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Unequal V_H Gene Rearrangement Frequency Within the Large V_H7183 Gene Family Is Not Due to Recombination Signal Sequence Variation, and Mapping of the Genes Shows a Bias of Rearrangement Based on Chromosomal Location[1]

G. Stuart Williams,* Alan Martinez,* Alina Montalbano,* Alan Tang,* America Mauhar,† Kisani M. Ogwaro,* Denise Merz,* Christophe Chevillard,[2†] Roy Riblet,† and Ann J. Feeney[3]*

Much of the nonrandom usage of V, D, and J genes in the Ab repertoire is due to different frequencies with which gene segments undergo V(D)J rearrangement. The recombination signal sequences flanking each segment are seldom identical with consensus sequences, and this natural variation in recombination signal sequence (RSS) accounts for some differences in rearrangement frequencies in vivo. Here, we have sequenced the RSS of 19 individual *V_H7183* genes, revealing that the majority have one of two closely related RSS. One group has a consensus heptamer, and the other has a nonconsensus heptamer. In vitro recombination substrate studies show that the RSS with the nonconsensus heptamer, which include the frequently rearranging *81X*, rearrange less well than the RSS with the consensus heptamer. Although *81X* differs from the other *7183-I* genes at three positions in the spacer, this does not significantly increase its recombination potency in vitro. The rearrangement frequency of all members of the family was determined in μMT mice, and there was no correlation between the in vitro recombination potential and V_H gene rearrangement frequency in vivo. Furthermore, genes with identical RSS rearrange at different frequencies in vivo. This demonstrates that other factors can override differences in RSS potency in vivo. We have also determined the gene order of all *V_H7183* genes in a bacterial artificial chromosome contig and show that most of the frequently rearranging genes are in the 3′ half of the region. This suggests that chromosomal location plays an important role in nonrandom rearrangement of the *V_H7183* genes. *The Journal of Immunology*, 2001, 167: 257–263.

The diversity of the Ab and TCR repertoires is due in large part to the combinatorial association of the large numbers of V, D, and J segments to create thousands of different heavy and light, or α and β, chain molecules, respectively. However, this combinatorial diversity is constrained by the fact that V, D, and J segments recombine at very different frequencies. An example of unusually high frequency of rearrangement is observed with the *81X* gene, which is the most 3′ functional V_H gene in the murine V_H locus. Many laboratories have observed that this V_H gene family rearranges very frequently early in ontogeny and also in the adult bone marrow (1–9). The overuse of the *7183* family, especially *81X*, gave rise to the chromosomal proximity hypothesis of Alt and colleagues, which proposed that the rearrangement mechanism preferentially rearranges genes that are in closer proximity in the chromosome (1, 10). Supporting the generality of this proximity observation, mice that lack the IL-7 receptor show decreased rearrangement of V_H genes, displaying a gradient of rearrangement throughout the entire V_H locus, ranging from almost normal levels of rearrangement of the *V_H7183* family to essentially no rearrangement of the distal *V_HJ558* and *V_HJ606* families (11). Also, overuse of V-proximal Jα genes has been observed in the *TCRα* loci in fetal and newborn thymi (12), and only later do the more distal J genes rearrange. Most strikingly, there is a strict chromosomally ordered gene rearrangement in the *Vγ* loci (13). However, other loci, such as the human *V_H* loci, do not display this phenomenon of chromosomally ordered rearrangements (14). It has been shown that many of the *V_H3* genes that are over-represented in rearrangements map in the middle or 5′ portion of the locus (15). However, the most 3′ human V_H gene, *V_H6*, is over-represented in rearrangements early in ontogeny (16). A direct tracking mechanism as an explanation for this phenomenon has been deemed unlikely, but the reason for these several examples of positionally graded rearrangement frequencies is not clear.

An alternative, or additional, explanation is that each individual gene controls the frequency of its rearrangement to a large extent by virtue of its relatively unique recombination signal sequence (RSS).[4] Most natural TCR and Ig RSS vary from the consensus RSS, and this variation could result in differences in rearrangement frequency (17). In these cases, the chromosomal location would be irrelevant, and thus could explain the inconsistent correlation of high frequency of rearrangement with proximal chromosomal location for only some, but not other, V and J loci. We and others,

*Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037; and †Torrey Pines Institute for Molecular Studies, San Diego, CA 92121

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[2] Current address: Institut National de la Santé et de la Recherche Médical, Unité 399, Immunology and Genetics of Parasitic Diseases, Faculty of Medicine, 27 boulevard J. Moulin, Marseilles 13385, France.

[3] Address correspondence and reprint requests to Dr. Ann J. Feeney, Department of Immunology, IMM-22, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail address: feeney@scripps.edu

[4] Abbreviation used in this paper: RSS, recombination signal sequence(s); BAC, bacterial artificial chromosome; FR, framework.

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particularly Wu and colleagues, have, in fact, shown that the natural variation in the RSS of V genes can affect rearrangement frequencies (18–23). Although the classic studies of Gellert and colleagues have shown that some positions in the heptamer and nonamer are critical for rearrangement, while others are less critical, those studies have not analyzed all possible changes in the RSS (24). We have shown that an allelic change involving a single substitution in the heptamer can lead to a 4.5-fold reduction in rearrangement frequency in a Vκ gene critical for an effective Ab response to *Haemophilus influenzae* type b (21, 25). Furthermore, Navajos, who have the allele with the nonconsensus heptamer, have a greatly increased susceptibility to *H. influenzae* type b disease. Thus, variations in RSS leading to relatively modest differences in rearrangement frequencies can alter the composition of the Ab repertoire and can have severe biological ramifications (18).

It has been proposed that the reason that *81X* rearranges so often is due to its RSS (23). Using miniloci recombination substrates transiently transfected into pre-B cells, the relative frequency of rearrangement of *81X* vs V_HJ558 genes was determined, and *81X* rearranged ~15 times more often than V_HJ558 (22, 23). However, the choice of a V_HJ558 gene as a competitor was likely to show a bias in favor of V_H7183, because V_HJ588 genes have a poor RSS. It stands to reason that if *81X* rearrangements comprise over half of the V_H7183 rearrangements, the other ~18 functional members of the V_H7183 family cannot all rearrange at that same high rate. Hence one would predict that *81X* would have a unique RSS if its high frequency of rearrangement were due to a particularly potent RSS.

Therefore, in this current study we have sequenced the RSS of 19 of 20 members of the V_H7183 family, and they fall into two similar groups. All but two genes share the same nonconsensus nonamer. Strikingly, over half of the V_H7183 genes have the same nonconsensus heptamer as *81X*, although *81X* has a unique spacer sequence. The other V_H7183 genes have RSS with a consensus heptamer and a slightly different spacer. Twelve of the 19 RSS are identical with one of the two prototypic RSS, and the other seven have a small number of changes. We determined the relative potential of the various RSS to support recombination in a recombination substrate assay, and, not surprisingly, the RSS with the consensus heptamer rearranged more often than RSS with the nonconsensus heptamers. Even the RSS of *81X*, with its unique spacer, supported less rearrangement than the V_H7183 RSS with the consensus heptamer.

We determined the chromosomal order of the V_H7183 genes from a bacterial artificial chromosome (BAC) contig covering the entire V_H7183 region of the IgH locus, and we also determined the relative frequency of rearrangement of each of the V_H7183 genes. All but one of the frequently rearranging genes were in the 3′ half of the region, and the rearrangement frequency fell off in gradient fashion 5′ of *81X*, with the exception of one poorly rearranging gene in the 3′ portion. Thus, these data clearly show that variation in RSS potency is not the major determinant in the nonrandom V_H usage of the V_H7183 gene family or in the overuse of *81X* in rearrangements, and that the RSS variation is overridden by other factors. It appears that chromosomal location may play a more major role in the over-rearrangement of many of the 3′ genes. Finally, despite identity in RSS and similarity in coding regions, similar genes rearrange at quite different frequencies in different parts of the locus, suggesting that the local region surrounding each V_H gene is a major determinant of the frequency of rearrangement.

Materials and Methods

Mice

μMT mice (26) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were bred at The Scripps Research Institute's animal facility.

PCR and sequencing

The following oligonucleotides were used, with engineered restriction enzyme sites underlined: 5′ primers: AF191, 5′-CGGGCTCGAGTTGGTTTTCCTTGTCC-3′ (leader); AF215, 5′-GAGGCTCGAGGAAACTCTCCTGTG-3′ (starts at bp 43 of the coding region); AF303, 5′-GGGGCTCGAGGAGTCTGGGGGA-3′ (starts at bp 6 of the coding region); AM3, 5′-GTCCGAATTCCTCTCCTGTGCAA-3′ (starts at bp 48 of the 50.1 coding region); AM6, 5′-AATAGGAATTCTGGTAGCAGGTAT-3′ (starts at bp 154 of the *68–5N* coding region); RR190, 5′-ATGCTGGTGGAGTCTGGG-3′ (starts at bp 7 of the coding region); *Ox2* 5′, 5′-GCTGAAGCTTTCAGGCAATGGCCA-3′; 3′ primers: AF192, 5′-CTCCGCGGCCGCTGCTGGTCCT-3′ (starts 16 bp 3′ of the RSS); AF13N, 5′-AAAAGCGGCCGCTTACCTGAGGAGACGGTCA-3′ (J_H); AF269, 5′-GGTCGCGGCCGCTCTGCAGGAGGTTT-3′ (starts 5 bp from the end of the nonamer of the RSS); AM5, 5′-GCAGAAGCTTTAGTTTGAGCTCA-3′ (starts in the middle of the spacer of the V_H7183 family RSS and extends through the nonamer); RR192, 5′-GACCTCAGACTGCTCATTTGC-3′ (starts at bp 243 of the coding region); *Ox2* 3′, 5′-GCGCGAATTCCTGATCATCTTTCTAGTCA-3′; and *E4.psi*.seq, 5′-ACTGATGGTAGTTTCA-3′ (starts at bp 157 of *E4.psi* coding region).

All oligonucleotides were supplied by Genosys (The Woodlands, TX) or Life Technologies (Gaithersburg, MD). For the repertoire analysis, newborn liver DNA was prepared using the method described by Schlissel et al. (27). AF303 and AF13N were used to amplify VDJ rearrangements from newborn liver DNA. Thirty-five cycles of 30 s at 94°C, 30 s at 57°C, and 1 min at 68°C, followed by one cycle of 10 min at 68°C, were performed using an Ericomp cycler (Ericomp, San Diego, CA). Elongase (Life Technologies) was used for all PCRs because it contains a proofreading polymerase. The resulting PCR fragments were digested with *Xho*I and *Not*I and cloned into pBluescript (Stratagene, La Jolla, CA). Individual colonies were miniprepped and sequenced with a T7 primer using Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, OH) sequencing kit and [^{35}S]ATP. For the repertoire analysis, once repeat sequences (i.e., the same V, D, and J segments and the same junctional sequence) were identified coming from the same PCR, no further sequences from that PCR were obtained. Identification of the V_H genes was routinely based on >150 bp of V_H sequence information from the 3′ half of the gene.

AF191 and AF192, AF191 and AF269, RR190 and RR192, or AF215 and AM5 were used for amplifying the genomic sequences from individual BAC DNA for the mapping analysis. Twenty-five cycles of 30 s at 94°C, 30 s at 55°C, and 1.5 min at 68°C, followed by one cycle of 10 min at 68°C, were performed using Elongase. The PCR fragments were digested with *Xho*I and *Not*I, or *Eco*RI and *Hin*dIII, depending upon the primers used. Clones were sequenced from both sides, and a minimum of two sequences from independent PCRs were determined for each V_H gene to ensure the absence of errors.

Additionally, in the assembly of the BAC contig (see below) each V_H7183 gene, except *7183.10*, *7183.11*, *81X*, *E4.psi*, *D6.96*, and *3:3.39*, has recently been sequenced directly from BAC DNA without PCR amplification, confirming the sequences obtained by PCR and cloning. Direct sequencing was performed on agarose gel eluates purified with the Qiaquick kit (Qiagen, Valencia, CA). Cycle sequencing used BigDye terminators (PE Biosystems, Foster City, CA) for one cycle of 95°C for 5 min, followed by 100 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min.

Recombination substrate assay

The competition recombination substrate assay has been described previously (21). The RSS and ~100–200 bp of flanking regions were cloned into a competition recombination substrate plasmid 5′ of the transcriptional stop, and an inverted *DSP2* segment was cloned into the 3′ side of the transcriptional stop using the following oligonucleotides (restriction enzyme sites are underlined): P50a, 5′-GGGTACGCGTGCCTCCAGGGAAG-3′; P50b,5′-CCTGGCGGCCGCGAGCACCCCTGCA-3′;P58A2,5′-CCCTGCGGCCGCAAATGAGCAGTC-3′; P61b, 5′-CTCTCGTCGACCCCTGCTGGTCCT-3′; P76b, 5′-TCTGGTCGACCAGAACACCCCTGCA-3′; P82, 5′-TTCAGGCGGCCGCCCTGAGAGCTGAGG-3′; P97a, 5′-TGAAGCGGCCGCCTGGACAGGGACTTG-3′; and P97b, 5′-TTCTGTCGACTCAATCCCAGTGC-3′.

P50a and P50b were used to amplify the $V_HS107/V1$ RSS for cloning into the external site; P82 and P76b were used for cloning the *V1* gene into the internal RSS cloning site. P58A2 and P61b were used to clone *81X*,

3.3.39 (representative 7183-I gene), and *37.1* (representative 7183-II gene) into the internal RSS cloning site. P97a and P97b were used to clone the V_H*J558* RSS into the internal RSS cloning site. The resulting constructs were sequenced to confirm that no mutations had been introduced during the PCR.

The RSS competition assay was performed as previously described (21). Briefly 18.8 Abelson-murine leukemia virus-transformed pre-B cells were transfected using a Bio-Rad GenePulser with 300 V and 960 μF capacitance (Bio-Rad, Hercules, CA). Transfected cells were cultured for 48 h in the presence of 0.5–1.0 mM caffeine (ICN Pharmaceuticals, Costa Mesa, CA), then the plasmids were recovered using a standard alkaline lysis protocol. Recovered plasmid was digested with *Spe*I, transformed into Electrocompetent XL1 Blue (Stratagene), and plated on Luria Bertoni agar containing isopropyl β-D-thiogalactoside and chloramphenicol. The bacterial colonies were screened using a PCR-based assay as previously described to determine the relative rearrangement of the *DSP* RSS to the external vs internal V_H RSS (21).

Assembly of V_H*7183 BAC contig*

The BAC library of the mouse strain 129/Sv (129S3) from the embryonic stem cell line CJ7 distributed by Research Genetics (Huntsville, AL) was screened using PCR assays for V_H*III*, D12Nds2 (V_H*S107/V1*), V_H*E4.psi*, and BAC ends CT7–19B1-T7 and CT7–057J13-Sp6 (C. Chevillard et al., manuscript in preparation). BAC ends were isolated by vector-hexamer PCR (28) and hybridized to Southern blots of *Hin*dIII-digested BAC DNAs to identify overlap patterns. High density BAC library membranes were probed with pooled V_H*7183* PCR products from several *7183*$^+$ BACs, and additional BACs were identified. All BACs were digested with *Eco*RI and blotted; replicate blots were probed with amplified inserts of *81X* and V_H*Q52* plasmids. The gene content of each hybridizing fragment was identified from at least two representative BACs by excision of the fragment from agarose gel and sequencing its V_H*7183* gene. At least one sequence in each case was obtained directly from eluted restriction fragment without PCR, with the exception of six genes, as noted above; confirmatory sequences were in some cases obtained from PCR amplification from eluted restriction fragments. Taken together, these data allowed the unambiguous ordering of BACS and V_H*7183* genes.

Results

Sequence of the germline genes

The RSS of *81X* is nonconsensus, but has been shown to be much more efficient at rearrangement than a V_H*J558* RSS (22). We wanted to determine the sequence of the RSS of each V_H*7183* gene to determine the extent of diversity of RSS within this V_H gene family and to determine whether the relative frequency of recombination of the individual family members correlated with the sequence of their RSS. To determine the sequence of the RSS of each V_H*7183* gene, we initially used a primer in the leader, and a primer 3′ of the RSS. The leader sequence is often very conserved within members of a V_H family, and the sequence just 3′ of the RSS is relatively conserved. Therefore, we used this primer set to amplify as many germline V_H*7183* genes as possible. PCRs were performed on the BAC DNA from 129/Sv mice, which, like BALB/c, are of the Igh^a haplotype. We were able to amplify 13 genes in this way, with multiple independent sequences of those genes. Because there were >13 *Eco*RI bands on Southern blots that hybridized with the V_H*7183*, and because some known genes were missing initially, we also amplified some of the BAC DNAs with framework 1 (FR1) primers and either AF269 (which overlaps the 3′ end of the RSS by 5 bp) or AM5 (which contains the nonamer and half the spacer) in an attempt to choose regions more likely to be conserved. *E4.psi* did not amplify with the primers, so we used a sequencing primer in FR3 of the published sequence of *E4.psi* to sequence the RSS. In other cases direct sequencing of DNA obtained from *Eco*RI bands of BAC DNA excised from agarose gels was performed for genes that we were unable to amplify from the BAC.

The sequence of the RSS and the adjacent coding end sequence for all members of the family are shown in Fig. 1 (full-length sequences are available from GenBank under accession no. AF290959–AF290972). The RSS of the V_H*7183* genes fell into two major groups, which we called *7183-I* and *7183-II*. *7183-I* RSS had the same nonconsensus heptamer as *81X* (CACA<u>A</u>TG; the one change underlined) and a nonconsensus nonamer (AC<u>T</u>A AAACC), while the *7183-II* RSS had the same nonconsensus nonamer as the *7183-I* genes, but had a consensus heptamer. The spacers were very similar, with the *7183-I* and *7183-II* prototypes differing only in three positions. Seven genes had the identical prototypic *7183-I RSS*, and five had the identical prototypic *7183-II RSS*. In addition to these genes, seven other genes were found to vary from the prototypes at a few positions in the RSS, predominantly in the spacer, including *81X*, which differed from the *7183-I* RSS by two changes in the spacer. Only two genes have heptamers that vary from these two prototypes, and they are classified as variants of *7183-I* or *-II* based on spacer sequence.

```
                                    RSS
              Consensus  CACAGTG                           ACAAAAACC

7183 I        7183.9     ----A--  AGGAAATGTTACTGTGAGCTCAA  --T------
              7183.9T    ----A--  -----------------------  --T------
              3:3.39     ----A--  -----------------------  --T------
              69.1       ----A--  -----------------------  --T------
              D6.96      ----A--  -----------------------  --T------
              68-5N      ----A--  -----------------------  --T------
              50.1       ----A--  -----------------------  --T------

        Ib    81x        ----A--  --C---A----------------  --T------
        Ic    7183.13    ----A--  --A---A------A---------  --T------
        Id    7183.19    ----A--  -----------T-----------  --T------
        Ie    7183.20    G---A--  -----------T-----------  -GT------

7183 II       E4.15/283  -------  --TG-------------------  --T------
              37.1       -------  --TG-------------------  --T------
              7183.14    -------  --TG-------------------  --T------
              98-3G      -------  --TG-------------------  --T------
              7183.10    -------  --TG-------------------  --T------

        IIc   E4.psi     -------  --TG-------------------  ---C-----
        IIb   61-1P      -------  ---G---A---T-----------  --T------
        IId   7183.12    ---T---  --TG------------T------  --T------

              7183.11    Unknown RSS

S107          V1         -------  --A-G-CG-C-T-------C--G  ---C-----

J558                     -------  TTGT--CCAC-TCC----TGTG  T--G-----
                     or: -------  TTGT--CCAC-TCC----TGTGT CAG---C--
```

FIGURE 1. RSS sequences of the V_H*7183* family members. Genes with a *7183-I*-type RSS are on the *top*; genes with a *7183-II* RSS are on the *bottom*. The variants are separated from the genes possessing prototypic RSS. The RSS of V_H*S107/V1* and V_H*J558* genes used in the competition recombination substrates are also shown. The RSS of *J558* is shown as if it has a 22-bp spacer on the *top line*, and the same RSS is written as though it has a 23-bp spacer on the *second line*. The full-length sequence of any previously unpublished sequences of these genes is available from GenBank under accession numbers AF290959–AF290972. Some of these sequences have previously been reported (1, 7, 29, 30).

Three new V_H*7183 genes*

During our analysis of the BAC contig we found a previously unknown functional member of the V_H*7183* family by using FR1 and FR3 primers. It is identical with *7183.9* in the coding region, other than two changes at the 5′ end and a single base pair change of a G to a T in FR3, so we call this gene *7183.9T*. We have extended the sequence through the RSS, and it is also identical with *7183.9* in that region, but its leader/intron region shows four differences between the two genes. We also identified a second new, apparently functional, gene that we have provisionally called *7183.19*. All these V_H genes will be renamed numerically once all the interspersed pseudogenes and V_H*Q52* genes are identified. It is only 94% homologous to the most similar V_H*7183* gene and has a *7183-I* variant RSS. A new presumably nonfunctional gene of the V_H*7183* family, called *7183.20*, was also identified. Its RSS was

related to the *7183-I* RSS; however, it contains a C to G mutation at the first position in the heptamer, an invariant position essential for RSS function (24). Therefore, this RSS is highly likely to be nonfunctional.

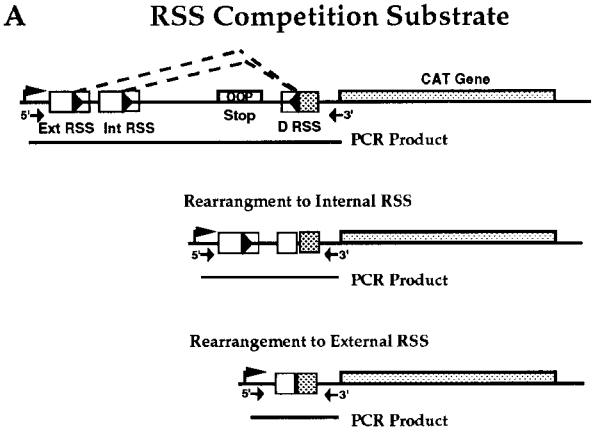
Several years ago we reported the sequence of four new *V_H7183* genes from BALB/c mice and also made a compilation of all the *V_H7183* genes described at that time in the BALB/c strain (also *Igh^a*) (29). The sequences obtained in this current study enable us to refine that list. We have added three new sequences as just described. We also now eliminate *MOPC21* as a germline gene; rather, it is almost certainly a *61-1P* gene with three somatic mutations. Also, we previously listed a sequence from the literature, *V-BK*, which only differs from *7183.12* by 3 bp, two of which are a GA dinucleotide instead of an AG dinucleotide in FR3. We never observed *V-BK* in rearrangements, nor did we find it in the BAC contig, and we assume that it is the same as the germline *7183.12* gene. Also, we never observed the published sequence for *283* (30). The published coding sequence of *283* is almost identical with that of *E4.15*, but they vary in the intron (1). *283* is the only published *V_H7183* gene that ends with . . .TA, so we conclude that the two published sequences, *283* and *E4.15*, really represent the same gene.

Analysis of the role of the RSS in the nonrandom V_H gene use

It has been shown that *81X* rearranges 7- to 30-fold more frequently than the *V_HJ558* gene in a competition substrate (22, 23). However, the *V_HJ558* genes have very poor RSS, and the nonamer is so different from the consensus that it is not clear whether they also have a shortened, less functional, 22-bp spacer (see Fig. 1) (24). Therefore, the first issue we wanted to address was whether the RSS of *81X* is much better than that of a more conventional RSS. We chose the *V_HS107* gene *V1* as a representative gene. This gene has a consensus heptamer, and the nonamer has one change from consensus. The spacer length is the optimal 23 bp, as are all of the *V_H7183* RSS. We wished to assess the relative recombination supported by *V1* vs *81X*, so we made competition recombination substrates comparing the relative frequency of recombination of these two RSS to recombine to a *DSP2* fragment. RSS fragments are made by PCR and are usually ~100–200 bp in size, containing the RSS as well as some of the 5′- and 3′-flanking regions. The substrates are transiently transfected into pre-B cells, and the recovered plasmid is plated on a chloramphenicol plate. Colonies are screened by PCR to determine the relative frequency of rearrangement of the internal vs the external V_H fragment (Fig. 2).

Fig. 2 shows that a control competition substrate containing *V1* in both internal and external positions demonstrates the minimal effect of position (internal vs external) of the two competing RSS fragments in the substrates. This is consistent with the controls we have previously published using this competition recombination system (18, 21). When we cloned a *V_HJ558* RSS fragment in the competition substrate, it was vastly disfavored, with 91% of the rearrangements occurring to *V1* instead of *J558* (Fig. 2). Thus, we conclude that *V_HJ558* genes do, in fact, have poor RSS, as predicted by inspection of their RSS. However, when the *J558* fragment is replaced by *81X* in the above substrate, the relative frequencies of recombination of *V1* and *81X* are similar. This shows that the *81X* RSS is only slightly better than that of a *V_HS107* gene and suggests that *V1* should rearrange almost as often as *81X* in vivo if the RSS is the major determinant of rearrangement frequency.

Within the *V_H7183* family, all V_H genes but two share an identical nonconsensus nonamer. Approximately one-third of the V_H genes have a consensus heptamer, while two-thirds have a nonconsensus heptamer, identical with that reported for *81X*. Based on classical studies with recombination substrates, one would expect



B

External	Internal	No. of colonies screened	% Rearrangements External : Internal
VI	VI	241	56 : 44
VI	J558	172	91 : 9
VI	81X	202	47 : 53
VI	7183-I	568	49 : 51
VI	7183-II	537	29 : 71

FIGURE 2. *A*, A linear map of the RSS competition substrate. The primers used to screen for internal vs external rearrangements are indicated. Amplified PCR products as the result of rearrangement to the internal or external RSS are shown. *B*, The results of the RSS competition substrate experiments are presented. For each competition recombination substrate, we describe the V gene/RSS in the external and the internal position, the total number of colonies assayed, and the percentage of rearrangements to internal and external RSS. The sequences of all RSS are given in Fig. 1.

that the *7183-II* RSS, with its consensus heptamer, might rearrange at a higher frequency than the *7183-I* RSS, with one change in the heptamer sequence (24, 31). *81X*, which rearranges at a very high frequency, has a *7183-I*-type RSS, but with a unique spacer sequence. It is possible that its variant spacer is the reason for its high frequency of recombination. Although the heptamer and nonamer are traditionally believed to be the most important regions of the RSS, we have previously shown that the spacer can also play a significant role in recombination frequency of *Vκ* genes (18). In that study we also showed the surprising result that a RSS with a nonconsensus nonamer but an optimal spacer rearranges more frequently than a consensus RSS with a poorer spacer, thus underscoring the necessity to actually test the recombination potential of each RSS experimentally. Therefore, to determine the relative frequency of recombination of prototypic *7183-I* and *7183-II* RSS, we replaced the internal gene fragment in our control recombination substrate with that of *3.3.39* (a representative *7183-I* gene) or *37.1* (a representative *7183-II* gene). As predicted, the *7183-II* gene, with it consensus RSS, is better than *V1* or the *7183-I* genes for supporting rearrangement in the recombination substrates (Fig. 2). Seventy-one percent of the rearrangements were to the *7183-II* gene, rather than to *V1*. Importantly, the *7183-I* gene rearranged much less well than the *7183-II* gene, and *81X* rearranged at only a marginally higher frequency. Therefore, for *81X*, the two changes in the spacer sequence did not significantly affect its recombination frequency. Thus, if the RSS plays a major role in controlling the relative rearrangement frequency of this V_H family in vivo, then one would predict that the *71813-II* genes would rearrange most frequently, and that *81X* rearrangement would occur at approximately the same frequency as that of the other *7183-I* genes.

Relative frequency of rearrangement of individual V_H7183 genes

Therefore, we analyzed the initial rearrangement frequency of the *V_H7183* gene family members, determining only the biases inherent to the VDJ recombination process and not subsequent biases imposed by preferential selection and clonal expansion. The μMT mice (26), which have a targeted deletion of the cytoplasmic tail of μ, have such an unbiased repertoire. Because these mice cannot express surface Ig nor can they signal through the pre-B receptor, there can be no selection of developing pre-B cells, and furthermore, there is a resulting block of B cell development at the pre-B to B cell transition step. Thus, neither selection nor environmental bias is able to shape the observed repertoire in μMT mice. As would be anticipated in such mice, two-thirds of the sequences are out-of-frame. Using these mice, we asked the following questions. Do the *7183-I* vs *7183-II* RSS competition substrate results reflect the rearrangement frequency of those genes in vivo? Do all prototypic *7183-I* (or all *7183-II*) genes rearrange at the same frequency?

To determine the *V_H7183* family repertoire we used a 5′ pan-*V_H7183* family-specific primer (AF303) located in FR1 in a region that is identical among all the *V_H7183* family members, and a 3′ J_H primer (AF13N). Rearranged *V_H7183* genes were amplified from newborn liver DNA and cloned into pBluescript, and individual clones were sequenced to determine their identities. The data in Fig. 3 represent 126 sequences derived from nine independent PCRs.

There is a wide range of rearrangement frequencies among the 20 genes. *81X* clearly dominates the *V_H7183* repertoire, comprising 59% of the rearrangements. Of the others, *7183.9*, *3:3.39*, *50.1*, *D6.96*, and *61-1P* rearrange the most frequently, while other genes rearrange at a lower range of frequencies. A comparison of the usage of the *7183-I* and *7183-II* genes clearly revealed the surprising observation that despite the fact that the *7183-II* RSS is better than *7183-I* RSS in the in vitro recombination substrate assay, the genes that rearrange the most in vivo were predominantly *7183-I* genes. The seven genes with prototypic *7183-I* RSS account for 62% of the non-*81X* rearrangements, while the five genes with prototypic *7183-II* RSS together account for only 21% of the non-*81X* rearrangements. This argues against the hypothesis that the RSS plays a predominant role in the nonrandom recombination frequency of the *V_H7183* genes. Also of importance, the range of rearrangement of genes with identical RSS (shown with one or two asterisks for *7183-I* or *7183-II* RSS, respectively, in Fig. 3) is quite large. Two of the *7183-I* genes showed minimal if any rearrangement (*69.1* and *68–5N*), while other *7183-I* genes were among the five most frequently rearranging non-*81X* genes. Surprisingly, the prototypic *7183-II* genes were all relatively poor at rearranging in vivo. Of the genes with variant RSS other than *81X*, only *61-1P* rearranged at a high frequency.

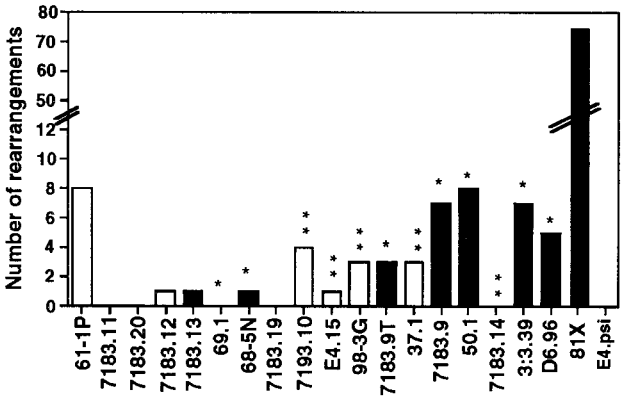


FIGURE 3. Repertoire analysis of rearranged *V_H7183* genes in newborn liver. ■, genes possessing *7183-I* RSS (prototypic and variant); □, those with *7183-II* RSS (prototypic and variant). ∗, Genes with prototypic *7183-I* RSS; ∗∗, those with a prototypic *7183-II* RSS.

Mapping of the chromosomal order of the V_H7183 genes

Because the frequency of recombination of individual genes did not correlate with variation in the RSS, we wished to determine whether there was a gradient of usage from 3′ to 5′ throughout the locus as predicted by the chromosomal location hypothesis. The ongoing assembly of a BAC contig of the *Igh* locus of strain 129 (P. Goebel, N. Janney, W. J. Romanov, C. Hurre, and A. J. Feeney, manuscript in preparation) enabled us to map all the *V_H7183* genes and correlate their relative positions with their frequency of rearrangement.

V_H7183 genes were identified and ordered using two strategies. In one approach, each BAC in a tiling path across the *V_H7183* region was analyzed by PCR amplification using sets of *V_H7183*-specific primers (primarily AF191 (leader) and AF192 (3′ of the RSS)), followed by cloning and sequencing multiple clones to determine the content of each BAC. This approach gave rise to most of the full-length sequences. In the second approach, hybridizing bands on the Southern blots were ordered by analysis of the overlapping BACs; the corresponding restriction fragments were isolated from agarose gel, PCR-amplified, and sequenced without cloning. This approach supplemented the first for genes that did not amplify with the external primers. Additionally, most of the genes were confirmed by direct sequencing from the agarose-isolated restriction fragment. Using all these approaches, we have identified almost all *Eco*RI bands that hybridize with *7183* probes, suggesting we have essentially completed this analysis. It is likely that most of the hybridizing bands that we have not been able to amplify represent pseudogenes, which may vary significantly from our primer sequences. A map of the BAC contig and the *V_H7183* gene order is shown in Fig. 4.

We found that there was no strict clustering of the *7183-I* or *7183-II* genes, although there are more *7183-I* genes than *7183-II* genes at the 3′ end, which may partially explain the higher frequency of recombination of *7183-I* genes. All the genes with variant RSS are at the 5′ end of the locus, with the exception of the two most 3′ genes, which are also variant. By comparing the genomic map (Fig. 4) with our repertoire data (Fig. 3) which is displayed with the genes in the correct chromosomal order, we identified an approximate gradient of rearrangement from the 3′ to the 5′ within the locus. *3.3.39*, *D6.96*, *7183.9*, and *50.1*, all located near *81X* at the 3′ end of the locus, are all over-represented. However, *61-1P*, which rearranges at a relatively high rate is located at the 5′ end of the locus, and *7183.14*, on the 3′ side, seldom rearranges.

Mapping of V_HOx2

The large *V_HQ52* family is interspersed with the *V_H7183* genes. One *V_HQ52* gene, *Ox2*, was shown to be greatly over-represented in rearrangements from fetal liver-derived Abelson-murine leukemia virus pro-B cell lines, and also from hybridomas from LPS-stimulated adult spleen cells (32). To determine whether this gene mapped near the frequently rearranging *81X* at the 3′ end of the locus, we mapped it using primers from the published sequence of *Ox2*, followed by cloning and sequencing. We mapped *Ox2* to the most 3′ BAC, 167C1, which contains *81X*, *D6.96*, and *E4.Psi* (data not shown). This supports the hypothesis that *V_H* genes at the 3′ end of the locus undergo frequent rearrangement.

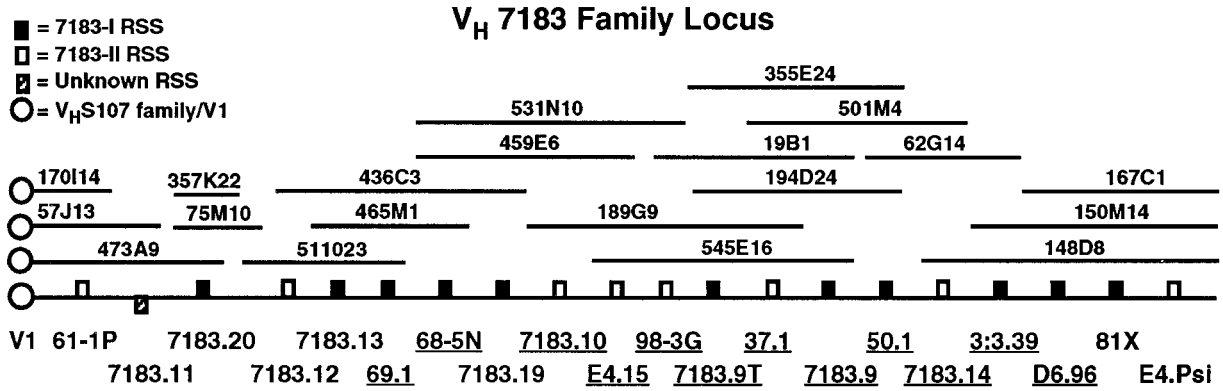


FIGURE 4. A map of the V_H7183 family locus. The genes are ordered from *V_HS107* proximal on the *left* to *D_H* proximal on the *right*. *Above* are lines representing each BAC clone, showing the *V_H7183* genes contained on each clone. ■, *7183-I* genes; □, *7183-II* genes. Genes with prototypic RSS are underlined. The RSS is unknown for *7183.11*.

Discussion

We describe here 20 members of the *V_H7183* family in strain 129 mice, and we have studied their rearrangement in vivo. This is the first analysis of the relative frequency of initial rearrangement events for all individual members of the *V_H7183* family independent of subsequent selection and clonal expansion biases. The repertoire results presented here are similar to those found previously by Holmberg and colleagues, who examined the *V_H7183* repertoire found in BALB/c mice (also Igh^a) at later stages of B cell development. They looked at both bone marrow pre-B and B cells, and splenic B cells, populations that have been subject to selection and clonal expansion by surrogate light chain-driven signaling at the pro-B to pre-B cell stage or Ag-driven expansion after the cells express surface Ig (7). This selection and expansion could have significantly altered the Ig repertoire, but nonetheless, they also observed significant over-representation of *81X* and, to a lesser extent, *7183.9*, *3.3.39*, and *D6.96*, similar to our data (Fig. 3). Together, their data and ours indicate that the nonrandom frequency of gene usage seen in the more mature B cell repertoire is significantly influenced by initial rearrangement frequencies.

We addressed two main hypotheses that have been proposed to explain the unequal frequency of rearrangement of V genes. One issue was the role of the natural variation in RSS in determining the frequency of rearrangement. We sequenced the RSS of 19 members of the *V_H7183* family and showed that 12 of these had one of two different closely related RSS, the prototypic *7183-I* and *7183-II* RSS. Most of the rest had a small number of changes from one of these two prevalent RSS. *81X*, which rearranges the most of all genes in this family, had a unique spacer, and variations in the spacer sequence can affect recombination frequency (18, 33). Thus, the hypothesis that the unique RSS of *81X* was responsible for its high frequency of rearrangement was a reasonable hypothesis until we performed recombination substrate experiments (Fig. 2). These experiments comparing the various RSS in competition recombination substrates reinforce previous observations that differences in the individual elements of the RSS can clearly influence the frequency of recombination. The *V_HJ558* RSS with its poor nonamer and shortened spacer was greatly disfavored compared with the much more conventional *$V_HS107/V1$* RSS. The *V_H7183-II* RSS containing a consensus heptamer rearranged better than the *V_H7183-I* RSS containing a nonconsensus heptamer. However, the *81X* RSS, which differed by only two nucleotides in the spacer from the prototypic *7183-I* RSS, rearranged only marginally better than the consensus *7183-I* RSS, showing that in this case, the spacer sequence variation did not influence recombination potential.

However, these in vitro results were not mirrored by rearrangement patterns in vivo. Firstly, *7183-II* genes have the better RSS as assayed in vitro, but rearrange less frequently in vivo. Secondly, the frequency of use of the various members of *7183-I* is unequal. For example, rearrangements of *7183.9*, *3:3.39*, *50.1*, and *D6.96* are found much more frequently than those of *68–5N* and *69.1*, yet both possess identical *7183-I*-type RSS. Previously we and others have found a strong correlation between the relative frequency of rearrangement of genes in vivo and the ability of their RSS to support recombination in vitro in recombination substrates. In some cases different loci (e.g., κ vs λ) or different genes within a locus (e.g., the three *VκIII* genes) were analyzed, so the correlation could possibly be deemed coincidental (18, 19). However, for the *VκA2* genes we showed that two alleles differing at one position in the heptamer rearrange at ~5-fold different frequencies in vivo and in vitro, and the alleles presumably are identical in chromosomal location and sequence (only three changes were found in 700 bp) strongly suggesting that indeed the RSS was responsible for the differences in rearrangement frequencies in vivo vs in vitro (21). Similarly, two alleles of *Vβ3* have been shown to differ drastically in their representation in the peripheral repertoire (8.1 vs 1.2%), and the only difference in their sequence is 1 bp in the spacer sequence (34). Thus, we believe that these two latter cases of allelic differences in which chromosomal position is not a factor coupled with the extensive in vitro recombination substrate data demonstrate that RSS differences can indeed significantly affect rearrangement frequencies (24, 31, 35). However, the data shown here indicate that factors other than the RSS can also have an important role in rearrangement frequency, and for the *V_H7183* genes these other factors appear to play a predominant role, overriding the variation in RSS potency.

The other previously proposed hypothesis that we were testing was that the location of a gene within the V, D, or J locus plays a key role in its accessibility for rearrangement, with the most proximal genes rearranging first or most often. Here we analyzed BAC clones to determine the gene order of the individual members within the *V_H7183* region. These data clearly show that there is increased frequency of rearrangement in the 3′ end of the locus. Also, we showed that the *V_HQ52* gene, which was previously shown to rearrange very frequently, was located on the same BAC as *81X*, again suggesting that this region at the 3′ end of the locus contains genes that rearrange more often. However, there are a few exceptions. *61-1P*, which rearranges frequently, is at the far 5′ end of the *V_H7183* region. Also, chromosomal location alone cannot account for the very high frequency of rearrangement of *81X*, nor

can its RSS. Thus, chromosomal location appears to bias the rearrangements in general, although location proximal to the *D* cluster is clearly not the only factor that affects recombination frequency.

All frequently rearranging genes other than *61-1P* were *7183-I* genes. These genes also are located in the 3′ half of the locus, so their location may contribute to their overuse. However, *7183.14*, a *7183-II* gene, is located within this region of higher recombination, but rearranges at a much lower level, suggesting that perhaps *7183-I* genes may be more recombinogenic than *7183-II* genes. However, any difference in *7183-I* and *7183-II* use would clearly not be a result of the RSS per se, but could perhaps be due to some other commonality among the *7183-I* vs *7183-II* genes in a region outside of the portion that we have sequenced. Perhaps the *7183-I* genes have a common flanking DNA element that influences their recombination frequency. Further analysis of the sequence of the flanking regions surrounding these genes should yield insight into genomic control regions potentially located in the V_H*7183* locus. Also, because not all *7183-I* genes rearrange at high frequency, this again suggests that individual genes control their rearrangement frequency by some flanking DNA. In accord with this concept, γδ T cells show strictly ordered rearrangement in fetal and adult thymus, and swapping of ~1 kb of 5′-flanking regions, including the promoters of the Vγ3 and Vγ2 genes, led to alteration of their recombination pattern (36). Also, we have recently shown that the transcription factors E2A and EBF show gene-specific preferences in their ability to induce rearrangements in transfected epithelial cells, suggesting that another factor influencing rearrangement frequency could be the presence and relative location of binding sites for transcription factors that promote accessibility to rearrangement.[5] Perhaps the 7183 genes, which do not rearrange as often as other genes with the same RSS, such as *7183.14*, might have a deleterious change in the sequence of the binding sites for factors that control accessibility, and thus be less accessible for rearrangement.

In conclusion, we determined the relative rearrangement frequency of all members of the V_H*7183* family and showed that the individual genes rearranged with a wide range of different frequencies. We compared individual V_H gene family usage with RSS efficiency and chromosomal location, two factors that have been proposed to be major contributors to rearrangement frequencies of a given gene segment in vivo. We determined that the RSS was clearly not the predominant reason for preferential rearrangement, but, rather, that other factors must override the relative efficacy of the RSS. Because the mapping of all the individual genes showed that most frequently rearranging genes were located in the 3′ half of the V_H*7183* region, this suggests that chromosomal location may have the most major influence on V_H*7183* gene rearrangement.

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