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Immunology

Position-dependent inhibition of class-switch recombination by PGK-*neo*^{r} cassettes inserted into the immunoglobulin heavy chain constant region locus

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ABSTRACT **The Ig heavy chain (IgH) constant region (C_H) genes are organized from 5′ to 3′ in the order Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, and Cα. Expression of C_H genes downstream of Cδ involves class-switch recombination (CSR), a process that is targeted by germ-line transcription (GT) of the corresponding C_H gene. Previously, we demonstrated that insertion of a PGK-*neo*^{r} cassette at two sites downstream of Cα inhibits, in cultured B cells, GT of and CSR to a subset of C_H genes (including Cγ3, Cγ2a, Cγ2b, and Cε) that lie as far as 120 kb upstream. Here we show that insertion of the PGK-*neo*^{r} cassette in place of sequences in the Iγ2b locus inhibits GT of and CSR to the upstream Cγ3 gene, but has no major effect on the downstream Cγ2a and Cε genes. Moreover, replacement of the Cε exons with a PGK-*neo*^{r} cassette in the opposite transcriptional orientation also inhibits, in culture, GT of and CSR to the upstream Cγ3, Cγ2b, and Cγ2a genes. As with the PGK-*neo*^{r} insertions 3′ of Cα studied previously, the Cγ1 and Cα genes were less affected by these mutations both in culture and in mice, whereas the Cγ2b gene appeared less affected *in vivo*. Our findings support the existence of a long-range 3′ IgH regulatory region required for GT of and CSR to multiple C_H genes and suggest that PGK-*neo*^{r} cassette insertion into the locus short circuits the ability of this region to facilitate GT of dependent C_H genes upstream of the insertion.**

Ig variable regions are encoded by component gene segments that are assembled during early B cell differentiation by V(D)J recombination (reviewed in ref. 1). The IgH locus contains eight different constant region (C_H) genes organized as follows: 5′ V(D)J–Cμ–Cδ–Cγ3–Cγ1–Cγ2b–Cγ2a–Cε–Cα–3′ (Fig. 1). Newly differentiated B lymphocytes produce complexes of μ heavy chains and Ig light chains referred to as IgM. Subsequently, mature B cells can change the class of Ig produced from IgM to IgG, IgE, or IgA through a second specific genomic rearrangement process termed heavy chain class-switch recombination (CSR). CSR results in the generation of a new transcription unit containing the same productively rearranged V(D)J exon together with the new downstream C_H exons (1, 2), accompanied by deletion of intervening DNA sequences. CSR involves recombination between large, repetitive sequences called switch (S) regions located upstream of individual C_H genes (reviewed in ref. 3).

CSR to particular C_H genes is directed by different combinations of activators and lymphokines (reviewed in refs. 4 and 5) via their ability to modulate germ-line transcription (GT) of a given C_H gene before CSR (2). For example, stimulation of B cells with bacterial lipopolysaccharide (LPS) induces GT of and CSR to Cγ2b and Cγ3, whereas inclusion of interleukin 4 (IL-4) with the LPS treatment inhibits GT of and CSR to Cγ2b and Cγ3 while inducing GT of and CSR to Cγ1 and Cε (1, 3, 5). In the context of a given form of activation, other lymphokines can induce CSR to other C_H genes. Primary germ-line C_H transcripts are processed to a form in which the I exon is spliced to the C_H exon to yield "sterile" transcripts that do not encode a protein. Therefore, it is likely that germ-line C_H transcription and/or transcripts play a direct role in the CSR process, a notion strongly supported by gene-targeted mutation experiments (6–14). Correspondingly, the elements that control germ-line transcription must be directly responsible for control of CSR.

Although germ-line C_H gene promoters contain consensus sequences that are responsive to signaling pathways activated by specific lymphokines, their properly regulated expression appears to require sequences beyond the proximal promoters (reviewed in ref. 3). One candidate was the intronic IgH enhancer region (iEμ); however, various studies suggest that the major function of this region may be to promote germ-line transcription through Sμ, as opposed to regulating downstream I region promoters (10–13, 15). Moreover, the expression of switched V(D)J-C_H transcripts in cell lines in the absence of iEμ suggested the existence of additional regulatory sequences (16, 17). The initial candidate for such a regulatory sequence was an enhancer sequence discovered about 15 kb downstream of Cα, referred to as the 3′ CαE (18–20). However, more recent studies showed that the 3′ Cα region comprises a series of DNase-hypersensitive sites/enhancers spread over approximately 40 kb including from 5′ to 3′: HS3a, HS1,2 (the original 3′ CαE), HS3b, and HS4 (21, 22). Cell line transfection studies suggested that combinations of the HS3a, HS1,2, HS3b, and HS4 elements may have locus control region (LCR)-like properties (21, 23).

It has been suggested that sequences in the 3′ IgH region may function as a long-range control region for regulation of CSR (24, 25) by regulating certain germ-line C_H promoters in the context of B cell activation (24). To further elucidate this putative regulatory region, we previously generated mice in which HS3a or HS1,2 was either deleted or replaced with a PGK-*neo*^{r} cassette (24, 25). None of these mutations had a measurable effect on V(D)J recombination, and none severely

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Abbreviations: CSR, class-switch recombination; LCR, locus control region; GT, germ-line transcript; ES, embryonic stem; RT-PCR, reverse transcription–PCR; IL-4, interleukin 4; LPS, lipopolysaccharide; LCR, locus control region; FACS, fluorescence-activated cell sorter; wt, wild type.

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impaired *in vitro* activation of mutant B cells to secrete IgM, IgG1, or IgA. The deletion mutations also had little or no effect on CSR to any C_H gene. However, mutant B cells with the PGK-*neo*^{r} insertions were severely deficient in their ability to switch to IgG2a, IgG2b, IgG3, and IgE after appropriate stimulation, and these defects correlated with corresponding defects in GT of and CSR to the affected C_H genes (24, 25). Therefore, the HS3a and HS1,2 replacement mutations inhibited CSR to different C_H genes spread over 120 kb. Similar long-range inhibitory effects of such insertion/replacement mutations also have been found in the context of targeted mutations of the β-globin LCR region (26–29) and, although studied in less detail, in the context of targeted mutations of other genetic loci (30–37).

The similarity of the HS3a and HS1,2 replacement mutation phenotypes, both of which were cis-acting, suggested that the inhibition of CSR resulted from effects of the inserted PGK-*neo*^{r} gene, potentially via competition of the inserted promoter for control elements in the putative 3′ IgH CSR regulatory locus. These findings also led to the suggestion that CSR recombination could be regulated, at least in part, by the relative ability of various germ-line C_H promoters, after activation, to compete for activities of this putative regulatory region (24, 25). Here, we report studies designed to test postulates of such a 3′ IgH locus regulatory model by examining the effects of insertion of the PGK-*neo*^{r} gene at two sites within the C_H locus on GT of and CSR to upstream and downstream C_H genes.

MATERIALS AND METHODS

Generation of Iγ2b and IgE C_H Mutant Mice. We previously have described (10) the generation of embryonic stem (ES) cells homozygous for a mutation in which the PGK-*neo*^{r} resistance gene was inserted in place of Iγ2b sequences in the same transcriptional orientation as the endogenous unit (referred to as Iγ2bN/N cells) and ES cells homozygous for a mutation in which the PGK-*neo*^{r} gene that replaces Iγ2b sequences was deleted on both alleles (referred to as Iγ2b$-/-$ cells). These homozygous mutant ES cells subsequently were assayed for effects of the mutations on CSR by the RAG-2-deficient blastocyst complementation as described previously (10, 24). For these analyses, chimeras were assayed for serum Ig isotypes at 10–12 weeks of age. For all other experiments, chimeras were between 1 and 8 months in age. The IgEN/N mice, homozygous for a mutation in which a PGK-*neo*^{r} gene was inserted in the opposite transcriptional orientation as the endogenous Cε1–4 exons, have been described previously (38). The germ-line IgE mutant mice were analyzed at 5–8 weeks. The general structure of the mutations analyzed is outlined in Fig. 1.

Splenocyte Cultures. Single-cell suspensions of spleen cells were prepared as described previously (39). Subsequently, cells were cultured and maintained at a concentration of 10^6 cells/ml in RPMI 1640/10% FCS medium [supplemented with 2-mercaptoethanol (50 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and glutamine (2 mM)] containing 20 μg/ml LPS with or without 20 ng/ml rIL-4 (R & D Systems), interferon γ (100 units/ml) (R & D Systems), or transforming growth factor β (1 ng/ml) (R & D Systems) as described previously (14, 24). Cells were harvested at day 4.5 for supernatant collection for Ig isotype analysis by ELISA and to analyze isotype cell surface expression by fluorescence-activated cell sorter (FACS).

FACS Analysis for Surface Ig Expression. Harvested splenocytes were stained for surface expression with fluorescein isothiocyanate-labeled anti-IgG1 (A85–1), anti-IgA (R5–140), anti-IgE (R35–72) (PharMingen), goat anti-mouse IgG2b (SBA), phycoerythrin (PE)-labeled goat anti-mouse IgM, goat anti-mouse IgG3 (SBA), streptavidin-PE (PharMingen), Cy-C labeled B220/CD45R (RA3–6B2), biotin-labeled anti-IgG2a (8.3), and anti-IgG2b^{a}2a^a (21–48.31) (PharMingen). FACS analysis of the cells was acquired on a FACSCaliber flow cytometer (Becton Dickinson) using CELLQUEST (Becton Dickinson) and analyzed using FLOWJO analysis software (Becton Dickinson).

ELISA Analysis. Supernatants from day 4 or 4.5 splenocyte cultures or sera were analyzed for the presence of different Ig isotypes by sandwich ELISA assay as previously described (14, 40) by using goat anti-mouse IgG2b, goat anti-mouse IgG3, goat anti-mouse IgG1, goat anti-mouse IgM, goat anti-mouse IgA coating (SBA), anti-IgE (R35–72) coating (PharMingen), alkaline phosphatase-labeled anti-mouse IgG2b (R12–3) (PharMingen), anti-mouse IgG3, anti-mouse IgG1, anti-mouse IgM, anti-mouse IgA (SBA) revealing, biotin labeled anti-IgE (R35–92) (PharMingen) revealing, and alkaline phosphatase-labeled streptavidin (SBA). Standards used were IgG2a, IgG3, IgG1, IgG2b, IgA (SBA), IgM (Zymed), and IgE (IgE-3) (PharMingen). Cultures were established in triplicate for each assay. Sensitivity of the assays ranged from 10 to 20 ng/ml.

Northern Blot Analysis. RNA was prepared using Trizol reagent (GIBCO/BRL) as per the instructions of the manufacturer. For Northern blot analyses (41), approximately 10–15 μg of total RNA was electrophoresed through a 1% agarose gel, transferred to Zetaprobe membrane (Bio-Rad), and assayed for hybridization with probes labeled by random hexamer priming with [α-^{32}P]dCTP. The Cγ2b probe is an $\approx$300-bp *Sac*I-*Sac*I genomic fragment of the C_H3 region of Cγ2b. The mb-1 probe was generated from a full-length 900-bp cDNA. The Cγ3 probe was a *Bam*HI 6-kb genomic fragment spanning most of Cγ3 and $\approx$4.5 kb 3′ of Cγ3 (42). The Cε probe was an $\approx$2.5-kb *Sac*I genomic fragment spanning all Cε exons. The Cμ probe is a 900-bp *Xba*I-*Bam*HI genomic fragment spanning 5′ of Cμ. The Iγ2b probe is a *Hin*dIII-*Xho*I 500-bp genomic fragment from the plasmid SKA (gift of S. Li, Columbia University).

PCR Amplification of Germ-Line Transcripts. Total RNA was isolated from 1×10^7 day 2 LPS (for γ3) or LPS + IL-4 (for Iε) stimulated splenocytes using the Trizol reagent (GIBCO/BRL) as per the manufacturer's instructions. We generated cDNA by using Superscript (GIBCO/BRL) according to the manufacturer's instructions. Germ-line Iε and Iγ3 transcripts were amplified as described previously (25). The cDNA samples were diluted into mouse genomic DNA to keep a consistent amount of 100 ng total DNA per reaction. Probes used for detection of PCR products were a *Bam*HI 6-kb Cγ3 genomic fragment (42), a 1.3-kb genomic *Pst*I Iε fragment, and a mb-1 probe generated from a full-length 900-bp cDNA.

RESULTS

Insertion of *PGK-neo*^{r} Genes into the Iγ2b Locus Inhibits CSR to the Cγ3 Gene. We employed the RAG-2-deficient blastocyst complementation method to generate chimeric mice containing B lymphocytes derived from Iγ2b mutant ES cells as described previously (10). Mutant ES cells assayed contained targeted mutations in which most of the Iγ2b exon was either deleted on both chromosomes (Iγ2b$-/-$) or replaced with a PGK-*neo*^{r} gene in the same transcriptional orientation as the endogenous locus on one (Iγ2b+/N) or both (Iγ2bNN) chromosomes (ref. 10; Fig. 1). Previously, we have shown that homozygous insertion of a PGK-*neo*^{r} gene in place of HS3a (HS3aN/N) or HS1,2 (HS1,2N/N) inhibits generation of serum IgG3 and IgG2a (refs. 24 and 25; Fig. 1). Likewise, ELISA measurement of serum Ig levels demonstrated severely reduced levels of IgG3 (30- to 50-fold) in Iγ2bN/N chimeras versus Iγ2b+/N or wild-type (wt) mice (Fig. 2 *Upper*). However, the serum levels of all other measured IgH isotypes, including IgG2a, were similar in Iγ2bN/N chimeras as compared with those of controls (Fig. 2).

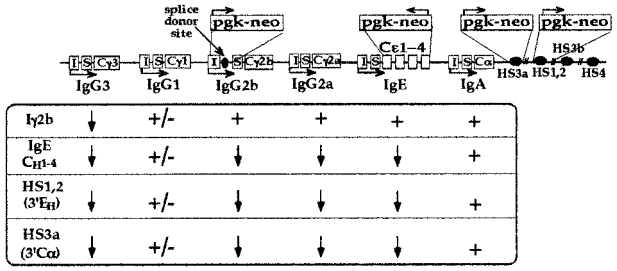

FIG. 1. Insertion of the PGK-*neo*^{r} gene in the IgH locus disrupts class switching upstream of the insertion. Summary of the effects on CSR in gene-targeting experiments within the IgH locus using the PGK-neo^r gene as a selectable marker. Iγ2b and IgE C_H1–4 summarize experiments presented here, and HS1,2 and HS3a summarize previously published studies (24, 25). The effect on CSR is noted as inhibition (downward-pointing arrow), partial inhibition (+/−), or CSR levels comparable to wt controls (+). Transcripts and transcriptional orientation are represented by horizontal arrows. ND, not determined. I, I exon; S, switch region; C, constant region genes for corresponding isotype.

We previously have observed CSR defects to a large set of C_H genes, including Cγ3, Cγ2b, Cγ2a, and Cε in HS3aN/N or HS1,2N/N B cells assayed *in vitro* (24, 25). As expected (10), LPS-stimulated Iγ2b−/− splenic B cells failed to switch to IgG2b, whereas Iγ2bN/N splenic B cells switched to IgG2b at approximately wt levels (Fig. 2). To determine whether the inserted PGK-*neo*^{r} gene affected CSR to other C_H genes, B cells from Iγ2bN/N, Iγ2b+/N, Iγ2−/−, and wt mice were stimulated in culture for 4 or 5 days with either LPS (for CSR to Cγ3 and Cγ2b, LPS plus IL-4 (for CSR to Cγ1 and Cε), LPS plus interferon γ (for CSR to Cγ2a), or LPS plus transforming growth factor β (for CSR to Cα) and then assayed by various methods for CSR. Class switching to all IgH isotypes other than IgG2b was similar to that of wt for cultured Iγ2b−/− B cells as measured by FACS for surface Ig isotype expression and by ELISA for supernatant Ig levels in the cultures (ref. 10; Fig. 2 *Lower*, and data not shown). For appropriately stimulated Iγ2bN/N B cells, surface expression and secretion of IgG2a, IgE, and IgA, isotypes encoded by C_H genes downstream of the insertion were at levels comparable to those of wt and Iγ2b+/N B cells, both as assayed by surface expression (not shown) and by supernatant secretion (Fig. 2). However, production of IgG3 (an isotype encoded by a C_H gene 5′ to the PGK-*neo*^{r} insertion) was reduced severely in Iγ2bN/N B cells after *in vitro* stimulation with LPS (Fig. 2 and data not shown). In contrast, production of IgG1 was only partially reduced and IgA was not greatly affected, similar to our observations with HS3aN/N and HS1,2 N/N B cells (refs. 24 and 25; Figs. 1 and 2).

Deficient CSR to Upstream C_H Genes in IgEN/N Mice. To determine whether PGK-*neo*^{r} insertions at other locations or in the reverse orientation within the C_H locus resulted in CSR defects, we assayed mice homozygous for a germ-line mutation in which exons 1–4 of the Cε gene were replaced with a PGK-*neo*^{r} gene inserted in the opposite transcriptional orientation as the endogenous locus (IgEN/N) (ref. 38; Fig. 1). These mice are not capable of producing IgE and previously had been reported to have normal serum IgG levels (38). However, in those studies, serum IgG levels were assayed with pan-IgG antibodies, which would have missed specific deficiencies in IgG3, IgG2b, and IgG2a (38). Analysis of the serum isotype levels of IgEN/N mice by ELISA revealed that these mutant mice had class-switch defects reminiscent of HS3aN/N or HS1,2N/N mice (Fig. 2 *Upper*). Thus, serum IgG3, IgG2b, and IgG2a levels were reduced significantly (from 10- to 100-fold), whereas IgM, IgG1, and IgA levels were comparable to normal. Likewise, cultured B cells from IgEN/N displayed class-switch defects essentially identical to those that we have observed previously for HS3aN/N and HS1,2N/N B cells (Fig. 2 *Lower*). Thus, production of IgG3, IgG2a, and IgG2b (and IgE) was reduced severely after activation by appropriate stimuli, whereas production of IgG1 was somewhat reduced and that of IgA was not affected substantially (Fig. 2).

CSR Defects in Iγ2b and Cε PGK-*neo*^{r} Insertion Mutant B Cells Correlate with Corresponding Defects in Germ-Line C_H Gene Transcription. To further elucidate the mechanisms by which class switching was blocked in Iγ2bN/N and IgEN/N B cells, we employed Northern blotting to assay expression of C_H transcripts in cultured mutant and control B cells after 2 or 5 days of the various stimulations. Most of the Northern analyses employed probes (C_H probes) that would detect both germ-line or mature [i.e., V(D)J containing] transcripts. Both Cμ and mb-1 probes were used to standardize the amount of B cell RNA assayed by Northern blotting (Fig. 3*A*). As detected by Northern blotting, the accumulation of Cγ3-hybridizing transcripts were inhibited severely after LPS treatment of Iγ2bN/N B cells, whereas the levels of other tested C_H-hybridizing transcripts in the variously treated cells were not substantially different from those of controls (Fig. 3*A*). On the other hand, levels of both Cγ3- and Cγ2b-hybridizing transcripts were markedly reduced in LPS-stimulated IgEN/N B cells as compared with controls (Fig. 3*A*).

To more specifically test the effect of the mutations on accumulation of germ-line transcripts, we assayed Northern blots for hybridization to an Iγ2b probe and also assayed for Iγ3-Cγ3 and Iε-Cε transcripts by reverse transcription–PCR (RT-PCR) (employing mb-1 as a control). These studies showed inhibition of the accumulation of Iγ2b-containing, Iγ3-Cγ3 transcripts and (as expected because of the deletion of Cε) Iε-Cε transcripts in IgEN/N mice, but inhibition of only the Iγ3-Cγ3 transcripts in the Iγ2bN/N B cells [ref. 10; data not shown and Fig. 3*B*). Therefore, the observed blocks in CSR to C_H genes upstream of the assayed PGK-*neo*^{r} cassette insertions directly correlate with inhibition of the generation of the corresponding germ-line transcripts.

DISCUSSION

Inhibition of CSR by Insertion of PGK-*neo*^{r} Genes into the Ig C_H Locus. We previously showed that mutations just 3′ of the IgH locus, in which HS3a and HS1,2 were replaced with a PGK-*neo*^{r} gene, impaired GT of and CSR to a subset of C_H genes in cultured B lymphocytes, including Cγ3, Cγ2a, Cγ2b, and Cε (24, 25). These findings suggested a model in which insertion of the PGK-*neo*^{r} gene interfered with the activities of a long-range CSR regulatory region that controls relative expression of certain C_H promoters and, as a result, CSR (3, 24). In support of this hypothesis, we now show that cultured B cells harboring a PGK-*neo*^{r} cassette in place of Iγ2b sequences are impaired in GT of and CSR to the upstream Cγ3 gene, but not to the downstream Cγ2a and Cε genes. Moreover, we show further that B cells in which the Cε exons have been replaced with the PGK-*neo*^{r} cassette, in the opposite transcriptional orientation to that of the other insertion mutations, are impaired in GT of and CSR to the upstream Cγ3, Cγ2b, and Cγ2a genes. Therefore, the observed inhibitory effects are not strictly dependent on the transcriptional orientation of the PGK-*neo*^{r} gene.

Together, our findings support the existence of a long-range 3′ IgH regulatory region required for GT of and CSR to multiple C_H genes and suggest that PGK-*neo*^{r} cassette insertion into the locus short circuits the ability of this region to facilitate GT of dependent C_H genes upstream, but not downstream, of the insertion site. Of note, replacement of the Eμ/MAR region (11) or a portion of the Cμ coding sequence (43) with PGK-*neo*^{r} did not appear to substantially affect switching to tested isotypes encoded by downstream C_H genes, even though these insertions were physically more proximal to

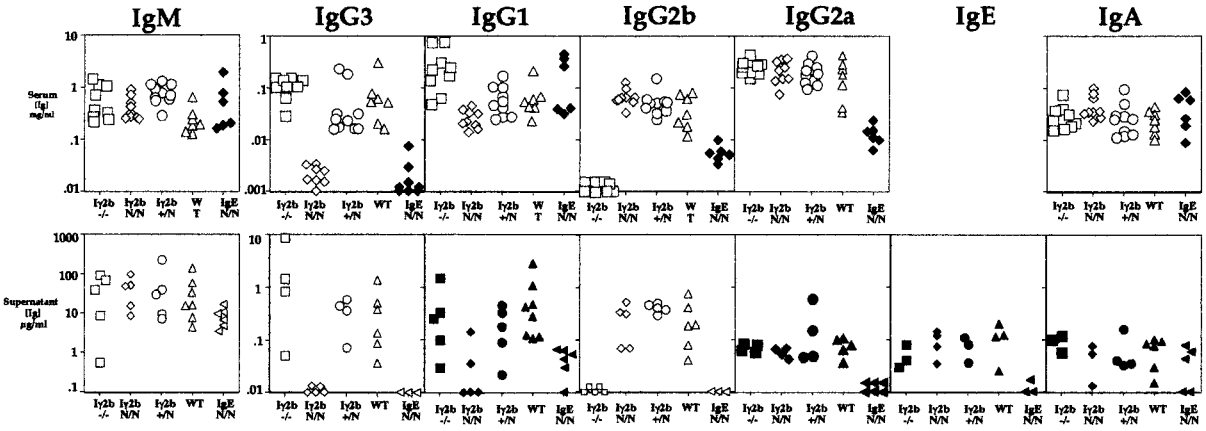

FIG. 2. Class-switching defects in Iγ2b N/N and IgE N/N mice. (*Upper*) Concentrations of specific Ig isotypes in sera from Iγ2b−/− (□), Iγ2bN/N (◇), Iγ2b +/N (○), wt (TC1/WT) (△), and IgE N/N (◆) mice as measured by ELISA. Serum from some *neo*^{r}-inserted mice contained undetectable levels of IgG3 (symbols on axis). (*Lower*) ELISA analyses of culture supernatants from splenic B cells from Iγ2b−/− (squares), Iγ2bN/N (diamonds), Iγ2b +/N (circles), wt (TC1/WT) (triangles), or IgE N/N (◁) mice stimulated *in vitro* for 4.5 days in the presence of LPS (open symbols) (IgM, IgG3, IgG2b), LPS plus interferon γ (closed symbols for IgG2a), LPS plus IL-4 (closed symbols for IgG1 and IgE), or LPS plus transforming growth factor β (closed symbols for IgA). For Iγ2bN/N cultures, IgG3 was reduced 40- to 200-fold and IgG1 was partially inhibited (reduced 10-fold) compared with controls. For IgEN/N cultures, IgG3 was down 40- to 200-fold, IgG2b was down 20- to 50-fold, IgG2a was down 5- to 20-fold, IgG1 was reduced slightly (down 10-fold), and IgE was reduced severely.

Iγ3 than the HS1,2 insertion—further supporting the notion that inhibition may be dependent on insertion of the PGK-*neo*^{r} gene between the affected I region promoter and a 3′ regulatory region. The overall findings regarding polarized effects of inserted PGK-*neo*^{r} genes on CSR supports the existence of a CSR regulatory locus 3′ of the IgH locus that could comprise the four known HS sites and/or elements that are yet to be determined. Because deletion of the HS3a or HS1,2 elements *per se* had no major effect on CSR, these elements must either be redundant in function for this regulatory region or, more likely, critical regulatory elements lie downstream of HS1,2.

PGK-*neo*^{r} Insertion Mutations Affect Only a Subset of C_H Genes. Neither the HS3a nor the HS1,2 PGK-*neo*^{r} insertion mutations substantially affected switching to the Cα gene either *in vivo* or in cultured B cells, suggesting that Cα expression may not be dependent on the 3′ regulatory sequences affected by these mutations (24, 25). Because our currently analyzed mutations did not dramatically affect switching to IgA, but also were upstream to the Cα gene, they offer no additional insights. Similarly, switching to IgG1 was only partially affected by the various analyzed mutations, consistent with the notion that GT of the Cγ1 gene may be influenced by elements that are, at least partially, independent of the putative 3′ IgH sequences (ref. 24 and 25; this study). Of note, we usually can detect at least low-level expression of even the most severely inhibited IgH isotypes in the serum of Iγ2bN/N chimeras or of IgEN/N, HS3aNN, and HS1,2N/N mice, suggesting that there may be alternative pathways for switching to some isotypes (e.g., IgG2b; refs. 24 and 25) or that the very low level switching observed *in vitro* is sufficient to allow accumulation of switched cells and/or observed serum Ig isotypes. In this regard, our detection of Cγ3-hybridizing transcripts in day 5 LPS-treated Iγ2bN/N cells, but not Iγ3-Cγ3 transcripts by RT-PCR assay of day 2 treated cells (Fig. 3), may reflect the low-level generation of IgG3-producing B cells followed by accumulation of transcripts during culture.

What Aspect of the Inserted PGK-*neo*^{r} Gene Causes the Inhibitory Effects? The β-globin LCR has been speculated to regulate differential β-globin locus gene expression via a promoter-competition mechanism (44–46), although other interpretations remain possible (44, 46–48). Likewise, one interpretation of our findings that PGK-*neo*^{r} insertion into the downstream portions of the IgH locus interferes with transcription from upstream I exon promoters is that PGK-*neo*^{r} competes with affected I region promoters for interactions with elements of the putative 3′ IgH regulatory region necessary for transcriptional activation. Comparable inhibitory effects have been described with respect to insertion of transcribed marker genes into the β-globin LCR, which is reminiscent in organization to the 3′ IgH enhancer region (21, 26, 27, 29, 49). Consistent with this model, insertion of the PGK-*neo*^{r} gene in the IgH locus renders the *neo*^{r} gene LPS-inducible (10, 25). However, although these findings are consistent with promoter-competition mechanisms to explain the negative effects of the PGK-*neo*^{r} cassette, they do not eliminate other conceivable long-range mechanisms. For example, rather than strict promoter competition, the PGK promoter could alter local chromatin structure in a manner that prevents access and interaction of the 3′ IgH regulatory region with upstream I exon promoters (47).

Whatever the absolute mechanism, the many similarities between the overall organization of the β-globin LCR and the 3′ IgH region suggest that these two loci have evolved similar strategies to regulate differential gene expression. However, it has not been proven formally in any system that the effects of the inserted PGK-*neo*^{r} cassette are mediated by its promoter; nor has it been shown exactly how promoters might function in such a "competition." Negative effects of PGK-*neo*^{r} gene insertion have been observed in several loci; it is conceivable that not all mechanisms of inhibition are the same (discussed in ref. 25). Several additional mechanisms besides promoter-based mechanisms have been considered to potentially explain inhibitory effects of the PGK-*neo*^{r} cassette inserted into the Ig κ locus (33, 34, 50, 51). However, given the great distance and polarity of the effects of the PGK-*neo*^{r} cassette in the IgH locus, a promoter-based mechanism seems most likely in this case. Further support for this notion comes from the recent finding that replacement of the Iα exon with a PGK-hypoxanthine phosphoribosyltransferase minigene inhibits CSR to several upstream C_H genes (13). Of note, not all promoters appear to have such strongly negative effects as PGK. For example, insertion of a *neo*^{r} selectable marker driven by the polyoma enhancer/herpes simplex virus thymidine kinase promoter did not lead to obvious inhibition of CSR to upstream C_H genes, although orientation and insertion site remain a variable in these experiments (6, 7, 14, 52). Finally, promoters other than PGK may cause such negative effects,

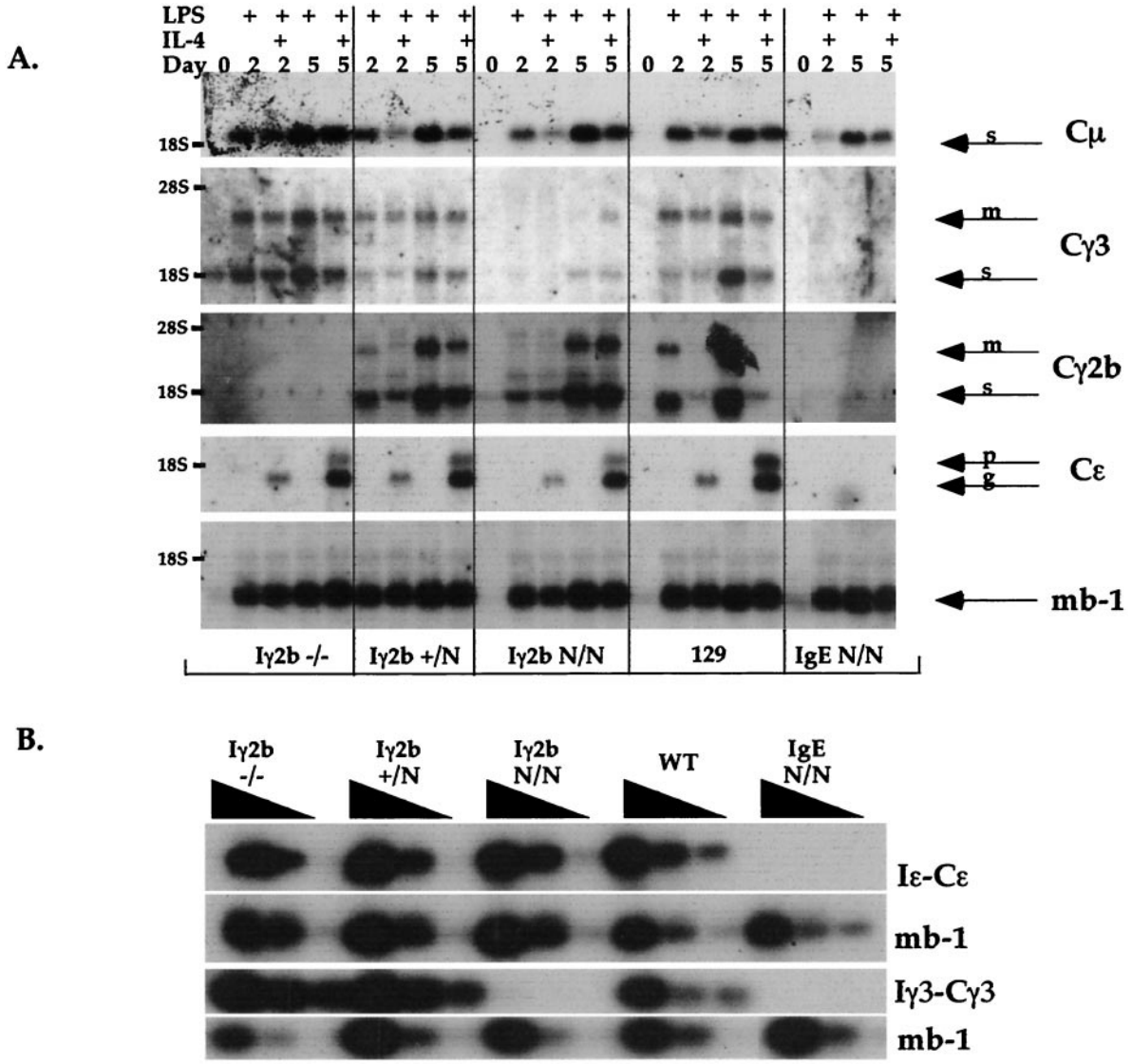

FIG. 3. Analysis of C_H germ-line transcripts in Iγ2bN/N- and IgEN/N-activated B cells. (*A*) Northern analysis of RNA from cells cultured with LPS or LPS + IL-4 for 2 or 5 days as indicated. Probes used were (in this order) Cγ2b C_H3 region, Cγ3, mb-1, Cε, and Cμ. Transcript sizes for productive (p) and germ-line (g) Cμ are 2 kb; for Cγ3 transcript, sizes are 3.3 membrane (m) and 1.9 secreted (s), where p and g transcripts are a similar size. P and g Cγ2b transcripts are 3.6 (m) and 1.7 (s) (53). P and g γ3 transcripts are 3.3 (m) and 1.9 (s) (54), the ε g transcript is 1.7, and the p is 2.3 (14). 129, Wild type. One representative experiment of three is shown. (*B*) RT-PCR for γ3 (LPS-stimulated) and ε (LPS + IL-4-stimulated) germ-line transcripts. cDNA derived from cells stimulated for 2 days in culture was diluted 1:1, 1:5, and 1:25 for all samples. Mb-1 RT-PCR was used as a control for amount of cDNA per reaction. Wild-type controls are indicated as WT. One representative experiment of three is shown.

because insertion of hygromycin or *neo*^{r} genes driven by the Friend virus long terminal repeat into the human β-globin LCR suppressed the expression of adult β-globin genes (26, 27).

Model for Control of IgH CSR. Our current findings are consistent with our earlier hypothesis that promoter "competition" may be a general mechanism employed for modulating GT and CSR activity in the IgH locus (24, 25). In this context, we propose that transcription from, at least a subset of, I region promoters is based on ability of the local I region promoter to "interact" with the putative 3′ regulatory region to achieve transcription. In the context of such a model, LPS treatment, for example, would lead to activation of the local Iγ2b and Iγ3 promoters, which, via interaction with the 3′ regulatory region, would initiate transcription and lead to CSR. On the other hand, addition of IL-4 along with LPS would activate the local Iγ1 and Iε promoters, which, based on this model, would out-compete the Iγ2b or Iγ3 promoters for 3′ regulatory regions, perhaps because of promoter strength, the more 3′ position, or both. In any case, such competition would lead to Iγ1 or Iε transcription and extinguish Iγ2b and Iγ3 transcription (via competition). Of potential relevance, recent studies of a transgenic β-globin locus showed that a second β-globin gene competed more efficiently with other genes to which it was LCR-proximal (45), supporting the looping model of LCR function in which one gene interacts with the LCR at a time (discussed in ref. 44). This general model for regulation of germ-line C_H promoter expression could extend to other C_H genes and other activation/lymphokine pathways, although some C_H genes clearly may be activated by independent mechanisms, at least under some conditions.

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