Analysis of Mice Lacking Dnasel Hypersensitive Sites at the 5′ End of the IgH Locus

Thomas Perlot1,9a, Inka Pawlitzky2,9b, John P. Manis3, Ali A. Zarrin1,9c, Peter H. Brodeur2, Frederick W. Alt1,9*

1 The Howard Hughes Medical Institute, The Children’s Hospital, Immune Disease Institute, and Department of Genetics, Harvard Medical School, Boston, Massachusetts, United States of America, 2 Immunology Program, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, Boston, Massachusetts, United States of America, 3 Children’s Hospital Boston and Joint Program in Transfusion Medicine, Harvard Medical School, Boston, Massachusetts, United States of America

Abstract

The 5′ end of the IgH locus contains a cluster of Dnasel hypersensitive sites, one of which (HS1) was shown to be pro-B cell specific and to contain binding sites for the transcription factors PU.1, E2A, and Pax5. These data as well as the location of the hypersensitive sites at the 5′ border of the IgH locus suggested a possible regulatory function for these elements with respect to the IgH locus. To test this notion, we generated mice carrying targeted deletions of either the pro-B cell specific site HS1 or the whole cluster of Dnasel hypersensitive sites. Lymphocytes carrying these deletions appear to undergo normal development, and mutant B cells do not exhibit any obvious defects in V(D)J recombination, allelic exclusion, or class switch recombination. We conclude that deletion of these Dnasel hypersensitive sites does not have an obvious impact on the IgH locus or B cell development.


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* E-mail: alt@enders.tch.harvard.edu

† These authors contributed equally to this work.

¤a Current address: IMBA, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria

¤b Current address: Division of Molecular Genetics, Netherlands Cancer Institute, Amsterdam, The Netherlands

¤c Current address: Department of Immunology, Genentech, San Francisco, California, United States of America

Introduction

The variable region of an immunoglobulin heavy chain (IgH) is assembled from V (variable), D (diversity), and J (joining) gene segments that lie upstream of several IgH constant (C) region exons in a process called V(D)J recombination [1]. The mouse IgH locus contains large numbers of V̄H segments and multiple D and J̄H segments but an individual IgH V(D)J exon is assembled from only one V̄H, one D, and one J̄H segment. V(D)J recombination of the IgH locus takes place in pro-B cells in an ordered way such that D to J̄H recombination precedes V̄H to DJ̄H recombination [2]. In this regard, activation of the IgH locus is thought to progress in a stepwise manner [3]. D to J̄H rearrangement efficiently occurs on both alleles, however, allelic exclusion ensures that V̄H to DJ̄H recombination results in expression of a functional heavy chain (HC) from only one of the two alleles [4].

Mature B-cells can undergo further alterations of their HCs. IgH class switch recombination (CSR) causes expression of different immunoglobulin isotypes which confer different effector functions. During this recombination process one of several sets of downstream C̄H exons replaces the C̄H exons and the intervening sequence is deleted from the chromosome, which results in expression of a new C region without changing the specificity of the IgH variable region [5].

A large effort has been made to elucidate mechanisms of IgH locus regulation and a number of cis-regulatory elements have been described and characterized. The IgH intrinsic enhancer (E4) resides in the JH – C̄H intron and was shown to be necessary for efficient V(D)J recombination by promoting both D to J̄H and V̄H to DJ̄H recombination [6,7]. Downstream of the C̄H genes at the very 3′ end of the IgH locus a cluster of Dnasel hypersensitive sites was described, termed 3′ IgH regulatory region (3′IgH RR). So far two main functions have been assigned to this regulatory region: the 3′ Igh RR plays an important role in promoting CSR to most IgH isotypes, and the 3′IgH RR was shown to be necessary for high level expression of the functionally assembled HC gene from the promoter 5′ of the V̄HDJ̄H exon [8].

An additional potential regulatory region was identified at the 5′ end of the IgH locus, consisting of four Dnasel hypersensitive sites [9]. One of these sites, HS1, was shown to be pro-B cell specific, the stage during which IgH V(D)J recombination takes place, and was suggested to include binding sites for the transcription factors PU.1, Pax5 and E2A [9]. These observations led to the suggestion that this region might represent a new regulatory region for IgH rearrangements. In this regard, the 5′ end of the IgH locus is an attractive location for a regulatory element because it would not be deleted during the course of V(D)J recombination, and it might explain control of several unresolved phenomena in the IgH locus. Among these is the regulation of V̄H germline transcripts as so far...
no cis-regulatory element has been identified that controls activity of the bulk of unarranged VH promoters. Furthermore, it is not known how it is achieved that proximal and distal VH segments are activated independently or why usage of distal versus proximal VH gene families varies significantly.

Here we report the targeted deletion of the pro-B cell specific 5′IgH HS1 as well as combined deletion of HS1, HS2, HSa,b in mice. We analyzed potential implications on B cell development, V(D)J recombination, and IgH CSR.

Methods

Targeted deletion of 5′IgH DNAseI hypersensitive sites in ES cells and generation of mutant mice

All mouse were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by Animal Research of Children’s Hospital Boston (Protocol # 08 11 1253R).

The RHS1 targeting vector was assembled in pLNTK (10). As a 5′ homology arm a 2.2 kb PCR product was generated with primers 5′ GTGACGGATTTAGAGGATTACAACAC 3′ and 5′ GTGACCTTGGATAACACAGGAATTCTG 3′ containing a SalI site at their 5′ ends, which facilitate cloning of the PCR product into the Sall site of pLNTK. As a 3′ homology arm a 7.3 kb AatII – ApaI fragment was blunt end cloned into the XhoI site of pLNTK. The R3′HSs targeting vector was generated by blunt end cloning a 4.4 kb EcoRI fragment into the Sall site of pLNTK as a 5′ homology arm, and a 7.0 kb Kpnl fragment into the XhoI site as the 3′ homology arm. Correct targeting events and cre – loxP deletion events were confirmed by Southern blotting (Fig 1).

Probe 1 is a 830 bp PCR product amplified with primers 5′ GCCTATGTCACTGCAGCTCAAC 3′ and 5′ CACGTGAGCACCTCGTTATGCTG 3′. Probe 2 is a 1.2 kb PstI – EcoRI fragment of HS2. Probe 3 is a 0.8 kb PstI – XbaI fragment about 11 kb 3′ of HS3b. To confirm single integration of the targeting vectors a 525 bp NeoR probe was used, amplified with primers 5′ GCAGCCATATGGGATCGGC 3′ and 5′ GACCTCCATCTTATGTCTG 3′.

Targeted deletion of 5′IgH DNaseI HSs/RHS1+HS2−HS3+ allele and, upon cre/loxP deletion, 340 bp BccI – AatII fragment, harboring HS1, HS2, HSa,b in mice. We analyzed potential implications on B cell development, V(D)J recombination, and IgH CSR.

PCR assay for V(D)J rearrangements

Pro-B cells (IgM+ B220+ CD43−), pre B-cells (IgM+ B220+ CD43−), and double positive T-cells (B220+ CD4+ CD8−) were isolated by FACs on a FACSAria (BD Biosciences) and genomic DNA was extracted. 30 ng DNA or 3-fold dilutions were analyzed by PCR for Dh-Jh, Vir-Djhs, Vc-Ja, and Vj-Jc rearrangements with primers listed in Table S1. Input DNA amounts were normalized upon PCR amplification within DLG5. PCR was performed at 95°C for 4′, 30 cycles of 95°C for 30′, 60°C for 90′, and 72°C for 2′, followed by 72°C for 3′. PCR products were transferred from ethidium bromide gels to nylon membranes and visualized with end labeled oligonucleotide probes (Table S1).

CDR 3 lengths were generated from IgH VDH rearrangements from mature B cells using oligonucleotides for V558 and JH4. PCR fragments were amplified using iProof (Bio-Rad) polymerase and cloned into Zero Blunt Topo vectors (Invitrogen), and sequenced.

IgH class switch recombination assay

CD43+ splenocytes were isolated by MACS, cultured with LPS or IL4/αCD40, and analyzed by flow cytometry as described previously [13].

RT-PCR analysis

RNA was extracted using TriPure Isolation Reagent (Roche). 200 ng–1 µg of total RNA was reverse transcribed for one hour at 50°C using random hexamers (Roche) and Superscript III (Invitrogen) reverse transcriptase. PCR was performed at 94°C for 4′, 30–39 cycles of 94°C for 30′, annealing temperature (Table S1) for 30′, 72°C for 30′, followed by 72°C for 5′. cdNA input amount was normalized upon PCR amplification of β-actin cDNA. PCR products were visualized on ethidium bromide gels and/or subsequently transferred to nylon membranes and visualized with end labeled oligonucleotide probes (Table S1).

Flow cytometry and cell sorting

Single cell suspensions from spleen, thymus, or bone marrow were stained in PBS 2% FBS with various antibodies: FITC-αCD3, PE-Cy5-αCD22, PE-αCD8a, PE-αCD43, FITC-αLy9.1, APC-αIgM, APC-CD7-αB220 (BD Pharmingen), PE-αAA4.1, FITC-αCD4 (eBioscience). FACs analysis was performed on a FACS Calibur (BD Biosciences) and a FACS Aria (BD Biosciences) apparatus. Cell sorts were performed on a FACS Aria (BD Biosciences) apparatus.

Results

Generation of mice with targeted deletion of 5′IgH

DNAseI hypersensitive sites

To determine the in vivo function of the cluster of DNAseI hypersensitive sites described at the 5′ end of the IgH locus [9] we first replaced a ~340 bp BccI – AatII fragment, harboring HS1, with a loxP flanked PGK-Neo cassette. All targeting experiments were performed in heterozygous IgH+/−EF1 ES cells which have the advantage that IgH+/− (129 strain) and IgH−/− (C57BL/6 strain) alleles can be distinguished by antibodies against the different allotypes or by detection of restriction fragment length polymorphisms (RFLP). Targeting vector homology arms were cloned from 129 strain genomic DNA, resulting in correct targeting events only on the IgH− allele. In heterozygous targeted ES cells, the IgH− allele always remained in the untargeted wildtype configuration.

Targetings were performed with the RHS1 targeting vector (Fig 1A) to obtain the RHS1 allele and, upon cre/loxP deletion,
Figure 1. Targeting strategy for the generation of the RHS1, ΔHS1, and ΔHSs alleles. (A) the wildtype (wt) IgH locus and its 5’ flanking region are shown. Vα, Dα, Jα indicate representative IgH V, D, and J segments. Exons 1, 2, and 3 of Zfp386 are shown as grey rectangles, DNaseI hypersensitive sites HS1, HS2, HS3a, and HS3b are shown as black ovals. Correct targeting events of the RHS1 targeting vector were identified by Southern blotting on BglII digested ES cell DNA using probe 1, which results in a 15.8 kb band (lane 1) in addition to the 14.2 kb wildtype band (lane 2). Cre – loxP (black triangles) mediated deletion of the PGK-NeoR cassette (NeoR) from the RHS1 allele results in the ΔHS1 allele. Deletions were identified by Southern analysis of SacI digested DNA utilizing probe 2. A targeted clone before Cre – mediated deletion exhibits a 6.7 kb RHS1 band and a 5.1 kb wildtype band (lane 3). Upon deletion of the PGK-NeoR cassette, a 4.8 kb ΔHS1 band and a 5.1 kb wildtype band are visible (lane 4). Lane 5 shows untargeted wildtype DNA. (B) The ΔHS1 allele was targeted with the R3'HSs targeting vector to introduce a PGK-NeoR cassette flanked by loxP sites. Correct targeting events were confirmed by Southern blotting on SphI digested ES cell DNA with probe 1, resulting in a 2.6 kb band for the wildtype IgHb allele and a 7.2 kb band for the ΔHSs allele (lane 5). Cre – mediated recombination between the first and the third loxP site generates the ΔHSs allele (14.1 kb, lane 8). Homozygous R3'HSs ES cells were generated under increasing concentrations of G418, resulting in a single 7.2 kb R3'HSs band (lane 7). Southern analysis on SphI digested DNA with probe 3 confirms correct targeting events of the R3'HSs targeting vector. ΔHS1 ES cells exhibit a 15.7 kb ΔHS1 band and a 26.3 kb band for the wildtype IgHb allele (lane 9), R3'HSs ES cells show a 11.1 kb ΔHSs band in addition to the 26.3 kb wildtype IgHb allele (lane 10). All targeting events occurred on the IgHb allele, whereas the IgHs allele remained in wildtype configuration. Drawings not to scale. B - BglII; S - SacI; P - SphI.

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A

bone marrow

wt  ΔHS1  RHS1  ΔHSs

B

spleen

wt  ΔHS1  RHS1  ΔHSs

C

thymus

wt  ΔHS1  RHS1  ΔHSs
the ΔH1S allele. Correct targetting events (Fig1) and single integration of the targeting vector (Figure S1) were confirmed by Southern blotting. Subsequently, targeted ES cells were injected into Rag2−/− blastocysts to obtain Rag-deficient blastocyst complementation (RDBC) chimeras, and into wildtype blastocysts to generate mice that carry the RH1S or ΔHS1 allele in their germline. In order to delete all four hypersensitivity sites (HS1, HS2, HS3a, and HS3b), ES cells containing the ΔHS1 allele were targeted with the R′3′HSs targeting vector to obtain the R′3′HSs allele (Fig. 1B). Cre/loxP recombination between the loxP site originating from the ΔHS1 allele and the loxP site 3′ of the PGK-Neo cassette results in the replacement of a 8.9 kb region, harboring all described 5'IgH DNaseI hypersensitive sites, with a single loxP site, referred to as the ΔHS1 allele. Germline transmission could not be achieved for either of the R′3′HSs or ΔHS1 heterozygous ES cell lines. Therefore, we placed ES cells containing the R′3′HSs allele under increasing concentrations of G418 to select for homozygous mutant ES cells. The homozygous mutant ES cells were subsequently subjected to cre/loxP recombination to delete the Neo' gene and generate ES cells homozygous for the ΔHS1 allele. The homozygous mutant ΔHS1 ES cells were injected into Rag2−/− blastocysts, and chimeras generated by RDBC and lymphocytes were analyzed.

Development of homozygous RHS1, ΔHS1, and ΔHSs lymphocytes

Lymphocytes of different developmental stages can be identified by FACS analysis of cells from lymphoid tissues such as bone marrow, thymus, or spleen. We analyzed 8 week old wildtype mice, homozygous RH1S1, and homozygous ΔHS1 mice that carry the mutant alleles in their germline, as well as lymphocytes from RDBC chimeras generated from homozygous ΔHSs ES cells (Fig. 2). In wildtype bone marrow, pro-B cells can be identified as IgM+ B220+ CD43lo and pre-B cells as IgM+ B220+ CD43hi, respectively. Defects in B-cell development can be revealed by the increase or decrease of certain lymphocyte populations. In this regard, impaired IgH V(D)J recombination leads to an accumulation of pro-B cells and to reduced numbers of pre-B cells [7]. We performed FACS analyses of bone marrow from three mice of each genotype to measure the percentage of pro- and pre-B cells in the lymphocyte gate. These analyses revealed the average percentage (± standard deviation) of pro-B and pre-B cells, respectively of B220+CD43lo events in the total lymphocyte gate were 14±2 and 50±20 for wildtype, 9±3 and 56±6 for ΔHS1, and 9±3 and 42±11 for RHS1 mice (Fig. 2A). Thus, there were no obvious differences in early B-cell development in wildtype and mutant mice. However we cannot exclude minor developmental defects not readily detectable by such analyses. Homozygous mutant ΔHS1 bone marrow cells were analyzed in a similar fashion, but only Ly9.1+ cells were included in the analysis. Ly9.1 is exclusively expressed on cells derived from the ΔHS1 ES cells but not on cells derived from the Rag2−/−- blastocyst. The presence of a large compartment of blastocyst derived Rag-deficient pro-B cells in the bone marrow can interfere with development of ES cell derived B-lymphocytes. However, FACS analysis of ΔHS1 bone marrow B cells indicated the presence of both pro- and pre-B cells and did not suggest a block in B-cell development (Fig. 2A).

Next we analyzed spleens for IgM+ B220+ AA4.1+ transitional B-cells and IgM+ B220+ AA4.1+ mature B-cells (Fig. 2B). In homozygous RH1S1, and homozygous ΔHS1 mice transitional (19.7%−27.5%) and mature (63.5%−72.7%) B-cell compartments similar to wildtype were identified, whereas, in spleens from RDBC chimeras generated from homozygous ΔHSs ES cells strongly reduced numbers of transitional B-cells were observed (6.44%). This reduction in the transitional B-cell compartment compared to the mature B-cell compartment (75%) might be due to overall reduced numbers of developing B cells in the obtained RDBC chimeras and to the accumulation of mature B-cells in the periphery of these mice and not to a defect in B cell development. Finally, we observed normal development of T-lymphocytes in the thymi of wildtype, homozygous RH1S1, and homozygous ΔHS1 mice as well as RDBC chimeras generated from homozygous ΔHSs ES cells (Fig. 2C).

The ΔHS1, RHS1, and ΔHSs alleles show no significant defect in V(D)J recombination

The data indicating that HS1 is pro-B cell specific and contains binding sites for the transcription factors PU.1, Pax5, and E2A led to the suggestion that HS1 could be involved in regulation of V(D)J recombination at the IgH locus [9]. We utilized a PCR based assay to assess V(D)J recombination efficiencies in developing lymphocytes from mice with homozygous deletion of HS1. FACS-sorted pro-B cells (IgM+ B220+ CD43lo) and pre-B cells (IgM+ B220+ CD43hi) from bone marrow and double positive (DP) T-cells (B220+ CD43+ CD4hi) from thymus were analyzed for D to JH, Vh to DJh, Vk to Jk and Vλ to Jλ rearrangements. Intensities of PCR bands for Dα4Q52 to JH (Fig. 3A) and DSP to JH rearrangements (Fig. 3B) were comparable in pro-B cells, pre-B cells, and DP T-cells from wildtype, homozygous RH1S1, and homozygous ΔHS1 mice indicating that deletion of the pro-B cell specific HS1 site does not detectably affect the D to JH recombination step. DNA input amounts were normalized to the presence of a genomic sequence within the murine DLG5 gene (Fig. 3H).

It was speculated that HS1 might regulate the differential activation of distal versus proximal Vh families [9]; therefore, we analyzed the rearrangement efficiencies of the proximal Vh7183 family (Fig. 3C), the distal Vh4558 family (Fig. 3D), and the distal most Vh1 segment Vh558.55 (Fig. 3E). We found that pro-B cells and pre-B cells from wildtype, homozygous RH1S1, and homozygous ΔHS1 mice rearrange the proximal Vh7183 family at similar levels (Fig. 3C). Also, the distal family Vh4558 (Fig. 3D) as well as the distal most Vh1 segment Vh558.55 (Fig. 3E) rearranged at comparable efficiencies in pro-B cells and pre-B cells from the three different genotypes. Vh1 to DJh recombination was absent in DP T-cells from wildtype, homozygous RH1S1, and homozygous ΔHS1 mice as the Vh1 to DJh recombination step is restricted to the B-lineage (Fig. 3C, D, E). These data show that HS1 is not
Figure 3. V(D)J recombination in ΔHS1 and RHS1 mice. Pro-B cells, pre-B cells and double positive (DP) T-cells from wildtype 129 mice, from homozygous ΔHS1, and homozygous RHS1 mice were sorted by FACS. 5-fold dilutions of genomic DNA were subjected to PCR analysis. IgH V(D)J recombination efficiencies were assessed using a reverse primer downstream of JH4 and a forward primer recognizing DQ52 (A), DSPs (B), the V\textsubscript{H}7183 family (C), the V\textsubscript{H}J558 family (D), or the V\textsubscript{H}J558.55 segment (E). Rearrangements can occur to JH1, JH2, JH3, or JH4 as indicated. GL indicates PCR product from germline configuration. Ig\textsubscript{k} rearrangements were quantified (F), rearrangements can occur to J\textsubscript{k}1, J\textsubscript{k}2, J\textsubscript{k}3, or J\textsubscript{k}4 as indicated. Ig\textsubscript{l} rearrangement efficiency was analyzed (G). Bands correspond to V\textsubscript{l}2-J\textsubscript{l}2, V\textsubscript{l}1-J\textsubscript{l}3, or V\textsubscript{l}1-J\textsubscript{l}1 rearrangements as indicated. DNA input was normalized to DLG5 PCR products (H).

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necessary for rendering the distal part of the \(V_H\) cluster accessible and, therefore, suggest that HS1 does not play a major role in regulation of usage or accessibility of distal versus proximal \(V_H\) families.

Recently, it has been shown that IgH and Igk loci can colocalize during B-cell development, mainly at the pre-B cell stage, and it was suggested that this colocalization induces decontraction of the IgH locus [14]. We therefore performed an assay to evaluate Igk (Fig. 3F) and Igl (Fig. 3G) V(D)J recombination efficiencies. Both Igk and Igk loci show similar V(D)J recombination levels in the analyzed developing B cells from wildtype, homozygous RHS1, and homozygous \(DHS1\) mice, while light chain rearrangements were absent in DP T-cells from the three different genotypes. Therefore, we conclude that deletion of HS1 does not markedly affect Ig light chain gene rearrangements.

As an independent method to evaluate D to JH and \(V_H\) to DJH recombination efficiencies, we generated clonal hybridoma lines from splenic B-cells of IgH\(^{ab}\) heterozygous RHS1, RHS1 mice carrying the mutant allele in their germline and of RDBC chimeras generated from heterozygous \(AHS1\) ES cells (Table 1). In each case the IgH\(^a\) allele was the mutant allele while the IgH\(^b\) allele was the wildtype allele. In splenic B-cells, one allele exists as a functional \(V_HJ_H\) rearrangement, while the second allele can either be in germline configuration, or it exists as a DJH or a nonproductive \(V_HDJ_H\) rearrangement. The rearrangement status of the second IgH allele was assessed by Southern blot analysis. Consequently, hybridomas expressing the mutant IgH\(^a\) allele can be analyzed for rearrangement efficiency of the wildtype IgH\(^b\) allele, and vice versa, in hybridomas expressing the wildtype IgH\(^a\) allele, the rearrangement status of the mutant IgH\(^b\) allele can be assessed.

Wildtype B cells undergo D to JH rearrangements on both alleles; but still, consistent with earlier studies, about 5% of hybridomas harbor an IgH allele in germline configuration which presumably originates from tripartite fusions involving non-B-cells [12] (not shown). The number of mutant alleles in germline configuration was not increased compared to wildtype indicating that RHS1, RHS1, and DHSs alleles can undergo efficient D to JH recombination (not shown). In 30–60% of wildtype B-cells the nonproductive allele is in DJH configuration; whereas in 40–50% the nonproductive allele is in \(V_HDJ_H\) configuration [13]. An increased percentage of DJH alleles could indicate less efficient VH to DJH recombination: in contrast, an increased percentage of \(V_HDJ_H\) alleles might indicate a break in allelic exclusion. IgMa expressing hybridomas generated from B-cells heterozygous for RHS1, RHS1, and DHSs were analyzed for their rearrangement status of the wildtype IgM\(^a\) allele and show ratios of DJH (56%–61%), and \(V_HDJ_H\) alleles (39%–44%) in the expected range (Table 1). IgM\(^b\) expressing hybridomas were analyzed for the

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### Table 1. \(AHS1\), RHS1, and DHSs hybridoma analysis.

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<th>DJ</th>
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<td>IgM(^a)</td>
<td>66 (61%)</td>
<td>43 (39%)</td>
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<tr>
<td>IgM(^b)</td>
<td>52 (57%)</td>
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<td>IgM(^a)</td>
<td>62 (56%)</td>
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<td>IgM(^b)</td>
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<td>IgM(^a)</td>
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<td>IgM(^b)</td>
<td>55 (69%)</td>
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Hybridomas were generated from heterozygous IgH\(^{ab}\) \(AHS1\), RHS1, and DHSs splenic B-cells. In each case, IgM\(^a\) is the wildtype allele and IgM\(^b\) is the mutant allele, IgM\(^a\) expressing hybridomas (IgM\(^a\)) and IgM\(^b\) expressing hybridomas (IgM\(^b\)) of each genotype were analyzed for the rearrangement status of their nonproductive allele. Numbers for D to JH rearranged alleles (DJ) and nonproductive \(V_H\) to DJH rearranged alleles (VDJ-) are shown.

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**Figure 4. IgM\(^a\) versus IgM\(^b\) expression in \(AHS1\), RHS1, and DHSs B cells.** Heterozygous IgM\(^{ab}\) B-cells from spleen (A) and bone marrow (B) of 129 wildtype (wt) or \(AHS1\), RHS1, and DHSs RDBC chimeras were analyzed IgM\(^a\) and IgM\(^b\) expression. In \(AHS1\), RHS1, and DHSs B-cells the IgM\(^a\) allele is in wildtype configuration whereas the IgM\(^b\) allele is the mutant allele.

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rearrangement status of their mutant IgM⁺ allele. RHS1, AHS1, and AHSs alleles do not show significantly increased or decreased (Fisher’s exact test) rearrangement ratios compared to wt alleles, as 52%–69% of mutant alleles were in DJH configuration while 31%–48% were in VHDJH configuration.

FACS analysis was performed on B-cells from spleens (Fig. 4A) and bone marrow (Fig. 4B) of RDBC chimeras generated from heterozygous RHS1, AHS1, and AHSs ES cells. IgM⁺ expressing populations, representing the targeted allele, and IgM⁺ expressing populations, representing the wt allele, were of similar size both in bone marrow and in spleen from RHS1, AHS1, and AHSs chimeras, suggesting that the RHS1, AHS1, and AHSs alleles can undergo V(D)J recombination at the IgH locus at similar efficiencies as wt alleles.

The AHS1, RHS1, and AHSs alleles do not affect allelic exclusion

FACS analysis of wt B cells from spleen (Fig. 4A) and bone marrow (Fig. 4B) shows distinct populations of similar size for B cells that are single positive for either IgH⁺ or IgH⁺, but intact allelic exclusion prevents the appearance of an obvious IgH⁺, IgH⁺ double producing population. Similarly, RDBC chimeras generated from heterozygous RHS1, AHS1, and AHSs ES cells exhibited IgH⁺ or IgH⁺ single positive B-cell populations of similar size in spleen (Fig. 4A) and bone marrow (Fig. 4B) but no IgH⁺, IgH⁺ double producing population. These data indicate that the deleted sequences of the targeted alleles do not contain a regulatory element that is necessary for implementation of allelic exclusion.

Furthermore, data from hybridoma analysis (Tab. 1) support this notion as in the case of a break in allelic exclusion increased numbers of hybridomas with VH to DJH rearrangements on both alleles would be expected. Such an increase compared to wt alleles could not be observed (Tab. 1), which indicates intact allelic exclusion of RHS1, AHS1, and AHSs alleles.

The 5’IgH DNaseI hypersensitive sites are not required for efficient class switch recombination

To assess a potential effect of the 5’IgH DNaseI hypersensitive sites on CSR, B-cells were stimulated to undergo CSR and analyzed by FACS (Fig. 5). Stimulation with LPS induces IgH isotype switching to γ3, while stimulation with IL4+ αCD40 promotes switching to γ1. B-cells from AID−/− mice served as negative controls, while wt B-cells represented a positive control and therefore switched to the appropriate isotypes under LPS or IL4+ αCD40 stimulation. CSR in homozygous AHSs B-cells occurs at similar levels as in wt B-cells implying that the cluster of 5’IgH DNaseI hypersensitive sites is not required for efficient CSR to γ1 (Fig. 5A) and γ3 (Fig. 5B).

Complex phenotypes without an obvious relation to the IgH locus in AHS1 mice

We performed targeted deletion experiments of the 5’IgH DNaseI hypersensitive sites to test their suggested function in IgH locus regulation. So far no major IgH related phenotype was identified. However, about 20% of homozygous AHS1 mice develop a complex neurological phenotype and die at 3–5 weeks of age.
age, likely due to a lack of food intake. These mice exhibit an abnormal limp grasping phenotype, i.e. mice clasp their front and hind feet almost immediately upon being lifted by their tail (Fig. 6A, B). Furthermore these mice develop a hydrocephalus, which is already visible at about one week of age and is enlarged over the following weeks (Fig. 6C, D). Histological analysis confirmed the presence of a hydrocephalus, revealed abnormal hindbrain development, and revealed retinal abnormalities (Fig. 6E, F, G). The wildtype retina is organized in a delicate layer system (Fig. 6E): stratum opticum and ganglionic layer (1), inner plexiform layer (2), inner nuclear layer (3), outer plexiform layer (4), outer nuclear layer (5), layer of rods and cones (6), pigment layer (7). In the ΔHS1 mutant mice, the organization of retinal layers is impaired in such a way that nuclei from the outer nuclear layer are aberrantly located in the layer of rods and cones (Fig. 6F). In some more severe cases rosette formation in the outer nuclear layer is evident (Fig. 6G). Currently, we do not know what causes these phenotypes, but we exclude that this phenotype is caused by a second integration of the targeting vector at an undefined site in the genome (Figure S1). The deletion in the ΔHS1 allele deletes 340 bp within intron 1 of Zfp386. Therefore, misregulation of that poorly described gene might cause the described phenotypes although other possibilities are conceivable.

**Discussion**

This study aimed for elucidating the potential regulatory functions of a cluster of recently described DNaseI hypersensitive sites at the 5’ end of the IgH locus [9]. We performed targeted deletion of either the pro-B cell specific site HS1 (ΔHS1) or deletion of the entire cluster of hypersensitive sites (ΔHSs) in mice or in their lymphocytes, respectively. A potential regulatory element at the 5’end of the IgH locus was speculated to regulate processes such as IgH allelic exclusion, V_{H} germline transcription, differential accessibility or usage of distal versus proximal V_{H} gene families. Furthermore, it was suggested that the 5’end of the IgH locus might play a role in positioning the IgH locus in distinct subnuclear compartments [16,17,18], and it was suggested to harbor insulator or boundary capacity [19].

B- and T-lymphocytes homozygous for the ΔHS1, RHS1, and ΔHSs alleles appear to proceed through lymphocyte development in an unimpaired way. Data from RDBC chimeras generated from...
heterozygous *AHSl*, *RHS1*, and *HSs* ES cells indicated that allelic exclusion is not affected in mutant B-cells and that mutant IgH alleles can undergo efficient V(D)J recombination of their IgH locus. Furthermore, data from PCR assays to analyze V(D)J recombination efficiency in mice with HS1 deleted on both alleles supports the notion that HS1 is not necessary for neither the D to JH nor the VH to DJH recombination step. Both proximal and distal VH families as well as the distal most VH segment VhJ530.55 rearrange as efficiently as on wildtype alleles. Similarly, IgL loci in HS1 deleted B-cells rearrange at the same efficiency as wildtype IgL loci. Analysis of IgH V(D)J rearrangement status in hybridomas generated from heterozygous *AHSl*, *RHS1*, and *AHs* B-cells also strengthens the idea that the deleted DNaseI hypersensitive sites would not regulate IgH V(D)J recombination. We tested for potential alterations associated with DNA end processing during V(D)J recombination by examining the CDR3 sequence obtained from homozygous *AHSl* B cells and found a distribution in length that was similar to wildtype B cells [20] (Figure S2).

We tested a potential effect of the cluster of DNaseI hypersensitive site on the process of IgH CSR. Assaying class switching upon different in vitro stimulations in wildtype and homozygous *AHs* B-cells let us conclude that the cluster of 5 IgH DNaseI hypersensitive sites does not play a crucial role in CSR.

The only observed phenotypes so far occurred in homozygous *AHSl* mice and seem to be independent of the IgH locus. *AHSl* mice show abnormal limp grasping indicating a neurological abnormality, *AHSl* mice can develop severe hydrocephalus and exhibit retinal impairments. A possible explanation for these phenotypes is a potential defect in regulation of the zinc finger protein *Zfp386*. *AHSl* deletes a 340 bp region from intron 1 of *Zfp386*. *AHSl* deletes a 340 bp region from intron 1 of *Zfp386* which might result in different splice forms, impaired expression levels, or expression patterns of this gene.

Overall, our analysis of the deletion of the pro-B cell specific site HS1 or the whole cluster of 5 IgH DNaseI hypersensitive sites did not support the existence of a cis-regulatory function of these elements regarding the IgH locus.

**References**

20. Brotman I, Williams for mouse work, and Natasha Barteneva for cell sorting.

**Author Contributions**

Conceived and designed the experiments: TP IP PB FWA. Performed the experiments: TP IP. Analyzed the data: TP IP JPM AZ PB. Wrote the paper: TP IP FWA.