Mechanism and Control of V(D)J Recombination at the Immunoglobulin Heavy Chain Locus

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Abstract

V(D)J recombination assembles antigen receptor variable region genes from component germline variable (V), diversity (D), and joining (J) gene segments. For B cells, such rearrangements lead to the production of immunoglobulin (Ig) proteins composed of heavy and light chains. V(D)J is tightly controlled at the Ig heavy chain locus (IgH) at several different levels, including cell-type specificity, intra- and interlocus ordering, and allelic exclusion. Such controls are mediated at the level of gene segment accessibility to V(D)J recombinase activity. Although much has been learned, many long-standing questions regarding the regulation of IgH locus rearrangements remain to be elucidated. In this review, we summarize advances that have been made in understanding how V(D)J recombination at the IgH locus is controlled and discuss important areas for future investigation.
INTRODUCTION

Lymphocytes can initiate specific immune responses against antigens by generating a nearly infinite diversity of antigen receptors within the constraints of a finite genome (1). This remarkable feat is achieved in large part by a somatic recombination process known as V(D)J recombination. Through V(D)J recombination, the variable region of antigen receptor genes is assembled from component V, D, and J gene segments. There are seven different loci that are rearranged to generate the antigen receptors of T and B lymphocytes. These include the immunoglobulin (Ig) heavy chain locus (IgH) and the Ig light chain (IgL) loci (Igκ and Igλ), which encode the antigen receptor and secreted antibodies of B cells, and the T cell receptor (TCR) β, δ, and α, γ loci. The rearrangement of these loci is tightly controlled in a lineage-, stage-, and allele-specific manner. Regulation of V(D)J recombination in these contexts requires a complex orchestration of DNA, chromatin structure, and trans-acting factors in collaboration with transcriptional activation and DNA breakage and repair mechanisms. As the immune system is not required for survival in a pathogen-free environment, the antigen receptor loci provide a system uniquely suited to advance our basic understanding of these fundamental cellular processes, as well as their roles in both normal and dysfunctional states. In this review, we examine advances in understanding regulation of V(D)J recombination at the IgH locus.

OVERVIEW OF THE BASIC V(D)J RECOMBINATION REACTION AND DIVERSITY GENERATION

The basic subunit of an Ig or antibody molecule is a pair of identical Ig heavy chains and a pair of identical Ig light chains. The N-terminal portion of the heavy chain and light chain has a variable and unique amino acid sequence (variable region), and is involved in specific antigen binding. By contrast, the C-terminal portion of these chains comes in only a few different forms and is termed the constant region, which prescribes the class and effector function of an antibody molecule. Variable regions of antigen receptors, including IgH and IgL chains and TCR chains, are assembled from germline V, D, and J gene segments through a site-specific recombination reaction known as V(D)J recombination (2, 3). V(D)J recombination only functions in developing T and B lymphocytes. The recombination-activating genes 1 and 2 (RAG1 and RAG2) together form the RAG endonuclease (RAG), which is sufficient in vitro (4, 5) and necessary in vivo (6, 7) to initiate the cleavage phase of V(D)J recombination. RAG introduces DNA double-strand breaks (DSBs) specifically at the borders between two coding segments and their flanking recombination signal sequences (RSs). RSs are comprised of a highly conserved heptamer and nonamer, separated by a relatively non-conserved spacer of either 12 or 23 base pairs (bp) (8). RAG function requires that one RS have a 12-bp spacer and the other a 23-bp spacer for efficient recognition and DSB formation (9, 10), a restriction referred to as the 12/23 rule (11). Following further processing of the ends, the resolved DSBs are repaired by ubiquitously expressed nonhomologous end joining proteins to generate coding and RS joints (12).
The D_H segments are located in a roughly 50 kb region upstream of the four J_H gene segments, which are, in turn, located within a 2 kb region starting approximately 700 bp downstream of DQ52, the most 3’ D_H gene segment. Starting with the C_μ exons that lie about 7 kb downstream of the J_H gene segments, the various constant region exons are spread across 200 kb (13). Although this review focuses primarily on the mouse IgH variable region locus, the human locus has a similar organization (Figure 1) (13). Based on past usage, we refer to a D_H to J_H rearrangement as DJH and a VH to DJH rearrangement as VHDJH. Germline VH and D_H gene segments are flanked by upstream transcriptional promoters, which initiate transcription in a developmentally regulated fashion and may be important for activation and regulation of IgH rearrangements (14–16). Assembly of the complete VHDJH variable region at the J_H locus places the germline VH exon promoter in close proximity to a strong enhancer element (referred to as iE_μ) that lies in the intron between the J_H and C_μ exons. Transcripts initiated from the promoter of the rearranged VH exon run through the C_μ constant region exons, and RNA splicing assembles the mature heavy chain mRNA with the variable region exon spliced to the C_μ constant region exons via splice donor and acceptor signals encoded 3’ to the J_H segment and 5’ to the first C_μ exon (Figure 2b) (17).

Several different mechanisms generate IgH variable region diversity with respect to V(D)J recombination (Figure 2). In assembled IgH variable exons, the bulk of the exon is encoded by the germline VH segment, which also contains two of the three complementarity determining (antigen contact) regions (CDRs). These two CDRs are different in different germline VH segments, which provides germline-encoded diversity. In addition, the VH,D_H,J_H junctional region encodes the third CDR (Figure 2b). Random assortment of the different germline VH, D_H, and J_H segments is therefore a source of somatic diversity. Finally, the combinatorial diversity generated by the different combinations of germline V, D, and J segments is further augmented by diversification of D to J and V to D junctions during V(D)J recombination through deletion of nucleotides and nontemplated nucleotide additions (N regions) by terminal transferase to generate a vast diversity of different IgH variable region gene exons (18). Although junctional diversification mechanisms typically only lead to deletion or addition of a limited number of nucleotides, the actual number is usually random. Therefore, given that the ATG translation initiation codon at the 5’ end of the VH exon is fixed within a triplet reading frame, and given that the genetically encoded in-frame splice junction between the J_H and the C_μ exon is also fixed, only a fraction of
Assembly and expression of IgH genes. (a) Variable (VH), diversity (DH), and joining (JH) gene segments are shown, along with their flanking recombination signal sequences (RSs). RS heptamers are depicted as yellow triangles, whereas RS nonamers are depicted as white triangles. Spacer lengths are indicated above the various RSs. (b) Location of the three complementarity determining regions (CDRs) on the assembled VH(JH) exon is shown. Transcription initiates upstream of the assembled VH(JH) exon and proceeds through the four C\(\mu\) exons and the membrane (m) and secreted (s) exons. Possible splicing events are indicated.

VH(JH) rearrangements place the VH ATG in-frame with the coding sequence of the JH gene segment to generate a \(\mu\) protein (termed productive; Figure 2). Out-of-frame rearrangements encode full-length and processed transcripts but cannot encode a full-length heavy chain protein and, therefore, are termed nonproductive. Nonproductive rearrangements, which figure significantly into mechanisms that regulate IgH locus V(D)J recombination, can therefore be considered a necessary by-product of the imprecise mechanisms that create junctional diversity.

VH and JH gene segments are flanked by 23 RSs, whereas DH gene segments are flanked on both sides by 12 RSs. The 12/23 rule therefore dictates that the vast majority of IgH locus rearrangements involving VH gene segments will contain a DH gene segment sandwiched between a VH and a JH gene segment (Figure 2a) (11, 19). Inversional DH to JH rearrangements, which are technically permitted by the 12/23 rule, have been described (20–22), but the vast majority of DH to JH joints occur by deletion (21, 22). Investigators have suggested that RS preferences may underlie this bias, as JH 23 RSs displayed a roughly 20-fold preference for 3' DH 12 RSs versus 5' DH 12 RSs in transfected recombination substrates (23, 24). Another
well-described example of rearrangement patterns driven by RS preference has been elucidated for the TCRβ locus, where Vβ segments are flanked by 23 RSs and Jβ segments are flanked by 12 RSs, whereas Dβ segments are flanked by 5′ 12 RSs and 3′ 23 RSs to mediate joining to the Vβ and Jβ gene segments, respectively. With this organization, Vβ segments could theoretically join directly to Jβ segments under the 12/23 rule, but they are prevented from doing so by a “beyond 12/23” restriction under which 3′ Dβ 23 RSs can mediate joining efficiently with the Jβ 12 RSs but the Vβ 23 RSs cannot (25, 26). In this regard, replacement of a Vβ 23 RS with a 3′ Dβ 12 3′ RS can greatly increase the frequency of Vβ utilization and can promote direct Vβ to Jβ rearrangement (27). Similar replacement experiments to exchange a 5′ DH 12 RS with a 3′ DH 12 RS could directly test whether beyond 12/23 restrictions also prevent inversional DH to JH rearrangements.

Progressive stages of early B cell development have been well defined on the basis of expression of various cell surface markers and ordered patterns of IgH and IgL chain gene rearrangements (28). Assembly of VH, DH, and JH segments during the pro-B cell differentiation stage into a complete VHDJH variable region gene generally occurs before that of IgL chain genes (29). Productive assembly of a VH-DH-JH exon leads to the generation of a μ heavy chain protein that signals differentiation to the pre-B cell stage by signaling through the pre-B cell receptor (pre-BCR, see below). RAG mRNA and protein expression are also shut off by various mechanisms once a productive IgH rearrangement occurs but are reexpressed during IgL rearrangement in subsequent developmental stages (30–32). Igκ and Igλ light chain genes are assembled in the pre-B cell stage, with Igκ rearrangement generally preceding that of Igλ (29). Productive IgL gene assembly leads to production of a light chain protein that can associate with the preexisting heavy chain and together be deposited on the cell surface in the form of a membrane-bound IgM surface antigen receptor known as the B cell receptor (BCR). The resulting immature B cells can migrate to the periphery where, upon appropriate stimulation with a cognate antigen, they can be activated to secrete their previously membrane-bound BCR as IgM antibodies. The shift from production of a membrane-bound IgM BCR to a secreted IgM antibody is mediated at the level of differential RNA processing of a set of membrane versus secreted exons at the 3′ end of the Cμ coding exons (Figure 2a) (33, 34).

**ORDERED REARRANGEMENT OF THE IgH LOCUS**

Early studies demonstrated that μ heavy chains are expressed before IgL chains during B cell development (35). Subsequently, investigators showed that this ordered expression of IgH versus IgL chains occurs because IgH genes rearrange before IgL genes (36–38). One set of studies showed that Abelson murine leukemia virus (A-MuLV)-transformed pro-B cell lines contain rearrangements of both JH gene segments in the absence of IgL rearrangements (39). Further studies showed that, in normal ex vivo sorted cell populations, early B cells similarly rearrange both of their IgH alleles before proceeding to rearrange their IgL genes (40). Such studies therefore demonstrated that rearrangement of IgH versus IgL loci is ordered during B cell development, a process that is thought to, at least in part, have evolved to allow proper regulation for variable region assembly in the context of feedback regulation (41).

Ordered Ig gene rearrangement also extends to the order in which the gene segments of the IgH variable region are assembled. Thus, IgH DH to JH joints can occur in some developing T cells, whereas VH to DJH joints do not (42). Subsequent studies of a large number of immature and mature B cell lines, with findings confirmed for sorted normal B cell populations, clearly demonstrated that DH to JH joining occurs on both IgH
Alleles before VH to DJH joining on one allele, as nearly all the cell lines analyzed harbored DJH/DJH, VH/DJH/DJH, or VH/DJH/VH/DJH rearrangements at their two IgH loci (43). Furthermore, only one VH/DJH rearrangement in each cell line produced a functional μ chain (43). Moreover, although VH to DH rearrangements (in the absence of DH to JH joining) are technically permitted by the 12/23 rule, they were not observed (43). Taken together, these analyses showed that IgH locus rearrangements are ordered, with DH to JH joining occurring on both alleles before the initiation of VH to DJH joining (Figure 3). These observations, coupled with the findings of DH to JH but not VH to DJH rearrangement in T cells (42), led to the suggestion that the VH to DJH step is the one that is regulated in the context of lineage specificity and, as discussed below, in the context of feedback regulation and allelic exclusion (38, 43). Later studies showed that assembly of Vβ, Dβ, and Jβ gene segments at the TCRβ locus is similarly ordered, with DJβ joining taking place before Vβ to DJβ joining (44), despite the fact that Vβ to DJβ joining would be permitted by the 12/23 rule, and that Vβ to DJβ rearrangement also appears to be the regulated step (45).

ALLELIC EXCLUSION OF IgH LOCUS REARRANGEMENTS

The clonal selection theory of acquired immunity is based on the assumption that, despite the fact that two alleles of every antigen receptor locus are theoretically available for rearrangements in every developing lymphocyte, B and T cells of the adaptive immune system each express a unique antigen receptor. This unique expression results in a functional allelic exclusion of the nonexpressed alleles. The clonal selection theory further postulates that specific binding between an antigen receptor and its cognate antigen stimulates cellular proliferation and, ultimately,
To help understand about the content of the SIBC611-gene rearrangement, students may read the paper from page 1-6.
increased production of the receptor molecule (46). Conversely, the clonal selection theory also suggests a solution to the related problem of autoimmunity, whereby antigen receptor recognition of self-antigens results in elimination of self-reactive lymphocytes. The observation of allelic exclusion supports this theory, as over 99% of peripheral B cells express a single antigen receptor composed of a unique heavy chain generated from only one of two IgH alleles, and a unique light chain generated from only one of several IgL (two for Igκ and four for Igλ) alleles (47, 48). At the IgH locus, cells expressing two different μ chains occur at an estimated frequency of less than 1 in 10^4 (49). Other antigen receptor loci that are similarly allelically excluded include the Igκ and Igλ light chain loci, as well as the TCRβ locus in T cells.

Models for Allelic Exclusion

Despite intense investigation, a precise mechanistic understanding of the enforcement of allelic exclusion remains elusive (50). Although there have been many models for allelic exclusion, two general models have prevailed, the regulated models (43, 51) and the unregulated, stochastic models (52–54). All these theories must accommodate the fact that V(D)J rearrangements are inherently imprecise owing to mechanisms that maximize junctional diversity. Junctional diversification mechanisms dictate that only about one in three V(D)J joints will align the joined gene segments in the correct translational reading frame, although this fraction is merely an estimate for the IgH locus, as some VH segments have crippling germline mutations (13), and many DJH joints cannot form functional μ chains owing to in-frame stop codons within the DJH joint or counterselection of certain DJH joints (55, 56). At the Igκ locus in immature B cells that are undergoing receptor editing, nonfunctional Vκ to Jκ joints can be replaced by “leapfrogging” of an upstream Vκ to a downstream Jκ, a process in accord with the 12/23 rule (57). As Vκ to DJκ joining deletes all intervening Dκ segments, however, VκDJκ rearrangements are usually fixed because of the 12/23 incompatibility of direct Vκ to Jκ joining. A minority of nonfunctional VκDJκ joints may be rescued by Vκ editing mechanisms that use an internal Vκ heptamer to join an upstream germine Vκ to an assembled VκDJκ (58, 59), thereby potentially rescuing a nonproductive rearrangement (60).

Of the prevailing theories, much evidence supports a regulated model for the establishment of IgH allelic exclusion (Figure 3) (50). A regulated model was first invoked to explain control of IgL rearrangements, and proposed that IgL chain V gene assembly must proceed on one chromosome at a time and that protein products generated from a functional IgL (i.e., that could associate with the preexisting heavy chain) rearrangement mediate allelic exclusion via feedback inhibition of further IgL assembly (51). This model was based, in part, on the observation that Igκ-expressing B cells could be divided into a subset of about 40% that had rearranged both Igκ alleles (one nonfunctionally) and another subset of about 60% that had rearranged only one allele and left the other in germline configuration (52, 53). The observation that IgH locus rearrangements are ordered revealed a potential parallel between feedback regulation of both IgL and IgH rearrangements. Therefore, because both IgH alleles are always rearranged in B lineage cells in the form of DJH rearrangements, if the DJH rearrangement is considered equivalent to an Igκ germline allele, then Vκ to DJκ joining occurs in the same patterns and proportions of productive, nonproductive, and nonrearranged alleles as described for Igκ alleles (Figure 3) (43). These findings led to the notion that for both IgL and IgH chain rearrangements, control of V segment rearrangement is the allelically excluded step. Under a regulated model for IgH allelic exclusion, analogous to that originally proposed for Igκ rearrangement, pro-B cells that make a productive V(D)J rearrangement on the first attempt do not initiate Vκ to DJκ joining on the second allele, which freezes
a DJH on the second allele (Figure 3). Further, this model predicts that cells that make a nonfunctional VDJH joint on the first allele will subsequently rearrange VH to DJH on the second allele, leading to the population of mature lymphocytes carrying VDJH rearrangements on both alleles (one productive and one nonproductive). B cells that are nonproductively rearranged VH on both alleles cannot express Ig on their surface and therefore generally do not survive (61), although some may be rescued by VH replacement mechanisms (see below).

Approximately 50%–60% of mature B cells have one IgH allele rearranged as a VHJH and one allele frozen at DJH, whereas about 40%–50% have two VDJH rearrangements in productive/nonproductive configuration (Figure 3) (43, 50). These percentages are consistent with a productive rearrangement level of 33% or less and with feedback regulation by a μ chain to inhibit further rearrangement specifically at the VH to DJH joining step on the second allele (Figure 3) (43, 53). This proportion of productive versus nonproductive rearrangements is referred to as the 60/40 ratio for historical reasons, even though this ratio is an estimate owing to DJH reading frame incompatibility and VH pseudogenes (56). As noted above, further evidence that VH to DJH joining is the allelically excluded and regulated step comes from studies showing that DJH joints occur in thymocytes, whereas VHJH joints do not (42, 62, 63); in addition, IgH alleles that are DJH rearranged can continue to undergo DJH joining during IgL chain rearrangement, whereas VHJH joining does not occur at this stage (64). The fact that rearrangement patterns of Igκ alleles also fit the 60/40 ratio supported the notion that Vκ to Jκ rearrangements are feedback regulated in a similar fashion (16, 53). Finally, the TCRβ locus is also allelically excluded and undergoes ordered rearrangement in a pattern similar to the IgH locus, consistent with feedback regulation by productive TCRβ chains (65). In summary, control of V gene segment rearrangement is the regulated step under a feedback model for allelic exclusion.

An alternative stochastic model to explain IgL allelic exclusion has proposed that the first allele to rearrange is selected by chance, and that the V(D)J rearrangement process is so inefficient that the likelihood of generating two productive rearrangements in the same cell is vanishingly low (52, 53). One recent extension of this proposal to explain the basis for Igκ allelic exclusion is that variegated, low-probability activation of a locus for V(D)J recombination, in conjunction with allelic competition for a limited number of activating proteins (such as transcription factors), can result in allelic exclusion (54). This model is based on data generated from a Igκ reporter allele that suggested only a small percentage of cells transcriptionally activate the Igκ locus and that those cells go on to rearrange their Igκ loci (54). However, one prediction of this model is that, among normal pre-B cells, a very large population should be unrearranged on both alleles, which thus far has not been found (66). In any case, if the inefficient rearrangement model were to be applied to the IgH locus, it would have to account for the fact that all IgH alleles undergo DJH to JH rearrangements, indicating that DJH to JH joining is quite efficient. The inefficiency would therefore have to be speculated to occur at the VH to DJH rearrangement stage. However, as for Vκ to Jκ joining at the Igκ locus, the observation that nearly half of all mature B cells contain nonproductive VH to DJH rearrangements (43) suggests that, in its simplest form, an inefficient rearrangement model cannot fully explain IgH locus allelic exclusion (50). Yet, there are still scenarios whereby these rearrangement patterns might be rationalized with inefficiency (50), and additional experiments are needed to fully address this model. Therefore, although aspects of both stochastic and regulated models of allelic exclusion with regard to the rearrangement patterns that are observed at the IgH locus could apply, most current evidence strongly supports a feedback regulated model.
The precise mechanistic relationship between ordered rearrangement and allelic exclusion remains to be fully elucidated. However, it is worth noting that allelically excluded antigen receptor loci containing D gene segments, including the IgH and TCRβ loci, rearrange in an ordered, regulated manner, whereas the TCRδ locus, which also contains V, D, and J segments, does not undergo ordered rearrangement and is not allelically excluded (67). These observations are consistent with the idea that ordered rearrangement is important to allow time for a V to DJ rearrangement to be functionally evaluated by a feedback mechanism (43). This long-standing hypothesis has not been directly tested, although in principle it could be evaluated by generating gene-targeted mutations that break the order of rearrangement and allow VH segments to join directly to unrearranged DI segments or to rearrange directly to Ji segments. If the timing of Vi gene segment rearrangements is directly linked with the order in which DI to Ji and Vi to DJi rearrangements take place, such a mutation would be predicted to disrupt allelic exclusion.

Feedback Regulation of IgH Locus Rearrangements: Evidence and Open Questions

Studies on the regulation of IgL recombination indicated that functional light chain protein production—as opposed to light chain gene expression—is required to block further light chain rearrangements (51). This line of reasoning was extended to test the functional requirements for feedback regulation of IgH locus rearrangements and, in particular, to see if a membrane-bound μ chain is required to signal allelic exclusion. Transgenic studies first provided direct experimental evidence for regulated allelic exclusion, where expression of a functional antigen receptor protein partially blocked the rearrangement of endogenous loci (68–70). In most of these early studies, however, both the secreted and membrane-bound forms of the μ chain were expressed (69), necessitating further work to address whether cell surface expression of the μ chain is necessary for feedback regulation. Direct evidence for this proposal has subsequently come both from additional transgenic studies (71), which showed that transmembrane but not secreted μ chains blocked endogenous IgH rearrangements, and from endogenous deletion of the heavy chain transmembrane exon (72), which resulted in allelic inclusion owing to inability to shut off rearrangements of the other allele. Thus, the requirement for cell surface expression of a μ chain for allelic exclusion directly supports a feedback regulated model for IgH rearrangements. Similar experiments also showed that it was the membrane-bound form of the μ chain that directs progression from the pro-B to the pre-B stage, where IgL rearrangement is initiated (64).

In this regard, the observation that, in accordance with a feedback regulated model for rearrangements, roughly 40% of mature B cells that have completed all V(D)J rearrangements are ViD Ji rearranged on one allele and remain DJi rearranged on the second allele provides strong evidence that continued Vi to DJi rearrangements do not occur at the pre-B cell stage (43). DSBs at 5′ DI 12 RSs, which would correspond to Vi to DJi joining, are only present in pro-B cells and are not present in pre-B cells, when IgL loci rearrange (73). This retargeting of RAG appears to be specific for the Vi to DJi joining step, however, as studies in early B cell lines have demonstrated that a DJi joint can be replaced by successive DJi joining during Vk to Jκ joining (64), further supporting the notion that Vi to DJi joining is the allelically excluded step in IgH locus recombination. Thus, the available evidence strongly supports the idea that Vi to DJi joining is limited to the pro-B cell stage and does not occur during the pre-B cell stage even though RAG is reexpressed to rearrange the IgL loci, implying that Vi to DJi joining has been rendered inaccessible to RAG relative to IgL joining and Di to Ji joining (see below).
The fact that IgH rearrangements usually occur before IgL rearrangements and that surface expression of \( \mu \) chains in mature B cells depends on IgL expression (74) suggested that an alternative complex is involved in pairing with newly generated heavy chains. Subsequent studies demonstrated that production of a functional \( \mu \) chain at the pro- to pre-B cell transition leads to its cell surface expression in association with protein products of the V pre-B and \( \lambda 5 \) genes (75–77), which together form the surrogate light chain (78). Ability to pair with the surrogate light chain is a quality control parameter by which \( \mu \) chains are assessed. For example, a study of rare B cells with two productive V\( \mu \)DJ\( \mu \) rearrangements demonstrated that in every case, only one of the two expressed \( \mu \) chains was capable of pairing with the surrogate light chain (79). However, allelic exclusion is normal in animals with deletion of the known surrogate light chain elements (78), suggesting that other functionally redundant elements can take the place of surrogate light chain in its absence. Although normal light chains do rearrange during IgH rearrangement in a small fraction of developing B cells (80), it is unlikely that BCRs frequently take the place of pre-BCRs because insertion of a productive Ig\( \kappa \) rearrangement into the Ig\( \kappa \) locus cannot fully rescue B cells in the absence of surrogate light chain (81). Therefore, although the proteins that can substitute for surrogate light chain elements in their absence remain unknown, existing data strongly support the model that cell surface expression of the pre-BCR produces a feedback signal that shuts off further IgH rearrangements and promotes development to the pre-B cell stage.

Although production of a functional, full-length \( \mu \) chain results in positive B cell selection, generation of a shortened \( \mu \) chain can subvert this process. The production of truncated \( \mu \) chains was first demonstrated to occur in certain early B cell lines undergoing IgH rearrangements (82), and these shortened chains were later shown to be membrane-bound and encoded by a DJ\( \mu \) joint and the \( \mu \) constant region; these “D\( \mu \)” chains are encoded in one of the three possible D\( \mu \) reading frames (14). Further work showed that the production of D\( \mu \) chains is counterselected during B cell development owing to arrest of V\( \mu \) to DJ\( \mu \) joining (55). In addition, generation of mice transgenic for a D\( \mu \) chain has shown that D\( \mu \) cannot allow developing B cells to transition from the pro-B to the pre-B cell stage (83). However, the reasons D\( \mu \) protein production should be evolutionarily conserved remain unclear, as do the qualitative differences between \( \mu \) chain and D\( \mu \) chain signaling.

Additional details regarding downstream signaling effectors of feedback regulation remain to be characterized, but some of the proximal events have been identified. The pre-BCR pairs with the signal-transducing transmembrane proteins Ig\( \alpha \) and Ig\( \beta \) (84, 85) to generate signals that ultimately shutdown further IgH rearrangements and drive progression to the pre-B cell stage, where IgL is rearranged (86). Association of the pre-BCR with Ig\( \alpha \) and Ig\( \beta \) is required for pre-BCR signaling because mutations blocking \( \mu \) chain association with Ig\( \alpha \) and Ig\( \beta \), as well as mutations in the cytoplasmic domains of either of these molecules, disrupt allelic exclusion and pre-B cell development (87). Stimulation through Ig\( \alpha \) and Ig\( \beta \) leads to activation of various members of the Src family kinases (86). Thus, concomitant deletion of Syk and ZAP-70 disrupts allelic exclusion at the IgH locus (88), and deletion of the analogous kinase SLP-76 disrupts allelic exclusion at the TCR\( \beta \) locus (89), suggesting that at least the proximal elements of pathways signaling allelic exclusion are conserved between both B and T cell lineages. Ultimately, activation of these kinases probably triggers a phosphorylation cascade to signal developmental progression to the pre-B cell stage in cells that express a functional pre-BCR. In T cells, there appear to be separate signals that cause feedback regulation of TCR\( \beta \) rearrangements versus proliferation and onset of TCR\( \alpha \) rearrangements (90, 91). It remains to be shown whether promotion of developing B cell proliferation and IgL rearrangements can be similarly
dissociated from signals for IgH feedback regulation. Such approaches may help to elucidate the ultimate signals that model accessibility of the IgH and IgL loci (see below).

IgL rearrangements occur in the pre-B cell stage, after expression of a pre-BCR. Several lines of experimental evidence suggest that heavy chain expression activates IgL rearrangements, as first inferred from the observation that IgH loci rearrange before IgL loci (39). For example, B cell lines that do not make endogenous heavy chain because they have rearranged V_{H}D_{H} nonproductively on both IgH alleles can undergo V_{κ} to J_{κ} rearrangements when transfected with an expression vector encoding a membrane-bound μ chain, but not when transfected with a vector encoding a secreted μ chain (64). Moreover, initiation of IgL rearrangements in these contexts appears to depend on signaling through the pre-BCR (92). Membrane-bound heavy chain expression is not absolutely required for IgL rearrangement, however, as some transformed human pre-B cell lines can express IgL in the absence of V_{H} to DJ_{H} joining (93); moreover, animals that express only secreted μ chains (80) or harbor deletion of the J_{H} gene segments (94) both initiate a low level of V_{κ} to J_{κ} joining. The overall impact of this “disordered” rearrangement is not yet understood, as the developmental arrest incurred by such mutations may enhance this alternative pathway for IgL rearrangements (29). The weight of evidence therefore supports the idea that a membrane-bound μ chain signals both the cessation of further V_{H} to DJ_{H} joining and progression to the pre-B cell stage and the initiation of IgL rearrangement. In this regard, there have been several transformed cell lines (95) as well as in vitro culture models derived from normal early B cells (96) that reproduce this ordered program of IgH and IgL rearrangement.

Feedback models of allelic exclusion at the Igκ locus were based on the premise that one allele is rearranged and tested for productivity before the other is rearranged (51). The asynchrony required for this process has been subdivided into two phases: initiation and maintenance (50). With respect to allelic exclusion of the IgH locus, feedback regulation can explain maintenance of a frozen DJ_{H} upon a productive V_{H}D_{H}J_{H} rearrangement on the other allele, but it cannot explain how cells initiate V_{H} to DJ_{H} rearrangements on only one allele first (51, 97) over a period in which the feedback signal can take place. Many potential general mechanisms have been proposed as to how this might occur, including differential nuclear localization and locus contraction (29, 98, 99) and allelic marking associated with early replication and demethylation (97, 100), although the potential mechanistic roles of these phenomena remain to be directly tested. Regardless, these findings still would not explain how the second allele becomes activated for V_{H} to DJ_{H} rearrangement in those cells that have made a nonproductive V_{H}D_{H}J_{H} rearrangement on the first allele. Two mechanisms in this regard—permissive or instructive—are theoretically possible, or a combination of these two mechanisms may be at work. First, a permissive model would postulate that both IgH alleles in a pro-B cell operate on a recombination clock, where one allele has a head start and has initiated rearrangement first. In this scenario, the second allele eventually undergoes V_{H} to DJ_{H} rearrangement, unless a signal from the pre-BCR prevents it from doing so (54, 97). Conversely, in an instructive model, a nonproductive rearrangement somehow informs the cell that it is necessary to initiate V_{H} to DJ_{H} rearrangement on the second allele. V_{H} to DJ_{H} rearrangement on the second allele would, thus, only occur upon production of a nonproductive V_{H}D_{H}J_{H} joint on the first allele. Investigators have argued that such a mechanism also operates in activating Igλ for V(D)J recombination upon deletion or nonproductive rearrangement of both Igκ alleles (29). Within the context of permissive and instructive models for activating V_{H} to DJ_{H} rearrangement on the second allele, we should also consider the implications that both models hold for the maintenance of IgH allelic
exclusion when RAG is reactivated for IgL rearrangement, which takes place after progression to the pre-B cell stage. On one hand, a permissive model predicts that, while initiating V_{H} to DJ_{H} asynchronously, both alleles are destined to rearrange V_{H} to DJ_{H} in the absence of a pre-BCR signal. This model therefore would postulate that the pre-BCR sends a signal that actively shuts down a DJ_{H} allele with respect to V_{H} to DJ_{H} rearrangements at the transition between the pro- to pre-B cell stage. On the other hand, an instructive model predicts that a DJ_{H} allele will only rearrange V_{H} to DJ_{H} upon a signal that the first allele has generated a nonproductive V_{H}DJ_{H} joint. If the first allele generates a productive V_{H}DJ_{H} joint, therefore, such a signal would never be sent, and the DJ_{H} allele would never be available for V_{H} to DJ_{H} rearrangement. In either scenario, both permissive and instructive models for activating V_{H} to DJ_{H} rearrangement on the second allele imply that DJ_{H}-rearranged alleles are unavailable for V_{H} to DJ_{H} joining events at the pre-B cell stage. By contrast, the second population of B cells that transit to the pre-B cell stage have terminally rearranged both IgH alleles V_{H}DJ_{H}/V_{H}DJ_{H} (in productive/nonproductive configuration) and would likely not require such mechanisms to block further rearrangements (Figure 3).

**Accessibility Control of IgH Locus Rearrangements**

As outlined above, V(D)J recombination at the IgH locus is strictly controlled with respect to cell-type specificity (complete rearrangements in B cells but not in T cells), intralocus order (DJ_{H} to J_{H} on both alleles before V_{H} to DJ_{H}), interlocus order (no continued V_{H} to DJ_{H} rearrangement during light chain rearrangement), and feedback regulation by productive V_{H}DJ_{H} joints in the context of allelic exclusion. Although the mechanisms of IgH locus ordered rearrangement and feedback regulation remain to be fully elucidated, both processes are likely based on differential accessibility of V_{H}, D_{H}, and J_{H} gene segments to the RAG recombinase (16, 101). The accessibility model for control of V(D)J recombination in all the above contexts initially was hypothesized based on the discovery that murine V_{H} gene segments are transcribed and produce germline (i.e., noncoding and unarranged) transcripts in cell lines and normal early B lineage cells that are actively undergoing V_{H} to DJ_{H} joining (15). However, such transcripts are not found in mature cell lines or adult spleen or in newborn or adult thymus, all of which are lymphoid tissues where V_{H} to DJ_{H} joining does not occur. These germline transcripts initiate from V_{H} promoters, are spliced, and are observed in the cytoplasm, where they could theoretically be translated into V_{H} chains (15), although the endogenous production of such chains has never been reported.

Proof for the accessibility model for regulation of V(D)J recombination, along with evidence for its association with higher-order controls that act to selectively open or close antigen receptor loci, came from the demonstration that transfected TCR_{β} gene segments and endogenous IgH gene segments rearranged in a pro-B cell line, whereas endogenous TCR_{β} gene segments did not (102). Furthermore, transfected TCR_{β} gene segments and endogenous IgH gene segments in this cell line were sensitive to DNase I, whereas endogenous TCR_{β} gene segments were not, which directly correlated recombinational potential with a physical correlate of open chromatin with respect to the various gene segments (102). The ability of antigen receptor gene segments to undergo V(D)J recombination has therefore been broadly defined as physical accessibility of these segments to the recombinase machinery. In a further demonstration of this general principle, RAG-mediated cleavage events occur in a cell- and stage-specific manner both in ex vivo lymphocytes (73) and in nuclei isolated from lymphocytes in vitro (103). In addition, a large number of studies have demonstrated that known transcriptional control elements, most extensively studied for
transcriptional enhancers, function to target V, D, and J gene segments for recombinational accessibility, both in transgenic constructs and in endogenous loci (104). Although a considerable amount of recent effort has been invested in defining the nature of accessibility and the role of transcriptional regulatory elements in controlling V(D)J recombination of different Ig and TCR loci (104), we focus primarily on progress related to the IgH locus.

**CONTROL OF D<sub>H</sub> TO J<sub>H</sub> REARRANGEMENT**

Various cis-acting elements associated with transcription have been studied for their potential roles in activating D<sub>H</sub> to J<sub>H</sub> rearrangement. The intronic μ enhancer (iEμ) is located within the intron between the last J<sub>H</sub> gene segment and the Cμ exons about 0.8 kb downstream of J<sub>H</sub>4 (105, 106). Ectopic expression of E2A and EBF, factors that bind to iEμ, activates D<sub>H</sub> to J<sub>H</sub> rearrangement in nonlymphoid cells (107). In addition, it has been shown that iEμ can activate D to J rearrangement in a transgenic recombination substrate (108). Prior to D<sub>H</sub> to J<sub>H</sub> rearrangement, μ0 sterile transcripts are initiated upstream of DQ52, the most J<sub>H</sub>-proximal D<sub>H</sub> gene segment (109, 110). Transcription also initiates upstream of other germline D<sub>H</sub> gene segments and generates higher levels of steady-state transcript upon D<sub>H</sub> to J<sub>H</sub> joining, a property that is thought to depend on proximity to iEμ (14, 109, 110). At the endogenous locus, replacement of iEμ with a highly transcribed pgk-Neo’ element blocks all detectable V(D)J recombination of the J<sub>H</sub> gene segments (111, 112). However, clean deletion of iEμ results in a partial defect in D<sub>H</sub> to J<sub>H</sub> joining, deletion of iEμ results in a stronger inhibition of V<sub>H</sub> to DJ<sub>H</sub> joining (112–114).

This partial inhibition of D<sub>H</sub> to J<sub>H</sub> joining on alleles harboring the iEμ deletion, along with complete inhibition of D<sub>H</sub> to J<sub>H</sub> rearrangements on alleles harboring replacement of iEμ with a pgk-Neo’ cassette, together suggest that additional elements are required to activate D<sub>H</sub> to J<sub>H</sub> rearrangement in vivo. As a potential example of such an element, a promoter/enhancer element has been identified upstream of DQ52 (115), although deletion of this sequence together with the DQ52 gene segment does not completely abolish μ0 transcription and also does not appear to dramatically inhibit overall levels of D<sub>H</sub> to J<sub>H</sub> joining (116). In this regard, the pgk-Neo’ cassette is known to inhibit the activities of the long-range 3′ IgH regulatory region (RR) (117) that lies 200 Kb downstream of the J<sub>H</sub> segments, just beyond the last set of constant region exons, and contains enhancers (hs3b and hs4) that are important for activating transcription from I promoters that flank switch regions upstream of the various constant regions. The 3′ IgH RR functions to enhance germline constant region transcription, to regulate germline constant region genes, and to influence expression of rearranged V<sub>H</sub>DJ<sub>H</sub> exons assembled upstream at the J<sub>H</sub> region (118). The pgk-Neo’ cassette appears to affect the 3′ IgH RR through promoter competition (117). Thus, the 3′ IgH RR may well be another candidate for an element that works in addition to the iEμ element to influence V(D)J recombination at the J<sub>H</sub> locus (112). Although deletion of the 3′ IgH RR in isolation does not result in an obvious defect in D<sub>H</sub> to J<sub>H</sub> (or V<sub>H</sub> to DJ<sub>H</sub>) rearrangement (118), iEμ may contribute overlapping activities. Therefore, inactivating both the 3′ IgH RR and the DQ52 promoter/enhancer element, or some other elements, including unknown elements within the V<sub>H</sub> locus or upstream, in conjunction with deleting iEμ, is relevant to see if these various elements
cooperate with iEμ to enhance Dμ to Jμ rearrangements. In addition to the study of endogenous transcriptional control elements in the initiation of Dμ to Jμ joining, a number of chromatin modifications, many first identified in transcriptional studies as markers of “open” chromatin, correlate with V(D)J recombination accessibility at this locus. Thus, the genomic region comprising the Dμ and Jμ gene segments is associated with hyperacetylated chromatin in RAG-deficient pro-B cells, which should be poised to rearrange but are unable to initiate V(D)J rearrangements (119–121). Additional studies of transformed RAG-deficient pro-B cell lines have further shown that the Dμ and Jμ gene segments are also associated with BRG1, the catalytic subunit of the chromatin remodeling complex SWI/SNF, suggesting that chromatin remodeling may be important for accessibility of genomic DNA to the RAG recombinase machinery (121). Perhaps the most direct genetic evidence that such chromatin modifications control V(D)J recombinational accessibility comes from studies of TCR β locus gene segments, which also undergo ordered rearrangement and feedback regulation in vivo (65). Thus, a TCR β rearrangement substrate in which a H3-K9 methyltransferase is tethered in close proximity to a Dβ promoter has increased local methylation of H3-K9 and decreased transcription through and V(D)J recombination of the Dβ gene segment (122). Further work using such creative approaches at endogenous loci may shed light on whether transcription and its associated chromatin modifications are a cause or effect of an antigen locus primed for rearrangement.

In this regard, it is instructive to note basic differences between the requirements for transcription during V(D)J recombination and during class switch recombination (CSR). CSR changes the receptor effector function of a VμDμJμ exon through site-specific recombination between large, repetitive switch regions that lie upstream of the various constant region exons. The AID (activation-induced deaminase) enzyme is required for this process (123), and deaminates cytidine residues in single-stranded DNA generated within S regions undergoing transcription (124). The deaminated cytidines eventually yield strand lesions that may ultimately lead to DSBs by mechanisms that are still under intense investigation (125). By contrast, it appears that, during V(D)J recombination, transcription may only be required to render gene segments accessible to RAG, rather than playing a direct mechanistic role in the recombination reaction (101). In support of this idea, recent experiments have shown that, in transgenic V(D)J recombination substrates, the presence of a transcriptional promoter upstream of a recombining gene segment can activate its rearrangement independent of its orientation (126). Thus, although the precise role of transcription in regulating V(D)J recombination remains to be fully understood, it appears that, in contrast to CSR, transcription through recombining V, D, and J gene segments may be dispensable for this process, and the role of transcriptional control elements may instead be to provide more general accessibility via modifications in chromatin structure.
promoter competition for necessary activating factors (117). In addition, deletion of iEμ severely disrupts VH to DJH joining and generates a distribution skewed away from cells that are rearranged VlDJ/μ/VHDJH on both IgH alleles and toward VlDJ/μ/DJH rearranged cells (112–114). Finally, various endogenous mutations of RAG2 specifically decrease Vl to DJH joining. In this regard, the core RAG mutations have been identified as the minimal regions necessary to support RAG-mediated cleavage in vitro, although these truncated proteins clearly lack functions that may be important during V(D)J recombination in vivo. On the one hand, targeted replacement of the RAG2 gene with the core RAG2 mutation in vivo resulted in a dramatic defect in Vl to DJH rearrangement, along with a lesser but significant disruption of DJH to JH rearrangement (127, 128). On the other hand, replacement of the endogenous RAG1 gene with the core RAG1 mutation caused an overall decrease in efficiency of rearrangement, without any obvious differential effect on DJH to JH versus Vl to DJH joining (129). With respect to requirements for Vl to DJH joining, it is worth noting that clones harboring unrearranged JH alleles were identified at increased frequency among mature B cell hybridomas generated from animals harboring the two core RAG mutations (128, 129), as well as from those with the iEμ deletion (112, 114). This finding demonstrates that DJH to JH joining is not required on both IgH alleles to activate Vl to DJH joining. However, whether or not DJH to JH joining is required in cis to activate Vl to DJH joining on the same allele remains an open question.

The different phenotypes of the two RAG core mutations may perhaps be explained by different functions encoded in the non-core regions of the RAGs; for example, the non-core region of RAG2 contains a PHD (plant homeodomain) finger, a domain found in many chromatin-associated proteins, raising the tantalizing possibility that RAG2 may be involved in directly modulating chromatin structure during V(D)J recombination (130). Consistent with this potential general role for RAG2 is the finding that the noncore portion of RAG2 binds various histones through residues that also appear to be critical for Vl to DJH rearrangement, although these residues lie outside the PHD finger (131). However, further work is needed to show that RAG2 physically targets chromatin modification activities in vivo, either by itself or in conjunction with additional factors.

**Molecular Correlates of VH Gene Segment Rearrangement**

As the VH gene segment cluster is spread across three megabases of DNA, elucidation of how these segments are brought into DJH joints should illuminate mechanisms of large-scale chromosomal interactions in other gene regulatory processes. Moreover, understanding how one allele is chosen to rearrange Vl to DJH first is critical for a complete understanding of the processes underlying allelic exclusion, although no mechanism has yet been shown to be directly involved in controlling this process. Asynchronous, early replication of one IgH allele is closely correlated with rearrangement of that allele about 90% of the time (100), although the precise link between the two processes remains unclear. In addition, although allele-specific demethylation serves as a mark for preferential rearrangement at the Igκ locus (97), it has not to date been reported for the IgH locus and, moreover, has not been clearly demonstrated to be mechanistically related to allele-specific choice. By analogy with the Igκ locus (97), one might expect that allele-specific demethylation or some other allele-specific mark, if one exists, will localize to the region between the Vl and DJH gene segments, and further work should address this possibility. In this regard, a recent study of the Igκ locus followed various stages of Igκ activation from pro-B cells through pre-B cells to outline steps of nuclear relocalization, histone modification, monoallelic heterochromatinization, and monoallelic...
demethylation (132). Similar comprehensive studies of the IgH locus may help elucidate how one allele is first chosen to initiate V_{H} to DJ_{H} joining.

The observations of chromosomal relocalization of the IgH locus and IgH locus contraction and looping in pro-B cells have been recent visual correlates of V_{H} to DJ_{H} joining (62, 99, 133–135). Transport to a localized recombination center in the nucleus, followed by export out of such a center upon the cessation of rearrangement, is an attractive means by which cell stage-specific control of V(D)J recombination might be established (98). In this regard, recent studies have shown that in pro-B cells, which are actively undergoing IgH locus rearrangement, both IgH alleles are centrally located (133). In non-B cells, however, both IgH loci are located at the nuclear periphery (134). In addition, the phenomena of IgH locus contraction and looping, as visualized by 3D-FISH, are thought to represent the linking of distal V_{H} gene segments with DJ_{H} complexes (62, 99, 135). Importantly, IgH locus looping occurs in the absence of RAG2 (62), suggesting that the relocalization of V_{H} gene segments in three-dimensional space occurs prior to the assembly of a RAG-RS complex. Thus, further understanding the control of IgH locus localization, contraction, and looping may provide important insights into the determination of stage-specific accessibility in the context of ordered rearrangement and allelic exclusion.

In another correlate of the accessibility mechanisms that drive V_{H} gene segment recombination, V_{H} segments are transcribed prior to their rearrangement (15, 136). More recently, researchers have also shown that antisense transcripts are generated in the vicinity of unrearranged V_{H} gene segments (137). Like sense germline V_{H} transcripts, antisense transcripts are found in pro-B cell populations that are actively undergoing V_{H} to DJ_{H} joining, and are switched off in cells that have successfully completed V_{H} to DJ_{H} joining (137). Moreover, antisense V_{H} transcripts appear to be generated in wide regions of the V_{H} gene segment cluster, including in intergenic regions. In the context of IgH rearrangements, antisense transcription may be a property specific to the V_{H} cluster, as it was not observed near J_{H} gene segments (137). Although the functional significance of these transcripts is not yet clear, several possibilities were proposed, including remodeling of IgH locus chromatin and generation of double-stranded RNA (by the coordinated generation of sense and antisense transcripts) to target the RNA interference machinery and thereby recruit other proteins, such as histone methyltransferases, that may be important for V_{H} gene segment rearrangement (137). Strikingly, about 80% of the clones expressed antisense germline transcripts monoallelically, as would be expected for a mechanism involved in choosing one allele to rearrange first (137), although we do not know whether the allele expressing antisense transcripts actually rearranged first. Further work to define antisense promoters and/or enhancers may elucidate whether antisense V_{H} transcripts play a role in rendering V_{H} gene segment chromatin accessible to RAG or, alternatively, are a nonspecific by-product of opening the locus by some other means. Regardless, it is striking that a sense/antisense transcription profile appears to operate at many known mammalian genes, perhaps to mediate gene silencing through degradation of sense transcripts by targeting RNA interference proteins (138), and understanding the control of V_{H} gene segment rearrangement may shed light on the general functional importance of this phenomenon.

**Preferential Rearrangement of Proximal V_{H} Gene Segments**

A striking feature of murine V_{H} gene segment recombination is that V_{H} gene segments proximal to the D_{H} gene segments are preferentially rearranged (139, 140). Although the functional significance of this phenomenon remains unresolved, it has been suggested that rearrangements using proximal V_{H} gene segments generate self-reactive antibodies and
that such antibodies may play some role in shaping the adult antibody repertoire (141). In the human IgH locus, by contrast, although the two most proximal VH gene segments are highly rearranged (142, 143), other frequently rearranged VH gene segments are scattered throughout the human V H cluster (144), as is also observed for the Igκ and TCRβ loci. The mechanistic basis for this difference between murine and human VH gene segment utilization remains unclear and may depend on various factors, including RS strength and differential accessibility of individual VH gene segments versus VH gene segment families.

Preferential rearrangement of proximal VH gene segments at the murine IgH locus was first demonstrated in fetal liver hybridomas and in A-MuLV-transformed pre-B cell lines (139, 140). Although this process was (and by some still is) considered representative of developmental programming of VH gene rearrangement with proximal V H segments rearranging earlier in ontogeny than distal VH segments, this possibility was ruled out by studies showing that the newly generated repertoire was similarly biased in both fetal liver and adult bone marrow B lineage cells (145). Thus, 3′ VH genes are preferentially rearranged at all stages of development. However, before reaching the periphery, the VH repertoire appears to be normalized by selection mechanisms such that the population of rearranged VH genes expressed by peripheral B cells roughly correlates with the number of members in each VH gene segment family (146), although the degree to which this is achieved may depend on other factors, such as the number of nonfunctional gene segments within a particular family (147, 148).

An example of one process that may be involved in normalizing the VH repertoire comes from studies that show V H81X, the most proximal and most frequently rearranged VH gene segment in the murine 129 strain, is often counterselected, most likely because of its relative inability to pair with the surrogate light chain (149, 150). The VH repertoire might therefore be normalized through a VH replacement mechanism (139). In this regard, rearranged V H/DJH joints can undergo VH replacement through recombination between a germline VH gene segment and a cryptic heptamer within the rearranged VH coding sequence (58, 59). However, how often VH replacements occur in vivo remains unclear, and they likely occur infrequently (60).

**Possible Mechanisms Involved in Preferential VH Gene Segment Rearrangement**

The mechanistic basis for the greater recombinational potential of proximal VH gene segments has remained an outstanding question in IgH locus V(D)J recombination. Several factors, either singly or in combination, may be responsible for the preferential rearrangement of proximal VH gene segments. Such factors may include more potent RSs flanking proximal versus distal VH gene segments and/or increased accessibility of proximal VH gene segments to the RAG proteins. Regarding RS strength, recombination substrate transfection assays have demonstrated that a consensus V H7183 RS, derived from a proximal VH family, can mediate higher levels of rearrangement than a consensus V HJ558 RS, derived from a distal VH family, can mediate higher levels of rearrangement than a consensus V H7183 RS. However, other studies have shown that among proximal VH gene segments, in vivo rearrangement frequencies are correlated with a more proximal position and not with RS potency (152). A definitive answer in this regard thus likely awaits genetic swaps between proximal and distal VH gene segments and/or RSs. However, the likelihood that RS differences can play a role is reinforced by the results of such an experiment done in the context of the TCRβ locus, which showed a major increase in the rearrangement frequency and change in preference for a V β gene segment by replacing its 23 RS with a 3′ Dβ1 23 RS (27).

Various mutations in factors and signaling pathways that may act in *trans* with respect to the IgH locus appear to demonstrate a
specific defect in distal V\textsubscript{H} rearrangement. Several of these involve elements of the IL-7 signaling cascade. Deletion of the IL-7 receptor causes a developmental block very early in B cell development in vivo (153). In addition, pro-B cells sorted from IL-7R\textsuperscript{-/-} mice are deficient in distal \textit{V}\textsubscript{H}558 germline transcription and rearrangements, as detected by PCR (154). Consistent with a role for IL-7 signaling in the activation of distal \textit{V}\textsubscript{H}J558 gene segments, \textit{V}\textsubscript{H}J558 gene segments have increased levels of histone acetylation and nuclease sensitivity upon IL-7 treatment (119, 155). In further support of a role for IL-7 signaling in recombinational activation of distal \textit{V}\textsubscript{H} gene segments, STAT5, an IL-7-responsive transcription factor, appears to be important for distal \textit{V}\textsubscript{H}J558 germline transcription and rearrangement (156, 157). In addition to elements of the IL-7 signaling cascade, B-cell-specific deletion of Ezh2, a histone methyltransferase, results in a decrease in \textit{V}\textsubscript{H}J558 rearrangement (again as measured by PCR on pro-B cell DNA), but in the presence of normal germline transcripts and histone acetylation; instead, methylation of H3-K27 appears to be impaired in Ezh2\textsuperscript{-/-} pro-B cells (158).

Pax5 is a transcription factor that has been extensively analyzed for its role in controlling the rearrangement of \textit{V}\textsubscript{H} gene segments and for its more general role in B cell commitment and differentiation (159). In this regard, Pax5-deficient pro-B cells appear to have a specific defect in distal \textit{V}\textsubscript{H} gene rearrangement, as detected by PCR on pro-B cell DNA (160). This defect is not correlated with differences in certain measures of accessibility, as germline transcription and histone acetylation are normal at both proximal and distal \textit{V}\textsubscript{H} gene segments from Pax5-deficient pro-B cells (160); conversely, demethylation of H3-K9 appears to be globally disrupted across the \textit{V}\textsubscript{H} gene segment cluster in pro-B cells lacking Pax5 (161). However, contraction of the IgH locus, which has been correlated with rearrangement of distal \textit{V}\textsubscript{H} gene segments, is only observed in pro-B cells that express Pax5 (62). Recent studies have reported the striking finding that ectopic expression of Pax5 in developing T cells results in (a) the relocalization of IgH alleles to the center of the nucleus (62), (b) induction of proximal and distal \textit{V}\textsubscript{H} germline transcription (62), and (c) rearrangement of proximal versus distal \textit{V}\textsubscript{H} gene segments (62, 63). Thus, with respect to V(D)J recombination of the IgH locus, early T cells expressing Pax5 appear to be phenotypically nearly identical to early B cells lacking Pax5 by all measures so far examined. One interpretation of the available data on Pax5, therefore, is that Pax5 is sufficient in early T cells for the rearrangement of proximal \textit{V}\textsubscript{H} gene segments and necessary in early B cells for the rearrangement of distal \textit{V}\textsubscript{H} gene segments, which may suggest the existence of another factor in B cells that is required for IgH locus contraction and distal \textit{V}\textsubscript{H} gene segment rearrangement (62).

Taken together, experiments on roles of IL-7R signaling and Pax5 in driving distal versus proximal \textit{V}\textsubscript{H} gene segment rearrangement are compelling studies that warrant further investigation. However, there may be additional explanations than simply activating distal versus proximal \textit{V}\textsubscript{H} gene segments that could also, at least in part, account for the data. For example, mutations related to IL-7 signaling, Pax5 function, or other genetic modifications, including transgenes, which appear to differentially affect distal versus proximal \textit{V}\textsubscript{H} genes, may also reflect changes in B cell development that result in an unnormalized repertoire. Notably, proximal \textit{V}\textsubscript{H} gene segments are preferentially rearranged in all developing B cells (145); thus, preferential rearrangement of the 3' \textit{V}\textsubscript{H} gene segments in different transgenic and knock-out models could, theoretically, reflect the normally biased patterns found in the newly generated repertoire. Further work, perhaps aimed at directly measuring recombinational accessibility of proximal versus distal \textit{V}\textsubscript{H} gene segments in different contexts, may help to distinguish among these possibilities.
Transgenic Studies of \(V_H\) Feedback Regulation and Proximal versus Distal \(V_H\) Rearrangement

In accordance with a feedback-regulated model for IgH rearrangement control, expression of transgenic constructs that contain membrane-bound prerearranged \(V_HDJ_H\) \(\mu\) chains results in a substantial decrease of endogenous \(V_H\) to \(DJ_H\) rearrangements \(69, 71\). Such studies may be confounded, however, by evidence that suggests \(\mu\) transgenes drive artificially accelerated B cell development. Because the \(V_H\) to \(DJ_H\) joining step is the allelically excluded step, one would predict that, if \(\mu\) transgenes signal normal allelic exclusion, virtually all B cells from \(\mu\)-transgenic mice would contain \(DJ_H/DJ_H\) rearrangements on both alleles. In fact, a significant fraction of hybridomas generated from \(\mu\)-transgenic B cells retain at least one of their endogenous \(J_H\) clusters in unrearranged configuration, which is not observed in normal B cells \(69, 162\). Further, in \(\mu\)-transgenic B cells, \(DJ_H\) to \(J_H\) rearrangements do not begin until the pre-B cell stage, during normal light chain rearrangement \(162\). Studies on other membrane \(\mu\) transgenes, however, have reported that levels of \(DJ_H\) to \(J_H\) rearrangements are normal, based on PCR assays \(163, 164\). Such differences in interpretation may be due to some aspect of transgene expression levels or the methods used to detect rearrangements.

Although the majority of \(V_H\) to \(DJ_H\) rearrangements are abrogated in \(\mu\)-transgenic mice, some \(V_H\) to \(DJ_H\) rearrangements do occur \(163, 165, 166\). As in normal mice, the \(V_H\) to \(DJ_H\) rearrangements in \(\mu\)-transgenic mice are skewed toward proximal \(V_H\) gene segments \(163, 166\), a finding that has been interpreted to suggest that proximally located \(V_H\) gene segments may be less regulated with respect to allelic exclusion \(99, 163\). Given that proximal \(V_H\) gene segments are preferentially rearranged in both fetal liver and adult bone marrow \(145\), however, a possible alternative explanation of these findings might be that, in the context of accelerated B cell development, proximal \(V_H\) gene segment rearrangements are observed preferentially because they are normally more likely to occur. Thus, shortening the time available for IgH rearrangements in general to occur might nonspecifically select for proximal \(V_H\) to \(DJ_H\) rearrangements by decreasing the overall level of \(V_H\) to \(DJ_H\) rearrangements. Taken together, the data suggest that results generated using \(\mu\) transgenes should be interpreted with some caution, as should similar experiments generated with TCR\(\beta\) transgenes \(45, 90\).

Receptor Editing of \(V_HDJ_HJ_H\) Joints

Receptor editing refers to the process by which secondary V(D)J rearrangements can replace a nonfunctional or self-reactive variable region exon with another one \(57\). In B cells, this general phenomenon can occur frequently at the Igk locus, as V\(k\) gene segments are allowed to rearrange, by the 12/23 rule, to available downstream J\(k\) gene segments. However, for the IgH locus, V\(H\) replacements appear to take place much less frequently because a V\(H\) to DJ\(H\) rearrangement deletes all remaining D\(H\) segments, which are required for \(V_H\) gene segment recombination that obeys the 12/23 rule. However, V\(H\) replacement events did occur in murine cell lines through a V\(H\) to V\(HDJ_H\) recombination reaction that was hypothesized to employ a conserved heptamer sequence in the downstream body of the rearranged V\(H\) segments \(58, 59\). More recently, V\(H\) replacement was also shown to occur in human B cell lines \(167\). Furthermore, a database generated from normal human mature B cells was screened for a V\(H\) replacement footprint, revealing that 5%-10% of V\(H\)DJ\(H\) joints may have resulted from V\(H\) replacement events \(167\). However, mature B cells represent a selected population, and there still remains no evidence that murine V\(H\) replacement has any significant role in gross normalization of primary V\(H\) repertoires \(168\). In models in which self-reactive V\(H\)DJ\(H\) joints have been targeted to replace the J\(H\) gene segments, initiation of
cRS: cryptic recombination signal sequence

V_{H} editing events appear to take place during the immature B cell stage, after the completion of IgL rearrangements (169).

Recombination substrate studies confirmed that V_{H} replacements involve the usage of a cryptic RS (cRS) that contains only an isolated heptamer and is located near the 3' end of most V_{H} gene segments, which mediates V(D)J recombination with the 23 RS of an upstream V_{H} segment (170). The cRS element is highly conserved from mammals to cartilaginous fish, suggesting that it has been selected during the course of evolution (168). Moreover, the cRS can mediate RAG-induced DSBs in cell lines and in cleavage reactions in vitro, although at a lower frequency relative to RS-RS cleavage (167). Thus, the fact that V(D)J recombination efficiently occurs only in the context of two complete RSs suggests that immature B cells that undergo editing may have some way of relaxing the specificity of RAG, perhaps through the expression of as yet undefined factors, to accommodate rearrangements between a complete RS and a cRS. Notably, the Igκ light chain locus sometimes may be inactivated during editing by joining a Vκ to an isolated heptamer in the Jκ-Cκ intron (57), again raising the possibility of relaxed RAG specificity during editing.

The regulation of V_{H} replacements may have implications for certain aspects of the allelic exclusion process. In this regard, it seems likely that in V_{H}DJ_{H}/V_{H}DJ_{H} rearranged B cells expressing a self-reactive μ chain, V_{H} replacement may be specifically targeted to occur on the V_{H}DJ_{H} allele, whereas V_{H} genes on the DJ_{H} rearranged allele are kept in an inaccessible state to maintain allelic exclusion. Thus, receptor editing occurs preferentially on the rearranged allele as opposed to the germline allele in the Igκ locus (73, 171). Furthermore, in V_{H}DJ_{H}/V_{H}DJ_{H} rearranged B cells that express a self-reactive μ chain from one IgH allele and are nonproductively rearranged on the other IgH allele, V_{H} replacement is likely similarly targeted specifically to the self-reactive V_{H}DJ_{H} joint to maintain allelic exclusion. By contrast, such considerations do not seem relevant for B cells that harbor two nonfunctional V_{H}DJ_{H} rearrangements and attempt to salvage a productive μ chain through V_{H} replacement. Understanding the mechanisms by which various alleles choose to pursue V_{H} replacement events may also be valuable in understanding allelic exclusion and feedback regulation.

PERSPECTIVE

Research into the mechanisms underlying V(D)J recombination at the IgH locus appears poised to generate many exciting insights over the next several years, as emerging technologies should facilitate the study of IgH rearrangements. Longstanding issues include how one allele is chosen to first rearrange V_{H} to DJ_{H}, how the second allele is activated to rearrange V_{H} to DJ_{H} if the first is nonproductive, and what the signals are that redirect the RAG proteins to the κ locus at the pre-B cell stage. To address these questions, new approaches are likely required. Improvements in genomic engineering technology should allow for simplification of the V_{H} gene segment cluster (i.e., deleting it down to a few V_{H} gene segments), which would allow for detailed analyses of the requirements for accessibility at individual V_{H} gene segments, provided such large-scale deletions are normally regulated. Such an approach would also make it easier to characterize chromatin modifications and factors such as antisense transcripts and their potential function. Another long-standing issue is the function of ordered Dt oJH and VH to DJH rearrangement. To address the role of ordered rearrangement, it is important to disrupt the normal timing and/or order of events to determine effects on feedback regulation. Investigators must also have more direct measurements of accessibility throughout the locus. One such approach could be to use reporters to measure transcription [as has been done for the κ locus, (54)], an approach that could be particularly useful for analyzing antisense transcription. Another approach might be the insertion of V(D)J...
recombination reporter cassettes into specific locations within endogenous antigen receptor loci to assess whether these locations are available to RAG in various stages and contexts.

Further elucidation of the implications of IgH locus contraction and looping may provide additional mechanistic insights. Recent reports have described the role of nuclear actin in activating transcription by helping to form a preinitiation complex (172), and additional reports have shown that actin is important for chromosome association during meiosis (173), suggesting that the potential role of actin and other such proteins in relocalization of IgH alleles should be examined. In addition, IgH locus contraction may involve the spatial reorganization of chromatin to facilitate physical interaction between the different VH genes and the DJH region. Thus, some of the known regulatory elements, as well as some that perhaps await discovery, might function as a locus control region (LCR) to coordinate such looping events in bringing together DJH rearrangements and germline VH gene segments for rearrangement. Such a putative element might function analogously to the way the β-globin LCR (174) and the Tβ2 cytokine LCR (175) regulate stage-specific gene expression. Many or all of the future advances in our understanding of V(D)J recombination at the IgH locus likely hold broad implications for the control of gene expression in different systems.

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