Brief report

Genomic deletion of the whole IgH 3′ regulatory region (hs3a, hs1,2, hs3b, and hs4) dramatically affects class switch recombination and Ig secretion to all isotypes

Christelle Vincent-Fabert,[1] Remi Fiancette,[1] Eric Pinaud,[1] Véronique Truffinet,[1] Nadine Cogné,[1] Michel Cogné,[1] and Yves Denizot[1]

[1]Unité Mixte de Recherche Centre National de la Recherche Scientifique, Université de Limoges, Limoges, France

The immunoglobulin heavy chain locus (IgH) undergoes multiple changes along B-cell differentiation. In progenitor B cells, V(D)J assembly allows expression of μ heavy chains. In mature B cells, class switch recombination may replace the expressed constant (C)μ gene with a downstream C_H gene. Finally, plasma cell differentiation strongly boosts IgH transcription. How the multiple IgH transcriptional enhancers tune these changes is unclear. Here we demonstrate that deletion of the whole IgH 3′ regulatory region (3′RR) allows normal maturation until the stage of IgM/IgD expressing lymphocytes, but nearly abrogates class switch recombination to all C_H genes. Although plasma cell numbers are unaffected, we reveal the role of the 3′RR into the transcriptional burst normally associated with plasma cell differentiation. Our study shows that transcriptional changes and recombinations occurring after antigen-encounter appear mainly controlled by the 3′RR working as a single functional unit. (*Blood*. 2010;116(11):1895-1898)

Introduction

The immunoglobulin heavy chain locus (IgH) undergoes multiple changes along B-cell differentiation, affecting transcription and accessibility to V(D)J or class switch recombination (CSR). The *iE*μ enhancer upstream of Cμ mostly promotes V(D)J recombination,[1] whereas IgH 3′RR enhancers (hs3a, hs1,2, hs3b, and hs4) have controversial roles. A role in CSR was suggested by replacing mouse hs1,2 with a neomycin resistance gene, thus affecting germline transcription and CSR to γ2a, γ2b, γ3, and ϵ.[2] However, deletion of this neo cassette restored CSR.[3] hs3a, hs3b, and hs4 also proved individually dispensable for CSR.[3-5] Enhancer redundancies might explain that their individual deletion only results in minor effect. Indeed, joint hs3b/hs4 deletion impaired germline transcription and CSR to most isotypes except μ and γ1.[6] Reporter genes also demonstrated synergies between 3′RR enhancers,[7] which altogether promote CSR into large transgenes.[8] The 3′RR is followed with DNase hypersensitive sites (hs5-7) lacking enhancer activity but binding CCCTC-binding factor and constituting the 3′ locus boundary.[9] To reconcile the controversial phenotypes of focal mutations, potentially attenuated by functional redundancies, we evaluated IgH expression and CSR after deleting the whole 30-kb extent of the 3′RR.

Methods

Experimental details are available in the supplemental data (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Studies have been reviewed and approved by Centre National de la Recherche Scientifique and University review committee. Generation of 3′RR-deficient mice is described in supplemental Figure 1. B-cell compartments, B-cell proliferation and apoptosis, and CSR were investigated by flow cytometry.[4,5,10] Germline and hybrid transcripts were detected by reverse-transcribed polymerase chain reaction (RT-PCR).[6] Amounts of the various Ig classes were assessed by enzyme-linked immunosorbent assay.[4-6] Mice immunizations were as reported.[11]

Results and discussion

Generation of 3′RR-deficient mice

The gene-targeting vector replaced the 30-kb genomic fragment encompassing the 3′RR with a *lox*P/*neo*^{R} cassette. Neo mutant mice allowed the derivation of 3′RR-deficient animals after cre-deletion of *neo* (supplemental Figure 1).

B-cell development and activation in 3′RR-deficient mice

In contrast to the iEμ deletion impairing B-cell differentiation,[1] progenitor and peripheral B-cell compartments appeared normal in 3′RR-deficient animals (supplemental Figure 2). Surface IgM was normal, contrary to hs4- and hs3b/hs4-deficient mice,[5,6] suggesting that the complete deletion might remove some inhibitory elements left after the hs4 deletion. Lipopolysaccharide (LPS) or anti-CD40-induced proliferation, and apoptosis were not affected either (supplemental Figure 3).

Submitted January 20, 2010; accepted May 25, 2010. Prepublished online as *Blood* First Edition paper, June 10, 2010; DOI 10.1182/blood-2010-01-264689.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Ig synthesis in 3′RR-deficient mice

After in vitro stimulation with LPS and appropriate cytokines, 3′RR-deficient B cells showed an 85% reduced IgM secretion and more than or equal to 99% decrease for other isotypes (Table 1). This was not related to a defect in plasma cell maturation because amounts of Blimp/Xbp1 transcripts and $CD138^+$ plasmablasts after 3 days in vitro LPS activation were normal (supplemental Figure 3). Similar amounts of $CD138^+$ cells in spleen of 3′RR-deficient mice and wild-type animals suggested that the deletion did not impede plasma cell differentiation (supplemental Figure 2), whereas μ IgH transcripts were globally decreased in splenocytes from 3′RR-deficient mice (supplemental Figure 4). Probably because of variable half-lives, serum Ig levels were heterogeneously affected, most strongly lowered, whereas IgG_1 and IgG_{2a} were preserved (Table 1). However, investigation of specific antibody levels on challenge with thymus-dependent (ovalbumin) or -independent (DNP-Ficoll) antigens confirmed strong IgM reduction, whereas all 4 IgG subclass antibodies were undetectable (supplemental Figures 5-6).

Hybrid and germline transcripts in 3′RR-deficient mice

Overall, 3′RR-deficient mice exhibited a globally decreased Ig secretion, culminating in a blockade for class-switched isotypes. To determine whether this resulted from decreased CSR or only from decreased expression of class-switched genes, we appreciated the number of cells switching to a particular isotype after in vitro stimulation. 3′RR-deficient mice splenocytes showed decreased switching to all isotypes (Figure 1A-E). Parallel quantification of *I*μ-Cx hybrid transcripts (x being any constant gene) showed a more than 100-fold decrease of *I*μ-Cγ2b and *I*μ-Cγ3 transcripts in LPS-treated splenocytes, of *I*μ-Cε and *I*μ-Cγ1 on LPS/IL-4 stimulation, of *I*μ-Cα on LPS/TGF-β-stimulation, and of *I*μ-Cγ2a on LPS/interferon-γ stimulation (Figure 1F). 3′RR-deficient mice thus exhibited a severe blockade of CSR. Germline transcription of C_H gene, a known prerequisite of CSR, finally showed a more than 5-fold decrease of *I*μ-Cμ, *I*γ1-Cγ1, *I*γ2a-Cγ2a, *I*γ2b-Cγ2b, and a more than 25-fold decrease of *I*γ3-Cγ3, *I*ε-Cε, and *I*α-Cα (Figure 1G). Although semiquantitative RT-PCR may be imperfect and although its extent varied between isotypes, a global decrease of germline transcription was observed and did not spare μ and γ1 as in hs3b/hs4-deficient mice.[6] Interestingly, and by contrast to previous focal alterations of the 3′RR, the Iμ-Cμ transcription unit, including the donor Sμ region, was also affected. Confirming CSR defect, excised circle transcripts were lowered in 3′RR-deficient mice compared with wild-type animals (supplemental Figure 7).

Table 1. Ig synthesis in IgH 3′RR-deficient mice

	Ig isotype	Wild-type mice	IgH 3′RR-deficient mice	*P*
Splenocyte supernatant, ng/mL*				
LPS	IgM	21 333.3 ± 8855.5	1652.5 ± 685.0	.009
LPS	IgG_3	445.3 ± 259.2	0 ± 0	< .001
LPS	IgG_{2b}	155.4 ± 71.8	4.8 ± 2	.001
LPS + IL-4	IgG_1	2402.5 ± 1040.6	13.5 ± 0.7	< .001
LPS + IL-4	IgE	14.6 ± 6.4	0 ± 0	< .001
LPS + IFN-γ	IgG_{2a}	4.8 ± 2.8	0 ± 0	.001
LPS + TGF-β	IgA	353.5 ± 191.2	0 ± 0	< .001
Serum, μg/mL†				
	IgM	283.5 ± 52.4	23.7 ± 2.4	< .001
	IgG_3	9.7 ± 1.9	0 ± 0	< .001
	IgG_1	59.2 ± 18.4	37.7 ± 9.5	.45
	IgG_{2b}	1.9 ± 0.1	0.9 ± 0.1	< .001
	IgG_{2a}	63.2 ± 32.8	82.1 ± 19.0	.41
	IgE	2.3 ± 0.7	0 ± 0	< .001
	IgA	33.6 ± 5.8	2.9 ± 0.7	< .001

*ELISA analysis of IgM, IgG_3, IgG_1, IgG_{2b}, IgG_{2a}, IgE, and IgA in supernatants of B cells cultured for 4 days with LPS with or without cytokines. Data are mean ± SEM of 9 independent experiments (Mann-Whitney U test for significance).

†ELISA analysis of IgM, IgG_3, IgG_1, IgG_{2b}, IgG_{2a}, IgE, and IgA in 8-week-old mouse sera of *wt* and mutant mice. Data are mean ± SEM of 7 animals in each group (Mann-Whitney U test for significance).

In conclusion, there have been several previous focal genomic alterations of the 3′RR.[2-5] We generated mice lacking the whole 3′RR, thus extending and clarifying several findings from these previous models. Mice developed normally abundant B cells in bone marrow, spleen, and blood, in agreement with the concept that 3′IgH enhancers play their most crucial role at late stages of B-cell maturation. Functional redundancies of 3′RR enhancers have long kept their role quite elusive. The complete 3′RR deletion dramatically affects CSR and Ig secretion for all isotypes. Hybrid transcripts from class switched loci were more strongly reduced than germline transcripts, suggesting that the 3′RR promotes CSR not only by fostering germline transcription. BAC transgene models also showed the 3′RR to control CSR, except to the Cα gene.[8] 3′RR-deficient mice demonstrate that only Cγ1 transcription and CSR are partly independent from this control. Similar observations were made after deletion of the distal part of the 3′RR region (hs3b/hs4),[6] altogether suggesting that elements other than the 3′RR (such as the hs5-7 sites lying downstream?) are supported by γ1 transcription. As suggested by Wuerffel et al,[12] Eμ enhancer might also contribute. How the 3′RR affects CSR can be inferred from 3C experiments showing that S-S synapsis was promoted by the close spatial proximity of Eμ and 3′RR elements forming a unique chromosomal loop configuration.[12,13] The germline transcript promoter association with the Eμ-3′RR complex might create an architectural scaffolding, promoting S-S synapsis and further CSR.[12] Although Eμ appeared as dispensable, integrity of the 3′RR was mandatory for such conformations of the locus as judged from experiments with B cells lacking the 2 distal 3′RR enhancers.[12] Although plasma cells normally differentiated in 3′RR-deficient animals, the IgH defect resulted in depressed secretion of all Ig, including IgM. Although Cμ is more than or equal to 200 kb upstream of the 3′RR, Cμ transcripts severely dropped. Finally, the 3′RR deletion strongly alters CSR and afflicts expression of all IgH genes in plasma cells, including Cμ. The remaining IgH expression, CSR, and γ1 germline transcription suggest that additional elements cooperate with the 3′RR to stimulate I_X transcription in activated B cells and pV_H transcription in plasma cells. The iEμ enhancer is a suitable candidate because its deletion slightly impacts CSR and Ig synthesis[1] and because physical interactions have been documented between Eμ and the 3′RR.[12,13] Double mutant lacking both Eμ and the 3′RR would be the best tool for addressing this issue.

Acknowledgments

The authors thank S. Desforges and B. Remerand for help with animal care.

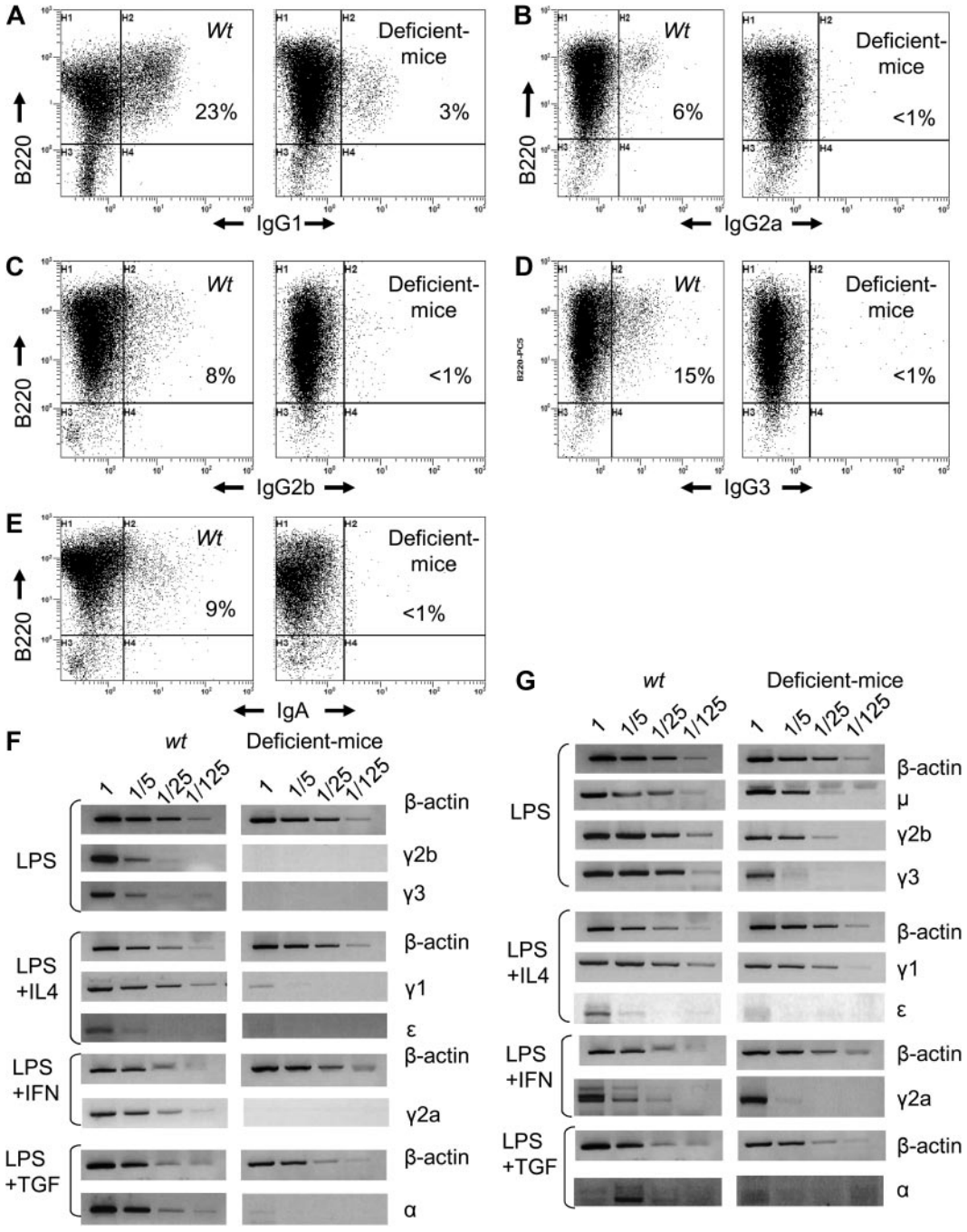

Figure 1. Altered class-switch recombination in IgH 3′RR-deficient mice. B cells were cultured for 4 days with LPS plus or minus cytokines at 1×10^6 cells/mL and stained with anti-B220-PC5 and anti-isotype fluorescein isothiocyanate antibodies: anti-IgG_1 (A), anti-IgG_{2a} (B), anti-IgG_{2b} (C), anti-IgG_3 (D), and anti-IgA (E). Representative results from 6 IgH 3′RR-deficient mice and wild-type mice are shown. (F) $I\mu$-Cx hybrid transcripts (x being any constant gene after CSR) induced in stimulated splenocytes from wild-type and IgH 3′RR-deficient mice. Total RNA was isolated on day 3. RT-PCR for $I\mu \rightarrow \gamma 2b$, $I\mu \rightarrow \gamma 3$ was performed on LPS-induced splenocytes RNA; $I\mu \rightarrow \gamma 1$ and $I\mu \rightarrow \epsilon$ on LPS plus IL4, $I\mu \rightarrow \alpha$ on LPS plus TGF-β; and $I\mu \rightarrow \gamma 2a$ on LPS plus interferon-γ. Fivefold dilutions of cDNA were used per assay. β-Actin amplification was used as control. (G) Germinal transcription in stimulated splenocytes from wild-type and IgH 3′RR-deficient mice. Total RNA was isolated on day 3. RT-PCR for germline μ, γ2b, γ3 transcripts was performed on LPS-induced splenocytes RNA; germline γ1 and ϵ on LPS plus IL4; and germline α on LPS plus TGF-β. Fivefold dilutions of cDNA were used per assay. β-Actin.

This work was supported by Ligue Contre le Cancer (comité départemental de la Haute-Vienne et de la Corrèze), Conseil Régional du Limousin, and Lions Club de la Corrèze, Zone 33 district 103 Sud. C.V.-F. was supported by Association pour la Recherche sur le Cancer.

Authorship

Contribution: C.V.-F., R.F., V.T., E.P., and Y.D. actively participated in the experimental design of the study (vector construction,

embryonic stem targeting, embryonic stem screening, cell proliferation analysis, apoptose analysis, flow cytometric analysis, Ig measurements, CSR experiments, RT-PCR analysis); M.C. and Y.D. participated in the scientific discussion for manuscript writing and obtained financial grants and agreement of the ethics committee of our institution to perform the study; and N.C. generated transgenic animals and played a key role in genotyping mice.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Michel Cogné, Centre National de la Recherche Scientifique Unité Mixte de Recherche 6101, Laboratoire d'Immunologie, 2 rue du Dr Marcland, 87025 Limoges Cedex, France; e-mail: cogne@unilim.fr; or Yves Denizot, Centre National de la Recherche Scientifique Unité Mixte de Recherche 6101, Laboratoire d'Immunologie, 2 rue du Dr Marcland, 87025 Limoges Cedex, France; e-mail: yves.denizot@unilim.fr.

References

1. Perlot T, Alt FW, Bassing CH, Suh H, Pinaud E. Elucidation of IgH intronic enhancer functions via germ-line deletion. *Proc Natl Acad Sci U S A*. 2005;102(40):14362-14367.
2. Cogné M, Lansford R, Bottaro A, et al. A class switch control region at the 3′ end of the immunoglobulin heavy chain locus. *Cell*. 1994;77(5):737-747.
3. Manis JP, van der Stoep N, Tian M, et al. Class switching in B cells lacking 3′ immunoglobulin heavy chain enhancers. *J Exp Med*. 1998;188(8): 1421-1431.
4. Bébin AG, Carrion C, Marquet M, et al. In vivo redundant function of the 3′ IgH regulatory element HS3b in the mouse. *J Immunol*. 2010; 184(7):3710-3717.
5. Vincent-Fabert C, Truffinet V, Fiancette R, Cogné N, Cogné M, Denizot Y. Ig synthesis and class switching do not require the presence of the hs4 enhancer in the 3′ IgH regulatory region. *J Immunol*. 2009;182(11):6926-6932.
6. Pinaud E, Khamlichi AA, Le Morvan C, et al. Localization of the 3′ IgH locus elements that effect long-distance regulation of class switch recombination. *Immunity*. 2001;15(2):187-199.
7. Chauveau C, Pinaud E, Cogné M. Synergies between regulatory elements of the immunoglobulin heavy chain locus and its palindromic 3′ locus control region. *Eur J Immunol*. 1998;28(10):3048-3056.
8. Dunnick WA, Collins JT, Shi J, et al. Switch recombination and somatic hypermutation are controlled by the heavy chain 3′ enhancer region. *J Exp Med*. 2009;206(12):2613-2623.
9. Garrett FE, Emelyanov AV, Sepulveda MA, et al. Chromatin architecture near a potential 3′ end of the IgH locus involves modular regulation of histone modifications during B-cell development and *in vivo* occupancy at CTCF sites. *Mol Cell Biol*. 2005;25(4):1511-1525.
10. Truffinet V, Pinaud E, Cogné N, et al. The 3′ IgH locus control region is sufficient to deregulate a c-myc transgene and promote mature B cell malignancies with a predominant Burkitt-like phenotype. *J Immunol*. 2007;179(9):6033-6042.
11. Le Morvan C, Pinaud E, Decourt C, Cuveillier A, Cogné M. The immunoglobulin heavy-chain locus hs3b and hs4 3′ enhancers are dispensable for VDJ assembly and somatic hypermutation. *Blood*. 2003;102(4):1421-1427.
12. Wuerffel R, Wang L, Grigera F, et al. S-S synapsis during class switch recombination is promoted by distantly located transcriptional elements and activation-induced deaminase. *Immunity*. 2007; 27(5):711-722.
13. Ju Z, Volpi SA, Hassan R, et al. Evidence for physical interaction between the immunoglobulin heavy chain variable region and the 3′ regulatory region. *J Biol Chem*. 2007;282(48):35169-35178.