REGULATING ANTIGEN-RECEPTOR GENE ASSEMBLY

Mark S. Schlissel

The genes encoding antigen receptors are unique because of their high diversity and their assembly in developing lymphocytes from gene segments through a series of site-specific DNA recombination reactions known as V(D)J rearrangement. This review focuses on our understanding of how recombination of immunoglobulin and T-cell receptor gene segments is tightly regulated despite being catalysed by a common lymphoid recombinase, which recognizes a widely distributed conserved recombination signal sequence. Probable mechanisms involve precise expression of the lymphoid-restricted recombination-activating genes *RAG1* and *RAG2*, and developmentally regulated epigenetic alterations in template accessibility, which are targeted by transcriptional regulatory elements and involve chromatin-modifying enzymes.

RECOMBINATION SIGNAL SEQUENCES (RSSs). Short, conserved DNA sequences that flank all rearranging gene segments and serve as the recognition elements for the recombinase machinery.

Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, USA.
e-mail:mss@uclink4.berkeley.edu
doi:10.1038/nri1225

It has been nearly 25 years since Tonegawa and colleagues[1] shattered one of the basic assumptions of molecular biology, the inviolate structure of the genome, and in so doing solved a fundamental puzzle in immunology — the generation of antigen-receptor diversity. The discovery that a complete immunoglobulin gene is generated from component gene segments through a series of site-specific DNA recombination reactions known as V(D)J recombination was followed over the ensuing decades by the elucidation of the structures of the seven rearranging antigen-receptor loci (immunoglobulin μ, κ and λ; and T-cell receptor (TCR) α, β, γ and δ), the identification of cis-acting DNA sequences essential for recombination — the RECOMBINATION SIGNAL SEQUENCES (RSSs) — the discovery of two lymphocyte-restricted genes, *RAG1* and *RAG2*, which encode the lymphocyte-specific components of the recombinase, and a thorough description of the recombination-reaction pathway[2–4] (BOX 1). What remains to be understood, however, are the mechanisms involved in the regulation of this remarkable site-specific DNA recombination reaction. This review focuses on the regulation of V(D)J recombination from the perspective of both the recombinase and its substrates.

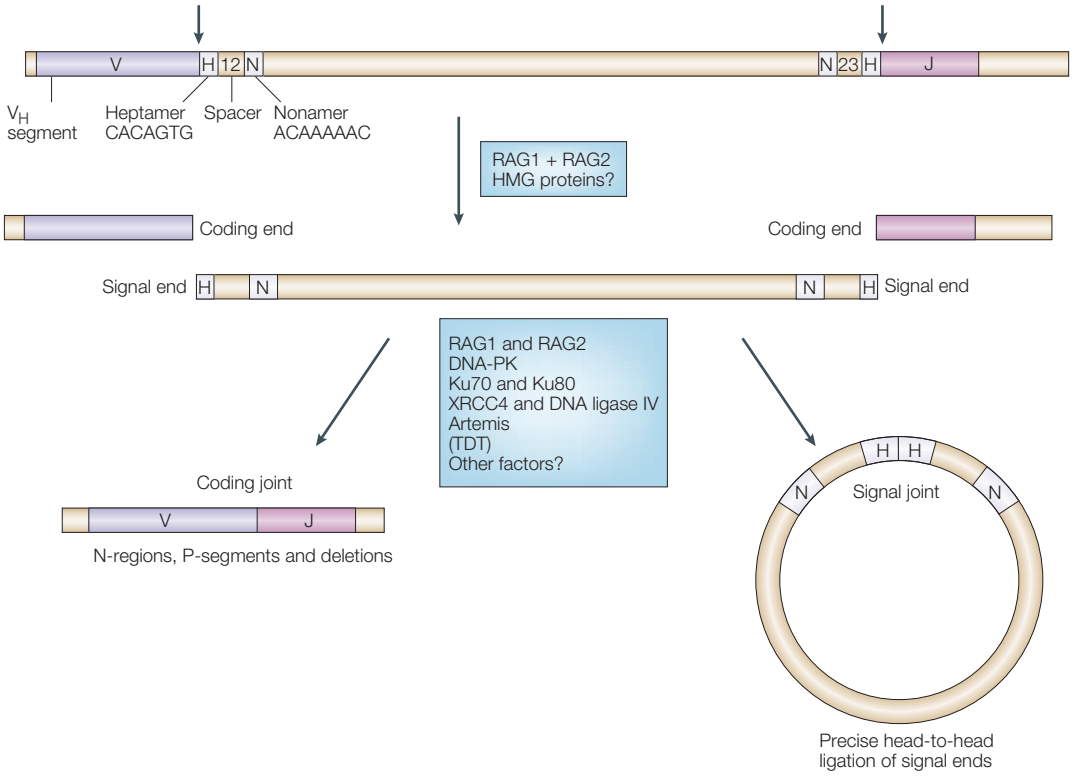
V(D)J recombination: levels of regulation

V(D)J recombination has three types of regulation — lineage specificity, order within a lineage and allelic exclusion. Transcriptional regulation limits the expression of RAG proteins to the progenitor stages of B- and T-cell development, accounting for the lymphoid lineage specificity of recombination *per se*. However, immunoglobulin genes fully rearrange in B cells only and TCR genes rearrange in T cells only, so additional regulatory mechanisms must exist to enforce B- versus T-cell lineage specificity. Moreover, in a given lineage, rearrangement is ordered; with immunoglobulin heavy-chain (IgH) or TCRβ locus D-to-J rearrangement preceding V-to-DJ rearrangement, followed most often by rearrangement of immunoglobulin light chain (IgL) or TCRα chains (FIGS 1 and 2). Finally, recombination at most loci is regulated such than an individual B or T cell generates only one functional allele at each locus. This phenomenon — allelic exclusion — results in lymphocytes expressing only a single receptor for antigen[5].

As noted earlier, all rearranging gene segments are flanked by conserved RSSs and all V(D)J recombination events seem to require the same trans-acting factors — the lymphoid-specific RAG1 and RAG2 proteins and

Box 1 | **The V(D)J recombination pathway: signals, intermediates, products and factors**

Rearranging gene segments are flanked by recombination signal sequences (RSSs), which consist of a conserved heptamer and nonamer separated by a non-conserved spacer of either 12 or 23 nucleotides (+ or – 1 nucleotide) in length. Gene segments of a particular type, variable heavy-chain (V_H) gene segments for example, are all flanked by RSSs with the same spacer length (23 nucleotides, in this case). Only gene segments that are flanked by RSSs with dissimilar spacer lengths can recombine with one another due to a limitation known as the 12/23 rule. Recombinase-activating gene 1 (RAG1) and RAG2 form a heteromultimer that recognizes and cleaves a 12/23 pair of RSSs precisely at the heptamer–RSS junction, generating reaction intermediates that consist of four DNA ends. The consensus heptamer and nonamer sequences are shown. High mobility group 1 (HMG1) — a prevalent non-histone chromosomal protein — stimulates this reaction *in vitro*, but its role *in vivo* remains undefined. Coding ends are covalently closed DNA hairpins and signal ends are blunt and 5′ phosphorylated. The latter stages of V(D)J recombination require the promiscuously expressed non-homologous end-joining (NHEJ) proteins DNA-dependent protein kinase (DNA-PK), Ku, X-ray repair cross complementing protein 4 (XRCC4), DNA ligase IV and Artemis in addition to the RAG proteins. Signal ends are joined by DNA ligase IV to form a signal joint and coding ends are first opened by the nuclease activity of Artemis, processed, then joined by DNA ligase IV to form a coding joint. Coding joints are imprecise and contain short deletions, palindromic duplications (P-segments) or non-templated nucleotide additions (N-regions) (introduced by terminal deoxynucleotidyl transferase, TDT). Ku70 is a DNA end-binding protein that interacts with Ku80. Ku80 in turn recruits DNA-PK, a protein kinase that is required to activate the nuclease activity of Artemis. XRCC4 increases the activity of DNA ligase IV. Null mutations in any of these proteins blocks the formation of coding joints, whereas mutation of DNA-PK spares signal-joint formation. As the joining step has not been recapitulated *in vitro* using purified components, it remains possible that additional factors might be required. Similarly, it is possible that specific factors might enhance RSS recognition or cleavage by the RAG proteins *in vivo*. (See REF. 4 for a recent review of the V(D)J recombination pathway).



the broadly expressed non-homologous end-joining (NHEJ) DNA break repair proteins[6] (BOX 1). The 12/23 rule prevents variable heavy-chain (V_H) gene segments from rearranging with one another and targets their rearrangement to diversity heavy-chain (D_H) gene segments (FIG. 1) and recent work indicates that the preferential pairing of RSSs might promote ordered V(D)J recombination (BOX 2). But the question remains as to how a common lymphoid recombinase that recognizes a conserved DNA sequence nonetheless results in highly regulated patterns of V(D)J recombination. An early clue to this conundrum came from observations made by Alt and colleagues[7,8], which were subsequently confirmed and extended by others, that rearranging gene segments are transcribed before or coincident with their activation for rearrangement. It was proposed that the generation of these germline transcripts either directly cause or correlate with changes in chromatin structure that increase the accessibility of the recombinase to a subset of its potential substrates[9]. Further experimentation has

POSITIVE SELECTION
Developing T cells are selected for survival and developmental progression based on the avidity of interaction between their T-cell receptors and the ligands expressed by the thymic microenvironment.

FOOTPRINTING ASSAYS
Assays that precisely map DNA–protein interactions by taking advantage of the fact that DNA sequences that are specifically complexed with protein are differentially susceptible to cleavage by nucleases or chemical modifying agents.

validated this 'accessibility hypothesis' and probed its mechanisms (see later). This review first considers what we know about regulation of the RAG genes and proteins and then addresses the regulation of recombinase-substrate accessibility.

Regulation of RAG1 and RAG2 activity

Patterns of RAG activity. The expression of RAG begins in a fraction of haematopoietic cells known as early lymphoid progenitors (ELPs), which can give rise to B-, T- and NK-cell precursors[10] (FIG. 2). In the early B- and T-cell lineages, RAG expression is modulated during developmental progression[11,12]. Levels of expression are high early in development during the assembly of a complete IgH or TCRβ chain. The levels of RAG transcripts and proteins drop sharply during a period of rapid pre-B- and pre-T-cell proliferation that follows assembly of the pre-B-cell receptor (BCR) and pre-TCR[13]. Pre-B and pre-T cells then exit the cell cycle and once again express RAG transcripts and proteins during the period of IgL and TCRα chain gene assembly. As noted earlier, POSITIVE SELECTION results in the loss of RAG expression in the thymus[14], although the corresponding event in B cells remains to be identified. Expression of a complete BCR or TCR on the cell surface of a developing lymphocyte ultimately results in the inactivation of RAG expression and recombinase activity. In developing B cells, however, if the initially produced BCR recognizes a self-antigen, then recombinase expression continues. This results in a tolerance mechanism known as receptor editing in which ongoing light-chain gene rearrangement replaces the initial light-chain gene in an attempt to alter receptor specificity[15,16]. In developing thymocytes, many $CD4^+CD8^+$ double-positive cells express low levels of TCR, but rearrangement of the TCRα locus continues until the cell generates a receptor that mediates positive selection and inactivation of RAG expression[17].

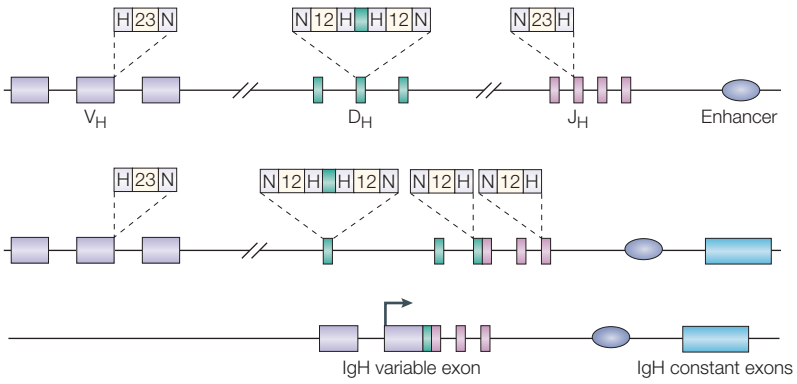


Figure 1 | **A schematic diagram of the mouse immunoglobulin heavy-chain (IgH) locus showing the positions of the V_H, D_H and J_H gene segments and the IgH intronic enhancer.** The top line indicates the germline conformation, the second line a partial DJ-rearranged allele, and the third line a complete, functional V(D)J rearrangement encoding the variable heavy-chain (V_H) exon (the arrow indicates the V_H promoter). The positions of the recombination signal sequences (RSSs) are indicated, showing the heptamer (H) immediately adjacent to the coding segment, the nonamer (N), and the length of the RSS spacer (12 or 23 nucleotides). Note that in the middle diagram, mechanisms must exist to promote the rearrangement of a V_H segment to the DJ recombinant rather than to an intervening unrearranged diversity heavy-chain (D_H) gene segment. J, joining.

Several groups reported that RAG expression, and perhaps V(D)J recombination, could be reactivated in peripheral B cells that participate in an immune response and remained active in the B-1 subset of mature B cells[18–20]. Subsequent studies using a RAG–green fluorescent protein (GFP) fusion construct generated by gene targeting showed that RAG-expressing germinal-centre B cells were likely to be recent bone-marrow emigrants that are prematurely sent to the periphery in response to systemic inflammatory signals[21]. The presence and function of RAG expression by peritoneal B-1 cells has also been questioned[22]. There is evidence of continuing gene rearrangement in peripheral T cells in a transgenic mouse system[23], but the generality and significance of this observation remains uncertain. Most evidence at present indicates that RAG expression does not have a role in the function of lymphocytes after receptor selection.

The cessation of V(D)J recombination during the proliferative expansion of early pre-B and pre-T cells is associated with both transcriptional and post-translational regulation of the *RAG* genes.

Post-translational regulation of RAG proteins. RAG2 is subject to cell-cycle regulated phosphorylation by a cyclin-dependent kinase that results in its degradation through a ubiquitin-dependent pathway[24]. As the half-life of RAG1 is prolonged by association with RAG2, this pathway probably contributes to the degradation of both proteins[25]. It has been proposed that limiting V(D)J recombination to the G0/G1 phase of the cell cycle might be crucial for genomic stability and for IgH and TCRβ locus allelic exclusion. Surprisingly, transgenic expression of a mutant RAG2 protein that is not subject to this mode of regulation had no obvious effect on regulated gene rearrangement, lymphocyte development or genomic stability[26]. Interestingly, however, misregulated expression of *RAG* genes through transgenesis leads to markedly abnormal lymph-node architecture[27].

Transcriptional regulation of RAG1 and RAG2. The highly unusual genomic structure of the *RAG* genes and their ability to catalyse transposition *in vitro*, has led to the suggestion that they evolved from a primitive transposable element system[28]. *RAG1* and *RAG2* are physically linked in the genomes of all organisms in which they have been studied, are convergently transcribed (their promoters 'point' at each other) and their entire open reading frames are contained in single large exons (FIG. 3).

Promoter elements have been identified for both genes in the mouse and human systems[29–32]. The *RAG1* promoter shows promiscuous expression in transient-transfection reporter construct assays, whereas the *RAG2* promoter seems to be more cell-type specific[32]. Binding sites for various transcription factors have been identified in each promoter including PAX5, MYB, SP1, LEF1, NF-Y, C/EBP and GATA3 (REFS 29–38) (FIG. 3). In several cases, the relevance of these proteins and their binding sites to promoter activity have been confirmed by *in vivo* FOOTPRINTING ASSAYS or chromatin immunoprecipitation[32,38].

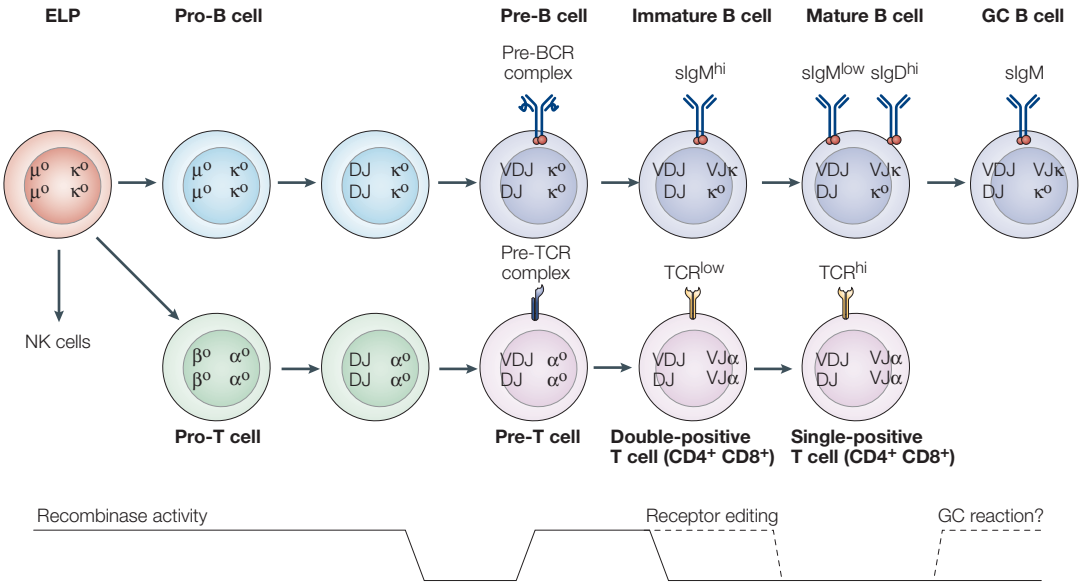


Figure 2 | **Regulation of V(D)J recombinase activity during lymphocyte development.** Successive stages of B- and T-cell development and the relative levels of recombinase expression at each stage are indicated. BCR, B-cell receptor; D, diversity; ELP, early lymphoid progenitor; GC, germinal centre; J, joining; NK, natural killer; TCR, T-cell receptor; V, variable.

As might be expected, distal elements are also involved in regulating RAG1 and RAG2 expression. In one particularly novel set of experiments, Nussenzweig and colleagues[39] developed a bacterial artificial chromosome (BAC) transgenic reporter construct system in which either the *RAG1* or *RAG2* open reading frames were replaced with a fluorescent protein complementary DNA. These investigators reported that *RAG* expression by $CD4^{-}CD8^{-}$ double-negative T cells and developing B cells was independent of sequences that were 5′ of *RAG1* and required only sequences within 10 kb 5′ of the *RAG2* promoter. This result was independently confirmed by another group using a $RAG2^{-/-}$ embryonic stem cell transfection assay[40]. Expression of the transgenic *RAG* locus BAC in double-positive T cells required sequences that extend towards a region as far as 90 kb 5′ of *RAG2*. A second group independently identified a putative *RAG* locus enhancer known as D3 that was ~8 kb 5′ of the *RAG2* first exon[41]. This element was associated with cell-type-specific DNASE HYPERSENSITIVITY and contains binding sites for several lymphoid transcription factors including E2A, MYB, RUNX1, C/EBP and LYF1.

Recently, my lab described a new *RAG* locus enhancer element known as *ERAG* located ~22 kb 5′ of the *RAG2* promoter, which is highly conserved between an array of mammalian species[42]. Targeted disruption of this sequence in the mouse germline results in a partial block in B-cell development but no T-cell phenotype. In addition, it was found that E2A-family transcription factors bound a set of conserved E-boxes in *ERAG* and were important for its activity. Of note, B-cell development is blocked in E2A-deficient mice at the stage of D-to-J IgH gene assembly[43]. The *ERAG* sequence is dispensable for the activity of the BAC reporter element described earlier, leading us to conclude that BAC transgenesis has limitations as an assay for gene-regulatory sequences.

The *RAG* genes reside in a large region of synteny between mice and humans. In this region, my lab identified ~20 conserved non-coding sequences (CNSs) of 200 base pairs or longer, lacking a significant open reading frame with 80% or greater homology between the two species (FIG. 3). Such conserved elements are often involved in gene regulation[44], therefore the regulation of *RAG* expression is likely to be even more complex than we understand at present. Why the regulation of *RAG* expression is so complex and depends on different sequences in B- and T-cell lineages is not known.

Regulation of substrate accessibility

It is firmly established that the targeting of V(D)J recombination to specific loci is highly dependent on developmentally regulated aspects of chromatin structure (BOX 3). Numerous examples exist of situations in which recombinase activity is present in a cell, but only a subset of potential substrates undergo V(D)J recombination. This might be due to either the inability of RAG proteins to recognize RSSs in the context of inaccessible chromatin structure or to the failure of RSSs, once associated with RAG complexes, to synapse effectively with one another. This type of regulation is most marked in pre-B and pre-T cells in which IgL or TCRα chain recombination proceeds, while the IgH or TCRβ chain loci no longer rearrange. Similarly, IgL rearrangement does not occur in T cells and TCRα rearrangement does not occur in B cells. In addition, transfection of *RAG1* and *RAG2* expression vectors into non-lymphoid cell lines is sufficient to activate rearrangement of an episomal reporter construct, but not endogenous immunoglobulin and TCR loci[45]. Endogenous loci can be made accessible

DNASE HYPERSENSITIVITY
A technique that allows the location of sites of gene-regulatory sequences because of their increased susceptibility to nucleases within nuclear structure.

in non-lymphoid cells, however, with the forced expression of lymphoid-lineage transcription factors such as E2A or early B-cell factor (EBF), which presumably result in changes in chromatin structure[46]. Finally, the role of chromatin structure was shown directly through experiments that used recombinant RAG proteins to cleave RSSs in either purified genomic DNA or chromatin[47]. Whereas RSSs associated with each rearranging locus could be cleaved in a naked DNA substrate, RSS cleavage in chromatin depended on the cell type of origin. So, chromatin from pre-B cells was cleaved *in vitro* by the RAGs at Jκ RSSs but not at TCRβ RSSs, for example.

Linking transcription and accessibility. Given the correlation between germline transcription and V(D)J recombination, much attention has been focused on transcriptional regulatory elements, such as enhancers and promoters, as potential regulators of chromatin structure and recombinase accessibility[7,48]. These studies show that in loci with a single transcriptional enhancer (TCRα and β), deletion of that enhancer by gene targeting markedly impairs recognition and cleavage by the recombinase. Loci with many regulatory elements are more complex. In the Igκ locus for example, targeted disruption of either the intronic or 3′ enhancers has only a modest effect on κ rearrangement whereas deletion of both elements nearly abrogates these rearrangements[49]. Germline transcript promoter elements also effect recombinase targeting. Deletion of PDβ1 — a promoter located just 5′ to the Dβ1 gene segment — markedly diminishes the use of that Dβ segment[50]. The situation is more complex in the TCRα locus where a promoter element that is 5′ of the most V-proximal Jα gene segment, known as TEA (T early α), functions to target recombination to adjacent Jα RSSs on each TCRα allele in a pre-T cell. Successive replacement rearrangements can then occur on both alleles using upstream Vα segments and downstream Jα segments to reduce the locus until a particular TCRα chain is made which contributes to a TCR that can promote positive selection[51,52]. Other downstream Jα region germline transcript promoters might be involved in these successive rearrangement events, or alternatively, the rearranged VJα promoter might serve this function. Consistent with a crucial role for replacement rearrangements, recent data have shown that use of more 3′ Jα gene segments depends on the lifespan of the pre-T cell[53].

The Igκ and TCRα loci efficiently rearrange only after productive IgH or TCRβ chain rearrangement and pre-BCR or pre-TCR signalling. Several groups have shown that pre-BCR or pre-TCR signalling results in the regulated activation of germline Igκ or TCRα transcription associated with changes in the pattern of transcription-factor binding to transcriptional enhancers associated with each locus[54,55]. Dysregulated expression of pre-BCR and pre-TCR signalling pathway components such as RAS, RAF or EGR can bypass the requirement for productive IgH or TCRβ gene rearrangement, presumably by similarly inducing transcriptional activation of these loci[56–58]. Cytokine signalling can also result in the activation of recombinase accessibility. Interleukin-7 receptor (IL-7R) signalling causes activation of signal transducer and activator of transcription 5 (STAT5) and its binding to DNA sequences in the TCRγ locus, activating germline transcription and Vγ-to-Jγ rearrangement[59,60].

Recent studies have been focused on understanding the molecular basis of chromatin accessibility in V(D)J recombination. Three potential mechanisms have been scrutinized: DNA methylation, histone modification and nuclear localization.

DNA methylation. Methyl-cytosine is the most common chemical modification of DNA in higher eukaryotes[61]. It is found almost exclusively in CpG dinucleotides, which tend to cluster in and around actively expressed regions of the genome (so-called CpG islands). This modification is introduced by enzymes known as DNA methyltransferases (DNMTs). Some of these enzymes are capable of *de novo* methylation whereas others act on hemi-methylated DNA sequences to perpetuate an established pattern of modification. In many systems, DNA CpG methylation has been correlated with gene inactivity. DNA methylation has been proposed as a regulatory mechanism affecting accessibility of RSSs to the V(D)J recombinase. This has been particularly well studied in the Igκ and TCRβ loci where developmentally regulated demethylation correlates with active recombination[50,62]. However, it remains unclear whether such demethylation is the direct cause of increased accessibility or whether it is simply a correlate. It is clear, however, that methylated loci within chromatin structure are less susceptible to recombinase cleavage than unmethylated loci[63], and that

Box 2 | Regulatory role of recombination signal sequences (RSSs)

Early experiments indicated that RSSs, although they varied in efficiency of recombination, were not likely to have a determining role in regulated recombination[90] with the possible exception of the κ versus λ decision in pre-B cells[91]. Recent studies have returned to this somewhat overlooked possibility and found that certain RSSs prefer to pair with one another in such a way as to promote ordered assembly of gene segments (D-to-J preceding V-to-DJ) within the TCRβ locus[92,93]. The biochemical basis of this mode of regulation known as 'beyond 12/23 regulation' is under intense study. The distribution of RSSs in the immunoglobulin heavy-chain (IgH) locus is both an obvious contributor to and a paradoxical component of ordered rearrangement in that locus (FIG. 1). The fact that both variable heavy-chain (V_H) and joining heavy-chain (J_H) gene segments are flanked by RSS-23 elements prevents direct V-to-J rearrangement and requires the involvement of a diversity heavy-chain (D_H) gene segment, flanked both upstream and downstream by RSS-12 elements, for proper variable exon assembly. Curiously, both V-to-D and D-to-J rearrangements are almost always deletional rather than inversional. The observation that D-to-J rearrangement precedes V-to-DJ rearrangement in the heavy-chain locus might be due to regulated V_H gene-segment accessibility, but it is uncertain why V_H gene segments prefer to rearrange to the 5′ RSS-12 associated with partially assembled DJ rather than unrearranged D_H gene segments (FIG. 1). One possibility is that the 3′ end of the D_H RSS-12 binds RAG proteins more avidly than the 5′ end of D_H RSS-12 and binding 3′ of D_H prevents binding 5′ of D_H by steric hindrance. Reporter construct experiments indicate that the 5′ end of D_H RSS-12 is 'stronger' than the 3′ end of D_H RSS-12 making it unlikely that RSS preferences alone account for ordered rearrangement in the IgH locus, however[94]. New computer algorithms have been developed, which might shed further light on the role of RSSs that are associated with different rearranging gene segments[95].

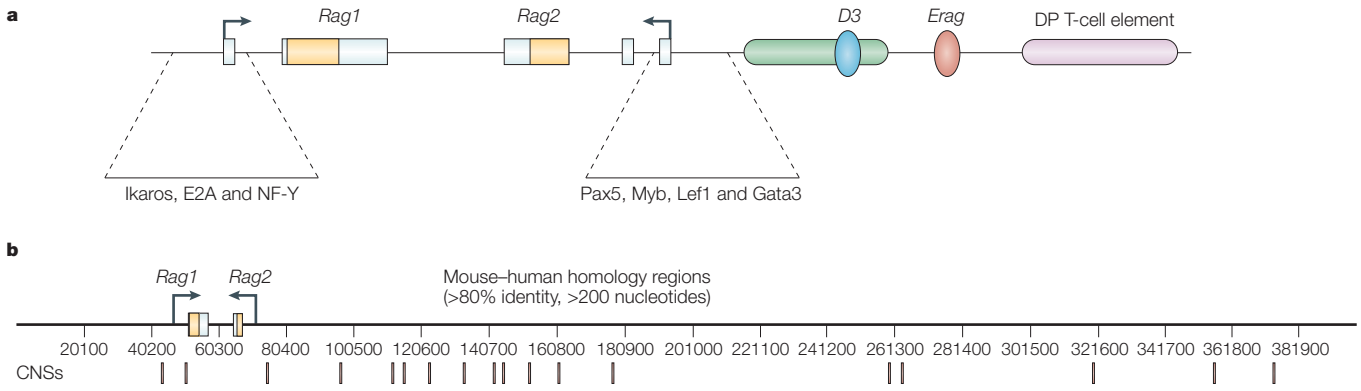


Figure 3 | **The recombinase-activating gene locus. a** | A schematic diagram of the mouse *Rag* locus indicating the positions of *Rag1* and *Rag2*. Boxes denote exons, with yellow indicating open reading frames and the arrows identifing promoters. The ovals show the positions of transcription regulatory regions. Transcription factors that are known or suggested to bind each promoter are indicated below each element. **b** | The distribution of conserved non-coding sequences (CNSs), identified through the comparison of mouse and human *Rag* locus sequences, is shown relative to the positions of *Rag1* and *Rag2*. CNSs are defined as sequences that are 200 base pairs or greater in length, and 80% or more homologous between mouse and human genomes.

rare RSSs, which themselves contain methylated CpG dinucleotides, are poor recombinase substrates *in vitro*[50]. Interestingly, inducible inactivation of the main DNMT in thymocytes fails to alter V(D)J recombination[64] and in wild-type mice the TCRα locus undergoes V(D)J rearrangement despite marked CpG methylation[65]. These observations indicate that other factors must be involved, including, most probably, effects of DNA methylation on chromatin structure. Methylated DNA is recognized by a protein known as methyl C binding protein, which can recruit histone-modifying enzymes that alter local chromatin structure[66].

Nucleosome structure and recombinase cleavage. A complex of recombinant CORE RAG1 AND RAG2 proteins can efficiently recognize and cleave RSSs *in vitro* on either simple substrates (oligonucleotides, plasmids or PCR-amplified DNA fragments) or purified genomic DNA. When a genomic DNA substrate is provided in the form of chromatin, this no longer holds true. All RSSs are essentially inaccessible in non-lymphoid chromatin whereas chromatin purified from lymphoid cells shows regulated RSS accessibility in a pattern that matches the known pattern of recombinase targeting in the cell type from which the chromatin was purified[47]. To test the idea that the fundamental element of chromatin structure — the nucleosome — imposes a barrier to sequence recognition by the V(D)J recombinase, several groups probed the ability of RAG1 and RAG2 to cleave RSSs assembled onto the surface of a nucleosome[67–69]. In general, it was found that the nucleosome markedly (or in some cases completely) prevented RSS cleavage by the RAG proteins. These observations have led to the hypothesis that nucleosomes must either be moved out of the way or modified in some manner so as to allow the RAG proteins to recognize their targets in chromatin structure. Although the structure of the nucleosome is known at atomic resolution and it is appreciated that the average distance between adjacent nucleomes is 40 to 60 nucleotides, the precise structure of higher order nucleosomal arrays is uncertain.

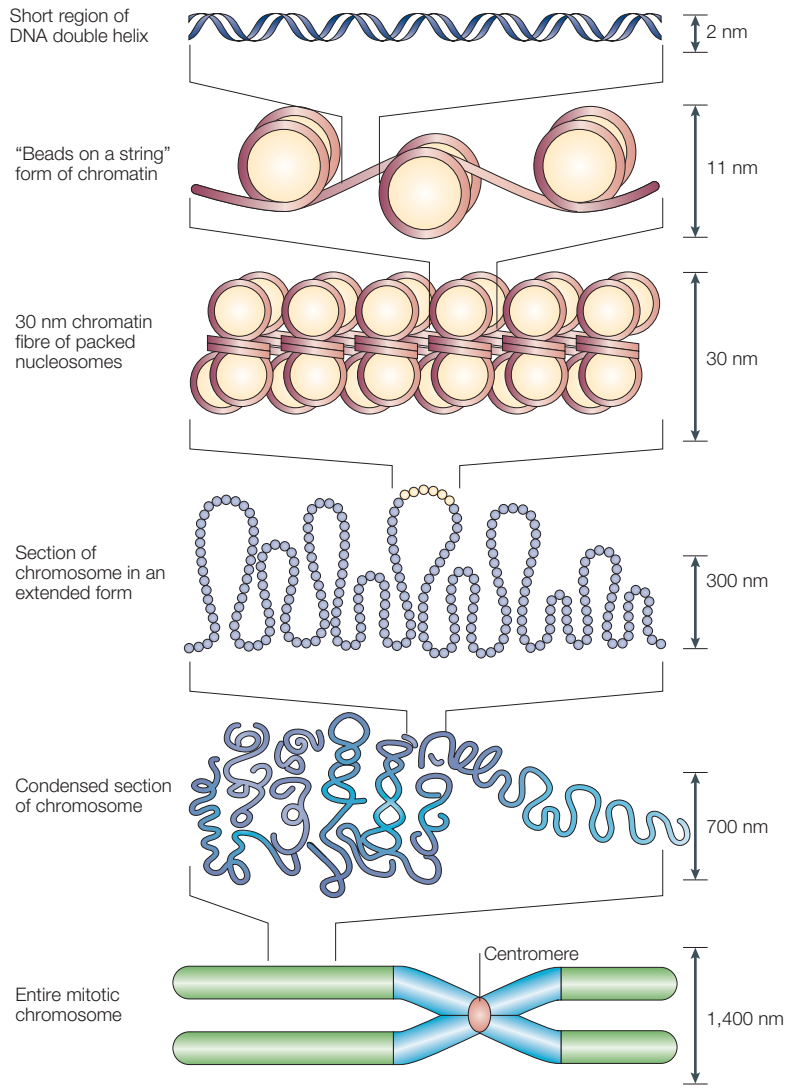
There has been an large amount of information in the past several years about how histones within chromatin structures can be covalently modified by acetylation, methylation and phosphorylation, or 'remodelled' by ATP-dependent multiprotein chromatin-remodelling complexes[70] (BOX 3). It was shown in an *in vitro* system that either histone acetylation or chromatin-remodelling complexes can enhance the ability of recombinant RAG proteins to cleave RSSs that are assembled on mononucleosomes[68,71]. In addition, several groups have found that rearranging IgH, TCRβ and TCRα/δ gene segments are packaged in chromatin structures that contain modified histones in a pattern similar to that found on actively transcribed genes[72,73,79] (BOX 3). These modifications extend over large domains (tens to hundreds of kilobases) and require intact enhancer activity for their establishment, so indicating a mechanism for the link between germline transcription and recombinase accessibility[74]. In addition, it was shown recently that mutation of a factor known to have a role in the regulation of chromatin structure, EZH2, results in a decrease in histone methylation, diminished V-to-DJ_H rearrangement and a partial block in B-cell development[75]. The emerging concept of the HISTONE CODE might prove to be key to understanding the regulation of V(D)J recombination through alterations in chromatin structure[76]. Precise introduction and removal of specific histone modifications might dictate chromatin accessibility to the recombinase. Experiments using transgenic recombination reporter constructs are consistent with the idea that individual transcription factors, such as E2A, that are required for recombinase accessibility but not for germline transcription, might have key roles in recruiting the recombinase to specific loci apart from their role in activating transcription, perhaps through a mechanism that involves chromatin modification[77].

CORE RAG1 AND RAG2
The minimal domains of recombinase-activating gene 1 (RAG1) and RAG2 that can activate the rearrangement of a reporter construct. Unlike the full-length proteins, the core domains are soluble and have been used for all biochemical analysis of this reaction.

HISTONE CODE
The concept that site-specific post-translational modification of histones might function to regulate gene activity precisely; the code is determined by which amino acids are acetylated, methylated or phosphorylated.

Box 3 | **Chromatin structure**

The basic element of chromatin structure is the mononucleosome, an octamer of histones (2 copies each of H2A, H2B, H3 and H4) with 146 base pairs of DNA wrapped nearly twice around. Adjacent nucleosomes are separated by between ~20 and 60 nucleotides of DNA referred to as linker or internucleosomal DNA. A fifth histone, H1, binds to DNA as it exits the nucleosome and interacts with the linker DNA. H1 is thought to be essential for the condensation of polynucleosomes into higher order structures. These structures include a solenoid of helically arrayed nucleosomes and then some superhelical twisting of solenoidal loops, the bases of which are attached to a non-histone protein scaffold. Although high resolution X-ray crystallographic data exist that confirm the precise structure of the mononucleosome and its associated DNA, higher order structures are inferred from electronic microscopic observations and various indirect biochemical assays. Individual histones undergo post-translational modification by phosphorylation, methylation and acetylation in ways that are expected to alter the local properties of chromatin structure, enhancing or inhibiting access of proteins to specific DNA sequences. These modifications are targeted by sequence-specific DNA-binding proteins (often transcription factors), which recruit modifying enzymes through protein–protein interactions. Finally, the positions of individual nucleosomes need not be static — they might be able to slide along the DNA, transiently exposing different sequences in the linker regions between nucleosome cores. Some ATP-dependent chromatin-remodelling complexes are thought to increase this sliding and thereby promote accessibility. Diagram is modified from REF. 96 **with permission from Nature © 2003.**



Despite the evidence indicating a role for histone modification in regulating V(D)J recombination, several experiments in the recent literature indicate that histone modification alone might not be determinative. Using a chromosomal reporter construct in a RAG-expressing cell line, Oltz and co-workers[78] showed that histone acetylation and germline transcription were not sufficient for reporter-construct recombination. This might indicate that either histone acetylation is not sufficient to allow RAG proteins to bind to RSSs or that additional modification might be required to allow synapsis and cleavage of bound RAG–RSS complexes. Furthermore, these workers found that the precise position of a promoter element relative to the rearranging gene segment was more important than that promoter's orientation, indicating that passage of RNA polymerase through an RSS is not required for a promoter to influence recombinase accessibility. Another group confirmed the observation that histone acetylation was not sufficient for recombinase accessibility[79]. In this case, distal V_H gene segments in PAX5-deficient pro-B cells failed to undergo V(D)J recombination even though they were packaged in an acetylated chromatin structure. So, histone acetylation is not sufficient to create accessible chromatin.

Nuclear choreography. Work in various model systems has shown that transcriptionally active and inactive DNA sequences often occupy different domains in the nucleus. Such nuclear localization might have a regulatory role in V(D)J recombination. Fisher and colleagues[80] reported that in splenic B cells the unexpressed IgH and IgL alleles were co-localized with heterochromatic (inactive) repetitive DNA domains, whereas the expressed alleles were not. Singh and co-workers[81] found that in non-lymphoid cells and T cells, immunoglobulin alleles were localized in peripheral (although not heterochromatic) nuclear areas, whereas in pro-B cells that were competent for IgH rearrangement, they were localized in central nuclear areas. Notably, the V and D-J-C regions of the IgH locus were closer to one another in pro-B cells where they rearrange than in T-cell progenitors where they do not. These observations indicate that the proximity of sub-domains of a rearranging locus might have a regulatory role in V(D)J recombination.

Allelic exclusion

Although developmentally regulated changes in chromatin structure might explain certain aspects of lineage specificity and the order of rearrangement, it is not immediately clear how such changes can enforce allelic exclusion. For example, both IgH alleles in pro-B cells undergo D-to-J_H rearrangement, but examples of B cells with two productive heavy chain V-to-DJ rearrangements are exceptionally rare (most examples of IgH 'allelic inclusion' are due to heavy chains that cannot efficiently pair with surrogate light chains to form a pre-BCR[82,83]). One can hypothesize that the pre-BCR signal results in chromatin structural changes that prevent further V-to-DJ rearrangement, but what prevents near simultaneous rearrangement of both heavy-chain alleles in pro-B cells? Twenty years ago, Coleclough *et al.*[84]

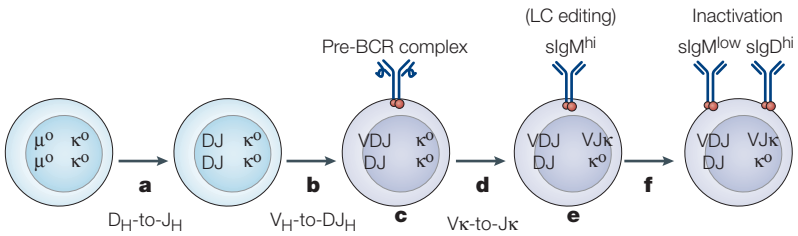


Figure 4 | **A model for the mechanisms of allelic exclusion of immunoglobulin gene expression.** Key regulated events at each developmental stage or transition are indicated. Different molecular mechanisms are responsible for allelic exclusion at successive stages of development. **a** | Both heavy-chain alleles are accessible to the recombinase, resulting in efficient, bi-allelic D-to-J rearrangement. **b** | Although both heavy-chain alleles are accessible to the recombinase as evidenced by bi-allelic D-to-J rearrangement, inefficient V-to-DJ rearrangement decreases the likelihood of bi-allelic in-frame heavy-chain gene rearrangement. **c** | Productive rearrangement results in the assembly of a pre-B-cell receptor (BCR), inactivation of recombinase-activating gene (*RAG*) expression and three to five rounds of cell division. Chromatin remodelling occurs during these rounds of cell division, preventing further variable heavy-chain (V_H) region accessibility. **d** | Low levels of light-chain enhancer-binding transcription factors result in the rare and stochastic activation of germline transcription and accessibility of the light-chain locus. Transcribed alleles are the favoured substrate for the recombinase, although silent alleles can undergo recombination less efficiently. **e** | Self-reactivity in immature B cells results in the continued expression of the *RAG* products and light-chain receptor editing. **f** | Expression of the membrane form of IgM on the cell surface in the absence of self-reactivity results in the inactivation of *RAG* expression, enforcing light-chain allelic exclusion.

presented the hypothesis that recombination was inefficient, so limiting the possibility of near-simultaneous allelic rearrangements. A recent study has provided data in support of the idea that V_H and Vβ RSSs in particular are relatively poor substrates for recombinase and that this might result in the unique inefficiency of V-to-DJ rearrangement, so contributing to IgH allelic exclusion[85]. Other data have indicated that there is a bias in rearrangement between allelic loci associated with the timing of their replication[86]. In pro-B cells, for example, one IgH allele replicates earlier in S phase than the other. It is possible that this difference in replication timing gives the early replicating allele a different, and perhaps more favourable, chromatin structure than the late allele and biases rearrangement in its favour.

Recent studies of the Igκ locus uncovered a similar allelic bias in recombinase activity, but through a distinct mechanism. As noted earlier, germline transcription is tightly correlated with recombinase accessibility[9]. Recent results from my lab indicated that during B-cell development, the likelihood that a κ allele would undergo high levels of transcription (and become accessible to the recombinase) was no more than 5%, and the likelihood that a cell would have two active κ alleles would be 0.25% (H.E. Liang and M.S.S., unpublished observations). There is experimental precedent for transcriptional enhancers to affect the likelihood rather than the level of gene activity[87]. Other workers have reported that Jκ region DNA is methylated in non-lymphoid tissues and in pro-B cells, but undergoes monoallelic demethylation in ~10% of bone marrow pre-B cells, consistent with the GFP reporter results noted earlier[62]. Conflicting data exist, however. Using single-cell polymerase chain reaction after reverse transcription (RT-PCR) and primary transcript fluorescence *in situ* hybridization (FISH), Chess and co-workers[88] found biallelic expression of the germline κ locus in pre-B cells. This approach, however, which relies on at least 70 cycles of PCR, might detect basal levels of transcription below those analysed in our own studies.

A model of allelic exclusion. To assure allelic exclusion, a developing lymphocyte must have a way to sense successful gene rearrangement (a feedback mechanism of some kind) and allelic loci must not rearrange simultaneously (or faster than the time-lag of the feedback system) (FIG. 4). Pre-B and pre-T cells sense productive IgH or TCRβ chain rearrangement through the pre-BCR and pre-TCR that result in signals that alter patterns of gene expression, enforce a period of proliferative expansion, and cause changes in chromatin structure that alter accessibility[13]. I propose that the second factor, prevention of near simultaneous rearrangement, is due to allelic differences in chromatin structure associated with rare and stochastic enhancer activation or allelic differences in the timing of DNA replication. It is worth noting that such allelic differences do not need to impose an absolute block to rearrangement, just a relative one. This notion is consistent with the observation that a significant fraction of B cells have two V(D)J-rearranged IgH or κ alleles, only one of which is in frame. If the favoured allele rearranges non-productively, then given an adequate amount of time the unexpressed allele might also undergo rearrangement. Successful rearrangement causes developmental progression and in the case of the light chain, inactivation of RAG expression.

Remaining challenges

The most important remaining questions in the field of V(D)J recombinase regulation are precisely how chromatin structure targets recombinase activity and how these regulatory chromatin structures are established and remodelled during development. Whereas chromatin structure is thoroughly understood at the level of the mononucleosome[89], our knowledge of higher order chromatin structure remains woefully incomplete. Such understanding will be required to decipher the functional consequences of histone modifications and chromatin-remodelling activities. Finally, these studies should lead us to a generally valid model of how enhancers regulate chromatin structure — an issue with implications far broader than this elegant solution to the diversity problem in immune recognition.

1. Tonegawa, S. Somatic generation of antibody diversity. *Nature* **302**, 575–581 (1983).
2. Schatz, D. G., Oettinger, M. A. & Schlissel, M. S. V(D)J recombination: molecular biology and regulation. *Ann. Rev. Immunol.* **10**, 359–383 (1992).
3. Hesse, J. E., Lieber, M. R., Mizuuchi, K. & Gellert, M. V(D)J recombination: a functional definition of the joining signals. *Genes Dev.* **3**, 1053–1061 (1989).
4. Fugmann, S. D., Lee, A. I., Shockett, P. E., Villey, I. J. & Schatz, D. G. The RAG proteins and V(D)J recombination: complexes, ends, and transposition. *Annu. Rev. Immunol.* **18**, 495–527 (2000).
5. Pernis, B. G., Chiappino, G., Kelus, A. S. & Gell, P. G. H. Cellular localization of immunoglobulins with different allotype specificities in rabbit lymphoid tissue. *J. Exp. Med.* **122**, 853–875 (1965).
6. Taccioli, G. E. *et al.* Impairment of V(D)J recombination in double-strand break repair mutants. *Science* **260**, 207–210 (1993).
7. Sleckman, B. P., Gorman, J. R. & Alt, F. W. Accessibility control of antigen-receptor variable-region gene assembly: role of cis-acting elements. *Ann. Rev. Immunol.* **14**, 459–481 (1996).
8. Yancopoulos, G. & Alt, F. Developmentally controlled and tissue-specific expression of unrearranged V_H gene segments. *Cell* **40**, 271–281 (1985).

9. Schlissel, M. & Baltimore, D. Activation of immunoglobulin κ gene rearrangement correlates with induction of germline κ gene transcription. *Cell* **58**, 1001–1007 (1989).
10. Igarashi, H., Gregory, S. C., Yokota, T., Sakaguchi, N. & Kincade, P. W. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity* **17**, 117–130 (2002).
11. Wilson, A., Held, W. & MacDonald, H. R. Two waves of recombinase gene expression in developing thymocytes. *J. Exp. Med.* **179**, 1355–1360 (1994).
12. Grawunder, U. *et al.* Downregulation of RAG1 and RAG2 gene expression in pre-B cells after functional immunoglobulin heavy chain rearrangement. *Immunity* **3**, 601–608 (1995).
This paper describes in detail the pattern of expression of recombination-activating gene (RAG) messenger RNA and protein in primary developing B cells.
13. Muljo, S. A. & Schlissel, M. S. Pre-B and pre-T-cell receptors: conservation of strategies in regulating early lymphocyte development. *Immunol. Rev.* **175**, 80–93 (2000).
14. Brandle, D., Muller, C., Rulicke, T., Hengartner, H. & Pircher, H. Engagement of the T-cell receptor during positive selection in the thymus downregulates RAG-1 expression. *Proc. Natl Acad. Sci. USA* **89**, 9529–9533 (1992).
15. Tiegs, S. S., Russell, D. M. & Nemazee, D. Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med.* **177**, 1009–1020 (1993).
16. Gay, D., Saunders, T., Camper, S. & Weigert, M. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J. Exp. Med.* **177**, 999–1008 (1993).
17. Turka, L. A. *et al.* Thymocyte expression of RAG-1 and RAG-2: termination by T cell receptor crosslinking. *Science* **253**, 778–781 (1991).
18. Qin, X. F. *et al.* Secondary V(D)J recombination in B-1 cells. *Nature* **397**, 355–359 (1999).
19. Hikida, M. *et al.* Reexpression of RAG-1 and RAG-2 genes in activated mature mouse B cells. *Science* **274**, 2092–2094 (1996).
20. Han, S. *et al.* V(D)J recombinase activity in a subset of germinal center B lymphocytes. *Science* **278**, 301–305 (1997).
21. Monroe, R. J. *et al.* RAG2:GFP knockin mice reveal novel aspects of RAG2 expression in primary and peripheral lymphoid tissues. *Immunity* **11**, 201–212 (1999).
22. Kuwata, N., Igarashi, H., Ohmura, T., Aizawa, S. & Sakaguchi, N. Cutting edge: absence of expression of RAG1 in peritoneal B-1 cells detected by knocking into RAG1 locus with green fluorescent protein gene. *J. Immunol.* **163**, 6355–6359 (1999).
23. McMahan, C. J. & Fink, P. J. RAG reexpression and DNA recombination at T cell receptor loci in peripheral $CD4^+$ T cells. *Immunity* **9**, 637–647 (1998).
24. Lin, W. C. & Desiderio, S. Cell cycle regulation of V(D)J recombination-activating protein RAG-2. *Proc. Natl Acad. Sci. USA* **91**, 2733–2737 (1994).
25. Grawunder, U., Schatz, D. G., Leu, T. M., Rolink, A. & Melchers, F. The half-life of RAG-1 protein in precursor B cells is increased in the absence of RAG-2 expression. *J. Exp. Med.* **183**, 1731–1737 (1996).
26. Lee, J. & Desiderio, S. Cyclin A/CDK2 regulates V(D)J recombination by coordinating RAG-2 accumulation and DNA repair. *Immunity* **11**, 771–781 (1999).
These authors describe the mechanism by which recombinase activity is limited to cells in the G1 stage of the cell cycle. A cyclin-dependent kinase (CDK) phosphorylates Thr490 in RAG2 resulting in its ubiquitin-dependent degradation.
27. Wayne, J. *et al.* A regulatory role for recombinase activating genes, RAG-1 and RAG-2, in T cell development. *Immunity* **1**, 95–107 (1994).
28. Agrawal, A., Eastman, Q. M. & Schatz, D. G. Transposition mediated by RAG1 and RAG2 and its implications for the evolution of the immune system. *Nature* **394**, 744–751 (1998).
29. Zarrin, A. A., Fong, I., Malkin, L., Marsden, P. A. & Berinstein, N. L. Cloning and characterization of the human recombination activating gene 1 (RAG1) and RAG2 promoter regions. *J. Immunol.* **159**, 4382–4394 (1997).
30. Kurioka, H. *et al.* Isolation and characterization of a TATA-less promoter for the human RAG-1 gene. *Mol. Immunol.* **33**, 1059–1066 (1996).
31. Fuller, K. & Storb, U. Identification and characterization of the murine Rag1 promoter. *Mol. Immunol.* **34**, 939–954 (1997).
32. Lauring, J. & Schlissel, M. S. Distinct factors regulate the murine RAG-2 promoter in B- and T-cell lines. *Mol. Cell. Biol.* **19**, 2601–2612 (1999).
33. Kishi, H. *et al.* Cooperative binding of c-Myb and Pax-5 activates the RAG-2 promoter in immature B cells. *Blood* **99**, 576–583 (2002).
34. Jin, Z. X. *et al.* Lymphoid enhancer-binding factor-1 binds and activates the recombination-activating gene-2 promoter together with c-Myb and Pax-5 in immature B cells. *J. Immunol.* **169**, 3783–3792 (2002).
35. Kishi, H. *et al.* Lineage-specific regulation of the murine RAG-2 promoter: GATA-3 in T cells and Pax-5 in B cells. *Blood* **95**, 3845–3852 (2000).
36. Fong, I. C., Zarrin, A. A., Wu, G. E. & Berinstein, N. L. Functional analysis of the human RAG 2 promoter. *Mol. Immunol.* **37**, 391–402 (2000).
37. Brown, S. T. *et al.* Regulation of the RAG-1 promoter by the NF-Y transcription factor. *J. Immunol.* **158**, 5071–5074 (1997).
38. Wang, Q. F., Lauring, J. & Schlissel, M. S. c-Myb binds to a sequence in the proximal region of the RAG-2 promoter and is essential for promoter activity in T-lineage cells. *Mol. Cell. Biol.* **20**, 9203–9211 (2000).
39. Yu, W. *et al.* Coordinate regulation of RAG1 and RAG2 by cell type-specific DNA elements 5′ of RAG2. *Science* **285**, 1080–1084 (1999).
Using a *RAG* locus bacterial artificial chromosome (BAC) transgenic reporter construct, these authors show that sequences required for developmentally regulated transcription of *RAG* reside 5′ of *RAG2* and that different sequences are important for expression at distinct stages of B- and T-cell development.
40. Monroe, R. J., Chen, F., Ferrini, R., Davidson, L. & Alt, F. W. RAG2 is regulated differentially in B and T cells by elements 5′ of the promoter. *Proc. Natl Acad. Sci. USA* **96**, 12713–12718 (1999).
41. Wei, X. C. *et al.* Characterization of chromatin structure and enhancer elements for murine recombination activating gene-2. *J. Immunol.* **169**, 873–881 (2002).
42. Hsu, L.-Y. *et al.* A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. *Immunity* **19**, 105–117 (2003).
This paper describes the discovery and analysis by targeted deletion of a new *RAG* locus transcriptional enhancer. Deletion of this element affects B- but not T-cell development.
43. Bain, G. *et al.* E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell* **79**, 885–892 (1994).
44. Loots, G. G. *et al.* Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. *Science* **288**, 136–140 (2000).
45. Oettinger, M. A., Schatz, D. G., Gorka, C. & Baltimore, D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science* **248**, 1517–1523 (1990).
A classic paper reporting the cloning of *RAG2* and its synergy in V(D)J recombination with *RAG1*. The unusual structure of the *RAG* locus is also shown.
46. Romanow, W. J. *et al.* E2A and EBF act in synergy with the V(D)J recombinase to generate a diverse immunoglobulin repertoire in nonlymphoid cells. *Mol. Cell* **5**, 343–353 (2000).
Stable expression of either E2A or EBF (lymphoid-lineage transcription factors) in non-lymphoid cells is sufficient to allow transfected *RAG* expression vectors to activate rearrangement of endogenous immunoglobulin genes.
47. Stanhope-Baker, P., Hudson, K., Shaffer, A., Constantinescu, A. & Schlissel, M. Cell type-specific chromatin structure determines the targeting of V(D)J recombinase activity *in vitro*. *Cell* **85**, 887–897 (1996).
These investigators used purified nuclear substrates and recombinant RAG proteins to directly show developmentally regulated changes in recombination signal sequence (RSS) accessibility within chromatin.
48. Schlissel, M. S. & Stanhope-Baker, P. Accessibility and the developmental regulation of V(D)J recombination. *Semin. Immunol.* **9**, 161 (1997).
49. Inlay, M., Alt, F. W., Baltimore, D. & Xu, Y. Essential roles of the κ light chain intronic enhancer and 3′ enhancer in κ rearrangement and demethylation. *Nature Immunol.* **3**, 463–468 (2002).
50. Whitehurst, C. E., Schlissel, M. S. & Chen, J. Deletion of germline promoter PDβ1 from the TCRβ locus causes hypermethylation that impairs dβ1 recombination by multiple mechanisms. *Immunity* **13**, 703–714 (2000).
51. Mauvieux, L., Villey, I. & de Villartay, J. P. T early α (TEA) regulates initial TCRVAJA rearrangements and leads to TCRJA coincidence. *Eur. J. Immunol.* **31**, 2080–2086 (2001).
52. Villey, I., Caillol, D., Selz, F., Ferrier, P. & de Villartay, J. P. Defect in rearrangement of the most 5′ TCR-J α following targeted deletion of T early α (TEA): implications for TCRα locus accessibility. *Immunity* **5**, 331–342 (1996).
53. Guo, J. *et al.* Regulation of the TCRα repertoire by the survival window of $CD4^+CD8^+$ thymocytes. *Nature Immunol.* **3**, 469–476 (2002).
54. Hernandez-Munain, C., Sleckman, B. P. & Krangel, M. S. A developmental switch from TCR delta enhancer to TCRα enhancer function during thymocyte maturation. *Immunity* **10**, 723–733 (1999).
55. Shaffer, A. L., Peng, A. & Schlissel, M. S. *In vivo* occupancy of the κ light chain enhancers in primary pro- and pre-B cells: a model for κ locus activation. *Immunity* **6**, 131–143 (1997).
56. Shaw, A. C., Swat, W., Davidson, L. & Alt, F. W. Induction of Ig light chain gene rearrangement in heavy chain-deficient B cells by activated Ras. *Proc. Natl Acad. Sci. USA* **96**, 2239–2243 (1999).
57. Iritani, B. M., Forbush, K. A., Farrar, M. A. & Perlmutter, R. M. Control of B cell development by Ras-mediated activation of Raf. *EMBO J.* **16**, 7019–7031 (1997).
58. Dinkel, A. *et al.* The transcription factor early growth response 1 (Egr-1) advances differentiation of pre-B and immature B cells. *J. Exp. Med.* **188**, 2215–2224 (1998).
59. Ye, S. K. *et al.* Differential roles of cytokine receptors in the development of epidermal γδ T cells. *J. Immunol.* **167**, 1929–1934 (2001).
60. Ye, S. K. *et al.* Induction of germline transcription in the TCRγ locus by Stat5: implications for accessibility control by the IL-7 receptor. *Immunity* **11**, 213–223 (1999).
61. Jaenisch, R. & Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genet.* **33 Suppl**, 245–254 (2003).
62. Mostoslavsky, R. *et al.* κ chain monoallelic demethylation and the establishment of allelic exclusion. *Genes Dev.* **12**, 1801–1811 (1998).
The authors found that DNA in the Igκ locus was CpG methylated in non-lymphoid and pro-B cells, but underwent progressive, monoallelic demethylation with the progression of B-cell development. They proposed that methylation was important for allelic exclusion.
63. Goldmit, M., Schlissel, M., Cedar, H. & Bergman, Y. Differential accessibility at the kappa chain locus plays a role in allelic exclusion. *EMBO J.* **21**, 5255–5261 (2002).
64. Lee, P. P. *et al.* A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* **15**, 763–774 (2001).
65. Villey, I., Quartier, P., Selz, F. & de Villartay, J. P. Germ-line transcription and methylation status of the TCR-Jα locus in its accessible configuration. *Eur. J. Immunol.* **27**, 1619–1625 (1997).
66. Nan, X. *et al.* Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**, 386–389 (1998).
67. Golding, A., Chandler, S., Ballestar, E., Wolffe, A. P. & Schlissel, M. S. Nucleosome structure completely inhibits *in vitro* cleavage by the V(D)J recombinase. *EMBO J.* **18**, 3712–3723 (1999).
68. Kwon, J., Imbalzano, A. N., Matthews, A. & Oettinger, M. A. Accessibility of nucleosomal DNA to V(D)J cleavage is modulated by RSS positioning and HMG1. *Mol. Cell* **2**, 829–839 (1998).
69. McBlane, F. & Boyes, J. Stimulation of V(D)J recombination by histone acetylation. *Curr. Biol.* **10**, 483–486 (2000).
70. Felsenfeld, G. & Groudine, M. Controlling the double helix. *Nature* **421**, 448–453 (2003).
71. Kwon, J., Morshead, K. B., Guyon, J. R., Kingston, R. E. & Oettinger, M. A. Histone acetylation and hSWI/SNF remodeling act in concert to stimulate V(D)J cleavage of nucleosomal DNA. *Mol. Cell* **6**, 1037–1048 (2000).
72. McMurry, M. T. & Krangel, M. S. A role for histone acetylation in the developmental regulation of VDJ recombination. *Science* **287**, 495–498 (2000).
Using a chromatin immunoprecipitation assay, the authors uncovered developmentally regulated changes in the post-translational modification of histones, which correlate with RSS accessibility.
73. Chowdhury, D. & Sen, R. Stepwise activation of the immunoglobulin μ heavy chain gene locus. *EMBO J.* **20**, 6394–6403 (2001).
74. Mathieu, N., Hempel, W. M., Spicuglia, S., Verthuy, C. & Ferrier, P. Chromatin remodeling by the T cell receptor (TCR)-β gene enhancer during early T cell development: implications for the control of TCR-β locus recombination. *J. Exp. Med.* **192**, 625–636 (2000).
75. Su, I. H. *et al.* Ezh2 controls B cell development through histone H3 methylation and IgH rearrangement. *Nature Immunol.* **4**, 124–131 (2003).
76. Jenuwein, T. & Allis, C. D. Translating the histone code. *Science* **293**, 1074–1080 (2001).
77. Fernex, C., Capone, M. & Ferrier, P. The V(D)J recombinational and transcriptional activities of the immunoglobulin heavy-chain intronic enhancer can be mediated through distinct protein-binding sites in a transgenic substrate. *Mol. Cell. Biol.* **15**, 3217–3226 (1995).

78. Sikes, M. L., Meade, A., Tripathi, R., Krangel, M. S. & Oltz, E. M. Regulation of V(D)J recombination: a dominant role for promoter positioning in gene segment accessibility. *Proc. Natl Acad. Sci. USA* **99**, 12309–12314 (2002).
Using a stably transfected recombination reporter construct, this study explored the roles of promoter sequences and germline transcripts in V(D)J recombination. They found that the position of the promoter was more important than its orientation, and that histone acetylation was not sufficient for recombinase accessibility.
79. Hesslein, D. G. *et al.* Pax5 is required for recombination of transcribed, acetylated, 5′ IgH V gene segments. *Genes Dev.* **17**, 37–42 (2003).
80. Skok, J. A. *et al.* Nonequivalent nuclear location of immunoglobulin alleles in B lymphocytes. *Nature Immunol.* **2**, 848–854 (2001).
81. Kosak, S. T. *et al.* Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. *Science* **296**, 158–162 (2002).
82. ten Boekel, E., Melchers, F. & Rolink, A. G. Precursor B cells showing H chain allelic inclusion display allelic exclusion at the level of pre-B cell receptor surface expression. *Immunity* **8**, 199–207 (1998).
83. Sonoda, E. *et al.* B cell development under the condition of allelic inclusion. *Immunity* **6**, 225–233 (1997).
84. Coleclough, C., Perry, R. P., Karjalainen, K. & Weigert, M. Aberrant rearrangements contribute significantly to the allelic exclusion of immunoglobulin gene expression. *Nature* **290**, 372–378 (1981).
85. Liang, H. E. *et al.* The 'dispensable' portion of RAG2 is necessary for efficient V-to-DJ rearrangement during B and T cell development. *Immunity* **17**, 639–651 (2002).
86. Mostoslavsky, R. *et al.* Asynchronous replication and allelic exclusion in the immune system. *Nature* **414**, 221–225 (2001).
87. Walters, M. C. *et al.* Enhancers increase the probability but not the level of gene expression. *Proc. Natl Acad. Sci. USA* **92**, 7125–7129 (1995).
88. Singh, N., Bergman, Y., Cedar, H. & Chess, A. Biallelic germline transcription at the κ immunoglobulin locus. *J. Exp. Med.* **197**, 743–750 (2003).
89. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F. & Richmond, T. J. Crystal structure of the nucleosome core particle at 2. 8 A resolution. *Nature* **389**, 251–260 (1997).
90. Feeney, A. J. Factors that influence formation of B cell repertoire. *Immunol. Res.* **21**, 195–202 (2000).
91. Ramsden, D. A. & Wu, G. E. Mouse κ light-chain recombination signal sequences mediate recombination more frequently than do those of λ light chain. *Proc. Natl Acad. Sci. USA* **88**, 10721–10725 (1991).
92. Bassing, C. H. *et al.* Recombination signal sequences restrict chromosomal V(D)J recombination beyond the 12/23 rule. *Nature* **405**, 583–586 (2000).
These investigators used a gene-targeting strategy to show the roles of particular RSSs in regulating rearrangement in the T-cell receptor β (TCRβ) locus. Before this study, it was thought that RSS involvement in recombinase regulation was limited to the restrictions in pairing imposed by the 12/23 rule.
93. Sleckman, B. P. *et al.* Mechanisms that direct ordered assembly of T cell receptor β locus V, D, and J gene segments. *Proc. Natl Acad. Sci. USA* **97**, 7975–7980 (2000).
94. Pan, P. Y., Lieber, M. R. & Teale, J. M. The role of recombination signal sequences in the preferential joining by deletion in D_H-J_H recombination and in the ordered rearrangement of the IgH locus. *Int. Immunol.* **9**, 515–522 (1997).
95. Cowell, L. G., Davila, M., Yang, K., Kepler, T. B. & Kelsoe, G. Prospective estimation of recombination signal efficiency and identification of functional cryptic signals in the genome by statistical modeling. *J. Exp. Med.* **197**, 207–220 (2003).
96. Felsenfeld, G. & Groudine, M. Controlling the double helix. *Nature* **421**, 448–453 (2003).

Acknowledgements
I would like to acknowledge my students and fellows, past and present, who have contributed to advancing our knowledge of V(D)J recombination. Work in my lab is funded by the National Institutes of Health and the Arthritis Foundation.

Online links

DATABASES
The following terms in this article are linked online to:
LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
C/EBP | EBF | EZH2 | GATA3 | IL-7R | LEF1 | LYF1 | MYB | NF-Y | PAX5 | *RAG1* | *RAG2* | RUNX1 | SP1 | STAT5

FURTHER INFORMATION
Mark Schlissel's laboratory: http://mcb.berkeley.edu/faculty/imm/schlisselm.html
Access to this interactive links box is free online.