Cell, Vol. 109, S45–S55, April, 2002, Copyright ©2002 by Cell Press

# The Mechanism and Regulation of Chromosomal V(D)J Recombination

Review

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**V(D)J recombination is of fundamental importance to the generation of diverse antigen receptor repertoires. We review our current understanding of the V(D)J recombination reaction and how it is regulated during lymphocyte development. We also discuss how defects in the mechanism or regulation of V(D)J recombination can lead to human disease.**

## Introduction

The exons which encode immunoglobulin (Ig) or T cell receptor (TCR) variable regions are assembled in developing B or T lymphocytes from germline variable (V), diversity (D), and joining (J) gene segments (Tonegawa, 1983; Hesslein and Schatz, 2001). The TCR β and δ chain and Ig heavy chain (IgH) variable region exons are assembled from V, D, and J segments, while TCR α and γ chain and Ig κ and λ light chain (IgL) exons are assembled from V and J segments. V(D)J recombination is of fundamental importance to the generation of diverse antigen receptor repertoires, as such diversity derives in large part from the multiple combinations of possible joining events and through an inherent imprecision in the joining reaction. V(D)J recombination occurs only in lymphocytes, where it is regulated in the context of lineage specificity (e.g., complete TCR gene assembly in T cells but not B cells and complete Ig gene assembly in B cells but not T cells), developmental stage specificity (e.g., assembly of TCRβ genes before TCRα genes and IgH genes before IgL genes), and in the context of allelic exclusion. Allelic (and IgL isotypic) exclusion was discovered based on the observation that any given mature B cell carrying allotypically distinguishable IgH or IgL alleles expresses, on its surface, products of only one of its two IgH and one of its multiple IgL alleles (Gorman and Alt, 1998). Analyses of TCRβ gene rearrangements in transgenic mice suggested a similar scenario (Kisielow and von Boehmer, 1995). The reason for allelic exclusion of Ig and TCRβ gene expression remains speculative, but it likely prevents one or more potentially adverse immunological consequences of clonal lymphocytes expressing several diverse antigen receptors. Finally, the different antigen receptor gene segments are assembled by the same basic V(D)J "recombinase"; therefore, regulation of V(D)J recombination must occur in large part via a higher order process, referred to as accessibility (Sleckman et al., 1996). Accessibility control, for example as related to transcription, replication, and repair, is a broadly relevant biological process. Herein, we review our current understanding of V(D)J recombination and how it is regulated. We also discuss how defects in the mechanism or regulation of V(D)J recombination can lead to disease.

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## The V(D)J Recombination Reaction

V(D)J recombination is a site-specific recombination process that occurs only in developing lymphocytes and only between Ig and TCR gene segments flanked by conserved recombination signal (RS) sequences. RSs are composed of a conserved palindromic heptamer and an AT-rich nonamer separated by nonconserved 12 or 23 bp spacers. V(D)J recombination occurs only between two gene segments flanked, respectively, by RSs that contain 12 (12-RS) and 23 (23-RS) bp spacers, referred to as the 12/23 rule (Figure 1). V(D)J recombination is initiated via introduction of DNA double-strand breaks (DSBs) between the V, D, and J segments and flanking RSs; subsequently, RS ends are precisely joined, while coding ends are modified via a process that involves potential nucleotide loss and potential nucleotide addition. Joining can result either in inversion or deletion of intervening sequences depending on the relative orientation of recombining segments (Tonegawa, 1983; Figures 1 and 2). The joining phase of the V(D)J recombination reaction is carried out primarily by ubiquitously expressed nonhomologous DNA end-joining (NHEJ) proteins (Figures 1 and 2).

The recombination activating genes-1 and -2 (*RAG-1* and *RAG-2*) were identified based on ability to synergistically confer V(D)J recombination to nonlymphoid cells (Oettinger et al., 1990). Both RAG-1 and RAG-2 are absolutely required to initiate V(D)J recombination, as demonstrated by a complete block in B and T cell development at the progenitor stage in RAG-1- or RAG-2-deficient mice (Table 1). In addition, RAG-1 and RAG-2 (subsequently, together referred to as RAG) bind to RSs and can initiate V(D)J recombination on DNA containing RSs in vitro (McBlane et al., 1995), allowing the initiation phase of V(D)J recombination to be rigorously studied (Fugmann et al., 2000a). RAG-1 has an active site similar to those of transposases and retroviral integrases (Kim et al., 1999; Landree et al., 1999; Fugmann et al., 2000b); however, precise RAG-1 and RAG-2 functions remain under investigation. RAG first introduces single-strand nicks between two participating coding sequences and their flanking RSs (Figure 1). This is followed by RAG catalysis of a *trans*-esterification reaction in which the 3′OH of the coding strand invades the opposite DNA strand to form closed hairpin coding ends and blunt 5′ phosphorylated RS ends (van Gent et al., 1996a). The four RAG-liberated DNA ends remain associated with RAG in a stable postcleavage synaptic complex (PSC) (Fugmann et al., 2000a); RAG, in the context of this complex, appears important for the joining phase of the reaction (Schultz et al., 2001). Besides revealing mechanistic details, in vitro studies confirmed that the 12/23

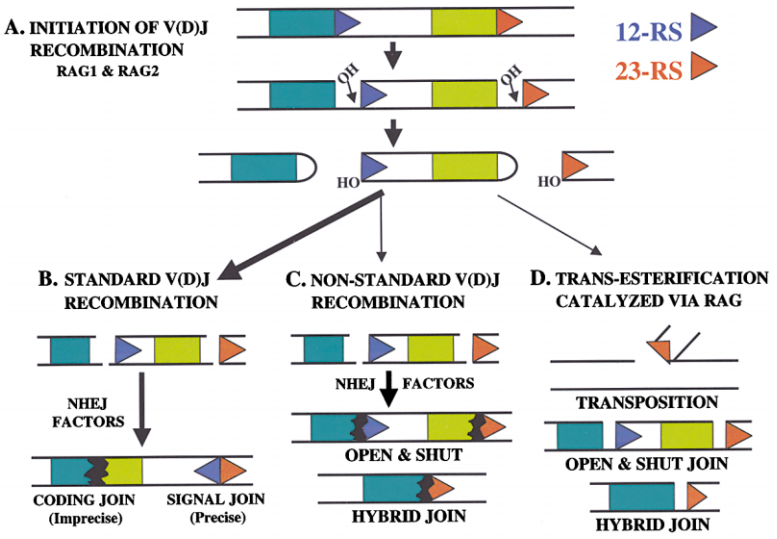


Figure 1. RAG-Mediated DNA Rearrangements

(A) RAG initiation of V(D)J recombination.
(B) Standard inversional V(D)J recombination reaction catalyzed via RAG and NHEJ proteins to form coding and RS joins.
(C) Nonstandard reactions catalyzed via RAG and NHEJ proteins to form "open and shut" or hybrid joins.
(D) RAG-catalyzed *trans*-esterification reactions occur in the absence of NHEJ proteins to form either transposition-like products, incomplete "open and shut," or incomplete hybrid joins. See text and Sekiguchi et al., 2001, for details and nomenclature. (Adapted from Sekiguchi et al., 2001).

rule is enforced at the level of RAG recognition and cleavage (Eastman et al., 1996; van Gent et al., 1996b) and showed that RAGs, in the absence of NHEJ proteins, can catalyze nonstandard V(D)J reactions including hybrid joins, open and shut joins, and transpositions (Agrawal et al., 1998; Hiom et al., 1998; Melek et al., 1998; Figure 1).

As full-length RAGs are largely insoluble, in vitro studies have employed minimal RAG-1 and RAG-2 regions (referred to as core RAGs) sufficient to direct rearrangement of extrachromosomal V(D)J substrates. However, noncore RAG regions are evolutionarily conserved and can influence both reaction efficiency and products generated as well as potentially serving important accessory and/or regulatory functions in chromosomal V(D)J recombination (Fugmann et al., 2000a; Sekiguchi et al., 2001). Therefore, future analyses, particularly those aimed at studying regulation, will likely attempt to incorporate full-length proteins.

There have been several promising reports of complete V(D)J recombination in vitro (Fugmann et al., 2000a); however, in the absence of follow-up studies, the relationship of these reactions to bona fide V(D)J recombination remains unclear. An in vitro NHEJ reaction has been shown to be dependent on known NHEJ proteins (Baumann and West, 1998); however, it has not yet been coupled to V(D)J recombination. Clearly, this remains a major challenge for the near future. Thus, most of what we know or speculate regarding the role of NHEJ in V(D)J recombination comes from known biochemical properties of NHEJ proteins and from characterization of reaction steps impaired in NHEJ-deficient cells and mice. Clearly, hair-pinned coding ends and blunt RS ends provide distinct substrates for the joining phase of the reaction. Sealed coding ends must be opened and further processed before joining, whereas the RS ends can be directly fused. Hairpin coding ends normally are opened at the apex or at points nearby, the latter resulting in overhanging flaps, which if incorporated into the join form P (palindromic) elements (Lewis,

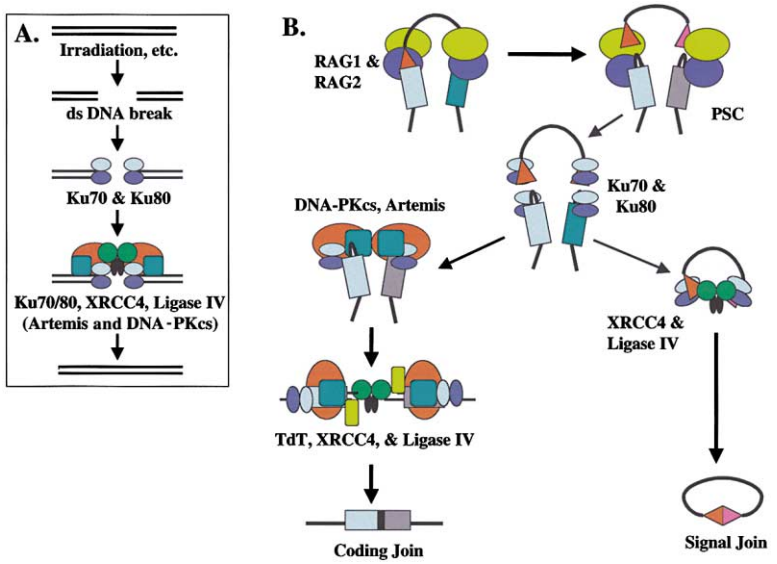


Figure 2. NHEJ Proteins Repair DSBs and Join RAG-Liberated Coding and Signal Ends

(A) NHEJ can repair DSBs via the concerted actions of the Ku proteins, DNA-PKcs, Artemis, XRCC4, and Ligase IV. Parentheses indicate different cell types may exhibit variable dependence upon Artemis and DNA-PKcs.
(B) The Ku proteins, XRCC4, and Ligase IV are required for both coding and RS joins, while DNA-PKcs and Artemis are more important for coding joins. The RAGs, in the context of the PSC, are also important for joining both coding and RS ends. See text and references for details.

Table 1. The Phenotypes of Mice Deficient in Proteins Required for V(D)J Recombination

| Genotype | Phenotypes | References |
|---|---|---|
| TdT deficiency | Normal B and T cell Development<br>Reduced junctional diversity (no N nucleotides) | Gilfillan et al., 1993; Komori et al., 1993 |
| RAG-1 or RAG-2 deficiency | Complete block in B and T cell development<br>No other phenotypes | Mombaerts et al., 1992; Shinkai et al., 1992 |
| DNA-PKcs deficiency (classical SCID) | Leaky block in B and T cell development (coding joining)<br>Ionizing radiation sensitivity (variable)<br>Normal size mice; no cell proliferation defects | Bosma and Carroll, 1991; Gao et al., 1998a; Taccioli et al., 1998; Kurimasa et al., 1999 |
| Ku70 or Ku80 deficiency | Leaky block in B and T cell development (RS and coding joining)<br>Increased IR sensitivity<br>Small size mice; cell proliferation defects<br>Increased neuronal apoptosis | Nussenzweig et al., 1996; Zhu et al., 1996; Gu et al., 1997, 2000; Ouyang et al., 1997 |
| XRCC4 or Ligase 4 deficiency | Block in B and T cell development (RS and Coding joining)<br>Increased IR sensitivity<br>Cell proliferation defects<br>Late embryonic lethality and servere neuronal apoptosis (rescued by p53 deficiency) | Barnes et al., 1998; Frank et al., 1998, 2000; Gao et al., 1998b |

The phenotypes of mice deficient in V(D)J recombinase components are listed in order of increasing severity. See text and references for details.

1994). The coding and hairpin-opening activity has been proposed to be carried out by RAG and/or Mre11 (Fugman et al., 2000a; Paull, 2001); although the recently discovered Artemis protein now appears to be the prime physiological candidate (Ma et al., 2002; see below). In addition, "N-region" nucleotides may be added de novo to joins by terminal deoxynucleotydl transferase (TdT; Table 1). Nucleotides are also deleted from junctional sequences; this, along with N-regions, comprises a major source of diversity beyond the germline-encoded V, D, and J repertoire. Coding joins can be promoted by microhomologies near the ends of the segments being joined; in this case, the formation of particular junctions ("canonical joins") is increased, and repertoire diversity is restricted. This effect is particularly notable in repertoires formed in the absence of TdT (e.g., fetal repertoires), the activity of which tends to obviate this restriction (Lewis, 1994; Table 1).

Analyses of the V(D)J recombination potential of ionizing radiation (IR)-sensitive SCID mouse and Chinese hamster ovary cells led to the discovery that the NHEJ proteins are employed by V(D)J recombination to repair RAG-initiated DSBs (Bosma and Caroll, 1991; Taccioli et al., 1993). Three such NHEJ proteins are subunits of the DNA-dependent protein kinase (DNA-PK), which consists of the Ku70 and Ku80 DNA binding subunits and a large catalytic subunit (DNA-PKcs) related to PI3 kinases (Khanna and Jackson, 2001). Activation of DNA-PKcs requires binding of the Ku70/Ku80 complex (Ku) to DSBs. Ku-deficient cells are severely impaired in both coding and RS joining, but DNA-PKcs-deficient cells are severely impaired only for coding joining (Table 1). These findings, plus the differential IR sensitivity of Ku- versus DNA-PKcs-deficient ES cells (Gao et al., 1998a), show that Ku functions independently of the DNA-PK holoenzyme. The structure of the Ku-DNA cocomplex (Walker et al., 2001) is consistent with previous notions that Ku may protect broken ends in yeast, synapse ends for joining, remodel ends, or recruit factors besides DNA-PKcs (Doherty and Jackson, 2001). The precise role of DNA-PKcs has been a mystery, as its in vivo kinase substrates were unknown; however, its function in NHEJ has been speculated to involve (directly or indirectly) end-processing, including hairpin opening, as suggested by the unusually large P elements in DNA-PKcs-deficient cells (Lewis, 1994). However, the identification of the Artemis protein (discussed below) appears to have provided a major insight into DNA-PKcs function. Artemis is related to proteins that repair interstrand DNA cross-links and, like DNA-PKcs, is required primarily for coding as opposed to RS joining (Moshous et al., 2001). In this context, in vitro studies have demonstrated that DNA-PKcs forms a complex with and phosphorylates Artemis, leading to the activation of an endonuclease activity that can cleave RAG-generated hairpins (Ma et al., 2002). Based on the in vitro data and on the Artemis-mutant phenotype, it has now been argued compellingly that Artemis is the only important hairpin-opening activity in V(D)J recombination (Ma et al., 2002), although this function still requires direct in vivo confirmation. The other known NHEJ proteins include XRCC4, isolated on the basis of its absolute requirement for NHEJ and V(D)J recombination (Li et al., 1995), and DNA Ligase 4 (Lig4), implicated based on its ability to form a complex with XRCC4 (Critchlow et al., 1997; Grawunder et al., 1997). XRCC4 or Lig4 deficiency results in essentially identical impairments, including blocked coding and RS joining, consistent with the ability of the Lig4/XRCC4 complex to catalyze this ligation step (Table 1). In this regard, other known mammalian ligases, including Lig1 or Lig3, normally do not substitute for Lig4 in V(D)J recombination (Grawunder et al., 1998).

There are knockout mice for all known V(D)J recombination factors except Artemis (Table 1). TdT deficiency does not cause overt immunodeficiency or lead to defects outside the immune system, although potential effects of restricted repertoires are under investigation. RAG-1- or RAG-2-deficient mice lack B or T lineage cells

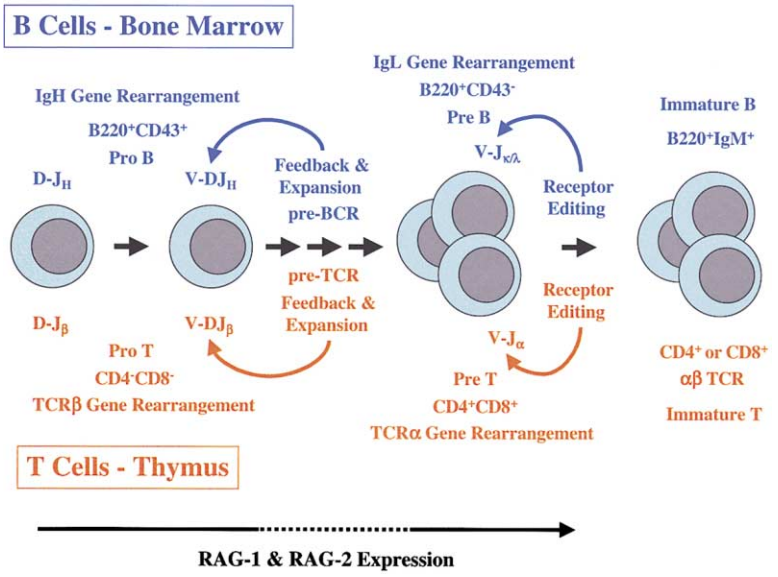


Figure 3. Parallels between Early B and T Cell Development

Developing B and αβ T cells share developmental strategies that include ordered gene rearrangement and feedback mechanisms that link antigen receptor protein expression from productive rearrangements to further developmental progression. See text and references for details.

beyond progenitors and thus have a severe combined immune deficiency (SCID). As V, D, and J segments are never cut, the RAG-deficient SCID is not "leaky." RAG-deficient mice have no other phenotypic defects, consistent with the highly specific role of RAGs in V(D)J recombination. DNA-PKcs-deficient mice (classical SCID mice) have a SCID that is leaky (Table 1). This is because RAGs cut V, D, and J segments, which can then be assembled at low efficiency into functional V(D)J joins by residual NHEJ or some other repair pathway. Other than variable cellular IR sensitivity, DNA-PKcs-deficient mice have no other consistent phenotype. Ku-deficient mice also have a "leaky" SCID, probably for the same reason as DNA-PKcs-deficient mice (Table 1). However, in accord with the idea that Ku functions independently of DNA-PK, Ku-deficient mice also are small and have increased apoptosis of newly generated neurons; their cells show growth defects, premature senescence, and consistent IR sensitivity (Table 1). XRCC4 and Lig4 deficiency leads to embryonic lethality accompanied by a major increase in neuronal apoptosis (Table 1). It is unclear whether the apoptosis, which is more severe than that in Ku-deficient embryos, causes death. In addition to cellular defects analogous to those of Ku-deficient mice, XRCC4- and Lig4-deficient mice have a complete SCID. In fact, their neuronal death and embryonic lethality, but not V(D)J recombination defects, are fully rescued by p53 deficiency, distinguishing the role of NHEJ in neuronal development from that in V(D)J recombination (Frank et al., 2000; Gao et al., 2000). Resulting XRCC4/p53 or Lig4/p53 double mutant mice appear identical to Ku/p53 double mutant mice (Difilippantonio et al., 2000) in that they are small, retain their SCID, and are prone to pro-B cell lymphomas. Thus, the major differences in Ku versus XRCC4- or Lig4-deficient phenotypes are likely to be quantitative, perhaps due to the greater "leakiness" in NHEJ and, potentially, a lower level of apoptotic cell death in Ku-deficient mice.

## Parallels between B Cell and α/β T Cell Differentiation

Developing B and α/β T lymphocytes share certain developmental strategies which include ordered gene rearrangement and feedback mechanisms that link antigen receptor protein expression from productive rearrangements to further developmental progression (Figure 3; Rajewsky, 1996; Gorman and Alt, 1998; Kisielow and von Boehmer, 1995).

In developing B cells, IgH variable region exons are assembled before those of IgL genes, while in α/β T cells, TCRβ variable region exons are assembled before those of TCRα genes. Even at the level of individual gene segments, IgH and TCRβ genes are assembled via an ordered process: D-to-J rearrangements usually occur first, followed by appendage of a V segment to a preexisting DJ complex. Due to random junctional diversification, only about 1 of 3 V(D)J rearrangements are in frame and, thus, are productive. Progenitor cells that first make nonproductive rearrangements go on to rearrange their second allele; thus, the majority of differentiating lymphocytes achieves productive rearrangements. Productive V(D)J rearrangements generate IgH μ or TCRβ chains, which, respectively, associate with surrogate LC (λ5/v-pre-B) or surrogate TCRα chains (pre-Tα) to form "prereceptor" complexes. The generation of prereceptor complexes in $B220^+/CD43^+$ progenitor B cells and $CD4^-CD8^-$ (DN) progenitor T cells signals expansion and differentiation, respectively, to the $B220^+/CD43^-$ pre-B cell stage and the $CD4^+/CD8^+$ (double positive, DP) thymocyte stage. The progenitor to precursor transition also is accompanied by cessation of further IgH or TCRβ gene rearrangement, effecting allelic exclusion, and by activation of IgL or TCRα gene rearrangement.

Functional rearrangement and expression of TCRα or IgL chains allows precursor B or T lymphocytes to develop into the immature lymphocytes that express IgM or α/β surface receptors. For the Igκ and Igλ loci, allelic exclusion also is effected via feedback regulation following the production of a functional IgL protein (Gorman and Alt, 1998), although receptor editing (see below) adds a further complexity. On the other hand, TCRα gene rearrangement is not allelically excluded. Ultimately, downregulation of RAG expression in most mature lymphocytes prohibits further V(D)J rearrange-

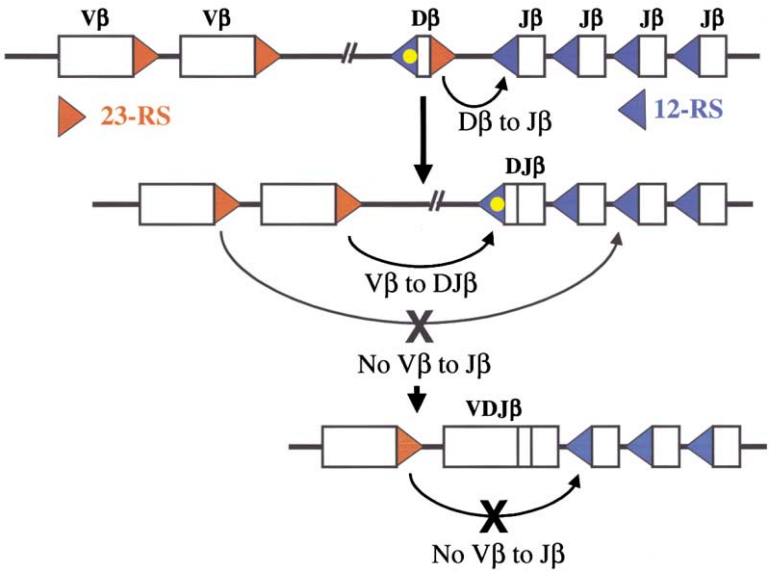


Figure 4. The 5′Dβ1 12-RS Targets Vβ Rearrangement beyond Simple 12/23 RS Compatibility

Despite 12/23 compatibility, direct Vβ-to-Jβ rearrangements seldom occur. Thus, the 5′D12-RS (blue with a yellow dot), but not the Jβ12-RSs (blue), efficiently targets the various Vβ 23-RSs (red). See text and references for details.

ments. Finally, Ig loci are subject to additional genomic alterations in the periphery including somatic hypermutation (Jacobs and Bross, 2001) and IgH class switch recombination (Manis et al., 2002).

## RSs Direct V(D)J Recombination beyond 12/23 Restriction

The 12/23 restriction helps ensure proper assembly of Ig and TCR variable region genes. For example, wasteful V-to-V and J-to-J rearrangements are prevented, as all V and J segments within a given locus are flanked by RSs of the same spacer length. Within the IgH locus, $V_H$s are flanked with 23-RSs, $D_H$s with 5′ and 3′ 12-RSs, and $J_H$s with 23-RSs. Since direct $V_H$-to-$J_H$ rearrangement is prohibited, the IgH RS structure ensures $D_H$ utilization and, consequently, enhances IgH repertoire diversification. V(D)J recombination substrate studies demonstrated that reaction efficiency is influenced by RSs and adjacent coding sequences (Lewis, 1994; Hesslein and Schatz, 2001). Such influences could exert an effect at a number of levels including RAG binding, synapsis, coupled cleavage, and/or resolution of coding ends. Therefore, RS and coding sequence effects could have important consequences, such as influencing rearrangement frequency of particular Vs, Ds, or Js (Lewis, 1994; Livak et al., 2000).

Gene targeted mutation of the TCRβ locus indicated that RS differences can influence physiological V(D)J recombination in a much more specific manner than previously anticipated. Vβs are flanked with 23-RSs and Jβs with 12-RSs, while Dβs have 5′12-RSs and 3′23-RSs (Figure 4). Yet, despite 12/23 compatibility, Vβs do not commonly rearrange directly to the Jβs. Resolution of this paradox came from findings that the 5′Dβ1 12-RS, but not the Jβ 12-RSs, specifically targets rearrangement of a diverse Vβ repertoire in a precise and position-independent manner (Bassing et al., 2000; Sleckman et al., 2000). The molecular basis for this "beyond 12/23 restriction" (B12/23 restriction) is unknown. Sequence determinants could provide *cis*-acting physical constraints that promote synapsis between Vβ and 5′Dβ RSs and 3′Dβ and Jβ RSs, but not between Vβ and Jβ RSs. Alternatively, sequence determinants may serve as binding sites for generally expressed and/or lymphoid-specific *trans* factors that drive synapsis of only particular 12/23 RS pairs. Whatever the mechanism, B12/23 RS restriction of V(D)J recombination has potentially important implications for regulation of variable region gene assembly and repertoire development. However, neither 12/23 nor B12/23 restrictions, per se, can fully explain ordered rearrangement or feedback regulation, nor can they fully account for tissue-, lineage-, and developmental stage-specific Ig and TCR gene rearrangement (Sleckman et al., 2000).

## RAG Expression and Accessibility Regulation

Lymphocyte-specific RAG expression limits V(D)J recombination activity to developing lymphocytes and, within developing lymphocytes, to nonproliferating compartments (Hesslein and Schatz, 2001; Figure 3). However, controlled RAG expression, alone, also cannot explain regulation of V(D)J recombination at endogenous loci. For example, even though RAG is downregulated in the expansion phase following IgH or TCRβ gene expression in pro-B or pro-T cells, it must be reactivated for IgL or TCRα gene assembly without further rearranging IgH and TCRβ loci to ensure allelic exclusion. Thus, regulation of antigen receptor gene assembly must be achieved by modulating accessibility of chromosomal Vs, Ds, and Js to the common V(D)J recombinase (Yancopoulos and Alt, 1985; Stanhope-Baker et al., 1996).

Receptor editing is a process by which B and T lineage cells undergo secondary rearrangement of their IgL or TCRα genes, without further IgH or TCRβ rearrangement (Nemazee and Weigert, 2000). The genomic organization of Igκ and TCRα loci permit successive V-to-J rearrangements, supporting the notion that editing evolved as a mechanism to change the specificity of self-reactive B and self-reactive or nonselected T cells. Editing is possible because of continued or induced RAG expression in newly generated B or T lymphocytes (Nemazee and Weigert, 2000). In this context, receptor

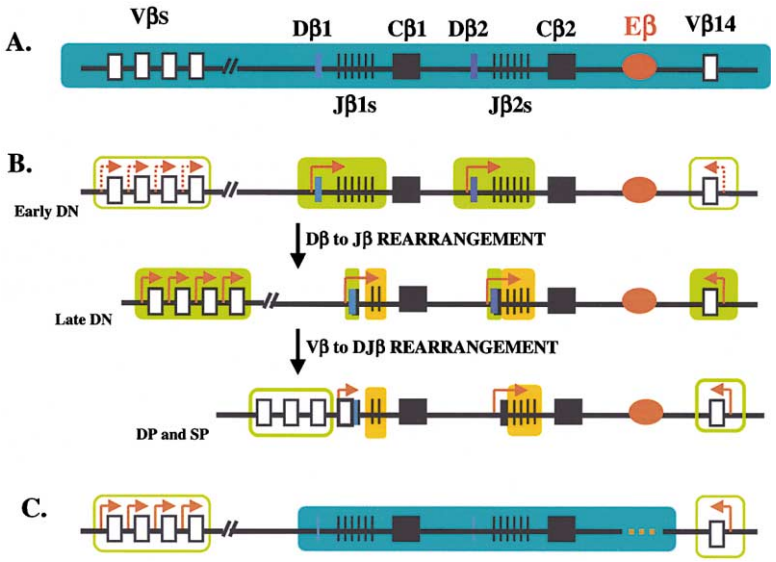


Figure 5. Developmental Regulation of TCRβ Locus Accessibility

(A) In non-T cells, Eβ is inactive and all TCRβ gene segments are inaccessible (shaded blue).

(B) In early DN thymocytes, Eβ directs recombinational accessibility (shaded green) and transcription of the Dβ and Jβ genes (red arrows). In this stage, the status of the Vβ genes, with regard to recombinational accessibility (open green) and transcriptional activity (broken red arrows), is uncertain. In late DN thymocytes, the Vβs are accessible and transcribed; thus, Vβ-to-DJβ rearrangement occurs as the 5′Dβ 12-RSs are also accessible. Direct Vβ-to-Jβ rearrangement is B12/23 restricted (shaded orange). In DP and SP thymocytes, only TCRβ VDJ or DJ rearrangements, and not germline Vβs, are clearly transcribed. Further Vβ rearrangements do not occur, as the remaining 5′Dβ 12-RSs, and possibly the germline Vβ genes, are not accessible due to allelic exclusion.

(C) In Eβ-deleted DN thymocytes, the Dβ and Jβ genes are not accessible and also not transcribed. The Vβ genes are actively transcribed via Eβ-independent elements; however, it remains to be established whether they are recombinationally accessible. See text for references and details.

editing poses unique issues related to accessibility regulation. Thus, accessibility must operate in this context to direct RAG activity to the appropriate rearranged loci and, thus, ensure allelic exclusion (Constantinescu and Schlissel, 1997). RAG expression in splenic B cells suggested that induced RAG expression functions to promote secondary rearrangement in mature B cells (Han et al., 1996; Hikida et al., 1996). Such secondary rearrangements might occur in a subset of splenic B cells following antigenic stimulation and facilitate whole-scale "mutation" of the IgL V regions to allow more ready selection for a highly specific antibody via "receptor revision" (Nemazee and Weigert, 2000). However, the notion of peripheral RAG reinduction has been questioned based on findings that splenic B cells of reporter mice failed to undergo RAG induction (Yu et al., 1999). In fact, most RAG-expressing spleen cells are pre- and pro-B cells, of unknown significance, that likely come from the bone marrow (Monroe et al., 1999).

### Transcriptional Control Elements Direct Recombinational Accessibility in *cis*

Transcriptional activity was the first correlate of V(D)J recombinational accessibility. Subsequently, transgenic substrate studies and targeted mutation of endogenous loci proved that transcriptional enhancers and promoters generate tissue-, lineage-, and developmental stage-specific V(D)J recombinational accessibility (Sleckman et al., 1996; Krangel et al., 1998). Thus, deletion of Ig or TCR locus enhancers resulted either in a complete block in all rearrangement events, a selective block in one rearrangement step, or a reduction in all rearrangement events within the targeted locus. In the latter context, enhancer redundancies and/or additional *cis* elements contribute in some loci to the establishment of an accessible locus (Gorman and Alt, 1998). Targeted mutations also showed that enhancers mediate recombinational accessibility of multiple gene segments, some of which may be located at large distances (80 kb or more) from the enhancer (Sleckman et al., 1997). However, potential enhancer functions that direct V segment accessibility remain elusive, as inhibitory effects of enhancer deletions on recombination could be due to a bona fide decrease in V segment recombinational accessibility or merely reflect (D)J inaccessibility (Figure 5). Mutation of endogenous germline D and J promoters affects recombinational accessibility, but only of proximal flanking segment(s) (Villey et al., 1996; Whitehurst et al., 1999). Although germline V promoters have been implicated in accessibility control (Baker et al., 1998), this has not yet been confirmed by targeted mutation of endogenous loci.

Analysis of transfected and/or transgenic recombination substrates suggested that additional *cis* elements, including matrix attachment regions (MARs), locus control regions (LCRs), silencers, and insulators may influence recombinational accessibility (Hesslein and Schatz, 2001). However, targeted deletion of endogenous elements has raised additional questions. For example, enhancer-associated MARs are required for high-level, position-independent transcription of rearranged Igκ and IgH transgenes (Scheuermann and Garrard, 1999), yet deletion of these MARs had no obvious effect on rearrangement or transcription of endogenous Igk or IgH loci (Sakai et al., 1999; Yi et al. 1999). In this regard, traditional transgenic studies might reveal activities for certain elements not observed via "knockouts" due to redundancies within endogenous loci; on the other hand, transgenes could yield misleading results (for example, via artifacts associated with composition or integration site). Likewise, simple knockouts of one element may obviate the possibility of assaying contributory activities of elements activated downstream from the first. Firm conclusions regarding the putative functions of various types of *cis* elements in the control of chromosomal V(D)J recombination eventually may be facilitated by using targeted mutation to tailor simplified endogenous loci in which specific properties of putative elements can be more readily tested.

## Molecular Mechanisms of Accessibility

In all endogenous loci, Vs rearrange to actively transcribed (D)Js, yet there is no unequivocal evidence that transcription itself is required for chromosomal V(D)J recombination. Thus, while transcriptional enhancers and promoters direct recombinational accessibility, they may do so via transcription-related mechanisms, recruitment of putative "accessibility factors" and/or RAG, or a combination of mechanisms. In addition, one study implicated an unexpected role for enhancers in the joining phase of V(D)J recombination (Hempel et al., 1998). Clearly, elucidation of cause and effect with respect to all molecular correlates of recombinational accessibility will require development of appropriate in vitro and in vivo systems.

For transcriptional enhancement, enhancers facilitate assembly of basal transcription machinery on promoters via mechanisms that involve general chromatin "opening" associated with histone acetylation, CpG demethylation, recruitment of transcriptional coactivators, and repositioning of promoter bound nucleosomes (Blackwood and Kadonaga, 1998). These processes likely are interdependent as, for example, transcriptional coactivators often contain histone acetylase activities. Many of these events are also correlates of V(D)J accessibility. For example, CpG demethylation was recognized as an attribute of V(D)J accessible loci in early studies, although, by itself, it is not sufficient and/or required for V(D)J recombination (Hesslein and Schatz, 2001). Enhancers direct developmental stage-specific acetylation of histones in chromatin over antigen receptor genes in a pattern that strongly correlates with V(D)J accessibility (McMurry and Krangel, 2000; Mathieu et al., 2000), but histone acetylation also may not be not sufficient to generate full accessibility (Senoo et al., 2001). Nucleosomal RS packaging in vitro inhibits V(D)J recombination and, in some instances, can be alleviated via histone acetylation and/or the actions of nucleosome-remodeling complexes (Kwon et al., 1998, 2000; Golding et al., 1999). However, it remains uncertain whether nucleosomes inhibit RAG access in vivo, as full-length RAG-2 contains a PHD domain that is conserved in many chromatin remodeling proteins (Hesslein and Schatz, 2001) and an activity, perhaps related to its PHD domain, that may direct endogenous $V_H$ access (Kirch et al., 1998). Finally, observed rearrangement patterns at some antigen receptor loci cannot be explained easily by simple domain-wide "open" chromatin structure. Thus, enhancer-mediated acetylation of histones in chromatin over the entire Jα cluster fails to account for the ordered progression of Jα utilization upon successive Vα gene rearrangements (Krangel et al., 1998). Theoretically, additional covalent histone modifications might direct V(D)J accessibility of distinct chromatin regions via other mechanisms; for example, covalent histone modification may comprise a "histone code" (Strahl and Allis, 2000) that could specify V(D)J recombinational accessibility.

The mechanisms that direct and stabilize synapsis of V and (D)J RSs located over large (1–2 Mb) chromosomal distances also remain to be determined. As random collision between RAG-bound RSs seems inefficient, mechanisms that actively couple RAG-accessible V and (D) RSs are likely (Hesslein and Schatz, 2001). Covalent modification of histone tails via acetylation, methylation, and phosphorylation provides docking sites for proteins, similar to the manner in which phosphotyrosine residues interact with SH2 domains (Strahl and Allis, 2000). In this context, (D)J promoter and/or enhancer-bound transcriptional coactivators could, theoretically, interact with the acetylated tails of histones in the chromatin over V segments to facilitate the juxtaposition of V and (D)J RSs and, thus, couple V gene rearrangement with active (D)J germline transcription. Furthermore, (D)J RS-bound RAG-2 might facilitate V segment rearrangement by interacting with V segment chromatin directed via its PHD domain.

## Ordered Rearrangement and Feedback Regulation

Despite intense investigation, the precise mechanisms responsible for ordered rearrangement and allelic exclusion remain elusive. IgH and TCRβ ordered rearrangement appears mediated via stage-specific D versus V accessibility, as D to J rearrangement occurs at an earlier developmental stage than V rearrangement (Alt et al., 1984; Tourigny et al., 1997). Specific deletion of the IgH intronic enhancer substantially blocked $V_H$-to-$DJ_H$ but not D-to-$J_H$ joining, indicating $D_H$ and $J_H$ accessibility may be mediated by elements distinct from the intronic enhancer, possibly the distal 3′ IgH regulatory region and/or $D_H$-proximal elements (Sakai et al., 1999). In contrast, deletion of Eβ results in a complete block of both Dβ-to-Jβ and Vβ-to-DJβ rearrangement (Bories et al., 1996; Bouvier et al., 1996; Figure 5). As Dβ-to-Jβ rearrangement, per se, is not required for Vβ-to-Dβ joining, neither assembly of a DJβ complex nor deletion of sequences between Dβ1 and the Jβs are necessary to promote Vβ rearrangement (Sleckman et al., 2000). Therefore, ordered TCRβ gene rearrangement may be effected via Eβ-independent transcriptional elements, such as germline Vβ promoters (Mathieu et al., 2000; Figure 5), that direct Vβ accessibility and are activated subsequent to germline Dβ promoters. Alternatively, Dβ-to-Jβ rearrangement may enhance Vβ rearrangement through deletion of the 3′Dβ 23-RSs that could theoretically be a significant higher affinity site for RAG than the Vβ RSs.

Allelic exclusion is an actively regulated process that, for the IgH locus, is maintained at levels sufficient to limit double expression to as little as 0.01% (Barreto and Cumano, 2000). For both the IgH and TCRβ loci, allelic exclusion occurs at the progenitor to precursor transition via feedback control of the V-to-DJ joining step (Alt et al., 1984; Uematsu et al., 1988). In this context, D-to-J joining appears unregulated as it occurs on both alleles. Thus, ordered assembly of IgH and TCRβ V, D, and J segments may be a mechanistic requirement for feedback regulation. In accord with this notion, TCRδ V(D)J joining is not ordered (Krangel et al., 1998) and not allelically excluded (Sleckman et al., 1998). In this context, TCRβ allelic exclusion could be mediated via a signal that modulates RAG access to the 5′Dβ1 (5′$DJ_H$) 12-RS, as opposed to influencing Vβ ($V_H$) accessibility. Allelic exclusion via feedback regulation must initially be enforced through a rapid intracellular signal initiated upon expression of prereceptor complexes or IgM. In

developing T cells, the signal(s) that mediate TCRβ feedback regulation appears distinct from those that direct rapid expansion and differentiation of pro-T cells (Gartner et al., 1999; Iritani et al., 1999).

A prerequisite for an efficient feedback mechanism is a process by which a cell could test for productive rearrangement of one allele before the next was rearranged (Alt et al., 1980). In this context, various asynchronous allelic rearrangement mechanisms have been proposed ranging from differential nuclear localization to asynchronous replication (Alt et al., 1992; Gorman and Alt, 1998). Notably, Igκ loci exhibit asynchronous replication and, in pre-B cells, the early replicated and demethylated Igκ allele preferentially rearranges first (Mostoslavsky et al., 1998, 2001). However, the majority of B lymphocytes make nonproductive rearrangements on their first attempt and then go on to rearrange their second allele (Rajewsky, 1996). Thus, in the context of such an asynchronous rearrangement mechanism, a major question would be how the second Igκ allele would be efficiently activated. In addition, such a mechanism would require additional attributes to extend to IgH and TCRβ loci, which undergo D-to-J rearrangements on both alleles. Recent findings also support differential nuclear localization of rearranging loci (Skok et al., 2001). In any case, it remains to be established whether feedback regulation serves to render the second allele inaccessible or prevent it from becoming accessible (Mostoslavsky et al., 2001). Finally, the molecular mechanisms that enforce IgH and TCRβ allelic exclusion may be distinct from those of Ig κ and λ loci, as rearrangement of the IgH and TCRβ genes must be actively repressed during IgL or TCRα gene assembly/editing.

## V(D)J Recombination in Disease

Defects in V(D)J recombination underlie a wide range of diseases ranging from immunodeficiencies and autoimmunity to cancer. Human SCIDs result either from defects in T cell development (70%–80% of SCIDs) or defects in both T and B cell development (20%–30% of SCIDs). The latter disease, $T^-B^-$ SCID, generally results from V(D)J recombination defects. Null mutations in RAG-1 or RAG-2 underlie approximately half of the human $T^-B^-$ SCIDs (Schwarz et al., 1996). Furthermore, hypomorphic mutations in RAG-1 or RAG-2 cause Omenn syndrome (Villa et al., 1998), a rare autosomal recessive SCID associated with an oligoclonal T cell expansion and symptoms suggestive of aberrant T cell-mediated cytokine production (Santagata et al., 2000). Thus, incomplete V(D)J recombination defects can affect the delicate balance between peripheral B and T lymphocytes. The remaining human $T^-B^-$ SCIDs have normal RAG genes, but their cells are defective in V(D)J recombination and exhibit increased IR sensitivity. This subset of $T^-B^-$ SCID (RS-SCID) is caused by mutation of the Artemis gene (Moshous et al., 2001). No human SCID has been shown to involve genes encoding any of the five previously identified NHEJ proteins (Notarangelo et al., 1999). This is particularly curious in the context of DNA-PKcs, as murine DNA-PKcs deficiency has a comparatively mild phenotype that is not dissimilar to Artemis deficiency in humans. In this context, however, recent studies have shown that Ku80 deficiency may have a much more severe phenotype in human versus murine cells (Li et al., 2002).

Aberrant V(D)J recombination can unleash oncogenic activities via chromosomal translocations involving antigen receptor loci. RAG-dependent translocations can arise from rare intermolecular V(D)J recombination events targeted by antigen receptor locus RSs and cryptic RSs on another chromosome (Raghavan et al., 2001). However, as many translocations occur in the absence of cryptic RSs on the second chromosome, RAG-dependent translocations also were proposed to arise via improper joining between RAG-liberated ends and random lesions on a second chromosome. Clearly, defects in the joining phase of V(D)J recombination can promote translocations involving RAG-initiated DSBs. Thus, on a p53-deficient background, mice deficient for Ku80, XRCC4, Lig4, or DNA-PKcs reproducibly developed pro-B cell lymphomas that, where analyzed, contained $J_H$ translocations to chromosome 15 in the general vicinity of *c-Myc* (Ferguson and Alt, 2001).

Cell cycle-regulated RAG-2 expression serves to restrict V(D)J recombination to G1 and ensures repair of RAG-liberated ends by NHEJ prior to reentry of the cell cycle (Lee and Desiderio, 1999). In this context, proteins such as ATM that monitor DNA damage and initiate the repair of DNA prior to continued cell cycle progression are critical. Although ATM, a serine/threonine kinase that is a master regulator of the DNA damage response, is not required for V(D)J recombination, ATM-deficient mice develop RAG-dependent thymic lymphomas with TCR translocations at a high frequency (Liao and Van Dyke, 1999). Mutations in the human genes encoding ATM, NBS1, and Mre11 cause, respectively, ataxia telangiectasia (AT), Nijmegen breakage syndrome (NBS), and ataxia telangiectasia-like disorder (ATLD). Each of these disorders is characterized by genomic instability and genetic predisposition to lymphoid malignancies with translocations (Khanna and Jackson, 2001). Finally, NBS1 and the H2AX histone, two proteins phosphorylated via ATM or ATM-related kinases, form RAG-dependent foci along the TCRα/δ locus during thymocyte development (Chen et al., 2000). This observation led to the proposal that these proteins may sense RAG-initiated DNA damage and generate signals that may suppress oncogenic translocations.

Translocations also have been proposed to occur via RAG-mediated transposition events, whereby a RAG-liberated RS end nonspecifically attacks another chromosome (Hiom et al., 1998; Figure 1). Evidence supporting this model is lacking, however, leading to various postulated mechanisms that could account for the suppression of RAG-mediated transposition events in vivo (Fugmann et al., 2000a; Melek and Gellert, 2000). In this context, it is important to note that current evidence for RAG-mediated transposition activities come from in vitro studies employing core RAGs. In NHEJ-deficient cells, core RAGs, but not wild-type RAGs, lead to accumulation of hybrid joins sealed on only one strand (Sekiguchi et al., 2001), analogous in structure to hybrid joins catalyzed by core RAGs in vitro via a reaction analogous to transposition (Melek et al., 1998; Figure 1). Thus, it is possible that the full-length RAGs may have evolved to suppress such potentially detrimental activities. If so,

certain RAG mutations theoretically could activate a potential to catalyze transpositions, especially in a NHEJ-deficient background.

**Acknowledgments**

We thank G. Rathbun, J. Sekiguchi, D. Jung, D. Ferguson, J. Chaudhuri, and R. Mostoslavsky for helpful discussions. C.H.B. is a Research Associate of the Howard Hughes Medical Institute. F.W.A. is an Investigator of the Howard Hughes Medical Institute. We apologize for not citing all relevant publications due to space constraints. However, we have referenced both recent and earlier in-depth reviews that provide these references.

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