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

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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 were unknown. Here we show that not only does the 3′RR affect transcription at distant sites within the *IgH* variable region but also 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.

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 family, by far the largest, and the proximal V_H family. The V_H genes are followed by a dozen 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 and 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 cell lineage specificity, V_H-DJ_H rearrangement is regulated by allelic exclusion, which enables monoallelic 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 to support the notion that V_H-DJ_H rearrangement is the regulated step in *IgH* allelic exclusion and its maintenance through a feedback mechanism (4, 5), although the molecular mechanisms through which feedback 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 recombinations of the distal and proximal domains are regulated very differently (4, 6, 7). Additionally, allelic exclusion of the distal V_H genes is more stringent than that of the most proximal V_H genes (4).

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

The established role of the 3′RR in *IgH* locus expression in late B cells and the lack of an effect on repertoire diversity led to the notion that 3′RR activity is restricted to the late stages of B cell maturation (19, 20). However, various studies described long-

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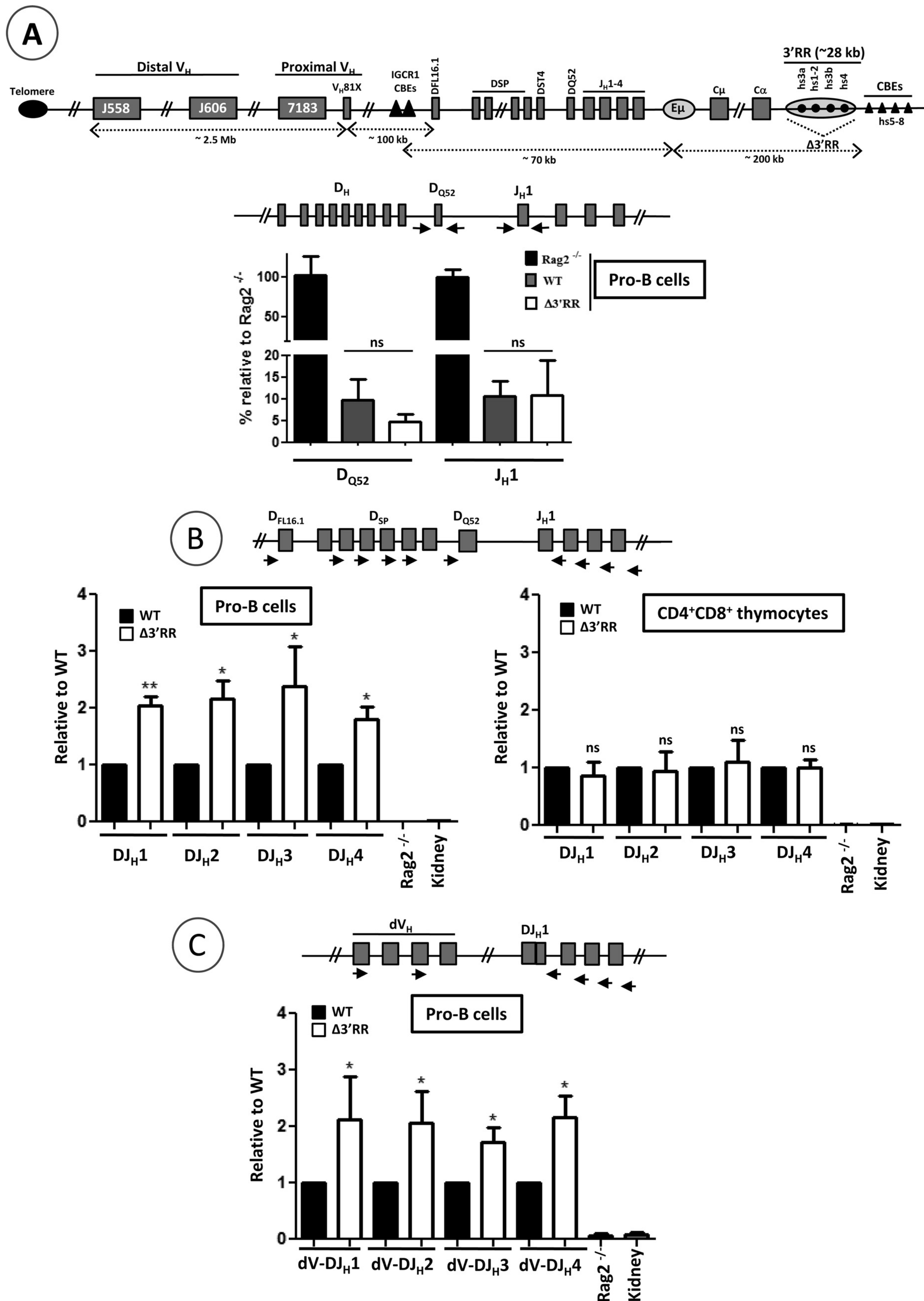

FIG 1 V(D)J recombination in mutant mice. (A) The schematic at the top shows the mouse *IgH* locus (not to scale). CBEs, CTCF-binding elements. Not all CBEs are shown. Genomic DNAs were prepared from sorted WT and Δ3′RR pro-B cells and subjected to qPCR to amplify unrearranged D_{Q52} and J_{H1} gene segments. The relative positions of the primers are indicated in the schematic above the graph. Genomic DNA from *Rag2*$^{-/-}$ mice was used as a control. The hs5 sequence was used for normalization ($n = 4$). (B) Genomic DNAs from sorted pro-B cells and double-positive thymocytes were subjected to qPCR to quantify D-J_{H1}, D-J_{H2}, D-J_{H3}, and D-J_{H4} recombination events. Rag2$^{-/-}$ and kidney DNAs were used as negative controls ($n \geq 4$). (C) Quantification of distal (dV_H) V_H-DJ_H recombination events in pro-B cells by qPCR ($n \geq 4$). **, $P < 0.01$; *, $P < 0.05$; ns, not significant. Error bars indicate SEM.

range interactions between the 3′RR and various upstream sequences, including the Eμ enhancer (15, 16, 21, 22), though their functional significance was unclear. Strikingly, deletion of either Eμ or the 3′RR had no effect on long-range interactions mediating locus contraction of the *IgH* locus, leading to the proposal that the activity of these elements may be restricted to the ~270-kb D-C_H region (22).

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

TABLE 1 D segment usage[a]

Junction	No. of sequences			
	Total	D_{FL16}	D_{SP}	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

[a] 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 to be one. Within the limits of our data set, there was no obvious anomaly with regard to D gene segment usage or to the number of inserted or deleted nucleotides.

MATERIALS AND METHODS

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

Antibodies. Allophycocyanin (APC)-conjugated anti-B220 and fluorescein isothiocyanate (FITC)-conjugated anti-IgM were purchased from BioLegend. Phycoerythrin (PE)-conjugated anti-CD43, FITC-conjugated anti-Igκ, PE-conjugated CD4, and FITC-conjugated CD8 were obtained from BD-Pharmingen.

FACS analyses. Bone marrows from 6- to 8-week-old mice were prepared by standard techniques. A total of 5×10^5 cells/assay were stained with anti-B220, anti-CD43, and anti-IgM or anti-Igκ and gated on either the IgM^- or $Ig\kappa^-$ population. Data were obtained for 2.0×10^4 viable cells by using a BD FACSCalibur flow cytometer. Fluorescence-activated cell sorter (FACS) acquisitions included isotype controls and exclusion of dead cells by labeling 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 were labeled 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 analysis (>95%) and by the rearrangement status of the Igκ locus. The $CD4^+$ $CD8^+$ thymocytes were sorted as described previously (16). Genomic DNAs from the sorted pro-B cells ($B220^+$ $Ig\kappa^-$ $CD43^{high}$ 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 in the supplemental material.

Reverse transcription-quantitative PCR (RT-qPCR). RAG-2-deficient pro-B cells were sorted using CD19 magnetic microbeads (Miltenyi). RAG-2-deficient thymuses were prepared as described previously (25). $B220^-$ $CD4^+$ $CD8^+$ thymocytes were sorted as described previously (16). Pro-B and pre-B cells ($B220^+$ $Ig\kappa^-$ $CD43^{low}$ or $B220^+$ IgM^- $CD43^{low}$) were sorted as described above. Unstimulated splenic B cells were sorted by using CD43 magnetic microbeads (Miltenyi) and activated by culturing for 48 h in the presence of 20 μg/ml lipopolysaccharide (Sigma) and 2 ng/ml anti-IgD–dextran (Fina Biosolutions). Total RNAs were reverse transcribed (Fermentas) and subjected to semiquantitative PCR, using SYBR green I (Invitrogen) and ImageQuant software as described previously (26), or to qPCR, using Sso Fast Eva Green (Bio-Rad). The relative transcription levels were normalized using β-*actin* and *Gapdh* RNAs as controls.

Statistical analysis. Results are expressed as means ± standard errors of the means (SEM) (GraphPad Prism), and overall differences between values for wild-type (WT) and mutant mice were evaluated by the Student *t* test. The difference between means is considered significant if the *P* value is <0.05, very significant if the *P* value is <0.01, and extremely significant if the *P* value is <0.001.

RESULTS

The 3′RR downmodulates 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 proportions 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 unrearranged D_{Q52} and J_{H1} segments on the mutant alleles was comparable to that for 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 D segments (Fig. 1B, schematic) or distal V_H genes (Fig. 1C, schematic) and specific backward primers located downstream of each J_H segment (Fig. 1B and C, schematics). We did not analyze the recombination of proximal V_H genes because the mutant allele is derived from 129Ola ES cells, which bear an ~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 of an overrepresentation 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 enhancement of V(D)J recombination (see Discussion).

The 3′RR does not affect the order of rearrangements. As mentioned previously (see the 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-

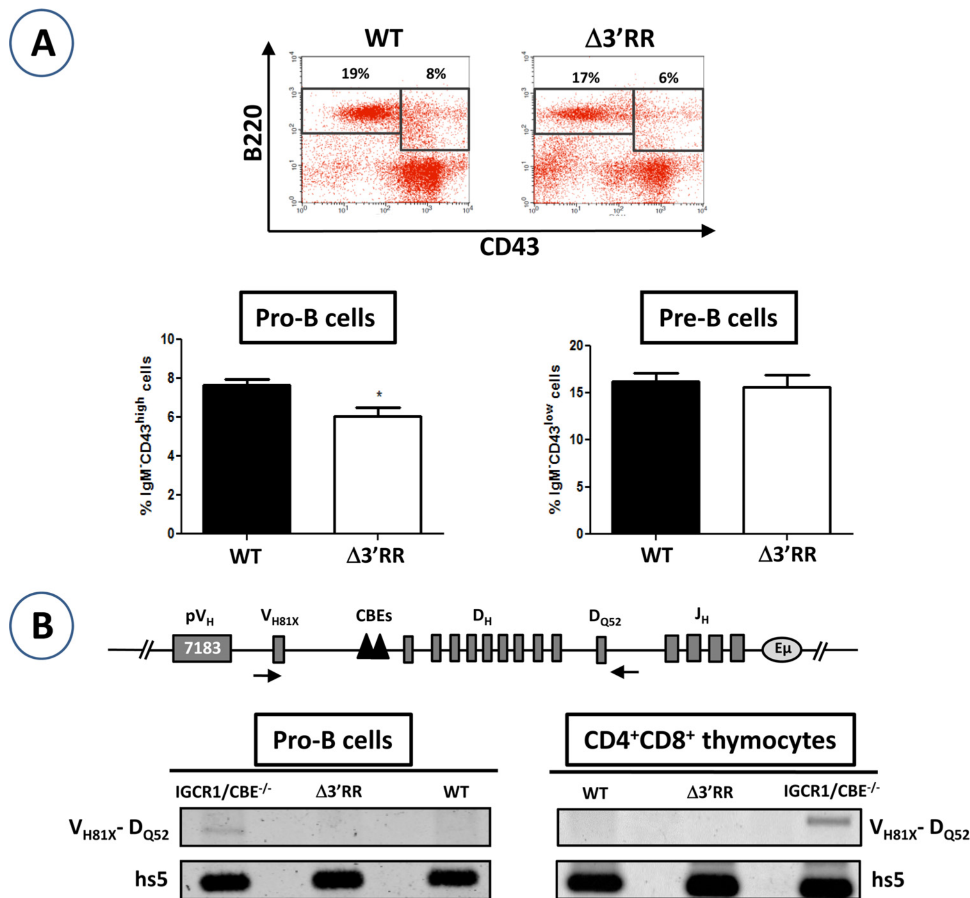

FIG 2 Early B cell development and order of rearrangements. (A) To determine the distribution of pro-B and pre-B cell populations, single-cell suspensions from the bone marrows of WT and Δ3′RR mice were stained with anti-B220, anti-CD43, and anti-IgM and gated on the IgM^- population ($n = 11$). *, $P < 0.05$. Data are presented as means and 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}-D_{Q52} rearrangement ($n = 2$).

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 the 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 the 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 of V_HD amplicons 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 downregulates sense and antisense 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 unrearranged genes, we first introduced the Δ3′RR mutation into the RAG-2-deficient background, which precludes V(D)J recombination. We mainly focused on the D-Cμ and distal V_H domains, for which high levels of transcription are detected (7, 16).

Germ line transcription within the D-Cμ domain, but not that within the distal V_H domain, is regulated by the Eμ enhancer (8–12, 29). In contrast, the effect of the 3′RR is unknown. We found no obvious effect on Iμ or μ0 sense transcripts (derived from the Eμ enhancer and the D_{Q52} promoter, respectively) or on D_{SP} antisense transcripts (derived from the Eμ region and/or an ill-known promoter upstream of the D_{ST4} segment [30]) (Fig. 3A). Concordantly, normal levels of Iμ, μ0, and D_{SP} GL transcripts were found in RAG-2-deficient thymuses and RAG-2-proficient $CD4^+$ $CD8^+$ thymocytes (Fig. 3A). Thus, within the D-Cμ domain, sense and antisense transcription was not altered in the absence of the 3′RR, indicating that the Eμ-mediated control of germ line transcription in the D-Cμ domain does not require the 3′RR.

Remarkably, the distal V_H region yielded increased levels of both spliced, sense transcripts and unspliced, antisense transcripts in Δ3′RR mice (Fig. 3B). To exclude any bias potentially introduced by the increased level of primary V_H sense transcripts, we quantified intergenic, antisense germ line transcript levels within the V_{HJ558} and V_{HJ606} clusters. The levels of these exclusively antisense transcripts were also increased (Fig. 3C). In contrast, the Pax5-activated intergenic repeat 4 (PAIR4) antisense germ line transcripts (31) were unaltered (Fig. 3C), suggesting that regulation of these transcripts, derived from the PAIR4 promoter/en-

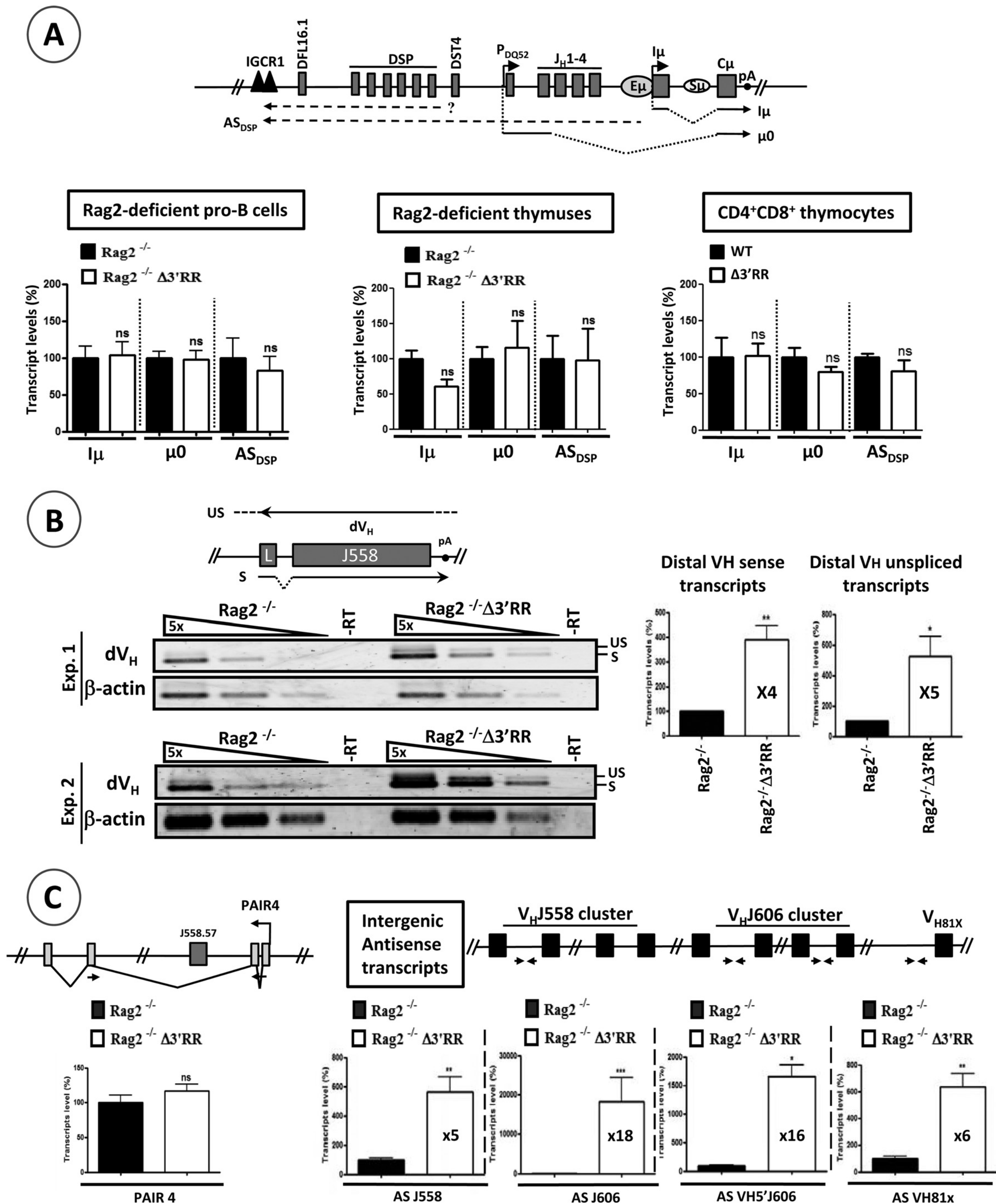

FIG 3 Sense and antisense transcription in the *IgH* variable locus. (A) The schematic at the top shows the germ line transcripts analyzed in the D-Cμ domain. The Iμ and μ0 sense transcripts are derived from Eμ and the D_{Q52} promoter, respectively, while antisense transcripts originate from the Eμ region and an ill-defined promoter around the D_{ST4} segment (30). Dots indicate that the initiation and termination sites of the indicated transcripts were not precisely mapped. pA, polyadenylation site. Germ line transcripts in RAG-2-deficient pro-B cells (left; $n \geq 6$) and thymuses (middle; $n \geq 3$) and RAG2-proficient $CD4^+$ $CD8^+$ thymocytes (right; $n \geq 3$) were quantified by RT-qPCR. (B) (Left) Analysis of distal (dV_H) germ line transcripts by semiquantitative RT-PCR. 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 schematics at top show the relative positions of the primers used along the *IgH* variable domain. The histograms display the antisense transcript levels as measured by RT-qPCR ($n \geq 6$). AS, antisense. (D) The schematic at top indicates the relative positions of the analyzed germ line transcripts along the *Ig*κ 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 the Iμ exon and a reverse primer that pairs with the Cμ1 exon were used to quantify μ and Iμ cDNAs ($n \geq 6$). (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. Error bars indicate SEM.

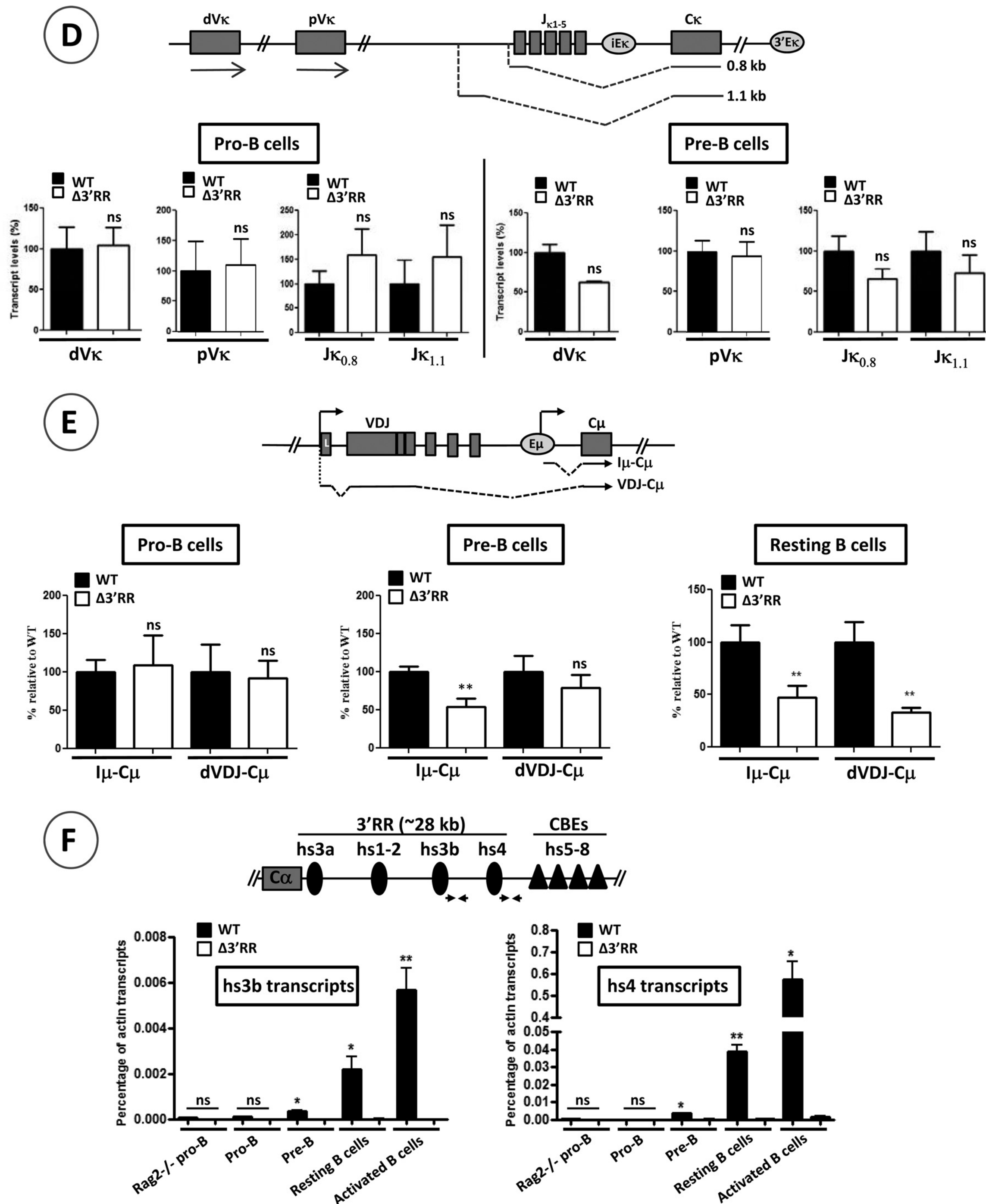

FIG 3 continued

hancer element (31), is 3′RR independent. Thus, within the distal V_H domain (except for PAIR elements), the 3′RR affects both sense and antisense transcription.

Within the proximal V_H domain, we also found increased antisense transcription upstream of V_{H81X} (the most 3′ functional V_H gene segment, which is not encompassed by the ~120-kb deletion in Δ3′RR mice [22]) (Fig. 3C), suggesting a variable region-wide effect of the 3′RR.

Overall, and within the limits of the transcripts analyzed, the data show that the 3′RR downregulates sense and antisense germ line transcription along the remote *IgH* variable domain.

Transcription of some loci could be regulated by elements located on a different chromosome (32). Specifically, it was suggested that the *Igκ* locus and its 3′ enhancer (on chromosome 6) are involved in directing the *IgH* locus (on 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

that it was unchanged (Fig. 3D), excluding, at least with regard to the *Ig*κ 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 the 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). Downregulation 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 (in pre-B cells) activity correlated with the appearance of 3′RR enhancer 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 it correlates with the onset of 3′RR transcription.

DISCUSSION

We report here the first demonstration of a stepwise shift in the transcriptional activity of a long-range regulatory element in higher eukaryotes. The downregulating activity of the 3′RR targets multiple upstream sense and antisense promoters in the remote *IgH* variable region, but it 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 controlled mainly by the 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; this is not the case for RAG-2-deficient mice, which are derived from strain 129Sv. Thus, although we cannot formally exclude the possibility that the 120-kb deletion within the proximal V_H domain affected distal V_H germ line transcription and V(D)J recombination, we think that it is unlikely, for various reasons. Multiple studies clearly showed that the proximal and distal V_H domains are differentially regulated. Thus, recombination of distal but not proximal V_H genes is inhibited in mice deficient in the histone-modifying enzyme EZH2 and in 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 chromosome conformation capture-derived (4C-seq) analyses to strongly or minimally interact with the 3′RR correlated well with our transcriptional analyses. For instance, antisense transcription upstream of the V_{H81X} gene (which is intact in the 129Ola background) was enhanced in the absence of the 3′RR (present study), and this gene was efficiently contacted by the 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 reference 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′CBE chromatin domain (15, 16, 21, 22).

Determining 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 means through which recombination regulates the 3′RR transcriptional activity. Alternatively, though not mutually exclusive, the correlation between the triggering of 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 downmodulating 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 have found that transcription and V(D)J recombination can be mediated by distinct activities of accessibility control elements, including the Eμ enhancer (43; reviewed in reference 44), and there is some evidence that recombination can be recapitulated *in vitro* in the absence of transcription (45).

By quantifying the proportions of the D_{Q52} and J_{H1} segments in their unrearranged 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 an 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 that is restricted to pro-B cells, i.e., an increased number of recombination events in a "fixed" time window. One simple explanation is that the process runs faster in the absence of the 3′RR. Recent evidence highlighted the importance of spatial confinement for the kinetics of V(D)J recombination and the time of 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 affecting germ line transcription within the V_H domain, while Eμ is focused on DJ_H transcription (40).

Why does the 3′RR mediate 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 germ line transcription is likely the primary event during allelic exclusion (42). In the absence of the 3′RR, we found increases of germ line transcription within the distal V_H domain (with the exception of PAIR promoter/enhancer-derived transcripts) and of 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

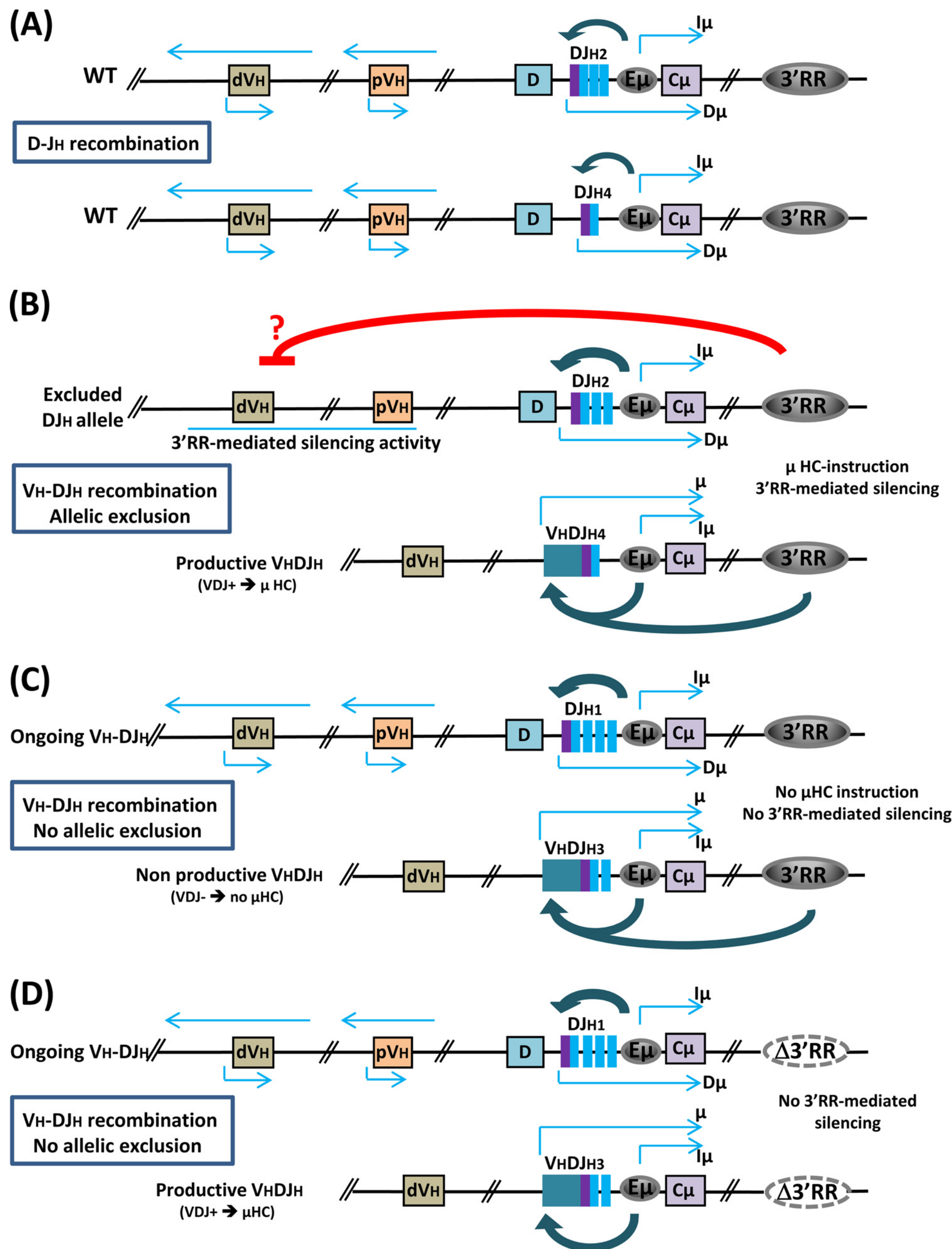

FIG 4 Speculative model linking 3′RR-mediated silencing activity to allelic exclusion. This model stipulates an interplay between *cis*-acting elements and μ heavy chain (HC) signaling. Among the *cis*-regulatory elements which play a role in allelic exclusion, only the interactions between the Eμ enhancer and the 3′RR are shown. (A) Upon D-J_H recombination, the Eμ enhancer upregulates DJ_H transcription (Dμ transcripts), and sense and antisense 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 the 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 downregulation of sense and antisense transcription and V_H-DJ_H recombination. Cooperation between Eμ and the 3′RR on the productive allele upregulates 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 recombination can therefore occur on the second allele. (D) In the absence of the 3′RR, the link between μ HC instruction and germ line 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.

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 (Fig. 4) in which a productive rearrangement on one allele instructs the 3′RR on the second allele to downregulate 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 allele (and subsequent surface expression of the 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 can we 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 germ line transcription within the variable domain, and therefore V_H-DJ_H recombination. Thus, 3′RR-mediated inhibition of V_H germ line 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 germ line transcription and V_H-DJ_H frequency, and we found this result in the present study. Another prediction is that if a heavy chain is expressed prematurely [that is, prior to V(D)J recombination], the 3′RR will be instructed to inhibit V_H germ line transcription, and the outcome will be an impairment of V_H-DJ_H recombination; this 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 of 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 may 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 such transcripts were undetectable in pro-B cells. This implies that the 3′RR-mediated silencing activity correlates with a lack of 3′RR transcription. A likely explanation is that the 3′RR exploits 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 the 3′RR and its target promoters into close proximity.

How the 3′RR can 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 factor-mediated loops, posttranslational modifications of factors such as CTCF, and the 3′RR epigenetic modifications and recruitment of transcription factors and corepressors (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 also have the potential to mediate transcriptional silencing activity, which may depend on the developmental stage, lineage specificity, interacting partners, and 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.

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F.-Z.B. and C.C. performed research and analyzed data; M.M., Y.D., and M.C. contributed new reagents or analytic tools; and A.A.K. designed research, analyzed data, and wrote the paper.

We declare that we have no conflicts of interest.

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