

# Localization of the 3' IgH Locus Elements that Effect Long-Distance Regulation of Class Switch Recombination

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## Summary

Four transcriptional enhancers lie downstream of the immunoglobulin heavy chain locus: *Cα3'/hs3a*, *hs1,2*, *hs3b*, and *hs4*. Although individually weak, these elements have strong transcriptional synergies when combined and they altogether behave as a locus control region. Previous knockout experiments in the 3' region have shown that both *hs3a* and *hs1,2* are dispensable for normal expression and rearrangement of the IgH locus but that their replacement with a transcribed *neo* gene severely affects class switch recombination. Here we show that even in the absence of a *neo* gene, joint deletion of the last two 3' enhancers, *hs3b* and *hs4*, severely impairs germline transcription and class switching to most isotypes and may in addition affect  $\mu$  gene expression in resting B cells.

## Introduction

A complex interplay of multiple regulatory elements is responsible for the tissue-specific and stage-specific regulation of both transcription and rearrangements of the IgH locus. Germline transcription of  $C_{\mu}$ , initiation of DJ and VDJ rearrangements, expression of rearranged  $\mu$  genes, and opening of the  $S_{\mu}$  region to class switch recombination (CSR) mainly rely on upstream regulatory elements such as VH promoters, the DQ52 promoter/enhancer, and the  $E_{\mu}$  intronic enhancer (reviewed in Ernst and Smale, 1995; Henderson and Calame, 1998). In addition to upstream elements, a 3' regulatory region located downstream the locus has been shown to include four lymphoid-specific transcriptional enhancers: *hs3a*, *hs1,2*, *hs3b*, and *hs4* (see map on Figure 1) (Pettersson et al., 1990; Dariavach et al., 1991; Lieberson et al., 1991; Matthias and Baltimore, 1993; Michaelson et al., 1995; Chen and Birshtein, 1997; Mills et al., 1997; Pinaud et al., 1997). The *hs1,2* element is *trans*-activated by mitogens and by cross-linking of the BCR or of CD40 in activated B lymphocytes and plasma cells (Arulampalam et al., 1994; Grant et al., 1995, 1996; reviewed in Khamlichi et al., 2000). It is flanked by inverted repeats

including two copies of a weak enhancer (*hs3a* and *hs3b*) (Chauveau and Cogné, 1996). Although *hs3a* and *hs3b* have been considered poorly responsive to stimulations by mitogens, these B-specific elements carry binding sites for octamer factors and for Bach2 and Maf family proteins, which altogether may either mediate repression of the 3' elements in lymphocytes or activation in plasma cells (Muto et al., 1998). By contrast, the distant *hs4* enhancer, lying some 26 kb downstream of the  $\alpha$  gene, seems to be active throughout B cell differentiation (Michaelson et al., 1995). Its activity in pre-B cells may rely on the presence of oct and  $\kappa$ B sites; BSAP binds *hs4* in a differential manner, apparently repressing the enhancer in pre-B cells and boosting its activity in mature B cells (Michaelson et al., 1996).

Based on transient transfection experiments, it was concluded that the four enhancers are rather weak when they individually drive transcription of reporter genes. However, their combinations displayed strong transcriptional synergies, especially when their normal palindromic arrangement was respected, and they also synergistically act as powerful coenhancers of  $E_{\mu}$  when driving transcription of reporter genes (Mocikat et al., 1995; Chauveau et al., 1998; Ong et al., 1998). Moreover, in stably transfected plasmacytoma cell lines, a cassette bearing *hs1,2*, *hs3b*, and *hs4* could confer high-level, tissue-specific expression of a linked *c-myc* gene in a position-independent and copy-dependent manner, strongly suggesting that the 3' regulatory region could act as a locus control region (LCR) (Madisen and Groudine, 1994). Similarly, transgenic mice bearing a VH-driven  $\beta$ -globin gene linked to the four 3' enhancers displayed high-level, position-independent, and strict B cell-specific expression, although no correlation regarding copy number dependence could be established (Chauveau et al., 1999).

The role of the 3' LCR in processes that affect the IgH locus such as rearrangement, transcription, and CSR has remained elusive. In a differentiated B cell line, spontaneous deletion of a large part of the 3' LCR was associated with a decreased transcription of an IgH  $\alpha$  gene controlled by the sole  $E_{\mu}$  (Gregor and Morrison, 1986). In a pre-B cell line, a large deletion including both *hs3a* and *hs1,2* had no effect on  $\mu$  expression (Saleque et al., 1999). Targeted replacement of 3' $\alpha$ E(*hs1,2*) in a mature B cell line also affected transcription of a rearranged  $\gamma$ 2a gene lacking  $E_{\mu}$  (Lieberson et al., 1995).

The function of the 3' LCR during B cell maturation has been approached by knockout experiments in ES cells. Targeted replacement of *hs1,2* with a neomycin gene first suggested its major involvement in the germline transcription of several downstream IgH constant region genes and in CSR (Cogné et al., 1994). Essentially the same phenotype resulted from replacement of *hs3a* with a *neo* gene (Manis et al., 1998). However, in both cases, further deletion of the *neo* gene with *cre/lox* restored normal CSR (Manis et al., 1998), thus showing that *hs3a* and *hs1,2* are both individually dispensable for this process. Given that the four enhancers act synergistically for high-level transcription, one might specu-

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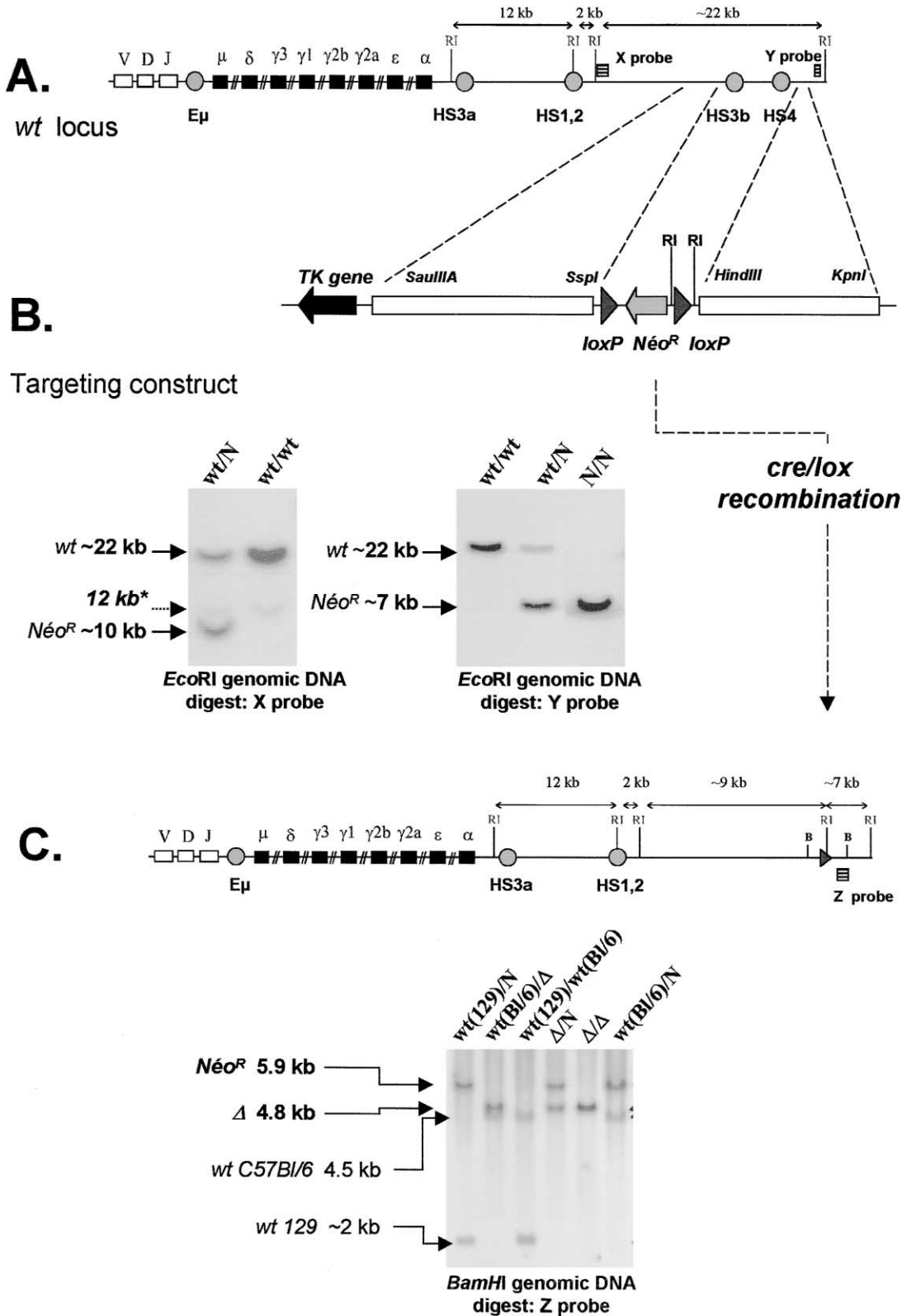


Figure 1. Targeting of the *hs3b* and *hs4* IgH Enhancers

(A) Map of the mouse IgH 3' regulatory region. Closed circles stand for transcriptional enhancers. (B) Targeting construct and Southern blot of knockout ES cells or animals with *Neor* insertion (N). The 5' probe (X, 0.4 kb XbaI fragment) detects a genomic EcoRI band of 22 kb and a 10 kb band after homozygous recombination; the 12 kb\* extra band corresponds to hybridization with the partially homologous 5' part of the LCR. The 3' probe (Y, 0.7 kb HindIII-EcoRI fragment) detects the same genomic 22 kb band and

late that deletion of only one weak enhancer will have no drastic effects on germline transcription and CSR. Whether the lack of a marked phenotype in the previous deletion experiments is merely due to the redundancy of the 3' enhancers or if it does reflect the very mechanism by which the LCR controls CSR is presently unclear. In regard to the "neo effect," the inserted *neo* gene may somehow compete with or isolate germline transcription promoters from the downstream part of the LCR.

We therefore undertook gene targeting experiments where the 3' part of the LCR encompassing *hs3b* and *hs4* was either replaced with a *neo* gene or deleted by the Cre/loxP system. In this study, we focus on the effect of the joint deletion on class switching, in order to assess the hypothesis that the 3' part of the LCR and the distal enhancers *hs3b* and *hs4* may indeed influence germline transcription of IgH constant genes and class switch recombination.

## Results

### Replacement and Deletion of the *hs3b* and *hs4* Enhancers within the 3' IgH LCR in ES Cells and Generation of Mutant Animals

A gene-targeting vector was constructed in order to replace a 6 kb SspI-HindIII genomic fragment encompassing *hs3b* and *hs4* with a loxP2/neomycin resistance gene cassette. The *neo* gene was driven by a *tk* promoter and flanked by two loxP sites. This construct allowed the growth of E14 ES cell clones through the positive-negative selection technique (Mansour et al., 1988). Southern blotting and hybridization with two flanking probes located outside of the construct allowed the selection of 3 out of 700 clones, which gave hybridizing fragments of the size expected for the "N*hs3b4*" mutation (N), i.e., replacement of *hs3b* and *hs4* with the *neo* gene (Figure 1; data not shown).

Two independent heterozygous N*hs3b4* mutant ES clones were injected into C57/B6 blastocysts, which were implanted into foster mothers to derive somatic chimeras. Chimeras were bred either with wild-type animals to obtain heterozygous F1 mice carrying either the heterozygous N*hs3b4* (wt/N) mutation, or with Ella-cre transgenic 129sv mice (Lakso et al., 1996) to yield heterozygous  $\Delta$ *hs3b4* (wt/ $\Delta$ ) mutant after cre-deletion of the *neo* gene. Further breeding and Southern blot testing allowed the derivation of two homozygous lines of N*hs3b4* (N/N) and  $\Delta$ *hs3b4* ( $\Delta$ / $\Delta$ ) mutant animals.

### B Cell and Lymphoid Tissue Development in Mutant Animals

Pathological studies of tissues from homozygous animals and their littermates were carried out. Spleens and Peyer's patches were of normal sizes in both the N/N and  $\Delta$ / $\Delta$  mutant animals and showed germinal centers of normal morphologies (data not shown). Numbers of B cells (IgM<sup>+</sup>, B220<sup>+</sup>) in peripheral compartments (pe-

ripheral blood, spleen, and Peyer's patches) were normal (data not shown).

### IgM Expression in B Cells Carrying a Homozygous Deletion of the *hs3b* and *hs4* Elements

IgM class BCR expression was estimated by flow cytometric analysis of freshly isolated splenic or Peyer's patch cells by double staining with anti-CD19 and anti- $\kappa$  or anti-B220 and anti- $\mu$  antisera. In both organs, although not drastic, surface IgM expression in the CD19<sup>+</sup> or B220<sup>+</sup> populations was reproducibly decreased when  $\Delta$ / $\Delta$  mutant cells were compared to wt (Figure 2).

### *Hs3b* and *hs4* Deletion May Affect $\mu$ Gene Expression in Resting B Cells

The question of whether such a decrease in surface IgM expression reflects a decrease in the corresponding transcript was approached by Northern blot analysis. Total RNA from unstimulated splenocytes or after stimulation with LPS plus or minus cytokines (IL4 or IFN $\gamma$ ) was hybridized with either a probe specific for the  $\mu$  membrane exons, a probe specific for C $\mu$ 1-3 exons, or a C $\kappa$  probe. Although amounts of  $\kappa$  transcripts were roughly equivalent, both the membrane and secreted forms of  $\mu$  transcripts from unstimulated mutant splenocytes were clearly decreased when compared to the wild-type (Figure 3). However, LPS stimulation appeared to restore full  $\mu$  expression.

Altogether, these results suggest that deletion of *hs3b* and *hs4* may affect  $\mu$  gene expression in unstimulated B cells and that 3' elements somehow interact with E $\mu$  to regulate  $\mu$  gene expression. They also suggest that, although more decreased in unstimulated B cells,  $\mu$  gene transcription is still LPS inducible and can achieve a relatively normal level upon activation.

### Immunoglobulin Isotype Production in B Cells Carrying a Homozygous Replacement or a Homozygous Deletion of the *hs3b* and *hs4* Elements

Previous knockouts of upstream elements in the LCR affected CSR, but only when *hs3a* or *hs1,2* were replaced with a *neo* cassette (Cogné et al., 1994; Manis et al., 1998). To determine whether replacement or deletion of *hs3b/hs4* affects CSR, immunoglobulin production was assessed in vitro through stimulation of splenocytes with LPS and/or cytokines. B cells from six  $\Delta$ / $\Delta$  and six N/N animals exhibited similar defects (Figure 4A). LPS-stimulated secretion of IgG2b and IgG3 was significantly reduced below wild-type (eight mice), showing a 15-fold ( $p < 0.05$ ) and 100-fold ( $p < 0.01$ ) reduction, respectively. LPS- plus IFN $\gamma$ -stimulated IgG2a secretion was cut by about 10-fold, as was LPS- plus IL4-induced IgE production, which fell in most experiments below the ELISA detection threshold; LPS- plus TGF $\beta$ -induced IgA production was cut by about 3-fold. By contrast, IgG1 and IgM secretion by  $\Delta$ / $\Delta$  cells was normal, while the N/N mutation caused a slight and

a 7 kb band in the targeted locus.

(C) Map of the cre-deleted targeted locus. Southern blot on knockout animals DNA. The 3' Z probe (1.8 kb HindIII-BamHI fragment) distinguishes BamHI fragments from *NeoR* (N) (5.9 kb), cre-deleted ( $\Delta$ ) (4.8 kb), and wt mouse strain allelic polymorphism (4.5 kb, C57Bl/6, and ~2 kb, 129).

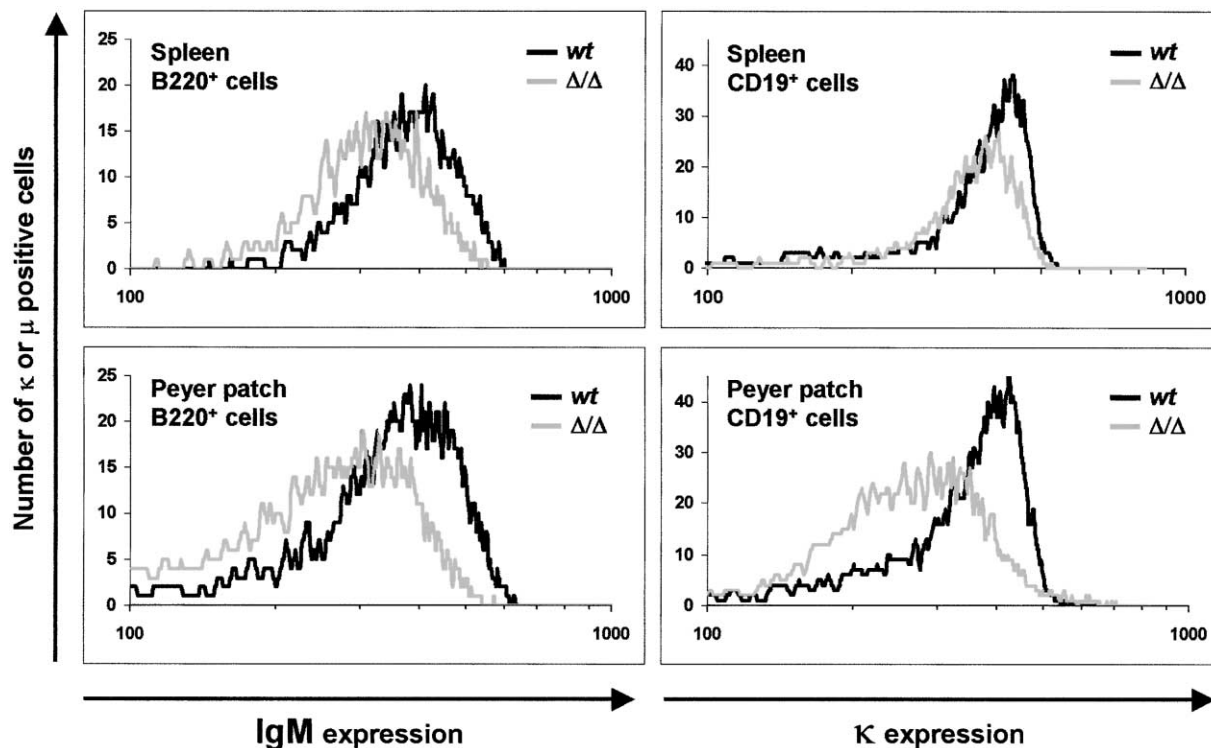


Figure 2. Effect of *hs3b* and *hs4* Deletion on Surface BCR Expression

Freshly isolated splenic or Peyer's patch cells were isolated and stained with PE and FITC antibodies. B cells were gated as B220 or CD19 expressing cells were gated and histograms of surface  $\kappa$  (A) and IgM (B) expressing B cells were compared. Representative results from *wt/wt* (in black) and  $\Delta/\Delta$  (in gray) animals are shown.

nonsignificant decrease of IgM and a 4-fold decrease of IgG1.

Although similar, deficiencies also appeared to be more severe in N/N than in  $\Delta/\Delta$  animals when immunoglobulin production was evaluated in vivo. Serum levels were strongly reduced in both N/N and  $\Delta/\Delta$  animals for IgG2a (15-fold and 4-fold reduction, respectively), IgG2b (5-fold and 10-fold reduction), IgG3 (50-fold and 10-fold), and IgE (at least 300-fold, i.e., down to undetectable levels, and 4-fold, respectively) (Figure 4B). In addition and only in the N/N mutants, significant reduction was observed for serum levels of IgA and IgG1 (15-fold) and even IgM (4-fold).

#### Differential Effect of *hs3b/hs4* Deletion on CSR as Estimated by Flow Cytometry

To determine whether decreased secretion of certain isotypes by stimulated splenocytes resulted from decreased frequency of CSR or only from decreased expression of class-switched genes, we wished to appreciate the numbers of cells that had switched to a particular Ig isotype. Flow cytometric analysis allowed the counting of cells and the study of surface expression of class-switched isotypes on LPS only, LPS plus IL4, or LPS plus TGF $\beta$  activated splenic B cells from either wild-type, N/N, or  $\Delta/\Delta$  mutant mice. A pattern clearly mirroring that found in splenocyte supernatants upon in vitro stimulation was obtained (Figure 5). In particular, there was a major depression on surface expression of IgG3 in LPS-stimulated cells and of IgA in LPS plus

TGF $\beta$ -stimulated cells. Class switching and membrane expression of the IgG1 isotype in LPS plus IL-4-stimulated cells was roughly normal in  $\Delta/\Delta$  mutant mice (Figure 5).

#### Deletion of *hs3b* and *hs4* Affects Expression of Class-Switched Transcripts in Stimulated Splenocytes

We then checked if a decrease of class-switched transcripts parallels that of surface Ig expression. Northern blot allowed the assaying of transcription of IgH constant region genes in splenic B cells. Total RNA from appropriately stimulated splenocytes was hybridized either to a  $C\gamma 2b$  probe that cross-hybridized with all the  $C\gamma$  genes or with a  $\gamma 1$  membrane form mRNA-specific probe (Figure 3). A drastic depletion of  $\gamma$  transcripts was found in LPS-stimulated splenocytes from N/N and  $\Delta/\Delta$  mice (Figure 3). In other conditions, only a partial blockade was observed: for membrane form  $\gamma 1$  transcription stimulated by LPS plus IL4, the decrease paralleled that found by FACS analysis of IgG1 surface expression (Figure 5), i.e., a more severe decrease in N/N  $\gamma 1$  transcription level, whereas that of  $\Delta/\Delta$  mice was only slightly affected (Figure 3). When we looked at  $\gamma 2a$  transcription stimulated with LPS + IFN $\gamma$ , we found a substantial decrease in transcription level for both N/N and  $\Delta/\Delta$  mice as compared to wt mice (Figure 3).

It has been shown that  $I_{\mu-Cx}$  hybrid transcripts (x being any constant gene after CSR) could be detected in B cells that have undergone CSR (Li et al., 1994). A semiquantitative RT-PCR was thus used to individually

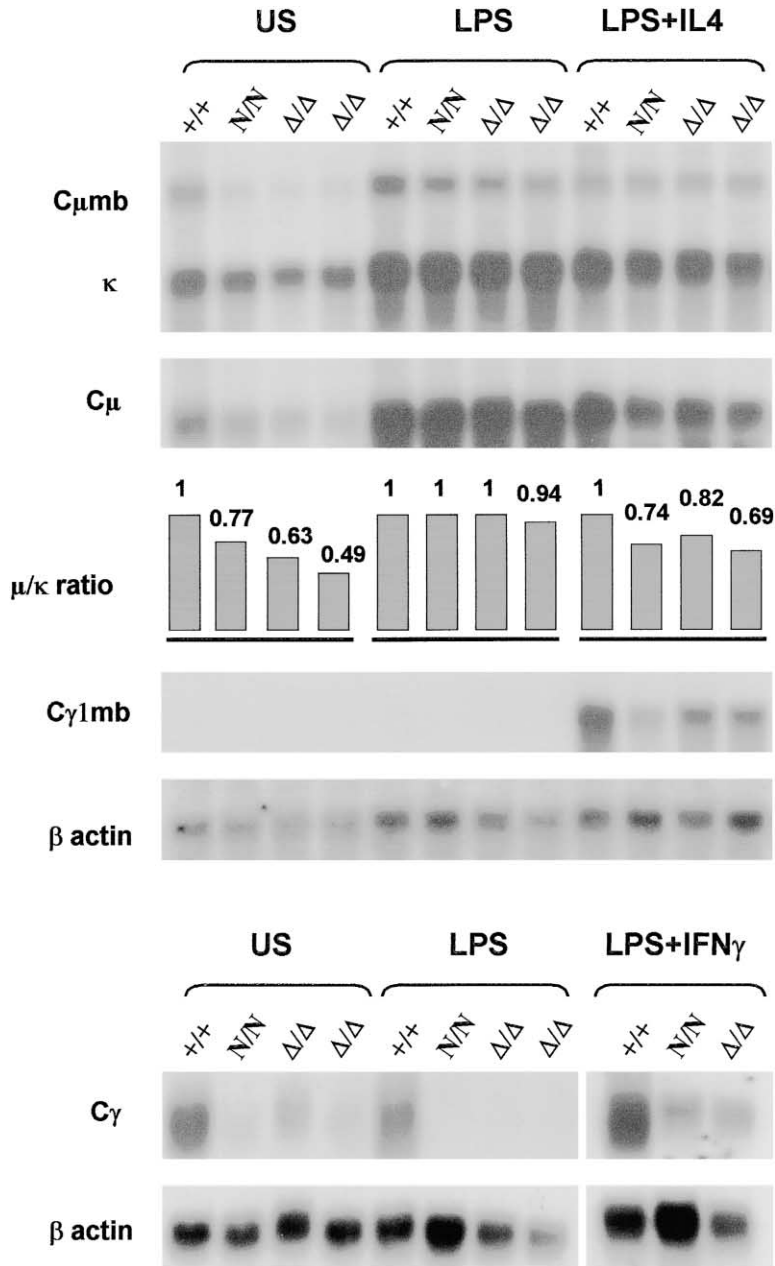


Figure 3. Northern Blot Analysis of Immunoglobulin Constant Gene Transcription

Total RNA was isolated from *wt*, *N/N*, and  $\Delta/\Delta$  unstimulated (US) splenocytes and after 3 days of stimulation with LPS, LPS plus IL4, or LPS plus IFN $\gamma$ . Northern blot hybridizations were assayed with several probes detecting membrane form of  $C_{\mu}$  and  $C_{\gamma}1$  transcripts and secreted forms of  $C_{\mu}$  (specific) and  $C_{\gamma}2b$  (cross hybridizing with all  $C_{\gamma}$ ) transcripts. In parallel,  $\kappa$  gene, and  $\beta$ -actin hybridization were performed as internal transcription controls.

assay transcription of each C gene in stimulated B cells by specifically amplifying  $I_{\mu}-C_{\alpha}$  transcripts. In agreement with Northern blots, when LPS-stimulated splenocytes were studied, a dramatic decrease in  $I_{\mu}-C_{\gamma}3$  and  $I_{\mu}-C_{\gamma}2b$  transcripts was found in *N/N* and  $\Delta/\Delta$  mice. The same holds true for  $I_{\mu}-C_{\alpha}$  transcripts upon addition of TGF $\beta$  to LPS-activated splenocytes, respectively. In contrast, upon stimulation of splenocytes with LPS/IL4, no dramatic decrease in  $I_{\mu}-C_{\gamma}1$  transcripts was detected in *N/N* mice and less so in  $\Delta/\Delta$  mice, whereas a slight decrease for  $I_{\mu}-C_{\epsilon}$  was observed in mutant mice (Figure 6).

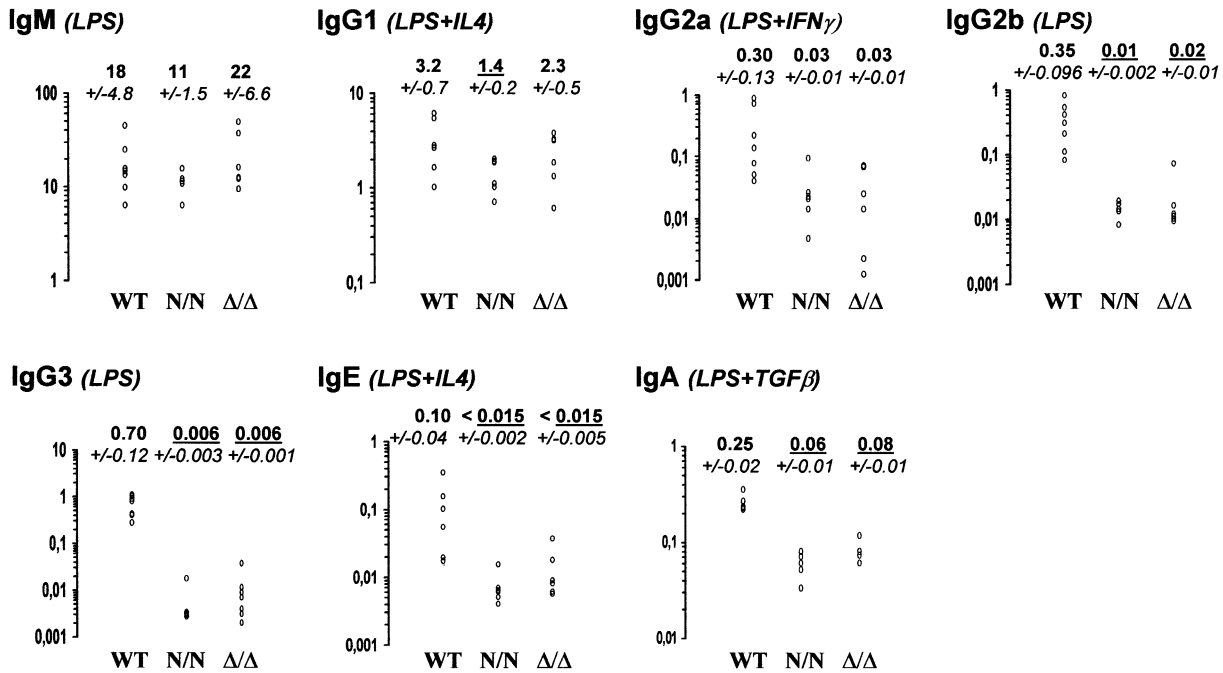
**Differential Expression of the Mutated and the Wild-Type Locus in Heterozygous Animals**

Normal soluble IgM production by  $\Delta/\Delta$  cells contrasted with decreased membrane IgM in resting B cells. Since

a non specific maturation defect in B cells expressing the mutated IgH locus may potentially result in a global defect in CSR, we wished to check expression of a mutated locus in competition with a *wt* allele in heterozygous IgH  $a^{\Delta hs3b4}/b^{wt}$  animals. Alleles were compared in regard to the expression of bone marrow  $VDJ-C_{\mu}$  transcripts, the majority of which should originate from bone marrow plasma cells. Transcription of both loci was assessed by cloning  $\mu$  cDNA obtained from  $a^{\Delta hs3b4}/b^{wt}$  bone marrow and further subtyping individual clones by PCR with IgH<sup>a</sup> or IgH<sup>b</sup> allotype-specific primers. Expression of both alleles did not markedly differ in this assay and 15 out of 41 clones originated from the mutated locus in heterozygous bone marrow, showing that full maturation of heterozygous B cells occurred whether they expressed the deleted or the *wt* IgH locus.

In homozygous  $\Delta/\Delta$  animals, IgA concentration was

## A. Supernatant [Ig] $\mu\text{g/mL}$



## B. Serum [Ig] $\mu\text{g/mL}$

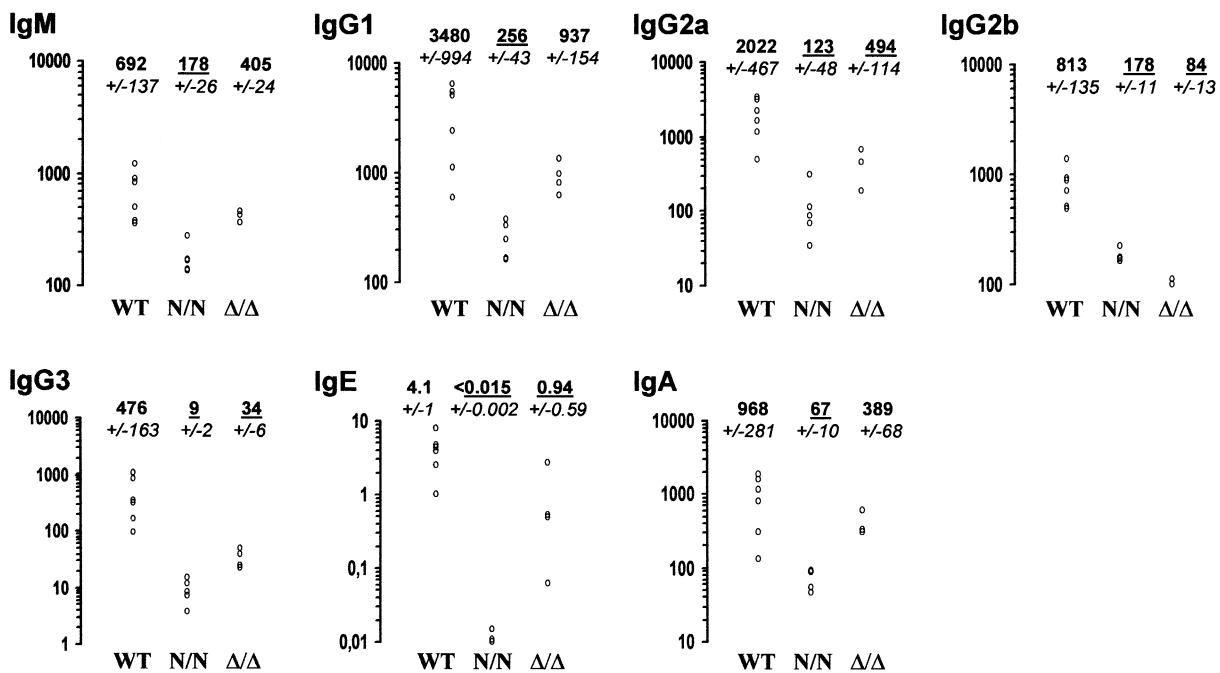


Figure 4. Alterations of Immunoglobulin Secretion in Mutant Animals

(A) ELISA analysis of immunoglobulin secretion by LPS (and/or appropriate cytokines)-stimulated splenocytes supernatants. *wt/wt* littermates are shown on the left part of graphs, followed by homozygous *N/N* and  $\Delta/\Delta$ . Mean Ig levels from all experiments ( $\pm$  standard errors in italics) are indicated and underlined for mutant mice that significantly differ from *wt* animals (with  $p < 0.05$ ).

(B) ELISA analysis of immunoglobulin secretion in 8-week-old mice sera. *wt/wt* littermates are shown on the left part of graphs, followed by homozygous *N/N* and  $\Delta/\Delta$ .

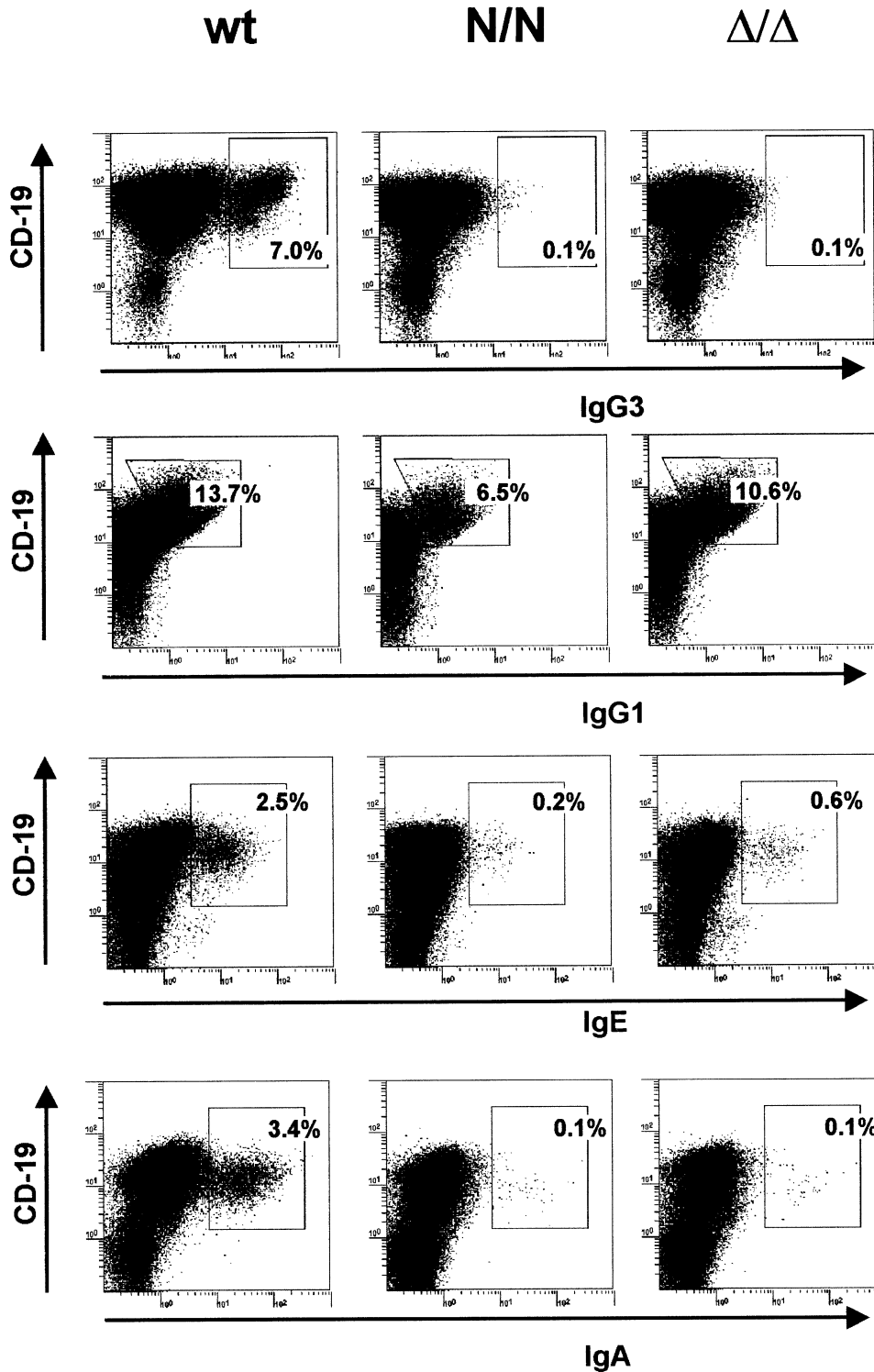


Figure 5. Altered Surface Immunoglobulin Expression in Activated Splenic B Cells  
Splenic B cells were cultured for 4.5 days with LPS at  $6 \times 10^6$  cells/ml and stained with anti-CD19 and anti-isotype fluorescent antibodies. Representative results from *wt/wt*, homozygous *N/N*, and  $\Delta/\Delta$  animals are shown.

virtually unaffected in serum but significantly decreased in splenocyte supernatants upon *in vitro* LPS/TGF $\beta$  stimulations; we thus used the same strategy as for IgM by exploring the *in vivo*  $\alpha$  gene expression in Peyer's patches

of the mutant locus when it "competes" with a wild-type locus in either heterozygous  $\alpha^{\Delta hs3b4}/b^{wt}$  animals or  $\alpha^{Nhs3b4}/b^{wt}$  animals. In contrast to the  $\mu$  gene, we observed that cloned  $\alpha$ -cDNA mostly originated from the wild-type allele (19

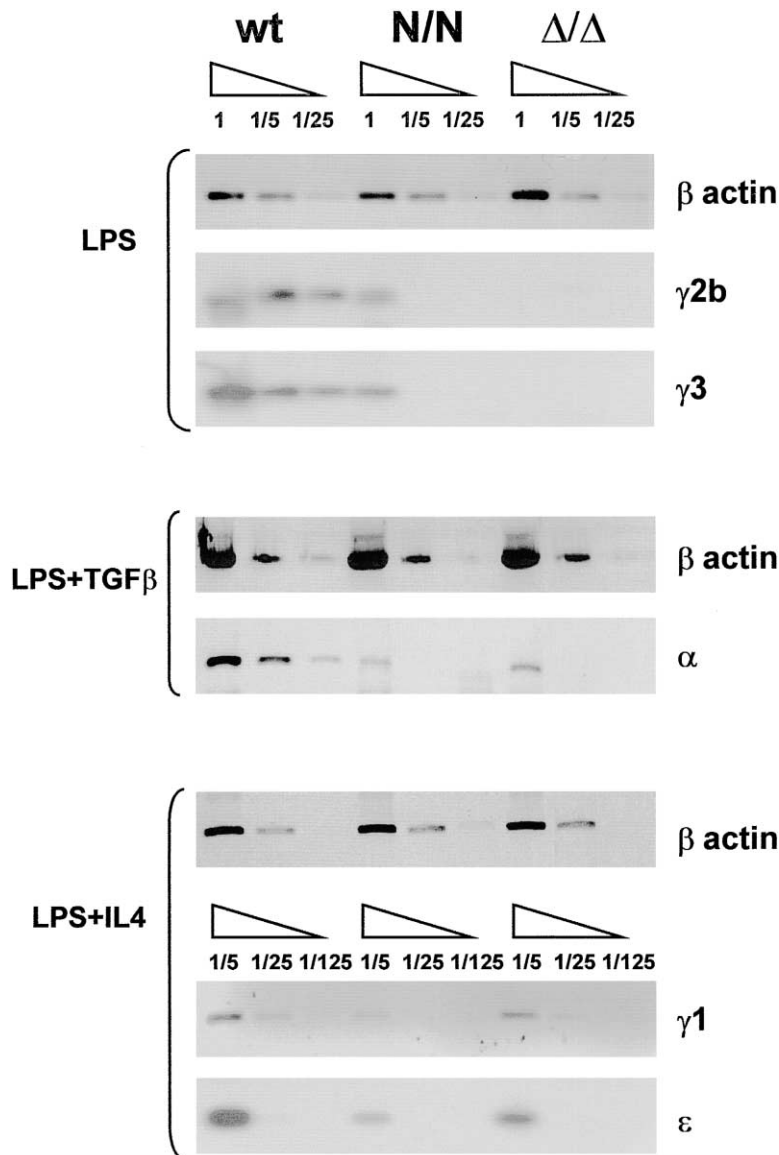


Figure 6. *I<sub>μ</sub>-C<sub>x</sub>* Hybrid Transcripts (x Being Any Constant Gene after CSR) Induced in Stimulated Splenocytes from wt/wt, Homozygous N/N*hs3b4*, and Δ/Δ*hs3b4* Animals

Total RNA was isolated on day 3. RT-PCR for *I<sub>μ</sub>→γ2b*, *I<sub>μ</sub>→γ3* transcripts was performed on LPS-induced splenocytes RNA; *I<sub>μ</sub>→γ1* and *I<sub>μ</sub>→ε* on LPS plus IL4 and *I<sub>μ</sub>→α* on LPS plus TGFβ. 5-fold dilutions of cDNA were used per assay, amplification was performed as described in Experimental Procedures. Transcripts were revealed after Northern blot hybridization with a cDNA fragment encompassing *I<sub>μ</sub>-C<sub>μ</sub>*.

IgH<sup>b</sup> out of 21 clones from a<sup>Δ*hs3b4*</sup>/b<sup>wt</sup> animals and 19 IgH<sup>b</sup> out of 20 clones from a<sup>N*hs3b4*</sup>/b<sup>wt</sup> animals, compared to 9 IgH<sup>b</sup> out of 20 clones from a<sup>wt</sup>/b<sup>wt</sup> control animals).

Differential production of IgG2b by both alleles was also checked in heterozygous a<sup>Δ*hs3b4*</sup>/b<sup>wt</sup> animals and a<sup>N*hs3b4*</sup>/b<sup>wt</sup> animals by comparison to wild-type a/b animals. ELISA determinations were done with an a allo-type-specific assay and showed IgG2b<sup>a</sup> levels about 40-fold lower than normal in both mutant lines (data not shown).

Altogether, these findings in homozygous and heterozygous animals suggest that deletion of *hs3b/hs4* barely affects IgM and IgG1 secretion by B cells undergoing an overall normal maturation but severely affects expression of most other isotypes. These data thus point to a critical role of the distal enhancers in CSR. A likely explanation for this defect could be a depressed germline transcription of certain switch regions. We therefore sought to analyze germline transcripts of the different isotypes.

#### Germline Transcription of IgH Constant Region Genes in Stimulated Splenocytes

Germline transcription of IgH constant genes is generally a prerequisite of CSR, and induction of transcription correlates with further DNA recombination in stimulated B cells. We thus assayed germline transcription of each IgH constant region gene by specific RT-PCR in semi-quantitative conditions (Figure 7). When LPS-stimulated splenocytes were studied, *I<sub>μ</sub>-C<sub>μ</sub>* transcripts of normal abundance were found in N/N and Δ/Δ animals. By contrast, *I<sub>γ3</sub>-C<sub>γ3</sub>* and *I<sub>γ2b</sub>-C<sub>γ2b</sub>* transcripts were severely reduced in cells from mutant animals. In LPS plus TGFβ stimulation experiments, a strong decrease of *I<sub>α</sub>-C<sub>α</sub>* germline transcripts was noticed in splenocytes from both strains of mutant animals. In LPS plus IL4 stimulation experiments, *I<sub>γ1</sub>-C<sub>γ1</sub>* germline transcripts appeared to be unaffected for N/N and Δ/Δ animals, while *I<sub>ε</sub>-C<sub>ε</sub>* transcripts were slightly less abundant in cells carrying either mutation than in cells from wild-type animals.



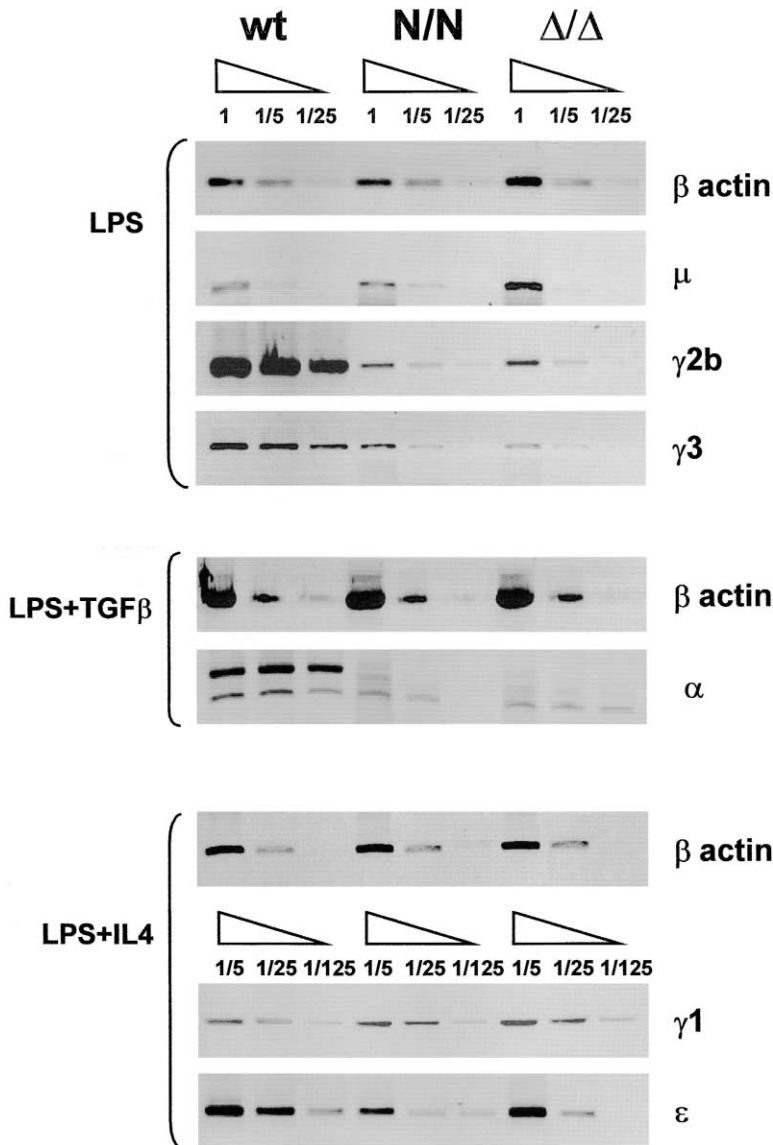


Figure 7. Germinal Transcription Induced in Stimulated Splenocytes from *wt/wt*, Homozygous *N/N*, and  $\Delta/\Delta$  Animals

Total RNA was isolated on day 3. RT-PCR for germline  $\mu$ ,  $\gamma 2b$ ,  $\gamma 3$  transcripts was performed on LPS-induced splenocytes RNA; germline  $\gamma 1$  and  $\epsilon$  on LPS plus IL4; and germline  $\alpha$  on LPS plus TGF $\beta$ . 5-fold dilutions of cDNA were used per assay; amplification was performed as described in Experimental Procedures.

The most likely explanation is that the *hs3b4* mutation impairs CSR by altering germline transcription of the target genes. Therefore, *hs3b* and *hs4* enhancers control CSR to a given isotype by activating the corresponding germline transcription under appropriate stimulation.

#### Discussion

There has been considerable interest and conflicting data in the past few years about the potential role of the 3' IgH region in CSR. Knockout experiments first implicated this region in the process of CSR by showing that replacement of either the *hs1,2* or the *hs3a* enhancer with an expressed *neo* gene resulted in CSR blockade to most Ig classes and subclasses, although  $\gamma 1$  and  $\alpha$  were minimally affected (Cogné et al., 1994; Manis et al., 1998). *Neo* replacement also implicated *hs1,2* in transcriptional regulation of an expressed  $\gamma 2a$  gene in a B cell line lacking *E $\mu$*  (Lieberson et al., 1995). The observation of a hybridoma with a joint deletion of *hs3a* and *hs1,2*, still switching to IgG1 at a rate similar to other

hybridomas, further confirmed that CSR to  $\gamma 1$  was somehow independent from these enhancers (Saleque et al., 1999). More strikingly, *Cre/lox* experiments allowed the assaying of deletions of *hs3a* or *hs1,2* in the absence of an inserted *neo* gene: CSR appeared normal, either denying any role to these enhancers in CSR or assigning them with redundant functions (Manis et al., 1998). The observation that mice carrying large human IgH transgenes can undergo some level of CSR in the absence of 3' regulatory element is more difficult to interpret in the absence of comparison with a normal endogenous locus (Taylor et al., 1992, 1994; Wagner et al., 1994, 1996). Referring to human, *hs1,2* alleles with duplicated NF- $\kappa$ B sites have been correlated with higher serum IgA levels in patients (Aupetit et al., 2000; Denizot et al., 2001). It is noteworthy that CSR defects were reported in mice deficient in NF- $\kappa$ B components, whose targets in the IgH locus have mostly been reported within *hs1,2* and *hs4* (Sha et al., 1995; Köntgen et al., 1995; Snapper et al., 1996).

The data reported herein now show definitely that

joint deletion of the *hs3b/hs4* enhancers resulted in a severe CSR defect even in the absence of an inserted *neo* gene. While IgM and IgG1 were only slightly affected, the mutation resulted in a severe defect of IgG2b and IgG3 both in vivo and in vitro. IgG2a, IgE, and IgA classes were also reduced but to a lesser extent. Concerning IgA, although the serum concentration in  $\Delta/\Delta$  animals was close to normal, germline transcription of the  $\alpha$  gene and IgA secretion were decreased upon in vitro stimulation by LPS plus TGF $\beta$ . In addition, in vivo studies of heterozygous animals showed that  $\alpha$  transcripts mostly originated from the unmutated allele.

Apart from the CSR defect, the phenotype observed in  $\Delta/\Delta$  animals was marked by an apparently normal B cell differentiation, with roughly normal numbers of B cells, normal production of IgM in serum, and a grossly equivalent usage of both IgH alleles in heterozygous  $\Delta/hs3b4/wt$  IgM producing cells. Surface expression of IgM was decreased on resting B lymphocytes as estimated by flow cytometry and Northern analysis, although LPS-stimulated B cells transcribed the  $C_{\mu}$  gene and secreted IgM at roughly the normal level. This situation is reminiscent of that in the 3'  $E_{\kappa}$  enhancer knockout, where  $\kappa$  expression was reduced in resting B cells but restored in activated cells (Gorman et al., 1996). This observation suggests that either *hs3b*, *hs4*, or both somehow interact with  $E_{\mu}$  in regulating  $\mu$  gene expression in resting cells. As discussed before (Khamlichi et al., 2000), several data support *hs4* being the candidate for such an interaction: whereas *hs4* is active throughout B cell development, *hs3a*, *hs1,2*, and *hs3b* are active only at late B cell stages. Deletion of *hs3a* in mouse did not affect  $\mu$  gene expression. *Hs3a* and *hs3b* are 97% identical so that deletion of only one leaves intact the inverted copy. Moreover, *hs3b* does not exist in human. Both *hs3a* and *hs1,2* were deleted in the 70Z/3 pre-B cell line without affecting  $\mu$  expression. It is tempting to speculate that the position of *hs4* outside the palindrome does allow such a long-range interaction with  $E_{\mu}$ .

In heterozygous mice, we found that the  $\mu$  allele usage of mutated/wt was between one-third and one-half. With regard to CSR, we cannot formally exclude that a slight decrease in  $\mu$  gene expression may contribute to the CSR defect, but this mechanism alone is unlikely to account for the CSR alterations we found. It should be noted that  $\mu$  gene expression raised roughly normal levels in activated B cells. Bearing in mind that activation of B cells is a prerequisite to class switching (reviewed in Rajewsky, 1996), an alteration at the target gene level is more likely to accommodate most of the facts regarding CSR. The relative preservation of  $\gamma 1$  expression at both the germline transcription and CSR levels also provides an internal control, further showing that full B cell maturation is achieved in mutant B cells and that the CSR machinery is intact.

Previous reports of targeted replacements or spontaneous deletions have suggested that the 3' IgH LCR is somehow involved in controlling CSR to and transcription of most IgH constant genes, i.e.,  $\gamma 3$ ,  $\gamma 2b$ ,  $\gamma 2a$ ,  $\epsilon$ , and, to a lesser extent,  $\alpha$ . Our data clearly show that the downstream part of the 3' LCR affects CSR by altering germline transcription from I promoters of the target IgH genes. That  $\alpha$  expression is less affected could merely reflect physical proximity of  $I_{\alpha}$  (and VH promoter after

CSR) to the remaining 3' enhancers. In LP1-2, an IgA-producing line lacking the whole 3' LCR but with intact  $E_{\mu}$ , a dramatic decrease of  $\alpha$  transcription was detected compared to the parental cells with intact LCR (Gregor and Morrison, 1986). By contrast, the  $\gamma 1$  gene appears to be transcribed and undergo CSR at a fairly high level despite the *hs3b/hs4* deletion. Similarly,  $\gamma 1$  transgenes have been suggested to be regulated mainly by  $E_{\mu}$  and regulatory elements located close to  $\gamma 1$  (Xu and Stavnezer, 1992; Cunningham et al., 1998a, 1998b; Adams et al., 2000). Such a VDJ $\mu\delta\gamma 1$  transgene has also been reported to yield full expression of the  $\mu$  gene in the absence of the 3' IgH LCR (Cunningham et al., 1998a). Although our experiments confirm that the  $\mu$  gene may be transcribed independently of *hs3b* and *hs4* in activated B cells from  $\Delta/\Delta$  animals, the reduced expression in resting B cells was unexpected. This observation suggests complex control pathways with synergistic interactions between 5' and 3' regulatory elements that may vary along B cell maturation.

An important finding stemming from transient, stable transfection, and transgenic studies was the transcriptional synergies between the 3' enhancers themselves and the 3' enhancers and  $E_{\mu}$  (Mocikat et al., 1995; Chauveau et al., 1998; Collins and Dunnick, 1999). If we assume that such synergies do occur in vivo as the data presented here suggest, one might expect that deletion of only one 3' enhancer would have no drastic effect as was shown for *hs3a* and *hs1,2*. In contrast, the more enhancers that are deleted, the more drastic the effect would be. One might thus speculate that deletion of the whole LCR would have a dramatic effect on IgH transcription and probably on VDJ recombination as well.

The finding that the 3' LCR exerts its effect at the transcriptional level is reminiscent of the  $\beta$ -*globin* LCR. Deletion of individual *hs* had no remarkable phenotype though replacement KOs had severe effects (reviewed in Martin et al., 1996; Fiering et al., 1995; Hug et al., 1996; Bender et al., 2000a). Deletion of the whole LCR also severely affected transcription of the *globin* genes but not chromatin opening or DNase sensitivity (Bender et al., 2000a, 2000b). For the IgH locus, one might also speculate that 5' elements are responsible for opening the locus and establishing DNase I sensitivity whereas the 3' LCR would further tune expression of IgH genes.

It may be noteworthy that the phenotype resulting from the presence of the *neo* gene in the N/N animals described herein is generally more drastic than for  $\Delta/\Delta$  animals. Indeed, they show a more severe reduction of serum IgE and also carry a combined in vitro and in vivo defect of IgM, IgG1, and IgA production, contrasting with the near preservation of all three classes in  $\Delta/\Delta$  animal sera. This phenotype is also more severe than for previously reported *neo* replacements of upstream enhancers within the 3' IgH LCR, where IgM and IgG1 production were unaffected (Cogné et al., 1994; Manis et al., 1998). Mechanisms for *neo* effects may involve competition between germline I region promoters and the inserted *neo* promoter, affecting switching in a similar way as other insertions of *neo* within the IgH locus (Seidl et al., 1999). The *neo* gene would then constitute a decoy site for factors normally controlling IgH transcription. Alternatively, in a "linking model" of LCR func-

tion (as reviewed in Bulger and Groudine, 1999), the *neo* effect may rely on the constitutively active *neo* promoter behaving as an insulator element and precluding activity of the downstream part of the LCR on all IgH locus promoters. In the *Nhs3b4* mutation reported herein, such a model would imply that other regulatory elements lie downstream of *hs4*, whose function is impeded by the inserted *neo* stuck between them and their target promoters.

In conclusion, we have shown that *hs3b* and *hs4* control CSR to IgH genes by regulating the corresponding germline transcription, clearly pointing to a critical role of the 3' LCR in this process. Our data also suggest that 3' enhancers participate in regulating  $\mu$  gene expression.

### Experimental Procedures

#### Gene Targeting

An *hs3b/hs4* targeting construct was generated using a plasmid containing an  $\sim 7$  kb long 5' arm (SauIIA-SspI fragment) and an  $\sim 5$  kb 3' arm (HindIII-KpnI fragment normally located downstream *hs4*). A neomycin resistance gene (*tk-neo*) flanked by *loxP* sites was stuck in between. At the 3' end, a phosphoglucokinase promoter-Herpes Simplex Virus thymidine kinase gene (HSVtk) was included to permit negative selection against random integration. Cells of the embryonic stem (ES) cell line E14 were transfected with linearized vector by electroporation and selected using G418 (400  $\mu$ g/ml) and gancyclovir (2  $\mu$ M). Southern blot analysis with two probes 5' (XbaI fragment: Figure 1 X probe) and 3' (EcoRI-HincII fragment: Figure 1 Y probe) of the construct (shown in Figure 1B) identified recombinants. Blots were autoradiographed or directly analyzed on an Instant-Imager apparatus (Packard, CT). Two ES cell clones showing homologous recombination of *hs3a/hs4* were injected into C57Bl/6 blastocysts, and the resulting male chimeras were mated with C57Bl/6 females. Germline transmission in heterozygous and homozygous mutant mice was assessed by coat color, and the presence of the disrupted IgH allele was checked by Southern blot. In parallel, mutant mice were mated with Ella-cre transgenic mice. The progeny was checked by Southern blot for the occurrence of a Cre-mediated deletion, yielding a 5.9 kb band for the *neo* replacement and a 4.8 kb BamHI band for the cre-deleted allele with a probe internal to the construct (a HindIII-BamHI fragment located downstream *hs4*; Figure 1 Z probe).

Ella-cre mice were a kind gift of Dr. Heiner Westphal. They were used under a noncommercial research license agreement from DUPONT PHARMA (Wilmington, DE).

#### Spleen Cell Cultures and ELISA Assays

Single-cell suspensions of spleen cells were cultured 4 to 5 days at  $6 \times 10^5$  cells/ml in RPMI medium supplemented with 10% FCS and 20  $\mu$ g/ml LPS, with or without addition of cytokines: 1 ng/ml mouse recombinant IL4 (PeproTech, Rocky Hill, NJ), 1 ng/ml TGF $\beta$  (PeproTech, Rocky Hill, NJ) or 100 units/ml murine recombinant IFN $\gamma$  (PeproTech, Rocky Hill, NJ). In total, six independent culture experiments were performed, employing a total of eight wt mice, and six *N/Nhs3b4* and six  $\Delta/\Delta$ *hs3b4* mutant animals.

#### ELISA Assays

Supernatants from spleen cell cultures (harvested after 5 days of stimulation) and sera from heterozygous mutant mice, homozygous mutant mice, and normal littermates were analyzed for the presence of the various immunoglobulin classes and subclasses by ELISA. All immunoglobulin evaluations were done in duplicate.

ELISA assays were performed in polycarbonate 96 multiwell plates (Maxisorp, Nunc, Denmark), coated overnight at 4°C (100  $\mu$ l/well) with suitable capture antibodies diluted in 0.05 M Na $_2$ CO $_3$  buffer (2  $\mu$ g/ml for IgM, IgG1, IgG2a, IgG2b; 3  $\mu$ g/ml for IgG3; and 4  $\mu$ g/ml for IgE and IgA). After three successive washing steps in 0.1% Tween20/PBS buffer (washing buffer), a blocking step was performed for 30 min at 37°C in 3% BSA/0.2% Sodium Azide/PBS

buffer. After three washing steps, 50  $\mu$ l of sera (first diluted to 1:50), supernatants, or isotypic standard immunoglobulins was diluted into successive wells in 1% BSA/PBS buffer and incubated for 2 hr at 37°C. The mouse standard panel included antisera specific for IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA (Southern Biotechnologies, Birmingham, AL) and for IgE (Pharmingen, San Diego, CA). After three washing steps, 100  $\mu$ l/well appropriate conjugated antibodies diluted in PBS were added and adsorbed during 1.5 hr at 37°C. Alkaline phosphatase-conjugated goat antisera specific for mouse immunoglobulin classes (Southern Biotechnologies, Birmingham, AL) were used in 0.1% Tween20, PBS buffer, either at 1  $\mu$ g/ml for IgM, IgG1, IgG2a, IgG2b, and IgG3, or at 2  $\mu$ g/ml for IgE and IgA. After washing, phosphatase alkaline activity was assayed on 1 mg/ml AP substrate (Sigma, St. Louis, MO), and blocked with addition of 3 M NaOH; optical density was measured at 400 nm in a Spectracount photometer (Packard, Meriden, CT). Repeated ELISA determinations were submitted to a statistical analysis by using the Student t test and the Statviews software (Alsym, Meylan, France).

#### Flow Cytometric Analysis

Single-cell suspensions from peripheral blood, fresh organs, and in vitro stimulated spleen cells were washed in PBS 5% FCS and stained ( $5 \times 10^5$  cells per assay) with various antibodies (Southern Biotechnologies, Birmingham, AL): anti-B220 conjugated with SpectralRed, anti-immunoglobulin subclasses conjugated with fluorescein-isothiocyanate, and anti-CD19 conjugated with phycoerythrin. Cells were analyzed on a Coulter XL apparatus (Beckman Coulter, Fullerton, CA).

#### Northern Blots

Total cellular RNA was prepared from either 0 and 3 days stimulated splenocytes of wild-type, *N/N*, and  $\Delta/\Delta$  mice by homogenization of the cells in TriPure (Roche GmbH, Germany). Northern blotting was carried out by migrating 10  $\mu$ g of total RNA on a 1% agarose denaturing gel, followed by transfer on nylon sheets (Amersham, Buckinghamshire, UK). Probes used for hybridization were the following: for  $\mu$  membrane transcripts, a 0.4 kb KpnI-XbaI genomic fragment containing the murine  $\mu$  membrane exons; for  $\kappa$  transcripts, a 2 kb AfIII genomic fragment containing the murine  $C\kappa$  gene; for  $C\mu$  transcripts, a 1.2 kb XbaI-HindIII genomic fragment containing the murine  $C\mu 1$  to  $C\mu 3$  region; for membrane  $\gamma 1$  transcripts, a 0.35 kb PCR fragment containing the specific  $\gamma 1$  cytoplasmic tail and for  $C\gamma$  transcripts, a 0.8 kb XhoI-KpnI genomic fragment containing the murine  $C\gamma 2b 2$  to  $C\gamma 2b 4$  region and cross hybridizing with all  $\gamma$  constant transcripts.

#### RT-PCR Analysis of Germline Transcription

Total RNA was extracted from stimulated splenocytes of wild-type, *N/N*, and  $\Delta/\Delta$  mice. Reverse transcription was carried out for 1 hr with Superscript (Life Technologies, France), starting with 1  $\mu$ g of total RNA. Expected sizes of amplified products corresponding to spliced germline transcripts were as described by Muramatsu et al. (2000):  $I\mu-C\mu$  (Imf/cmf primers), 245 bp;  $I\gamma 3-C\gamma 3$  (ig3f/cg3r), 323 bp;  $I\gamma 1-C\gamma 1$  (ig1f/cg1r), 429 bp;  $I\gamma 2b-C\gamma 2b$  (ig2bf/cg2br), 371 bp;  $\epsilon-C\epsilon$  (ief/cer), 392 bp;  $I\alpha-C\alpha$  (iaf/car), 497 and 357 bp. RT-PCR amplification of spliced transcripts from exons 3 and 4 of the  $\beta$ -actin gene was used as an internal loading control. Amplification of germline transcripts was done in either 32 cycles with hybridization at 50°C for  $\beta$ -actin, 32 cycles with hybridization at 55°C for  $\mu$ ,  $\gamma 1$ ,  $\gamma 2b$ , and  $\gamma 3$  transcripts, 32 cycles with hybridization at 59°C for  $\alpha$  transcripts, or 39 cycles with hybridization at 59°C for  $\epsilon$  transcripts. Semiquantitative assessment of germline transcription was obtained by assaying in parallel serial dilutions of each cDNA. For LPS and LPS plus TGF $\beta$  stimulations, cDNA were assayed undiluted, diluted 1/5, and diluted 1/25. For LPS plus IL4 stimulations, cDNAs were assayed diluted 1/5, 1/25, and 1/125.

#### Allotype-Specific PCR Analysis

Bone marrow cells and Peyer's patches from heterozygous mice carrying IgHa/b allotypes were used to prepare DNA from two different types of mice:  $a^{wt}/b^{wt}$  or  $a^{\Delta/hs3b4}/b^{wt}$ . Assignment of IgH alleles was done by Southern blotting with BamHI digests and the HindIII-BamHI 3'/*hs4* probe, which allowed identification of the  $a^{wt}$  allele as a 2 kb

band, the b<sup>wt</sup> allele as a 4.5 kb band, and the a<sup>Δns3b4</sup> as a 4.8 kb band. In a first round of RT-PCR, consensus primers were used to clone expressed μ or α transcripts originating from either a or b allotype loci. Fragments cloned in the pTOPOII vector (Invitrogen, Groningen, The Netherlands) were then further characterized through a second round of PCR with a or b allotype-specific primers. That PCR allotype typing was indeed correct was confirmed by sequencing several cloned cDNAs. PCR conditions for the a or b allotypes of μ transcripts were 15 cycles with the hybridization temperature at 66°C, followed with 10 cycles with the hybridization temperature at 65°C. For the a or b allotypes of α transcripts PCR conditions were 35 cycles with the hybridization temperature at either 61°C for amplification of both the a and b allotype transcripts or 67°C for specific amplification of a allotype transcripts.

#### PCR Primers

I<sub>μ</sub>f, 5'CTCTGGCCCTGCTTATGTTTG3'; C<sub>μ</sub>r, 5'GAAGACATTTGGGAAGGACTGACT3'; I<sub>γ</sub>3f, 5'TGGGCAAGTGGATCTGAACA3'; C<sub>γ</sub>3r, 5'CTCAGGGAAGTAGCCTTTGACA3'; I<sub>γ</sub>1f, 5'GGCCCTCCAGATCTTTGAG3'; C<sub>γ</sub>1r, 5'GGATCCAGAGTCCAGGTCAGT3'; I<sub>γ</sub>2bf, 5'CACTGGGCTTTCCAGAATA3'; C<sub>γ</sub>2br, 5'CACTGAGCTGCTCATAGTGTAGAGT3'; I<sub>ε</sub>f, 5'TGGGATCAGACGATGGAGAATAG3'; C<sub>ε</sub>r, 5'CCAGGGTCATGGAAGCAGT3'; I<sub>α</sub>f, 5'CCTGGCTGTCCCTATGAA3'; C<sub>α</sub>r, 5'GAGCTGGTGGGAGTGTCAAGT3'; VH1183, 5'CGGTACCAAGAASAMCCTGTWCCTGCAAATGASC3'; CH2<sub>μ</sub>(rev), 5'CATTGGGGTTCATCTCTGCG3'; CH1<sub>μ</sub>(a)rev, 5'ATGGGCACATGCAATGATCT3'; CH1<sub>μ</sub>(b)rev, 5'AATGGGCACATGCAATGATCT3'; ALPHMURCH2, 5'GCAGGTCCTCAAGAGCTGGC3'; ALPHMURCH2A, 5'GCTGGGCTGGCAGGAAGGAAT3'; Acti-4, 5'TACCTCATGAAGATCC TCA3'; and Acti-5, 5'TTCGTGGATGCCACAGGAC3'.

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