Mechanism and Control of V(D)J Recombination versus Class Switch Recombination: Similarities and Differences

Darryll D. Dudley,[1] Jayanta Chaudhuri, Craig H. Bassing, and Frederick W. Alt

Howard Hughes Medical Institute, The Children's Hospital Boston, CBR Institute for Biomedical Research, and Harvard Medical School, Boston, Massachusetts 02115

Abstract 43
1. Overview: V(D)J and Class Switch Recombination 44
2. Antigen Receptor Gene Rearrangement 47
3. Regulation of V(D)J Recombination 61
4. Class Switch Recombination Employs Distinct Mechanisms for V(D)J Recombination 69
5. CSR-Related Diseases 93
6. Concluding Remarks 95
References 97

Abstract

V(D)J recombination is the process by which the variable region exons encoding the antigen recognition sites of receptors expressed on B and T lymphocytes are generated during early development via somatic assembly of component gene segments. In response to antigen, somatic hypermutation (SHM) and class switch recombination (CSR) induce further modifications of immunoglobulin genes in B cells. CSR changes the IgH constant region for an alternate set that confers distinct antibody effector functions. SHM introduces mutations, at a high rate, into variable region exons, ultimately allowing affinity maturation. All of these genomic alteration processes require tight regulatory control mechanisms, both to ensure development of a normal immune system and to prevent potentially oncogenic processes, such as translocations, caused by errors in the recombination/mutation processes. In this regard, transcription of substrate sequences plays a significant role in target specificity, and transcription is mechanistically coupled to CSR and SHM. However, there are many mechanistic differences in these reactions. V(D)J recombination proceeds via precise DNA cleavage initiated by the RAG proteins at short conserved signal sequences, whereas CSR and SHM are initiated over large target regions via activation-induced cytidine deaminase (AID)–mediated DNA deamination of transcribed

[1]Present address: Immunobiology and Cancer Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104.

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target DNA. Yet, new evidence suggests that AID cofactors may help provide an additional layer of specificity for both SHM and CSR. Whereas repair of RAG-induced double-strand breaks (DSBs) involves the general nonhomologous end-joining DNA repair pathway, and CSR also depends on at least some of these factors, CSR requires induction of certain general DSB response factors, whereas V(D)J recombination does not. In this review, we compare and contrast V(D)J recombination and CSR, with particular emphasis on the role of the initiating enzymes and DNA repair proteins in these processes.

1. Overview: V(D)J and Class Switch Recombination

The lymphoid arm of the vertebrate immune system has evolved to respond and protect against a diverse set of antigens constantly encountered by the host. Lymphocytes generate a nearly limitless diversity of antigen receptors via processes that direct somatic rearrangements and mutations into the germline DNA sequences of antigen receptor genes. Variable region exons of antigen receptors expressed on B and T lymphocytes are generated via somatic assembly of component variable (V), diversity (D), and joining (J) gene segments in a process called V(D)J recombination. As the usage of particular gene segments for a given locus is to a certain extent stochastic, this combinatorial joining process generates a highly diverse set of antigen receptors from a limited number of germline gene segments. B cells are capable of undergoing two additional forms of genetic alteration that enhance the ability of an antigen-specific B cell to recognize and respond to its cognate antigen. Somatic hypermutation (SHM) introduces a high rate of mutations into the germline DNA sequences of assembled immunoglobulin heavy (IgH) and light (IgL) chain variable region exons and allows the selection of B cells with receptors that have increased affinity for a given antigen. IgH class switch recombination (CSR) adjoins a rearranged variable region exon initially associated with the Igμ constant region (Cμ) exons to one of several downstream sets of C_H exons (referred to as C_H genes) through the deletion of intervening germline DNA sequences. This allows expression of an antibody with the same antigen-binding specificity but with altered C_H effector function.

Initiation of the V(D)J recombination reaction requires the products of recombination activating genes 1 and 2 (RAGs) (Oettinger *et al.*, 1990; Schatz *et al.*, 1989), which are expressed only in developing lymphocytes (Chun *et al.*, 1991; Mombaerts *et al.*, 1992). RAGs were identified by their ability to confer recombinational activity to a fibroblast cell line harboring a drug-selectable recombination substrate (Oettinger *et al.*, 1990; Schatz *et al.*, 1989). Deficiency in either RAG-1 or RAG-2 leads to a complete block in lymphocyte development at progenitor stages, the first stages at which V(D)J

recombination normally takes place (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992). RAGs introduce a DNA double-strand break (DSB) precisely between a variable region gene-coding segment and an associated recombination signal (RS) sequence (reviewed in Fugmann *et al.*, 2000a; Jung and Alt, 2004). Each RS is made up of conserved heptamer and nonamer sequences and an intervening spacer sequence that is either 12 or 23 bp in length. RAGs will mediate recombination only between antigen receptor gene segments that have RS spacer sequences of 12 and 23 bp, referred to as the 12/23 rule. RAG-induced DNA breaks are repaired by ubiquitously expressed nonhomologous end-joining (NHEJ) proteins, forming precise signal end joints (SJs) and imprecise coding end joints (CJs) (reviewed in Bassing *et al.*, 2002b; Jung and Alt, 2004).

Lymphoid-specific expression of RAGs limits V(D)J recombination to B and T lymphocytes (reviewed in Nagaoka *et al.*, 2000). However, to ensure that T cell receptor (TCR) genes are rearranged to completion only in T cells and that immunoglobulin genes are rearranged to completion only in B cells, the regulation of V(D)J recombination also involves the lineage-specific accessibility of gene segments (Yancopoulos and Alt, 1985). Such regulated accessibility of antigen receptor gene segments directs developmental stage-specific rearrangement. In developing B cells IgH genes are assembled before IgL genes, whereas in developing αβ T cells TCRβ genes are assembled before TCRα genes (reviewed in Willerford *et al.*, 1996). Regulated accessibility also likely contributes to the ordered rearrangement of IgH and TCRβ genes, wherein D-to-J rearrangements proceed to completion before the onset of V-to-DJ rearrangements (Alt *et al.*, 1984; Born *et al.*, 1985; Sleckman *et al.*, 2000). Recombinational accessibility correlates with transcriptional activity of a given antigen receptor locus, as eliminating transcriptional enhancers often ablates rearrangement of associated gene segments (reviewed in Bassing *et al.*, 2002b; Sleckman *et al.*, 1996).

CSR and SHM, unlike V(D)J recombination, are dependent on activation-induced cytidine deaminase (AID), a protein expressed only in activated germinal center B cells (Muramatsu *et al.*, 2000). Conversely, CSR and SHM do not require the presence of RAGs, as B cells derived by site-specific targeting of rearranged IgH and IgL transgenes into the corresponding endogenous loci of RAG-deficient mice undergo normal levels of CSR (Lansford *et al.*, 1998) and SHM (Zheng *et al.*, 1998). AID was identified via subtractive cloning of a cell line capable of switching from IgM to IgA on appropriate cellular stimulation (Muramatsu *et al.*, 1999). The absence of AID results in the loss of CSR and SHM in humans and mice and eliminates gene conversion in chickens, a process related to SHM that allows gene diversification in some animals (Arakawa *et al.*, 2002; Muramatsu *et al.*, 2000; Revy *et al.*, 2000). In addition, expression of AID in nonlymphoid cell lines induces CSR and

SHM of transfected substrates, implying that AID is the only lymphoid-specific factor necessary to effect these processes (Okazaki *et al.*, 2002; Yoshikawa *et al.*, 2002). Evidence demonstrates that AID deaminates cytidines of single-stranded DNA (ssDNA), thereby introducing DNA lesions that effect CSR and SHM (Bransteitter *et al.*, 2003; Chaudhuri *et al.*, 2003; Petersen-Mahrt *et al.*, 2002; Pham *et al.*, 2003; Sohail *et al.*, 2003; Yu *et al.*, 2004). Multiple DNA repair pathways including base excision repair (BER), mismatch repair (MMR), and NHEJ appear to be required for the processing and resolution of the AID-initiated DNA lesions during SHM and CSR (Chaudhuri and Alt, 2004; Petersen-Mahrt *et al.*, 2002). The NHEJ factors Ku and DNA-PKcs appear to be required for normal levels of CSR (Casellas *et al.*, 1998; Manis *et al.*, 1998a, 2002a) and may be involved in the resolution of DNA lesions, including DNA DSB intermediates induced by AID (Bross *et al.*, 2000; Chen *et al.*, 2001; Papavasiliou and Schatz, 2000; Wuerffel *et al.*, 1997).

In contrast to the site-specific RSs that target V(D)J recombination, CSR is targeted to large regions (1–12 kb) of repetitive DNA sequences, known as switch (S) regions, located upstream of all C_H genes except Cδ (which is regulated at the level of alternate RNA splicing) (Davis *et al.*, 1980; Honjo and Kataoka, 1978; Kataoka *et al.*, 1980). Likewise, SHM mutates nonconserved sequences of rearranged V_HDJ_H and V_LJ_L exons (reviewed in Harris *et al.*, 1999; Jacobs and Bross, 2001). CSR requires the transcription of S region target sequences, as disruption of specific S region transcriptional units eliminates CSR to the corresponding isotype (reviewed in Manis *et al.*, 2002b), whereas transcription has not been shown to be directly (i.e., mechanistically) involved in V(D)J recombination. In this regard, the transcriptional orientation of an S region is important, as inverted S regions are impaired in their ability to mediate CSR *in vivo* (Shinkura *et al.*, 2003), in accord with a direct role of transcription in the process of CSR, as opposed to V(D)J recombination, which clearly involves a different mechanism. Thus, although enhanced Vκ germline transcription *in vivo* enhances Vκ rearrangement (Casellas *et al.*, 2002), germline promoter location, rather than transcription through gene segments, may target gene segment accessibility via chromatin remodeling in a polymerase-independent manner (Sikes *et al.*, 1998).

The identification of RAG-1 and -2 was instrumental in elucidating the V(D)J recombination mechanism, which is now understood in some detail (Fugmann *et al.*, 2000a). Likewise, the identification of AID has led to rapid advances in our understanding of SHM and CSR mechanisms (reviewed in Honjo *et al.*, 2002; Kenter, 2003; Manis *et al.*, 2002b). This review compares and contrasts the targeting, initiation, and resolution of V(D)J recombination and CSR.

2. Antigen Receptor Gene Rearrangement

2.1. Genomic Organization of Murine Antigen Receptor Loci

The antigen receptor expressed on the surface of a B cell normally consists of four polypeptides that are made up of two identical IgH chains and two identical IgL chains, with IgL chains being derived from the rearrangement of either Igκ or Igλ genes (reviewed in Gorman and Alt, 1998). T cells express surface receptors made up of either αβ or γδ heterodimers (reviewed in Kisielow and von Boehmer, 1995). The assembly of the variable region exons of Igκ and Igλ in developing B cells, as well as the assembly of the variable region exons of TCRα and TCRγ in developing T cells, involves the rearrangement of V and J gene segments (reviewed in Bassing *et al.*, 2002b). In contrast, IgH, TCRβ, and TCRδ variable region exons are assembled from component V, D, and J gene segments, thus increasing the level of diversification of rearranged products (reviewed in Bassing *et al.*, 2002b). The variable region exons of all antigen receptors are then linked to constant region exons via RNA splicing and subsequently expressed at the cell surface (Fig. 1).

The murine IgH locus consists of some several hundred different V gene segments distributed throughout an approximate 1-Mb region beginning about

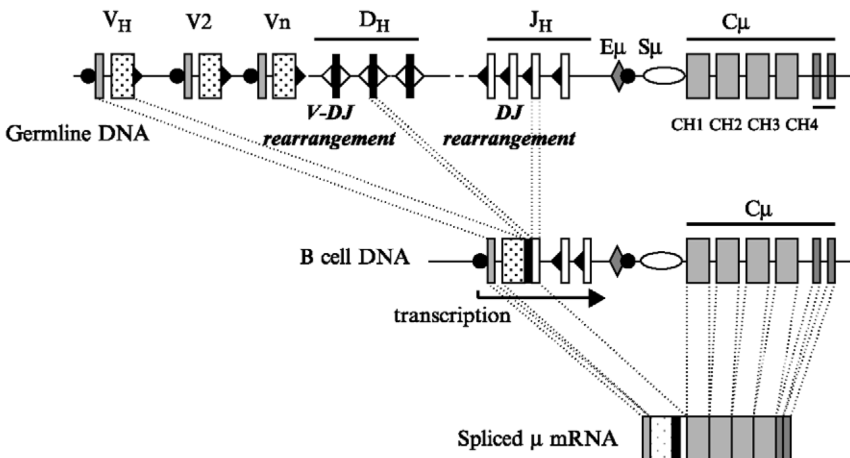

Figure 1 Schematic diagram of the murine IgH locus before and after V(D)J recombination. The V_H, D_H, and J_H gene segments are depicted as rectangles. The 12-bp RS sequences are shown as open triangles, and the 23-bp RS sequences as solid triangles. The μ constant region exons are shown as shaded rectangles, and the switch μ region as an oval. The position of the iEμ enhancer is indicated by a shaded diamond. The positions of the V_H and I exon promoters are shown as solid circles. Distances between the various elements are not drawn to scale.

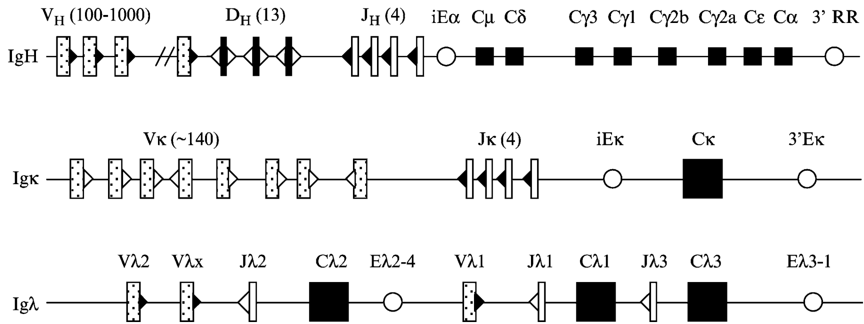

Figure 2 Schematic diagram of the murine B cell receptor loci. The V, D, and J gene segments are depicted as rectangles. The 12-bp RS sequences are shown as open triangles, and the 23-bp RS sequences as solid triangles. Only functional constant region exons are shown, represented by squares. The positions of various enhancer elements are indicated by circles. The estimated number of antigen receptor gene segments for the V_H and V_κ loci is indicated above each locus. Distances between the various elements are not drawn to scale. Adapted from Hesslein and Schatz (2001).

100 kb upstream of Cμ on chromosome 12 (reviewed in Honjo and Matsuda, 1995) (Fig. 2). Four J gene segments are positioned in a cluster about 7.5 kb upstream of Cμ, and 13 known D gene segments are dispersed between the V_H and J_H gene segments (reviewed in Hesslein and Schatz, 2001). The V_H gene segments are flanked at their 3′ ends with RSs containing 23-bp sequences (23-bp RS), as are the J_H gene segments at their 5′ ends (reviewed in Hesslein and Schatz, 2001). The D_H gene segments, on the other hand, are flanked on both sides by RSs with 12-bp spacer sequences (12-bp RS) (reviewed in Hesslein and Schatz, 2001). Thus the 12/23 rule prohibits direct V_H-to-J_H joining and ensures the usage of D_H gene segments during normal V(D)J rearrangement, augmenting junctional diversification.

The Igκ locus spans approximately 3 Mb of chromosome 6 and contains about 140 Vκ gene segments that can rearrange to 1 of 4 functional Jκ gene segments positioned just upstream of a single Cκ gene (reviewed in Gorman and Alt, 1998; Schable *et al*., 1999) (Fig. 2). There is also one nonfunctional Jκ gene segment (reviewed in Hesslein and Schatz, 2001). Unlike the IgH and Igλ loci, Vκ gene segments are found in both transcriptional orientations and thus allow for rearrangement by both deletion and inversion of intervening sequences (reviewed in Gorman and Alt, 1998). Vκ gene segments are flanked by 12-bp RSs, and Jκ segments by 23-bp RSs (reviewed in Gorman and Alt, 1998).

The Igλ locus in most mouse strains spans about 200 kb on chromosome 16 and has only three functional Vλ gene segments, each with a flanking 23-bp RS

(reviewed in Gorman and Alt, 1998; Selsing and Daitch, 1995; Fig. 2). There are three functional and one nonfunctional Cλ genes, each of which is associated with an upstream Jλ gene segment flanked by a 12-bp RS (reviewed in Gorman and Alt, 1998; Selsing and Daitch, 1995). Two of the Vλ gene segments are located upstream of all four Jλ–Cλ units whereas Vλ1 is positioned upstream of only the two 3′-most Jλ–Cλ units and is therefore restricted in potential rearrangements (reviewed in Gorman and Alt, 1998; Selsing and Daitch, 1995).

The TCRβ locus contains two Cβ genes, each associated with a single Dβ and six functional Jβ gene segments positioned upstream (reviewed in Glusman *et al*., 2001) (Fig. 3). The entire locus spans nearly 700 kb of mouse chromosome 6 (reviewed in Glusman *et al*., 2001; Fig. 3). The Jβ gene segments are associated with 12-bp RSs, whereas the Dβ segments are flanked on the 5′ side by 12-bp RSs and on the 3′ side by 23-bp RSs (reviewed in Hesslein and Schatz, 2001). There are about 34 Vβ gene segments flanked by 23-bp RSs located upstream of the DJβ clusters, 14 of which appear to be nonfunctional pseudogenes (reviewed in Hesslein and Schatz, 2001). There is also one Vβ segment, Vβ14, found 3′ of Cβ2 that rearranges by inversion (reviewed in Hesslein and Schatz, 2001). The gene segments and associated RSs of the TCRβ locus are organized in such a way that according to the 12/23 rule, direct Vβ-to-Jβ rearrangement should be allowed, yet such rearrangements do not normally occur (Bassing *et al*., 2000a; Davis and Bjorkman, 1988; Ferrier *et al*., 1990). Additional constraints, referred to as beyond 12/23 restriction, ensure that Dβ

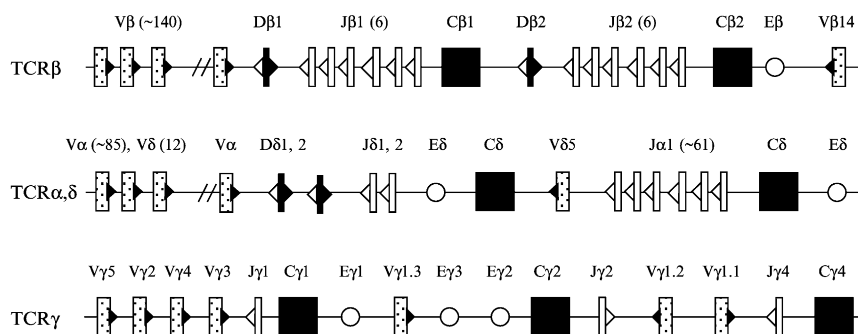

Figure 3 Schematic diagram of the murine T-cell receptor loci. The V, D, and J gene segments are depicted as rectangles. The 12-bp RS sequences are shown as open triangles, and the 23-bp RS sequences as solid triangles. Only functional constant region exons are shown, represented by solid squares. The positions of various enhancer elements are indicated by circles. The estimated number of antigen receptor gene segments for each locus is indicated above each locus. Distances between the various elements are not drawn to scale. Adapted from Hesslein and Schatz (2001).

gene segments are utilized during Vβ(D)Jβ rearrangement of the TCRβ locus and limit direct Vβ-to-Jβ joining (Bassing *et al.*, 2000a; Jung *et al.*, 2003; Sleckman *et al.*, 2000).

Both TCRα and TCRδ gene segments are spread throughout a region spanning more than 1.3 Mb of mouse chromosome 14 (reviewed in Glusman *et al.*, 2001; Fig. 3). The single Cδ, two Dδ, and two Jδ gene segments are positioned between the 3′-most Vα and 5′-most Jα segments, and thus are deleted after Vα-to-Jα rearrangement (reviewed in Hesslein and Schatz, 2001). There are more than 85 Vα and 12 Vδ gene segments, each adjoined by a 3′ 23-bp RS, some of which can function as either Vα or Vδ gene segments, located upstream of the Dδ segments (reviewed in Hesslein and Schatz, 2001). There is also one Vδ positioned 3′ of Cδ that has a promoter in the opposite transcriptional orientation and undergoes inversional recombination (reviewed in Hesslein and Schatz, 2001). Like the Dβ gene segments, the Dδ gene segments have 5′ 12-bp RSs and 3′ 23-bp RSs (reviewed in Hesslein and Schatz, 2001).

Furthermore, Jδ gene segments have 5′ 12-bp RSs that according to the 12/23 rule might allow for direct Vδ-to-Jδ joining, although, as with the TCRβ locus, this does not normally occur. At least 60 Jα gene segments are found upstream of a single Cα gene, each associated with a 5′ 12-bp RS (reviewed in Hesslein and Schatz, 2001).

The TCRγ locus is distributed across a region spanning approximately 200 kb of mouse chromosome 13 (reviewed in Glusman *et al.*, 2001; Fig. 3). There are seven Vγ gene segments and one Vγ pseudogene segment interspersed among three functional Jγ–Cγ units and one nonfunctional Jγ–Cγ unit (reviewed in Hesslein and Schatz, 2001). All gene segments are positioned in the same transcriptional orientation, with Vγ segments flanked by 23-bp RSs and Jγ gene segments flanked by 12-bp RSs (reviewed in Hesslein and Schatz, 2001).

2.2. Initiation of V(D)J Recombination

2.2.1. Recombinant-Activating Genes 1 and 2

RAGs were identified by transfecting cDNAs into a fibroblast cell line carrying the stable integration of a V(D)J recombination substrate that can confer drug resistance on successful completion of an RS-directed rearrangement (Schatz and Baltimore, 1988). RAG-1 and RAG-2 are each encoded within a single coding exon, and the RAG genes are located within 20 kb of one another in the opposite transcriptional orientation (Oettinger *et al.*, 1990). The close proximity of the two RAG genes, the lack of introns in their coding sequences, and

their inverted orientation led to the hypothesis that the RAGs were once part of a transposable element that integrated into the vertebrate genome (Agrawal *et al*., 1998; Lewis and Wu, 1997; Spanopoulou *et al*., 1996; Thompson, 1995; van Gent *et al*., 1996a). In support of this theory, RAGs have been shown to carry out transposition of target sequences *in vitro* (Agrawal *et al*., 1998; Hiom *et al*., 1998) and have been implicated in mediating translocations that occur *in vivo* (Messier *et al*., 2003; Zhu *et al*., 2002).

Null mutations in RAGs cause a severe combined immune deficiency (SCID) in humans (Schwarz *et al*., 1996) and mice (Mombaerts *et al*., 1992; Shinkai *et al*., 1992) caused by a complete block in B-and T-cell development. The block in lymphocyte development occurs at the B and T progenitor stages (Mombaerts *et al*., 1992; Shinkai *et al*., 1992), the stages at which B cells normally rearrange IgH genes and T cells rearrange TCRβ, γ, and δ genes (reviewed in Fehling *et al*., 1999; Willerford *et al*., 1996). Furthermore, mutations that lead to partial RAG activity in humans cause Omenn syndrome (Villa *et al*., 1998, 1999; Wada *et al*., 2000), an SCID disorder characterized by hepatosplenomegaly, lymphadenopathy, eosinophilia, elevated IgE, lack of circulating B cells, and a variable number of oligoclonal T cells (reviewed in Notarangelo *et al*., 1999; Villa *et al*., 2001).

Aberrant RAG activity has been implicated in translocations between immunoglobulin or TCR and oncogenes such as c-Myc, Bcl-2, and Bcl-6 among human T and B lineage lymphomas (reviewed by Mills *et al*., 2003; Roth, 2003). Some such translocations may involve interchromosomal V(D)J recombination involving cryptic RSs in the oncogene loci; whereas others may involve aberrant joining of RAG-initiated DSBs at antigen receptor loci to general DSBs on other chromosomes. Clear evidence for the latter process has come from studies of mouse pro-B lymphomas that arise in an NHEJ- and p53-deficient background (Difilippantonio *et al*., 2002; Guidos *et al*., 1996; Zhu *et al*., 2002). In addition, work has suggested that RAGs initiate translocations by introducing ssDNA nicks, which can be converted to DSBs, at cryptic RS or other non-B form DNA structures at various chromosomal locations, such as around the major breakpoint cluster region of human Bcl-2, and, thereby, initiate translocations (Lee *et al*., 2004; Raghavan and Lieber, 2004; Raghavan *et al*., 2004).

2.2.2. RAGs Recognize Site-Specific Target Sequences

RAGs recognize and bind to site-specific RSs positioned adjacent to all antigen receptor gene-coding segments (reviewed in Tonegawa, 1983). Each RS consists of a conserved 7-bp sequence (heptamer; consensus, 5′-CACAGTG), a conserved 9-bp sequence (nonamer; consensus, 5′-ACAAAAACC), and an intervening, relatively nonconserved 12 ± 1 or 23 ± 1 bp spacer sequence

(Early *et al.*, 1980; Hesse *et al.*, 1989; Max *et al.*, 1979; Sakano *et al.*, 1979). Although overall highly conserved, there is variation between heptamer and nonamer sequences of individual RSs, with those most closely resembling the consensus sequences being the most efficiently rearranged (reviewed in Lewis, 1994a). Moreover, not all of the positions within the conserved heptamer and nonamer sequences appear to be important for RAG-mediated cleavage. Whereas changes in the first three nucleotide positions of the heptamer or in the sixth or seventh positions of the nonamer greatly reduce RAG-mediated cleavage of plasmid substrates, changes at other positions are better tolerated (Hesse *et al.*, 1989). The spacer sequences also play an essential role in V(D)J recombination, as RAG-mediated cleavage will occur only when an RS with a 12-bp spacer sequence is paired in complex with an RS with a 23-bp spacer sequence, a constraint referred to as the 12/23 rule (Eastman *et al.*, 1996; Sakano *et al.*, 1981; van Gent *et al.*, 1996b). The 12/23 rule appears to be enforced at the level of binding and assembly of RAGs to paired RSs (Hiom and Gellert, 1998; Mundy *et al.*, 2002) as well as the subsequent cleavage step (West and Lieber, 1998; Yu and Lieber, 2000). Although much less conservation exists in RS spacer sequences compared with heptamer and nonamer sequences, these sequences have also been shown to influence RAG-mediated cleavage and RS usage (Jung *et al.*, 2003; Nadel *et al.*, 1998). As described above, like gene segments (e.g., all V segments) for any given antigen receptor locus are each associated with RSs with the same length spacer sequences, and thus the 12/23 rule prevents nonproductive V-to-V or J-to-J joining.

The configuration of the heavy chain locus ensures that D gene segments flanked with 12-bp RSs will be utilized in all successful V_HDJ_H rearrangements, as V_H and J_H gene segments all have 23-bp RSs (Fig. 2). In contrast, the configuration of the TCRβ locus, with 23-bp RSs flanking the Vβs and 12-bp RSs flanking the Jβs, should allow direct Vβ-to-Jβ rearrangement according to the 12/23 rule, yet this rarely occurs *in vivo* (Bassing *et al.*, 2000; Sleckman *et al.*, 2000; Wu *et al.*, 2003) (Fig. 3). Even when Dβ1 was deleted on both alleles in mice, Vβ-to-Jβ1 rearrangements rarely took place and subsequent αβ T-cell development was severely impaired (Bassing *et al.*, 2000). Several studies have shown that this so-called beyond 12/23 restriction is enforced at the level of specific RSs (Bassing *et al.*, 2000; Jung *et al.*, 2003; Tillman *et al.*, 2003). Indeed, when a Vβ 23-bp RS was replaced by the 3′ Dβ1 23-bp RS, the "beyond 12/23 restriction" was broken, and direct Vβ-to-Jβ rearrangement was detected (Wu *et al.*, 2003). The strength and efficiency with which the 3′ Dβ1 23-bp RS mediates rearrangement imply that this RS might contribute to ordered rearrangement in which Dβ1-to-Jβ rearrangement takes place before the onset of Vβ-to-DJβ rearrangement.

In a coupled cleavage reaction involving both 12- and 23-bp RSs, RAGs introduce DNA DSBs between the heptamers and flanking coding sequences, followed by subsequent ligation of the two blunt RS ends and two modified coding ends. Recombination that takes place between RSs found in the opposite chromosomal orientation will therefore result in the deletion of intervening DNA sequences in the form of covalently sealed DNA circles (Fujimoto and Yamagishi, 1987; Okazaki *et al*., 1987; Sakano *et al*., 1979). Subsequent rounds of cell division result in the permanent loss of these sequences from the genome (Kabat, 1972; Sakano *et al*., 1979; Tonegawa *et al*., 1977). On the other hand, recombination between RSs that are in the same chromosomal orientation leads to an inversion of intervening DNA sequences and retention of these sequences in the genome (Alt and Baltimore, 1982; Lewis *et al*., 1982; Malissen *et al*., 1986; Weichhold *et al*., 1990; Zachau, 1993). As the presence of an accessible RS is all that is necessary to render a piece of DNA susceptible to RAG-mediated cleavage, plasmid substrates have been engineered that retain either the RS or coding ends, allowing detailed analysis of each type of DNA junction (Hesse *et al*., 1987; Lewis *et al*., 1985).

2.2.3. Assembly of Precleavage Complex

In vitro, RAGs are found to cooperatively associate with 12- and 23-bp RSs and their flanking coding gene segments to form a synaptic complex (Bailin *et al*., 1999; Hiom and Gellert, 1997; Leu and Schatz, 1995). Contacts between RAG-1 and nonamer sequences are essential for RS binding, whereas interactions with the heptamer or coding sequences appear to help provide specificity to the RAG-binding complex and to promote efficient DNA cleavage (Difilippantonio *et al*., 1996; Roman and Baltimore, 1996). RAG-1 binding to nonamer sequences involves the region between residues 376 and 477 of RAG-1, with a GGRPR motif (amino acids 389–393 of murine RAG-1) that is also found in members of the bacterial DNA invertase family forming the main site of interaction (Difilippantonio *et al*., 1996; Spanopoulou *et al*., 1996). Independently, RAG-1 binds only weakly to heptamer sequences; however, the presence of RAG-2 has been shown to help stabilize this interaction (Aidinis *et al*., 2000; Akamatsu and Oettinger, 1998; Fugmann and Schatz, 2001; Spanopoulou *et al*., 1996; Swanson and Desiderio, 1999). The region of RAG-1 that makes contact with the heptamer has been mapped to residues 528–760 and also appears to contain the main site of RAG-2 interaction (Arbuckle *et al*., 2001; Peak *et al*., 2003). Although the RAG-2 protein does not bind DNA independently, RAG-2 does make contact with the RS heptamer sequence when in a complex with RAG-1 (Difilippantonio *et al*., 1996; Spanopoulou *et al*., 1996; Swanson and Desiderio, 1999).

Synaptic complex assembly begins *in vitro* with the binding of RAGs to a single 12-bp RS referred to as a single complex (SC), followed by integration of the companion 23-bp RS target DNA into a paired complex (PC) (Jones and Gellert, 2002; Mundy *et al.*, 2002; Swanson, 2002b). The DNA-bending proteins HMG1 and HMG2 facilitate the integration of the 23-bp RS and assembly of the SC (Rodgers *et al.*, 1999; Swanson, 2002a) and appear to promote RAG-mediated cleavage (Swanson, 2002a; van Gent *et al.*, 1997). The coordinated assembly of the PC and subsequent coupled cleavage requires the presence of Mg^{2+} divalent cation, whereas *in vitro* the presence of Mn^{2+} allows cleavage to take place on single RS-containing substrates (van Gent *et al.*, 1996b). By replacing Mn^{2+} or Mg^{2+} divalent cations with Ca^{2+} *in vitro*, DNA cleavage by RAGs is blocked and the SC consisting of RAGs bound to a single RS can be isolated as an intermediate of the reaction (Hiom and Gellert, 1997). This made it possible to detect two distinct complexes that form on a single RS, single complex 1 (SC1) and single complex 2 (SC2) (Mundy *et al.*, 2002; Swanson, 2002b). The number of RAG-1 subunits in the SC1, SC2, and PC appears to be the same, although whether there are two (Swanson, 2002b) or more (Mundy *et al.*, 2002) molecules of RAG-1 per complex is still not clear. Studies have consistently found that the slower migrating SC1 contains two subunits of RAG-2, whereas only a single subunit of RAG-2 exists in SC2 (Mundy *et al.*, 2002; Swanson, 2002b). However, crystallization of the complex may ultimately be required to unequivocally ascertain the stoichiometry.

2.2.4. Biochemistry of the Cleavage Reaction

After assembly of the PC, RAGs introduce a single-strand nick in the DNA between the border of the RS heptamer and the gene-coding segment in a coupled cleavage reaction that *in vivo* requires the presence of both a 12-bp RS and a 23-bp RS (McBlane *et al.*, 1995; van Gent *et al.*, 1996b). This creates a 3′-OH on one DNA strand of the gene-coding segment and a 5′-phosphate group on the corresponding RS-containing DNA strand (Fig. 4). The 3′-OH then acts as a nucleophile in attacking the opposite DNA strand in a *trans*-esterification reaction, forming a covalently sealed hairpin coding end and a blunt, 5′-phosphorylated RS end (McBlane *et al.*, 1995; Roth *et al.*, 1992) (Fig. 4). After cleavage, the DNA ends are held together in a postcleavage complex that includes the RAGs and all four DNA ends (Agrawal and Schatz, 1997; Hiom and Gellert, 1998; Jones and Gellert, 2001; Qiu *et al.*, 2001; Tsai *et al.*, 2002; Yarnell Schultz *et al.*, 2001).

Mutational studies have identified active catalytic residues in RAG-1 that when mutated result in defects in DNA nicking and hairpin formation, although these residues do not appear to be required for assembly of the PC (Fugmann *et al.*, 2000b; Kim *et al.*, 1999; Landree *et al.*, 1999). The three

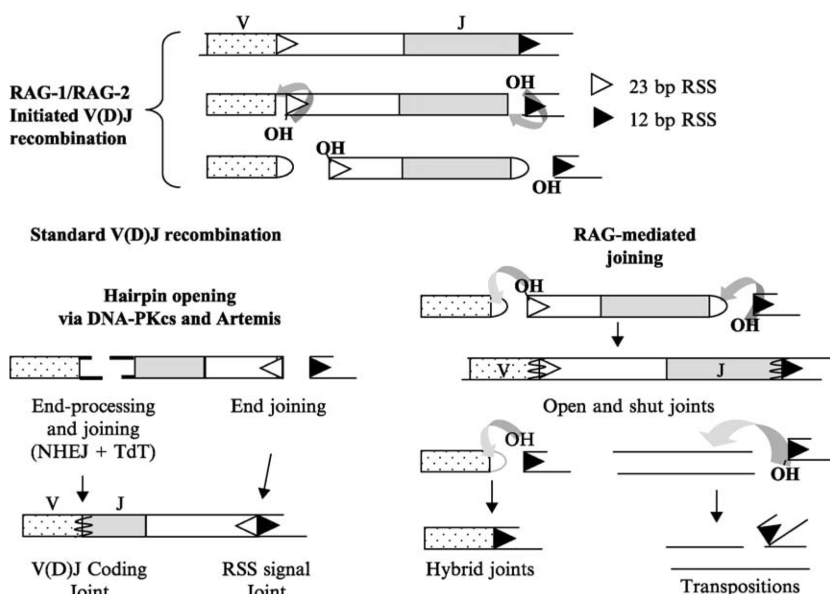

Figure 4 Biochemistry of V(D)J recombination. Standard V(D)J recombination results in the formation of precise signal joints and modified coding joints. Products of aberrant V(D)J recombination include hybrid joints, open and shut joints, and transposition events. The rectangles represent V, D, or J gene segments and the solid and open triangles represent 12- and 23-bp RSs, respectively. RAG cleavage and subsequent processing and joining via the NHEJ pathway leads to the standard V(D)J recombination products shown on the left. Hybrid joints can form when the 3′-OH of an RAG-liberated RS end attacks the hairpin-coding end of the partner gene segment in the coupled reaction, as shown in the center. RAG-mediated transposition of a liberated 3′-OH into an intact piece of double-stranded DNA is depicted on the right.

identified acidic residues (D600, D708, and E782) are all contained within the active core RAG-1 protein and likely constitute a DDE motif similar to that found in many integrase/transposase family proteins (reviewed in Haren *et al*., 1999). The DDE triad is thought to function in coordinating two divalent metal ions (Mg^{2+}) that facilitate the *trans*-esterification reaction, one acting as a general base and the other as a general acid (reviewed in Haren *et al*., 1999). The presence of the DDE motif is consistent with the theory that RAGs started out as components of a transposable element that integrated into the vertebrate genome (Agrawal *et al*., 1998; Spanopoulou *et al*., 1996; Thompson, 1995; van Gent *et al*., 1996a).

Full-length RAG proteins are relatively insoluble, and therefore elucidation of the biochemistry behind RAG-mediated V(D)J recombination has largely made use of highly truncated "core" RAG proteins (Kirch *et al*., 1996;

McBlane *et al.*, 1995; Sadofsky *et al.*, 1993, 1994; Sawchuk *et al.*, 1997). Although truncated core RAG proteins are capable of mediating complete V(D)J recombination *in vitro*, the core RAGs carry out the reaction at reduced efficiency both *in vitro* and *in vivo* (Akamatsu *et al.*, 2003; Dudley *et al.*, 2003; Kirch *et al.*, 1998; Liang *et al.*, 2002). In the absence of the C-terminal portion of RAG-2, V-to-DJ rearrangements appear more severely affected than D-to-J rearrangements, suggesting that the noncore region of RAG-2 may play a specific role during ordered rearrangement of IgH and TCR genes (Kirch *et al.*, 1998; Roman *et al.*, 1997).

In addition to normal CJs and SJs (see below), RAGs can mediate open and shut joints, hybrid joints (HJs), and transpositions both *in vitro* and *in vivo* (Fig. 4) (Agrawal *et al.*, 1998; Lewis *et al.*, 1988; Messier *et al.*, 2003; Morzycka-Wroblewska *et al.*, 1988; Sekiguchi *et al.*, 2001). An HJ is defined as the joining of the liberated RS end from one coding segment to the partner hairpin-coding end participating in the recombination reaction (Fig. 4) (Lewis *et al.*, 1988). A transposition event is similar to a hybrid joint, with insertion of the liberated RS end into a double-stranded DNA target sequence instead of joining with an RAG-generated coding end (Fig. 4) (Agrawal *et al.*, 1998; Hiom *et al.*, 1998). The truncated core RAGs mediate an increased rate of HJs in NHEJ-deficient cells compared with full-length RAGs, suggesting that the noncore regions normally function to suppress such aberrant joining events (Sekiguchi *et al.*, 2001). Furthermore, there is an increase in the frequency of transposition events in core RAG-2–expressing cells compared with controls (Elkin *et al.*, 2003; Tsai and Schatz, 2003), which form by a similar mechanism as that of hybrid joints. Taken together, these studies imply that the noncore regions of RAGs may have evolved to ensure that RAG-liberated DNA ends are properly joined, thus preventing transposition and other deleterious or ineffective recombination reactions (Agrawal and Schatz, 1997; Elkin *et al.*, 2003; Hiom *et al.*, 1998; Messier *et al.*, 2003; Sekiguchi *et al.*, 2001; Steen *et al.*, 1999; Tsai and Schatz, 2003).

2.2.5. Postcleavage Complex

After cleavage, the RAGs remain associated with the four DNA ends in a postcleavage complex, possibly playing a role in the protection of DNA ends from degradation, the juxtaposition of ends before rejoining, or recruitment and activation of end-joining factors for both CJ and SJ formation (Fig. 5) (Agrawal and Schatz, 1997; Hiom and Gellert, 1998; Jones and Gellert, 2001; Qiu *et al.*, 2001; Tsai *et al.*, 2002; Yarnell Schultz *et al.*, 2001). Stability of the postcleavage complex may also function to inhibit DSB-induced cell cycle arrest and apoptosis, as well as to prevent potentially deleterious transposition events (Jones and Gellert, 2001; Perkins *et al.*, 2002). However, studies have

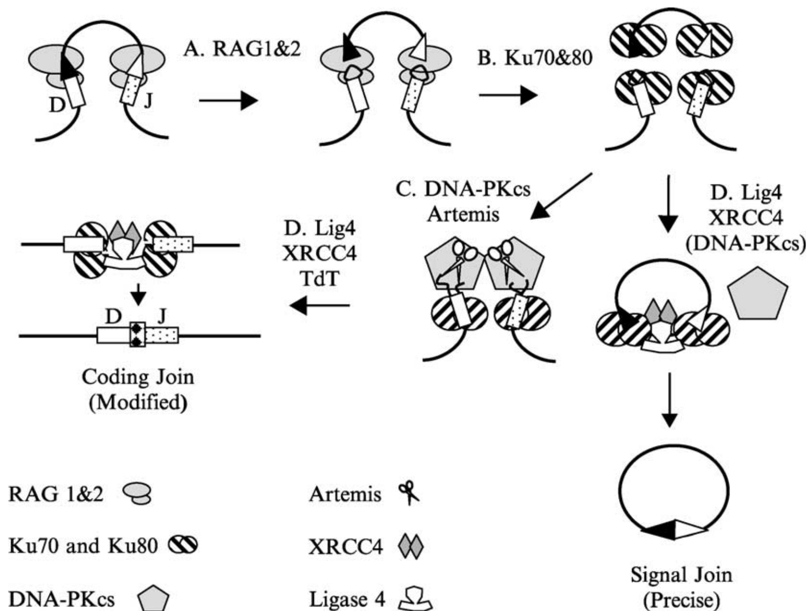

Figure 5 Joining of RAG-mediated DNA double-strand breaks. (A) RAG-1 and RAG-2 cleavage occurs between RS and coding segments. (B) Ku70 and Ku80 bind to the broken DNA ends. (C) DNA-PKcs and Artemis facilitate the opening and processing (opening) of covalently sealed hairpin coding ends. (D) TdT adds random nucleotides to opened coding ends. XRCC4 and Lig4 seal the blunt signal ends and processed coding ends to produce precise signal joints and modified coding joints. In addition, DNA-PKcs functions independently of Artemis to form normal signal joints.

shown that signal ends must be deproteinized before rejoining by NHEJ factors *in vitro* (Leu *et al*., 1997; Ramsden *et al*., 1997). In this regard, it was demonstrated that the N terminus of RAG-1 has E3 ubiquitin ligase activity (Yurchenko *et al*., 2003), suggesting a function for RAG-1 in steps beyond recognition and DNA cleavage. For instance, once the appropriate end-joining proteins have been recruited or have performed their function, RAG-1–mediated ubiquitination could tag RAG-2 or NHEJ proteins within the complex for proteasomal degradation, thus promoting disassembly of the complex and ligation of the DNA ends.

2.2.6. Coding and Signal Joint Formation

RAG-mediated cleavage generates hairpin-coding ends that must be opened and processed before rejoining, whereas the RS ends do not require any additional processing and are religated by NHEJ proteins to form precise

SJs (reviewed in Fugmann *et al*., 2000a; Fig. 5). Although studies have demonstrated that the RAGs themselves can mediate hairpin opening *in vitro* (Besmer *et al*., 1998; Ma *et al*., 2002; Shockett and Schatz, 1999), evidence has shown that the NHEJ protein Artemis, in association with DNA-PKcs, likely performs this role *in vivo* (Ma *et al*., 2002; Rooney *et al*., 2002, 2003) (Fig. 5). Hairpin-coding ends are opened via the introduction of a DNA nick, usually within four or five nucleotides 3′ of the apex of the hairpin (reviewed in Fugmann *et al*., 2000a; Lieber, 1991; Nadel *et al*., 1995). Once the hairpins are opened, the 3′ overhangs can be filled in via DNA polymerases, thus generating short stretches of palindromic sequences at the junctions of CJs, referred to as P nucleotides (Lafaille *et al*., 1989; Lewis, 1994b; reviewed in Lewis, 1994a; Lieber, 1991). Alternatively, nucleases can chew back the 3′ overhangs, resulting in a loss of germline nucleotides at the junction of CJs (reviewed in Fugmann *et al*., 2000a; Lieber, 1991; Nadel *et al*., 1995). To further diversify junctions, the lymphoid-specific protein terminal deoxynucleotidyltransferase (TdT) adds random, nontemplated nucleotides to 3′ coding ends and introduces so-called N-nucleotide additions, further increasing the diversity of antigen receptor variable regions (Alt and Baltimore, 1982). Moreover, a splice variant of TdT appears to function to remove nucleotides from coding junctions (Thai *et al*., 2002). Although TdT is not required for either V(D)J recombination or lymphocyte development, it does affect overall repertoire diversification (Gilfillan *et al*., 1993; Komori *et al*., 1993). Finally, DNA polymerase μ (polμ)–deficient mice have a significant reduction in the length of Vκ-to-Jκ junctions, suggesting polμ plays a role in maintaining CDR3 length of Igκ chains (Bertocci *et al*., 2003). It is still unclear whether polμ regulates the processing of coding ends by protecting them from exonucleolytic attack or by filling in 3′ overhangs (Bertocci *et al*., 2003). Notably, polμ shares homology with TdT.

2.3. Joining of RAG-Mediated DNA Double-Strand Breaks

DNA DSBs can be induced by a variety of agents including ionizing radiation (IR), oxidative stress incurred during normal cellular metabolism, and RAGs during V(D)J recombination. Mammalian cells have evolved two different pathways to repair such potentially catastrophic lesions. Homologous recombination (HR) is a high-fidelity process that repairs breaks, using a homologous chromosome as a DNA template (reviewed in Hoeijmakers, 2001). NHEJ repairs broken DNA ends in the absence of long stretches of homology, allowing both the loss and addition of nucleotides at the repair junction (reviewed in Khanna and Jackson, 2001). HR takes place predominantly in S and G_2 phases of the cell cycle, when homologous templates are both

available and in close proximity (reviewed in Hoeijmakers, 2001). Conversely, NHEJ appears to be the preferred repair pathway during the G_1 phase of the cell cycle, corresponding to the stage at which RAGs are both expressed and active for recombination (reviewed in Jackson, 2002; Lin and Desiderio, 1995). However, it is clear that NHEJ can function outside of the G_1 phase and complement the repair activities of HR (Couedel *et al*., 2004; Mills *et al*., 2004). Studies involving the transfection of recombination substrates into IR-sensitive cell lines implicated several members of ubiquitously expressed NHEJ proteins as having direct roles in V(D)J recombination (reviewed in Bassing *et al*., 2002b; Taccioli *et al*., 1993). Members of the NHEJ repair pathway known to be involved in V(D)J recombination include Ku70, Ku80, DNA-PKcs, XRCC4, ligase 4 (Lig4), and Artemis (reviewed in Mills *et al*., 2003; Rooney *et al*., 2004). Cells isolated from patients that are unable to complete RAG-initiated V(D)J recombination of transiently transfected plasmid substrates and exhibit IR sensitivity have implicated a seventh potential member of the NHEJ group, as genetic analyses have ruled out defects in Ku70, Ku80, DNA-PKcs, Artemis, XRCC4, or Lig4 (Dai *et al*., 2003).

2.3.1. Ku70 and Ku80

Ku70 and Ku80 form a heterodimer (Ku) that directly associates with DNA DSBs as well as telomeric regions of chromosomes (reviewed in Critchlow and Jackson, 1998). Ku-deficient cell lines are IR sensitive and defective in both CJ and SJ formation of transiently transfected recombination substrates, demonstrating that these proteins are essential for normal V(D)J recombination (Gu *et al*., 1997; Nussenzweig *et al*., 1996; Taccioli *et al*., 1993, 1994; Zhu *et al*., 1996). Potential functions for Ku during NHEJ include (1) the protection of DNA ends generated during V(D)J recombination from unwanted processing or degradation (Boulton and Jackson, 1996; de Vries *et al*., 1989; Getts and Stamato, 1994), (2) the juxtaposition of DNA ends produced by RAG cleavage before religation (Boulton and Jackson, 1996; Cary *et al*., 1997), and (3) the recruitment or activation of DNA repair or DNA damage-sensing proteins (Gottlieb and Jackson, 1993; Lieber *et al*., 1997; Ramsden and Gellert, 1998; West *et al*., 1998).

2.3.2. DNA-PKcs and Artemis

DNA-PKcs is a serine/threonine kinase and member of the phosphatidylinositol-3-kinase (PI-3 kinase) family that includes the DNA damage response proteins ataxia telangiectasia mutated (ATM) and ataxia telangiectasia related (ATR) (reviewed in Smith and Jackson, 1999). DNA-PKcs–deficient cell lines display varying degrees of IR sensitivity (Gao *et al*., 1998; Lees-Miller *et al*.,

1995; Taccioli *et al.*, 1998). Furthermore, cell lines derived from SCID mice, which harbor a mutation in DNA-PKcs (Blunt *et al.*, 1995), are severely impaired in their ability to form CJs, although SJ formation is relatively unaffected in these cells (Blackwell *et al.*, 1989; Lieber *et al.*, 1988; Malynn *et al.*, 1988). DNA-PKcs–deficient embryonic stem (ES) cells fail to make CJs but make fully normal SJs (Gao *et al.*, 1998); however, mouse embryonic fibroblasts from DNA-PKcs–deficient mice, which are also fully deficient for CJ formation, are also somewhat impaired in SJ formation, with many RS joins in such cells harboring abnormal deletions (Bogue *et al.*, 1998; Errami *et al.*, 1998; Fukumura *et al.*, 1998, 2000; Gao *et al.*, 1998; Priestley *et al.*, 1998). Therefore, although not fully required, DNA-PKcs has some unknown role in SJ formation, and this role is independent of Artemis (see below) and may be substituted by other factors (e.g., in ES cells). Finally, hairpin-coding ends were shown to accumulate in lymphocytes derived from DNA-PKcs–deficient mice (Roth *et al.*, 1992), thus implicating a function for DNA-PKcs in the processing of these V(D)J intermediates.

More recently, DNA-PKcs has been shown to phosphorylate Artemis, another member of the NHEJ repair pathway required for the formation of CJs but not SJs (Ma *et al.*, 2002; Nicolas *et al.*, 1998; Rooney *et al.*, 2002). The phosphorylated form of Artemis has an endonuclease activity that *in vitro* is capable of opening DNA hairpins produced by RAGs (Ma *et al.*, 2002). Sequences of CJ junctions generated from both DNA-PKcs– and Artemis-deficient cells show an increased rate of P-nucleotide additions (Rooney *et al.*, 2003) consistent with aberrant opening of the hairpin ends (Kienker *et al.*, 1991; Lewis, 1994b; Rooney *et al.*, 2002; Schuler *et al.*, 1991). In contrast to DNA-PKcs deficiency, SJ formation is normal (both in quantity and quality) in all types of Artemis-deficient cells examined (Noordzij *et al.*, 2003; Rooney *et al.*, 2003), again supporting a non-Artemis–mediated role for DNA-PKcs in V(D)J recombination and NHEJ.

2.3.3. XRCC4 and DNA Ligase 4

The role of XRCC4 in V(D)J was discovered by expression cloning via complementation of an IR-sensitive, V(D)J recombination-defective hamster cell line with a human cDNA library, which was shown to completely complement all IR sensitivity and V(D)J recombination defects of this line (Li *et al.*, 1995). XRCC4 was then shown to associate with DNA Lig4 *in vitro* (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997). XRCC4- and Lig4-deficient cells were both shown to exhibit IR sensitivity and an inability to generate either SJs or CJs (Frank *et al.*, 1998; Gao *et al.*, 1998). Thus DNA Lig4 in association with XRCC4 rejoins the four broken DNA ends generated by RAG-mediated cleavage and likely has a similar role in NHEJ in general.

3. Regulation of V(D)J Recombination

3.1. RAG-1 and RAG-2 Expression

3.1.1. Lymphoid-Specific Expression of RAGs

The expression of RAG-1 and RAG-2 is predominantly limited to developing lymphocytes, although low levels of RNA transcripts have been detected in the murine central nervous system and in peripheral lymphoid tissues (reviewed in Nagaoka *et al.*, 2000). In fact, transcription of the RAG-1 locus is detected in the earliest lymphocyte progenitors isolated thus far from the bone marrow of mice (Igarashi *et al.*, 2002). However, the only known defect in RAG-deficient mice is a complete block in lymphocyte development, and therefore the RAGs do not appear to play a role in the development or function of the central nervous system (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992).

The RAG-2 promoter is lymphoid specific and differentially regulated in B and T cells, whereas the basal RAG-1 promoter in both mice and humans does not impart lymphoid specificity (Lauring and Schlissel, 1999; Monroe *et al.*, 1999a). A more distal element 5′ of RAG-2 directs the coordinate and lymphoid-specific expression of fluorescently tagged RAG-1 and RAG-2 from a bacterial artificial chromosome (BAC) transgene integrated into mice (Yu *et al.*, 1999a). Moreover, RAG transcription appears to be regulated by different *cis* elements in B and T lymphocytes (Hsu *et al.*, 2003), with both a silencer and antisilencer important for proper tissue- and stage-specific expression (Yannoutsos *et al.*, 2004).

Several studies have detected RAG expression in mature B and T cells, leading to the hypothesis that RAGs could function to maintain self-tolerance via secondary rearrangements of autoreactive receptors (Han *et al.*, 1996; Hikida *et al.*, 1996; Papavasiliou *et al.*, 1997). However, targeted replacement of the RAG-2 gene with sequences encoding a RAG-2:GFP (green fluorescent protein) fusion protein demonstrated that the low level of RAGs detected in the periphery likely came from immature lymphocytes that had not completely shut off RAG expression and yet migrated to the peripheral lymphoid tissues (Kuwata *et al.*, 1999; Monroe *et al.*, 1999b; Yu *et al.*, 1999b). Moreover, RAG-2:GFP expression was not detected when GFP-negative lymphocytes isolated from the periphery of RAG-2:GFP mice were adoptively transferred into RAG-1–deficient host animals after immunization (Yu *et al.*, 1999b), thus indicating that RAG-2 was not reexpressed in mature lymphocytes.

3.1.2. Allelic Exclusion and Feedback Regulation

Coincident with the onset of RAG expression, IgH rearrangements begin in B220^{+}CD43^{+}ckit^{+}CD19^{+} progenitor B cells (pro-B), and TCRβ, TCRγ, and TCRδ rearrangements take place in CD4^{-}CD8^{-} double-negative (DN) T cells

(reviewed in Willerford *et al.*, 1996). Because of the inherently imprecise nature of CJs, only one in three rearrangements will be in-frame and capable of expressing a functional protein. Theoretically, lymphocytes could make a different receptor chain for each allele and express multiple receptors, each of a different specificity. However, almost all B cells express the functional products of only one IgH allele and one IgL allele, and in mature αβ T cells only one TCRβ allele is functionally rearranged and expressed, a process referred to as allelic exclusion (reviewed in Gorman and Alt, 1998; Kisielow and von Boehmer, 1995; Melchers *et al.*, 1999). Thus for the IgH, Igκ, Igλ, and TCRβ loci, only those cells in which the first V(D)J rearrangement is nonproductive go on to rearrange their second allele, preventing the assembly of multiple antigen receptors in a single cell (Alt *et al.*, 1984; Yancopoulos and Alt, 1985). Both stochastic and regulated models have been proposed to explain allelic exclusion, but the absolute mechanism, which may involve different mechanistic aspects for different loci, remains enigmatic; although it seems clear that there must be some form of feedback regulation to prevent opening of the second allele (reviewed by Mostoslavsky *et al.*, 2004). In this context, epigenetic factors, such as asynchronous replication, monoallelic demethylation, and variegated, monoallelic transcriptional activation, which render only a single allele capable of V-to-(D)J rearrangement initially (Liang *et al.*, 2004; Mostoslavsky *et al.*, 1998), may contribute to initiation of allelic exclusion before the feedback signals that maintain allelic exclusion in the face of continued expression of RAG.

The product of a functionally rearranged IgH gene, μIgH, and expression of a TCRβ chain initiate signals that enforce feedback regulation at nonrearranged IgH and TCRβ alleles, respectively, thus preventing further rearrangements (reviewed in Gorman and Alt, 1998; Kisielow and von Boehmer, 1995; Melchers *et al.*, 1999). In this regard, μIgH associates with the surrogate light chain proteins, Vpre-B and λ5, to form the pre-B cell receptor (pre-BCR) (Melchers *et al.*, 1993). A productive TCRβ chain then associates with the pre-Tα protein to form the pre-T cell receptor (pre-TCR) (Saint-Ruf *et al.*, 1994). Expression of the pre-BCR and pre-TCR provides the necessary signals to mediate feedback regulation, cellular expansion, and differentiation to the B220^{+}CD43^{lo}/ckit^{-}CD19^{+} pre-B and CD4^{+}CD8^{+} double-positive (DP) T-cell stages, respectively (reviewed in Kisielow and von Boehmer, 1995; Rolink *et al.*, 2001; von Boehmer *et al.*, 1999). Thus, introduction of rearranged IgH (Spanopoulou *et al.*, 1994; Young *et al.*, 1994) or TCRβ (Shinkai *et al.*, 1993) transgene into an RAG-deficient background promotes development to the pre-B or DP T-cell stages, respectively.

RAG expression is terminated during the phase of cellular proliferation that occurs during the transition from pro-B to pre-B in developing B cells, and

from DN to DP in developing T cells (Grawunder *et al.*, 1995). To facilitate this, RAG-2 is specifically tagged for degradation by cell cycle–dependent phosphorylation and ubiquitination (Lin and Desiderio, 1993; Mizuta *et al.*, 2002). After the cellular proliferation signaled by expression of the pre-BCR or pre-TCR, RAGs are once again expressed, thus allowing rearrangement of IgL and TCRα genes in pre-B and DP T cells, respectively (reviewed in Nagaoka *et al.*, 2000). During this second wave of RAG expression, further rearrangement of the IgH and TCRβ loci does not occur, and therefore these loci have somehow been rendered inaccessible to the RAGs (reviewed in Krangel, 2003).

Individual B cells produce immunoglobulin containing either Igκ or Igλ light chains, but not both, referred to as IgL isotype exclusion (reviewed in Mostoslavsky *et al.*, 2004). In pre-B cells, the Igκ locus is the first to rearrange, with subsequent rearrangement of Igλ genes usually occurring only in cells that have failed to generate a productive Igκ gene rearrangement (reviewed in Gorman and Alt, 1998). The mechanism of sequential Igκ versus Igλ rearrangement and whether it is regulated or stochastic also remain unsolved problems (reviewed in Mostoslavsky *et al.*, 2004). Once a productive IgL chain is formed, it pairs with the previously rearranged IgH chain to create a mature BCR in the form of IgM that is expressed at the surface of the developing B cell. Surface expression of IgM provides further feedback regulation and allelic exclusion, as well as signaling for the differentiation to the immature B cell stage and beyond (reviewed in Rolink *et al.*, 2001; Willerford *et al.*, 1996). IgL rearrangements sometimes result in Igκ and Igλ protein products that fail to associate with μIgH, and thus are functionally nonproductive (Alt *et al.*, 1980).

Similarly, productive TCRα rearrangement in DP T cells allows for expression of a functional TCRα/β complex that induces progression through positive and negative selection steps that lead to the development of mature $CD4^+$ or $CD8^+$ single-positive (SP) T cells (reviewed in Kisielow and von Boehmer, 1995; Willerford *et al.*, 1996). Unlike other antigen receptor gene loci, the TCRα, γ, and δ loci do not undergo feedback regulation or allelic exclusion at the level of gene rearrangement (Casanova *et al.*, 1991; Davodeau *et al.*, 1993; Malissen *et al.*, 1988, 1992; Padovan *et al.*, 1993; Sleckman *et al.*, 1998). Preferential pairing of one TCRα chain to the expressed TCRβ chain essentially prevents the expression of more than one antigen-specific receptor at the surface of αβ T cells carrying two productive TCRα rearrangements (reviewed in Fehling and von Boehmer, 1997; Malissen *et al.*, 1992).

If a newly generated B cell expresses a productive but self-reactive receptor, signaling via the Ig receptor appears to prolong, or possibly reactivate, RAG expression and allow further rearrangement of IgL chains (reviewed in

Nemazee and Weigert, 2000; Nussenzweig, 1998). This may lead to the replacement of a self-reactive receptor with a non-self-reactive one in a process termed receptor editing (Gay *et al.*, 1993; Radic *et al.*, 1993; Tiegs *et al.*, 1993). There also is some evidence that suggests potential editing of TCRβ genes in peripheral T cells (reviewed by Mostoslavsky and Alt, 2004). In contrast, both alleles of the TCRα locus appear to rearrange concomitantly in T cells and continue to rearrange throughout the DP T cell stage until positive selection on self-MHC has successfully occurred (Borgulya *et al.*, 1992; Malissen *et al.*, 1988; Petrie *et al.*, 1995).

3.1.3. Effect of Deregulated RAG Expression

Tight regulatory control over RAG expression is important for normal lymphocyte development. Overall numbers of B and T cells are dramatically reduced in transgenic mice expressing RAGs continually during all stages of lymphocyte development (Barreto *et al.*, 2001; Wayne *et al.*, 1994a). The reduction in T-cell numbers reflects a selective reduction in αβ T cells, with γδ T cell development appearing relatively normal (Barreto *et al.*, 2001). As αβ T cells undergo a burst of proliferation after productive TCRβ rearrangement and expression of the pre-TCR, the impairment would be consistent with p53-induced cell cycle arrest or apoptosis caused by an abundance of RAG-generated DNA DSBs. Surprisingly, allelic exclusion is maintained even in mice continually expressing RAGs throughout lymphocyte development, indicating that the regulation of antigen receptor gene accessibility is an extremely efficient process (Barreto *et al.*, 2001; Wayne *et al.*, 1994b). Finally, transgenic mice that ubiquitously express RAGs die prematurely and are significantly smaller than control littermates, although it is unclear why (Barreto *et al.*, 2001).

3.2. Regulated Accessibility of Antigen Receptor Gene Segments

3.2.1. Regulated Accessibility and V(D)J Recombination

IgH and TCRβ rearrangements take place in an ordered fashion, with D_H-to-J_H (Alt *et al.*, 1984) and Dβ-to-Jβ (Born *et al.*, 1985) rearrangements proceeding to completion on both IgH and TCRβ alleles, respectively, before the onset of subsequent V-to-DJ rearrangements. Lymphoid-restricted RAG expression limits V(D)J recombination to developing B and T lymphocytes but cannot account for the ordered or stage-specific rearrangement of immunoglobulin and TCR loci (Yancopoulos and Alt, 1985). Regulated gene accessibility imparts ordered rearrangement of antigen receptor genes such that IgH genes assemble before IgL genes in developing B cells, and TCRβ genes assemble before TCRα genes in developing αβ T cells (reviewed by Krangel,

2003; Mostoslavsky *et al.*, 2003; Yancopoulos and Alt, 1986). Moreover, during IgH and TCRβ gene assembly, D-to-J rearrangements precede V-to-DJ rearrangements and take place on both alleles before the onset of V-to-DJ rearrangements (Alt *et al.*, 1984; Born *et al.*, 1985). In this regard, when nuclei isolated from RAG-deficient lymphocytes are incubated with RAGs *in vitro*, RS cleavage reflects the lineage and stage specificity of the cell from which the nuclei were isolated (Stanhope-Baker *et al.*, 1996). Moreover, the efficiency of RAG-mediated cleavage of RS-containing extrachromosomal substrates was found to be substantially reduced when the substrate was incorporated into a nucleosome compared with the same substrate in the form of naked DNA (Golding *et al.*, 1999; Kwon *et al.*, 1998). Thus higher order chromatin structure plays an integral role in the regulated accessibility of antigen receptor gene rearrangement (reviewed in Bassing *et al.*, 2002b; Hesslein and Schatz, 2001; Krangel, 2003).

3.2.2. Transcription and V(D)J Recombination

Recombinational activity of integrated immunoglobulin or TCR transgenes is often largely dependent on the presence and function of associated transcriptional enhancer elements (reviewed in Ferrier *et al.*, 1990; Krangel, 2003; Raulet *et al.*, 1985; Sleckman *et al.*, 1996). As the site of integration and number of integrated copies can influence the expression of transgenes, gene-targeted ablation of regulatory *cis* elements at endogenous loci was used to assess more directly their function in recombination.

There are transcriptional enhancer and promoter elements associated with all antigen receptor loci (reviewed in Hempel *et al.*, 1998; Hesslein and Schatz, 2001). Early observations that V(D)J recombination generally correlated with the appearance of germline transcripts at various antigen receptor loci implicated regulatory *cis* elements as playing a role in recombinational accessibility (Alessandrini and Desiderio, 1991; Fondell and Marcu, 1992; Goldman *et al.*, 1993; Schlissel and Baltimore, 1989; Yancopoulos and Alt, 1985). In the IgH locus, gene-targeted deletion of the intronic IgH enhancer (iEμ) substantially reduced V_H-to-DJ_H but not D_H-to-J_H rearrangement in developing B lymphocytes (Chen *et al.*, 1993; Serwe and Sablitzky, 1993). In the Igκ locus, elimination of both the intronic Igκ (iEκ) and 3′ (3′Eκ) enhancers completely abolished Vκ-to-Jκ rearrangement (Inlay *et al.*, 2002) and separate deletion of one or the other led to a substantial reduction in Vκ-to-Jκ rearrangement (Gorman *et al.*, 1996; Xu *et al.*, 1996).

In T cells, deletion of the TCRβ enhancer (Eβ) substantially reduced the level of TCR DJβ and VβDJβ transcripts, although some Vβ-associated germline transcripts were still present (Bories *et al.*, 1996; Bouvier *et al.*, 1996; Mathieu *et al.*, 2000). A corresponding decrease in levels of DJβ and VβDJβ

rearrangements in E$\beta^{-/-}$ lymphocytes was also found, and overall αβ T-cell development was severely impaired (Bories *et al.*, 1996; Bouvier *et al.*, 1996). Gene-targeted ablation of the TCRα enhancer (Eα) resulted in a severe reduction in germline Jα transcripts and TCRα rearrangement in developing T cells (Sleckman *et al.*, 1997). On the other hand, deletion of Eα did not substantially alter the level of TCRδ rearrangements in these same cells, even though TCRδ gene segments are distributed among TCRα gene segments in this locus (Sleckman *et al.*, 1997). In contrast, mice carrying a deletion of the TCRδ enhancer (Eδ) had substantial impairment in VδDJδ rearrangements but were normal for TCRα/β development (Monroe *et al.*, 1999c). Whereas Eδ was required for TCRδ transcripts in DN thymocytes, TCRδ transcripts were unaffected in the few γδ T cells that developed in the absence of Eδ (Monroe *et al.*, 1999c). Thus Eδ differentially regulates early but not late γδ T-cell processes.

Antigen receptor gene segment–associated germline promoters have also been shown to affect V(D)J recombination. Deletion of the germline Dβ1 promoter substantially reduced germline Dβ1 transcripts and Dβ-to-Jβ1 rearrangement levels but did not affect transcription or rearrangement involving Dβ2/Jβ2 gene segments (Whitehurst *et al.*, 1999). Similarly, deletion of the T early α (TEA) germline promoter upstream of the 5′-most Jα gene segments eliminated germline transcripts associated with upstream Jα gene segments and reduced Vα-to-Jα rearrangements corresponding to these same Jα segments (Villey *et al.*, 1996). However, germline Jα transcripts initiating downstream of TEA were detectable in thymocytes lacking TEA, and overall levels of TCRα rearrangements were normal (Villey *et al.*, 1996).

Regarding lineage specificity, targeted replacement of the TCRβ enhancer with iEμ promoted transcription and rearrangement of the TCRβ locus at levels substantially higher than in developing T cells with Eβ deleted (Bories *et al.*, 1996; Bouvier *et al.*, 1996), demonstrating that iEμ can function outside of the IgH locus to promote accessibility of heterologous sequences. VβDJβ rearrangements did not occur at appreciable levels in B lineage cells carrying iEμ in place of Eβ, although germline JβCβ transcripts were readily detectable in these same cells (Bories *et al.*, 1996). However, when a larger region encompassing Cβ2 and Eβ was replaced with iEμ, significant levels of Dβ-to-Jβ rearrangements were detected in splenic B cells (Eyquem *et al.*, 2002), suggesting that elements within the larger region have the ability to suppress TCRβ accessibility in B lineage cells. When Eβ was replaced with the TCRα enhancer (Eα), there was a significant reduction in Dβ germline transcripts in $CD25^{+}CD44^{-}CD4^{-}CD8^{-}$ (DN3) thymocytes, the stage at which rearrangements of TCRβ gene segments normally take place. However, levels of germline Dβ transcripts were normal in $CD4^{+}CD8^{+}$

thymocytes carrying the Eβ-to-Eα replacement, the stage during which TCRα is normally active. Thus Eα affected TCRβ transcription in a manner corresponding to its normal functional stage. However, levels of Vβ germline transcripts in DN3 thymocytes appeared normal, and overall levels of DJβ and VβDJβ rearrangements were only modestly reduced, demonstrating that Eα could function to promote VβDJβ recombination differently than it would normally at the TCRα locus. Finally, TCRα rearrangements were dramatically reduced when Eα was replaced with either the TCRδ enhancer (Eδ) or iEμ (Bassing *et al.*, 2003b), demonstrating that Eα must carry elements important for the regulated rearrangement of TCRα genes that cannot be replaced by other enhancer sequences. Thus, promoter/enhancer interactions clearly influence transcription as well as RAG-mediated recombination at specific sites within antigen receptor loci (reviewed in Bassing *et al.*, 2000; Krangel, 2003).

3.2.3. Chromatin Modifications

Chromatin is made up of complexes of protein and DNA that allow the packaging of approximately 1- to 10-cm lengths of unwound chromosomal DNA into a nucleus with a diameter of only 3–10 μm (Alberts *et al.*, 1983). The structure of chromatin begins with 146 bp of DNA wrapped around a complex of histone proteins that form a nucleosome. Each nucleosome consists of eight histone molecules, two copies each of histone family proteins H3, H4, H2A, and H2B (Alberts *et al.*, 1983). The histone protein H1 then links nucleosomes into the higher ordered structure of 30-nm fibers, which are condensed even further during interphase of the cell cycle (reviewed in Belmont *et al.*, 1999). The regulation and control of higher order chromatin is an integral component of transcriptional activation and repression of eukaryotic genes (reviewed in Udvardy, 1999), as well as DNA replication (reviewed in Gerbi and Bielinsky, 2002). The epigenetic regulation of chromatin accessibility is associated with histone acetylation, phosphorylation, methylation, and ubiquitination (reviewed in Berger, 2002), as well as DNA methylation (reviewed in Richards and Elgin, 2002).

Histone modifications have been associated with actively recombining extrachromsomal substrates and endogenous antigen receptor loci (reviewed in Krangel, 2003; Oettinger, 2004). Treatment with histone deacetylase inhibitors has been shown to induce RS accessibility and V(D)J recombination within the Igκ, TCRγ, and TCRβ loci of cells otherwise inaccessible because of higher order chromatin (Agata *et al.*, 2001; Mathieu *et al.*, 2000; McBlane and Boyes, 2000). In addition, deletions of *cis*-regulatory elements necessary for endogenous V(D)J recombination have been linked to reduced levels of histone acetylation of antigen receptor locus–associated sequences (Agata *et al.*,

2001; Mathieu *et al.*, 2000). Furthermore, chromatin near the Dβ1Jβ1 region of Eβ-deficient thymocytes, which have reduced levels of TCRβ rearrangements (see above), contained alterations in histone acetylation, methylation, and phosphorylation (Spicuglia *et al.*, 2002). These data are consistent with the hypothesis that such combinatorial interactions and modifications lead to an epigenetic regulatory system or a so-called "histone code" that in some manner may promote recombinational accessibility at antigen receptor loci (reviewed in Bassing *et al.*, 2002b; Jenuwein and Allis, 2001; Oettinger, 2004; Strahl and Allis, 2000). Methylation of specific lysine residues on histone H3 has also been shown to correlate with recombinational activity at the IgH and TCRβ loci (Morshead *et al.*, 2003; Ng *et al.*, 2003; Spicuglia *et al.*, 2002; Su *et al.*, 2003). However, targeted recruitment of a histone methyltransferase to chromosomal recombination substrates blocks transcription and recombination of nearby segments (Osipovich *et al.*, 2004), illuminating the complexity of such regulation.

DNA hypomethylation at CpG dinucleotides has been shown to correlate with transcription in general (Razin and Riggs, 1980), as well as with specific antigen receptor gene segments (Kelley *et al.*, 1988; Mather and Perry, 1981, 1983). Regarding recombinational accessibility, methylation at a single CpG site within the 3′ RS of Dβ1 did not allow cleavage by RAGs (Whitehurst *et al.*, 2000). In addition, Vκ-to-Jκ rearrangements appear limited to hypomethylated alleles, and thus DNA methylation may also play a role in allelic exclusion (Mostoslavsky *et al.*, 1998). However, as not all actively recombining antigen receptor loci display hypomethylated status (Villey *et al.*, 1997), DNA methylation does not always result in elevated recombinational accessibility (Cherry *et al.*, 2000). Therefore, the overall state of recombinationally accessible antigen receptor gene segments likely involves a variety of interacting modifications involving chromatin and DNA (reviewed in Krangel, 2003).

3.2.4. H2AX

Chromatin modifications along antigen receptor loci are also important for monitoring the chromosomal V(D)J recombination reaction to ensure the normal NHEJ-mediated repair of RAG-generated DSBs. There are three subfamilies of histone H2A, of which H2AX comprises 10–15% of total H2A protein in most mammalian cells (Mannironi *et al.*, 1989). In response to IR-induced DNA DSBs, H2AX is phosphorylated on Ser-139, thus producing γ-H2AX. γ-H2AX is found in discrete foci at the site of DSBs, and these foci occur at a frequency comparable to the number of induced DSBs (Rogakou *et al.*, 1999). Several DNA repair factors including Rad50, Rad51, and Nijmegen breakage syndrome protein (NBS1) have been shown to colocalize with γ-H2AX after the induction of DSBs (Chen *et al.*, 2000; Paull *et al.*, 2000).

Both DNA-PKcs and ATM are able to phosphorylate H2AX *in vitro* and/or *in vivo* (Burma *et al.*, 2001; Rogakou *et al.*, 1998; Stiff *et al.*, 2004).

γ-H2AX and NBS1 have been shown to undergo RAG-dependent colocalization with the TCRα locus in normal thymocytes (Chen *et al.*, 2000), and H2AX-deficient mice have a slight reduction in overall numbers of B and T lymphocytes (Bassing *et al.*, 2003a; Celeste *et al.*, 2002). Nevertheless, the formation of V(D)J-associated CJs and SJs was found to be normal in H2AX-deficient mice (Bassing *et al.*, 2003a; Celeste *et al.*, 2002) and from recombination substrates transiently transfected into H2AX-deficient ES cells (Bassing *et al.*, 2002a). Clearly, H2AX is not essential for the repair of RAG-induced DNA DSBs. However, approximately 4% of nontransformed αβ T cells from H2AX-deficient mice contain potential TCRα/δ locus translocations (Celeste *et al.*, 2002) and H2AX-deficient mice may exhibit an increased predisposition to thymic lymphomas with potential TCRα/δ locus translocations (Bassing *et al.*, 2003a). Thus, H2AX likely serves a critical role in the suppression of aberrant V(D)J recombination, possibly through the proposed "anchoring" function of H2AX in forming a nucleation site for a number of DNA–protein–protein–DNA interactions that might serve to stabilize synaptic complexes of chromosomal RAG-cleaved antigen receptor loci (Bassing and Alt, 2004).

4. Class Switch Recombination Employs Distinct Mechanisms for V(D)J Recombination

4.1. Overview of Class Switch Recombination and Somatic Hypermutation

The consequence of successful V(D)J recombination of IgH and IgL chains in developing B cells is the surface expression of IgM and/or IgD. Activation by antigen in the context of certain cytokine stimuli can induce the process of CSR, whereby the V(D)J_H exon initially associated with Cμ exons is adjoined to one of several groups of downstream C_H exons (e.g., Cγ, Cε, and Cα, referred to as C_H genes) (Fig. 6). Recombination takes place between repetitive sequences, termed S regions, which lie just upstream of the various C_H genes (reviewed in Chaudhuri and Alt, 2004). The exchange in C_H genes alters the isotype of expressed antibody from IgM to either IgG, IgE, or IgA, along with associated changes in effector function, while maintaining antigen-binding specificity (reviewed in Manis *et al.*, 2002b). DNA sequences located between the recombining S regions can be detected in the form of circularized DNA that has been excised from the genome of the effected B cell (Iwasato *et al.*, 1990). The liberation of circular DNA in CSR is consistent with the participation of DSB intermediates, analogous to excised SJs in V(D)J

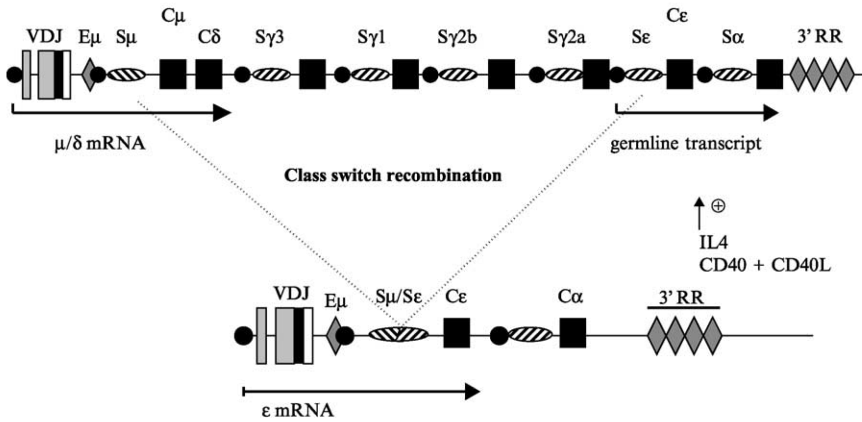

Figure 6 Schematic diagram of the murine IgH locus before and after class switch recombination between Sμ and Sε. The V_H gene segments are depicted as shaded rectangles, the D_H segments as solid rectangles, and the J_H segments as open rectangles. The Sμ regions exons are shown as striped ovals and constant region exons as solid squares. The position of the iEμ and 3′ RR enhancers are indicated by diamonds. The positions of the V_H and I exon promoters are shown as solid circles. Distances between the various elements are not drawn to scale.

recombination that require synapsis and repair over appreciable chromosomal distances. CSR is a mature B-cell–specific process and, unlike V(D)J recombination, does not occur in T lineage cells.

In addition to CSR, activation of mature B cells in the context of a germinal center reaction can introduce mutations at a high rate (10^{-3} to 10^{-4} per base pair per generation) into assembled IgH and IgL variable region exons via a process called SHM. Selection of B cells in which mutated V regions create an antigen receptor of higher affinity than the original results in "affinity maturation" and the generation of a more effective immune response.

CSR and SHM rely on the activity of the *aicda* gene, which encodes activation-induced deaminase (AID), which in turn deaminates cytidine residues on DNA and, thus, forms dU/dG mismatched DNA base pairs (Bransteitter *et al.*, 2003; Chaudhuri *et al.*, 2003; Dickerson *et al.*, 2003; Petersen-Mahrt *et al.*, 2002; Pham *et al.*, 2003; Sohail *et al.*, 2003; Yu *et al.*, 2004). CSR and SHM likely proceed via subsequent excision of the mismatched dU by the base excision repair protein uracil DNA glycosylase (UNG). UNG creates an abasic site, and differential repair of this lesion apparently leads to either SHM or CSR (Di Noia and Neuberger, 2002; Petersen-Mahrt *et al.*, 2002; Rada *et al.*, 2002b). The mismatch repair (MMR) proteins Msh2/Msh6 can also bind and process the

dU:dG mismatch and contribute to the CSR and SHM. In this regard, UNG and MMR deficiencies can impair CSR and alter the spectrum of mutations sustained by V genes during SHM. Intriguingly, however, some UNG mutants that have lost the uracil glycosylase activity retain the ability to mediate CSR in mice, leading to the suggestion that UNG may participate in an as yet unidentified manner in CSR that extends beyond its known enzymatic activity (Begum *et al.*, 2004a).

Transcription of target S region or variable region target sequences is essential for both CSR (Bottaro *et al.*, 1994; Zhang *et al.*, 1993) and SHM (Bachl *et al.*, 2001; Betz *et al.*, 1994; Fukita *et al.*, 1998; Peters and Storb, 1996). Each C_H gene is organized into a transcriptional unit differentially regulated by cytokine-specific transcription factors, thus providing the necessary specificity for directing isotype-specific switching (Fig. 6) (reviewed in Manis *et al.*, 2002b; Stavnezer, 2000). AID deaminates cytidines of ssDNA *in vitro* (Bransteitter *et al.*, 2003; Chaudhuri *et al.*, 2003; Dickerson *et al.*, 2003; Pham *et al.*, 2003) but requires transcription of double-stranded dsDNA to generate an appropriate ssDNA substrate (Chaudhuri *et al.*, 2003; Ramiro *et al.*, 2003). Thus, transcription provides an appropriate target substrate for AID to act on (Chaudhuri *et al.*, 2003; Ramiro *et al.*, 2003). The remainder of this review focuses on CSR, although contrasts will be made between CSR and SHM where appropriate.

4.2. Organization of Heavy Chain Constant Region Genes

In mice there are eight C_H genes located on chromosome 12 and positioned downstream of the antigen receptor gene segments in a region spanning approximately 200 kb (Fig. 6). Cμ and Cδ are the most J_H-proximal group of C_H genes, and Cμ is the first to associate with a functionally rearranged $V(D)J_H$ exon, and therefore IgM is the first isotype expressed on the surface of a B cell. Later in development, expression of IgD occurs via alternative RNA splicing of the $V(D)J_H$ exon to the Cδ exons. Thus, IgM and IgD are often simultaneously expressed on the surface of the same B cell. Expression of all other immunoglobulin isotypes requires CSR between Sμ and downstream S region sequences (e.g., Sγ, Sε, or Sα), with subsequent loss of the intervening Cμ and Cδ genes (reviewed in Chaudhuri and Alt, 2004). All C_H genes, except Cδ, are integrated into transcriptional units consisting of an intervening (I) exon (Lutzker and Alt, 1988), S region, C_H exons, and downstream polyadenylation signal sequences corresponding to the membrane and secreted versions of immunoglobulin. Finally, a region downstream of Cα containing enhancer elements, referred to as the 3′ regulatory region (3′ RR), is important

for the production of germline transcripts (GTs) and CSR to the various C_H genes except Cγ1 (reviewed in Manis *et al*., 2002b).

4.3. Regulation of Class Switch Recombination

4.3.1. B-Cell Activation and Class Switch Recombination

V(D)J recombination and early B-cell development take place in the bone marrow. On successful expression of surface immunoglobulin in the form of IgM, immature B cells migrate from the bone marrow to peripheral lymphoid tissues located in the spleen, lymph nodes, and gut-associated lymphoid tissue. Here, at discrete anatomic sites referred to as germinal centers, and often in association with T cells, B cells encounter antigen and undergo antigen-driven clonal expansion that can lead to CSR and/or SHM; although CSR (Macpherson *et al*., 2001) and SHM (William *et al*., 2002) can also take place outside of germinal centers as well.

CSR is induced *in vivo* by both T-dependent (TD) and T-independent (TI) antigens. B-cell activation by TD antigens requires interaction of CD40 ligand expressed on activated T cells and CD40 on the surface of B cells. T-independent antigens can activate B cells in the absence of direct T- and B-cell interactions. Type 1 TI antigens, such as lipopolysaccharide (LPS), can act as polyclonal B-cell activators at high concentrations and are able to activate B cells in the complete absence of T cells. Type 2 TI antigens, on the other hand, which usually consist of highly repetitious molecules, do not require direct B- and T-cell interactions to induce B-cell activation or CSR, although B-cell activation and CSR occur inefficiently in the absence of T-cell–derived cytokines. TD antigen stimulation can be mimicked *in vitro* by culturing B cells in the presence of anti-CD40 along with specific cytokines, and TI activation can be mimicked by treatment with LPS plus or minus the addition of specific cytokines (reviewed in Manis *et al*., 2002b; Stavnezer, 2000). In concert with antigen-dependent activation, cytokine-induced signaling provides specificity to CSR (reviewed in Manis *et al*., 2002b; Stavnezer, 2000). For instance, LPS induces isotype switching to IgG2b and IgG3, whereas LPS plus interleukin 4 (IL-4) induces isotype switching to IgG1 and IgE (reviewed in Manis *et al*., 2002b; Stavnezer, 2000).

4.3.2. Germline C_H Transcripts

Isotype switching to a particular C_H gene is preceded by transcription of the corresponding germline sequences (reviewed by Chaudhuri and Alt, 2004; Manis *et al*., 2002b; Stavnezer, 2000). Transcripts initiate upstream of I exons found 5′ of each C_H gene and terminate at polyadenylation sites located

downstream of C_H genes (Lutzker and Alt, 1988). These transcripts undergo RNA splicing between the I and C_H exons to form processed GTs in which the intervening S region sequences are deleted (reviewed in Chaudhuri and Alt, 2004). Transcription is regulated by I region promoter sequences that are activated by CD40-, LPS-, and cytokine-mediated signals (Fig. 6) (reviewed in Stavnezer, 2000).

Deletion of I region promoters and subsequent loss of recombination involving the associated C_H gene demonstrated the dependence on S region transcription for CSR (Jung *et al*., 1993; Lorenz *et al*., 1995; Zhang *et al*., 1993). Furthermore, targeted replacement of I promoters with heterologous, constitutively active promoters rescues S region transcription and directs isotype switching to the corresponding C_H genes (Bottaro *et al*., 1994; Lorenz *et al*., 1995; Seidl *et al*., 1998). GTs do not encode functional proteins, but several studies have implicated RNA splicing and processing as potentially playing a role in the CSR process, as targeted mutations in specific splice sites strongly inhibited CSR to the corresponding C_H gene (Hein *et al*., 1998; Lorenz *et al*., 1995). However, it now seems clear that the major role of GTs is to provide the appropriate ssDNA substrate for AID (see Sections 4.4 and 4.5, below) (reviewed in Chaudhuri and Alt, 2004).

In the IgH locus, V_H-to-DJ_H rearrangements are impaired in the absence of the intronic enhancer (iEμ) that lies in the intronic region between the J_H gene segments and Cμ just uptream of Sμ; thus it appears that iEμ serves an essential role in promoting V_H-to-DJ_H rearrangement in developing B cells (Sakai *et al*., 1999a; Serwe and Sablitzky, 1993). In this regard, CSR is also reduced in the absence of iEμ, which serves a promoter function for Iμ-Cμ–containing GTs (Bottaro *et al*., 1998; Gu *et al*., 1993; Sakai *et al*., 1999a; Su and Kadesch, 1990). Conceivably, CSR that occurs in the absence of iEμ may be mediated by transcription of Iμ sequences initiated by heterologous promoters such as those associated with V_H or D_H gene segments (Gu *et al*., 1993; Kuzin *et al*., 2000). Notably, removal of iEμ along with a large region of potential upstream promoters resulted in reduced Sμ recombination, but left substantial recombination within downstream S regions, which manifest as internal S region deletions (Gu *et al*., 1993). Thus, iEμ does not appear to function as an essential enhancer element for promoting CSR to downstream C_H genes, which may be more dependent on the activity of the 3′RR (see below).

The production of GTs is influenced by enhancer-like elements located in the region found approximately 15 kb downstream of Cα. The 3′RR is an ~40-kb region composed of four elements corresponding to hypersensitivity sites (5′-HS3a-HS1,2-HS3b-HS4-3′), which in various combinations have been shown to possess locus control region (LCR)–like activity (reviewed in

Madisen and Groudine, 1994; Manis *et al.*, 2002b). Targeted replacement of HS1,2 with a *pgk*-driven neomycin resistance gene cassette (pgk neo^r) disrupts the production of GTs and CSR to all C_H genes except IgG1 and IgA, some of which are located as far as 120 kb upstream (Cogne *et al.*, 1994). This suggests that either I promoter–driven transcription of C_H genes is dependent on this site, or that *pgk neo*^{r} blocked the activity of an unknown downstream element (Cogne *et al.*, 1994; Manis *et al.*, 1998b). In support of the latter possibility, production of GTs and CSR was rescued when the *neo*^{r} cassette was deleted from the locus, leaving a *loxP* site in place of HS1,2 (Manis *et al.*, 1998b). Similar studies demonstrated that HS3a is also dispensable for CSR (Manis *et al.*, 1998b). Furthermore, targeted insertion of the *pgk neo*^{r} cassette at several locations throughout the IgH locus inhibited the production of GTs and CSR of C_H genes located upstream of the *pgk neo*^{r} insertion, but not downstream (Seidl *et al.*, 1999). Thus the *pgk neo*^{r} cassette appears to preferentially compete with C_H promoters for an enhancer activity found in the region downstream of HS1,2. Clean deletion of HS3b and HS4 together has the identical effect as the targeted replacement of HS1,2 or HS3a with the *pgk neo*^{r} cassette, namely a significant reduction in the production of all immunoglobulin isotypes except IgM and IgG1, with a modest reduction in IgA (Pinaud *et al.*, 2001). Finally, insertion of the *pgk neo*^{r} cassette downstream of HS4 did not reduce CSR or the production of GTs, which, together with the other data, strongly implies that the enhancer activity is contained within the HS3b or HS4 sequences, and possibly both (Manis *et al.*, 2003).

4.3.3. Class Switch Recombination and Somatic Hypermutation Are Region-Specific Events

CSR is targeted to S regions, found upstream of each C_H gene, that consist of 1–12 kb of repetitive sequences (Fig. 6) (Kataoka *et al.*, 1980). Each unique S region is made up of tandem repeat units varying in length between 5 bp (Sμ) and 80 bp (Sα) (reviewed in Honjo *et al.*, 2002) with some varying degrees of overall homology (reviewed in Stavnezer, 1996). In contrast to the site-specific cleavage mediated by RAGs at RSs in V(D)J recombination, CSR between two S regions can occur throughout, and even outside, the core repeat sequences (Dunnick *et al.*, 1993; Lee *et al.*, 1998; Luby *et al.*, 2001). Sequencing the break points of CSR junctions failed to identify a consensus target sequence either in relation to the overall S regions or in the context of the short pentameric repeats, confirming that CSR is a region-specific rather than site-specific event (Dunnick *et al.*, 1993; Lee *et al.*, 1998).

The lack of consensus sequences at CSR junctions suggests that rather than depending on sequence-specific recognition, the CSR "recombinase" might instead be targeted via the formation of S region–specific higher order

structures. The S regions of most vertebrates are rich in G and C nucleotides, with a bias for purine nucleotides on the nontemplate strand of DNA (Shinkura *et al.*, 2003). Such properties have been shown to promote the formation of RNA–DNA hybrids (Mizuta *et al.*, 2003; Reaban and Griffin, 1990; Reaban *et al.*, 1994; Tian and Alt, 2000) that could lead to the generation of structures such as R loops (Tian and Alt, 2000; Shinkura *et al.*, 2003; Yu, 2003) and G quartets (Dempsey *et al.*, 1999; Sen and Gilbert, 1988). In contrast, the S region sequences in *Xenopus* contain approximately 60% A and T nucleotides, consistent with the overall nucleotide composition of the *Xenopus* genome (Muβmann *et al.*, 1997). However, *Xenopus* S regions are highly repetitive and contain palindromic sequences similar to those that target SHM, which conceivably could effect CSR in the absence of potential higher order structures. (Muβmann *et al.*, 1997; Zarrin *et al.*, 2004).

Deletion of the core tandem repeat sequences of Sμ significantly reduced CSR in mice (Luby *et al.*, 2001). The remaining level of CSR detected in these mice might be due to the retention of a considerable amount of G-rich and short palindromic sequences left upstream of Cμ (Luby *et al.*, 2001), although complete deletion of all Sμ tandem repeats further reduces, but does not eliminate, CSR (Khamlichi *et al.*, 2004). Thus, transcriptional activation from the iEμ enhancer may by itself be enough to induce low levels of recombination (Khamlichi *et al.*, 2004). On the other hand, complete deletion of Sγ1 sequences in mice essentially blocks CSR to the deleted allele (Shinkura *et al.*, 2003). Finally, the lack of an associated S region apparently prevents the usage of the ψCγ gene in humans, as it is located in the correct transcriptional orientation and lacks mutations in its coding sequence that result in frameshift or stop codons or would otherwise prevent functional expression (Bensmana *et al.*, 1988). Therefore, the presence of an S region appears critical for normal CSR. Potential functions of mammalian S regions are discussed in more detail below.

SHM is also region specific, as mutations begin in the region just downstream of an IgH or IgL V promoter, are found throughout the variable region exons, and are detected as far as 2 kb downstream of V promoters within the intronic region between J and C exons (reviewed in Harris *et al.*, 1999). However, most of the introduced mutations occur within the assembled variable regions that form the antigen-binding portion of an antibody molecule or in nearby flanking sequences (reviewed in Harris *et al.*, 1999; Papavasiliou and Schatz, 2002b). Mutations are frequently associated with RGYW sequence motifs (where R is A or G, Y is C or T, and W is A or T). The most common changes are point mutations, with transitions being slightly favored over transversions, although small deletions and duplications are also detected. Targeting to RGYW sequences likely reflects specific recruitment and

specificity of the recombinase machinery (Chaudhuri *et al.*, 2004; see below), whereas mutations concentrated in sequences making up antigen-binding regions are partly the result of the selection for high-affinity antigen receptors in response to antigen during affinity maturation (Griffiths *et al.*, 1984).

4.3.4. Induced Mutations in and Around S Region Sequences

Sequencing of CSR junctions reveals frequent DNA alterations in the form of single-nucleotide mutations and small deletions (Dunnick *et al.*, 1993; Lee *et al.*, 1998). These mutations are AID dependent (Petersen *et al.*, 2001) and can be found both 5′ and 3′ of the Sμ region in wild-type B cells activated for, but that have not undergone, CSR (Dudley *et al.*, 2002; Nagaoka *et al.*, 2002; Petersen *et al.*, 2001). CSR is therefore frequently associated with mutations that resemble those induced by SHM, likely reflecting the common AID deamination event in the initiation of both CSR and SHM. Thus, the resolution of a common DNA lesion generated by AID by different downstream repair pathways could result in either DNA recombination or mutation (reviewed in Chaudhuri and Alt, 2004).

4.4. Activation-Induced Cytidine Deaminase

4.4.1. Discovery and Isolation of Activation-Induced Cytidine Deaminase

The discovery of AID and the subsequent demonstration of its essential role in CSR and SHM have led to rapid advances toward the elucidation of the mechanisms that effect CSR and SHM (reviewed in Kenter, 2003; Reynaud *et al.*, 2003). AID was isolated via a subtractive cloning screen from a murine B-cell line (CH12) that on activation switches from IgM to IgA (Muramatsu *et al.*, 1999). Expression of AID is limited to developing germinal center B cells (Muramatsu *et al.*, 1999) and can be induced *in vitro* by culturing splenic B-cells in the presence of activating stimuli known to induce CSR (Muramatsu *et al.*, 1999). AID deficiency completely abrogates CSR and SHM in both humans (Revy *et al.*, 2000) and mice (Muramatsu *et al.*, 2000), and expression of AID in nonlymphoid cell lines induces at least limited CSR (Okazaki *et al.*, 2002) and SHM (Martin *et al.*, 2002b; Yoshikawa *et al.*, 2002). Furthermore, overexpression of AID in bacteria can lead to mutations in several transcribed genes (Petersen-Mahrt *et al.*, 2002b; Ramiro *et al.*, 2003). Thus, analogous to RAGs with respect to V(D)J recombination, AID is both necessary and sufficient to effect CSR and SHM in the context of proteins expressed in nonlymphoid cells. It is to be noted, however, that the rate of CSR and the

spectrum of mutations observed in nonlymphoid cells on artificial substrates do not faithfully recapitulate that observed at endogenous loci in B cells, suggesting that B-cell–specific factors and/or AID modifications (see below) contribute to these processes.

4.4.2. Activation-Induced Cytidine Deaminase Expression

High-level expression of AID via stable integration of an AID transgene can induce mutations within actively transcribed nonimmunoglobulin genes, including the AID transgene itself, both in lymphoid and nonlymphoid cell lines (Martin and Scharff, 2002b; Yoshikawa *et al*., 2002). Constitutive and ubiquitous expression of AID in mice via a transgene leads to T-cell lymphomas and adenosarcomas (Okazaki *et al*., 2003) and deregulated AID expression has been detected in several types of human non-Hodgkin lymphomas (Greeve *et al*., 2003; Hardianti *et al*., 2004a,b). Moreover, mature human and mouse B lineage tumors often have translocations that fuse S regions with oncogene loci (reviewed by Mills *et al*., 2003). Work has shown, in mouse models, that such translocations are dependent on AID (Ramiro *et al*., 2004). Thus, tight regulatory control of AID expression is necessary to prevent generalized genomic mutations and genomic instability.

AID is expressed in activated B lymphocytes in the context of a germinal center reaction, precisely in those cells that undergo SHM and CSR *in vivo* (Muramatsu *et al*., 1999). Expression of AID is modulated by inhibitors of differentiation (Id) proteins, as ectopic expression of Id2 or Id3 reduces AID expression in activated splenic B cells and inhibits CSR (Gonda *et al*., 2003; Sayegh *et al*., 2003). Id proteins are best known as antagonists of the E family of transcription factors (E proteins), a class of basic helix–loop–helix proteins that bind DNA at conserved E box sites as homo- and heterodimers (reviewed in Quong *et al*., 2002; Sun, 2004). Id proteins form heterodimers with E proteins that are unable to bind DNA, thus negatively regulating transcriptional activation by E proteins (Benezra *et al*., 1990a,b; Christy *et al*., 1991; Riechmann *et al*., 1994; Sun *et al*., 1991). Id proteins have also been shown to interact with members of Pax and Ets families of transcription factors, likewise inhibiting their DNA-binding functions (Roberts *et al*., 2001; Yates *et al*., 1999). E12, E47, and Pax5 are vital for B-cell development (Bain *et al*., 1994; Urbanek *et al*., 1994; Zhuang *et al*., 1994), and their expression is highly induced in mature B cells by CSR-inducing stimuli (Gonda *et al*., 2003; Quong *et al*., 1999). E47 and Pax5 have both been shown to bind regulatory elements upstream of AID *in vivo* (Gonda *et al*., 2003; Sayegh *et al*., 2003), and the Pax5 element was shown to be essential for AID gene expression (Gonda *et al*., 2003).

4.4.3. Activation-Induced Cytidine Deaminase Deaminates dC Residues of Single-Stranded DNA Substrates: Interaction with Replication Protein A

AID is a ssDNA cytidine deaminase (Bransteitter *et al*., 2003; Chaudhuri *et al*., 2003; Dickerson *et al*., 2003; Pham *et al*., 2003; Sohail *et al*., 2003) and such ssDNA substrates are probably revealed during transcription through the S regions (reviewed in Chaudhuri and Alt, 2004). Transcription of S regions, in their physiological orientation, generates ssDNA in the context of R loops *in vitro* (Shinkura *et al*., 2003; Tian and Alt, 2000; Yu *et al*., 2003) and *in vivo* (Yu *et al*., 2003) and such transcribed DNA can serve as targets of AID deamination *in vitro* (Chaudhuri and Alt, 2004; see below).

In addition to R loops, other mechanisms may operate to target AID activity to S regions (see below). In this regard, variable region exons do not have repetitive sequences or unusual GC content that would lead to R loop formation and do not form R loops when transcribed *in vitro* (reviewed in Chaudhuri and Alt, 2004; Papavasiliou and Schatz, 2002b), yet they are targeted by AID during SHM. These observations suggested a specific cofactor to target AID during SHM and such a cofactor was identified (Chaudhuri *et al*., 2004) as replication protein A (RPA), a heterotrimeric ssDNA-binding protein involved in replication, recombination, and repair (reviewed in Wold, 1997). RPA stabilizes ssDNA (Wold, 1997) and can bind short stretches of ssDNA bubbles and recruit nucleotide excision and base excision repair proteins (reviewed by Binz *et al*., 2004; Matsunaga *et al*., 1996). AID forms a specific complex with RPA that facilitates AID-induced DNA deamination of transcribed RGYW-containing substrates (Chaudhuri *et al*., 2004). The efficiency of substrate binding and deamination by RPA·AID complexes was dependent on the number of RGYW motifs, and deamination was observed at or around these sequences (Chaudhuri *et al*., 2004). Thus RPA likely functions to target AID to transcribed SHM hot spots found in V region exons of IgH genes.

Significantly, the AID·RPA complex is B-cell specific, and this specificity appears regulated, at least in part, by the phosphorylation status of AID in B cells (Chaudhuri *et al*., 2004). Other findings support the notion that the AID·RPA complex may also be involved in CSR, particularly in the context of *Xenopus* S regions that lack R loop-forming ability but contain regions of RGYW motifs (Zarrin *et al*., 2004; see below). Also, it is possible that RPA that remains bound to the deaminated mammalian S region substrate, as proposed for SHM, also can actively recruit proteins that are downstream of deamination, such as UNG and MMR proteins, to the site of initial DNA lesions in CSR.

4.4.4. Apobec1 and Other Deaminase Family Members

AID shares significant sequence homology with Apobec-1 (34% amino acid identity), a known cytidine deaminase (Muramatsu *et al*., 1999), and has been shown to catalyze the deamination of free CTP nucleotides *in vitro* (Muramatsu *et al*., 1999; Papavasiliou and Schatz, 2002a). Apobec-1 functions as an RNA-editing enzyme, inducing a C-to-U conversion at position 6666 of the ApoB mRNA transcript, hence changing Gln-2153 into an in-frame stop codon (reviewed in Chan *et al*., 1997). The edited transcript encodes ApoB-48, a protein that, although colinear with the N-terminal 2152 residues of full-length ApoB-100, has significantly altered biological function (reviewed in Chan, 1992). The shared homology with Apobec-1 led to the proposal that AID may edit an mRNA transcript of unknown function, thus generating a novel class switch recombinase and/or V region mutator (Muramatsu *et al*., 2000). This model, which contrasts with most data arguing for a DNA deamination activity for AID in CSR and SHM, was supported, albeit quite indirectly, by the finding that *de novo* protein synthesis is required for AID to induce CSR (Begum *et al*., 2004b; Doi *et al*., 2003).

The cytidine deaminase ApoBec3G acts as an inhibitor of the human immunodeficiency virus type (HIV-1) retrovirus, not by mutating the genomic viral RNA or RNA transcripts, but by introducing dG-to-dA mutations into the newly synthesized viral DNA (reviewed in Neuberger *et al*., 2003). Furthermore, although RNA is the physiological substrate for Apobec-1, AID (Bransteitter *et al*., 2003; Chaudhuri *et al*., 2003; Dickerson *et al*., 2003; Ramiro *et al*., 2003) and other Apobec-1 family members (Harris *et al*., 2002; Petersen-Mahrt and Neuberger, 2003) can deaminate dC residues of nontranscribed ssDNA or transcribed dsDNA substrates *in vitro*. Furthermore, overexpression of Apobec-1 in bacteria, as well as other Apobec-1 family members including AID, can lead to dC-to-dG mutations in bacterial DNA (Harris *et al*., 2002; Petersen-Mahrt *et al*., 2002). These mutations were dependent on the catalytic function of the transfected deaminase vectors, as mutations in Zn^{+} coordination motifs required for deaminase activity abolished this mutagenic effect (Harris *et al*., 2002). Thus Apobec-1 family members including AID, but not Apobec-1 itself, function via a DNA deamination process that is dependent on ssDNA, rather than the previously proposed RNA-editing model, further weakening the argument that AID acts via RNA editing. Notably, Apobec-1 is unable to induce CSR or SHM when overexpressed in B cells, as does AID (Eto *et al*., 2003; Fugmann *et al*., 2004), although Apobec-1 can function *in vitro* to deaminate DNA; it is conceivable that lack of such complementing activity may, in part, reflect inability to recruit cofactors such as RPA. Overall, current evidence suggests the possibility that

AID may well have evolved from a family of DNA-editing proteins, with Apobec-1 being the outlying protein that may have evolved a new RNA-editing function.

4.5. Mechanism of Class Switch Recombination and Somatic Hypermutation

4.5.1. S Region Transcription and Activation-Induced Cytidine Deaminase Substrate Formation

Mammalian S regions display a strong G-rich nontemplate strand bias (reviewed in Manis *et al.*, 2002b). In this regard, inversion of the Sγ1 region *in vivo* significantly reduces recombination of the corresponding allele (Shinkura *et al.*, 2003). This strongly suggests that CSR, at least to Cγ1, is influenced by the transcriptional orientation of S region sequences *in vivo*. Targeted replacement of Sγ1 sequences with randomly generated purine- or pyrimidine-rich sequences supported these findings (Shinkura *et al.*, 2003). Replacement of Sγ1 with a 1-kb sequence that when transcribed produces a highly purine-rich transcript was able to target recombination to Cγ1, albeit at a reduced level compared with the endogenous Sγ1 sequences (Shinkura *et al.*, 2003). The reduction in CSR efficiency is likely due in significant part to the difference in overall length of available target sequences (1 kb for the synthesized regions compared with 12 kb for the endogenous Sγ1 region) (A. Zarrin, and F. Alt, unpublished data). When this 1-kb sequence was inverted, so that pyrimidine-rich instead of purine-rich transcripts are generated, recombination was reduced to levels comparable to that of an allele completely lacking Sγ1 (Shinkura *et al.*, 2003).

In vitro, transcription of substrates with pyrimidine-rich sequences on the template strand produces purine-rich transcripts that form stable RNA–DNA heteroduplexes (R loops and collapsed R loops) with the DNA template strand and result in stretches of ssDNA that have been shown to exist *in vitro* (Fig. 7) (Mizuta *et al.*, 2003; Reaban and Griffin, 1990; Reaban *et al.*, 1994; Tian and Alt, 2000). Furthermore, R loop formation is orientation dependent, as substantial levels of R loops do not form when these same sequences are transcribed in the opposite transcriptional orientation (Shinkura *et al.*, 2003; Tian and Alt, 2000). Thus R loop structures form under circumstances that also lead to CSR *in vivo* and promote stretches of ssDNA that could provide the necessary substrate for AID deamination (Fig. 7). Notably, such stable R loop structures were demonstrated to occur within endogenous S regions when they were transcribed *in vivo*, indicating that they may well serve the physiological function of providing an AID substrate that was generated from *in vitro* studies (Yu *et al.*, 2003).

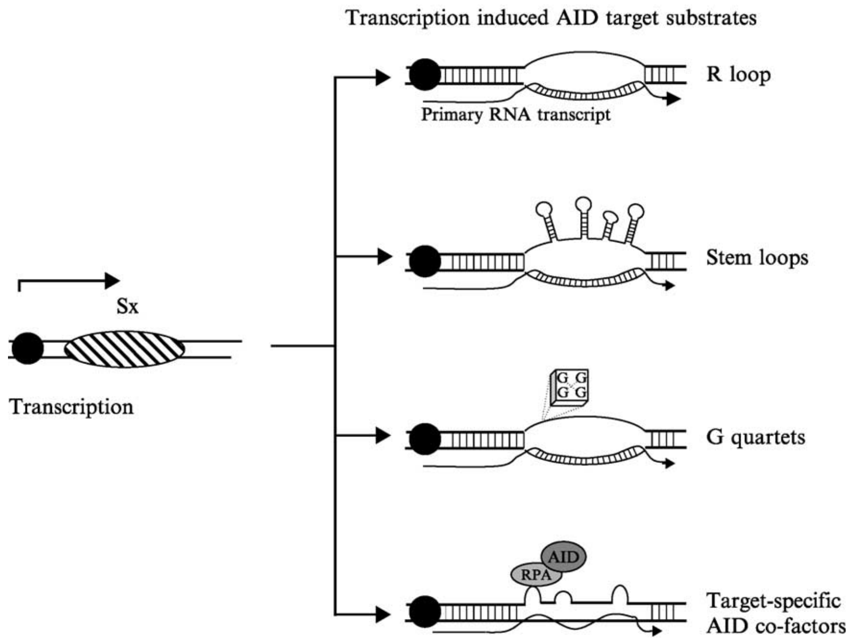

Figure 7 Transcription of DNA results in the formation of secondary structures that provide the target substrate for AID. The indicated secondary structural changes induced into S regions (Sx) by transcription have all been proposed to play a role in CSR and/or SHM. R loops, which have been demonstrated to form *in vivo*, occur when RNA transcripts stably interact with the DNA template strand. AID deaminates cytidine residues preferentially on the coding strand, thus leading to DNA lesions that effect CSR and SHM. Adapted from Chaudhuri and Alt (2004).

Inversion of endogenous Sγ1 sequences did not completely abolish recombination *in vivo* (Shinkura *et al.*, 2003). This would imply that either RNA–DNA hybrid structures are still generated at a reduced level when Sγ1 is transcribed in the nonphysiological orientation, or there are other means of providing the necessary substrates for AID and CSR. In addition, as *Xenopus* S regions are A-T rich instead of G-C rich, the transcription of *Xenopus* S region sequences would not be predicted to form R loops (Muβmann *et al.*, 1997). Transcription of palindrome-containing sequences found in S regions is also prone to the formation of stem–loop structures (Kataoka *et al.*, 1981; Muβmann *et al.*, 1997; Tashiro *et al.*, 2001). Unlike R loops, stem–loop structures should form on transcription of palindromic sequences regardless of transcriptional orientation. However, like R loops, stem loops can promote the formation of short stretches of ssDNA that could provide appropriate substrates for AID. Therefore, there may be several ways in which the unusual

sequence content of S regions could lead to the generation of structures containing ssDNA that would be a substrate for AID activity. Finally, all S regions (from *Xenopus* to mammals) have a high concentration of the consensus SHM motif (most notably AGCT), and evidence suggests that this sequence could target AID in CSR in the context of an AID·RPA complex as it does in SHM (Zarrin *et al*., 2004). It has been proposed that S regions may have evolved from sequences containing large numbers of SHM motifs (e.g., AGCT) in lower organisms to the form found in mammals in which high levels of such motifs remain (to target AID in the context of RPA), but in mammalian S regions, ssDNA generation may have been further augmented via evolution of ability to form R loops (Zarrin *et al*., 2004).

4.5.2. AID-Induced Cytidine Deamination

Cytidine deaminases catalyze the conversion of dC to dU residues via the hydrolytic removal of the amino group at the fourth position of the pyrimidine ring of cytidine (Betts *et al*., 1994). The deamination mechanism is likely to resemble that of adenosine deaminases as both involve a zinc atom in the active site (Betts *et al*., 1994; Harris *et al*., 2002). Deamination of dC residues by AID thereby induces dU/dG mismatches in DNA, the type of DNA lesions normally corrected by the base excision repair and MMR pathways (reviewed in Lindahl, 2000).

4.5.3. Base Excision Repair and Uracil DNA Glycosylase

The base excision repair pathway has evolved to provide protection against structural alterations that can occur in DNA as a result of various endogenous alkylating agents and metabolic reactive oxygen species (reviewed in Lindahl, 2000). Such alterations lead to the formation of aberrant nucleotide residues that are frequently recognized by DNA glycosylases (reviewed in Krokan *et al*., 1997). These glycosylases recognize the aberrant nucleotide and remove it from the DNA backbone, leaving behind an abasic site (reviewed in Krokan *et al*., 1997). In the case of cytidine deamination, this function is performed by UNG. The abasic site is then cleaved by an apyrimidic (AP) endonuclease, followed by nucleotide replacement via the action of a polymerase, which is normally polymerase β (polβ) (reviewed in Lindahl, 2000). The final repair step is ligation, likely involving DNA ligase III (reviewed in Lindahl, 2000).

4.5.4. DNA Deamination Model

The DNA deamination mechanism for CSR and SHM was initially proposed on the basis of the observation that AID expression in bacteria caused mutations that somewhat resemble those induced by SHM (Petersen-Mahrt *et al*.,

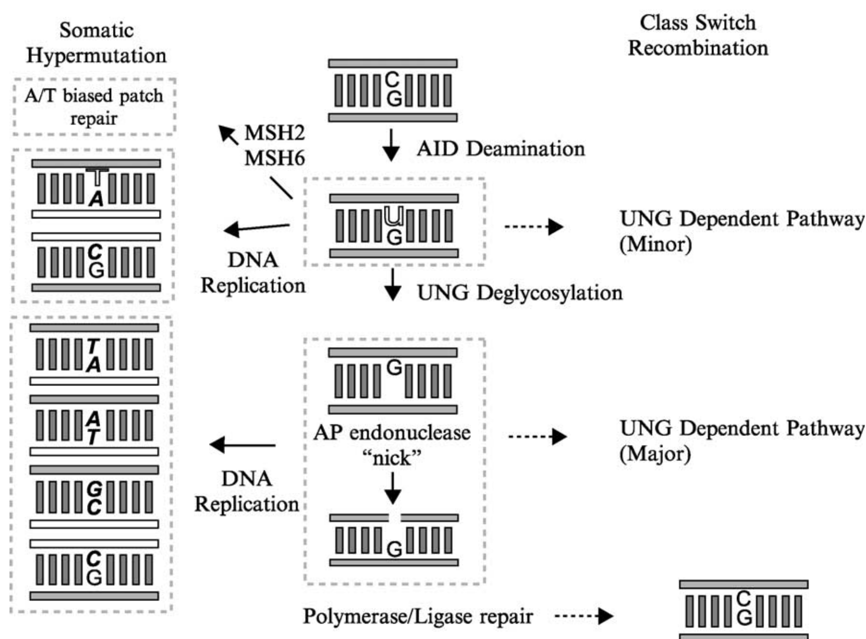

Figure 8 The DNA deamination model for CSR and SHM. AID deaminates cytidine residues in S regions, and in the variable region exons of IgH and IgL genes. Steps that lead to SHM are shown on the left and those that effect CSR on the right. UNG, uracil deglycosylase; AID, activation-induced cytidine deaminase. The minor pathway for CSR refers to CSR in the absence of UNG, likely involving mismatch repair (MMR). Adapted from Rada *et al.* (2002) and Di Noia and Neuberger (2002).

2002). In this model, replication or repair of dU-containing DNA strands leads to both CSR between S regions and SHM of immunoglobulin V_H and V_L exons (Fig. 8; Petersen-Mahrt *et al.*, 2002). Mutations could be generated if the dU residue resulting from AID-induced cytidine deamination is not removed by base excision repair before DNA replication, wherein the dU is read as a dT, thus resulting in dC-to-dT and dG-to-dA transitions (Fig. 8; Petersen-Mahrt *et al.*, 2002). Alternatively, if the abasic site produced by the function of UNG undergoes replication via a translesional polymerase (reviewed by Chaudhuri and Alt, 2004; Reynaud *et al.*, 2003), subsequent repair of the abasic site would lead to both transitions and transversions (Fig. 8; Petersen-Mahrt *et al.*, 2002). Moreover, if an error-prone polymerase were recruited to the abasic site during replication, then mutations could be introduced both in and around the initial deaminase-induced lesion, explaining mutations that arise at non-dC

or non-dG residues in SHM (reviewed in Reynaud *et al.*, 2003). The dU:dG mismatch could also be recognized and processed by components of the MMR machinery, ultimately leading to the generation of DNA breaks.

Regarding CSR, if multiple cytidine residues are deaminated in close proximity and on opposite strands, then base excision repair could lead directly to staggered DNA breaks (Petersen-Mahrt *et al.*, 2002). Processing of the ends of staggered DNA breaks could then result in the formation of DNA DSBs that are either blunt or have short overhangs (reviewed in Reynaud *et al.*, 2003). The subsequent repair of DNA DSBs induced in two different S regions by the NHEJ pathway could thus result in CSR. In addition, there are models whereby MMR could also lead to DSBs after AID deamination (reviewed in Martin and Scharff, 2002a).

In support of the DNA deamination model with respect to SHM, the pattern of hypermutations observed in the hypermutating chicken cell line DT40 changes from transversion-dominated mutations to transitions when UNG function is inhibited (Di Noia and Neuberger, 2002). Furthermore, there is a dramatic shift in the SHM pattern to transitions in immunoglobulin V genes of murine UNG-deficient B cells (Rada *et al.*, 2002b). As transversions are primarily dependent on the removal of dU residues by UNG, these data provide further support for the DNA deamination model. With respect to class switching, CSR is substantially reduced in UNG-deficient mice and humans, in accordance with the predictions of the model (Rada *et al.*, 2002b). In addition, as mentioned above, AID has been shown to be capable of direct deamination of DNA (Bransteitter *et al.*, 2003; Chaudhuri *et al.*, 2003; Dickerson *et al.*, 2003; Petersen-Mahrt *et al.*, 2002; Pham *et al.*, 2003; Ramiro *et al.*, 2003; Sohail *et al.*, 2003; Yu *et al.*, 2004). Finally, the observation that AID associates with transcribed S regions (Chaudhuri *et al.*, 2004; Nambu *et al.*, 2003) provides strong support for the DNA deamination model.

As mentioned above, work has questioned the precise role of UNG in CSR and SHM, as certain UNG mutants that are catalytically inactive in U removal activity *in vitro* are still proficient in mediating CSR and SHM *in vivo*, suggesting that the role of UNG in CSR and SHM is beyond its DNA glycosylase activity (Begum *et al.*, 2004a). These results were surprising given that human patients with similar mutations have profound defects in CSR (Imai *et al.*, 2003a), leading to the speculation that there may be secondary mutations in these patients that contribute to the phenotype (Begum *et al.*, 2004a) or that there is some undetected UNG activity *in vivo* in the mouse mutants; overall, these apparently conflicting findings await further experimentation for full resolution.

4.5.5. Class Switch Recombination versus Somatic Hypermutation Specific Factors

Hyper-IgM syndrome (HIGM) is caused by defects in CSR leading to reduced levels of IgG, IgA, and IgE (reviewed in Durandy, 2001). Until recently, the known causes of HIGM have been thought to affect both CSR and SHM (reviewed in Durandy, 2001). Some patients with HIGM have now been identified with a novel form of HIGM that results in impaired CSR but normal levels of SHM (Imai *et al*., 2003b). B cells isolated from the peripheral blood of HIGM4 patients were activated for CSR *in vitro* and shown to express substantial levels of AID and GTs, yet failed to secrete detectable levels of IgE or IgG (Imai *et al*., 2003b). Furthermore, using a ligation-mediated polymerase chain reaction (LM-PCR) assay, DNA DSBs corresponding to Sμ region sequences were readily detectable in DNA isolated from activated HIGM4 and control B cells (Imai *et al*., 2003a). Thus HIGM4 may arise from defects in processes downstream of DNA deamination that are distinct between CSR from SHM.

CSR and SHM share the requirements for AID-induced DNA deamination; however, AID mutants have now been identified that can differentially effect CSR or SHM. Mutations in the C terminus of AID retain SHM activity but are unable to promote CSR in $AID^{-/-}$ B cells (Barreto *et al*., 2003; Ta *et al*., 2003). AID C-terminal mutants retained DNA deamination function (Barreto *et al*., 2003; Ta *et al*., 2003), and loss of CSR was not due to failure in nuclear transport (Barreto *et al*., 2003). Furthermore, several mutations in the N terminus of AID had nearly normal CSR activity but were unable to mediate SHM of a retroviral GFP expression construct (Shinkura *et al*., 2004). Although some of these C-terminal mutants had defects in nuclear import, their ability to effect CSR suggests inefficient nuclear transport is not the cause of defective SHM (Shinkura *et al*., 2004). AID mutations that uncouple the related but distinct processes of CSR and SHM suggest specific cofactors might exist that interact with these different domains of AID, with RPA being one such potential factor. In this regard, the C terminus of AID has been shown to contain a nuclear export sequence that facilitates nuclear export in a CRM1-dependent pathway (McBride *et al*., 2004). Although AID must be present in the nucleus to effect CSR and SHM, AID is predominantly found in the cytoplasm (Rada *et al*., 2002a). Thus AID cofactors such as RPA may play a role in the retention of AID in the nucleus as well as target specificity.

4.5.6. AID-Induced Double-Strand Breaks

Past studies have documented DNA DSBs in S regions of cells stimulated for CSR (Chen *et al*., 2001; Wuerffel *et al*., 1997) and in variable regions of B cells stimulated for SHM (Bross *et al*., 2000; Papavasiliou and Schatz, 2000). These

findings led to the proposal that DSBs may be intermediates in these processes. On identification of AID, early efforts focused on determining whether AID functioned in the induction or resolution of DNA DSBs (reviewed in Chua *et al.*, 2002). The IgH locus has been shown to colocalize with NBS-1 and γH2AX foci, which are normally associated with DSBs, following CSR activation of wild-type but not AID-deficient B cells (Begum *et al.*, 2004b; Petersen *et al.*, 2001). Colocalization was interpreted to reflect the induction of DNA DSBs in S regions; and, therefore, these studies concluded that AID directly participates in the formation of DNA DSBs during CSR (Petersen *et al.*, 2001). However, H2AX was also found to be associated with V genes (Woo *et al.*, 2003), and, therefore, it is not clear whether the H2AX foci indeed represent breaks at S regions or those at V genes, particularly given that H2AX foci can extend for up to 1 Mb from a DSB (Rogakou *et al.*, 1999). In support of DSB intermediates in CSR, LM-PCR assays have detected both AID- and UNG-dependent S region breaks (Catalan *et al.*, 2003; Imai *et al.*, 2003a).

In several studies, DNA breaks in V gene segments were found at similar frequencies in both wild-type and AID-deficient B cells stimulated to undergo SHM (Bross *et al.*, 2002; Papavasiliou and Schatz, 2002a). These results led to the suggestion that AID was not involved in the induction of DNA DSBs during SHM, and thus it was speculated that AID might instead be somehow involved in the repair of these DSBs (Papavasiliou and Schatz, 2002a). However, it remains unclear whether the DSBs observed in these studies were actually related to SHM; so the significance of the findings remains unclear (reviewed in Chua *et al.*, 2002). Overall, it seems likely that CSR works via a DSB intermediate, whereas SHM does not; this interpretation has been reinforced by the requirement for factors involved in the DSB response (H2AX, DNA-PKcs, Ku, 53BP1, etc.) in CSR but not SHM (see below).

4.5.7. Internal S Region Deletions Are Analogous to Class Switch Recombination

A high frequency of internal S region (intra-S) deletions is detected in the Sμ region of normal B cells and B cell lines activated for CSR (Alt *et al.*, 1982; Bottaro *et al.*, 1998; Hummel *et al.*, 1987; Winter *et al.*, 1987). Intra-Sμ deletions are largely AID dependent, can occur in the absence of an acceptor S region, and are accompanied by mutations in 3′ flanking sequences analogous to those seen in CSR junctions (Dudley *et al.*, 2002). Thus an intra-S region deletion probably reflects the normal CSR mechanism, but in which recombination has taken place within homologous sequences of a single S region rather than between two heterologous S regions. This could result

via the failure to induce DNA lesions in downstream S regions or in the absence of S region synapsis.

4.5.8. S Region Mutations

AID mutants with N-terminal alterations that result in selective loss of SHM activity, but retain the ability to mediate CSR, also generate mutations in Sμ (Shinkura *et al.*, 2004). Mutations in AID that selectively lose SHM activity could be the result of failure to target the AID mutant to V region sequences, for instance because of inability to interact with RPA (Chaudhuri *et al.*, 2004). In this regard, induction of Sμ mutations by SHM-defective AID mutants would simply reflect the differential targeting of AID and not SHM-versus CSR-specific functions of AID. However, loss of AID-targeting activity does not preclude an SHM-specific function of AID not directly related to a separate CSR-specific activity.

Separate studies involving human or murine C-terminal mutants of AID that promote SHM and gene conversion but not CSR have given conflicting results regarding the induction of Sμ mutations (Barreto *et al.*, 2003; Shinkura *et al.*, 2004). CSR-defective mutants of murine AID induced normal levels of Sμ mutations, whereas most human CSR-defective AID mutants were unable to promote Sμ lesions. Sμ mutations induced by CSR-defective murine AID mutants (Barreto *et al.*, 2003) could be due to the retention of low-level CSR activity as suggested by an analogous human C-terminal AID mutant, and thus be independent of SHM activity (Shinkura *et al.*, 2004). This would imply that AID contains distinct functions for promoting SHM and CSR; or that the CSR-defective mutant forms of AID fail to target S regions. Alternatively, the CSR-defective AID mutants may properly target S regions and effect DNA lesions, as evidenced by the ability to induce mutations in Sμ sequences but fail to complete actual CSR. In this scenario, CSR-defective mutants might be unable to interact with cofactors essential for DNA repair or to facilitate S region synapsis (reviewed by Chaudhuri and Alt, 2004).

4.6. Class Switch Recombination and S Region Synapsis

Recombination between two different S regions takes place over large chromosomal distances (up to ~175 kb), and these regions must be juxtaposed before being joined. Adjoining of S regions could be mediated via association with transcriptional promoters, enhancers, chromatin factors, DNA repair proteins, or AID-associated factors, or by interactions involving the S region sequences themselves.

4.6.1. Promoter/Enhancer Interactions

CSR is reduced in the absence of iEμ, even though the level of steady-state transcription through Sμ appeared unaffected (Bottaro *et al.*, 1998). Promoter/enhancer interactions between iEμ and downstream I promoters could effect juxtaposition of Sμ with the S regions of downstream C_H genes. However, as Sμ transcription detected in the absence of iEμ could be driven by V_H or D_H promoters, normal levels of CSR may be dependent on the specific site of transcriptional initiation rather than overall levels of transcription (Bottaro *et al.*, 1998). In this regard, replacement of iEμ with a *pgk* promoter returns the rate of CSR to approximately normal levels (Bottaro *et al.*, 1998). Similarly, although deletions of HS3b and HS4 in the 3′ RR located downstream of the IgH locus result in the reduction of GTs, a role for these sites in the synapsis of S region sequences cannot be excluded (Pinaud *et al.*, 2001).

4.6.2. H2AX

Effective long-range synapsis of S regions likely relies on chromatin modifications and associated factors, as indicated by studies of H2AX deficiency (Reina-San-Martin *et al.*, 2003). As noted above, AID-dependent H2AX foci are found at the IgH locus in conjunction with IgH CSR (Petersen *et al.*, 2001). SHM is unaffected in H2AX-deficient mice, whereas CSR is substantially impaired (Reina-San-Martin *et al.*, 2003). Intra-S region deletions were detected in H2AX-deficient B cells activated for CSR, demonstrating that accessibility of S regions to the CSR machinery and the basic joining mechanism required for CSR is not impaired by the absence of H2AX (Reina-San-Martin *et al.*, 2003). The recruitment and assembly of repair factors at sites of DNA DSBs by γ-H2AX has been proposed to facilitate the juxtaposition of broken DNA ends and subsequent repair by NHEJ proteins (Bassing and Alt, 2004). Thus γH2AX might similarly promote long-range S region synapsis for the efficient recombination between heterologous S regions. In this regard, H2AX-deficient mice, in the absence of the cell cycle checkpoint protein p53, have been shown to undergo translocations involving S region sequences, perhaps indicating that proper synapsis of S regions during CSR is important for genome stability as well as CSR (Bassing *et al.*, 2003a). The finding that another protein, 53BP1, proposed to work in the H2AX anchoring mechanism, is also required for CSR (but not SHM) further supports this general model (Manis *et al.*, 2004).

4.6.3. DNA-PKcs

Pro-B cells that lack DNA-PKcs are defective for switching to the IgE isotype (Rolink *et al.*, 1996). However, significant levels of CSR to all immunoglobulin isotypes were detected in a study involving SCID mice reconstituted with

rearranged IgH and IgL transgenes, which carry a catalytic mutation in DNA-PKcs that abrogates the kinase activity of DNA-PKcs (Bosma *et al.*, 2002; Cook *et al.*, 2003). In contrast, DNA-PKcs–deficient mice had a significant reduction in CSR to all isotypes except IgG1 (Manis *et al.*, 2002). Expression of DNA-PKcs, albeit catalytically inactive, can be detected in cells from SCID mice, leading to the intriguing possibility that serine/threonine kinase activity of DNA-PKcs is dispensable for CSR, whereas the presence of a noncatalytic DNA-PKcs can provide a necessary function for CSR (Bosma *et al.*, 2002). In the context of this model, why there should still be CSR to IgG1 in DNA-PKcs–deficient mice remains a mystery. The fact that DNA-PKcs–deficient B cells switch to IgG1 and not other isotypes implies that recombination between Sμ and Sγ1 may be mechanistically different than that of CSR between Sμ and other S regions. Alternatively, a general reduction in CSR efficiency in the absence of DNA-PKcs could result in the preferential detection of IgG1 simply because it occurs the most efficiently because of its large size. Whatever the case, it is notable that DNA-PKcs is able to promote synapsis of broken DNA ends *in vitro* (DeFazio *et al.*, 2002), consistent with such a function in CSR. In this regard, transformation/transcription domain-associated protein (TRRAP), a distantly related member of the PI-3 kinase family found in humans with homologs in both yeast and *Caenorhabditis elegans*, apparently lacks kinase activity and appears to instead function as a scaffolding protein during chromatin remodeling (McMahon *et al.*, 1998).

4.6.4. Mismatch Repair

Mlh1- and Pms2-deficient mice have a modest reduction in CSR activity, and sequences isolated from S junctions of Mlh1- and Pms2-deficient B cells have an increased rate of microhomologies compared with wild-type B cells (Ehrenstein *et al.*, 2001; Schrader *et al.*, 2002). Yeast homologs of PMS2 and MLH1 can bind two different DNA molecules simultaneously (Hall *et al.*, 2001), leading to the proposal that PMS2 and MLH1 might facilitate S region synapsis during CSR (Schrader *et al.*, 2003).

4.6.5. Other Factors

LR-1 is a B-cell–specific heterodimeric protein composed of nucleolin and heterogeneous nuclear ribonucleoprotein D (hnRNP D), in which each subunit is capable of low-affinity binding to S region–specific duplex sequences, and with high affinity to sequences in the form of G quartets or G4 DNA (Dempsey *et al.*, 1999; Williams and Maizels, 1991). Consequently, it has been proposed that LR-1 might bind and capture DNA from two different S regions and facilitate their synapsis, thus contributing to CSR (Dempsey *et al.*, 1999).

4.7. Class Switch Recombination and Double-Strand Break Repair

The detection of closed circles of DNA composed of intervening sequences between two different S regions implied that intermediates of CSR occur in the form of DNA DSBs (reviewed in Iwasato *et al.*, 1990; Kenter, 2003). Sequences from CSR junctions demonstrate little or no sequence homology between donor and acceptor S regions, and CSR junctions frequently contain short deletions or insertions of nucleotides, all of which are consistent with the NHEJ pathway of DNA DSB repair (Dunnick *et al.*, 1993). Lending further support for DSBs as intermediates in CSR, deficiencies in assayed NHEJ proteins reduce CSR in mice (Casellas *et al.*, 1998; Manis *et al.*, 1998a, 2002a). Finally, deficiency for 53BP1, a DNA damage-sensing protein that becomes activated in response to DSBs and is found associated with H2AX, also leads to significantly reduced levels of CSR (Manis *et al.*, 2004; Ward *et al.*, 2004).

4.7.1. Ku

Ku-deficient mice do not develop B or T cells; therefore rearranged IgH and IgL genes must be introduced into these animals to derive mature B cells (Casellas *et al.*, 1998; Manis *et al.*, 1998a). The only detectable IgH isotype in the serum of these mice is IgM, and splenic B cells isolated from these animals and stimulated *in vitro* to undergo specific CSR fail to secrete anything other than IgM (Casellas *et al.*, 1998; Manis *et al.*, 1998a). The presence of GTs from downstream C_H genes and DSBs detected in Sγ3 sequences suggested that the defect in CSR was not due to an inability to initiate the process (Casellas *et al.*, 1998; Manis *et al.*, 1998a). However, as Ku-deficient B cells are also defective in proliferation, the lack of CSR could be explained by decreased survival of activated B cells (Manis *et al.*, 1998a). Potentially countering this argument, cells that have undergone several rounds of cell division still do not undergo CSR (Reina-San-Martin *et al.*, 2003), although it is not clear whether these cells might represent those that have failed to be completely activated.

4.7.2. DNA-PKcs and Artemis

DNA-PKcs–deficient mice have significantly reduced levels of serum isotypes (Manis *et al.*, 2002a), whereas SCID mice that carry DNA-PKcs kinase inactive mutations undergo CSR at nearly normal levels (Bosma *et al.*, 2002; Cook *et al.*, 2003). In this regard, CSR occurs normally in the absence of Artemis (Rooney *et al.*, submitted), which is activated on phosphorylation by DNA-PKcs (Ma *et al.*, 2002), whereas Artemis is essential for opening the hairpin-coding ends

generated during V(D)J recombination (Rooney *et al.*, 2002). Therefore, DNA-PKcs may provide functions for the repair of DNA damage induced during CSR, such as stabilization of the repair complex, synapsis of target sequence (see above), or recruitment of other essential proteins to the site of DNA breaks, outside its role in Artemis activation that is required for V(D)J CJ formation.

4.7.3. Ataxia Telangiectasia Mutated

Human patients with ataxia telangiectasia mutated (ATM) have normal levels of SHM in their V region sequences, although an overall reduction in serum immunoglobulin isotypes and an increase in homology at S region junctions suggest that ATM does influence CSR (Pan *et al.*, 2002; Pan-Hammarstrom *et al.*, 2003; Waldmann *et al.*, 1983; reviewed in Regueiro *et al.*, 2000). ATM is activated by DNA damage, thereby phosphorylating and activating cell cycle control proteins p53 and Chk2, and thus inducing cell cycle arrest in cells containing DSBs (reviewed in Khanna and Jackson, 2001; Shiloh, 2001). However, ATM likely functions beyond sensing DNA damage and cell cycle regulation, as indicated by its ability to phosphorylate the DNA repair protein NBS1 (Gatei *et al.*, 2000; Lim *et al.*, 2000; Wu *et al.*, 2000; Zhao *et al.*, 2000). In addition to the increase in homology at CSR junctions, there are fewer mutations and insertions in the sequences around CSR junctions of ATM-deficient B cells than are found in control B cells (Pan *et al.*, 2002). Thus it appears that ATM may function during the repair phase of CSR, although secondary effects caused by defects in B- and T-cell development and survival could also contribute to the observed immunodeficiencies in patients with ATM. In this regard, ATM-deficient mice initially were not found to have clear-cut defects in the production of serum IgH isotypes (Barlow *et al.*, 1996; Xu *et al.*, 1996). However, more detailed analyses have now clearly shown a defect in CSR but normal internal Sμ deletions similar to what is seen in H2AX deficiency, which supports a role for the DNA DSB response in this process and potentially synapsis (Reina-San-Martin *et al.*, 2004; see below).

4.7.4. 53BP1

The role of NHEJ proteins and the likely generation of DNA DSBs during CSR imply the need to sense and respond to such DNA lesions. 53BP1 was found to interact with the DNA damage response and cell cycle checkpoint protein p53 (Xia *et al.*, 2001). 53BP1 was rapidly phoshphorylated in response to IR (Anderson *et al.*, 2001) and was found in foci that are thought to represent sites of DNA damage (Anderson *et al.*, 2001; Schultz *et al.*, 2000). Furthermore, 53BP1 colocalized with γ-H2AX in nuclear foci that appear after

DSB induction (Rappold *et al.*, 2001; Rogakou *et al.*, 1999). 53BP1-deficient B cells were dramatically impaired for CSR; although the production of germline transcripts and induction of AID expression were normal (Manis *et al.*, 2004; Ward *et al.*, 2004). In contrast, both V(D)J recombination and SHM occurred normally in 53BP1-deficient mice (Manis *et al.*, 2004; Ward *et al.*, 2004). Thus CSR is highly dependent on DNA damage-sensing proteins downstream of AID induction and, thus, likely to influence the DNA repair/S region joining phase. Finally, rare Sμ-Sγ1 switch junctions amplified from 53BP1-deficient B cells are qualitatively similar to wild-type junctions, demonstrating that 53BP1 does not mechanistically affect CSR (Manis *et al.*, 2004). In the context of these observations, it has been suggested that 53BP1 may work with H2AX for S region synapsis via an anchoring mechanism (Bassing and Alt, 2004; Manis *et al.*, 2004).

4.7.5. H2AX

Phosphorylation of H2AX on Ser-139 occurs within minutes after treatments that introduce DNA DSBs in yeast and mammalian cells (Downs *et al.*, 2000; Rogakou *et al.*, 1998). γ-H2AX appears in discrete nuclear foci that correlate in frequency and nuclear location with induced DSBs (Rogakou *et al.*, 1999). The rapid appearance of γ-H2AX foci after the induction of DSBs precedes that of DNA repair proteins, suggesting that γ-H2AX may be involved in the recruitment of specific repair factors such as BRCA1, MRE11, RAD50, and NBS1 to sites of DNA damage (Paull *et al.*, 2000). Whereas H2AX is required for efficient CSR and AID-dependent foci formation at the IgH locus (see above), it is not required for the process of intra-S region deletions and has been suggested to be therefore involved in long-range synapsis (Reina-San-Martin *et al.*, 2003), which might occur via an anchoring mechanism as outlined above (Bassing and Alt, 2004; Manis *et al.*, 2004).

4.7.6. NBS1

NBS1 is a DNA repair protein associated with the hMre11/hRad50/NBS1 complex that forms nuclear foci in response to DSB-inducing DNA damage and is a target of ATM-mediated phosphorylation (Carney *et al.*, 1998; Maser *et al.*, 1997; Nelms *et al.*, 1998; Wu *et al.*, 2000; Zhao *et al.*, 2000). In yeast, *scmre11* and *scrad50* mutants have defects in NHEJ and have been linked genetically to the same NHEJ pathway as *yku70* and *lig4*, and *Mre11*, also implicated in microhomology-mediated DNA break repair (reviewed in Critchlow and Jackson, 1998; Paull and Gellert, 2000). Furthermore, NBS1 has been detected at nuclear foci that colocalize with the IgH loci in B cells activated to undergo CSR, and this colocalization was dependent on the

presence of AID (Petersen *et al*., 2001). However, the levels of most serum isotypes in patients with Nijmegan breakage syndrome (NBS) are not substantially reduced (reviewed in Shiloh, 1997). B cells of patients with NBS do have an increased frequency of S region junctions with "imperfect" microhomology (four or more nucleotides, with only one mismatch) compared with controls, albeit from a limited number of samples (Pan *et al*., 2002). Thus NBS1 may play a role in the DNA repair phase of CSR or, given its association with γ-H2AX, it could be involved in S region synapsis.

4.7.7. Mismatch Repair

During DNA replication, MMR proteins recognize improperly paired nucleotide base pairs and mediate the removal and reinsertion of the correct nucleotide based on the DNA template strand (Buermeyer *et al*., 1999). Several studies have found an overall decrease in the rate of CSR in the absence of certain MMR proteins (Ehrenstein and Neuberger, 1999; Schrader *et al*., 1999, 2002). CSR junctions were found to occur more frequently in consensus GAGCT and GGGGT sequences, reminiscent of "hot spot" targeting of SHM in the absence of Msh2 (Ehrenstein and Neuberger, 1999; Phung *et al*., 1998). Moreover, CSR junctions isolated from B cells of Msh2-deficent mice were found to have slightly decreased lengths of microhomology (Schrader *et al*., 2002). This would be consistent with the DNA deamination model of CSR, as MMR proteins can extend the region of mutations beyond the original dU residue induced by the function of AID and UNG (Petersen-Mahrt *et al*., 2002). These results are consistent with Msh2 playing a more important role in end processing, specifically the removal of 3′ nonhomologous overhangs outside potential regions of microhomology (Schrader *et al*., 2002). In contrast, there was an increase in microhomology length detected in the CSR junctions of Mlh1- and Pms2-deficient B cells (Schrader *et al*., 2002). The increase in microhomology at CSR junctions of Pms2- or Mlh1-deficient B cells might reflect a role for stabilizing CSR intermediates or for S region synapsis, thus requiring increased sequence homology in their absence for adequate base pair interactions (Schrader *et al*., 2002, 2003).

5. CSR-Related Diseases

5.1. Hyper-IgM Syndrome Types 1 and 3

Hyper-IgM (HIGM) syndromes are immunodeficiencies caused by genetic defects that result in abrogation or impairment in CSR (reviewed in Durandy and Honjo, 2001). The first described was X-linked hyper-IgM, or

HIGM type 1 (HIGM1), caused by mutations in the gene encoding the CD40 ligand (CD40L), a membrane glycoprotein expressed on activated T cells (Allen *et al.*, 1993; Aruffo *et al.*, 1993; DiSanto *et al.*, 1993). CD40L interacts with CD40, a member of the tumor necrosis factor receptor family, that is constitutively expressed on B cells and is variably expressed on T cells, monocytes, basophils, dendritic cells, and endothelial cells (reviewed in Grammer and Lipsky, 2000). CD40L binds to CD40 and induces B cell proliferation (Nishioka and Lipsky, 1994; Tohma and Lipsky, 1991), AID induction (Muramatsu *et al.*, 1999) and the production of some immunoglobulin GTs (Fujita *et al.*, 1995; Jumper *et al.*, 1994; Warren and Berton, 1995). Removal of either CD40 or CD40L through the use of anti-CD40L antibodies (Foy *et al.*, 1993, 1994) blocks germinal center formation, SHM, and CSR in response to T-dependent antigens. Genetic defects in CD40 lead to an autosomal recessive form of hyper-IgM, HIGM3, similar to that caused by the absence of CD40L (Ferrari *et al.*, 2001). Thus HIGM1 and HIGM3 are caused by the ablation of upstream signaling pathways leading to CSR and SHM activation.

5.2. Hyper-IgM Syndrome Type 2

Autosomal recessive hyper-IgM syndrome type 2 is caused by mutations abrogating the expression or function of AID (Revy *et al.*, 2000). Patients lacking AID have enlarged lymph nodes with correspondingly expanded germinal centers (Revy *et al.*, 2000), a characteristic also seen in AID-deficient mice (Muramatsu *et al.*, 2000). These oversized germinal centers likely reflect the presence of activated B cells that are unable to effect CSR or SHM, and thus accumulate in B-cell follicles of the peripheral lymph tissue.

5.3. Hyper-IgM Syndrome Type 4

Patients with HIGM4 are substantially impaired for CSR, whereas SHM can be detected in V_H regions at levels comparable to that of controls (Imai *et al.*, 2003b). Defects in AID, UNG, or in the expression of GTs were eliminated as possible causes of HIGM4. Evidence for the existence of a factor differentially involved in CSR versus SHM is in keeping with the DNA deamination model, in which CSR is effected via DSB intermediates, whereas SHM can be induced in the absence of DSBs (Petersen-Mahrt *et al.*, 2002). Thus HIGM4 is likely caused by defect(s) in factors associated with the targeting of AID to S regions that affect the synapsis of S regions and/or that are involved in an aspect of DNA DSB repair (reviewed in Manis and Alt, 2003).

5.4. X-Linked Hypohydrotic Ectodermal Dysplasia

Another human disease, X-linked hypohydrotic ectodermal dysplasia (XHM-ED), is characterized by hyper-IgM immunodeficiency caused by missense mutations in the gene encoding NF-κB essential modulator (NEMO) (Doffinger *et al*., 2001; Jain *et al*., 2001). NEMO interacts with NF-κB kinases IKK1 (IκB kinase 1) and IKK2 and is essential for NF-κB activation (Yamaoka *et al*., 1998). Engagement of CD40 on the surface of B cells with T-cell–expressed CD40L leads to the induction of NF-κB family transcription factors (Berberich *et al*., 1994; Francis *et al*., 1995; Lalmanach-Girard *et al*., 1993). NF-κB family transcription factors mediate the production of Iγ1-Cγ1 (Lin and Stavnezer, 1996; Lin *et al*., 1998) and Iε-Cε (Iciek *et al*., 1997) GTs. Not all immunoglobulin GTs are dependent on NF-κB; thus mutations affecting NF-κB signaling would be predicted to abrogate CSR to some but not all C_H genes. However, patients with XHM-ED have undetectable levels of all serum IgGs, and B cells activated *in vitro* with anti-CD40 fail to effect CSR (Jain *et al*., 2001). Thus NF-κB signaling in B cells, as with upstream CD40- and CD40L-mediated signaling, is likely involved in overall activation of CSR, perhaps as an activator of AID, although an affect on AID expression in these patients has yet to be reported. The developmental aspects of XHM-ED syndrome can be attributed to defective NF-κB signaling through tumor necrosis factor (TNF) family receptors expressed on embryonic and fetal ectoderm-derived tissues (Doffinger *et al*., 2001). Thus genetic mutations that affect CD40, CD40L, or CD40-mediated downstream signaling molecules all lead to immunodeficiencies with hyper-IgM characteristics.

6. Concluding Remarks

V(D)J recombination and CSR (and the related process of SHM) lead to the direct alteration of DNA sequences and content in cells of the vertebrate immune system. V(D)J recombination occurs both in developing B and T lineage cells; whereas CSR and SHM occur only in mature B lineage cells. The potential for deleterious or catastrophic consequences during the manipulation of a cell's genetic material is obvious; and aberrant V(D)J recombination and CSR, and perhaps SHM, have all been implicated in translocations and other genetic alterations that underlie T lineage [V(D)J recombination] and B lineage [V(D)J recombination, CSR, and SHM] lymphomas. Therefore, all three of these potentially dangerous genomic alteration processes require tight regulatory control mechanisms. In this context, the proteins that initiate these genetic alterations, namely RAGs for V(D)J recombination and AID for CSR

and SHM, are expressed in tissue- and lineage-specific fashion and are subject to strict control via posttranslational regulatory processes. Likewise, there is control of the substrate DNA, RS sequences for V(D)J recombination, and S regions for CSR, such that its availability to the initiating enzymes is largely limited to the appropriate cell types and sequences. Among the major outstanding questions is the issue of precisely what aspects of target RS or S region DNA in chromatin make them good substrates for RAG or AID in the appropriate cell types, lineages, and/or activation stages.

One fundamental difference between CSR and V(D)J recombination is in the nature of the target sequences recognized by the "recombinase." RAG-mediated cleavage at the junction of antigen receptor gene-coding segments is site specific and dependent on short, well-defined *cis*-acting RSs. In contrast, AID deamination of cytidine residues, which appears to initiate CSR (and SHM), is targeted to large S regions that lie upstream of C_H genes in the IgH locus, with recombination occurring throughout the 1- to 12-kb repetitive sequences. Thus, CSR is region specific rather than site specific. Moreover, AID does not appear to recognize specific target sequences with the same degree of specificity as RAGs, which, in general, recognize specific RS sequences. Instead, AID has been thought to rely on transcription-dependent DNA structures such as R loops that are formed when sequences with certain base compositions are actively transcribed. Yet, S regions are composed of tandem repeat units with frequent repeats of specific motifs favored by SHM. Thus, in this context, there may still be specific sequences, such as the SHM consensus, that are preferentially targeted by AID in conjunction with its RPA partner to provide a further degree of specificity in CSR. Although we now have some idea about how AID is targeted, there is still much to be learned about how AID targeting is so specific for S regions and variable regions and why there is not more wide-scale deamination leading to a higher level of mutation and translocations involving other genes in activated B cells.

In both V(D)J recombination and CSR, the initiating lesion by RAG and AID ultimately appears to lead to a DSB and, subsequently, to employ DSB repair pathways, most likely NHEJ pathways, for the resolution of the DNA breaks. Clearly, the classic NHEJ pathway seals both coding and signal joints in the context of V(D)J recombination. Some evidence suggests this pathway is also responsible for ligating CSR junctions, although more evidence on this point is needed. A significant difference in the joining phase of V(D)J recombination and CSR lies in their relative reliance on the DNA DSB response. Thus, V(D)J recombination occurs relatively unimpaired in the absence of DSB response factors such as H2AX and 53BP1. However, the absence of these factors dramatically impairs CSR. One possible explanation is that the factors are somehow involved in the long-range synapsis of S regions in

the context of CSR via a general anchoring mechanism proposed to hold DSBs together in chromatin before their joining via NHEJ. In contrast, RAG-generated DSBs appear to be held together in a postcleavage synaptic complex by the RAGs themselves, which then recruit the NHEJ factors to complete the reaction. In both V(D)J recombination and CSR, however, we still know little about the actual process of synapsis and how the involved proteins contribute to it.

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