Impaired V(D)J Recombination and Lymphocyte Development in Core RAG1-expressing Mice

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Abstract

RAG1 and RAG2 are the lymphocyte-specific components of the V(D)J recombinase. In vitro analyses of RAG function have relied on soluble, highly truncated "core" RAG proteins. To identify potential functions for noncore regions and assess functionality of core RAG1 in vivo, we generated core RAG1 knockin (RAG1<sup>c/c</sup>) mice. Significant B and T cell numbers are generated in RAG1<sup>c/c</sup> mice, showing that core RAG1, despite missing ~40% of the RAG1 sequence, retains significant in vivo function. However, lymphocyte development and the overall level of V(D)J recombination are impaired at the progenitor stage in RAG1<sup>c/c</sup> mice. Correspondingly, there are reduced numbers of peripheral RAG1<sup>c/c</sup>B and T lymphocytes. Whereas normal B lymphocytes undergo rearrangement of both J<sub>H</sub> loci, substantial levels of germline J<sub>H</sub> loci persist in mature B cells of RAG1<sup>c/c</sup> mice, demonstrating that DJ<sub>H</sub> rearrangement on both IgH alleles is not required for developmental progression to the stage of V<sub>H</sub> to DJ<sub>H</sub> recombination. Whereas V<sub>H</sub> to DJ<sub>H</sub> rearrangements occur, albeit at reduced levels, on the nonselected alleles of RAG1<sup>c/c</sup>B cells that have undergone D to J<sub>H</sub> rearrangements, we do not detect V<sub>H</sub> to D<sub>H</sub> rearrangements in RAG1<sup>c/c</sup>B cells that retain germline J<sub>H</sub> alleles. We discuss the potential implications of these findings for noncore RAG1 functions and for the ordered assembly of V<sub>H</sub>, D<sub>H</sub>, and J<sub>H</sub> segments.

Key words: antigen receptor • DNA cleavage • RS • hybrid joint • immune deficiency

Introduction

Ig and TCR variable region genes are assembled during early lymphocyte development from component variable (V), diversity (D), and joining (J) gene segments. V(D)J recombination is initiated by the RAG1 and RAG2 proteins via introduction of DNA double strand break (DSBs) between the V, D, and J coding segments and flanking recombination signal (RS) sequences. RAG1 and RAG2 are necessary in vivo and sufficient in vitro for initiation of V(D)J recombination (for review see reference 1). After DNA cleavage, RAG-cleaved coding and RS ends are joined by ubiquitously expressed, nonhomologous end-joining (NHEJ) proteins (for review see reference 2).

V(D)J recombination is tightly regulated during lymphocyte development within the context of lymphocyte lineage specificity, developmental stage specificity, and feedback regulation of allelic exclusion (for reviews see references 2, 3). Furthermore, the developmental progression of lymphocytes requires the productive assembly and expression of antigen receptor genes. In developing B lymphocytes, IgH genes are assembled before IgL genes; whereas in developing TCR genes, TCR<sub>α</sub> genes are assembled before TCR<sub>β</sub> genes (for review see reference 3). Both IgH and TCR<sub>β</sub> genes are assembled via an ordered process in which D to J rearrangements proceed to completion on both alleles, followed by V segment rearrangement to a preexisting DJ complex (4, 5). Productive rearrangement and expression of IgH μ chains in ckit<sup>+/−</sup>B220<sup>+/−</sup>/CD43<sup>−/−</sup>/CD25<sup>−/−</sup>/CD19<sup>−/−</sup> progenitor (pro–)B cells and TCR<sub>β</sub> chains in CD4<sup>−</sup>CD8<sup>−</sup>

Abbreviations used in this paper: CyC, cytochrome C; DN, double negative; DP, double positive; DSB, double strand break; NHEJ, nonhomologous end-joining; OS, Omenn Syndrome; pro, progenitor; RS, recombination signal; RSS, RS sequence.
(double negative [DN]) pro–T cells induces cellular expansion and differentiation, respectively, to the corresponding cdT+/B220+/CD43+/CD25−/CD19+ pre–B cell stage and the CD4−/CD8+ (double positive [DP]) thymocyte stage (6, 7). Functional rearrangement and expression of IgL or TCRα chains as part of surface IgM or αβ TCR signals precursor B or T lymphocytes to develop into mature peripheral lymphocytes (for reviews see references 8, 7).

Biochemical activities of the RAG1 and RAG2 proteins have been extensively characterized in vitro using purified proteins and defined DNA substrates (for review see reference 9). Full-length RAG proteins are largely insoluble when overproduced; consequently, in vitro studies have used truncated “core” RAG proteins. Core RAG1 (aa 384–1,008 of 1,040 residues) and core RAG2 (aa 1–383 of 527 residues) comprise the minimal regions of RAG1 and RAG2 necessary for recombination of extrachromosomal substrates in nonlymphoid cells (10–13). Whereas core RAGs mediate the basic V(D)J recombination reaction in vitro, the activities of these truncated proteins are not identical to those of full-length RAGs. In this context, noncore RAG regions influence both V(D)J recombination efficiency and products formed when assayed in cell lines (14–19).

Noncore RAG regions are evolutionarily conserved, suggesting these regions may serve important functions (11). Studies of A-MuLV–transformed pre–B cells have implicated noncore RAG regions in accessory and/or regulatory functions in chromosomal V(D)J recombination (18, 19). Thus, NH2-terminal elements of RAG1 are required for complete D to J rearrangement in A-MuLV transformants (19), whereas the COOH terminus of RAG2 may be more important for V to DJ rearrangement than for D to J rearrangement (18). In this regard, mice expressing core RAG2 in place of endogenous RAG2 (which we will refer to here as RAG2<sup>c/c</sup>) display impaired lymphocyte development at the pro–B and pro–T cell stages, accompanied by decreased levels of V to DJ rearrangement (20, 21). Inactivating mutations in RAG1 or RAG2 lead to a complete block in lymphocyte development in mice (22, 23) and are a cause of human T−B− SCID (24, 25). However, missense mutations in RAG1 or RAG2 that result in reduced, and possibly altered, V(D)J recombinase activity lead to Omenn Syndrome (OS), a disease characterized by lack of B cells and a reduced, oligoclonal T cell repertoire (24, 26). In addition, frameshift mutations in noncore RAG1 regions can result in NH2-terminal RAG1 truncations that lead to OS-like immunodeficiencies (27, 28). To address the role of noncore RAG1 regions in vivo, we have generated mice containing specific replacement of the full-length endogenous RAG1 gene with a gene encoding the mouse core RAG1 protein.

Materials and Methods

Antibodies and Flow Cytometry. Single cell suspensions from lymphoid tissues were stained with antibodies conjugated to FITC, PE, and cytochrome C (CyC) by standard procedures. The following antibody conjugates (BD Biosciences and South-ern Biotechnology Associates, Inc.) were used: CyC anti-CD44, PE anti-CD25, CyC anti-CD4, FITC anti-CD8, FITC anti-CD43, FITC anti-GR1, FITC anti-TCRγδ, CyC and FITC anti-β220, and PE anti-IgM. A total of eight RAG<sup>c/c</sup> mice, six RAG<sup>+/−</sup>, and six RAG<sup>+/+</sup> mice between three and five wk of age were analyzed. pro- and pre–B cell populations were sorted based on CD43 and B220 expression after gating out all cells staining with IgM. Cell sorting was performed on independent BM samples from five RAG1<sup>c/c</sup>, one RAG1<sup>+/−</sup>, and four RAG1<sup>+/+</sup> litters.

Generation and Analysis of B Cell Hybridomas. Single cell splenocyte suspensions from RAG1<sup>c/c</sup>, RAG1<sup>+/−</sup>, and RAG1<sup>+/+</sup> mice were cultured with anti-CD40 (1 μg/ml) and IL-4 (10 ng/ml) for 4 d and activated B cells fused to the NS-1 fusion partner (ATCC TIB-18). Hybridomas were screened for isotype secretion by sandwich ELISA using isotype-specific antibodies from SBA. Genomic DNA from each Ig-secreting hybridoma was assayed for Ig rearrangements by Southern blotting. J<sub>E</sub> rearrangement status and the number of alleles was determined via a J<sub>E</sub>-specific probe on Stul– or EcoRI-digested DNA (29). V<sub>H</sub> to DJ<sub>E</sub> rearrangements were detected with a probe from 5′ of DFL16; V<sub>H</sub> to DI<sub>E</sub> rearrangements will result in deletion of this fragment (hybridomas nos. 10 and 12); whereas nonrearranged alleles or D to J<sub>E</sub> rearranged alleles will retain this band. DFL16 to J<sub>E</sub> joins will result in a band of unique size. Nonrearranged J<sub>E</sub> alleles were confirmed by Southern blot analyses using a probe from 3′ of DQ52 (30). D to J<sub>E</sub> rearrangements result in deletion of this fragment; thus, alleles that have not undergone D<sub>H</sub> to J<sub>E</sub> rearrangements will retain this germline band. Cell lines with only one detectable IgH allele originating from B cells were not further analyzed.

Purification of B and T Cell Fractions. Single cell suspensions of splenocytes from RAG1<sup>c/c</sup>, RAG1<sup>+/−</sup>, and RAG1<sup>+/+</sup> were cultured with anti-CD40 and IL4 for B cell activation or ConA and IL-2 for T cell activation. Activated B cells from day 2 cultures were purified using biotinylated anti-CD19–specific antibodies in conjunction with streptavidin-conjugated MACS microbeads and MACS Separation Columns (Miltenyi Biotech). T cells cultured with ConA + IL-2 for 6 d were purified by Lympholyte-M sedimentation (Cedarlane). Purity of separated B and T cells was analyzed by flow cytometry and found to be at least 90%. DP T cell DNA was isolated by staining thymocytes with FITC anti-CD4 and PE anti-CD8 antibodies and sorting on a MO-Flo (Becton Dickinson). DN3 thymocyte DNA was isolated by first depleting CD4<sup>+</sup> thymocytes using anti-CD4–conjugated MACS microbeads and staining the unbound cell fraction with CYC anti-CD44, PE anti-CD25, and a combination of FITC anti-CD4, -CD8, -B220, -Mac1, -GR1, and -TCRγδ antibodies and sorting on live-gated FITC<sup>−</sup>, CYC<sup>−</sup>, and PE<sup>−</sup> cells.

PCR Analysis of IgH and TCR Rearrangements. Genomic DNA from sorted pro– and pre–B cell populations and purified CD4<sup>+</sup>CD8<sup>+</sup> DP or CD4<sup>+</sup>CD8<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup>CD25<sup>−</sup> DN3 T cells was analyzed for IgH and TCRβ rearrangements by PCR amplification (31, 32). LMPCR for SE breaks were performed as described previously (33). Oligos and PCR primers are listed in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20030627/DC1). Briefly, PCR reactions contained 90, 30, 10, and 3.3 ng or 250, 50, 10 and 2 ng of genomic DNA for IgH and TCRβ amplifications, respectively, 20 pmol of each primer, 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 2.5 mM MgCl<sub>2</sub>, and 0.5 U QIAGEN Taq polymerase. Amplification conditions were as follows: 94°C for 45 s, 60°C for 1 min, 72°C for 2.5 min for 30 cycles. Amount of input genomic DNA was normalized by PCR amplification of a coding exon of the ATM.
gene. PCR products were analyzed by agarose gel electrophoresis, transferred to Zetaprobe membrane, and probed with nested oligonucleotide probes to detect rearrangements. PCR analyses were performed at least three times on genomic DNA samples from sorted B cells and purified T cells, respectively, isolated from at least three RAG1+/− mice and either RAG1+/+ or RAG1+/- littermates.

Generation of Core RAG1 Mice. The pMS127 plasmid containing the core RAG1 coding sequence and three copies of a human c-Myc tag at the COOH-terminal end was provided by M. Sadofsky (Albert Einstein College of Medicine) (11). The core RAG1 coding sequence contains a single nucleotide change (A to G) that results in a methionine to valine substitution at position 484. A similar form, with an additional 9-residue Histidine COOH-terminal tag, has been used extensively in vitro to define the biochemical and V(D)J recombination activities intrinsic to the RAG recombinase (34–36). 2 kb of homologous sequence immediately 5' of RAG1 was cloned along with core RAG1 coding sequence into the SalI site of pLNTK (37). A 3.3-kb MluI to BamHI fragment was cloned into the XhoI site to complete the core RAG1 replacement vector. TC1 embryonic stem cells (provided by P. Leder, Harvard Medical School) were transfected with the targeting vector as described previously (38). A positively selected recombinant was identified by Southern blotting using the 5' and 3' targeting probes (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20030627/DC1). The LoxP flanked neomycin resistance (Neo') gene was deleted by transiently transfecting the ES cell clone with a plasmid-based Cre recombinase expression construct (37). Subclones of this ES cell line were injected into C57Bl/6 blastocysts to generate chimeric mice. RAG1+/- mice were intercrossed to produce 129sv RAG1+/- mice.

Results

The Generation of Mice that Express the Core RAG1 Protein. To evaluate in vivo functions of noncore RAG1 regions, we used gene targeting to replace the endogenous RAG1 gene with a recombinant gene encoding the mouse

<table>
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<td>CD4</td>
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<td>5.9 ± 2.3 x 10^7</td>
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<td>CD8</td>
<td>12 ± 2</td>
<td>83 ± 4</td>
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<tr>
<td>CD44</td>
<td>42 ± 7</td>
<td>45 ± 3</td>
<td>42 ± 7</td>
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<td>CD25</td>
<td>27 ± 6</td>
<td>30 ± 9</td>
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<td>Spleen</td>
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<tr>
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<td>22.8 ± 9</td>
<td>16.4 ± 6</td>
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Figure 1. Flow cytometric analysis of T cell development in RAG1+/- mice. (A) FACS® analysis was performed on thymocytes isolated from RAG1+/+, RAG1+/-, and RAG1+/- littermates stained with CyC anti-CD4 and PE anti-CD8. (B) Double negative thymocytes stained with PE anti-CD44 and FITC anti-CD25 after electronically gating on CD4, CD8, TCRγδ, B220, and GR-1 positively staining cells. (C) Spleocytes from RAG1+/-, RAG1+/-, and RAG1+/- littermates were stained with CyC anti-CD4 and PE anti-CD8 antibodies. (D) Thymocytes were stained with FITC anti-TCRβ after electronically gating on CD4+CD8+ DP cells. Mice were analyzed at 3–5 wk of age; representative data are shown. Total thymus cell counts are indicated over the panels in A, and total spleen cell counts are indicated over the panels in C.
Core RAG1 Mice Exhibit Impaired Lymphocyte Development.

We found reduced thymic cellularity of RAG1/c/c mice compared with RAG1+/c and RAG1+/+/ control mice (average thymocyte count for RAG1/c/c thymuses = 29% ± 11% of controls; Fig. 1 A). FACSm analyses of RAG1/c/c thymocytes demonstrated an almost normal distribution of CD4+/CD8+ (DP) and CD4-/CD8+ or CD4+/CD8+ (single positive) populations (Fig. 1 A). However, RAG1/c/c mice accumulated CD4-/CD8- (DN) pro–T cells with a strikingly high percentage of cells amassing at the CD25-/CD44- (DN3) stage compared with RAG1+/+ and RAG1+/+/ control mice (Fig. 1 B). TCRβ rearrangements are completed within the DN3 stage, with productive TCRβ expression inducing cellular proliferation, differentiation to the CD25+/CD44+ DN4 stage, and cessation of further VB to DJβ rearrangement (allelic exclusion) (for review see reference 39). Thus, the accumulation of RAG1/c/c DN3 thymocytes is consistent with diminished capacity of core RAG1 to efficiently mediate complete V(D)Jβ rearrangements.

TCRα chain expression is not required for the DN to DP transition (for reviews see references 7, 39). However, we found an increased ratio of TCRβ0 to TCRβ+ DP thymocytes in RAG1/c/c mice compared with controls (Fig. 1 D). This is consistent with delayed TCRα rearrangement, since DP thymocytes from mice that lack either the TCRα enhancer or TCRα genes likewise express low levels of TCRβ at their cell surface in the absence of TCRα chains (40, 41). We did not detect TCRβ- DP thymocytes in RAG1/c/c mice, confirming that delayed development due to decreased V(D)J recombination efficiency does not allow progression to the DP stage in the absence of TCRβ gene expression (Fig. 1 D). The absolute numbers of splenic αβ T cells in RAG1/c/c mice were reduced ~50% from that of RAG1+/+ and RAG1+/+ controls (Fig. 1 C). We conclude that overall T cell development in RAG1/c/c mice is impaired with respect to the pro– to pre–T cell transition, accompanied by a modest reduction in overall T cell numbers.

We detected a statistically significant reduction in the percentage and absolute number of CD43+B220+ pre–B cells in RAG1/c/c mice relative to RAG1+/+ and RAG1+/+ control mice (average pre–B cell count RAG1/c/c BM = 6.5 ± 2.9 × 10^5 versus 10.3 ± 1.3 × 10^5 in control littermates, P = .020 by paired t test; Fig. 2 A) (6). Correspondingly, the absolute splenic B cell numbers in RAG1/c/c mice were ~50% of those in control mice (Fig. 2 C). On the other hand, absolute BM CD43+B220+ pro–B cell numbers were relatively normal in RAG1/c/c mice (average pro–B cell count RAG1/c/c BM = 5.2 ± 1.7 × 10^5 versus 5.5 ± 1.5 × 10^5 in control littermates); and the percentages of Hardy fractions D, E, and F in the BM of RAG1/c/c and littermates were also comparable (Fig. 2, A and B) (6). Therefore, B cell development in RAG1/c/c mice shows a modest impairment in the pro– to pre–B cell transition and a corresponding reduction in overall B cell numbers.

Figure 2. Flow cytometric analysis of B cell development in RAG1/c/c mice. (A) FACSm analysis was performed on RBC-free BM cells isolated from both femurs of individual RAG1/c/c, RAG1+/+, and RAG1+/+ littersmates stained with CyC anti-B220, FITC anti-CD43, and PE anti-IgM antibodies and gated on the IgM- population. B220+/CD43+ pro–B and B220+/CD43- pre–B cell populations are shown. (B) B220+/CD43+ BM cells were further stained with PE anti-IgM to determine the percentage of BM cells in Hardy fractions D, E, and F. (C) Splenocytes from RAG1/c/c, RAG1+/+, and RAG1+/+ littersmates were stained with CyC anti-B220 and PE anti-IgM antibodies. Mice were analyzed at 3–5 wk of age; representative data are shown. Total spleen cell counts are indicated over the bottom panels.
The levels of PCR products representing most D/H92521 to J/H92521 rearrangements were reduced in DNA isolated from DN3 T cells of RAG1 c/c mice compared with controls (Fig. 3 A). Similarly, D/H92522 to J/H92522 levels were reduced and were accompanied by increased levels of germline D/H92522/J/H92522 bands (Fig. 3 A). In contrast, D/H92521 to J/H92522 rearrangement levels were comparable between RAG1 c/c and control mice (Fig. 3 A). We found a similar reduction in D/H92521 to J/H92521 and D/H92522 to J/H92522 rearrangements in DNA isolated from sorted RAG1c/c DP thymocytes, but D/H92521 to J/H92522 rearrangements again were not markedly changed (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20030627/DC1). There was also a diminished level of 3’/H11032 D/H9252 broken RS ends in DN3 thymocyte DNA from RAG1c/c mice, consistent with reduced RS cleavage by core RAG1 (Fig. 3 C).

We employed V/H92528- and V/H925210-specific primers in conjunction with 3’/H11032 J/H92521 or 3’/H11032 J/H92522 primers to determine whether Vβ rearrangement is altered in RAG1 c/c versus WT mice. Vβ8 and Vβ10 rearrangements to either Jβ1 or Jβ2 were dramatically reduced in RAG1c/c DN3 thymocytes (Fig. 3 B). Likewise, 5’Dβ RS ends were also reduced in RAG1c/c DN3 thymocytes, consistent with reduced RAG-mediated cleavage during Vβ to DJβ rearrangement (Fig. 3 C). Conversely, there was little or no reduction in Vβ8 or Vβ10 to Jβ2 rearrangements in RAG1c/c DP thymocytes (Fig. S2, A and B), as expected given selection for at least one (productive) Vβ(D)Jβ rearrangement in each DP cell. Therefore, the ability of core RAG1 to mediate TCR H9252 rearrangements in vivo appears compromised for both the D/H9252 to J/H9252 and V/H9252 to DJ/H9252 steps. In WT mice, TCRα genes rearrange on both alleles during the DP T cell stage; thus, all mature T cells will have deleted the sequences located between the 3’ most Vα and 5’ most Jα segments (42). Southern blot analysis using a probe from between Vα and Jα gene segments (probe 8) (40) demonstrated that both TCRα alleles rearrange to completion in mature T cells of RAG1c/c and WT mice (Fig. 3 D). Although this finding does not rule out a modest reduction in TCRα rearrangement in RAG1c/c DP cells, it shows that overall TCRα gene rearrangements are not substantially altered.

(32). The levels of PCR products representing most Dβ1 to Jβ1 rearrangements were reduced in DNA isolated from DN3 T cells of RAG1c/c mice compared with controls (Fig. 3 A). Similarly, Dβ2 to Jβ2 levels were reduced and were accompanied by increased levels of germline Dβ2/ Jβ2 bands (Fig. 3 A). In contrast, Dβ1 to Jβ2 rearrangement levels were comparable between RAG1c/c and control mice (Fig. 3 A). We found a similar reduction in Dβ1 to Jβ1 and Dβ2 to Jβ2 rearrangements in DNA isolated from sorted RAG1c/c DP thymocytes, but Dβ1 to Jβ2 rearrangements again were not markedly changed (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20030627/DC1). There was also a diminished level of 3’ Dβ broken RS ends in DN3 thymocyte DNA from RAG1c/c mice, consistent with reduced RS cleavage by core RAG1 (Fig. 3 C).

We employed Vβ8- and Vβ10-specific primers in conjunction with 3’ Jβ1 or 3’ Jβ2 primers to determine whether Vβ rearrangement is altered in RAG1c/c versus WT mice. Vβ8 and Vβ10 rearrangements to either Jβ1 or Jβ2 were dramatically reduced in RAG1c/c DN3 thymocytes (Fig. 3 B). Likewise, 5’Dβ RS ends were also reduced in RAG1c/c DN3 thymocytes, consistent with reduced RAG-mediated cleavage during Vβ to DJβ rearrangement (Fig. 3 C). Conversely, there was little or no reduction in Vβ8 or Vβ10 to Jβ2 rearrangements in RAG1c/c DP thymocytes (Fig. S2, A and B), as expected given selection for at least one (productive) Vβ(D)Jβ rearrangement in each DP cell. Therefore, the ability of core RAG1 to mediate TCRβ rearrangements in vivo appears compromised for both the D/H9252 to J/H9252 and V/H9252 to DJ/H9252 steps. In WT mice, TCRα genes rearrange on both alleles during the DP T cell stage; thus, all mature T cells will have deleted the sequences located between the 3’ most Vα and 5’ most Jα segments (42). Southern blot analysis using a probe from between Vα and Jα gene segments (probe 8) (40) demonstrated that both TCRα alleles rearrange to completion in mature T cells of RAG1c/c and WT mice (Fig. 3 D). Although this finding does not rule out a modest reduction in TCRα rearrangement in RAG1c/c DP cells, it shows that overall TCRα gene rearrangements are not substantially altered.
Rearrangements of IgH and Igκ in RAG1+/c Mice. We used a PCR approach to analyze D\textsubscript{H} to J\textsubscript{H} and V\textsubscript{H} to DJ\textsubscript{H} rearrangements in sorted CD43\textsuperscript{hi}B220\textsuperscript{int} pro–B and CD43\textsuperscript{lo}B220\textsuperscript{i} pre–B cells of RAG1 c/c and control mice. Normal B cells usually rearrange D\textsubscript{H} to J\textsubscript{H} on both IgH alleles (4, 6). Moreover, all pre–B cells, based on selection for IgH \(\kappa\) chain expression, will have a productive V\textsubscript{H}DJ\textsubscript{H} rearrangement on one of their two J\textsubscript{H} alleles (43). Approximately 40% of normal mature B cells have a nonproductive V\textsubscript{H}DJ\textsubscript{H} rearrangement on their second nonselected allele, with the remaining \(\sim 60\%\) having a DJ\textsubscript{H} rearrangement on the second nonselected allele (4). Therefore, the overall level of V\textsubscript{H} to DJ\textsubscript{H} rearrangements can be reduced by, at least, \(\sim 30\%\) in pre–B or mature B cell populations. Consequently, PCR methods cannot accurately quantify the effects on V\textsubscript{H} to DJ\textsubscript{H} rearrangement in these populations. However, PCR can be used to assay for overall reduction in the level of D\textsubscript{H} to J\textsubscript{H} recombination, which would be reflected by increased levels of germline alleles.

We employed PCR primers located 5’ of D\textsubscript{H}Q52 and 3’ of the J\textsubscript{H}4 gene segment to amplify D\textsubscript{H}Q52 to J\textsubscript{H}4 rearrangements. Assembled D\textsubscript{H}Q52J\textsubscript{H}4 complexes can be excised from the chromosome upon subsequent rearrangement of 5’ D\textsubscript{H} segments with 3’ J\textsubscript{H} gene segments (44, 45).

Therefore, the use of a 3’ primer downstream of J\textsubscript{H}4 permits specific amplification of D\textsubscript{H}Q52 to J\textsubscript{H}4 rearrangements retained within the IgH locus on alleles that lack V\textsubscript{H} to DJ\textsubscript{H} rearrangements. Joining of D\textsubscript{H}Q52 to J\textsubscript{H}4 segments, especially J\textsubscript{H}4, was reduced in both RAG1 c/c pro–B and pre–B cells (Fig. 4 A). Consistent with reduced DJ\textsubscript{H} joining, we observed increased levels of germline J\textsubscript{H} alleles, most notably in the pre–B cell population (Fig. 4 A). We conclude that RAG1 c/c pro–B and pre–B cells have reduced levels of D\textsubscript{H}Q52 to J\textsubscript{H}4 rearrangements. To examine other D\textsubscript{H} segments, we employed a degenerate 5’ D\textsubscript{H} RS primer (31) to amplify most DH to DJ\textsubscript{H} rearrangements. The level of DH to DJ\textsubscript{H}1 and DJ\textsubscript{H}4 rearrangements also was reduced in both RAG1 c/c pro– and pre–B cells, but DJ\textsubscript{H}3 rearrangements to J\textsubscript{H}2 and J\textsubscript{H}3 were present at control levels (Fig. 4 A). Notably, the level of 3’ DFL16 RS ends did not appear to be markedly reduced in RAG1 c/c pro–B cells (Fig. 4 D).

We also analyzed rearrangement of three frequently used V\textsubscript{H} gene segment families (V\textsubscript{H}7183, V\textsubscript{H}Q52, and V\textsubscript{H}558) in RAG1 c/c B lineage cells. We employed a 3’ J\textsubscript{H}4 primer along with 5’ primers specific to these V\textsubscript{H} families to amplify family-specific V\textsubscript{H}DJ\textsubscript{H}4 rearrangements (31). Rearrangement of all three V\textsubscript{H} families were reduced in RAG1 c/c pro–B cells (Fig. 4 B). However, in RAG1 c/c pre–B cells,
rearrangements of these same V<sub>H</sub> families were only modestly reduced (Fig. 4 B). As outlined above, this is not surprising because all pre-B cells will have at least one productive V<sub>H</sub>DJ<sub>H</sub> rearrangement. Overall utilization of tested V<sub>H</sub> families in pro– or pre-B cells was not dramatically altered (Fig. 4 B), indicating that, at least at a gross level, usage of endogenous V<sub>H</sub> families by core RAG1 is not obviously influenced by RS sequence [RSS] or V<sub>H</sub> chromosomal location. We also detected a reduction in the level of 5<sup>'</sup>DFL16 and 5<sup>'</sup>DQ52 RS ends in the DNA of pro–B cells, indicating a reduced capacity of core RAG1 to mediate V<sub>H</sub> to DJ<sub>H</sub> RS cleavage compared with full-length RAG1 (Fig. 4 D). Therefore, RAG<sup>1/c/c</sup> pro–B cells have reduced overall levels of V<sub>H</sub>DJ<sub>H</sub> rearrangements but relative utilization of different V<sub>H</sub> families is not markedly altered.

To analyze Ig<sub>k</sub> rearrangements, we performed PCR analyses on DNA isolated from sorted pro– and pre-B cells using a V<sub>k</sub>-specific primer in conjunction with a primer located downstream of J<sub>k</sub>4. We detected equivalent levels of V<sub>k</sub> to J<sub>k</sub> rearrangements in pre–B cells of RAG1<sup>c/c</sup> and control mice, indicating that the noncore regions of RAG1 are not required for accumulation of normal levels of V<sub>k</sub> to J<sub>k</sub> rearrangements (Fig. 4 C). We also found an equivalent level of 5<sup>'</sup>J<sub>k</sub>1 RS ends in pro–B cells of RAG<sup>1/c/c</sup> and control mice, consistent with the finding that V<sub>k</sub> to J<sub>k</sub> rearrangements are not substantially diminished (Fig. 4 D).

Mature B Cells with Germline J<sub>H</sub> Alleles in RAG1<sup>c/c</sup> Mice. Normally, DJ<sub>H</sub> to J<sub>H</sub> rearrangement occurs on both IgH alleles in developing pro–B cells before the onset of V<sub>H</sub> to DJ<sub>H</sub> rearrangement (6). However, our PCR analyses suggested possible retention of germline J<sub>H</sub> alleles in RAG<sup>1/c/c</sup> pre–B cells. To confirm this, we performed Southern blot analyses on genomic DNA from purified CD19<sup>+</sup> splenic B cells of RAG<sup>1/c/c</sup> and control mice using a J<sub>H</sub>-specific probe. Whereas RAG1<sup>+/+</sup> B cells had, as expected, little or no unarranged J<sub>H</sub> loci, RAG<sup>1/c/c</sup> B cells contained a substantial level of the germline-sized J<sub>H</sub> fragment (Fig. 5 and not depicted). To more precisely quantify the reduction in J<sub>H</sub> rearrangements on the nonproductive allele in RAG<sup>1/c/c</sup> B cells, we analyzed J<sub>H</sub> rearrangements in B cell hybridomas. Previous analyses have shown that a low percentage of hybridomas from WT mice retain germline J<sub>H</sub> alleles (4, 30), despite the fact that essentially no germline J<sub>H</sub> locus is detectable in purified splenic B cells (4, 46). These findings suggested that WT hybridomas with germline J<sub>H</sub> alleles most likely result from tripartite fusions involving non–B lineage cells. Consistent with prior studies (30), Southern blot analyses revealed that most control hybridomas had rearrangements of both J<sub>H</sub> alleles; although a few retained a germline-sized band (Table I). In dramatic contrast, 59% of the RAG1<sup>c/c</sup> B cell hybridomas contained a germline J<sub>H</sub> allele (Fig. 5, A and C; Table I). This very high level of retained germline J<sub>H</sub> alleles in RAG<sup>1/c/c</sup> B cell hybridomas confirms the findings with purified CD19<sup>+</sup> B cells (Fig. 5 and not depicted). Therefore, expression of core RAG1 in the absence of WT RAG1 results in a developmentally altered pattern of IgH locus rearrangement in which V<sub>H</sub> to DJ<sub>H</sub> rearrangements frequently occur in developing

**Figure 5.** IgH rearrangement status in RAG1<sup>c/c</sup> B cell hybridomas. Genomic DNA was isolated from RAG1<sup>c/c</sup> hybridoma cell lines shown to secrete Ig, and the rearrangements were examined by Southern blot analysis. (A). Southern blot analysis of hybridoma DNA using a J<sub>H</sub>-specific probe on StuI-digested DNA for determining J<sub>H</sub> rearrangement status. (B). Southern blot analysis of hybridoma DNA using a 5<sup>'</sup>DFL16-specific probe on EcoR1-digested DNA for determining V<sub>H</sub> to DJ<sub>H</sub> rearrangement status. (C). Southern blot analysis of hybridoma DNA using a 3<sup>'</sup>DQ52-specific probe on StuI-digested DNA for confirming germline IgH rearrangement status. Arrows indicate two hybridomas with V<sub>k</sub>DJ<sub>k</sub> rearrangements on both IgH alleles. The presence of two IgH alleles was confirmed by Southern blot hybridization with the J<sub>H</sub> probe on EcoR1-digested DNA. Representative data are shown.
RAG1<sup>c/c</sup> B lineage cells that have not yet completed D<sub>H</sub> to J<sub>H</sub> rearrangement on both alleles.

To determine the level of V<sub>H</sub>D<sub>H</sub> versus D<sub>H</sub>J<sub>H</sub> rearrangements on the rearranged nonselected J<sub>H</sub> allele of RAG1<sup>c/c</sup> B lineage cells, we also quantified the number of D<sub>H</sub>J<sub>H</sub> and V<sub>H</sub>D<sub>H</sub> rearrangements in control B cell hybridomas. Southern blotting with a probe that hybridizes between the V<sub>H</sub> and D<sub>H</sub> gene segments demonstrated that 26 out of 42 (62%) and 12 out of 42 (28%) of the second alleles contained, respectively, D<sub>H</sub>J<sub>H</sub> and V<sub>H</sub>D<sub>H</sub> rearrangements in control B cell hybridomas (Table I). In contrast, whereas the majority of RAG1<sup>c/c</sup> B cell hybridomas contained one allele in germline configuration, the remaining 23 out of 66 (35%) and 4 out of 66 (6%) of the nonproductive alleles contained, respectively, D<sub>H</sub>J<sub>H</sub> and V<sub>H</sub>D<sub>H</sub> rearrangements (Fig. 5 and Table I). Therefore, there is a significant reduction in the percentage of RAG1<sup>c/c</sup> B cells containing V<sub>H</sub> to D<sub>H</sub> rearrangements on the nonproductive allele.

All RAG1<sup>c/c</sup> B cell hybridomas with germline J<sub>H</sub> loci also contained hybridizing sequences between the most J<sub>H</sub>-proximal V<sub>H</sub> gene segment and the most 5′ D<sub>H</sub> gene segment (Fig. 5 C and Table I) (30), indicating lack of direct V<sub>H</sub> to D<sub>H</sub> rearrangements. In addition, we were unable to amplify direct V<sub>H</sub> to D<sub>H</sub> rearrangements in WT or RAG1<sup>c/c</sup> B cell hybridomas (Table I). Therefore, nearly 40% of the highly conserved RAG1<sup>c/c</sup> clones had P or N nucleotide additions (Fig. S4).

Similarly, using an upstream V<sub>H</sub>81X primer in conjunction with the D<sub>3</sub>R primer that anneals to the 3′ RS sequence of DSP and DFL gene segment families (31), we were unable to amplify direct V<sub>H</sub>81X to D<sub>H</sub> rearrangements (unpublished data). Thus, despite the notable reduction in D<sub>H</sub> to J<sub>H</sub> rearrangement, none of the RAG1<sup>c/c</sup> B lymphocytes appeared to have undergone direct V<sub>H</sub> to D<sub>H</sub> rearrangements on the nonselected second allele (Fig. 5 and Table I). Therefore, RAG1<sup>c/c</sup> mice exhibit a marked impairment in both D<sub>H</sub> to J<sub>H</sub> and V<sub>H</sub> to D<sub>H</sub> rearrangement.

**Discussion**

*Altered V(D)J Recombination in RAG1<sup>c/c</sup> Mice.* We demonstrate that lymphocyte development and V(D)J recombination is modestly reduced in mice that express the core RAG1 protein in place of the WT RAG1 protein. The occurrence of significant levels of lymphocyte development in RAG1<sup>c/c</sup> mice is in accord with findings that the RAG1 core protein maintains substantial levels of cleavage activity in vitro. Thus, nearly 40% of the highly conserved RAG1 protein is not required to access RSSs and initiate V(D)J recombination in the context of chromosomal DNA. In addition, noncore regions of RAG-1 are not absolutely necessary for recruitment of factors required to complete the V(D)J recombination reaction in vivo. Moreover, the ab-

Table I. IgH Status of Hybridomas Derived from Rag1<sup>+/+</sup> or Rag1<sup>c/c</sup> B Cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Experiment</th>
<th>V(D)J/DJ (%)</th>
<th>V(D)J/V(D)J (%)</th>
<th>V(D)J/germline (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAG&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Fusion 1</td>
<td>14 (61)</td>
<td>7 (30)</td>
<td>2 (9)</td>
<td>23</td>
</tr>
<tr>
<td>Fusion 2</td>
<td>7 (64)</td>
<td>3 (27)</td>
<td>1 (9)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Fusion 3</td>
<td>5 (63)</td>
<td>2 (25)</td>
<td>1 (12)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>26 (63 ± 2)</td>
<td>12 (27 ± 3)</td>
<td>4 (10 ± 2)</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>RAG&lt;sup&gt;c/c&lt;/sup&gt;</td>
<td>Fusion 1</td>
<td>7 (33.3)</td>
<td>1 (4.8)</td>
<td>13 (61.9)</td>
<td>21</td>
</tr>
<tr>
<td>Fusion 2</td>
<td>12 (32.4)</td>
<td>2 (5.4)</td>
<td>23 (62.2)</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Fusion 3</td>
<td>4 (50)</td>
<td>1 (12.5)</td>
<td>3 (37.5)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23 (38.6 ± 9.9)</td>
<td>4 (7.6 ± 4.3)</td>
<td>39 (53.9 ± 14.2)</td>
<td>66</td>
<td></td>
</tr>
</tbody>
</table>
solute numbers of prolymphocytes are not as substantially reduced in RAG1<sup>+/−</sup> mice, as is found in NHEJ-deficient mice, suggesting that DNA DSBs initiated by the core RAG1 protein are properly repaired (48, 49). However, we cannot rule out a modest diminution in joining activities. Overall, RAG1<sup>+/−</sup> mice clearly exhibit an impairment in both B and T cell development at the pro to prelymphocyte transition, the same stage at which RAG1<sup>−/−</sup> (and RAG2<sup>−/−</sup>) deficient mice exhibit a complete developmental block (22, 23).

In normal mice, D to J rearrangements take place on both TCRβ or IgH alleles before V to DJ rearrangement (4–6). However, RAG1<sup>+/−</sup> mice develop mature lymphocytes with germline <i>J<sub>H</sub></i> loci on their nonproductive alleles, indicating that the noncore regions of RAG1 are required for fully efficient D to J rearrangement. Retention of germline <i>J<sub>H</sub></i> loci occurs despite the fact that <i>D<sub>H</sub></i> to <i>J<sub>H</sub></i> rearrangement initiates at an earlier developmental stage than <i>V<sub>H</sub></i> to <i>D<sub>H</sub></i> rearrangement (6). Expression of core RAG1 in the absence of WT protein also results in a reduced frequency of <i>V<sub>H</sub></i>|<i>D<sub>H</sub></i>|<i>J<sub>H</sub></i> rearrangements on nonselected alleles that contain <i>D<sub>H</sub></i>|<i>J<sub>H</sub></i> rearrangements. Therefore, reduced frequency of both <i>D<sub>H</sub></i>|<i>H</i> and <i>V<sub>H</sub></i>|<i>D<sub>H</sub></i>|<i>J<sub>H</sub></i> rearrangements on the nonselected alleles in RAG1<sup>+/−</sup> mice likely reflects a decrease in overall V(D)J recombination efficiency. The decreased levels of 3′ <i>D<sub>B</sub></i> RS ends, which are associated with <i>D</i> to <i>J</i> rearrangements, along with the decreased levels of 5′ <i>D<sub>H</sub></i> and 5′ <i>D<sub>B</sub></i> RS ends, which are associated with <i>V</i> to <i>DJ</i> rearrangements, further supports this conclusion. Of note, previous studies showed that Igk and TCRα rearrangements were not reduced in RAG2<sup>+/−</sup> mice (20, 21) which was argued to reflect a correlation between recombination efficiency and specific RS sequence motifs (21). In this regard, we also find that <i>V<sub>α</sub></i> to <i>J<sub>α</sub></i> rearrangements are comparable between RAG1<sup>+/−</sup> and control mice and that the TCRα locus rearranges on both alleles of RAG1<sup>+/−</sup> T cells.

Although the above findings clearly demonstrate that overall V(D)J recombination is affected in RAG1<sup>+/−</sup> mice at the IgH and TCRβ loci, we cannot unequivocally rule out the possibility of more subtle effects of truncated core RAG1 on D to J versus V to DJ rearrangements. Notably, expression of core RAG1 in place of full-length RAG1 resulted in the same or even increased production of <i>D<sub>H</sub></i>|<i>J<sub>H</sub></i> hybrid joints suggesting that noncore regions of RAG1 may preferentially favor this form of hybrid D to J join. In this regard, it is possible that the noncore region of RAG1 may contribute to the proper assembly or stability of synaptic and/or postcleavage complexes, thereby increasing overall recombination efficiency while leading to a decreased frequency of aberrant recombination products. This would be consistent with previous studies showing an increased frequency of hybrid joins and transposition events by core RAGs (17, 50, 51). However, we note that we cannot rule out the possibility that the steady-state increase in <i>D<sub>H</sub></i>|<i>J<sub>H</sub></i> hybrid joins results from a decreased level of secondary <i>D<sub>H</sub></i>|<i>J<sub>H</sub></i> joins that would delete such rearrangements in normal preB cells.

<i>V<sub>H</sub></i> to <i>D<sub>H</sub></i> and <i>V<sub>B</sub></i> to <i>D<sub>B</sub></i>β rearrangements were dramatically reduced in developing lymphocytes of mice that express core RAG2 in place of full-length RAG2 (20, 21). One study concluded that D to J recombination at the IgH and TCRβ loci was unaffected in core RAG2-expressing mice and showed that the reduction in V to DJ rearrangement correlated with decreased RAG cleavage activity at the 5′ D and V RSSs (21). A separate study found a similar effect on V to DJ rearrangements but clearly demonstrated through direct hybridoma analyses that <i>D<sub>H</sub></i> to <i>J<sub>H</sub></i> rearrangement was also impaired in core RAG2-expressing mice (20). However, <i>V<sub>H</sub></i>|<i>D<sub>H</sub></i>|<i>J<sub>H</sub></i> rearrangements on nonselected alleles were not detected in core RAG2-expressing B cell hybridomas, suggesting that the homozygous core RAG2 mutation affects <i>V<sub>H</sub></i> to <i>D<sub>H</sub></i> rearrangement more substantially than <i>D<sub>H</sub></i> to <i>J<sub>H</sub></i> rearrangement (20, 21). In contrast, we have demonstrated <i>V<sub>H</sub></i>|<i>D<sub>H</sub></i>|<i>J<sub>H</sub></i> rearrangements do occur on nonselected alleles of RAG1<sup>+/−</sup> B cell hybridomas and find that reduction in D to J and V to DJ rearrangements are accompanied by reduced cleavage at both 5′ and most 3′ RSSs.

Implications of Altered V<sub>β</sub>/H<sub>9</sub> Rearrangement Patterns in RAG1<sup>+/−</sup> B Lineage Cells. Ordered assembly of IgH and TCRβ genes appears to be mediated through developmental stage-specific D versus V segment accessibility (4, 52). Our detection of mature B cells with nonrearranged IgH alleles demonstrates that the expression of core RAG1 in place of full-length RAG1 allows developing B cells to progress to a stage in which <i>V<sub>H</sub></i> to <i>D<sub>H</sub></i> rearrangement can take place in the absence of <i>D<sub>H</sub></i>|<i>J<sub>H</sub></i> rearrangements on both alleles. Thus, these studies indicate that there is no mechanistic requirement for <i>D<sub>H</sub></i> to <i>J<sub>H</sub></i> rearrangement on both alleles for progression to a developmental stage in which <i>V<sub>H</sub></i> gene segments become accessible. However, we did not detect direct <i>V<sub>H</sub></i> to <i>D<sub>H</sub></i> joins in the absence of <i>D<sub>H</sub></i>|<i>J<sub>H</sub></i> rearrangements in the RAG1<sup>+/−</sup> B cells. Although absence of direct <i>V<sub>H</sub></i> to <i>D<sub>H</sub></i> joins might be explained by recombination efficiency, it would also be consistent with a mechanistic requirement for a <i>D<sub>H</sub></i> to <i>J<sub>H</sub></i> deletional rearrangement before a subsequent <i>V<sub>H</sub></i> to <i>D<sub>H</sub></i> rearrangement on the same allele. If so, <i>V<sub>H</sub></i> to <i>D<sub>H</sub></i> rearrangement may require deletion of sequences between <i>D<sub>H</sub></i>|<i>Q52</i> and the <i>J<sub>β</sub></i> gene segment and/or be specifically targeted only to an assembled <i>D<sub>H</sub></i> complex versus a nonrearranged upstream germline <i>D<sub>H</sub></i> segment. Thus, rearrangement may allow establishment of <i>V<sub>H</sub></i> gene accessibility, for example, by activating the 5′ <i>D<sub>H</sub></i> RSS via the germline <i>D<sub>H</sub></i> promoter, or may facilitate the capture or synopsis of <i>V<sub>H</sub></i>-associated RS target sequences. In the latter context, it is notable that deletion of the 3′ <i>D<sub>B</sub></i> RSS allowed direct <i>V<sub>B</sub></i> to <i>D<sub>B</sub></i> joining in the absence of <i>D<sub>B</sub></i> to <i>J<sub>β</sub></i> joining (53), suggesting that the 3′ <i>D<sub>B</sub></i> RSS may be responsible for maintaining ordered rearrangement, perhaps by providing a high affinity RAG binding site.

Putative Functions of Noncore RAG1 Regions. The NH<sub>2</sub> terminus of RAG1 contains a zinc finger ring domain involved in homodimerization (54) and cysteine-containing elements with suggested multimerization functions (19). The ring finger domain has E3 ubiquitin ligase activity, al-
though a physiological function has yet to be linked to this activity (55). In addition, three basic regions within the NH$_2$ terminus of RAG1 associate with SRP1 (56), a protein known to interact with nuclear localization sequences and to be involved in facilitating nuclear transport (57). Although mutations in these basic regions affect binding to SRP1, nuclear localization is not dramatically altered because there are additional nuclear localization sequences within the RAG1 core region (56). In this regard, we show that core RAG1 is expressed at somewhat higher levels than WT RAG1, with the majority of protein being located in the nucleus. Elevated expression of core RAG1 compared with full-length RAG1 probably reflects the deletion of sequences regulating its degradation (13, 16, 19).

The observation that both DJ$_{H}$ and V$_{H}$DJ$_{H}$ rearrangements are affected in RAG1$^{c/c}$ mice suggests the core RAG1 protein simply may be inefficient at mediating V(D)J recombination. Likewise, our PCR analyses indicate that the relative efficiencies of certain V$_{B}$ to DJ$_{B}$ rearrangements in RAG1$^{c/c}$ DP T cells is very similar to that of core RAG2-expressing T cells (21). In this context, the modest reduction in peripheral lymphocyte numbers probably reflects a selection and expansion of the few B and T cells that productively rearrange their antigen receptor genes. Although the nature of the V(D)J recombination defect is unclear, one could speculate that multimerization motifs within the noncore regions of RAG1 could be important for synthesis between cleavage complexes and/or other proteins associated with distant RS/coding gene segments, with early disassembly of the cleavage complex occurring in their absence. E3 ubiquitin ligases have been linked to protein degradation, cell cycle control, and DNA repair (58) and that such an activity is contained within the NH$_2$-terminal region of RAG1 raises some intriguing possibilities. For instance, it was shown recently that RAG2 is targeted for degradation by ubiquitination (59). Ubiquitination mediated by RAG1 could ensure that a RAG-associated complex is disassembled and removed from coding gene segments to allow for efficient ligation by NHEJ proteins. Another possibility would be that RAG1-dependent ubiquitination of histones flanking RAG-initiated DSBs may alter chromatin structure to facilitate subsequent repair (55, 60).

**RAG Mutations and Immune Deficiencies.** Mutations in both RAG1 and RAG2 can result in partial recombination activity that in humans can lead to T$^-$B$^-$ SCID and OS. One group of frameshift mutations identified in OS patients leads to alternative ATG usage resulting in NH$_2$-terminally truncated RAG1 proteins (27, 28). In vitro experiments demonstrated that, like the core RAG1 protein, proteins encoded by these frameshift mutant alleles remain partially functional for V(D)J recombination; however, in vivo they lead to dramatically reduced lymphocyte numbers and to immune deficiency (27, 28). Although the NH$_2$-terminal truncation of the murine core RAG1 protein does not fully recapitulate the effects of the frameshift mutant alleles detected in these OS patients, RAG1$^{c/c}$ mice do exhibit some similar phenotypes. For example, germline DB-$\beta$ sequences have been isolated from the T cells of OS patients, demonstrating incomplete TCR$\beta$ rearrangement (61). The targeted core RAG1 replacement results in the deletion of a larger stretch of NH$_2$-terminal sequences than the human frameshift mutations. Yet RAG1$^{c/c}$ mice have only slightly reduced numbers of B and T lymphocytes compared with OS patients that have variable T cell numbers but virtually undetectable B cells (26, 62). Many human RAG1 frameshift mutations result in decreased protein expression and cellular mislocalization (27), but core RAG1 in RAG1$^{+/-}$ mice is expressed at levels higher than that of full-length RAG1 protein and is expressed predominantly in the nucleus. Therefore, development of OS in patients with NH$_2$-terminal frameshift mutations may not simply be due to the absence of the noncore RAG1 regions but may also be influenced by reduction in the overall amount or localization of the truncated RAG1 protein.

The generation of mice harboring mutations in RAG1 that directly mimic those found in human OS patients should provide insights into the relationship between RAG activity and the development of specific immune deficiencies.

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