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

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Analysis of Mice Lacking D Nasel Hypersensitive Sites at the 5' End of the IgH Locus

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Abstract

The 5’ end of the IgH locus contains a cluster of D Nasel 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 D Nasel 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 D Nasel hypersensitive sites does not have an obvious impact on the IgH locus or B cell development.

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 V segments and multiple D and J segments, but an individual IgH V(D)J exon is assembled from only one VH, one D, and one JH segment. V(D)J recombination of the IgH locus takes place in pro-B cells in an ordered way such that D to JH recombination precedes V to DJH recombination [2]. In this regard, activation of the IgH locus is thought to progress in a stepwise manner [3]. D to JH rearrangement efficiently occurs on both alleles, however, allelic exclusion ensures that VH to DJH 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 C exons replaces the C 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 intronic enhancer (E4) resides in the JH – C intron and was shown to be necessary for efficient V(D)J recombination by promoting both D to JH and V to DJH recombination [6,7]. Downstream of the C genes at the very 3’ end of the IgH locus a cluster of D Nasel 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 VH DJH exon [8].

An additional potential regulatory region was identified at the 5’ end of the IgH locus, consisting of four D Nasel 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 VH germline transcripts as so far...
no cis-regulatory element has been identified that controls activity of the bulk of unrearranged V_{H} promoters. Furthermore, it is not known how it is achieved that proximal and distal V_{H} segments are activated independently or why usage of distant versus proximal V_{H} 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, HS5a,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 mice 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’ GTCGACGGATTAGGAGGATACACAA 3’ and 5’ GTCGACCTTGGATAACACAGAAGTCTG 3’ containing a Sall site at their 5’ ends, which facilitate cloning of the PCR product into the Sall site of pLNTK. As a 3’ homology arm a 7.5 kb AaiI – Apal fragment was blunt end cloned into the Xhol 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 KpnI fragment into the Xhol 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 amplifying with primers 5’ GCTCTAGTGCACAAATCTGACTC 3’ and 5’ CAGCTGT-GACCTCCATTTGTGTCTG 3’. Probe 2 is a 1.2 kb PstI – EcoRI fragment of HS1 of IgHa/b. Probe 3 is a 0.8 kb PstI – XbaI fragment about 11 kb 3’ of HS3b. To confirm single integration of the targeting vector a 525 bp Neo\(^{\circ}\) probe was used, amplified with primers 5’ GCAGGCGATAATGGGATCGGC 3’ and 5’ GATCCCGCAGCGAGCCCGCC 3’.

EF1 heterozygous IgH\(^{\circ}\) embryonic stem (ES) cells, generated in the Alt laboratory, were transfected with PvuI linearized RHS1 targeting vector to obtain RHS1/+ ES cells. To obtain RHS1/+ ES cells, the PGK-Neo\(^{\circ}\) cassette was deleted by applying a Cre – expressing adeno-virus vector. RHS1/+ ES cells were transfected with PvuI linearized R3’HSs targeting vector to obtain R3’HSs/+ ES cells. R3’HSs/+ ES cells were selected for homozgyocity of the targeted allele through increasing concentration of G418 to obtain R3’HSs/+ R3’HSs ES cells. Cre – loxp mediated deletion of the PGK-Neo\(^{\circ}\) cassette resulted in RHS1/-HSs ES cells. Targeted ES cells were injected into Rag2\(^{-/-}\) blastocysts to obtain RDBC chimera progeny [11] or into C57BL/6 blastocysts to obtain chimera progeny that could be crossed to 129Sv mice to achieve germine transmission of the targeted allele.

**B cell hybridomas**

CD43\(^{\circ}\) splenocytes were isolated by MACS, cultured with LPS or IL4/\(\alpha\)-SCD40, and analyzed by flow cytometry as described previously [13].

**RT-PCR analysis**

RNA was extracted using TRIzol® Reagent (Roche). 200 ng–1 \(\mu\)g 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 \(\beta\)-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-\(\alpha\)IgM, PE-\(\alpha\)CD20, PE-\(\alpha\)CD8a, PE-\(\alpha\)CD43, FITC-\(\alpha\)Ly9.1, APC-\(\alpha\)IgM, APC-\(\alpha\)CD7-\(\alpha\)CD220 (BD Pharmingen), PE-\(\alpha\)AA4.1, FITC-\(\alpha\)CD4 (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 Bcl – AaiI fragment, harboring HS1, with a loxp flanked PGK-Neo\(^{\circ}\) cassette. All targeting experiments were performed in heterozygous IgH\(^{\circ}\) EF1 ES cells which have the advantage that IgH\(^{\circ}\) (129 strain) and IgH\(^{\circ}\) (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\(^{\circ}\) allele. In heterozygous targeted ES cells, the IgH\(^{\circ}\) 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 S’ flanking region are shown. VH, DH, JH indicate representative IgH V, D, and J segments. Exons 1, 2, and 3 of Zfp386 are shown as grey rectangles, DNasel 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 Spel 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 R3'HSs, the targeted IgHa allele (lane 6). 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 Spel 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 14.1 kb ΔHSs band and a 11.1 kb R3'HSs band (lane 10). All targeting events occurred on the IgHa allele, whereas the IgHb allele remained in wildtype configuration. Drawings not to scale. B - BglII; S - SacI; P - Spel.
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the ΔH51 allele. Correct targeting events (Fig 1) 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 RHS1 or ΔH51 allele in their germline. In order to delete all four hypersensitivity sites (HS1, HS2, HS3a, and HS3b), ES cells containing the ΔH51 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 ΔH51 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 D/Nasel hypersensitive sites, with a single loxP site, referred to as the ΔH51 allele. Germline transmission could not be achieved for either of the R′3′HSs or ΔH51 heterozygous ES cell lines. Therefore, we replaced 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 ΔH51 allele. The homozygous mutant ΔH51 ES cells were injected into Rag2−/− blastocysts, and chimeras generated by RDBC and lymphocytes were analyzed.

Development of homozygous RHS1, ΔH51, and ΔH5s 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 RHS1, and homozygous ΔH51 mice that carry the mutant alleles in their germline, as well as lymphocytes from RDBC chimeras generated from homozygous ΔH5s ES cells (Fig 2). In wildtype bone marrow, pro-B cells can be identified as IgM+ B220− CD43− and pre-B cells as IgM+ B220− CD43+ cells, 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 can be revealed by the presence of a genomic sequence within the murine DLG5 gene.

Figure 2. Development of homozygous ΔH5s, RHS1, and ΔH5s lymphocytes. (A) Bone marrow from wildtype (wt), homozygous ΔH5s, homozygous RHS1 mice, and RDBC chimeras generated from homozygous ΔH5s ES cells was subjected to FACS analysis. Gates were set on the lymphocyte population, Ly9.1 positive population, and on IgM negative population (upper three blots, left to right) to analyze pro-B cell (IgM+ B220− CD43−) and pre-B cell (IgM+ B220+ CD43+) populations (lower blots). (B) FACS analysis of splenocytes from wildtype (wt), homozygous ΔH5s, homozygous RHS1, and RDBC chimeras generated from homozygous ΔH5s ES cells. Gates were set on the lymphocyte population and on the IgM positive population (upper two blots, left to right) to analyze transitional B-cell (IgM+ B220− AA4.1+) and mature B-cell (IgM+ B220+ AA4.1+) populations (lower blots). (C) FACS analysis of thymocytes gated on the lymphocyte population (upper blot) from wildtype (wt), homozygous ΔH5s, homozygous RHS1 mice, and RDBC chimeras generated from homozygous ΔH5s ES cell populations (lower blots).

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The ΔH51, RHS1, and ΔH5s 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− CD43−) and pre-B cells (IgM+ B220+ CD43+) from bone marrow and double positive (DP) T-cells (B220+ CD43− CD8+) were isolated from thymus and analyzed for D to JH, VH to DJH, and V to DJH rearrangements. Intensities of PCR bands for D9Q52 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 RHS1, and homozygous ΔH51 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 3C).

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 VH17183 family (Fig 3C), the distal VH1558 family (Fig 3D), and the distal most VH segment VH1558.55 (Fig 3E). We found that pro-B cells and pre-B cells from wildtype, homozygous RHS1, and homozygous ΔH51 mice rearrange the proximal VH17183 family at similar levels (Fig 3C). Also, the distal family VH1558 (Fig 3D) as well as the distal most VH segment VH1558.55 (Fig 3E) rearranged at comparable efficiencies in pro-B cells and pre-B cells from the three different genotypes. VH10 to DJH recombination was absent in DP T-cells from wildtype, homozygous RHS1, and homozygous ΔH51 mice as the VH10 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 DQS2 (A), DSPs (B), the Vα7183 family (C), the VμJ558 family (D), or the VμJ558.55 segment (E). Rearrangements can occur to JH1, JH2, JH3, or JH4 as indicated. GL indicates PCR product from germline configuration. Igκ rearrangements were quantified (F), rearrangements can occur to Jκ1, Jκ2, Jκ3, or Jκ4 as indicated. Igλ rearrangement efficiency was analyzed (G). Bands correspond to Vλ2 - Jλ2, Vλ1 - Jλ3, or Vλ1 - Jλ1 rearrangements as indicated. DNA input was normalized to DLG5 PCR products (H).

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necessary for rendering the distal part of the VH cluster accessible
and, therefore, suggest that HS1 does not play a major role in
regulation of usage or accessibility of distal versus proximal VH
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 Igl 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 VH to DJH
recombination efficiencies, we generated clonal hybridoma lines
from splenic B-cells of IgH<sup>ab</sup> heterozygous RHS1, RHS1, and RHS1
splenic B-cells. In each case, IgM<sup>H</sup> is the wildtype allele and IgM<sup>H</sup> is the mutant
allele. IgM<sup>H</sup> expressing hybridomas (IgM<sup>H</sup><sup>+</sup>) and IgM<sup>H</sup> expressing hybridomas
(IgM<sup>H</sup><sup>+</sup>) of each genotype were analyzed for the rearrangement status of their
nonproductive allele. Numbers for D to J<sub>H</sub> rearranged alleles (DJ) and
nonproductive V<sub>H</sub> to DJ<sub>H</sub> rearranged alleles (VDJ-) are shown.

| Table 1. ΔHS1, RHS1, and ΔHSs hybridoma analysis. |

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<td>ΔHSs</td>
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Hybridomas were generated from heterozygous IgM<sup>H</sup><sup>ab</sup>, ΔHS1, RHS1, and ΔHSs
spleenic B-cells. In each case, IgM<sup>H</sup> is the wildtype allele and IgM<sup>H</sup> is the mutant
allele. IgM<sup>H</sup> expressing hybridomas (IgM<sup>H</sup><sup>+</sup>) and IgM<sup>H</sup> expressing hybridomas
(IgM<sup>H</sup><sup>+</sup>) of each genotype were analyzed for the rearrangement status of their
nonproductive allele. Numbers for D to J<sub>H</sub> rearranged alleles (DJ) and
nonproductive V<sub>H</sub> to DJ<sub>H</sub> rearranged alleles (VDJ-) are shown.

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Figure 4. IgM<sup>H</sup> versus IgM<sup>k</sup> expression in ΔHS1, RHS1, and ΔHSs B cells. Heterozygous IgM<sup>H</sup><sup>ab</sup> B-cells from spleen (A) and bone marrow (B) of
129 wildtype (wt) or ΔHS1, RHS1, and ΔHSs RDBC chimeras were analyzed IgM<sup>H</sup> and IgM<sup>k</sup> expression. In ΔHS1, RHS1, and ΔHSs B-cells the IgM<sup>H</sup> allele is in
wildtype configuration whereas the IgM<sup>k</sup> allele is the mutant allele.

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rearrangement status of their mutant IgMα 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 VDJH 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. IgMα expressing populations, representing the targeted allele, and IgMβ expressing populations, representing the wildtype 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 wildtype alleles.

The ΔHS1, RHS1, and ΔHSs 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 IgHα or IgHβ, but intact allelic exclusion prevents the appearance of an obvious IgHα, IgHβ double producing population. Similarly, RDBC chimeras generated from heterozygous RHS1, AHS1, and AHSs ES cells exhibited IgHα or IgHβ single positive B-cell populations of similar size in spleen (Fig. 4A) and bone marrow (Fig. 4B) but no IgHα, IgHβ 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 wildtype 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 wildtype 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 wildtype 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 ΔHS1 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 ΔHS1 mice develop a complex neurological phenotype and die at 3–5 weeks of age.

Figure 5. Ig class switch recombination in absence of the 5′IgH DNaseI hypersensitive sites. MACS purified splenic B-cells were stimulated in culture with LPS or IL4+ αCD40 as indicated. FACS analysis shows B-cells that underwent CSR as B220+ IgG1 or B220+ IgG3 cells, respectively. AID-/- B-cells served as negative controls, wildtype (wt) 129 B-cells as positive controls. Homozygous ΔHSs B-cells were isolated from RDBC chimeras.

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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 AHSI, RHSI, and AHSH 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 either the D to JH nor the VH to DJH recombination step. Both proximal and distal VJH families as well as the distal most VH segment VHJ550.55 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 AHSI, RHSI, and AHSH 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 AHSI 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 AHSH 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 AHSI mice and seem to be independent of the IgH locus. AHSI mice show abnormal limp grasping indicating a neurological abnormality, AHSI 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. AHSI deletes a 340 bp region from intron 1 of Zfp386. AHSH 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


Supporting Information

Figure S1 Single integration of the RHS1 targeting vector. The targeting vector (targeting vector RHS1), the targeted locus (RHS1), and the wildtype (wt) IgH locus with its 5’ flanking region are shown. VH, DH, JH indicate representative IgV H 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, the NeoR specific Southern probe as a black rectangle. X - XbaI. Southern analysis of XbaI digested genomic DNA from the targeted RHS1 clones 5 (lane 1) and 23 (lane 2) utilizing the NeoR specific probe shows a single 16.0 kb band. No bands are visible from untargeted wildtype ES cell DNA (lane 3). M - Fermentas 1 kb ladder.

Figure S2 CDR3 length in AHSH B cells. Peripheral B cells were isolated from one AHSH mouse and a wildtype mouse and amplified for V558-JH4 rearrangements. Heavy Chain CDR3 lengths were calculated as the number of nucleotides between the consensus Cys residue and the Trp residue. 23 individual sequences were analyzed from AHSI B cells and 10 from wildtype. Found at: doi:10.1371/journal.pone.0013992.s001 (0.17 MB TIF)

Table S1

Found at: doi:10.1371/journal.pone.0013992.s002 (0.05 MB PDF)

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Author Contributions

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