Functional anatomy of the immunoglobulin heavy chain 3’ super-enhancer needs not only core enhancer elements but also their unique DNA context

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ABSTRACT

Cis-regulatory elements feature clustered sites for transcription factors, defining core enhancers and have inter-species homology. The mouse IgH 3’ regulatory region (3’RR), a major B-cell super-enhancer, consists of four of such core enhancers, scattered throughout more than 25 kb of packaging ‘junk DNA’, the sequence of which is not conserved but follows a unique palindromic architecture which is conserved in all mammalian species. The 3’RR promotes long-range interactions and potential IgH loops with upstream promoters, controlling class switch recombination (CSR) and somatic hypermutation (SHM). It was thus of interest to determine whether this functional architecture also involves the specific functional structure of the super-enhancer itself, potentially promoted by its symmetric DNA shell. Since many transgenic 3’RR models simply linked core enhancers without this shell, it was also important to compare such a ‘core 3’RR’ (c3’RR) with the intact full-length super-enhancer in an actual endogenous IgH context. Packaging DNA between 3’RR core enhancers proved in fact to be necessary for optimal SHM, CSR and IgH locus expression in plasma cells. This reveals that packaging DNA can matter in the functional anatomy of a super-enhancer, and that precise evaluation of such elements requires full consideration of their global architecture.

INTRODUCTION

In mature B-cells, a major 3’ cis-regulatory super-enhancer controls accessibility of immunoglobulin heavy chain (IgH) constant (C) genes to transcription and to IgH changes initiated by activation-induced deaminase (AID) (1–3). In the mouse, this 3’ regulatory region (3’RR) is made up of a cluster of four core enhancers: hs3a, hs1.2, hs3b and hs4. It barely influences V(D)J recombination in B-cell progenitors, except for paradoxically silencing early transcription in pro-B-cells, and barely affects basal pVH transcription in resting B-cells (4,5). By contrast in plasma cells, the 3’RR strongly contributes to increased IgH transcription which physiologically marks the plasma cell stage (6). The 3’RR is also the master cis-regulatory region controlling conventional class switch recombination (CSR), (7,8) locus suicide recombination (LSR) (9) and somatic hypermutation (SHM) (10) by modulating germline transcription of C genes in activated B-cells, chromatin remodeling of switch (S) regions, AID activity on targeted sequences, and also probably the synthesis between broken S regions which terminates CSR (1,11). In addition, to being bound by specific transcription factors, 3’RR core enhancers are transcribed into eRNA (9), and regulated in their function by a distantly transcribed long non-coding RNA (lncRNA CSR) (3).

In all mammalians studied, the upstream 3’RR enhancers are embedded within a long and uniquely palindromic DNA region, with the last enhancer (hs4) lying outside and downstream of this palindrome (1,12); for example in the mouse, the hs3a, hs1.2 and hs3b core elements are found within a ~25-kb dyad symmetry, with hs1.2 at the center, flanked by inverted repeated intervening sequences (IRIS) and bound by inverted copies of the hs3a/hs3b enhancers (Figure 1A). In humans, the 3’RR is duplicated and each 3’RR is composed of only three core enhancers (hs3, hs1.2 and hs4) with hs1.2 also flanked by IRIS. Despite the modest extent of each of the 3’RR core enhancers in vitro, they altogether build a synergistic and potent super-enhancer notably efficient in transgenes, especially when a palindromic architecture is preserved (13). IRIS lack DNase I hypersensitivity and obvious sites for transcription factors and are mostly made up of non-coding repetitive DNA. Some of these ‘like switch’ repeats resemble S regions and likely pro-
mote IgH deletion events during LSR (9). Although highly divergent in different species, IRIS sequences always stand as inverted copies on both sides of hs1,2, thus preserving 3'RR symmetry and strongly suggesting that their architectural role might be required for IgH expression. We recently deleted the upstream side of the palindrome (the 10 kb-long 5'IRIS) in the endogenous locus in ΔIRIS knock-out mice where all 3'RR core enhancers were preserved and the downstream copy of the IRIS remained unaltered (14). This affected SHM and to a lesser extent CSR. It was, however, questionable whether this phenotype resulted from the loss of an unknown cis-acting sequence included in the 5'IRIS, from the decreased distance between hs3a and hs1,2, or from loss of the 3'RR symmetry. 3'RR activity is also often considered as simply the sum of all core enhancer activities, and combinations of all 3' core enhancers are thus widely used as 3'RR models in transgenes (15–19). It has recently been observed in the CH12 B-cell line, that replacement of the 28 kb-long 3'RR with a simple combination of all four core enhancers preserved or even increased IgH locus accessibility to Cα CSR (20). Since transgenes are susceptible to random insertion effects and since immortalized B-cell lines such as CH12 might be frozen with regards to chromatin marks and accessibility, we found it important to check the real effect of 3'RR replacement in the context of the endogenous IgH locus of primary B-cells. Compared with the recent ΔIRIS mouse lacking only the 5'IRIS, the herein reported ‘core 3'RR’ (c3'RR) mouse model completely lacks 3'RR packaging DNA. Interestingly, this complete elimination of packaging DNA around core enhancers strongly affects the main activities of the 3'RR, notably with regards to IgH expression in plasma cells, SHM and class switching. It, however, appears very similar to the phenotype of ΔIRIS mice, and thus further indicates that the 3'RR IRIS are only active when present in two copies, with a role that differs from that of a core enhancer, and is solely architectural: providing the palindromic framework that ensures optimal function of 3'RR core enhancers.

MATERIALS AND METHODS

Mice

One hundred twenty-nine wt mice (from Charles River Laboratories, France) Δ3'RR and c3'RR mice (in a 129 background) were used. Our study was approved by the local ethics committee review board (Comité Régional d’Ethique sur l’Expérimentation Animale du Limousin, Limoges, France).

Vector construction and embryonic stem cell screening

The vector used for homologous recombination assembled a previously described 3' homology arm (located 3.5 kb downstream of hs4) (8) followed by the thymidine kinase (Tk) gene, and a 5' arm homologous to the region
upstream of hs3a (amplified using primers listed in Supplementary Table S1). A floxed neomycin resistance gene (neoR) together with a synthetic 2.1 kb c3’RR cassette were inserted in between both arms, replacing the entire 3’RR. Two CRISPR vectors were created using the pX330 vector (Addgene, Cambridge, USA), for production of two guide RNAs able to target Cas9 recombinase at both ends of the desired 3’RR deletion. The various PCR primers and CRISPR sequences used are reported in Supplementary Table S1.

Cells of the embryonic stem (ES) cell line CK35 were transfected with 8 μg of linearized targeting vector and 0.5 μg of each CRISPR/cas9 vector by electroporation and selected using 300 μg/ml geneticin and 2 μg/ml gancyclovir.

**Immunization**

For immunization experiments, groups of 8-week-old mice (>5 mice per genotype) were immunized by intraperitoneal injection of 200 μl SRBC and analyzed 8 after days.

**Cell culture**

Splenocytes were collected, red blood cells were lysed and cells were CD43-depleted using CD43 microbeads (Miltenyi Biotec). Spleen B lymphocytes were cultured for 5 days in RPMI containing 10% FCS with either LPS (20 μg/ml) + IL4 (40 ng/ml) (Peprotech), or LPS (20 μg/ml) + IFNγ (2 ng/ml) (RD systems) or LPS (20 μg/ml) + TGFβ (2 ng/ml) (RD systems) (R) together with a synthetic 2.1 kb intron, Jk5 and Sj region from sorted B220+/GL7+ cells from Peyer’s patches. PCR products (100 ng) were fragmented using the Ion Shear Plus Reagents kit (Life Technologies), then barcodes and adaptors were ligated using Ion Xpress Plus Fragment (Life Technologies). Fragments around 200 pb were selected using 2% E-Gel size select (Life Technologies) and sequenced on an Ion Proton™ System. Raw fastq files were generated using Ion Torrent Suite (adapter- and barcode-trimmed) and mapped to the reference sequences using BWA-MEM (Li, 2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv:1303.3997 [q-bio]. Position-wise base counts were performed with IGVTools (21) and resulting wig files were processed to call somatic mutations along the reference sequence with ad hoc Python scripts (available on request). Sequences were deposited on the EMBL EBI ENA website, http://www.ebi.ac.uk/ena, under accession number: PRJEB19507.

**Statistical analysis**

Statistical tests were performed using GraphPad Prism (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

**RESULTS**

**Generation of c3’RR mice**

We generated a mouse model with an IgH locus carrying a miniaturized ‘core-3’RR’ (c3’RR). Our gene-targeting strategy replaced the entire 3’RR region (i.e. 30.3 kb) with the c3’RR (2.1 kb), which encompassed only the four core enhancers (hs3a, hs1.2, hs3a and hs4) (Figure 1A). To optimize gene targeting, we used CRISPR/cas9 in order to generate double-strand breaks at both ends of the 3’RR and initiate recombination. The targeting vector was co-transfected with CRISPR/cas9 vectors in CK35 ES cells. We obtained three positive clones out of 15 tested. After germline transmission, mice were bred with cre expressing mice to delete the NeoR cassette. In this study, c3’RR mice were compared to Δ3’RR (10) and ΔIRIS mice (14) (Figure 1B).

**B-cell development**

To study B-cell development in c3’RR mice, we generated heterozygous c3’RR+/wt mice by crossing homozygous c3’RR+/c3’RR+ (IgHΔ locus from the SV129 strain) mice with C57BL/6 mice (wt/wt). This allowed us to study the effect of the mutation by comparison with a wt IgH locus in the same mouse and in the conditions of an internal competition between cells expressing either the wt ‘b’ allele or the knocked-out ‘a’ allotype IgH locus. B-cell
development was barely affected in those B-cells expressing a rearranged c3'RR allele, although these IgM^a cells were slightly less abundant than in wt/wt^b mice (Supplementary Figure S1A). Analysis of c3'RR^a/wt (vs wt/wt) splenic B-cells similarly showed a slightly decreased a/b ratio among transitional (B220^b AA4.1^a), follicular (B220^b CD21^low CD23^high) and marginal zone (B220^b CD21^high CD23^low) B-cells expressing either the a or b allotype. (Supplementary Figure S1B).

3'RR core enhancers are sufficient to induce CSR, but with a partial defect

We explored Ig production in vitro and in vivo upon extracting 3'RR core enhancers from their packaging DNA. When first examining secreted Ig levels and plasma accumulation in vitro (Figure 2A), we observed that Ig classes with short-half-lives and notably IgM and IgA were present at lower levels than in wt animals, in contrast with roughly normal amounts of those IgG classes with long half-lives, IgG1 and IgG3 (which can accumulate, since they are most efficiently recycled after binding to the FcRn receptor) (22,23). Ig steady state plasma levels indeed integrate the rates of both production and catabolism, the latter being decreased in conditions of lowered Ig production and then notably resulting for IgG1, in a 3-fold increased half-life (22). It has accordingly been observed, by checking in vitro cultures, that in several cases of Ig production defects, that in vivo serum levels can be apparently preserved even when an Ig production defect is clearly present (8).

We next quantified Ig levels in conditions more directly reflecting Ig production, in supernatants of B-cells activated in vitro by LPS and appropriate cytokines. We compared c3'RR homozygous cells with either wt or homozygous Δ3'RR cells known to carry a strong defect in in vitro Ig secretion (6). An intermediate, but significant secretion defect was observed for c3'RR cells, affecting all IgG classes except IgG1, while for IgA and IgM, the trend towards a decrease was not significant (Figure 2B).

To better assess in vivo Ig production compared to lymphocyte counts, and the role of the 3'RR palindromic shell during immune activation, we quantified the boost in serum IgM and IgG1 after SRBC immunization in wt compared to homozygous 3'RR mutant mice. In pre-immune mice, normal counts of IgM^+ lymphocytes contrasted with less serum IgM at the basal state in c3'RR and Δ3'RR mice; this difference persisted after SRBC activation (although serum IgM levels then increased in all animals, either wt, c3'RR or Δ3'RR) (Figure 3, top panel). To further evaluate the partial independence of IgG1 from the c3'RR mutation, we measured in vivo class-switched IgG1^+ lymphocytes, which were normal in c3'RR mice at the basal state (Figure 3, bottom panel). The serum IgG1 level was also normal at the basal state, in conditions where accumulation and decreased IgG catabolism are known to occur in mice producing low amounts of IgG (22). By contrast, serum IgG1 was boosted in animals stimulated by SRBC injection, and then remained at a level significantly lower than in wt mice, this dynamic production of Ig being more sensitive to an Ig production defect (Figure 3, bottom panel, right).

Independently of Ig secretion, CSR can be measured more directly in vitro by following the change in BCR expression. To determine the impact of 3'RR miniaturization on CSR, we evaluated class switching to the various BCR isotypes after specific in vitro stimulation. Homozygous c3'RR/c3'RR cells were compared with either wt or homozygous Δ3'RR cells carrying a complete 3'RR deletion. Flow cytometry evaluated surface expression of class-switched isotypes on activated splenic B-cells from wt, c3'RR and Δ3'RR mice. As previously described (6), CSR in control Δ3'RR cells stimulated for 4 days, was barely detectable for all classes except for IgG1 (with some IgG1-producing cells still detectable). Although milder, a CSR phenotype reminiscent of the latter also appeared in c3'RR mice: class-switched IgG3 cells decreased to a level intermediate between that observed in Δ3'RR and wt mice (Supplementary Figure S2), while IgG1^+ cells were not affected. In agreement with cell cytometry, Q-PCR in c3'RR cells revealed an intermediate decrease in post-switch transcripts (PST), less significant than for the Δ3'RR cells and diversely affecting PST from Cγ2b (down to the same low levels as in Δ3'RR B-cells), Cε, and to a lesser extent Cγ3, Cγ2a and Cγ1 PST which tended to be lower but did not show a significantly lower level in these experiments (Figure 4A).

We also explored in vitro whether the absence of the 3'RR palindromic shell lowered or delayed CSR. We quantified PST daily during a 5-day stimulation and found a decreased level of PST in c3'RR animals for Cγ1, Cγ3, Cγ2a, Cγ2b at all time points compared to wt mice. (Supplementary Figure S3).

Germline transcription (GLT) of a C_H gene is a known prerequisite for CSR. We evaluated GLT in in vitro (LPS ± cytokines) activated splenic B cells from wt, c3'RR and Δ3'RR mice. All GLT were partially affected in c3'RR mice compared to wt and Δ3'RR mice (Figure 4B). Altogether, these results indicated that a core-3'RR made up of naked super-enhancer. Similar to Δ3'RR mice, where μ chain transcription is roughly normal in lymphocytes expressing membrane IgM but strongly declines (together with IgM secretion) in plasma cells, secreted-type μ transcripts (encoding secreted IgM) in c3'RR mice decreased to a level intermediate between wt and Δ3'RR mice (Figure 4C).

3'RR packaging DNA is mandatory for optimal SHM

Using high throughput next generation sequencing, we explored SHM downstream of rearranged V(D)J genes in the J_H4 intron as described (24) (using the rearranged Jk5 intron as a control) in wt, c3'RR and Δ3'RR mice to determine the mechanistic impact of 3'RR alterations. Germline central B-cells from Peyer’s patches were sorted from wt, c3'RR and Δ3'RR mice and analyzed for SHM. In these settings, normal mutation frequencies in wt mice at the IgH (J_H4 and S_H regions) and Igk loci were respectively 13.27‰, 7.7‰ and 11.8‰. As previously documented (10), Δ3'RR mice showed a strong SHM defect at the IgH J_H4 (1.48‰) and S_H regions (2‰) (Figure 5A and B, Supplementary Figure S4 for positions of mutations and ATGC tables). Strik-
Figure 2. In the absence of packaging DNA, core 3'RR enhancers partially support Ig secretion. (A) ELISA analysis of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA in plasma from Δ3’RR, c3’RR and wt mice. (B) ELISA analysis of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA in supernatants from LPS ± IL-4, INFγ and TGFβ stimulated splenocytes from Δ3’RR, c3’RR and wt mice. Mean ± SEM of two independent experiments with at least three mice and Mann Whitney test for significance.
Figure 3. In vivo immune stimulation in the absence of 3’RR packaging DNA. FACS analysis of IgM and IgG1 positive cells at basal state (left panel) and ELISA of IgM (top) and IgG1 (bottom) in plasma from Δ3’RR, c3’RR and wt mice before (NI) and after SRBC immunization (right panel). Mean ± SEM of two independent experiments with three mice each and Mann–Whitney test for significance.

ingly, a similar SHM defect affected IgH JH4 sequences obtained from c3’RR mice (1.48‰) indicating that the 3’RR stimulatory effect on IgH SHM needs not only core enhancers but also their palindromic packaging DNA. The same analysis was performed in the Sμ region, where we again observed a decreased SHM in c3’RR mice, at an intermediate level between wt and Δ3’RR mice (Figure 5A and B).

DISCUSSION

The 3’RR is a strong super-enhancer regulating remodeling of the IgH locus in mature B cells and was even recently shown to have trans-acting effects on the other IgH allele (1,25). Full deletion of the 3’RR was previously reported to strongly impact CSR, SHM and the IgH boosted expression which ensures high antibody secretion by plasma cells (6,10). By contrast, this deletion fully preserved early B-cell maturation and marginally impacted membrane IgM density and B-cell fate (toward slightly less marginal zone B-cells) (6,26).

As any super-enhancer, the 3’RR integrates core enhancers buried in packaging DNA. Since transcription factors bind DNAse1 hypersensitive regions of core enhancers, and since large DNA fragments cannot easily be handled, many studies devoted to enhancer function and many transgenes supposedly providing 3’RR models simply omitted this packaging DNA (15–19).

More than 90% of mammalian DNA is non-coding and is often considered as useless (27), while this DNA might in fact play multiple roles, notably architectural. The 3’RR case is very specific due to its unique palindromic structure featuring several kb-long IRISs that flank the central hsl1,2 core enhancer (12,28).

While alterations of the 3’RR made in the endogenous locus were initially shown to affect CSR (7), it was also observed that several precise deletions of a single core enhancer simply had no phenotype, provided the global structure of the 3’RR was maintained and no exogenous gene (such as a neo resistance cassette) was left inserted (29–31).

By contrast, large deletions affecting multiple enhancers (and more recently a large deletion of the 5’ half of the packaging palindromic DNA), while preserving core enhancers, all severely impacted class switching (8,14). In agreement with these stereotyped CSR alterations following partial knock-outs, deletion of the entire 3’RR resulted in an al-
most complete CSR defect (6). Since miniaturized 3’RR substitutes, linking all four 3’RR core enhancers, have been widely used and considered to provide all 3’RR features, we wished, in the present study, to replace the full-length super-enhancer with its miniaturized (c3’RR) counterpart. We then evaluated this replacement in the physiologic context of the endogenous locus. Strikingly, this replacement severely affected CSR to some (IgG3, IgG2a, IgG2b...) but not all Ig classes (IgG1 was notably respected). This phenotype was strikingly similar to that of ΔIRIS mice lacking one half of the 3’RR repetitive packaging DNA (14). The c3’RR mice also poorly supported SHM and the level of mutation in the rearranged JH4 intron was in fact similar to that in 3’RR-less B-cells (32). Again, this SHM phenotype was quite similar to that of ΔIRIS mice (14).

We finally observed decreased IgH expression in plasma cells indicated by decreased IgM plasma levels in mice and decreased amounts of secreted-type IgM transcripts, at a level intermediate between wt and Δ3’RR mice, which provided another similarity with the ΔIRIS mice (6,14).

Altogether, our data indicate that the DNA shell surrounding core enhancers within the IgH 3’RR is not made up of useless junk DNA but, rather, contributes actively to 3’RR function. The ability of 3’RR core enhancers to support CSR and AID recruitment has been demonstrated by several previous studies in transgenes and transfectants and by deletion of the 3’RR hs3b and hs4 core enhancers while respecting most of the 3’RR DNA shell (8,19). The present study shows that while core enhancers are mandatory for full activity of the 3’RR super-enhancer, the surrounding sequences do not constitute useless junk DNA, but instrumentally contribute to 3’RR activity.

These observations clearly deserve to be considered for the future design of transgenic, knock-in or any other mouse engineered for Ig production (notably humanized) if efficient class switching and affinity maturation through SHM is desired.

Regarding the functional anatomy of the 3’RR super-enhancer and given the very strong similarity between the phenotype of c3’RR mice and that of ΔIRIS mice, our data suggest that the major activity of the 3’RR DNA shell is architectural and related to a necessary large DNA dyad symmetry of the regions flanking hs1,2. In that regard, deleting either flank of the palindrome in ΔIRIS or the whole palindromic DNA shell in c3’RR mice somehow yields a similar phenotype where core enhancers are working on their own and lacking the amplifying effect of the 3’RR 3D-architecture. Inverted repeats might function by posi-
tioning the 3′RR in its optimal 3D-configuration, together with eRNA, lncRNA-CSR and transcription factors in order to stabilize a chromosomal organization that most efficiently recruits AID to mammalian IgH loci. Interestingly, we found no inverted repeats similar to those in the 3′RR around the mouse and human intronic Eμ or around Eκ and the 3′κ enhancer. We found no palindrome either around the zebrafish IgH intronic and 3′ regulatory elements (33). An attractiveness hypothesis is thus that the peculiar 3′IgH structure found in all mammals has been evolutionarily selected and contributes to the highly efficient class switching and somatic hypermutation that occurs in mammalian B cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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