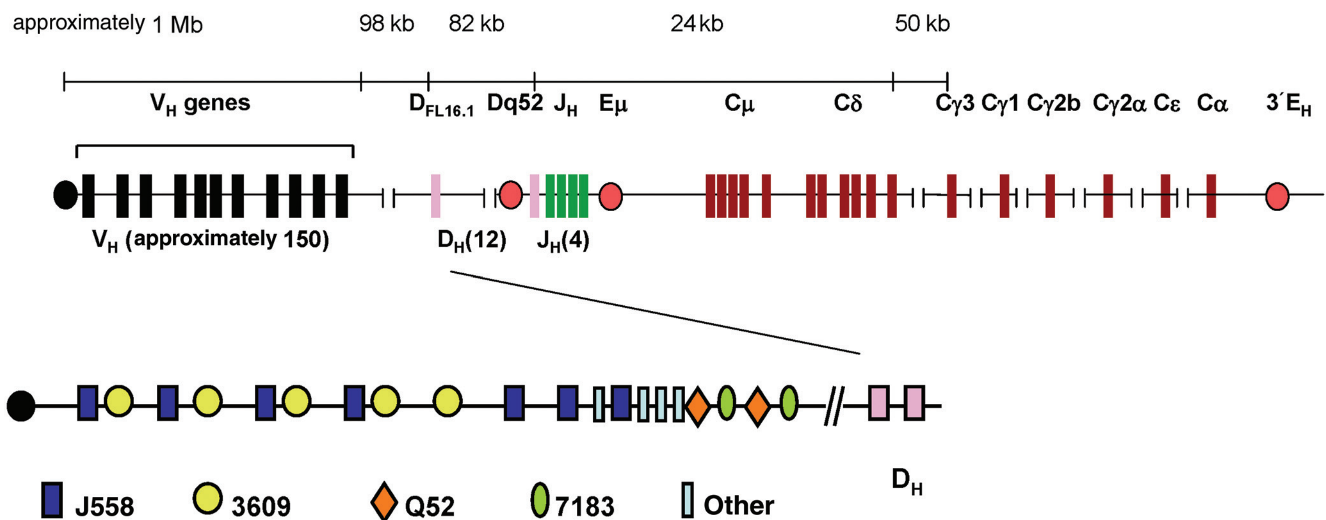


**Fig. 1. Immunoglobulin heavy-chain (IgH) locus organization.** A schematic representation of the murine IgH with approximate distances between the gene segments is shown on the top line (98). Several $V_H$ genes, the 5′-most $D_H$ gene segment (DFL16.1) and 3′-most (Dq52) $D_H$ gene segments are indicated along with the different constant region isotypes. Numbers of each gene family, $V_H$, $D_H$, or $J_H$, are written in parentheses below the line. Three known *cis*-regulatory elements, the intronic enhancer Eμ, the Dq52 promoter, and the 3′ locus control region (LCR) are depicted as red ovals. The second line is a detailed schematic of the $V_H$ locus showing the relative locations of the gene segments belonging to J558, 3609, Q52, and 7183 families. This part of the figure is adapted from Chevillard *et al.* (1). Black circle represents the telomere.

closest to the $V_H$ locus, DFL16.1, is located approximately 98 kb, 3′ of $V_H$81X. Eleven other $D_H$ gene segments are distributed over 60 kb, which end with Dq52. Interestingly, Dq52 is separated from the next upstream $D_H$ gene segment, DSp2.10, by an 18 kb gap beyond which the other $D_H$ genes are relatively regularly placed. 3′ of Dq52, there is less than a kilobase to the first of four $J_H$ gene segments. The exons encoding Cμ and Cδ are located in the next 12 kb, after which there is a 50-kb gap to the γ3 constant region exons. Coding regions for the remaining five isotypes are found in the next 50 kb. The entire locus encompassing the $V_H$ genes and extending to Cα is approximately 3 Mb. This review focuses on the regulation of IgH gene recombination in the mouse.

B cells develop in the adult from bone marrow-derived hematopoietic stem cells (HSCs). Pioneering work of Weissman and colleagues (4, 5) has led to a model where the HSCs give rise to two major progenitor populations, the common myeloid precursors (CMPs) and the common lymphoid precursors (CLPs), which is the source of B and T lymphocytes, natural killer cells, and dendritic cells. These early intermediates retain developmental flexibility, as indicated by the ability of a strong cytokine signal to redirect CLPs into the myeloid differentiation pathway (6). Furthermore, many genes of the 'wrong' lineage are expressed in both CLPs and CMPs suggesting that they are not irreversibly committed to a differentiation program (7). Kincade and colleagues have identified an early lymphocyte precursor (ELP) population in the bone marrow that reconstitutes both lymphoid lineages (8, 9). ELPs may be precursors to CLPs, because they do not express the interleukin-7 receptor α chain (IL-7Rα). Moreover, early T-cell precursors (ETPs), which are not derived from the CLPs, have been isolated from the blood (10). These observations are consistent with the idea that ELPs are the precursors for both CLPs and ETPs, with CLPs being more restricted to B-lineage differentiation. B-cell differentiation beyond the CLPs has been staged by the expression of cell-surface markers, as indicated in Fig. 2 (11).

## Regulation of $D_H$-to-$J_H$ recombination

The conceptual framework for regulation of antigen receptor gene rearrangements is provided by the accessibility model of Alt and colleagues (12,13). Originally proposed to account for the observation that Ig and T-cell receptor (TCR) genes contained identical recombination signal sequences (RSSs) and were therefore targeted by the same enzymatic machinery, the model proposes that the recombinase only acts on accessible genes. Thus, B-cell antigen receptor (BCR) genes are targets only in B-cell precursors, while TCR genes are targets only in T cells. Taken a step further, the accessibility model postulates that IgH genes are accessible earlier and light-chain genes are accessible later in the course of B-cell differentiation. Lineage-specificity and developmental timing of antigen receptor gene rearrangements have now been shown to be determined by gene-specific regulatory sequences, identified

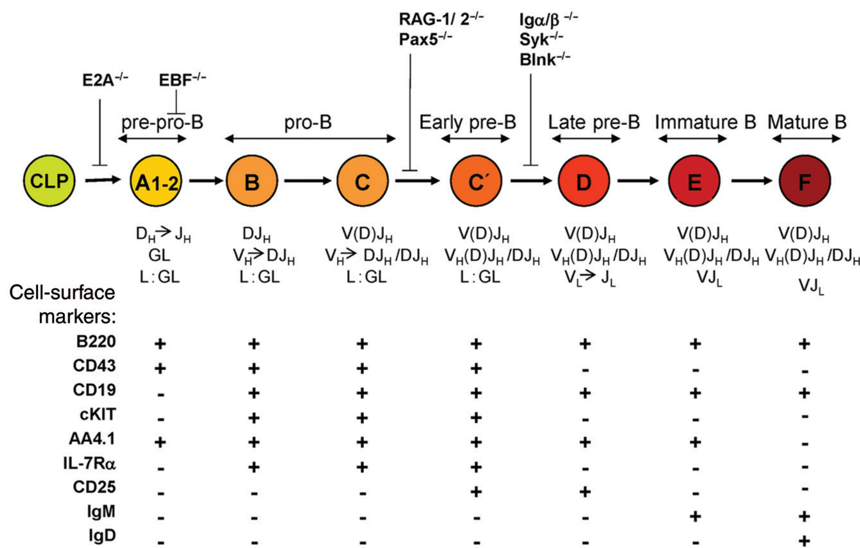


**Fig. 2. Schematic representation of B-cell differentiation.** Hardy classification of B-cell development is shown with a list of surface markers in the lower panel used to distinguish the different stages (11). The '+' indicates medium or high expression and '–' indicates low or no expression. All the fractions shown here are Ly6C negative (99). $D_H$-to-$J_H$ recombination is initiated in fraction A but occurs primarily in fraction B, where $V_H$-to-$DJ_H$ recombination is also initiated. Recombination at the immunoglobulin heavy-chain (IgH) locus stops after fraction C with light-chain recombination taking place in fraction D. B-cell differentiation is blocked at different stages (as indicated) in the absence of transcription factors (E2A, EBF, and Pax5), signaling molecules (Igα/β, Syk, and Blnk) and other factors like RAG-1/2 and Ezh2.

as transcription enhancers. Many of the properties attributed to transcriptional enhancers are also true of their function as recombination enhancers. In particular, enhancer location (distance from the RSSs) and orientation (relative to the RSSs) is flexible. The extent of flexibility, particularly with respect to distance from the site of recombination, varies significantly between enhancers.

The first step in the assembly of functional IgH genes is the rearrangement of a $D_H$ gene segment to one of four $J_H$ gene segments. IgH alleles are largely in germline configuration in the earliest $B220^+$ population [Hardy fraction (Fr.) A1/A2, or pre-pro-B cells], whereas significant $DJ_H$ recombination is evident at the next discernible stage (Fr.B, pro-B cells), suggesting that developmentally regulated activation of IgH recombination occurs in Fr. A cells. Low levels of $DJ_H$ recombination have also been noted in ELPs and CLPs (8, 14). However, fairly uniform expression and function of the recombination-activating gene 1 (RAG1) and RAG2 in CLPS together with inefficient $DJ_H$ recombination in this subset suggest that this is a minor pathway of IgH locus activation (14, 15). In addition to the precise timing of $D_H$-to-$J_H$ recombination, several other features of this recombination step await molecular explanations. In the following section, we briefly review the associated phenomena and discuss possible regulatory mechanisms based on available experimental data.

### Developmental timing

The recombination enhancer closest to the $J_H$ gene segments is the μ enhancer (Eμ) located in the intron between $J_H$ and Cμ exons (Fig. 1). However, deletion of this enhancer appears not to disrupt $D_H$-to-$J_H$ recombination (16–18). In all the studies, the rearrangement state of Eμ-deleted alleles was analyzed in mature heterozygote B cells; while levels of $DJ_H$ recombination appeared normal, greatly reduced levels of $V_H$-to-$DJ_H$ recombination were noted on the enhancer-deleted allele. The most straightforward interpretation of these observations is that Eμ is not involved in either developmental timing or efficiency of $D_H$-to-$J_H$ recombination. However, conclusions about the role of Eμ in timing and efficiency of the first recombination step must be taken with caution, because these analyses were carried out in mature B cells that have successfully negotiated several developmental checkpoints (and therefore have been selected). Moreover, the recombined alleles represent a steady-state population, and to determine whether these sequences affect recombination efficiency, experiments must be designed to assess the 'initial rates' of $D_H$-to-$J_H$ recombination in the presence or absence of putative regulatory elements.

#### Possible mechanisms for blockade of $V_H$-to-$DJ_H$ recombination in the absence of Eμ

The surprising block of $V_H$-to-$DJ_H$ recombination on Eμ-deleted alleles necessitates further comments. While the observation suggests that Eμ must be involved in activating $V_H$ gene recombination, this interpretation poses some difficulties. First, as evidenced by the presence of sterile Iμ transcripts, it is reasonable to believe that Eμ is activated very early in differentiation (19). If so, why does $V_H$ recombination not occur equally early? Second, there is no evidence that this enhancer can activate genes located, at the very minimum, more than a 100 kb away. While these are not insurmountable arguments, we were prompted to consider other plausible

models to explain the facts. One reason for the lack of $V_H$-to-$DJ_H$ recombination on Eμ-deleted alleles could be that the observed $D_H$-to-$J_H$ recombination occurs at a stage beyond that at which $V_H$ rearrangement is permitted. For example, if $D_H$-to-$J_H$ recombination in pro-B cells is inefficient in the absence of Eμ, then complete $VDJ_H$ recombination may occur on the unmutated allele before significant $DJ_H$ rearrangement on the Eμ-deleted allele. Cells that produce functional $VDJ_H$ alleles will thus differentiate into pre-B cells with little or no $DJ_H$ recombination on the mutated allele. Now $D_H$-to-$J_H$ recombination may be completed on the mutated allele together with light-chain gene rearrangements, when $V_H$ recombination is suppressed by feedback inhibition/allelic exclusion. Consequently, $V_H$-to-$DJ_H$ recombination would not occur on the mutated allele. In this scenario, Eμ is essential for activation of $D_H$-to-$J_H$ recombination at the appropriate developmental stage, but other compensatory mechanisms overcome its deficiency at later developmental stages.

Alternatively, $D_H$-to-$J_H$ recombination may proceed normally on both wildtype and Eμ-deleted alleles in Fr. A/B. However, $D_H$ recombination, which occurs largely by deletion, will remove a regulatory element associated with Dq52 from this region as shown in *Fig. 3*. This process leaves Eμ as the only identified recombination enhancer in the vicinity of the newly created $DJ_H$ junction. Several lines of evidence indicate that recombination enhancers are required close to both RSSs involved, even when the RSSs are not very far apart. For example, deletion of the TCR gene enhancer in mini-locus transgenic substrates preferentially reduces DNA breaks close to Jδ but not Vδ (20), and deletion of a sterile promoter close to Dβ1 in the TCRβ locus affects Dβ1 but not Dβ2 gene rearrangements (21). Finally, the work of Oltz and colleagues (22, 23) demonstrates the necessity of transcriptional enhancers close to RSSs separated by less than 2 kb for efficient recombination. Taken together, these observations imply that for $V_H$-to-$DJ_H$ recombination to proceed, $V_H$ RSSs and the 5′ $DJ_H$ RSSs must be independently activated by different enhancers. If Eμ is required to activate the RSSs of the $DJ_H$ allele for further $V_H$-to-$DJ_H$ recombination (*Fig. 3*), then $V_H$ recombination will be blocked on the Eμ-deleted allele. An assumption of this model is that local recombination enhancers are continuously required to activate RSSs of partially rearranged alleles. Its strength is that it does not invoke long distance $V_H$ gene activation by Eμ.

### Other $DJ_H$ recombination-activating enhancers

If Eμ is not involved in activating $D_H$-to-$J_H$ recombination, then what regulatory sequence activates this recombination step? The only other element in this part of the locus is a promoter located 5′ of Dq52 (24)(*Fig. 1*). Though Kohler and

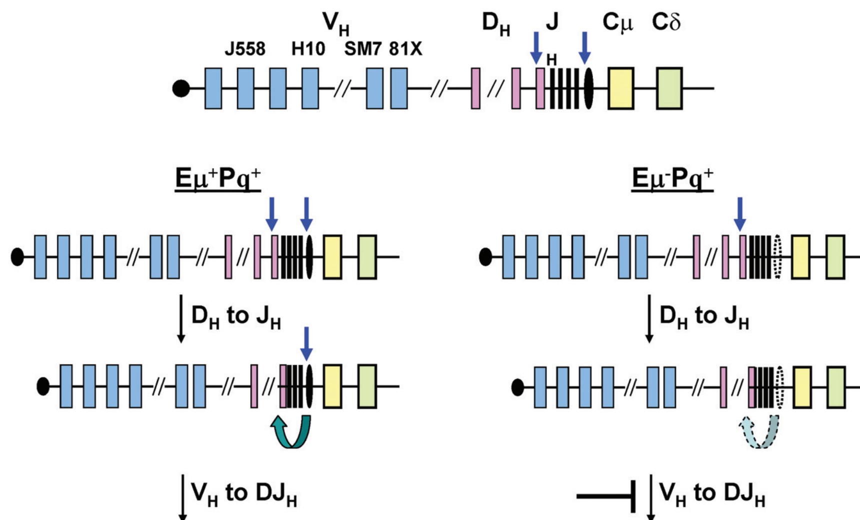


**Fig. 3. A model for lack of $V_H$-to-$DJ_H$ recombination on enhancer μ ($E\mu^-$) alleles.** The immunoglobulin heavy-chain (IgH) locus with $V_H$, $D_H$, and $J_H$ gene segments is shown on the top line. The two nuclease hypersensitive sites, the intronic enhancer (Eμ, black oval) and the Dq52 promoter (Pq) are marked by arrows. $D_H$-to-$J_H$ recombination proceeds normally on both wild-type ($E\mu^+$ $Pq^+$ left panel) and Eμ-deleted ($E\mu^-$ $Pq^+$, right panel) alleles. Pq, a potential recombination enhancer, may facilitate $D_H$-to-$J_H$ recombination of the Eμ-deleted allele. This recombination event deletes Pq leaving Eμ as the only known recombination enhancer near the newly created $DJ_H$ junction on normal alleles but no enhancer on $E\mu^-$ alleles (indicated by dotted oval in the right panel). We suggest that Eμ activates the 5′RSS of the $DJ_H$ allele (indicated by a blue curved arrow in the left panel) for $V_H$-to-$DJ_H$ recombination. In the absence of Eμ, this RSS remains inaccessible (indicated by a dotted curved arrow in the right panel), hence $V_H$-to-$DJ_H$ recombination is blocked. A central feature of the model is that $V_H$ and $DJ_H$ RSSs must be independently activated for recombination to proceed.

colleagues (24, 25) reported transcription enhancer activity associated with this region and we (26) mapped a DNase 1 hypersensitive site here, there is no direct evidence for it being a recombinational enhancer. However, genetic deletion of this region has a discernible effect on $D_H$-to-$J_H$ recombination (27). Specifically, $D_H$ rearrangement to $J_H3$ and $J_H4$ in the bone marrow is reduced approximately 50% with no effect on the utilization of $J_H1$ and $J_H2$ gene segments. In addition, coding joint processing is affected, and 5′ $D_H$ gene segments are used more frequently. These observations suggest that the Dq52 element may play a role in secondary rearrangements (to $J_H3/J_H4$), but it is compensated for by other regulatory sequences such as Eμ for primary rearrangements to $J_H1/J_H2$. After taking into account reduced recombination to $J_H3/J_H4$, loss of the Dq52 element has little, or no, effect on subsequent $V_H$-to-$DJ_H$ recombination, making it functionally dissimilar to Eμ in this regard. Analysis of mice deleted of both Eμ and the Dq52 element should soon provide insight into their function (Oltz, personal communication). We have also screened a large part of the $D_H$ locus and the Cμ-Cδ region without detecting any other DNase1 hypersensitive sites that may be relevant to this question. Thus, the identity of *cis*-regulatory sequences that determine developmental timing of $D_H$-to-$J_H$ recombination remains open.

### $D_H$ recombination by deletion or inversion

$D_H$ gene segments are sandwiched between RSSs that contain 12-base pair (bp) spacers, while $V_H$ and $J_H$ gene segments are flanked by 23-bp spacers. Direct $V_H$-to-$J_H$ recombination is thus prohibited by the 12/23 rule. This structure is in contrast to the TCRβ locus, where Vβ and Jβ RSSs are compatible for recombination by the 12/23 rule but disallowed by additional constraints imposed by the nucleotide sequence of the spacers (28). Although $D_H$-to-$J_H$ recombination can proceed by deletion or by inversion, rearranged IgH genes with inverted $D_H$ gene segments are rarely seen (29). Part of the explanation for the bias towards recombination by deletion is likely to be the sequence of the RSS and the adjacent coding nucleotides (30, 31). However, there is only a 20-fold preference for deletion over inversion in studies using extra-chromosomal substrates, which sets an approximate upper limit on sequence-intrinsic effects. Direct quantitation of $DJ_H$ junctions in spleen B cells shows more than 1000-fold preponderance of alleles generated by deletion compared to inversion (29). The basis for the 100-fold higher levels of $DJ_H$ junctions seen *in vivo* is unclear at present. Because much of the *in vivo* analysis was performed in splenic B cells, one possibility is that uninverted $DJ_H$ junctions are positively selected during B-cell development. Alternatively, $V_H$ gene recombination may proceed more efficiently on alleles generated by deletional $DJ_H$ joining or secondary $D_H$-to-$J_H$ recombinations may exaggerate the sequence-intrinsic bias towards deletion.

### Frequency of $D_H$ gene segment usage

The preference of different $D_H$ gene segments to undergo recombination has been examined previously. Studies from several laboratories indicate that DFL16.1, the 5′-most $D_H$ gene segment, and Dq52, the 3′-most $D_H$ gene segment, are utilized more frequently than the more numerous DSP2 gene segments that lie in between (32, 33). Recently, Nischke *et al.* (27) compared $D_H$ gene utilization in BALB/c mice and a genetic deletion of Dq52 and surrounding DNA. They too noted a high frequency of DFL16.1 rearrangements in wild-type mice, which was further accentuated in the absence of Dq52. Interestingly, they also noted that 3′ $D_H$ gene segments (other than Dq52) were under represented in Dq52$^{-/-}$ mice in favor of the 5′ $D_H$ gene segments.

$D_H$-to-$J_H$ recombination has also been analyzed in an inducible cell line transformed with a temperature-sensitive v-abl oncogene (34). IgH alleles that are in germline configuration at permissive temperatures initiate $D_H$-to-$J_H$ recombination upon switching off v-abl kinase activity at the non-permissive temperature. Taking care to study initial events, the authors noted that gene segments at either end of the $D_H$ region recombined most frequently. Recombination frequency of more centrally located $D_H$ gene segments dropped off with increasing distance from either end of the locus. This bias for $D_H$ gene segments at either extremity of the $D_H$ cluster has been explained in terms of RSS strength, coding region homologies, and chromosomal location. However, none of these models fully explain the observation as discussed in Chang *et al.* (35). A possible explanation for this pattern may lie in the chromatin structure of the $D_H$ cluster prior to the initiation of rearrangements (see below).

### Lack of $V_H$-to-$D_H$ recombination

A final feature of $D_H$ recombination is that rearrangement of $V_H$ genes to unrearranged $D_H$ gene segments has never been detected. There are at least two stages of development at which this could happen. First, rearrangements are initiated with the locus in germline configuration. As described fully below, chromatin structure-dependent inaccessibility of $V_H$ gene segments probably precludes $V_H$-to-$D_H$ recombination within the

germline IgH locus. However, after initial $D_H$-to-$J_H$ recombination, developing B lymphocytes may contain several unrearranged (upstream) $D_H$ gene segments. Each of these is flanked by RSSs that can recombine with $V_H$ RSSs. Yet this does not happen, despite $V_H$-to-$D_H$ recombination being more efficient than $D_H$-to-$J_H$ recombination in extra-chromosomal substrates (31). These observations imply that the 5′RSS of the $DJ_H$ junction is more recombinogenic than the RSSs of unrearranged $D_H$ gene segments.

One possibility is that proximity of the $DJ_H$ junction to a well-defined recombinational enhancer, such as Eμ, activates this RSS more than the RSSs of more distant unrearranged $D_H$ gene segments. This model draws upon the same idea invoked to explain the lack of $V_H$-to-$DJ_H$ recombination in $E\mu^-$ alleles. Whether germline $D_H$s revert to an inaccessible chromatin configuration concomitant with $DJ_H$ recombination, thereby further accentuating recombinogenicity of the 5′ $DJ_H$ RSS, is not known. An alternative possibility is that $DJ_H$ recombination leaves a 'mark' at the site (or vicinity) of recombination. Such a mark may include recombinase components that enhance utilization of a proximal RSS for the second recombination step. The two models are not mutually exclusive, with regulatory elements such as Eμ stabilizing or perhaps helping to establish the mark.

### Chromatin structure of the $D_H$-Cμ region

While the accessibility model provides a conceptual framework to understand regulatory aspects of V(D)J recombination, it does not define the features of chromatin that make a locus accessible or inaccessible to the recombinase. A long history of chromatin structure as it pertains to gene transcription has revealed hallmarks of genes that are transcriptionally active or readily activatable. Such genes are more sensitive to degradation by nucleases such as DNase 1 (DNase 1 sensitive) (36), often associated with DNase1 hypersensitive sites (short regions of heightened sensitivity to degradation) and usually hypo-methylated at CpG dinucleotides (37, 38). Conversely, genes that will not be transcribed in a particular cell are relatively insensitive to nuclease digestion, lack hypersensitive sites, and are hyper-methylated. These features are true of rearrangeable antigen receptor genes as well. Indeed, in the earliest studies with stably transfected rearrangement substrates, integrants that were DNase sensitive were found to rearrange preferentially (39, 40). These observations suggested that whatever mechanisms make genes accessible to RNA polymerase also make them susceptible to recombination, an idea that was bolstered by the presence of sterile transcripts from unrearranged antigen receptor loci in cells of the appropriate lineage and developmental stage. Though the strict correlation between transcription and recombination is no longer true (22, 41, 42), it is likely that transcriptionally active loci and recombinationally active loci share many common chromatin structural features.

In recent years, covalent modifications of histones have gained prominence as an epigenetic mark that alters the properties of associated DNA (43, 44). In a pioneering study, Hebbes *et al.* (45) showed that the domain of the chicken β-globin locus that was sensitive to DNase 1 digestion correlated perfectly with the region of the genome that was associated with acetylated histones H3 and H4. Within this domain, there are β-like genes that were transcriptionally active and others that were transcriptionally inactive, indicating that DNase sensitivity and histone acetylation correlate with a poised state. Felsenfeld and colleagues (46, 47) further demonstrated that the domain of acetylated histones matched exactly with the presence of histone H3 modified by methylation at lysine 4 (H3K4-Me) and depleted of histone H3 methylated at position 9 (H3K9-Me). Furthermore, this domain was established at the earliest stages of chicken erythropoiesis. Taken together with large numbers of studies in yeast, the current hypothesis is that histone modifications are the cause rather than the effect of marking a locus for expression (43, 48).

Analysis of histone modifications across the unrearranged IgH locus in pro-B cells from RAG-deficient mice reveals three important features (26, 49). First, activation-specific histone acetylation is apparent at the $D_H$ gene segments as well as within $J_H$ gene segments, Eμ, and the Cμ exons. This region is approximately 100 kb. Within it, the $J_H$ gene segments show somewhat higher levels of histone acetylation compared to other parts of the domain. We have speculated that this microdomain of elevated acetylation may help initiate V(D)J recombination by targeting recombinase or chromatin re-modeling activities to the $J_H$ gene segments. Such microdomains have also been detected in the murine β-globin locus where they colocalize with the expressed gene (47, 50). In addition, DFL16.1 is also hyper-acetylated compared to other intervening $D_H$ gene segments (Keyes, DC, and RS, manuscript in preparation). Perhaps accentuated histone acetylation at either end of the $D_H$ locus is partly responsible for biased $D_H$ gene recombination *in vivo*. Strikingly, the Cδ exons, which are located within 3 kb of Cμ, are not associated with hyper-acetylated histones. It must be kept in mind that a direct link between activating histone modifications and recombinase accessibility remains to be established. For the purposes of

this review, however, we refer to activation of a locus by the criteria of histone modifications being synonymous with availability for recombination.

Analysis of the $D_H$-Cμ domain has been extended to include a second activation-specific modification, H3K4-Me. Like the chicken β-globin locus, H3K4 methylation across the $D_H$-Cμ region mostly parallels the pattern of histone acetylation, with one important exception. Eμ, which is associated with hyper-acetylated histones, contains only moderate levels of H3K4-Me. This example is the only one where the two kinds of activation modifications do not correlate perfectly. Presumably, Eμ binding transcription factors do not effectively recruit histone methyl transferases to the genome. The functional implications of this difference are yet to be determined. Conversely, H3K9-methylation, a histone modification associated with inactive loci, is reduced in the $D_H$-Cμ region. Morshead *et al*. (51) have also examined phosphorylation of serine 1 of histone H4 (H4S1-phos) and association of the chromatin-remodeling complex BRG1 across the locus. BRG1 is broadly distributed across the accessible gene segments of the locus in a pattern similar to histone H3 acetylation. H4S1-phos, a modification whose functional correlates are not yet known, shows low levels in the $D_H$-Cμ region with peaks at the $V_H$81X gene segment, located approximate 100 kb 5′ of DFL16.1, and at Cγ3 exons, located 50 kb 3′ of Cδ.

A second important feature from the analysis of histone modifications across the unrearranged IgH locus is that none of the $V_H$ gene segments examined are in active chromatin configuration by the criterion of being associated with acetylated histones. It is interesting to recall that the majority of $CD19^+$ cells from RAG-deficient bone marrow classify as Fraction C, the population that undergoes $V_H$-to-$DJ_H$ recombination. Yet, the $V_H$ genes in these cells are in an inactive configuration with respect to histone acetylation, while normal Fr. C cells show elevated histone acetylation of representative $V_H$ genes across the locus (26, 52). These observations suggest that there is step-wise activation of the IgH locus. The first domain to be activated contains $D_H$ and $J_H$ gene segments and the Cμ exons. It is likely that $D_H$-to-$J_H$ recombination is initiated within this domain. Exclusion of $V_H$ gene segments from the activated domain provides a simple explanation for $D_H$-to-$J_H$ recombination preceding $V_H$-to-$DJ_H$ recombination.

A third observation is that the abrupt drop-off of histone acetylation between Cμ and Cδ demarcates one end of the active domain. In the chicken and murine β-globin loci domain boundaries are determined by *cis* elements that also have the property of being insulators. Such boundary elements are marked by DNase1 hypersensitive sites containing multiple binding sites for the transcription factor CTCF and prevent an enhancer located on one side from activating a promoter on the other. Unlike boundary elements in the globin loci, the region between Cμ and Cδ is not marked by a DNase1 hypersensitive site, suggesting that this boundary may be generated by a different mechanism. At the 5′ end of the $D_H$ cluster, Morshead *et al*. (51) have proposed a domain boundary very close to DFL16.1 that is marked by a peak of H3K4 methylation. This region also does not contain a DNase1 hypersensitive site (26), indicating that both extremities of the $D_H$-Cμ domain are not established by classical boundary elements/insulators. Perhaps the lack of an insulator at the 3′ end is because Cδ is expressed soon after immunoglobulin gene rearrangements. To ensure appropriate expression of Cδ, an insulator between Cμ and Cδ would have to be developmentally regulated, and there may be no need for such additional complexity. One mechanism by which this boundary may be established is by balancing histone acetylation spreading-3′ from the $J_H$-Cμ part of the locus with histone-de-acetylation spreading 5′ from downstream sequences. In this scheme, the sequence *per se* of the intergenic region does not determine the location of the boundary. Cδ expression may simply require tilting the balance in favor of acetylation so that the boundary gets pushed further 3′, and the active domain includes the Cδ exons. This outcome could be achieved for example by quantitatively or qualitatively altering histone acetyl transferases (HATs) loaded on to the genome at Eμ or by RNA polymerase II-associated HATs, once sufficient polymerase density is established on the gene.

Approximately, half of the $D_H$-Cμ domain has been examined by DNase 1 sensitivity assays, leading to the identification of only two DNase 1 hypersensitive sites. One site maps to intronic Eμ enhancer and the other close to Dq52, a region that contains a sterile promoter enhancer. Both sites have elevated histone acetylation, and the location of $J_H$ gene segments between them may be one explanation for the $J_H$-associated microdomain of accentuated histone acetylation. While juxtaposition of these two regulatory sequences may increase recombination efficiency at $J_H$, genetic deletion studies show that neither is essential. Furthermore, DNase 1 hypersensitive sites were not detected close to DFL16.1 or DSP2.4, indicating that a closely associated hypersensitive site and accompanying histone modification are not essential for $D_H$ recombination.

## Regulation of $V_H$ gene recombination

The most recent count places the number of murine $V_H$ genes in the mid-one hundred range, with the proviso that this is

likely to be an underestimate. Half or more of these genes belong to the J558 family. Most of the J558 genes are located in the 5′-telomere-proximal half of the $V_H$ locus, though several are also interspersed amongst genes in the 3′ half (*Fig. 1*). $V_H$ 3609 genes that comprise the next largest family are interspersed amongst J558 genes in the 5′ half of the locus. At the 3′ end of the locus, the $V_H$7183 and Q52 gene families also overlap significantly. Other smaller gene families are distributed between these 3′ genes and the 5′ half of the locus. The entire $V_H$ cluster spans approximately 1.5 Mb.

$D_H$-to-$J_H$ recombination in Fr. A/B is followed by $V_H$-to-$DJ_H$ recombination in Fr. C. As described in the preceding sections, the inactive state of all $V_H$ gene segments in $CD19^+$ bone marrow cells from RAG-deficient mice provides a plausible mechanism for the early onset of $D_H$-to-$J_H$ recombination. $V_H$ recombination follows once $DJ_H$ junctions are formed, except on alleles that lack Eμ (16–18), in cells from IgH transgenic mice (53, 54), and in thymocytes (55). The absence of $V_H$ recombination in these three situations likely reflects different aspects of the regulation of recombination. Possibilities for $E\mu^-$ alleles were discussed earlier. In thymocytes, it is likely that $V_H$ genes are never activated, and in IgH transgenic mice, $V_H$ recombination is probably suppressed by feedback inhibition. Activation and inactivation of $V_H$ genes is discussed below.

## Proximal versus distal $V_H$ gene recombination

A well-documented feature of $V_H$ recombination is the preferred rearrangement of $D_H$-proximal $V_H$ genes during fetal ontogeny, as is best exemplified by the over-representation of $V_H$7183 and $V_H$Q52 gene families in the fetal liver (56–58). Preferential rearrangement of these genes has been attributed to chromosomal position (proximity to $D_H$-Cμ), the nature of their associated RSSs (59, 60), and the role of coding-end microhomologies in facilitating recombination (61, 62). Briefly, these studies show that the $V_H$81X RSS mediates recombination more efficiently than a J558 RSS, and that microhomologies selectively direct in-frame fusion of $V_H$81X sequences in the fetal repertoire. While most of these studies compared the recombination frequencies of $V_H$81X to J558 genes, a recent analysis compared utilization and RSSs amongst 7183 family members (63). Feeney and colleagues (63) found that the RSSs of 7183 genes fell into two categories, but genes with weaker RSSs were utilized preferentially in neonatal bone marrow. Furthermore, with one exception, gene usage correlated better with placement within the locus than with RSS strength. That is, genes closer to the 3′ end were represented at a higher frequency. Interestingly, $V_H$Ox2, a $V_H$q52 family member that had been shown to rearrange preferentially (64, 65), also mapped close to the 3′ end of the locus, supporting the idea that proximity to $D_H$/Cμ positively influences rearrangement efficiency.

Though the bias towards $D_H$-proximal $V_H$ gene rearrangements is most obvious in the fetal repertoire, accumulated evidence suggests that these genes also recombine preferentially during B lymphopoiesis in the bone marrow (66, 67). Unlike fetal liver, however, the early developmental preference for proximal $V_H$ recombination is swamped out by the recombination of the more numerous J558 gene segments in the adult (68, 69). Continued under-representation of J558 genes in the fetal repertoire could be due to two reasons. First, recent studies indicate that fetal lymphopoiesis is less dependent on IL-7 (70, 71). Reduced IL-7 involvement in the fetal liver may result in inefficient activation of the distal $V_H$ genes, which are IL-7-dependent (see below). Second, it has been noted that microhomology-directed coding joint formations tend to produce out-of-frame V(D)J fusions in the case of J558 genes but in-frame fusions of 7183 genes (61, 72). This effect is particularly pronounced in fetal development, where the absence of terminal deoxynucleotidyl transferase-directed N nucleotide additions enforces functional joins to be created out of germline-encoded sequences. In the adult, by contrast, although J558 gene recombination follows that of the proximal genes, introduction of N regions allows greater chance of productive J558 rearrangements. Thus, the prevalence of these genes in the adult splenic repertoire is most likely determined by selection events beyond the regulation of recombination, such as the proliferative advantage of cells that express J558-bearing pre-BCRs.

Regulation of $D_H$-proximal $V_H$ genes in a manner distinct from the distal part of the locus is also evident in Pax 5- and Ezh2-deficient mice (73, 74). In both genetic backgrounds, $D_H$-to-$J_H$ recombination in pro-B cells occurs at normal levels, but $V_H$-to-$DJ_H$ recombination is significantly reduced. Among $V_H$ genes, the level of $V_H$81X recombination is comparable to that in normal pro-B cells, but utilization of more distal $V_H$ genes decreases with increasing distance from $V_H$81X, with the result that there is negligible recombination of the J558 genes. The rearrangement phenotype of $Pax5^{-/-}$ and $Ezh2^{-/-}$ strains resembles that of mice deleted for the gene encoding IL-7Rα (75). Indeed, connections between these three genes have been made. IL-7R signaling is proposed to be a positive regulator of Pax5 gene expression (75), and IL-7 treatment of bone marrow pro-B cells reduces H3-K27 methylation of the genome that may be mediated by the methyl transferase activity of Ezh2 (74). It is possible therefore that a common

mechanism links Pax5-, IL-7Rα-, and Ezh2-mediated regulation of $V_H$ gene recombination. However, this interpretation should be taken with caution. Recently, it has been shown that there is little adult B lymphopoiesis in the absence of IL-7 or in IL-7Rα$^{-/-}$ mice (71, 76). This finding suggests that the predominant $V_H$81X gene usage seen in IL-7Rα$^{-/-}$ mice likely reflects the fetal origin of these cells. Conversely, Pax5$^{-/-}$ B cells that contain 7183 gene rearrangements are unlikely to be of fetal origin, because there is undetectable B lymphopoiesis in the fetal liver of these animals (77). Thus, despite the similarity in $V_H$ gene utilization profile, the cell populations being analyzed are quite different and may not be directly comparable. Together, these observations suggest that the phenotype of IL-7Rα$^{-/-}$ mice is that of *normal* fetal lymphopoiesis, while that of Pax5$^{-/-}$ is of *abnormal* adult (or neonatal) lymphopoiesis. Regardless of the mechanism by which these shifts occur, the recombination data and the phenotype of the genetic knock-outs strengthen the idea that proximal $V_H$ genes are regulated differently than the distal $V_H$ genes.

## Distinguishing features of proximal and distal $V_H$ gene chromatin structure

### Proximal $V_H$ genes

An insight into the basis for biased $V_H$7183 rearrangements in the fetus comes from chromatin structural studies of Calame and colleagues (49). They compared the state of histone acetylation of unrearranged $V_H$ genes in IL-7 expanded cells from the fetal liver or the bone marrow of RAG$^{-/-}$ mice. Representative examples of $D_H$-proximal $V_H$ genes belonging to 7183, S107, Vgam, and V10 families were associated with hyper-acetylated histone H4 in fetal liver-derived cells but not bone marrow-derived cells. These observations indicate selective chromatin structure-dependent activation of unrearranged $D_H$-proximal $V_H$ genes in the fetal liver. Because IL-7-dependent distal $V_H$ genes may be inefficiently activated in the fetal liver, a combination of accentuated activation of proximal genes and reduced activation of distal genes could result in the biased $V_H$ usage noted in the fetal liver. Sequence-specific effects such as RSS strength and coding-end micro-homologies are presumably superposed on this skewed pattern of locus activation to further restrict the fetal repertoire.

In adult bone marrow cells from RAG$^{-/-}$ mice, neither proximal nor distal $V_H$ genes are significantly activated, as measured by histone acetylation. However, unrearranged proximal genes are hyper-acetylated in cell lines or primary bone marrow cells that carry $DJ_H$ recombined alleles (26, 78). Based on these observations, we have proposed that proximal $V_H$ genes are activated as a consequence of $D_H$-to-$J_H$ recombination during B lymphopoiesis in the bone marrow. Though the mechanism is not known, one simple possibility is that $DJ_H$ joining brings proximal $V_H$ genes under positive regulatory 'influence' of the $J_H$-Cμ part of the locus and associated enhancers/regulatory elements. The molecular basis for the differences in proximal $V_H$ gene activation in the fetus compared to the adult is not clear. Our working hypothesis is that the signals that turn on these genes are quantitatively 'stronger' in the fetal liver, so that the resultant activated state can be seen even in the absence of $DJ_H$ recombination. The same signals are 'weaker' in the adult bone marrow, thus requiring $DJ_H$ recombination-dependent proximity to be observed. Recent studies show that residual B-cell development in IL-7Rα-deficient mice is dependent on the Flt-3 receptor tyrosine kinase (79). Because B cells in IL-7Rα$^{-/-}$ mice are of fetal origin and carry a proximal $V_H$ repertoire, an attractive candidate for activating proximal $V_H$ genes is Flt-3 signaling.

Activation of proximal $V_H$ genes by movement closer to the $J_H$-Cμ part of the locus implies allele specificity. That is, $V_H$ gene recombination will be selectively activated on $DJ_H$ recombined alleles compared to germline alleles. This hypothesis was recently corroborated in mice carrying a core RAG1 gene knocked-in to the endogenous locus (80). Significant numbers of B cells in these mice contain one germline allele and one functionally recombined allele ($V_HDJ_H$). The origin of these cells is presumably through inefficient recombination by a defective enzyme that leads to $DJ_H$ recombination on one allele, rather than synchronously on both alleles as is usually observed. At this point, $V_H$ recombination to the $DJ_H$ junction is apparently more efficient than either $D_H$-to-$J_H$ recombination or $V_H$ recombination to unrearranged $D_H$ gene segments on the unrearranged allele.

### Distal $V_H$ genes

*In vitro* treatment of CD19$^+$ RAG$^-$ bone marrow cells with IL-7 leads to selective hyper-acetylation and enhanced accessibility of distal $V_H$J558 gene segments, showing that these genes are activated by IL-7R-initiated signals even in the absence of $DJ_H$ recombination (26, 49, 52). The region of acetylation corresponds closely to $V_H$ coding sequences indicating that the result of these signals is not a uniformly hyper-acetylated genomic domain. IL-7 responsiveness is not restricted to J558 genes, but it is also a property of 3609 family members located in the 5′ part of the $V_H$ locus. Conversely, J558 genes that fall in the 3′ half are not IL-7 responsive, indicating that chromosomal location rather than family ties determine IL-7 sensitivity of

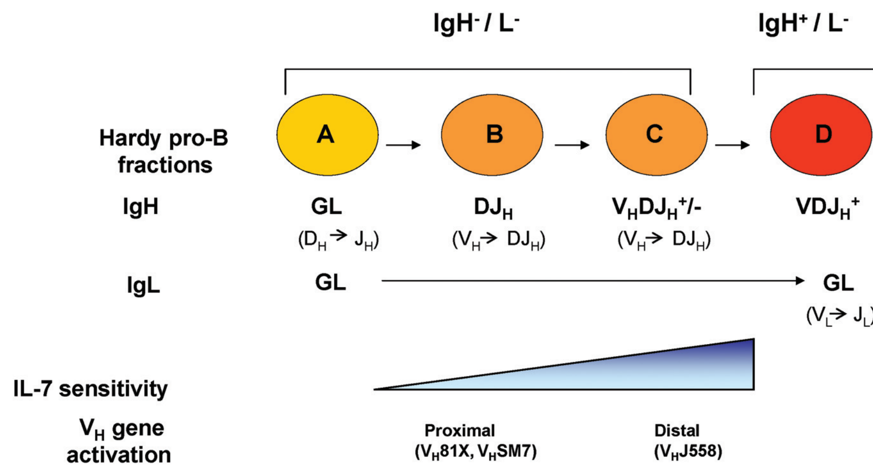


**Fig. 4. Mechanism of ordered $V_H$ recombination.** Pro-B cell subsets as defined by Hardy are shown in the circles. Genomic structure of the immunoglobulin (Ig) loci is shown in bold below each circle. Presumed ongoing rearrangements in each subset are indicated in brackets below genomic structure. Recombination at the Ig heavy-chain (IgH) locus is initiated in late A fraction cells; our observations suggest that only the $D_H$-Cμ part of the locus is accessible to recombinase at this stage resulting in $D_H$-to-$J_H$ recombination. Proximal $V_H$ gene families are activated on $DJ_H$ recombined alleles as described in the text. We propose that distal $V_H$ genes, which are activated by interleukin-7 (IL-7), lag behind the proximal $V_H$s, because IL-7 responsiveness is gained gradually during B-cell development (81) (indicated by a triangle). Preferential rearrangement of proximal $V_H$ genes is therefore a consequence of the late activation of the 5′ $V_H$ genes via the IL-7 receptor signals. GL, germline.

unrearranged $V_H$ gene segments (49). Thus, the $V_H$ genes can be broadly divided into two independently regulated chromosomal domains: an IL-7-responsive domain at the 5′ (telomere-proximal) end of the locus and the $J_H$-proximal $V_H$ genes at the 3′ end of the locus. A 100-kb region has been proposed to contain the boundary between the two domains (49).

Taken together, these observations lead to the following model for biased recombination of 7183 genes in the adult bone marrow (26) (Fig. 4). The IgH locus is activated for recombination in step-wise fashion. First, a region encompassing $D_H$ and $J_H$ gene segments is activated, presumably via regulatory sequences such as Eμ. At this stage, $V_H$ genes are in inactive chromatin configuration, as assessed by histone acetylation and nuclease accessibility. $D_H$-to-$J_H$ recombination is initiated within this domain. $DJ_H$ joining, in turn, activates recombination of proximal $V_H$ genes leading to preferential rearrangements of these genes. Recombination of distal $V_H$ genes may lag behind the proximal genes for two reasons. First, there is evidence that sensitivity of pro-B cells to IL-7 increases gradually during pro-B-cell differentiation, reaching a peak in the heightened responsiveness of pre-BCR-expressing pre-B cells (81, 82). The mechanism by which differentiating pro-B cells become more sensitive to IL-7 remains unclear, though higher levels of IL-7R expression probably play a role. However, IL-7R expression alone is unlikely to be the complete answer, because $RAG^{-/-}$ pro-B cells express high levels of the receptor yet require high concentrations of IL-7 to be activated (82). We proposed that at the time when the proximal $V_H$s are activated, the cells may be relatively insensitive to IL-7 and therefore maintain the distal $V_H$ genes in an inaccessible chromatin configuration. In addition, the RSSs of J558 genes have been found to be particularly poor compared to RSSs associated with 7183 genes in mediating recombination (60). Both mechanisms may contribute to give the proximal $V_H$ genes an early recombinational edge. However, as IL-7 sensitivity of differentiating pro-B cells increases, the distal $V_H$ genes are activated, and the more numerous J558 genes come to dominate the repertoire. There is no evidence that increased IL-7 sensitivity or proximal $V_H$ gene activation by $DJ_H$ recombination represent discrete steps of pro-B-cell differentiation. Rather, we view them as stages in a continuum that favors preferential rearrangement of proximal genes.

### Inactivation of $V_H$ genes

Successful V(D)J recombination produces an allele that encodes a complete IgH protein. Production of IgH in the pro-B cell is judged in the context a pre-BCR, which consists of IgH in a complex with the non-rearranging gene products λ5 and V-pre-B, and the signaling components Igα and Igβ (83). Pre-BCR expression results in several rounds of cell proliferation and differentiation of pro-B cells to pre-B cells. Immunoglobulin light-chain (IgL) gene recombination is initiated in pre-B cells. Several lines of evidence indicate that $V_H$ genes are refractory to further recombination in pre-B cells, a phenomenon referred to as feedback inhibition or allelic exclusion. Stringent regulation of $V_H$ gene recombination may have evolved to prevent loss of the functional IgH allele

by, for example, $V_H$ replacement or to prevent the generation of B cells with dual antigen receptor specificity. Feedback inhibition is also well documented at the TCRβ-chain gene locus (but is less evident at IgL or TCRα-chain loci) (84, 85).

The strongest evidence that feedback inhibition is an active process is the lack of $V_H$-to-$DJ_H$ recombination in B cells of IgH transgenic mice, though $D_H$-to-$J_H$ recombination occurs at normal levels (53, 54). While $D_H$ recombination may occur at the normal pro-B-cell stage in these mice, it is important to recognize that $D_H$-to-$J_H$ recombination continues in pre-B cells, as shown by Bosma and colleagues (86). However, breaks at the $5'D_H$ RSS, a telltale sign of $V_H$ recombination, are not seen in these cells (86, 87). These observations directly demonstrate that the $D_H/J_H$ region of the IgH locus is accessible to recombinase in pre-B cells, but the $V_H$ part of the locus is not accessible.

Lack of $V_H$ gene recombination in pre-B cells correlates well with the associated chromatin structure in these cells. Studies in primary cells as well as cell lines show that $V_H$ genes in pre-B cells are relatively insensitive to nucleases such as exogenously added RAG proteins, DNase 1, and restriction enzymes (26, 49, 52, 78, 87, 88). It must be kept in mind, however, that all these studies examined a limited number of $V_H$ gene segments, and more comprehensive analyses may reveal additional hitherto undiscovered patterns. Reduced accessibility of $V_H$ genes also correlates with reduced histone acetylation of unrearranged $V_H$ genes in primary pre-B cells (52). Two noteworthy features of $V_H$ gene histone modification are (i) that even though the level of acetylation is lower in pre-B cells than in pro-B cells, there is residual acetylation that is significantly above the background in non-lymphocytes and (ii) that the acetylation levels are comparable to those of $V_H$ genes in thymocytes, where $V_H$ genes do not normally recombine. Based on the similarity in histone modification pattern of $V_H$ genes in thymocytes and pre-B cells, we favor the hypothesis that partial de-acetylation from the levels seen in pro-B cells is sufficient to prevent recombinase access to these genes in pre-B cells. These observations in B-lineage cells closely mimic the reduced levels of histone acetylation of unrearranged TCR Vβ gene segments in allelically excluded $CD4^+CD8^+$ thymocytes (89).

How does one end of the IgH locus (or the TCRβ locus) get specifically inactivated during the pro-B to pre-B (or double-negative to double-positive) transition? The observation that the distal part of the IgH locus is activated by IL-7, a transient signal for developing B cells, first suggested the possibility that the activated state may be reversed upon loss of the activating signal. In other words, IL-7 signals turn on the distal $V_H$ genes, and loss of IL-7 signals allows unrearranged $V_H$ genes to revert to a hypoacetylated, less accessible, and non-recombinogenic state. Rearranged $V_H$ genes come under the influence of $J_H$-Cμ proximal regulatory sequences such as Eμ and remain transcriptionally active. A corollary of this model is that continued signals are required to maintain IL-7-responsive $V_H$ genes in an active configuration. Consistent with this idea, we found that the level of phosphorylated STAT5, a hallmark of IL-7 signals, is lower in pre-B cells compared to pro-B cells (52). It is not clear whether STAT5 itself mediates the effects of IL-7 on the J558 genes, but it serves as a convenient marker of IL-7 signaling. Finally, allelically excluded $V_H$ genes in splenic B cells are re-activatable by IL-7 signaling, as measured by histone modification and nuclease accessibility by IL-7 signaling. These observations showed that reduced IL-7 signaling coincides with the onset of allelic exclusion and the emergence of cells that contain $V_H$ genes in a hypo-acetylated state. However, it was difficult to distinguish whether hypo-acetylated $V_H$ genes in pre-B cells arose by selection of a small population of cells with reduced $V_H$ acetylation or active de-acetylation of a hyper-acetylated locus.

These possibilities were further investigated in a cell line model of pro-B to pre-B-cell differentiation. Abelson virus-transformed cell lines usually have a pro-B phenotype with ongoing transcription and recombination of IgH but not IgL genes. It is well known that switching off v-abl kinase induces pre-B-like characteristics including κ gene transcription and recombination (90, 91). In a temperature-sensitive v-abl transformed cell line, distal $V_H$ gene accessibility and acetylation progressively reduces upon inactivating the oncogene, closely mimicking the differences seen between pro-B and pre-B cells (52). In contrast, histone acetylation as well as nuclease accessibility at the $J_H$/Cμ end is unchanged. Because reduced acetylation occurs in the absence of cell division or significant cell death, selection is unlikely to play a role in this process, indicating that the hypo-acetylated state is the result of active de-acetylation mediated by histone de-acetylases (HDACs). These effects on $V_H$ genes are not observed if cells are treated with IL-7 during v-abl inactivation, suggesting that loss of IL-7-mimetic signals initiate hypoacetylation of the distal $V_H$ genes.

The role of IL-7 in regulating $V_H$ gene accessibility, inaccessibility, and cell proliferation during B-cell differentiation implies that IL-7 responsiveness must be strictly regulated. In early pro-B cells, IL-7 sensitivity must increase sufficiently to initiate distal $V_H$ gene hyper-acetylation and recombination yet not exceed a threshold that will push cells into the S phase of the cell cycle. This sensitivity may be achieved by cell-extrinsic mechanisms, such as limiting amounts of IL-7 in

the bone marrow, or by cell-intrinsic mechanisms, such as regulating IL-7R$\alpha$-chain expression or altering cellular sensitivity to IL-7. Maintaining pro-B cells in G1 allows V(D)J recombination to initiate and prospectively to generate functional IgH genes. Those cells that successfully generate a functional allele express a pre-BCR, and pre-BCR-induced extracellular regulated kinase 1 (Erk1) activation further sensitizes the cell to receive IL-7 signals, thus selecting them to undergo several rounds of IL-7-dependent proliferation (92). While IL-7 signals are active, the distal $V_H$ genes presumably remain in a hyper-acetylated and chromatin-accessible configuration. However, recombinase activity is reduced during this phase due to active degradation of RAG2 protein (93, 94), thus precluding recombination of $V_H$ (or $D_H$) gene segments. The proliferative burst of pro-B cells en route to becoming pre-B cells is tightly regulated in the range of 6–8 cell divisions by at least two mechanisms. First, because Erk1 activation is usually transient, the increased sensitivity to IL-7 probably does not last. Second, the pre-BCR may actively attenuate IL-7R signaling, as has been shown for the BCR-initiated signal (95). Thus, although the initial cellular response to generation of a pre-BCR is enhanced sensitivity to IL-7 with resultant proliferation, continued signals shift the balance towards de-sensitization of the IL-7R and cessation of proliferation. Small, resting pre-B cells that emerge do not express IL-7R and therefore cannot respond to IL-7 signals. The $V_H$ genes in these cells are associated with hypo-acetylated histones and are no longer available for recombination.

Cell proliferation may result in 'diluting out' of epigenetic histone modifications by random distribution of pre-existing nucleosomes and deposition of new nucleosomes on replicated DNA. Gene regulatory epigenetic marks can be re-established after cell division by appropriate interplay between histone modifying enzymes and regulatory sequence-bound transcription factors. Thus, during the proliferation phase that accompanies pre-B-cell differentiation, there is competition between diluting out of acetylated histones associated with the $V_H$ genes and re-acetylation of newly deposited histones by IL-7 signaling. It remains to be determined whether replication-dependent dilution accounts for the bulk of reduced $V_H$ gene acetylation that is characteristic of the allelically excluded state. Because germline $V_H$ genes can revert to a hypoacetylated state in the absence of cell division, albeit in a cell-line model, our working hypothesis is that HDACs play a role in establishing allelic exclusion. We propose that once IL-7-dependent positive activation by HATs is terminated by one of the mechanisms noted above, HDACs spread into the distal $V_H$ locus snuffing out acetylation and accessibility. An intriguing possibility is that inactivating HDACs associated with telomere-proximal heterochromatin may be poised continuously to strike at the distal $V_H$ genes from the 5′ flank.

### Allelic exclusion of 3′ (IL-7 non-responsive) $V_H$ genes

The model described in the preceding section provides a plausible mechanism for feedback inhibition of IL-7-responsive $V_H$ genes that make up more than half of all $V_H$ genes. How are the other $V_H$ genes down regulated in pre-B cells? A straightforward extrapolation of our model for IL-7-dependent genes leads to the hypothesis that it may be achieved by down-regulating transient signals that activate the proximal genes. As described above, such signals may be ones that cooperate with $DJ_H$ recombination to activate proximal $V_H$ gene recombination. However, because these signals have not yet been identified, the model cannot be directly tested.

Alternatively, it is possible that the proximal $V_H$ genes are intrinsically less susceptible to feedback inhibition. The most compelling evidence in support of this conjecture comes from the analysis of endogenous $V_H$ gene usage in IgH transgenic mice. Splenic B cells from mice that express a human IgM transgene show significant levels of proximal $V_H$ gene recombination of $V_H$7183 and $V_H$q52 gene families but very low levels of $V_H$J558 gene recombination (96). Similarly, double-producing splenic hybridomas generated from a different transgenic strain show that endogenous gene rearrangements largely use proximal $V_H$ genes (97). In both situations, presence of the transgene probably results in early expression of a pre-BCR and rapid transit of pro-B cells into pre-B cells. Endogenous IgH rearrangements that were scored in splenic B cells of these mice probably occurred at the pre-B-cell stage of differentiation, when $V_H$ genes are normally suppressed by feedback inhibition. Significant bias towards use of proximal $V_H$s in this situation is therefore consistent with these genes being less efficiently shut down compared with IL-7-responsive distal $V_H$ genes.

### Summary

The IgH locus is activated in discrete, independently regulated domains. The $D_H$-C$\mu$ domain that is activated first is characterized by a discrete boundary at the 5′ end just upstream of the DFL16.1 and a gradual transition to inactive chromatin at the 3′ end close to the C$\delta$ exons. Neither boundary is marked by a DNase 1 hypersensitive site in lymphoid cells. Within this domain, there is considerable additional chromatin fine structure. Of note, the four $J_H$ gene segments are encompassed in a

microdomain of accentuated histone modification, where we speculate that the recombinase machinery is first recruited to this gene. In addition, DFL16.1 and Dq52 gene segments at either extremity of the $D_H$ cluster also have higher levels of activation-specific histone modifications compared to the internal $D_H$ gene segments, which may explain, in part, the preferential use of these $D_H$s in B cells. Finally, the $D_H$-Cμ domain contains only two identified *cis*-regulatory sequences that may be involved in its activation. Deletion of each element, individually, has different rearrangement phenotypes suggesting that they are functionally non-identical.

$D_H$-to-$J_H$ recombination is followed by activation of $V_H$ genes in two independently regulated chromosomal domains. The $D_H$ proximal $V_H$ genes are particularly active in fetal liver-derived pro-B cells and may require $DJ_H$ recombination to be activated in adult bone marrow cells. $D_H$-distal $V_H$ genes are activated in response to IL-7. These features of independent control can be incorporated into plausible models that explain the predominant use of proximal $V_H$ genes in the fetal repertoire and their preferential rearrangement in adult pro-B cells. Identification of signals that turn on $V_H$ genes leads to a simple proposal for how they may be inactivated to impose feedback inhibition of IgH gene rearrangements. The chromatin landscape of the IgH locus may be viewed as a ground-state on which sequence-specific regulation of V(D)J recombination, such as the role of strong/weak RSSs and coding-end micro-homologies, are superposed to direct the formation of a diverse B-cell repertoire.

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