The B12/23 Restriction Is Critically Dependent on Recombination Signal Nonamer and Spacer Sequences

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*J Immunol* 2003; 171:6604-6610; doi: 10.4049/jimmunol.171.12.6604
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Ag receptor variable region gene assembly is initiated through the formation of a synaptic complex which minimally includes the recombination-activating gene (RAG) 1/2 proteins and a pair of recombination signals (RSs) flanking the recombing gene segments. RSs are composed of conserved heptamer and nonamer sequences flanking relatively nonconserved spacers of 12 or 23 bp. RSs regulate variable region gene assembly within the context of the 12/23 rule which mandates that recombination only occurs between RSs of dissimilar spacer length. RSs can exert additional constraints on variable region gene assembly beyond imposing spacer length requirements. At a minimum this restriction, termed B12/23, is imposed on the \( V_\beta \) to \( DJ_\beta \) rearrangement step by the 5' \( D_\beta \) RS and is enforced at or before the DNA cleavage step of the (V(D)J) recombination reaction. In this study, the components of the 5' \( D_\beta \) RS required for enforcing the B12/23 rule are assessed on chromosomal substrates in vivo in the context of normal murine thymocyte development and on extrachromosomal substrates induced to undergo recombination in nonlymphoid cell lines. These analyses reveal that the integrity of the nonamer sequence as well as the highly conserved spacer nucleotides of the 5' \( D_\beta \) RS are critical for enforcing the B12/23 restriction. These findings have important implications for understanding the B12/23 restriction and the manner in which RS synaptic complexes are assembled in vivo. The Journal of Immunology, 2003, 171: 6604–6610.

The cells of the adaptive immune response can recognize a near limitless number of foreign Ags. The basis for this diversity lies in the manner in which genes encoding Ag receptor chain variable regions are generated during lymphocyte development. Individual developing lymphocytes assemble Ag receptor variable region genes from component variable (V), joining (J), and, in some cases, diversity (D) gene segments by an enzymatic complex collectively referred to as the V(D)J recombinase. This process, termed V(D)J recombination, is regulated in several important contexts during lymphocyte development by constraints imposed on the V(D)J recombination reaction and through alterations in accessibility of gene segments to the V(D)J recombinase (1, 2).

V, D, and J gene segments are flanked by recombination signal (RS)\(^4\) sequences composed of conserved heptamers and nonamers flanking relatively nonconserved 12- or 23-bp spacers (hereafter referred to as 12RSs and 23RSs, respectively) (3). Recombination occurs only between gene segments flanked by RSs of dissimilar spacer length, a restriction known as the 12/23 rule (3). The V(D)J recombination reaction can be generally divided into DNA cleavage and joining steps. The DNA cleavage step is initiated through the formation of a synaptic complex that includes an appropriate RS pair and the recombining-activating gene (RAG) 1 and 2 proteins (4, 5). In the context of a synaptic complex, the RAG-1/2 proteins introduce DNA double-strand breaks at the RS coding segment border, resulting in the formation of blunt phosphorylated signal ends and hairpin-sealed coding ends. Processing and joining of these DNA ends is mediated by proteins of the nonhomologous end-joining pathway of DNA double-strand break repair (1).

RSs can impose significant constraints on variable region gene assembly beyond enforcing the 12/23 rule. This restriction, termed B12/23, has been defined in the TCR \( \beta \) locus (6, 7). TCR\( \beta \) variable region genes are assembled from \( V_\beta \), \( D_\beta \), and \( J_\beta \) gene segments. The murine TCR\( \beta \) locus is composed of \(~35\) \( V_\beta \) gene segments and two \( DJ_\beta \) gene segment clusters, each with a single \( D_\beta \) gene segment (\( DJ_1 \) and \( DJ_2 \)) and six functional \( J_\beta \) gene segments (6). \( V_\beta \) and \( J_\beta \) gene segments are flanked by 23RSs and 12RSs, respectively. \( D_\beta \) gene segments are flanked 5' by 12RSs and 3' by 23RSs. Although direct \( V_\beta \) to \( J_\beta \) rearrangement is permitted by the 12/23 rule, \( D_\beta \) gene segments are used in the assembly of essentially all TCR\( \beta \) variable region genes (8). This is due to a requirement for the 5'\( D_\beta \) 12RS to efficiently target rearrangement of \( V_\beta \) 23RSs beyond simply enforcing the 12/23 rule (6, 7). At a minimum, the B12/23 restriction ensures \( D_\beta \) gene segment utilization which is important for generating a diverse repertoire of functional TCR\( \beta \) chains (8).

The B12/23 restriction is imposed at or before the DNA cleavage step of the V(D)J recombination reaction and does not exhibit an absolute requirement for lymphoid-specific factors other than the RAG-1/2 proteins (9–11). Furthermore, the \( DJ_1 \) 12RS rearanges efficiently with \( V_\beta \) and \( J_\epsilon \) 23RSs, demonstrating that \( DJ_1 \) 12RSs enforce the B12/23 restriction in a manner that does not
require specific Vβ/DJ β RS synthesis (10). The 5’ Dβ1 12RSs have heptamers and nonamers that approximate consensus heptamer and nonamer sequences. Notably, a cross-species comparison of Dβ1 12RSs spacer sequences reveals a relatively high level of sequence conservation (see below). Finally, the mouse 5’ Dβ1 12RS contains a consensus TATA box used by the PDβ promoter for the initiation of germline (GL) Dβ1-Jβ1 gene segment cluster transcripts (12–14). Although this consensus TATA box is not conserved in other Dβ 12RSs, it is not known whether these RSs possess other transcript initiation sequences that could contribute to the B12/23 restriction.

In this study, we assess the requirements for different components of the 5’ Dβ RS in enforcing the B12/23 restriction. For this purpose, we generate mutant 5’ Dβ RSs and analyze their function in a TCRβ minilocus that undergoes DJ β to Jβ and Vβ to DJβ rearrangement in developing thymocytes and in extrachromosomal substrates that can recombine in nonlymphoid cell lines. Our findings have important implications for the manner in which rearrangement is regulated within the context of the B12/23 rule and for the assembly of RS synaptic complexes in vivo.

Materials and Methods

Southern blot analysis

Isolation of genomic DNA and Southern blot analysis were conducted as previously described using a 600-bp AccI fragment spanning the Vβ14 gene segment (probe P) (7, 15). Band intensities were determined using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Band intensities were corrected for background and the ratio of VDJ band intensity to DJ band intensity (VDJ/DJ ratio).

Cell culture

The M12 and ES cell lines were maintained and transfected with the TCRβSPF minilocus as previously described (7, 12).

S1 nuclease protection assay

mRNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). For the generation of S1 nuclease protection probes, 406-bp genomic DNA fragments spanning from immediately upstream of the 5’ Dβ1 RSs to the BγIII site 3’ of the Dβ1 gene segment were subcloned into pBSKK. The 471-bp S1 nuclease probes were then generated by PCR using the T7 primer and the ClaI/S1 primer: 5’-AGATCGATCTTTTAAAACAAAAC-3’. This results in S1 probes with ~65 bp of nonhomologous polynucleotide DNA followed by 406 bp that are homologous to the 5’ Dβ1 RSs and downstream region. Initial hybridizations were conducted with 40 μg whole cell RNA and excess end-labeled probes in 15 μl of 80% Formamid (Fluka, Buchs, Switzerland), 40 mM PIPES (pH 6.4), 400 mM NaCl, and 1 mM EDTA at 51°C for 2 h after incubation for 5 min at 100°C. S1 nuclease digestion was conducted in 300 μl of 37°C for 30 min with 300–400 U of S1 nuclease (Promega, Madison, WI). Samples were size fractionated on 7 M urea/8% polyacrylamide gels.

Transient recombination assays

Transient recombination assays using the pC substrate were performed and analyzed as previously described (10).

Results

Rearrangement of the TCRβSPF–Dβ1 minilocus in thymocytes

The TCRβSPF minilocus is composed of a single Vβ gene segment (Vβ14), Dβ gene segment (Dβ1), and two Jβ gene segments (Jβ1.1 and Jβ1.2) linked to the IgH intronic enhancer (Eμ) and constant region gene (Cμ; Fig. 1A) (7). Efficient DJβ and VDJβ rearrangement of this minilocus occurs during thymocyte development in chimeric mice generated by RAG-2-deficient blastocyst complementation (RDBC) using embryonic stem (ES) cells with integrated copies of the TCRβSPF minilocus (7). Furthermore, as is the case with the endogenous TCRβ locus, the 5’ Dβ1 12RS is required to efficiently target Vβ rearrangement in the minilocus during thymocyte development due to constraints beyond simply enforcing the 12/23 rule (7).

The TCRβSPF–Dβ1 minilocus was generated from the TCRβPF minilocus by introducing restriction sites (HpaI and Nhel) that flank the 5’ Dβ 12-RS, allowing for easy replacement of this RS with mutant RSs (Fig. 1A). Thus, the TCRβPF and TCRβSPF–Dβ1 miniloci are identical except for the introduction of these restriction sites. Chimeric mice were generated by RDBC using four

![FIGURE 1. Rearrangement of the TCRβSPF minilocus in thymocytes. A. Schematic of the TCRβPF and TCRβSPF miniloci showing the Vβ14, Dβ1, Jβ1.1, and Jβ1.2 gene segments, the IgH intronic enhancer (Eμ), and constant region gene (Cμ). 12RSs and 23RSs are shown as open and filled triangles, respectively. The TCRβPF and TCRβSPF miniloci differ by the nucleotide sequence modifications used to generate the HpaI and Nhel sites as indicated. The approximate positions of the BγIII (G) and BamHI (B) sites and the probe (P) used for Southern blot analyses are shown. The minilocus is not drawn to scale. B. Genomic DNA isolated from kidney (lane K) or ES cells (lane E) and thymocytes (numbered lanes) harboring the TCRβSPF-Dβ1 minilocus was digested with BamHI and BγIII and subjected to Southern blot analysis using probe P. Thymocytes from chimeric mice generated from four independently derived ES cell lines containing the TCRβSPF–Dβ1 minilocus (TCRβSPF–Dβ1–163, 169, 184, and 189) were analyzed. Shown is the analysis of thymocytes from one chimeric mouse, each generated from clones TCRβSPF–Dβ1–163 (lane 1), 169 (lane 2), 184 (lane 3), and three chimeric mice generated from clone TCRβSPF–Dβ1–189 (lanes 1–3). Bands corresponding to the minilocus in the unrearranged (GL), DJβ (DJ), and VDJβ (VDJ) configuration are indicated. The band generated by the unrearranged Vβ14 gene segment from the endogenous TCRβ locus is indicated (+). Molecular mass markers are also shown.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.6605)
independently derived ES cell lines (TCRβ\textsuperscript{SPF}:Dβ1–163, 169, 184, and 189) containing two to four stably integrated copies of the TCRβ\textsuperscript{SPF}:Dβ1 minilocus (Fig. 1B, Table I). Rearrangement of the TCRβ\textsuperscript{SPF}:Dβ1 minilocus was assayed by Southern blotting of BamHI- and BgII-digested thymocyte genomic DNA isolated from chimeric mice with \(>5 \times 10^7\) thymocytes and normal fractions of thymocyte subsets as assessed by flow cytometry (Fig. 1B and data not shown). Robust levels of Dβ to Jβ and Vβ to DIβ rearrangement of the TCRβ\textsuperscript{SPF}:Dβ1 minilocus were observed in thymocytes from chimeric mice generated from all four TCRβ\textsuperscript{SPF}:Dβ1 miniloci containing ES cells (Fig. 1B).

Bands generated by probe hybridization to miniloci in the unrearranged (GL) DJβ and VDJβ configurations were quantitated by densitometry and the percentage of rearranged miniloci in the VDJβ configuration was calculated as described in Materials and Methods (Table I). Since local integration effects would impact Dβ to Jβ and Vβ to DIβ rearrangement, the fraction of the minilocus in the VDJβ configuration (VDJβ/VDJβ + DIβ) was used to permit quantitative comparisons of the level of Vβ to DIβ rearrangement between thymocytes from chimeric mice generated from ES cell lines with distinct minilocus integrants. The fraction of rearranged TCRβ\textsuperscript{SPF}:Dβ1 miniloci in the VDJβ configuration was similar (37–54%) when comparing seven chimeric mice derived from four independent-derived ES cell lines (Table I). Together these data demonstrate that during thymocyte development, the TCRβ\textsuperscript{SPF}:Dβ1 minilocus undergoes consistently efficient Vβ to DIβ rearrangement.

### The 5' Dβ1 12RS nonamer impacts targeting of Vβ rearrangement

In the endogenous TCRβ locus, replacing the 5' Dβ1 12RS with the Jβ1.2 12RS results in a block in Vβ to Dβ rearrangement (6). The TCRβ\textsuperscript{SPF}:Jβ/H/N minilocus was generated by replacing 5' Dβ1 12RS of the TCRβ\textsuperscript{SPF}:Dβ1 minilocus with a chimeric RS (Jβ/H/N) composed of the Jβ1.2 heptamer/nonamer sequences and the 5' Dβ1 12RS spacer sequence (Figs. 2 and 3A). Thus, the TCRβ\textsuperscript{SPF}:Jβ/H/N and TCRβ\textsuperscript{SPF}:Dβ1 miniloci are identical except for the altered 5' Dβ1 12RS heptamer/nonamer sequences. Chimeric mice were generated by RDBC from two independently derived ES lines harboring the TCRβ\textsuperscript{SPF}:Jβ/H/N minilocus (TCRβ\textsuperscript{SPF}:Jβ/H/N-26 and -33; Fig. 3A). Southern blot analysis of thymocytes isolated from these mice revealed that the TCRβ\textsuperscript{SPF}:Jβ/H/N minilocus undergoes efficient Dβ to Jβ rearrangement but has a severe block in Vβ to DIβ rearrangement (Fig. 3A and Table I).

To investigate the individual contributions of the heptamer and nonamer, chimeric RSs composed of the Jβ1.2 heptamer and 5' Dβ1 spacer and nonamer (Jβ/H) or the Jβ1.2 nonamer with the 5' Dβ1 heptamer and spacer (Jβ/N) were generated (Figs. 2, 3, and 4A). Whereas the TCRβ\textsuperscript{SPF}:Jβ/H minilocus exhibits a modest reduction (≈2-fold) in VDJβ rearrangement, the TCRβ\textsuperscript{SPF}:Jβ/N minilocus exhibits profound reduction in VDJβ rearrangement (Fig. 3C and Table I). Together these findings demonstrate that the 5' Dβ1 12RS nonamer sequence is critical for enforcing the B12/23 restriction.

### The B12/23 restriction is dependent on the 5' Dβ1 RS spacer

The JβP 12RS is composed of the 5' Dβ1 12RS heptamer and nonamer flanking the Jβ1.2 spacer (Fig. 2). Strikingly, the TCRβ\textsuperscript{SPF}:JβP minilocus exhibits a reduction in Vβ to DIβ rearrangement of similar magnitude to that observed for the TCRβ\textsuperscript{SPF}:Jβ/N minilocus (Fig. 3A and Table I). Unlike the Jβ 12RS spacers, the 5' Dβ 12RS spacers exhibit a high degree of sequence homology across different mammalian species (Fig. 4).

<table>
<thead>
<tr>
<th>Minilocus</th>
<th>ES</th>
<th>Chm</th>
<th>GL</th>
<th>DJ</th>
<th>VDJ</th>
<th>DJ + VDJ</th>
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<td>184</td>
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<td>2</td>
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<td>8</td>
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<tr>
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<td>2</td>
<td>5</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>TCRβ\textsuperscript{SPF}:JβN</td>
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<td>1</td>
<td>12</td>
<td>2</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>TCRβ\textsuperscript{SPF}:JβH</td>
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<td>2</td>
<td>3</td>
<td>42</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>TCRβ\textsuperscript{SPF}:JβP</td>
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<td>15</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>TCRβ\textsuperscript{SPF}:CSM</td>
<td>14</td>
<td>17</td>
<td>4</td>
<td>5</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>TCRβ\textsuperscript{SPF}:TGT</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

The ES cell line number for independently derived ES cells harboring different TCRβ\textsuperscript{SPF} miniloci is indicated. The identification numbers of the individual chimeras (Chm) mice generated and analyzed from each ES cell line is also indicated. For example, chimera mouse 1 from ES line 14 harboring the TCRβ\textsuperscript{SPF}:Jβ/H minilocus is TCRβ\textsuperscript{SPF}:Jβ/H-14.1. The percentage of miniloci in the unrearranged (GL), DJβ (DJ), and VDJβ (VDJ) configuration is indicated and was determined as described in Materials and Methods. The percentage of rearranged miniloci in the VDJβ configuration is also indicated, VDJβ/DJ + VDJβ.

Five of the 12 nt are absolutely conserved and 2 additional nucleotides are conserved in all but single RSs (Fig. 4). To test whether these conserved nucleotides are important for enforcing the B12/23 restriction, the CSM 12RS was generated by replacing the
seven conserved nucleotides in the 5′ Dβ 12-RS with the corresponding bases in the Jβ1.2 spacer resulting in 4 bp changes (Fig. 2). Similar to what was observed for the TCRβSP:JβSP minilocus, a severe reduction in Vβ to DJβ rearrangement was observed for the TCRβSP:CSM minilocus, highlighting the importance of these conserved spacer nucleotides in enforcing the B12/23 restriction (Table I).

The 5′ Dβ1 12RS TATA box is not required to target Vβ rearrangement

As demonstrated above, targeting of Vβ rearrangement by the 5′ Dβ1 12 RS relies on features of the nonamer and spacer sequences. A consensus TATA box, utilized by the PDβ1 promoter, spans these sequences and is not present in the mutant 12RSs (JβSP, CSM, JβH/N, and JβJN) that fail to efficiently target Vβ rearrangement (Fig. 2). Unlike the 5′ Dβ1 12 RS, the 5′ Dβ2 12 RS does not contain a consensus TATA box sequence (Fig. 2). To determine whether this RS is capable of efficiently targeting the Vβ rearrangement, the TCRβSP:12-RS2 minilocus was generated in which the 5′ Dβ1 12 RS was replaced with the 5′ Dβ2 12 RS (Fig. 2). This minilocus undergoes efficient Vβ to DJβ rearrangement, demonstrating that the 5′ Dβ2 12 RS is capable of efficiently targeting Vβ rearrangement (Fig. 5 and Table I).

Although lacking a consensus TATA box, the 5′ Dβ2 12 RS may possess cryptic transcript initiation sequences. To investigate this possibility, the TCRβSP:12-RS1 and TCRβSP:12-RS2 miniloci were stably introduced into the M12 cell line and transcript initiation from the PDβ1 promoter was assayed by S1 nuclease protection (Fig. 6). These analyses revealed a predominant transcript initiating from the TCRβSP:12-RS1 minilocus approximately 20 bp downstream of the 5′ Dβ1 RS TATA (+20, Fig. 6). These transcripts are similar to PDβ1 promoter-specific transcripts previously observed in the endogenous locus during thymocyte development and in the TCRβ minilocus in the M12 cell line (12, 14). Transcripts that initiate at this position or others downstream of the 5′ Dβ2 RS were notably absent in M12 cells harboring the TCRβSP:12-RS:Jβ2 minilocus (Fig. 6). Together, these findings demonstrate that, unlike the 5′ Dβ1 12 RS, the 5′ Dβ2 12 RS contains neither a consensus TATA box nor sequences that mediate transcript initiation immediately downstream of the RS, yet the 5′ Dβ2 12 RS is capable of efficiently targeting the Vβ rearrangement.

The TGT 12 RS was generated by converting the TATATA TATA box in the 5′ Dβ1 12 RS to TGTAAA (Fig. 2). Analysis of transcripts initiating from the TCRβSP:TGT minilocus in M12 cells revealed a complete loss of the TATA-specific +20 transcripts observed in the TCRβSP:12-RS1 minilocus (Fig. 6). However, the TCRβSP:TGT minilocus exhibited only a mild reduction (~2-fold) in Vβ to DJβ rearrangement in developing thymocytes (Table I). Together, these findings demonstrate that a functional TATA box is required, or transcript initiation from, the 5′ Dβ 12 RSs is not required for enforcement of the B12/23 restriction.

The B12/23 nonamer and spacer sequences requirements are enforced on extrachromosomal substrates in nonlymphoid cell lines

The pC competitive extrachromosomal recombination substrate has three positions (P1, P2, and P3) for RSs cloning (Fig. 7) (10). The pC substrate containing appropriate RS combinations can undergo rearrangement in nonlymphoid cell lines that transiently express the RAG-1/2 proteins (10, 17). Rearrangement of the RSs cloned at P1 to RSs cloned at P2 or P3 deletes the transcriptional terminator, permitting expression of the chloramphenicol acetyltransferase (CAT) gene which allows quantitation of rearrangement efficiency after bacterial transformation as previously described (10, 17). Rearrangement of the RS at P1 to the RS at P2 or P3 is assayed by PCR.

The pC:V14DD substrate has the Vβ14 23 RS at P1 and the 5′ Dβ1 12 RSs at P2 and P3 (10). As previously demonstrated, an intrinsic bias for rearrangement of the Vβ14 23 RSs to the 5′ Dβ1 12 RSs at P2 or P3 is not observed in Chinese hamster ovary cells expressing the RAG-1/2 proteins (Table II) (10). The pC:V14DDH/N substrate was generated from the pC:V14DD substrate by replacing
the 5′ DJ1 12RS at P3 with the Jβ H/N 12RSS. This substrate exhibits a profound bias for rearrangement of the Vβ14 23RS to the 5′ DJ1 12RS at P2 over the Jβ H/N 12RSS at P3 similar in magnitude to that observed for the pC•V 1.2 substrate which has the Jβ1.2 12RS at P3 (Table II) (10). Analysis of extrachromosomal recombination substrates with the Jβ H (pC•V 1.2), and Jβ N (pC•V 1.1) RSs reveals that replacing the 5′ DJ β RS nonamer (Jβ N) has a more profound effect on Vβ RS targeting than replacing the heptamer (Jβ H) which had only a modest effect (Table II). Finally, the pC•V 1.2 substrate exhibits severely diminished levels of rearrangement of the Vβ 23RS to the CSM 12RS, as compared with the 5′ DJ β 12RS (Table II). Together these findings demonstrate that, similar to what was observed for rearrangement of the minilocus in vivo during thymocyte development, enforcing the B12/23 restriction on extrachromosomal substrates in nonlymphoid cells appears to be critically dependent on the 5′ DJ 1 12RSS nonamer and spacer.

**Discussion**

Assembly of TCRβ variable region genes during lymphocyte development is ordered with DJ to Jβ rearrangement preceding Vβ to DJβ rearrangement. Jβ 12RSS readily rearrange with 3′ DJβ 23RSS yet fail to rearrange efficiently with Vβ 23RSSs that rearrange efficiently with 5′ DJ β 12RSSs due to B12/23 constraints imposed on Vβ rearrangement by the 5′ DJ β 12RSS (6, 7). In this study, we show that the B12/23 restriction is critically dependent on the nonamer and spacer sequences of the 5′ DJ β 12RSS. This nonamer and spacer sequence requirement was observed for rearrangement both on chromosomal substrates in the setting of thymocyte development and on extrachromosomal substrates in nonlymphoid cell lines.

The CAC trinucleotide of the consensus heptamer sequence (CACAGTG) is essential for RS function (18, 19). As expected, this CAC trinucleotide is absolutely conserved in the 5′ DJ β and Jβ 12RSS heptamer sequences. With respect to the four remaining nucleotides, the DJ β 12RSS heptamers (CACAGTG) differ from consensus at one position whereas the Jβ 12RSSs, except for Jβ 1.1, differ from consensus at two or three of these positions (20). Deviations of these four nucleotides from consensus can affect the efficiency of recombination of extrachromosomal substrates (18, 19). However, the heptamer sequence differences between the 5′ DJβ and Jβ 12RSSs do not appear to be the critical factor for enforcing the B12/23 rule as evidenced by the modest reduction (~2-fold) in Vβ 23RSS rearrangement to the JβH 12RSS on chromosomal and extrachromosomal substrates. Thus, features of the 5′ DJ β 12RSS heptamer, other than the essential CAC trinucleotide, do not significantly impact the B12/23 restriction imposed on Vβ to DJβ rearrangement.

In striking contrast to the heptamer, replacing the nonamer of the 5′ DJ β 12RSS with the Jβ 1.2 nonamer (JβN RS) results in a profound block in Vβ to DJβ rearrangement of the TCRβ minilocus and on extrachromosomal substrates. Nonamer sequences vary widely with some deviating considerably from consensus (ACAA AAACC). Although there are no strict nonamer sequence requirements, alterations in one or both bases of the nonamer AA dinucleotide (ACAAAAACC) have led to reduced recombination efficiency on extrachromosomal substrates (18). This AA dinucleotide is intact in the 5′ DJ β, but not the Jβ 1.2, 12RSS nonamer. Although this difference may contribute to the B12/23 restriction, it is not likely to be the sole determinant of the nonamer requirement as many other Jβ 12RSS nonamers have the AA dinucleotide (20).

The strict requirement for RS spacer nucleotide length is well established (18, 19). Single nucleotide deviations in spacer nucleotide length result in a reduction in rearrangement efficiency, and a gain or loss of two or more nucleotides essentially abolishes RS function (18, 19). Although the importance of spacer nucleotide sequence is less well established, a low level of spacer sequence conservation has been noted upon analysis of a large number of RSs (20). Furthermore, spacer sequence variations have been implicated in affecting the efficiency of RS utilization (21–24). Perhaps most notably, Vκ RS spacer sequences affect rearrangement...
efficiency on extrachromosomal substrates with these effects likely impacting differential Vκ gene segment utilization in vivo (22). Analysis of the 5′ Dβ 12RS spacer sequences across species reveals a remarkable level of conservation with 5 of the 12 nt abutting the 5′ heptamer and spacer nucleotides with the corresponding Jβ1.2 12RS spacer nucleotides (29). Thus, it is possible that the nonamer and spacer sequences are required to enforce the B12/23 restriction. It is unlikely, though, that this requirement for the binding of lymphoid-specific factors to nonamer and/or spacer sequences since these sequences are required to enforce the B12/23 restriction on extrachromosomal substrates in a nonlymphoid cell line. Alternatively, and perhaps more plausibly, a common B12/23 mechanistic constraint relies on features of both the nonamer and spacer sequences. Notably, this is not due to a common B12/23 mechanistic constraint relies on features of both the nonamer and spacer sequences to enforce the B12/23 restriction. It is conceivable that independent mechanistic constraints rely on features of the nonamer and spacer sequences to enforce the B12/23 restriction. It is unlikely, though, that this requirement for the binding of lymphoid-specific factors to nonamer and/or spacer sequences since these sequences are required to enforce the B12/23 restriction on extrachromosomal substrates in a nonlymphoid cell line. Alternatively, and perhaps more plausibly, a common B12/23 mechanistic constraint relies on features of both the nonamer and spacer sequences. Notably, this is not due to a common B12/23 mechanistic constraint relies on features of both the nonamer and spacer sequences to enforce the B12/23 restriction.

Table II. Recombination of mutant 5′ Dβ 12RSs in Chinese hamster ovary cells

<table>
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<th>Substrate</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>% Recombination</th>
<th>% P2</th>
<th>% P3</th>
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<tr>
<td>pC:V14DD</td>
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<td>Dβ1</td>
<td>Dβ1</td>
<td>0.07 ± 0.04</td>
<td>58</td>
<td>42</td>
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<td>Dβ2</td>
<td>0.04 ± 0.02</td>
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<td>0.03 ± 0.03</td>
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<td>Vβ14</td>
<td>Dβ1</td>
<td>JβH</td>
<td>0.03 ± 0.02</td>
<td>76</td>
<td>24</td>
</tr>
<tr>
<td>pC:V14DJN</td>
<td>Vβ14</td>
<td>Dβ1</td>
<td>JβN</td>
<td>0.03 ± 0.01</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>pC:V14DDC</td>
<td>Vβ14</td>
<td>Dβ1</td>
<td>CSM</td>
<td>0.03 ± 0.01</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

*The Vβ14 23RS and the Dβ1 12RS were fixed at P1 and P2, respectively. Different mutant 5′ Dβ1 RSs were introduced at P3 as indicated. Rearrangement of each substrate was tested a minimum of three times. The percent recombination ± SD of the individual substrates is indicated as is the percent recombination to the 12RSs at P1 (% P1) and P2 (% P2).*

Functional synaptic complex formation in vitro occurs most efficiently between a RS bound by RAG-1/2 and an unbound RSS (32, 33). Thus, the 5′ Dβ 12RS may serve to “nuclease” the formation of a functional Vβ 23RS/Dβ 12RS synaptic complex in vivo by efficiently binding RAG-1/2 followed by complex formation with an unbound Vβ 23RS. In this regard, the Jβ 12RS may bind RAG-1/2 much less efficiently and as such be unable to readily nucleate functional synapsis with Vβ 23RSs. The notion that the Jβ 12RSs are generally less efficient at mediating recombination than the Dβ 12RSs is consistent with recent analyses of the functional effect of RS sequence variations (34, 35). However, rearrangement between the 3′ Dβ 23RS and Jβ 12RSs occurs efficiently in the endogenous TCRβ locus and on extrachromosomal recombination substrates (11). In this regard, it is conceivable that the 3′ Dβ 23RS functions to nucleate functional synapsis for Dβ to Jβ rearrangement. The notion that the 3′ Dβ 23RSs are generally more efficient at mediating recombination than the Vβ 23RSs is consistent with the observation that replacing the Vβ14 23RS with the 3′ Dβ 23RS in the endogenous TCRβ locus results in a dramatic increase in Vβ14 gene segment utilization (36). Thus, it is plausible that the B12/23 restriction may reflect a requirement that, at a minimum, one RS of a recombining pair has the capacity to nucleate functional synaptic complex formation through efficient and independent binding of the RAG-1/2 proteins. In this regard, specific nonamer and spacer sequence combinations may serve as optimal substrates for RAG-1/2 binding. Importantly, this would not preclude additional roles for these sequences in promoting synaptic complex stability once the complex forms (37). The general assembly of functional synaptic complexes in this manner in vivo has potentially important evolutionary implications because it would require that the sequence features permitting efficient nucleation of RAG-1/2 binding be strictly conserved at a single RS of a functional RS pair.

**Note added in proof.** Since the acceptance of this manuscript, three papers (38–40) with findings relevant to spacer and nonamer sequence function have been published.

**References**


