Developmental Switch in the Transcriptional Activity of a Long-Range Regulatory Element

Fatima-Zohra Braikia, Caroline Conte, Mohamed Moutahir, Yves Denizot, Michel Cogné, Ahmed Amine Khamlichi

Research article

A developmental switch in the transcriptional activity of a long-range regulatory element

Fatima-Zohra Braikia[1,2], Caroline Conte[1,2], Mohamed Moutahir[1,2], Yves Denizot[3,4], Michel Cogné[3,4,5] and Ahmed Amine Khamlichi[1,2,&]

[1] CNRS UMR5089; IPBS (Institut de Pharmacologie et de Biologie Structurale); FRBT ; 205 route de Narbonne, BP 64182, F-31077 Toulouse, France.

[2] Université de Toulouse; UPS; IPBS; F-31077 Toulouse, France.

[3] CNRS UMR 7276 ; 2 rue Marcland, F-87025 Limoges, France

[4] Université de Limoges, F-87025 Limoges, France

[5]Institut Universitaire de France, F-87025 Limoges, France.

Short title: *IgH* 3'RR-mediated silencing activity

[&]Corresponding author: Ahmed Amine Khamlichi, CNRS UMR 5089-IPBS, 205 route de Narbonne, 31077 Toulouse Cedex 4, France. Tel: +33 561 175 522, Fax: +33 561 175 997, Email: ahmed.khamlichi@ipbs.fr

Keywords: B lymphocyte / Enhancer / *IgH* locus / Transcriptional silencing / V(D)J recombination

Length: 25, 457 characters (excluding spaces)

Abstract

Eukaryotic gene expression is often controlled by distant regulatory elements. In developing B lymphocytes, transcription is associated with V(D)J recombination at immunoglobulin loci. This process is regulated by remote *cis*-acting elements. At the immunoglobulin heavy chain (*IgH*) locus, the 3' regulatory region (3'RR) promotes transcription in mature B cells. This led to the notion that the 3'RR orchestrates the *IgH* locus activity at late stages of B cell maturation only. However, long-range interactions involving the 3'RR were detected in early B cells, but the functional consequences of these interactions are unknown. Here we show that not only does the 3'RR affect transcription at distant sites within the *IgH* variable region, but that it conveys a transcriptional silencing activity on both sense and antisense transcription. The 3'RR-mediated silencing activity is switched off upon completion of V_H-DJ_H recombination. Our findings reveal a developmentally-controlled, stage-dependent shift in the transcriptional activity of a master regulatory element.

Introduction

The spatial and temporal control of gene expression in metazoans is effected by regulatory elements that are often located far from gene promoters (1). This pattern of gene expression regulation is a hallmark of antigen receptor loci whose expression involves both transcription and recombination. The mouse *IgH* locus contains ~195 variable (V_H) genes subdivided into domain-organized gene families, including the distal V_H, by far the largest, and the proximal V_H family. The V_H genes are followed by a dozen of diversity (D) segments, 4 joining (J_H) segments, and 8 constant (C_H) genes (2, 3) (Fig. 1A, top scheme). The assembly of an *IgH* variable region exon through V(D)J recombination occurs in early developing B cells in an ordered manner, first D to J_H then V_H to DJ_H. While the first recombination step (D-J_H) can also be detected in developing T cells, V_H-DJ_H recombination is strictly B cell-specific (4).

In addition to its B-lineage specificity, V_H-DJ_H rearrangement is regulated by allelic exclusion which enables mono-allelic expression of only one *IgH* locus by a given B cell (4, 5). In this process, a productive V(D)J rearrangement on one allele ultimately leads to surface expression of a μ heavy chain which signals arrest of V_H-DJ_H recombination on the second allele. If the first rearrangement is not productive (*i.e.* no μ heavy chain production), then the second allele can undergo V_H-DJ_H recombination (4, 5). There is considerable evidence in support of the notion that V_H-DJ_H rearrangement is the regulated step in *IgH* allelic exclusion and its maintenance through a feed-back mechanism (4, 5), although the molecular mechanisms through which feed-back signaling inhibits V_H-DJ_H recombination remain unclear.

Another level of regulation of V_H-DJ_H recombination relates to the physical location of V_H gene segments within the variable domain. Indeed, several gene targeted studies showed that recombination of the distal and the proximal domains is regulated very differently (4, 6, 7).

65 Additionally, allelic exclusion of the distal V_H genes is more stringent than that of the most
66 proximal V_H genes (4).

67 In developing B lymphocytes, sense and antisense transcription is associated with V(D)J
68 recombination in a cell-type and developmental-stage specific manner (7). The process is
69 regulated by distant *cis*-acting elements including enhancers, promoters and insulators (6, 7).
70 Three long-range regulatory elements were identified within the *IgH* locus and were shown by
71 targeted deletion studies to regulate the locus activity. The Eμ enhancer, located between the
72 variable and the constant regions, plays a critical role in V(D)J recombination and associated
73 germ-line transcription (8-12). Additionally, CTCF-binding elements (CBEs) with insulator
74 activity were identified in the V_H-D intergenic region (13-15). This regulatory region (called
75 IGCR1) is important for the order and lineage specificity of V(D)J rearrangements, and for
76 allelic exclusion of proximal V_H genes (16). A locus control region called the 3' regulatory
77 region (3'RR) contains four enhancers (hs3a, hs1-2, hs3b, hs4) (17) and was shown to
78 synergistically promote germ-line transcription of C_H genes during class switch
79 recombination in mature B cells and *IgH* expression in plasma cells (18, 19). Previous
80 targeted deletion studies showed that the 3'RR impacted μ heavy chain gene expression in
81 resting B cells (18, 19), but that it was dispensable for the repertoire diversity in pre-B cells
82 (20). In contrast, its role in allelic exclusion is still unknown.

83 The established role of the 3'RR in *IgH* locus expression in late B cells and the lack of
84 effect on repertoire diversity led to the notion that the 3'RR activity is restricted to the late
85 stages of B cell maturation only (19, 20). However, various studies described long-range
86 interactions between the 3'RR and various upstream sequences, including Eμ enhancer (15,
87 16, 21, 22) though their functional significance is unclear. Strikingly, deletion of either Eμ or
88 the 3'RR had no effect on long-range interactions mediating locus contraction of the *IgH*

89 locus, leading to the proposal that the activity of these elements may be restricted to the ~270
90 kb D-C_H region (22).

91 In this study, we used a mouse line devoid of the 3'RR (19) (hereafter called Δ3'RR) to
92 explore its role in V(D)J recombination and associated germ-line transcription which occurs
93 at distances ~220 kb to megabases far from the 3'RR. Here, we report the striking finding that
94 the 3'RR mediates a transcriptional silencing activity which is switched off after completion
95 of V_H-DJ_H recombination.

96

97

Materials and Methods

Mice. The generation of 3'RR-deleted mice was described (19). Throughout the study, RAG2-deficient mice used as controls are of 129Sv genetic background. The experiments on mice have been carried out according to the CNRS Ethical guidelines and approved by the Regional Ethical Committee.

Antibodies. APC-conjugated anti-B220 and FITC-conjugated anti-IgM were from BioLegend. PE-conjugated anti-CD43, FITC-conjugated anti-Igκ, PE-conjugated CD4 and FITC-conjugated CD8 were from BD-Pharmingen.

FACS analyses. Bone marrows from 6- to 8-week-old mice were prepared by standard techniques. $5x10^5$ cells/assay were stained with anti-B220, anti-CD43 and anti-IgM or anti-Igκ, and gated either on IgM^- or $Ig\kappa^-$ population. Data were obtained on 2.0 x 10^4 viable cells by using a BD FACSCalibur. FACS acquisitions included isotype controls and exclusion of dead cells by labelling with propidium iodide.

V(D)J rearrangement assays. B cells from bone marrows were sorted by using CD19-magnetic microbeads and LS columns (Miltenyi) and labelled with anti-B220, anti-CD43, and either anti-IgM or (as a cross-check) anti-Igκ. The purity of the sorted pro-B cell populations was checked by FACS (>95%) and by the rearrangement status of the Igκ locus. The $CD4^+CD8^+$ thymocytes were sorted as described (16). Genomic DNAs from the sorted pro-B cells ($B220^+\kappa^-CD43^{high}$ or $B220^+IgM^-CD43^{high}$) and $CD4^+CD8^+$ thymocytes were prepared by standard techniques, and diluted for the (q)PCR assays (23). Controls included genomic DNAs from kidney and RAG-2-deficient pro-B cells. The hs5 sequence located downstream of the 3'RR (24) was used for normalization. The primers are listed in table S1.

RT-qPCR. RAG-2-deficient pro-B cells were sorted using CD19-magnetic microbeads (Miltenyi). RAG-2-deficient thymuses were prepared as described (25). $B220^-$, $CD4^+CD8^+$ thymocytes were sorted as described (16). Pro-B and pre-B cells ($B220^+\kappa^-CD43^{low}$ or

123 B220^{+}IgM^{-}CD43^{low}) were sorted as described above. Unstimulated splenic B cells were sorted
124 by using CD43-magnetic microbeads (Miltenyi), and activated by culturing them for 48 h in
125 the presence of 20 µg/ml lipopolysaccharide (Sigma) and 2 ng/ml anti-IgD-dextran (Fina
126 Biosolutions). Total RNAs were reverse transcribed (Fermentas) and subjected to semi-
127 quantitative PCR, using SYBR green I (Invitrogen) and Image Quant software as described
128 (26) or to qPCR using Sso Fast Eva Green (BioRad). The relative transcription levels were
129 normalized using *β-actin* and *Gapdh* RNAs as controls.
130 **Statistical analysis.** Results are expressed as mean ± SEM (GraphPad Prism) and overall
131 differences between values from WT and mutant mice were evaluated by Student test. The
132 difference between means is significant if p value < 0.05 (*), very significant if p value < 0.01
133 (**), and extremely significant if p value < 0.001 (***).
134

Results

The 3'RR down-modulates V(D)J recombination. To analyze the effect of the 3'RR on V(D)J recombination, we performed sensitive qPCR-based V(D)J recombination assays (23) on genomic DNAs from sorted WT and Δ3'RR pro-B cells and $CD4^+CD8^+$ thymocytes.

We first quantified the proportion of the D_{Q52} and J_{H1} segments that had retained their germ-line configuration in purified pro-B cells (Fig. 1A). The total number of alleles with un-rearranged D_{Q52} and J_{H1} segments on the mutant alleles was comparable to WT controls (Fig. 1A), clearly indicating that there was no obvious delay in the onset of D-J_H recombination. Thus, any potential effect of the 3'RR on V(D)J recombination is likely to occur after the onset of the process.

We also quantified recombined DJ_H segments and fully rearranged V_HDJ_H segments. We used forward degenerate primers that recognize most of D segments (Fig. 1B, top scheme) or distal V_H genes (Fig. 1C, top scheme), and specific backward primers located downstream of each J_H segment (Fig. 1B, 1C, top schemes). We did not analyze recombination of proximal V_H genes as the mutant allele is derived from 129Ola ES cells which bear a ~120-kb internal deletion in the proximal V_H domain (22).

Surprisingly, a mild increase in DJ_H alleles was detected in mutant pro-B cells, but not in mutant $CD4^+CD8^+$ thymocytes (Fig. 1B). Interestingly, a similar increase was also detected for distal V_HDJ_H alleles (Fig. 1C), which could be due, at least in part, to accumulated DJ_H substrates. Inspection of D-J_H and V_H-D-J_H junction sequences in pro-B cells showed no evidence for an over-representation of a D gene segment family, or an anomaly regarding the number of inserted or deleted nucleotides (Table 1). The increase seen for V_HDJ_H alleles was not due to a block at the pro-B cell stage either as the pro-B compartment was rather slightly reduced (Fig. 2A). The data suggest an enhanced V(D)J recombination (see discussion).

The 3'RR does not affect the order of rearrangements. As mentioned previously (see introduction), V_H-DJ_H recombination is strictly B cell-specific and Eμ deletion impairs V(D)J recombination (8, 9, 12); additionally, mutation of IGCR1 CBEs affects the order of V(D)J rearrangements (16). Given the reported CTCF-mediated loop formation between IGCR1 CBEs and CBEs downstream of the 3'RR (15, 16, 21, 22), we asked whether deletion of the 3'RR, which would disrupt the stable Eμ-3'RR interaction (16) and potentially the architecture of the larger CTCF-mediated domain, would somehow deregulate the order of V(D)J rearrangements.

To this end, we attempted to detect V_HD amplicons, which result from a direct V_H-D recombination. Genomic DNAs were extracted from purified pro-B cells and $CD4^+CD8^+$ thymocytes, and assayed for V_H-D recombination by using a forward primer pairing with V_{H81X} gene and a backward primer downstream of the D_{Q52} segment (Fig. 2B). This sequence is deleted following any D-J_H rearrangement, but not if the V_{H81X} segment directly recombines with D_{Q52} segment. As a positive control, we used genomic DNAs from IGCR1 $CBE^{-/-}$ pro-B cells and $CD4^+CD8^+$ thymocytes, which undergo V_{H81X}-D_{Q52} recombination (16). We found no evidence for V_HD amplicon in Δ3'RR pro-B cells or in $CD4^+CD8^+$ thymocytes (Fig. 2B). Thus, the 3'RR does not affect the order of rearrangements.

The 3'RR down-regulates sense and anti-sense transcription in the distal variable region. Germ-line transcription in the variable region precedes V_H-DJ_H recombination (27, 28). To investigate the effect of the 3'RR on germ-line transcription of un-rearranged genes, we first brought the Δ3'RR mutation into RAG-2-deficient background which precludes V(D)J recombination. We mainly focused on the D-Cμ and the distal V_H domains where high levels of transcription are detected (7, 16).

185 Germ-line transcription within the D-Cμ domain, but not within the distal V_H domain, is
186 regulated by Eμ enhancer (8-12, 29). In contrast, the effect of the 3'RR is unknown. We
187 found no obvious effect on Iμ or μ0 sense transcripts (derived from Eμ enhancer and D_{Q52}
188 promoter respectively), or on D_{SP} antisense transcripts (derived from Eμ region and/or an ill-
189 known promoter upstream of D_{ST4} segment (30)) (Fig. 3A). Concordantly, normal levels of
190 Iμ, μ0 and D_{SP} GL transcripts were found in RAG-2-deficient thymuses and in RAG-2-
191 proficient $CD4^+CD8^+$ thymocytes (Fig. 3A). Thus, within the D-Cμ domain, sense and
192 antisense transcription was not altered in the absence of the 3'RR, indicating that the Eμ-
193 mediated control of germ-line transcription in the D-Cμ domain does not require the 3'RR.
194 Remarkably, the distal V_H region yielded an increase of both spliced, sense transcripts,
195 and unspliced, antisense transcripts in Δ3'RR mice (Fig. 3B). To exclude any bias potentially
196 introduced by the increased primary V_H sense transcripts, we quantified intergenic, antisense
197 germ-line transcript levels within the V_{HJ558} and V_{HJ606} clusters. These exclusively antisense
198 transcripts were also increased (Fig. 3C). In contrast, the Pax5-activated intergenic repeat 4
199 (PAIR 4) antisense germ-line transcripts (31) were unaltered (Fig. 3C), suggesting that
200 regulation of these transcripts, derived from the PAIR4 promoter/enhancer element (31), is
201 3'RR-independent. Thus, within the distal V_H domain (except for PAIR elements), the 3'RR
202 affects both sense and antisense transcription.
203 Within the proximal V_H domain, we also found increased antisense transcription upstream
204 of V_{H81X} (the most 3' functional V_H gene segment, which is not encompassed by the ~120 kb
205 deletion in Δ3'RR (22)) (Fig. 3C), suggesting a variable region-wide effect of the 3'RR.
206 Overall, and within the limits of the transcripts analyzed, the 3'RR downregulates sense
207 and antisense germ-line transcription along the remote *IgH* variable domain.
208 Transcription of some loci could be regulated by elements located on a different
209 chromosome (32). Specifically, it was suggested that the *Igκ* locus and its 3' enhancer (on

chromosome 6) were involved in directing the *IgH* locus (chromosome 12) to a repressive nuclear compartment and inducing *IgH* locus decontraction (33). To investigate whether the 3'RR can act in *trans*, we analyzed germ-line transcription along the *Igκ* locus and found it unchanged (Fig. 3D), excluding, at least with regard to the *Igκ* locus, any *trans*-effect of the 3'RR.

Switching off the 3'RR-mediated silencing activity coincides with the completion of V(D)J recombination. To elucidate precisely at which step the 3'RR-mediated silencing activity is turned off, we quantified the transcript levels of fully rearranged μ gene at various developmental stages. To avoid potential bias introduced by cellular selection and/or selective use of distal versus proximal V_H genes, we also measured Iμ transcript levels. We found normal levels of the distal V_H exon-containing μ transcripts ($dV_HDJ_HC\mu$) in pro-B cells (Fig. 3E). These transcript levels were unchanged in pre-B cells, but were clearly decreased in unstimulated splenic B cells (Fig. 3E). Down-regulation of Iμ transcripts was clearly detectable in pre-B cells and was more pronounced in unstimulated cells (Fig. 3E). Interestingly, the shift from a silencer (in pro-B cells) to an enhancer (pre-B cells) activity correlated with the appearance of 3'RR enhancers' transcripts (Fig. 3F) (34).

Therefore, the 3'RR-mediated silencing effect appears to be switched off upon completion of V(D)J recombination at the pro-B cell stage and correlates with the onset of 3'RR transcription.

Discussion

We reported here the first demonstration of a stepwise shift in the transcriptional activity of a long-range regulatory element in higher eukaryotes. The down-regulating activity of the 3'RR targets multiple upstream sense and anti-sense promoters in the remote *IgH* variable region, but spares known enhancers/promoters (Eμ and PAIR4). Specifically, the 3'RR does not affect sense and antisense transcription within the D-Cμ domain consistent with the notion that transcription within this domain is mainly controlled by Eμ enhancer (8, 10).

As mentioned previously, the Δ3'RR mouse line is derived from 129Ola ES cells which have a 120 kb internal deletion in the proximal V_H domain, which is not the case in the RAG-2-deficient mice which were 129Sv-derived. Thus, although we cannot formally exclude the possibility that the 120 kb deletion within the proximal V_H domain may have affected distal V_H germ-line transcription and V(D)J recombination, we think it is unlikely for various reasons. Multiple studies clearly showed that the proximal and the distal V_H domains were differentially regulated. Thus, recombination of distal but not of proximal V_H genes is inhibited in mice deficient in the histone-modifying enzyme EZH2 and different transcription factors involved in V(D)J recombination such as PAX5, YY1, and Ikaros (35-38). Mutations targeting various *cis*-acting elements at the *IgH* locus similarly showed a differential effect on germ-line transcription and recombination of proximal *versus* distal V_H genes (12, 16, 39-42). Importantly, deletion of the 3'RR in the context of the 120 kb deletion had no effect on long-range interactions across the *IgH* locus in RAG2-deficient pro-B cells (22). Additionally, within the *IgH* variable region, the viewpoints that were found by 4C-seq analyses to strongly or minimally interact with the 3'RR correlated well with our transcriptional analyses. For instance, antisense transcription upstream of V_{H81X} gene (which is intact in 129Ola background) was enhanced in the absence of the 3'RR (present study) and was efficiently contacted by hs3b enhancer of the 3'RR (22), whereas PAIR4 whose expression was not

affected by the 3'RR deletion (present study) did not significantly interact with the 3'RR (21, 22). Moreover, antisense transcription within the J606 family was increased (present study) which correlated well with an interaction of this region with the 3'RR-Eμ loop (21, 22, reviewed in 7). Finally, it is difficult to figure out how the 120 kb deletion would affect the 3'RR-mediated effect on D-J_H recombination which takes place in the stable IGCR1-*IgH* 3'CBEs chromatin domain (15, 16, 21, 22).

Whether the long-range 3'RR-mediated silencing activity is due to an unidentified developmentally-regulated silencer within the 3'RR itself or is mediated by interacting partners requires further investigations involving combined mutations. The strong correlation between the extinction of the 3'RR-mediated silencing activity and the completion of V(D)J recombination suggests that the interacting partner(s) should be deleted upon V_H-DJ_H recombination. Likely candidates could be the IGCR1 (16, 21, 22) and/or the newly identified interaction site upstream of IGCR1 (22). This could be a mean through which recombination regulates the 3'RR transcriptional activity. Alternatively, though not-mutually-exclusive, the correlation between the triggering of the 3'RR transcription and its enhancer activity suggests that the long-range activity of the 3'RR during B cell development may be modulated by its enhancers' transcripts.

The B cell-specific down-modulating effect of the 3'RR on D-J_H recombination suggests that 3'RR/Eμ interaction may affect Eμ-mediated control of recombination rather than transcription. Various studies found that transcription and V(D)J recombination could be mediated by distinct activities of accessibility control elements, including Eμ enhancer (43, reviewed in (44)), and there is some evidence that recombination could be recapitulated *in vitro* in the absence of transcription (45).

By quantifying the proportion of the D_{Q52} and J_{H1} segments in their un-rearranged configuration, we found no obvious delay in the onset of D-J_H recombination clearly

indicating that the effect of the 3'RR occurs after the initiation of V(D)J recombination. In contrast, there was a relative accumulation of DJ_H intermediates and fully recombined V_HDJ_H alleles with no obvious block at the pro-B cell stage at which V(D)J recombination at the *IgH* locus occurs. Thus, it appears that we are dealing with a specific phenomenon which is restricted to pro-B cells: Increased recombination events in a "fixed" time window. One simple explanation is that, in the absence of the 3'RR, the process runs faster. Recent evidence highlighted the importance of spatial confinement for the kinetics of V(D)J recombination and time encounter with regulatory elements (46). It is tempting to speculate that 3'RR interactions with its partners are a critical component of the mechanisms that regulate the kinetics of V(D)J recombination. Within a newly generated topological domain that forms upon DJ_H recombination, the 3'RR may for instance contribute to the control of the kinetics of V_H-DJ_H recombination by impacting germ-line transcription within the V_H domain, while Eμ is focused on DJ_H transcription (40).

Why does the 3'RR mediate a transcriptional silencing within the V_H domain? It should be noted that V_H-DJ_H recombination is the regulated step in allelic exclusion (4, 5), and that the control of germline transcription is likely the primary event during allelic exclusion (42). In the absence of the 3'RR, we found an increase of germline transcription within the distal V_H domain (with the exception of PAIR promoter/enhancer-derived transcripts) and in the proportion of distal V_HDJ_H alleles, with no obvious block at the pro-B cell stage. This increase could be due, at least in part, to accumulated DJ_H substrates. However, it may also indicate a disruption of allelic exclusion. Thus, our findings of enhanced V_H-DJ_H recombination and germ-line transcription may be explained by a speculative model (See Figure 4) in which a productive rearrangement on one allele instructs the 3'RR on the second allele to down-regulate antisense transcription, leading to the inhibition of V_H-DJ_H recombination. In the absence of the 3'RR, a productive V_HDJ_H rearrangement on the first

Molecular and Cellular Biology

allele (and subsequent surface expression of μ heavy chain) would not block V_H-DJ_H recombination on the second allele, leading to an overall accumulation of V_HDJ_H alleles.

In this regard, our model may fill a gap in the regulated/feed-back inhibition model of allelic exclusion. Indeed, how to explain that a productive rearrangement on the first allele inhibits V_H-DJ_H recombination on the second allele? Our interpretation is that surface expression of the μ heavy chain instructs the 3'RR to inhibit germline transcription within the variable domain and therefore V_H-DJ_H recombination. Thus, 3'RR-mediated inhibition of V_H germline transcription could be the missing link between surface expression of the heavy chain and effective allelic exclusion. One prediction of this model is that deletion of the 3'RR will result in increased V_H germline transcription and V_H-DJ_H frequency: That is what we found in the present study. Another prediction is that if a heavy chain were prematurely expressed (that is, prior to V(D)J recombination), the 3'RR would be instructed to inhibit V_H germline transcription and impairment of V_H-DJ_H recombination would be the outcome: That is indeed the case (42).

The wide impact of the 3'RR on sense and antisense germ-line transcription within the variable region raises the question as to whether the 3'RR targets sense and antisense promoters simultaneously. We favor the view that the 3'RR targets primarily antisense promoters and that the silencing of sense transcripts could be a downstream consequence of this primary effect. It should be noted that antisense transcripts are long and extend through multiple V_H genes (28). Thus, the control of a limited number of antisense promoters would be sufficient for a wide transcriptional impact. Nonetheless, the 3'RR must somehow reach its distant target promoters. The possibility of a long-range effect mediated by 3'RR transcripts was ruled out because they were undetectable in pro-B cells. This would rather imply that the 3'RR-mediated silencing activity correlates with the lack of its transcription. A likely explanation is that the 3'RR exploits the developmentally-regulated, 3'RR-independent (22),

mechanisms that allow compaction of the *IgH* locus. In particular, the large-scale reorganization of the distal variable region into rosette-like structures following D-J_H recombination and the compaction of the *IgH* locus in pro-B cells (47) may bring into close proximity the 3'RR and its target promoters.

How could the 3'RR mediate its silencing activity is presently unknown and may involve a developmental stage-dependent interplay between the topological reorganization of the *IgH* locus which may be modulated by CTCF insulators and transcription factors-mediated loops, post-translational modifications of factors such as CTCF, and the 3'RR epigenetic modifications and recruitment of transcription factors and co-repressors (48-51). Interestingly, there is some evidence that the human β-globin locus control region can in some contexts repress gene expression through transcriptional interference potentially involving transcripts initiating in flanking repetitive sequences and running through the β-globin locus (52). This suggests that, besides their established role in gene expression activation, locus control regions have also the potential to mediate transcriptional silencing activity which may depend on the developmental stage, lineage specificity, interacting partners and the chromatin context.

In conclusion, our study reveals a hitherto unsuspected function of the 3'RR during early B cell development. The 3'RR emerges as a master regulatory element that mediates a transcriptional silencing activity along the distant and large *IgH* variable region, leading to the inhibition of V_H-DJ_H recombination likely to promote allelic exclusion.

Acknowledgements

We thank F.W. Alt for providing genomic DNA from IGCR1-deficient mice and for advice. We also thank the animal facility staff at the IPBS and F. L'Faqihi/V. Duplan-Eche at Purpan CPTP platform for their excellent assistance.

Funding

This work was supported by "Fondation ARC" [Grant PJA 20141201647], "Agence Nationale de la Recherche », « Institut National du Cancer », « Ligue Contre le Cancer - Comité de Haute-Garonne », and « Cancéropôle Grand-Sud-Ouest ».

Author contributions

FZB and CC performed research and analyzed data; MM, YD and MC contributed new reagents or analytic tools; AAK designed research, analyzed data and wrote the paper.

Conflict of interest: The authors declare that they have no conflict of interest.

References

1. **Bulger M, Groudine M.** 2011. Functional and mechanistic diversity of distal transcription enhancers. Cell **144**:327-339.

2. **Johnston CM, Wood AL, Bolland DJ, Corcoran AE**. 2006. Complete sequence assembly and characterization of the C57BL/6 mouse Ig heavy chain V region. J Immunol **176:**4221-4234.

3. **Retter I, Chevillard C, Scharfe M, Conrad A, Hafner M, Im TH, Ludewig M, Nordsiek G, Severitt S, Thies S, Mauhar A, Blocker H, Muller W, Riblet R.** 2007. Sequence and characterization of the Ig heavy chain constant and partial variable region of the mouse strain 129S1. J Immunol **179:**2419-2427.

4. **Jung D, Giallourakis C, Mostoslavsky R, Alt FW**. 2006. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. Annu Rev Immunol **24:**541-570.

5. **Vettermann C, Schlissel MS**. 2010. Allelic exclusion of immunoglobulin genes: models and mechanisms. Immunol Rev **237:**22-42.

6. **Subrahmanyam R, Sen R.** 2012. Epigenetic features that regulate IgH locus recombination and expression. Curr Top Microbiol Immunol **356**:39-63.

7. **Stubbington MJ, Corcoran AE**. 2013. Non-coding transcription and large-scale nuclear organisation of immunoglobulin recombination. Curr Opin Genet Dev **23:**81-88.

8. **Perlot T, Alt FW, Bassing CH, Suh H, Pinaud E**. 2005. Elucidation of IgH intronic enhancer functions via germ-line deletion. Proc Natl Acad Sci USA **102:**14362-14367.

9. **Afshar R, Pierce S, Bolland DJ, Corcoran A, Oltz EM**. 2006. Regulation of IgH gene assembly: role of the intronic enhancer and 5'DQ52 region in targeting DHJH recombination. J Immunol **176:**2439-2447.

10. **Bolland DJ, Wood AL, Afshar R, Featherstone K, Oltz EM, Corcoran AE**. 2007. Antisense intergenic transcription precedes Igh D-to-J recombination and is controlled by the intronic enhancer Emu. Mol Cell Biol **27:**5523-5533.

11. **Perlot T, Li G, Alt FW.** 2008. Antisense transcripts from immunoglobulin heavy-chain locus V(D)J and switch regions. Proc Natl Acad Sci USA **105**:3843-3848.

12. **Chakraborty T, Perlot T, Subrahmanyam R, Jani A, Goff PH, Zhang Y, Ivanova I, Alt FW, Sen R**. 2009. A 220-nucleotide deletion of the intronic enhancer reveals an epigenetic hierarchy in immunoglobulin heavy chain locus activation. J Exp Med **206:**1019-1027.

13. **Degner SC, Wong TP, Jankevicius G, Feeney AJ**. 2009. Cutting edge: developmental stage-specific recruitment of cohesin to CTCF sites throughout immunoglobulin loci during B lymphocyte development. J Immunol **182:**44-48.

14. **Featherstone K, Wood AL, Bowen AJ, Corcoran AE**. 2010. The mouse immunoglobulin heavy chain V-D intergenic sequence contains insulators that may regulate ordered V(D)J recombination. J Biol Chem **285:**9327-9338.

15. **Degner SC(1), Verma-Gaur J, Wong TP, Bossen C, Iverson GM, Torkamani A, Vettermann C, Lin YC, Ju Z, Schulz D, Murre CS, Birshtein BK, Schork NJ, Schlissel MS, Riblet R, Murre C, Feeney AJ.** 2011. CCCTC-binding factor (CTCF) and cohesin influence the genomic architecture of the Igh locus and antisense transcription in pro-B cells. Proc Natl Acad Sci USA **108:**9566-9571.

16. **Guo C, Yoon HS, Franklin A, Jain S, Ebert A, Cheng HL, Hansen E, Despo O, Bossen C, Vettermann C, Bates JG, Richards N, Myers D, Patel H, Gallagher M, Schlissel MS, Murre C, Busslinger M, Giallourakis CC, Alt FW**. 2011. CTCF-binding elements mediate control of V(D)J recombination. Nature **477:**424-430.

419 17. **Khamlichi AA, Pinaud E, Decourt C, Chauveau C, Cogné M**. 2000. The 3' IgH
420 regulatory region: a complex structure in a search for a function. Adv Immunol **75:**317-345.
421 18. **Pinaud E, Khamlichi AA, Le Morvan C, Drouet M, Nalesso V, Le Bert M, Cogné M**.
422 2001. Localization of the 3' IgH locus elements that effect long-distance regulation of class
423 switch recombination. Immunity **15:**187-199.
424 19. **Vincent-Fabert C, Fiancette R, Pinaud E, Truffinet V, Cogné N, Cogné M, DenizotY**.
425 2010. Genomic deletion of the whole IgH 3' regulatory region (hs3a, hs1,2, hs3b, and hs4)
426 dramatically affects class switch recombination and Ig secretion to all isotypes. Blood
427 **116:**1895-1898.
428 20. **Rouaud P, Vincent-Fabert C, Fiancette R, Cogné M, Pinaud E, Denizot Y.** 2012.
429 Enhancers located in heavy chain regulatory region (hs3a, hs1,2, hs3b, and hs4) are
430 dispensable for diversity of VDJ recombination. J Biol Chem **287**:8356-8360.
431 21. **Guo C, Gerasimova T, Hao H, Ivanova I, Chakraborty T, Selimyan R, Oltz EM, Sen**
432 **R**. 2011. Two forms of loops generate the chromatin conformation of the immunoglobulin
433 heavy-chain gene locus. Cell **147:**332-343.
434 22. **Medvedovic J, Ebert A, Tagoh H, Tamir IM, Schwickert TA, Novatchkova M, Sun**
435 **Q, Huis In't Veld PJ, Guo C, Yoon HS, Denizot Y, Holwerda SJ, de Laat W, Cogne M,**
436 **Shi Y, Alt FW, Busslinger M**. 2013. Flexible Long-Range Loops in the VH Gene Region of
437 the Igh Locus Facilitate the Generation of a Diverse Antibody Repertoire. Immunity **39:**229-
438 244.
439 23. **Braikia F-Z, Chemin G, Moutahir M, Khamlichi AA.** 2014. Quantification of V(D)J
440 recombination by real-time quantitative PCR. Immunol Lett **162**:119-123.
441 24. **Garrett FE, Emelyanov AV, Sepulveda MA, Flanagan P, Volpi S, Li F, Loukinov D,**
442 **Eckhardt LA, Lobanenkov VV, Birshtein BK.** 2005. Chromatin architecture near a

443 potential 3' end of the IgH locus involves modular regulation of histone modifications during
444 B-Cell development and in vivo occupancy at CTCF sites. Mol Cell Biol **25**:1511-1525.

445 25. **Shinkai Y, Rathbun G, Lam K-P, Oltz EM, Stewart V, Mendelsohn M, Charron J,**
446 **Datta M, Young F, Stall AM, Alt FW.** 1992. RAG-2-deficient mice lack mature
447 lymphocytes owing to inability to initiate V(D)J recombination. Cell **68**:855-867.

448 26. **Haddad D, Oruc Z, Puget N, Laviolette-Malirat N, Philippe M, Carrion C, Le Bert**
449 **M, Khamlichi AA**. 2011. Sense transcription through the S region is essential for
450 immunoglobulin class switch recombination. EMBO J **30:**1608-1620.

451 27. **Yancopoulos GD, Alt FW**. 1985. Developmentally controlled and tissue-specific
452 expression of unrearranged VH gene segments. Cell **40:**271-281.

453 28. **Bolland DJ, Wood AL, Johnston CM, Bunting SF, Morgan G, Chakalova L, Fraser**
454 **PJ, Corcoran AE**. 2004. Antisense intergenic transcription in V(D)J recombination. Nat
455 Immunol **5:**630-637.

456 29. **Verma-Gaur J, Torkamani A, Schaffer L, Head SR, Schork NJ, Feeney AJ.** 2012.
457 Noncoding transcription within the Igh distal V(H) region at PAIR elements affects the 3D
458 structure of the Igh locus in pro-B cells. Proc Natl Acad Sci USA **109**:17004-17009.

459 30. **Giallourakis CC, Franklin A, Guo C, Cheng HL, Yoon HS, Gallagher M, Perlot T,**
460 **Andzelm M, Murphy AJ, Macdonald LE, Yancopoulos GD, Alt FW.** 2010. Elements
461 between the IgH variable (V) and diversity (D) clusters influence antisense transcription and
462 lineage-specific V(D)J recombination. Proc Natl Acad Sci USA **107**:22207-22212.

463 31. **Ebert A, McManus S, Tagoh H, Medvedovic J, Salvagiotto G, Novatchkova M,**
464 **Tamir I, Sommer A, Jaritz M, Busslinger M.** 2011. The distal V(H) gene cluster of the Igh
465 locus contains distinct regulatory elements with Pax5 transcription factor-dependent activity
466 in pro-B cells. Immunity **34**:175-187.

467 32. **Williams A, Spilianakis CG, Flavell RA.** 2010. Interchromosomal association and gene
468 regulation in trans. Trends Genet **26**:188-197.
469 33. **Hewitt SL, Farmer D, Marszalek K, Cadera E, Liang HE, Xu Y, Schlissel MS, Skok**
470 **JA.** 2008. Association between the Igk and Igh immunoglobulin loci mediated by the 3' Igk
471 enhancer induces 'decontraction' of the Igh locus in pre-B cells. Nat Immunol **9**:396-404.
472 34. **Péron S, Laffleur B, Denis-Lagache N, Cook-Moreau J, Tinguely A, Delpy L,**
473 **Denizot Y, Pinaud E, Cogné M.** 2013. AID-driven deletion causes immunoglobulin heavy
474 chain locus suicide recombination in B cells. Science **336**:931-934.
475 35. **Su IH, Basavaraj A, Krutchinsky AN, Hobert O, Ullrich A, Chait BT, Tarakhovsky**
476 **A.** 2003. Ezh2 controls B cell development through histone H3 methylation and Igh
477 rearrangement. Nature immunology **4**:124-131.
478 36. **Fuxa M, Skok J, Souabni A, Salvagiotto G, Roldan E, Busslinger M.** 2004. Pax5
479 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain
480 gene. Genes & development **18**:411-422.
481 37. **Liu H, Schmidt-Supprian M, Shi Y, Hobeika E, Barteneva N, Jumaa H, Pelanda R,**
482 **Reth M, Skok J, Rajewsky K.** 2007. Yin Yang 1 is a critical regulator of B-cell
483 development. Genes & development **21**:1179-1189.
484 38. **Reynaud D, Demarco IA, Reddy KL, Schjerven H, Bertolino E, Chen Z, Smale ST,**
485 **Winandy S, Singh H.** 2008. Regulation of B cell fate commitment and immunoglobulin
486 heavy-chain gene rearrangements by Ikaros. Nature immunology **9**:927-936.
487 39. **Volpi SA, Verma-Gaur J, Hassan R, Ju Z, Roa S, Chatterjee S, Werling U, Hou H**
488 **Jr, Will B, Steidl U, Scharff M, Edelman W, Feeney AJ, Birshtein BK.** 2012. Germline
489 deletion of Igh 3' regulatory region elements hs 5, 6, 7 (hs5-7) affects B cell-specific
490 regulation, rearrangement, and insulation of the Igh locus. J Immunol **188**:2556-2566.

40. **Puget N, Hirasawa R, Nguyen Hu NS, Laviolette-Malirat N, Feil R, Khamlichi AA**. 2015. Insertion of an imprinted insulator into the IgH locus reveals developmentally regulated, transcription-dependent control of V(D)J recombination. Mol Cell Biol **35:**529-543.

41. **Lin SG, Guo C, Su A, Zhang Y, Alt FW**. 2015. CTCF-binding elements 1 and 2 in the Igh intergenic control region cooperatively regulate V(D)J recombination. Proc Natl Acad Sci USA **112**:1815-20.

42. **Puget N, Leduc C, Oruc Z, Moutahir M, Le Bert M, Khamlichi AA.** 2015. Complete cis-exclusion upon duplication of Eμ enhancer at the immunoglobulin heavy chain locus. Mol Cell Biol. *In press*.

43. **Fernex C, Capone M, Ferrier P.** 1995. The V(D)J recombinational and transcriptional activities of the immunoglobulin heavy-chain intronic enhancer can be mediated through distinct protein-binding sites in a transgenic substrate. Mol Cell Biol **15**:3217-3226.

44. **Cobb RM, Oestreich KJ, Osipovich OA, Oltz EM.** 2006. Accessibility control of V(D)J recombination. Adv Immunol **91**:45-109.

45. **Du H, Ishii H, Pazin MJ, Sen R.** 2008. Activation of 12/23-RSS-dependent RAG cleavage by hSWI/SNF complex in the absence of transcription. Mol Cell **31**:641-649.

46. **Lucas JS, Zhang Y, Dudko OK, Murre C.** 2014. 3D trajectories adopted by coding and regulatory DNA elements: first-passage times for genomic interactions. Cell **158**:339-352.

47. **Jhunjhunwala S, van Zelm MC, Peak MM, Cutchin S, Riblet R, van Dongen JJ, Grosveld FG, Knoch TA, Murre C.** 2008. The 3D structure of the immunoglobulin heavy-chain locus: implications for long-range genomic interactions. Cell **133**:265-279.

48. **Giambra V, Volpi S, Emelyanov AV, Pflugh D, Bothwell AL, Norio P, Fan Y, Ju Z, Skoultchi AI, Hardy RR, Frezza D, Birshtein BK.** 2008. Pax5 and linker histone H1 coordinate DNA methylation and histone modifications in the 3' regulatory region of the immunoglobulin heavy chain locus. Mol Cell Biol **28**:6123-6133.

49. **Cobaleda C, Schebesta A, Delogu A, Busslinger M.** 2007. Pax5: the guardian of B cell identity and function. Nat Immunol **8**:463-470.

50. **Atchison ML.** 2014. Function of YY1 in long-distance DNA interactions. Front Immunol 5(45):1-11.

51. **Birshtein BK.** 2014. Epigenetic Regulation of Individual Modules of the immunoglobulin heavy chain locus 3' Regulatory Region. Front Immunol **5**(163):1-9.

52. **Feng YQ, Warin R, Li T, Olivier E, Besse A, Lobell A, Fu H, Lin CM, Aladjem MI, Bouhassira EE.** 2005. The human beta-globin locus control region can silence as well as activate gene expression. Mol Cell Biol. **25**:3864-3874.

Figure legends

Fig.1. V(D)J recombination in mutant mice.

(A) The top scheme shows the mouse *IgH* locus (not to scale). CBEs, CTCF-binding elements. Not all CBEs are shown. Genomic DNAs were prepared from sorted WT and Δ3'RR pro-B cells and subjected to qPCR to amplify un-rearranged D_{Q52} and J_{H1} gene segments. The relative position of the primers is indicated in the upper scheme. Genomic DNA from *Rag2*$^{-/-}$ mice was used as a control. hs5 sequence was used for normalization (n=4). **(B)** Genomic DNAs from sorted pro-B cells (left) and double-positive thymocytes (right) were subjected to qPCR to quantify D-J_{H1}, D-J_{H2}, D-J_{H3} or D-J_{H4} recombination events. Rag2$^{-/-}$ and kidney DNAs were used as negative controls (n≥4). **(C)** Quantification of distal (dV_H) V_H-DJ_H recombination events in pro-B cells by qPCR (n≥4). (**, p<0.01; *, p<0.05; ns, not significant; Data are presented ± SEM).

Fig. 2. Early B cell development and the order of rearrangements. (A). To determine the distribution of pro-B and pre-B cell populations, single-cell suspensions from the bone marrow of *WT* and Δ3'RR were stained with anti-B220+anti-CD43+anti-IgM, and gated on IgM$^-$ population (n=11) (*, p<0.05; ns, not significant; Data are presented ± SEM). **(B)** Genomic DNAs were prepared from sorted pro-B cells ($B220^+IgM^-CD43^{high}$) and $CD4^+CD8^+$ thymocytes and were assayed for V_{H81X} to D_{Q52} rearrangement (n=2).

Fig. 3. Sense and antisense transcription in the IgH variable locus. (A) The top scheme shows the germ-line transcripts analyzed in the D-Cμ domain. The Iμ and μ0 sense transcripts are derived from Eμ and D_{Q52} promoter respectively while antisense transcripts originate from Eμ region, and an ill-defined promoter around DST4 segment (26). Dots indicate that the initiation and termination sites of the indicated transcripts were not precisely mapped. pA,

polyadenylation site. Germ-line transcripts were quantified by RT-qPCR in RAG-2-deficient pro-B cells (left, n≥6) and thymuses (middle, n≥3), and in RAG2-proficient $CD4^+CD8^+$ thymocytes (right) (n≥3). **(B)** Analysis of distal (dV_H) germ-line transcripts by semi-quantitative RT-PCR (left). Results of two independent experiments are shown (n=4). S, spliced transcripts (sense); US, unspliced (antisense/primary sense) transcripts. Quantification of the bands is displayed in the histograms on the right. **(C)** The top schemes show the relative position of the primers used along the *IgH* variable domain. Bottom: The histograms display the antisense transcript levels as measured by RT-qPCR (n≥6). AS, antisense. **(D)** The upper scheme indicates the relative position of the analysed germ-line transcripts along the *Igκ* locus. The histograms display the transcript levels in pro-B and pre-B cells (n=3). **(E)** RT-qPCR analysis of μ ($V_HDJ_HC\mu$) and Iμ transcripts in pro-B, pre-B and unstimulated splenic B cells. Forward primers that bind the distal V_H (dV_H) genes or Iμ exon and a reverse primer that pairs with Cμ1 exon were used to quantify μ and Iμ cDNAs (n≥6). **(F)** RT-qPCR analysis of hs3b and hs4 transcripts at various stages of B cell development (n=3). (***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; ns, not significant; Data are presented ± SEM).

Fig. 4. A speculative model linking the 3'RR-mediated silencing activity to allelic exclusion. This model stipulates an interplay between *cis*-acting elements and μ heavy chain (HC) signalling. Among the *cis*-regulatory elements which play a role in allelic exclusion, only the interactions between Eμ enhancer and the 3'RR are shown. **(A)**. Upon D-J_H recombination, Eμ enhancer up-regulates DJ_H transcription (Dμ transcripts), and sense and anti-sense germ-line transcripts are detected at the *IgH* variable region. **(B)**. A productive rearrangement on one allele will lead to μ HC surface expression in association with VpreB and λ5 surrogate light chains and Igα/Igβ heterodimer, which will signal to the 3'RR on the second allele to mediate a transcriptional silencing activity within the V_H region, leading to

down-regulation of sense and antisense transcription and V_H-DJ_H recombination. Cooperation between Eμ and the 3'RR on the productive allele up-regulates rearranged μ HC gene expression, leading to the enforcement and maintenance of allelic exclusion. **(C)**. If the first rearrangement is not productive (not in frame and therefore no μ HC production), the 3'RR receives no signal to mediate its silencing activity and V_H-DJ_H can therefore occur on the second allele. **(D)**. In the absence of the 3'RR, the link between μ HC instruction and germline transcription in the *IgH* variable region is lost, and allelic exclusion is disrupted. pV_H, proximal V_H cluster; dV_H, distal V_H cluster.

Fig. 1

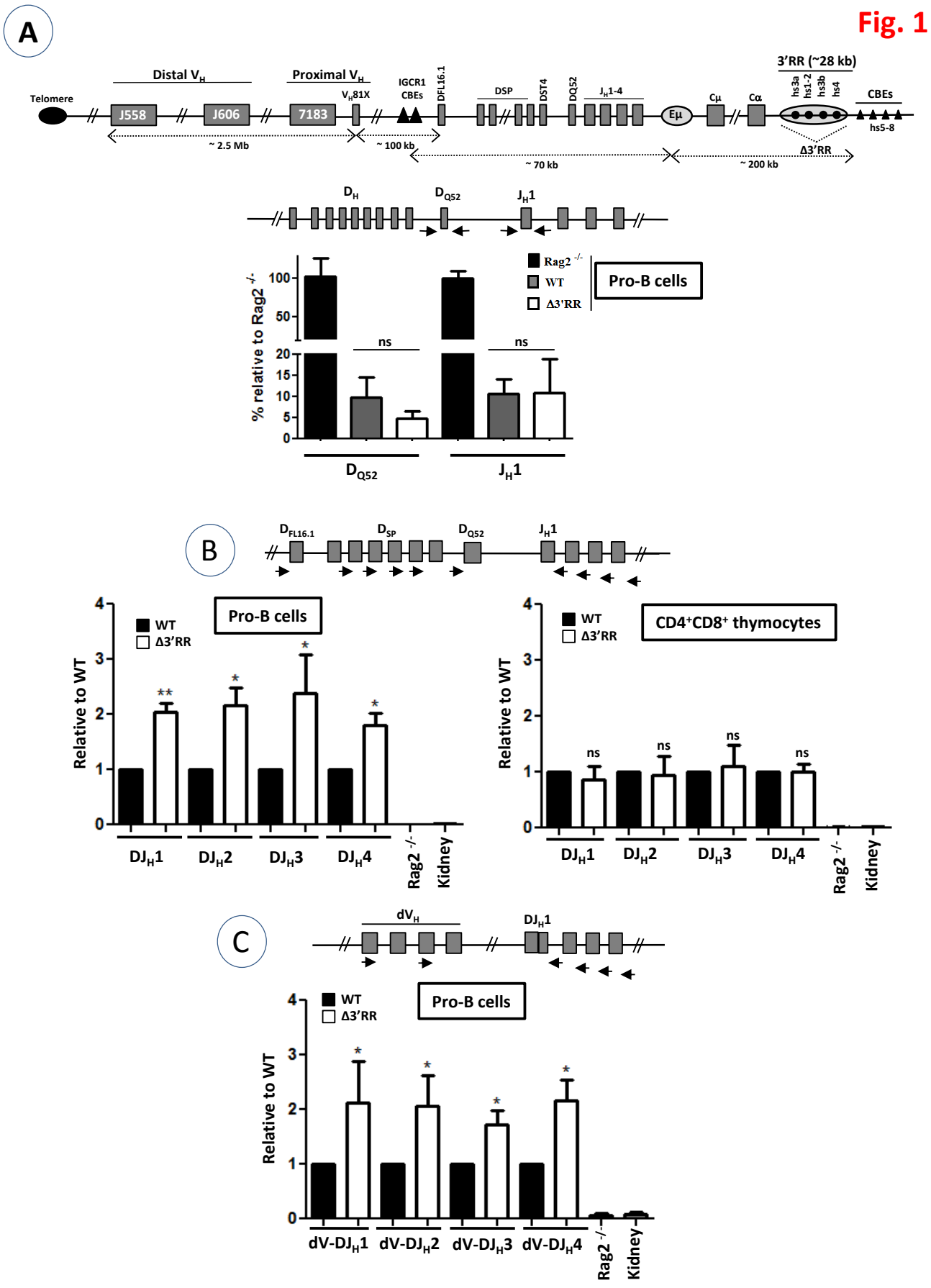

Fig. 2

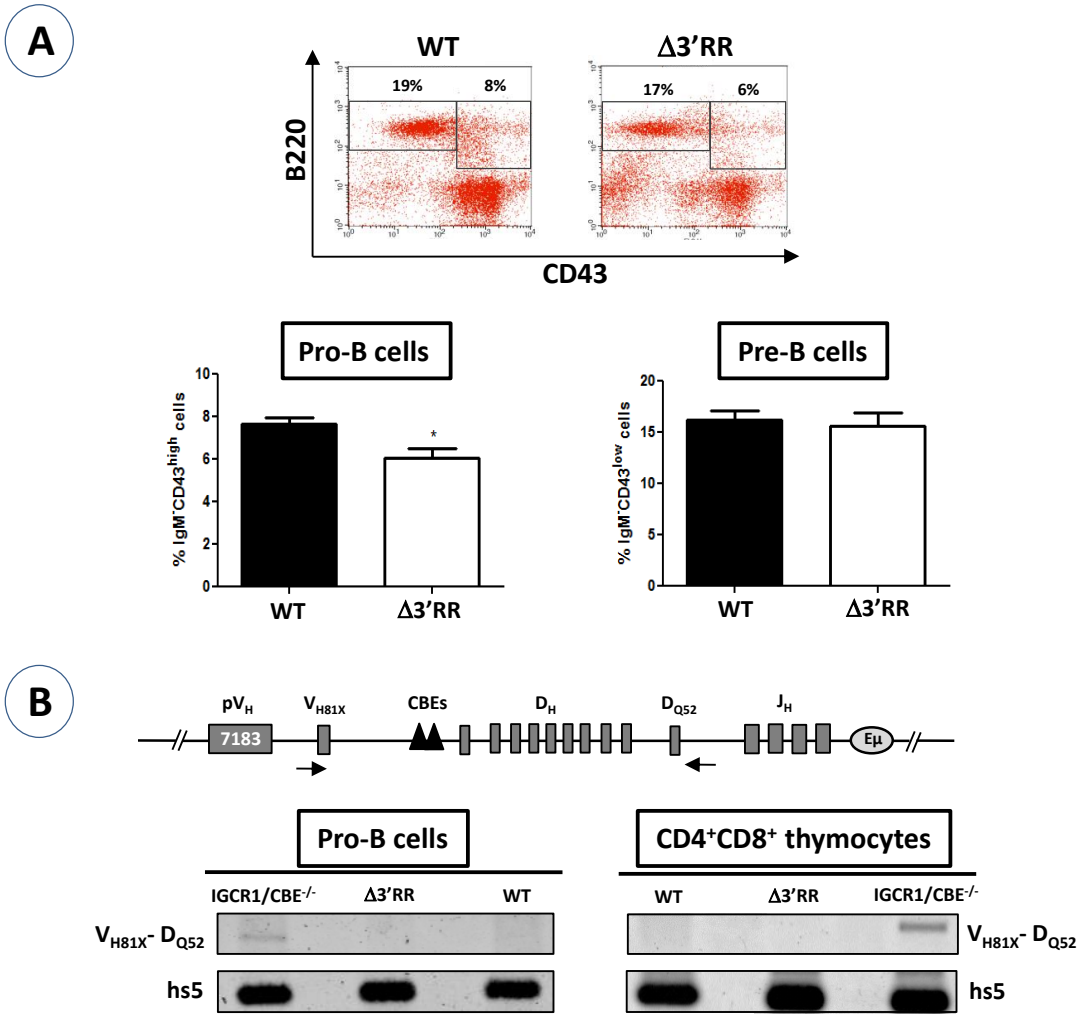

Fig. 3

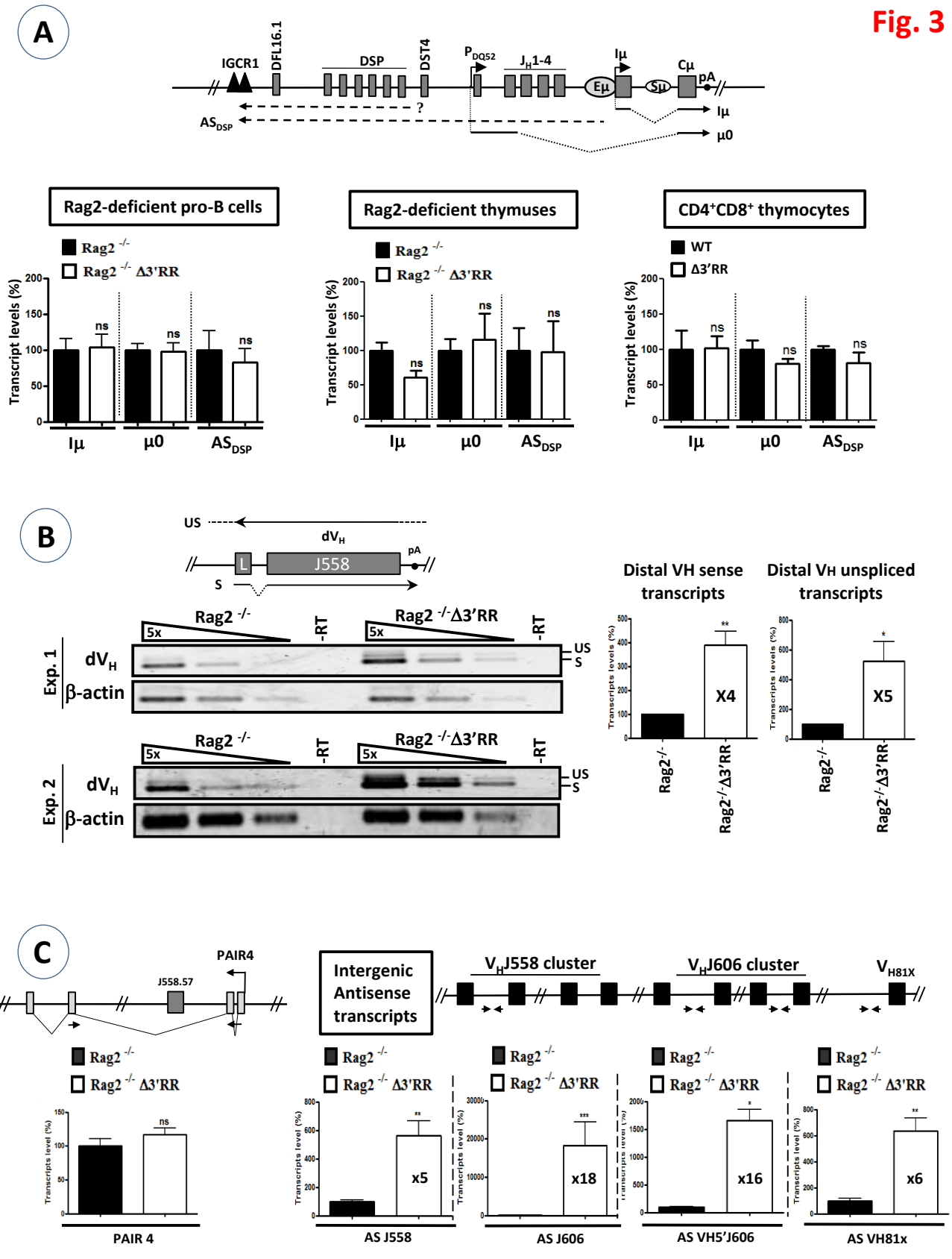

Fig. 3

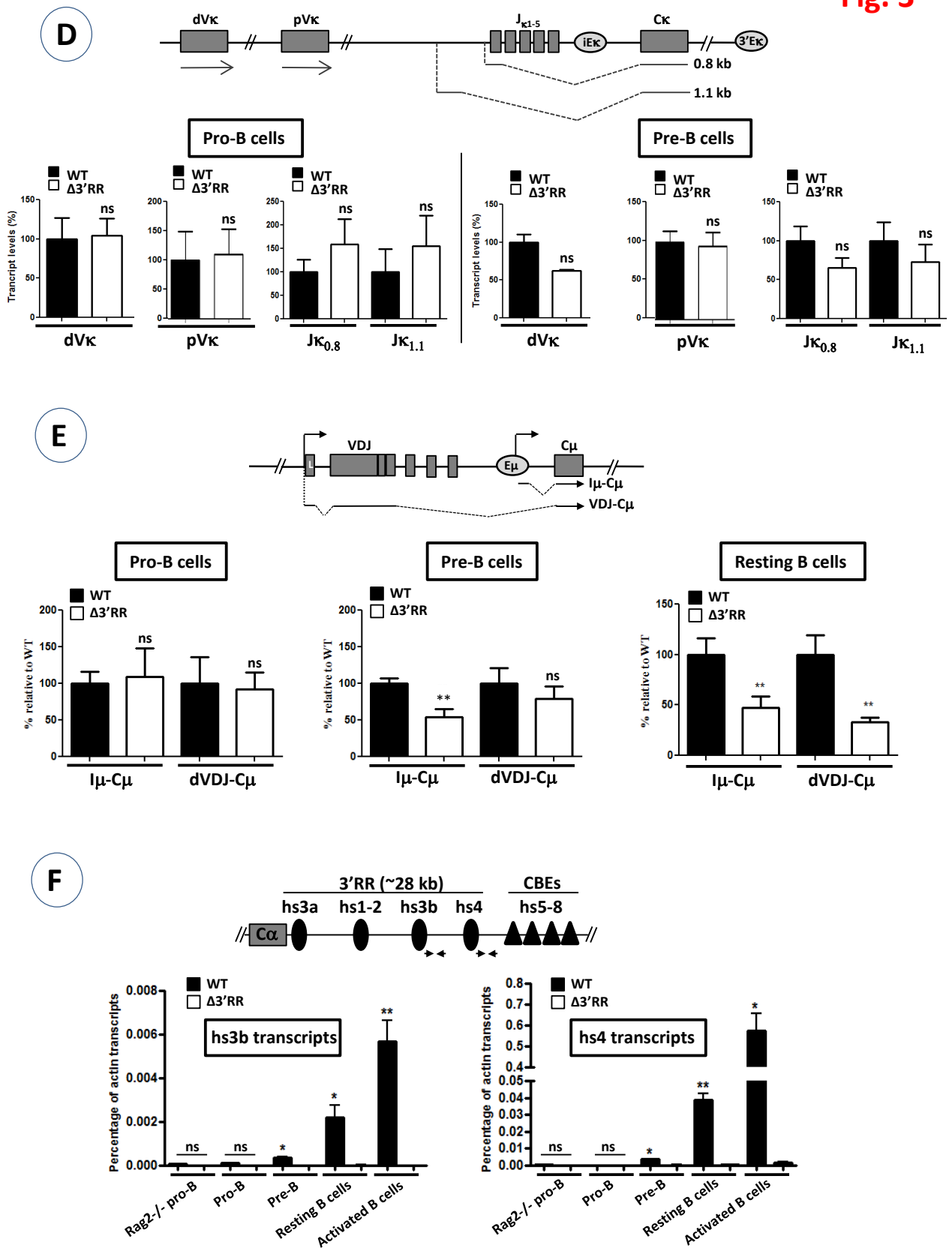

Fig. 4

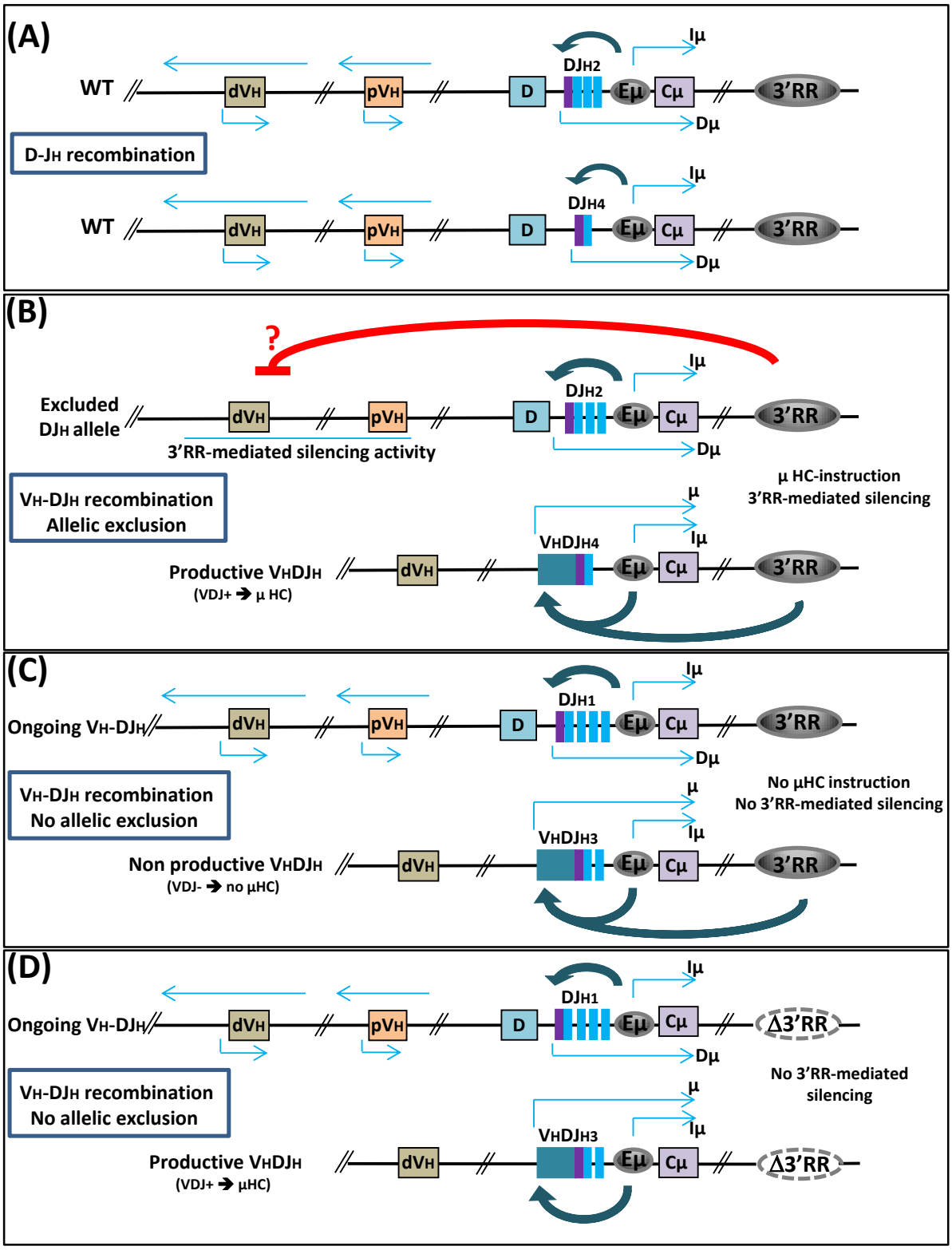

Table 1: D segment usage

Junction	Nb seq	D_{FL16}	DSP	D_{Q52}
D-J_H (WT)	39	9	29	1
D-J_H (Δ3'RR)	42	10	31	1
V_H-D-J_H (WT)	22	9	11	2
V_H-D-J_H (Δ3'RR)	23	8	14	1

Table 1. Genomic DNAs were purified from sorted WT and Δ3'RR pro-B cells, and subjected to PCR using degenerate primers that amplify DJ_H or V_HDJ_H segments. Amplicons were cloned and sequenced. The junctional diversity was used to check for the clonality of the sequences. Thus, sequences with identical insertions/deletions were considered as one. Within the limits of our data set, there is no obvious anomaly with regard to the D gene segments usage or to the number of inserted or deleted nucleotides.