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Biased V<sub>H</sub> Gene Usage in Early Lineage Human B Cells: Evidence for Preferential Ig Gene Rearrangement in the Absence of Selection<sup>1</sup>

Sambasiva P. Rao,* Jeffrey M. Riggs,* David F. Friedman,† Michael S. Scully,† Tucker W. LeBien,‡ and Leslie E. Silberstein<sup>2*</sup>

Certain V<sub>H</sub> genes are predominantly expressed in mature B cells. We hypothesized that several, mutually nonexclusive V<sub>H</sub>-dependent mechanisms operating at distinct stages during B cell development may be responsible for overrepresentation of these V<sub>H</sub> genes. In the present study, we have assessed whether one of the mechanisms involves preferential rearrangement at the pro-B cell stage. The frequency of individual V<sub>H</sub>4 and V<sub>H</sub>3 genes in rearrangement libraries from FACS-purified human CD34<sup>+</sup>/CD19<sup>+</sup> pro-B and CD34<sup>−</sup>/CD19<sup>+</sup> pre-B cells was assessed. The in-frame and out-of-frame rearrangements from both cell populations were analyzed using a high resolution PAGE system. The frequencies of individual V<sub>H</sub> gene segments among out-of-frame rearrangements from pro-B cells were determined, because these frequencies should reflect only processes before the translation of the μ-heavy chain and should not be biased by selection mechanisms. Our results demonstrate that, at the pro-B cell stage, the V4–34, V4–39, and V4–59 gene segments are the most frequently rearranged V<sub>H</sub>4 family genes, and the V3–23 and V3–30 gene segments are the most frequently rearranged V<sub>H</sub>3 family genes. This finding suggests that the predominant expression of these V<sub>H</sub> genes in peripheral mature B cells is determined to a significant degree by their preferential rearrangement during V-DJ recombination. The Journal of Immunology, 1999, 163: 2732–2740.

The differential expression of proteins in the cytoplasm and on the surface of B cells allows separation and definition of differentiation stages of bone marrow B cells in mice and humans (1–3). The first steps in the generation of the Ab repertoire include the process of combinatorial joining of V<sub>H</sub>, D, and J<sub>H</sub> gene segments to form the Ig heavy chain V domain in precursor pro-B cells. Cells that have productively rearranged VDJ genes may differentiate to the pre-B cell stage, where the pre-B cell receptor, consisting of μ/surrogate light chain complex, is generated (4). Further cellular differentiation to immature and mature B cells is represented by the progressive disappearance of the pre-B cell receptor and the expression of surface Ig (sIgM)<sup>3</sup> and IgD, respectively.

In a very recent study, it was found that the human V<sub>H</sub> locus on chromosome 14 consists of 123 V<sub>H</sub> gene segments grouped into seven families (5). Although the total number of V<sub>H</sub> segments is higher than that reported in an earlier study (6), only 44 genes were found to be functional as compared with 51 found in the earlier study (6). This difference in the number of functional genes reported in the two studies can be attributed to the insertion/deletion polymorphic region that, when present, results in the gain of some functional genes (6). V<sub>H</sub>3 is the largest family, followed by V<sub>H</sub>4 and V<sub>H</sub>1, and these three families contain 44 of the functional genes. It has been found that 13–14 gene segments from these families account for a very large percentage of expressed heavy chains in the peripheral repertoire (7–9). In the V<sub>H</sub>3 and V<sub>H</sub>4 families, which together have 34 functional genes, only about 10–12 genes are frequently rearranged and account for 70%–80% of the peripheral repertoire (7–11). Overall these studies indicate that only about 25% of the functional V<sub>H</sub> repertoire is utilized in the formation of the diverse B cell repertoire. The reasons for this biased representation of V<sub>H</sub> genes are unclear. In a recent study (9), we have shown that the biased usage of V<sub>H</sub>3 and V<sub>H</sub>4 family gene segments starts at an early sIg-negative pre-B cell stage (CD19<sup>+</sup>CD20<sup>−</sup>IgM<sup>−</sup>) and persists until the sIg<sup>+</sup> mature B cell stage. It has been postulated that during early B cell development, mechanistic processes leading to preferential Ig gene rearrangement and/or functional processes resulting in B cell selection could account for the nonrandom Ig repertoire in mature B cells.

The suggestion that some V<sub>H</sub> genes may be preferentially rearranged was derived from analysis of V<sub>H</sub> gene representation in pre-B and mature B cells (7–9, 12–15). In all these studies, including our previous study, the contribution of preferential V<sub>H</sub> gene rearrangement could not be definitively assessed because more than 70% of VDJ rearrangements were productive even in the sIg<sup>−</sup> pre-B cells. The relative high frequency of individual gene segments among productive rearrangements in these B lineage cells may thus have reflected both rearrangement frequency at the earlier pro-B cell stage as well as the contribution of selection mechanisms occurring at later stages of development, e.g., efficiency of μ-chain/surrogate light chain (SLC) pairing at the pre-B stage and/or ligand selection at the immature B cell stage.
The goals of the current study were 1) to examine the influence of VDJ rearrangement on V gene repertoire generation independent of the influence of cellular selection processes, and 2) to evaluate the changes in the VH gene repertoire during the transition of B cells from a selection-independent pro-B cell stage to a VH-dependent selection at the pre-B cell stage. Our approach was to analyze Ig V gene rearrangement libraries from a sorted pro-B cell population in which VDJ rearrangement has occurred (16), but more than 90% of the cells have not yet expressed the m protein (17), and from a population of pre-B cells, in which most of the cells express m-heavy chain protein. Using a high resolution (HR) PAGE system, we classified individual rearrangements as in frame (IF) or out of frame (OF) based on the length of HCDR3. OF rearrangements were considered nonproductive, because there is no functional m-heavy chain product or influence of VH-mediated selection. We determined the V gene usage among only OF rearrangements from pro-B cells to eliminate selection processes mediated by the product of the V gene, the variable portion of the m-heavy chain. These studies demonstrate that certain VH genes (e.g., V4–34, V3–23, and V3–30) may be preferentially rearranged, in the absence of selection, at the pro-B cell stage. In addition, when compared with the pro-B cell population, the change in the IF/OF ratio for rearranged Ig V genes in the pre-B cell population provides evidence for positive and negative selection mechanisms operating during the transition of cells from pro-B to pre-B cell stage.

Materials and Methods

B cell enrichment and cell sorting

A pool consisting of CD19/ surface μ B cell precursors and CD34+/CD19+ hemopoietic stem cells was initially isolated from the bone marrow of a fetus of 19–21 wk of gestation, by mAb/magnetic bead depletion, as previously described (18). CD34+/CD19+ pro-B cells and CD34+/CD19+ pre-B cells were then purified by FACS using biotinylated anti-CD19, detected with streptavidin-PE (Caltag, San Francisco, CA), and anti-CD34-FITC (Becton Dickinson Immunocytometry Systems, San Jose, CA). The cells were sorted on a FACSVantage (Becton Dickinson Immunocytometry Systems). Fig. 1 shows the light scatter profile of the B cell precursor/hemopoietic stem cell pool (A), the CD19/CD34 staining profile of the pool (B), the postsort purities of the CD34+/CD19+ pro-B cells (C), and the CD34+/CD19+ pre-B cells (D).

Construction of rearranged genomic DNA libraries

For the construction of libraries, amplification of the VH gene rearrangements from genomic DNA of pro-B and pre-B cells was performed in two steps using nested primers. In the first step, a 30-cycle amplification of VH 4 or VH 3 family genes was performed using family-specific leader primers V4-L, 5′-CCGAAATTCATGAAACACCTGTGGTTCTT-3′ and V3-L, 5′-CTGAATTCCATGGAGTTTTGGCTGAG-3′ (8) paired with a consensus 3′ JH primer, 5′-GCGAGCTCTAGACTTACCTGAGGAGACGGTGA-3′. The 5′ internal primers were complementary from
the −4 codon corresponding to the 3' regions of the leader sequence to the +4 codon in the FR1 region of their respective families. Construction of phage libraries and screening for individual genes using gene-specific probes were conducted as described previously (8, 9, 19–21). A family-specific gene probe was used to find the total number of positive clones. Gene-specific oligonucleotide hybridization (8, 9) was used to determine the frequency of each gene in the libraries. The validity of any data pertaining to the frequency of VH genes is based on the ability of the sense primers to amplify the individual VH genes with equal efficiency. The VH4 and VH3 5' primers used in the present study have been shown previously to amplify with equal efficiency all of the VH4 genes and ~25 VH3 genes, which possibly represent the entire VH3 component (8, 9, 21–24). Using the same primers, it was also determined that the frequency with which individual VH genes are recovered correlates with germline copy number (8). In control experiments, to confirm that the primers amplify all of the VH genes with identical efficiency, unarranged VH4 and VH3 genes from germline DNA were amplified using the same primers used previously for rearrangements and paired with a consensus family-specific FR3 3' primer (8). The individual genes were then identified with gene-specific oligonucleotides using the library screening approach. Furthermore, aliquots of PCR products were blotted on nylon membranes, and phosphor imaging analysis was conducted after gene-specific oligo hybridization. In both of the experiments, it was determined that all of the genes under study were amplified equally even after the second round of amplification (data not shown).

**Screening for IF and OF rearrangements**

We have developed a novel, HR acrylamide gel method to identify rearrangements as IF or OF. A size-standard DNA ladder was constructed consisting of 16 bands representing different HCDR3 sizes ranging from 24–69 bp. A smaller ladder consisting of five different lengths ranging from 24, 42, 57, 69, and 75 bp was also constructed. These PCR sequencing reactions were smaller in size than the constructs, but the PCR amplifications were performed in 100–μl reaction volumes containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 1 mM DTT, 0.2 mM dNTPs, 0.4 μM primer, 2.5 U of Taq polymerase, and 5 μl of the 1/10,000 dilution of the standard template. The template was initially denatured at 95°C for 3 min in Perkin-Elmer DNA Thermal Cycler (model 480; Norwalk, CT), and PCR was performed with 1-min denaturation at 95°C, 30-s annealing at 60°C, and 1-min extension at 72°C for 30 cycles, with a 5-min extension at 72°C in the last cycle. The products were ethanol precipitated, washed, vacuum dried, and resuspended in 35 μl of ethanol per well in 1× TBE buffer at a constant voltage of 750 V for a total of 18,000 V hours, typically about 21 h. As a control, an amplification product from VH4 rearrangements, for which the HCDR3 length and the frame had been determined by sequence analysis, was included with every run. The gel was stained in 2 μg/ml of ethidium bromide and photographed on a UV transilluminator (Fotodyne, Hartland, WI) after destaining in distilled water. Digital images of the gels were captured and saved as TIFF files using a two-camera (COHU, San Diego, CA).

Because codons consist of three bases, the length of segments of DNA that contain IF rearrangements will occur at an interval of three bases, and those that contain OF rearrangements will fall within those three base intervals (Fig. 2). The unknown DNA is loaded in greater quantity than the ladder controls, so they stand out visually as more intense bands superimposed on the less intense standards (Fig. 2). In a few samples, we found more than one band, possibly due to a PCR artifact or the presence of two rearrangements in a clone (9). In such samples, if both the bands in the unknown sample were OF, it was considered as one (Fig. 2B). Samples were not considered in the analysis for the VH gene assigned to that clone if IF and OF complementarily determining regions were present.

In some heavily loaded lanes, such as lane 22 in Fig. 2A and lanes 4 and 7 in Fig. 2B, the unknown band and the underlying marker band shift to a lower position on the gel. To confirm that this shift was due to the amount of DNA loaded, increased quantities of known IF rearrangements were loaded into adjacent lanes on an HR PAGE gel (not shown). The shift in band position was measured in pixels using the linear distance tool of the National Institute of Health Image software package. The degree of shift in band position was found to be proportional to the amount of DNA loaded, with the heaviest loading resulting in as much as 35% shortening of the distance between the overloaded band and the next lower marker band. Furthermore, the marker DNA comigrated with the unknown DNA, so that a single bright band, shifted to a lower position on the gel, was observed, rather than two bands. The presence of a single band was confirmed with a sequencing reaction. Accordingly, unknown bands were read as IF when a single heavy band shifted down on the gel was observed. In a few cases, heavily overloaded unknowns were repeated with reduced amounts loaded to confirm that only a single band was present. As an independent confirmation of the accuracy of the IF and OF assignments, a sample of 71 clones was sequenced to determine reading frame.

The assignments from the gel were found to be in 100% agreement with the sequence data (data not shown). Although some error could be made in scoring a rearrangement to be IF or OF due to reasons discussed above, our sequencing data strongly suggest that such errors are minimal.

**Statistical methods**

The χ2 tests were used to compare the distribution of the observed VH gene frequencies with the frequencies of individual VH genes, as expected by random usage based on the genomic complexity of the VH4 and VH3 genes. Test of significance was also performed to compare the average HCDR3 length of rearrangements from different repertoires, with the assumption that n1 is not equal to n2. Fischer's Exact test was used to compare the IF/OF ratios of individual genes between the pro-B and the pre-B cell stages with the null hypothesis that the frequency of IF rearrangements of a particular gene is similar at the pro- and pre-B cell stages. The p values equal to or less than 0.05 were considered significant.

**Results**

**Evidence for preferential rearrangement of certain VH genes at the pro-B cell stage**

Pro-B lineage (CD34+/CD19+) cells were isolated from a fetal bone marrow to assess 1) the frequency of individual VH4 and VH3 family gene segments among all rearrangements; 2) the proportion of IF vs OF rearrangements for individual gene segments; and 3) the distribution of individual genes among only the OF rearrangements. We chose to study Ig gene usage among only OF rearrangements from pro-B cells because we predicted, based on murine studies, that this B cell fraction would contain a large number of OF rearrangements and would exclude bias due to μ/SLC pairing or ligand selection.
Frequency of V_H^4 and V_H^3 gene rearrangements in pro-B cells

The V_H^4 and V_H^3 gene usage in CD34^+/CD19^+ pro-B cell population is presented in Table I. The frequency of individual V_H^4 genes was assessed by screening more than 600 independent rearrangements in two libraries (PV42a and PV42b) generated from independent PCRs (Table I). Because we used probes specific to all the known functional genes, including the pseudogene of the V_H^4 family, a definitive assignment to a V_H^4 gene segment was possible for 98% of the clones (641 of 657). V4–34 was the most frequent gene accounting for 32% of all the VH^4 repertoire. The gene segments V4–30, V4–39, and V4–59, and V4–4b were also overexpressed in the mature B cells (9) (D. F. Friedman, M. Scully, and L. E. Silberstein, unpublished data). V4–61 was found at the expected frequency, while other genes such as V4–4b and V4–28 were rare or absent. Interestingly, the pseudogene V4–55, which has never been reported in rearrangements from pre-B or mature B cells from earlier studies (7–9), was found in 4% of all rearrangements. Although some of the rearrangements of V4–55 were classified as being IF (see Table III), a functional protein cannot be made due to the presence of a stop codon in the V4–55 germline gene segment (5, 6).

The gene segments V4–30.4 and V4–30.2 were not identified in any of the rearrangements. These two V_H^4 genes, along with V3–30.3, are mapped to an insertion/deletion polymorphic region in...
the VH locus (6, 26). V4–30.4 and V3–30.3 contribute significantly to the VH repertoire in individuals having the insertion polymorphism (26). The fact that we did not detect V4–30.2, V4–30.4, and V3–30.3 rearrangements strongly suggests a deletion polymorphism (26). The fact that we did not detect V4–30.2, V4–30.4, and V3–30.3 rearrangements strongly suggests a deletion polymorphism in our sample. However, we were unable to confirm this by Southern blotting of genomic DNA because of the limited number of sorted cells.

We generated two (PV43a and PV43b) VH3 rearrangement libraries (Table I) from independent PCRs and screened 750 independent clones for eight VH3 genes. Table I shows that these eight genes accounted for 74.6% (567 clones) of the VH3 repertoire. V3–30 (24%) and V3–23 (20%) were the most frequently rearranged genes. The gene V3–30 has two alleles, V3–30b and V3–30.3. However, we did not detect V3–30.3 sequence in any of the rearranged clones for eight VH 3 genes. Table I shows that these eight genes accounted for 74.6% (567 clones) of the VH 3 repertoire.

The ratio of IF/OF rearrangements in pro-B cells suggests the absence of cellular selection

We hypothesized that the ratio of IF and OF rearrangements found in pro-B cells would be unaffected by selection mechanisms because VH-DJH rearrangement has occurred in this population of cells, but the μ protein is not yet produced. In a random rearrangement process without selection, two-thirds (66.7%) of all rearrangement events for any gene segment will be OF, and one-third (33.3%) will be IF, resulting in an IF/OF ratio of 0.5. As shown in Table II, of 997 VH4 and VH3 clones analyzed, 665 (66.7%) were OF and 332 (33.3%) were IF, resulting in an IF/OF ratio of 0.5, in agreement with the predicted value. Furthermore, the IF/OF ratio determined for most of the VH4 and VH3 genes individually (Tables III and IV) also approximated the expected ratio, consistent with an unselected population of progenitor B lineage cells. The sorted CD34+CD19+ pro-B cell population that we have analyzed contains a small number (3%) of CD34− pre-B cells (Fig. 1C). Cells that have synthesized cytoplasmic μ may be subject to a μ protein-dependent selection mechanism. It has been found from previous studies that 5–10% of the CD34+/CD19+ human pro-B cells are positive for cytoplasmic μ (17). However, the overall IF/OF ratio in the pro-B cell libraries indicates that the presence of a small number of cytoplasmic μ-positive B cells did not significantly alter the IF/OF ratios expected based on the random rearrangement process.

Table I. Frequency of VH4 and VH3 genes in pro-B cells

<table>
<thead>
<tr>
<th>VH4 genes</th>
<th>pV42a (n = 178)</th>
<th>pV42b (n = 479)</th>
<th>Total (n = 657)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>V4-4b</td>
<td>8.3</td>
<td>2</td>
<td>1.1</td>
</tr>
<tr>
<td>V4-28</td>
<td>8.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>V4-31</td>
<td>8.3</td>
<td>25</td>
<td>14.0</td>
</tr>
<tr>
<td>V4-34</td>
<td>8.3</td>
<td>59</td>
<td>33.1</td>
</tr>
<tr>
<td>V4-39</td>
<td>8.3</td>
<td>47</td>
<td>26.4</td>
</tr>
<tr>
<td>V4-55</td>
<td>8.3</td>
<td>6</td>
<td>3.4</td>
</tr>
<tr>
<td>V4-59</td>
<td>8.3</td>
<td>27</td>
<td>15.2</td>
</tr>
<tr>
<td>V4-61</td>
<td>8.3</td>
<td>10</td>
<td>5.6</td>
</tr>
<tr>
<td>HC15-8</td>
<td>8.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>176</td>
<td>98.8</td>
<td>465</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VH3 genes</th>
<th>pV43a (n = 379)</th>
<th>pV43b (n = 381)</th>
<th>Total (n = 760)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>V3-11</td>
<td>4.5</td>
<td>30</td>
<td>7.9</td>
</tr>
<tr>
<td>V3-15</td>
<td>4.5</td>
<td>15</td>
<td>4.0</td>
</tr>
<tr>
<td>V3-20</td>
<td>4.5</td>
<td>14</td>
<td>3.7</td>
</tr>
<tr>
<td>V3-23</td>
<td>4.5</td>
<td>74</td>
<td>19.5</td>
</tr>
<tr>
<td>V3-30</td>
<td>4.5</td>
<td>93</td>
<td>24.5</td>
</tr>
<tr>
<td>V3-33</td>
<td>4.5</td>
<td>16</td>
<td>4.2</td>
</tr>
<tr>
<td>V3-53</td>
<td>4.5</td>
<td>35</td>
<td>9.2</td>
</tr>
<tr>
<td>V3-73</td>
<td>4.5</td>
<td>5</td>
<td>1.3</td>
</tr>
<tr>
<td>Total</td>
<td>282</td>
<td>74.3</td>
<td>285</td>
</tr>
</tbody>
</table>

*a For each family, two libraries were constructed from independent PCR amplifications of DNA from pro-B cells. The frequency was calculated by dividing the number of clones identified with the gene-specific probe by the total number of clones identified with the family-specific probe prepared as described previously (9). The expected frequency for each family was calculated based on the number of functional genes (6, 50) in the respective families. The χ² test was performed to assess whether the observed frequencies were different from the expected.

*b Significantly different from the expected (p < 0.05).
are found at lower frequency than expected (see Tables I and II).

In the previous study on sIg2 pre-B cells (9) as well as in the current study of fetal pro-B cells, the gene V4–28 was not found in any of the rearrangements. Thus, this gene is not rearranged at all or is rearranged at such low frequency that it was not identified in the sample of 641 clones from the VH 4 library.

Comparison of Ig gene repertoires during ontogeny

The frequency of most individual V gene segments determined in the fetal bone marrow in the present study is similar to that observed in fetal liver (12–14, 30, 31) and adult bone marrow repertoires (9, 15). However, V4–34 and V4–39 rearrangements are either rare or absent in fetal liver B cells, although they predominate in fetal and adult bone marrow B cells. An additional difference between fetal bone marrow and fetal liver B cells is the average HCDR3 size of the VH rearrangements. We found that the average lengths of the HCDR3 in VH 4 and V H 3 rearrangements from fetal bone marrow are significantly longer than from the fetal liver (p < 0.001) and closer to the average adult HCDR3 size, although still significantly different from adult (p < 0.05). Taken together, these observations suggest that the fetal bone marrow VH repertoire shares more features with the adult B cell repertoire than with the fetal liver repertoire.

Comparison of IF/OF ratio between pro- and pre-B cells

The IF/OF ratios of members of V H 4 and V H 3 families are summarized in Tables III and IV. The ratio of IF/OF at the pro-B cell stage in both of the families is 0.5 (Table II), which is similar to the expected ratio based on the random rearrangement process. This is also characteristic for VH segments at the pro-B cell stage in mouse bone marrow populations (28). In general, the IF/OF ratio of individual members of VH 3 and V H 4 families was low, indicating the predominance of OF rearrangements at the pro-B cell stage. At the pre-B cell stage, however, a significant increase (p < 0.01) in the IF/OF ratio was observed. V H 4 genes and some members of the V H 4 family such as V4–34, V4–59, and V4–39 show an increase in the IF/OF ratio, indicating the selection of cells...
bearing productively rearranged genes. In contrast, among V4–31 rearrangements, the predominance of OF rearrangements persists in the pre-B library with an IF/OF ratio of 0.3, which is lower than the expected value of 2.3 (32–34). The V4–61 gene was infrequently rearranged at the pro-B cell stage relative to other VH4 genes, which possibly explains its relative low frequency at the later, more mature B cell stages (9).

**Discussion**

The suggestion that some VH genes may be preferentially rearranged during VDJ recombination has been derived from analyses of VH representation using cDNA from pre-B cells from fetal liver (13, 14, 30, 31, 35), adult bone marrow (15), and mature B cells from PBL (11, 36). In all of these studies, the majority of the rearrangements were IF, indicating that cells were already subjected to selection. Furthermore, analyses of VH genes from cDNA may not capture all rearrangements, excluding OF rearrangements that predominate in the earliest precursor B cells (29, 37). Additionally, the transcripts of OF rearrangements are considered to be more unstable compared with productive transcripts (13), which may result in the underrepresentation of OF rearrangements in cDNA-based libraries. Therefore, these studies do not give a true estimate of the relative frequency of rearrangement of individual VH genes.

IF and OF rearrangements have been analyzed in mature B cells (7, 24), in which OF rearrangements occur in mature B cells solely by virtue of their coexistence in cells with productive rearrangements of the second VH allele. However, in these studies it was difficult to draw definitive conclusions regarding the preferential rearrangement of VH gene elements due to the small number of observed OF rearrangements. In addition, a large proportion of rearrangements may not be observed in the mature B cell repertoire because both positive and negative selection may have occurred during B cell maturation (38). In the present study, however, more than 90% of the cells are unaffected by μ-chain-mediated selection mechanisms, permitting an unbiased sample of all rearrangement events. This analysis of OF rearrangements suggests that, at the pro-B cell stage, the V4–34, V4–39, and V4–59 gene segments are the most frequently rearranged VH4 family genes, and the V3–23 and V3–30 gene segments are the most frequently rearranged VH3 family genes during the recombination process.

The molecular mechanisms that result in the preferential rearrangement of particular VH elements during Ig gene assembly are not known. However, inherent differences in accessibility to recombination machinery (39), a more favorable spacer in the RSS (13), a more efficient cleavage in the RSS (12, 40–42), and proximity between elements (43–46) have all been suggested as possible explanations. More recently, the complete nucleotide sequence of the VH locus, including the heptamer/nonamer and the 5′ regulatory sequences for all VH genes, has been reported (5). For the genes analyzed in the present study, including the

Table IV. **Comparison of IF/OF ratio of VH3 genes between pro-B and pre-B cells**

<table>
<thead>
<tr>
<th>Genea</th>
<th>Pro-B Cell Libraries</th>
<th>Pre-B Cell Libraryb</th>
<th>Avg. Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PV43a</td>
<td>PV43b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IF/OF Ratio</td>
<td>IF/OF Ratio</td>
<td>IF/OF Ratio</td>
</tr>
<tr>
<td>V3-11</td>
<td>10/18 0.56</td>
<td>11/16 0.69</td>
<td>0.62</td>
</tr>
<tr>
<td>V3-20</td>
<td>3/11 0.27</td>
<td>4/3 1.33</td>
<td>0.5</td>
</tr>
<tr>
<td>V3-23</td>
<td>19/51 0.37</td>
<td>26/43 0.60</td>
<td>0.48</td>
</tr>
<tr>
<td>V3-30</td>
<td>29/59 0.49</td>
<td>26/65 0.40</td>
<td>0.44</td>
</tr>
<tr>
<td>V3-11</td>
<td>11/5 2.2c</td>
<td>37/10 3.7c</td>
<td></td>
</tr>
</tbody>
</table>

* IF/OF data obtained from one library after screening for individual genes.
* IF/OF analysis was performed on only four VH3 genes.
* Significantly (*p* ≤ 0.01) higher than pro-B cells.

**FIGURE 3.** Comparison of average HCDR3 size among different repertoires. FL, fetal liver; *n* = 61. Data pooled from (13, 14, 30); FBMVH4, fetal bone marrow VH4; *n* = 194 (this study). FBMVH3, fetal bone marrow VH3, *n* = 128 (this study). ABM, adult bone marrow, *n* = 54 (15); PBL, *n* = 84 (7). The values in the columns represent average HCDR3 lengths. The error bar on each column represents the SD. FL, SD ± 6.74; FBMVH4, SD ± 10.35; FBMVH3, SD ± 11.96; ABM, SD ± 18.62; PBL, SD ± 14.4.
pseudogene (V4–55) and the unrearranged V4–28 gene segment, the 5’ regulatory regions, spacers, splice sites, and heptamer/nonamer are all intact; no structural defects have been detected (5). The differences in the frequency of rearrangement of the V4i genes may be due to the influence of yet unknown factors outside the V4i locus, as previously suggested (23, 47). The position of genes within the V4i locus has also been suggested to contribute to the preferential rearrangement of some genes. In mice, the most D-proximal V4i segment, V(4i)81X, a member of V4i7183, predominates early in ontogeny (44, 48). In humans, however, proximity to J4i does not appear to influence the rearrangement frequency of V4i genes in early or later stages of ontogeny (14, 31, 49, 50).

While the current data demonstrate the preferential rearrangement of certain V4i genes in early lineage B cells of fetal bone marrow, we have not shown that this process also occurs in early B lineage cells of adults. However, based on the comparison of HCDR3 size (Fig. 3) and V4i gene usage, it appears that the Ig repertoire of fetal bone marrow more closely resembles that of adult bone marrow (9, 15) than that of fetal liver (13, 14, 30).

We demonstrate that during the transition from pro-B to pre-B cell stage, there is reversal of the IF/OF ratio, reflecting a predominance of IF rearrangements of frequently rearranged genes such as V4–34–34. In contrast, the IF/OF ratio of V4–31 is not reversed during the pro- to pre-B cell transition. The differential ability of V4(1) μ-chains to pair with surrogate light chain (51–53) may explain this difference. For example, μ-chains that contain V4i gene products such as V4–34 and V3–23, which are preferentially rearranged at the pre-B cell stage, may form stable complexes with surrogate light chain at the pre-B cell stage fostering clonal expansion and maturation of B cells expressing these V4i genes. In contrast, V4i genes such as V4–31, which is also frequently rearranged at the pre-B cell stage, may not pair efficiently with surrogate light chain. Consequently, they would not have a survival advantage and would occur less frequently in mature B cell populations, similar to the V4i81X gene in the mouse (51, 54).

In summary, the current experiments provide evidence that certain V4i gene segments are preferentially rearranged during VDJ recombination at the pre-B cell stage. Further studies are needed to determine whether selection mechanisms at later B cell developmental stages also contribute to the biased V4i gene usage of mature peripheral B cells.

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References

cells is dependent on the V<sub>H</sub> structure of the α/surrogate L chain receptor. EMBO J. 15:1524.