Enhancers Located in Heavy Chain Regulatory Region (hs3a, hs1,2, hs3b, and hs4) Are Dispensable for Diversity of VDJ Recombination*

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Background: We examined the effect of deletion of the heavy chain regulatory region (RR) on VDJ recombination in B-cells.
Results: V, D, and J usage is unaffected by the absence of the IgH RR.
Conclusion: The IgH RR is dispensable for V(D)J diversity.
Significance: This region only orchestrates IgH locus activity during late stages of B-cell differentiation.

V(D)J recombination occurs during the antigen-independent early steps of B-cell ontogeny. Multiple IgH cis-regulatory elements control B-cell ontogeny. IGCR1 (intergenic control region 1), the DQ52 promoter/enhancer, and the intronic Emu enhancer, all three located upstream of Cmu, have important roles during V(D)J recombination, whereas there is no clue about a role of the IgH regulatory region (RR) encompassing the four transcriptional enhancers hs3a, hs1,2, hs3b, and hs4 during these early stages. To clarify the role of the RR in V(D)J recombination, we totally deleted it in the mouse genome. Here, we show that V(D)J recombination is unaffected by the complete absence of the IgH RR, highlighting that this region only orchestrates IgH locus activity during the late stages of B-cell differentiation. In contrast, the earliest antigen-independent steps of B-cell ontogeny would be under the control of only the upstream Cmu elements of the locus.

Lymphopoiesis is coupled with programmed accessibility of Ig genes to transcription and to several major transcription-dependent DNA remodeling events (1, 2). During the antigen-independent step of B-cell ontogeny, the V(D)J recombination process within the IgH locus allows the assembly and expression of the functional heavy chain gene (3). Multiple cis-regulatory elements located 5’ and 3’ of constant genes control B-cell ontogeny. The intronic 5’ Eμ element is reported as a master control element of V(D)J recombination (4). Deletion of the 5’ DQ52 element leads to a minor effect on V(D)J recombination and altered D gene usage (5, 6). Recently, IGCR1 (intergenic control region 1), which lies between the Vγ and D clusters, was reported to promote rearrangement of distal rather than Dμ-proximal Vγ segments (7, 8). The IgH 3’ regulatory region (3’RR)6 that encompasses the four transcriptional enhancers hs3a, hs1,2, hs3b, and hs4 has been reported as the master element controlling class switch recombination (2, 9) and is also important for oncogene deregulation in B-cell lymphomagenesis (10, 11). Whereas physical chromosomal interactions have been reported in pro-B-cells between the 5’ Eμ enhancer and the 3’RR (12, 13) and whereas GFP transgenic mice highlighted that combination of 5’ Eμ and the 3’RR mimics the B-specific endogenous expression pattern of IgH genes from pro-B-cells to mature B-cells (14, 15), the potential contribution of the 3’RR to V(D)J recombination control has never been studied. Although bacterial artificial chromosome transgenes have been used to explore the role of the 3’RR in class switch recombination and somatic hypermutation (16, 17), they are inappropriate to test its role in V(D)J recombination. Because antigen receptor gene accessibility studies need the context of endogenous loci, only animals with genomic deletions are suitable for such investigations. To clarify the role of the 3’RR in V(D)J recombination, we analyzed 3’RR-deficient mice lacking the whole 30-kb extent (from hs3a to hs4) of the 3’RR; we recently reported a severe class switch recombination defect and an associated IgH transcription defect at the plasma cell stage in these mice (9).

MATERIALS AND METHODS

Mice—Generation of 3’RR-deficient mice has been described previously (7).

B-cell Lineage Enrichment—Pre-B-cells were purified using CD25-coupled beads (Miltenyi Biotech). B-splenocytes were purified using CD43-coupled beads (Miltenyi Biotech).

Amplification Procedures—Genomic DNA was extracted from the bone marrow CD25+ population (including precursor B-cell fractions C’ and D) (18) from 3’RR-deficient and 129 WT mice (three groups of five mice). Repertoire diversity was measured using the Immun’Ig® test (ImmunID Technologies, Grenoble, France). Multiplex PCR was performed as described (19) using an upstream primer specific to all functional members of a given V family and a downstream primer specific to a

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4 The abbreviation used is: 3’RR, 3’ regulatory region.
given J segment. This assay allows the simultaneous detection and resolution of 92 V-J rearrangements. For semiquantitative analysis, PCRs were stopped at their exponential step.

**Cloning and Sequencing**—Genomic DNA from B-splenocytes was amplified by PCR using the following primers: forward, 5'-GCGAAGCCTARGCCTGGGRCTTCAGTG-AAG-3' (complementary to the V_{H}J_{558} segment); and backward, 5-AGGCTCTGAGATCCCTAGACAG-3' (found in the J_{4} segment). PCR products were cloned into the pGEM®-T Easy vector system (Promega) and sequenced using an ABI PRISM automated laser fluorescent sequencer (PerkinElmer Life Sciences).

**Flow Cytometry Analysis**—Bone marrow cells from heterozygous IgH α^{3'}RR/b^{WT} mice were labeled with various antibodies conjugated with FITC and Spectral Red (PC5, SouthernBiotech): anti-B220-PC5, anti-IgMa-FITC, and anti-IgMb-FITC. Control experiments included irrelevant isotype-matched antibodies. Cells were analyzed on a Beckman Coulter XL apparatus (9, 20).

**RESULTS AND DISCUSSION**

3'RR-deficient IgH Allele Is Normally Used in Heterozygous IgH α^{3'}RR/b^{WT} Mice—3’RR-deficient mice have been described previously (9). Because the disruption was carried out in a 129 embryonic stem cell line, it resulted in a mutated a allo-type IgH locus. Mice were thus bred with C57BL/6 animals to derive heterozygous IgH α^{3'}RR/b^{WT} mice. Cell cytometry analysis of bone marrow cells from α^{3'}RR/b^{WT} mice with anti-IgM allotype-specific antibodies indicated that similar amounts of B-cells expressed either the a or b allotype (Fig. 1). This can be taken as an indication that the 3’RR-deficient allele underwent V(D)J recombination at a rate similar to the WT allele.

V(D)J Usage Is Not Affected in 3’RR-deficient Mouse Pre-B-cells—We investigated V(D)J usage in 3’RR-deficient mice compared with 129 WT mice. Pre-B-cells express a broad random repertoire of V_{H} genes, whereas mature B-cells dominantly express only some sets of V_{H} genes due to ligand selection (21). To bypass a bias generated by antigen selection, V(D)J usage was investigated in the bone marrow CD25^{+} population (including mostly fraction C’ and D pre-B-cell precursors) (18). Ninety-two PCR experiments were done, exploring a wide diversity of V_{H} segments (from the most upstream to the most downstream) and the four J_{4} segments. The diversity of rearrangement (ratio of observed rearrangement to theoretical rearrangement) was similar (p = 0.8, Mann-Whitney U test) between 3’RR-deficient mice (83.3 ± 3.8%, mean ± S.E.) and 129 WT mice (85.9 ± 2.7%) (Fig. 2A). The J_{1-4} usage was not affected by the 3’RR deletion (Fig. 2B). Finally, the vast majority (21/23) of V segments were used in a similar manner between 3’RR-deficient and WT mice (Fig. 2C). Taken together, these results suggest that V(D)J rearrangements normally occurred in bone marrow CD25^{+} B-cells of 3’RR-deficient mice, and there was no indication of any gross alteration in the V(D)J recombination process.

V(D)J Junctions Have Normal Structures in 3’RR-deficient Mice—To appreciate the potential effect of 3’RR deletion on D usage, V(D)J junction sequences from CD25^{+} pre-B-cells from 3’RR-deficient and 129 WT mice were amplified, cloned, and sequenced. Of 95 sequences from WT mice and 157 sequences from 3’RR-deficient mice, we found that there was no significant difference with regard to the percentage of functional sequences, CDR3 (complementarity-determining region 3) length, and number of N nucleotides inserted at the V-D-J junc-

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**FIGURE 1. Expression of IgMa and IgMb in heterozygous IgH α^{3'}RR/b^{WT} mice.** Femoral bone marrow cells were labeled with anti-B220, anti-IgMa, and anti-IgMb antibodies. One representative experiment of five is reported.
D usage in a 3’RR-deficient mouse appeared similar compared with a WT mouse. The D segments were used with frequencies that did not differ from WT mice, including the most upstream (DFL16,3) and the most downstream (DQ52) segments. There was thus no indication of any gross alteration in the DJ recombination process. The extent of N insertions was normal in 3’RR-deficient animals, suggesting that these rearrangements normally occurred at a stage where pro-B-cells express terminal deoxynucleotidyltransferase activity. Similar results were found after investigation of V(D)J

FIGURE 2. V(D)J recombination in 3’RR-deficient mice. A, diversity of V(D)J rearrangements in 3’RR-deficient and 129 WT mice. Experiments were done with bone marrow CD25+ pre-B-cells (five mice in each group). One representative experiment of three is shown. B, J1 usage is not altered in 3’RR-deficient mice. Data represent the mean ± S.E. of three experiments.
juxtaposition sequences from B-splenocytes (i.e. after interaction with cognate antigens) from homozygous 3’RR-deficient mice (89 sequences) and WT mice (100 sequences) (Table 2).

Conclusion—Despite its physical interaction with Eμ at early stages of B-cell development and, de facto, its eventual link to the proximity of rearranging V, D, and J segments (14, 15), it appears that the 3’RR is dispensable for the process of V(D)J recombination in B-cell progenitors. Although the 3’RR controls class switch recombination in differentiated B-cells (9), it appears to be dispensable for the process of V(D)J recombination in B-cell progenitors. These results extend those obtained with hs3b/hS4-deficient mice (22). They clearly contrast with those obtained with Eμ-, DQ52-, and IGCR1-deficient animals (4, 8) and suggest that, among cis-activating elements of the IgH locus, those located in the 5’ part of the locus are fully sufficient for the optimal completion of V(D)J recombination.

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TABLE 1
Pattern of VDJ junctions cloned from pre-B-cells of 3’RR-deficient and WT mice

| D usage         | WT mice | 3’RR-deficient mouse |
|-----------------|---------|----------------------|
|                 | %       | No.                  | %     | No.                  |
| DFL16,3         | 5.00    | 5                    | 3.37  | 3                    |
| DST4,2          | 6.00    | 6                    | 0.00  | 0                    |
| DFL16,1         | 28.00   | 28                   | 22.47 | 20                   |
| DFL16,1         | 9.00    | 9                    | 7.87  | 7                    |
| DFL16,3         | 3.00    | 3                    | 2.25  | 2                    |
| DFL16,2         | 1.00    | 1                    | 11.24 | 11                   |
| DFL16,2         | 16.00   | 16                   | 7.87  | 7                    |
| DFL16,2         | 4.00    | 4                    | 12.36 | 12                   |
| DFL16,2         | 2.00    | 2                    | 3.37  | 3                    |
| DFL16,2         | 0.00    | 0                    | 2.25  | 2                    |
| DST4            | 4.00    | 4                    | 4.49  | 4                    |
| DQ52            | 7.00    | 7                    | 4.49  | 4                    |

TABLE 2
Pattern of VDJ junctions cloned from B-splenocytes of 3’RR-deficient and WT mice

| D usage         | WT mice | 3’RR-deficient mouse |
|-----------------|---------|----------------------|
|                 | %       | No.                  | %     | No.                  |
| DFL16,3         | 5.00    | 5                    | 3.37  | 3                    |
| DST4,2          | 6.00    | 6                    | 0.00  | 0                    |
| DFL16,1         | 28.00   | 28                   | 22.47 | 20                   |
| DFL16,1         | 9.00    | 9                    | 7.87  | 7                    |
| DFL16,3         | 3.00    | 3                    | 2.25  | 2                    |
| DFL16,2         | 1.00    | 1                    | 11.24 | 11                   |
| DFL16,2         | 16.00   | 16                   | 7.87  | 7                    |
| DFL16,2         | 4.00    | 4                    | 12.36 | 12                   |
| DFL16,2         | 2.00    | 2                    | 3.37  | 3                    |
| DFL16,2         | 0.00    | 0                    | 2.25  | 2                    |
| DST4            | 4.00    | 4                    | 4.49  | 4                    |
| DQ52            | 7.00    | 7                    | 4.49  | 4                    |

V(D)J Diversity in IgH 3’RR-deficient Mice
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