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# A selective defect in IgG2b switching as a result of targeted mutation of the Iγ2b promoter and exon

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**LPS stimulation of B lymphocytes induces germline transcription of and subsequent switching to the γ2b gene. Mature germline transcripts contain an I exon (non-coding) spliced to the Cγ2b exons. To investigate the role of germline transcription and/or transcripts in heavy chain class switching, we have replaced the germline Iγ2b promoter and I exon in ES cells with an expressed neomycin resistance gene. The mutated chromosome retains the downstream target sequence for switch recombination (S regions) and all sequences necessary for expression of a switched γ2b gene. Wild-type or mutant ES cells were injected into RAG-2 deficient blastocysts to generate somatic chimeras in which all lymphocytes were ES-cell derived. Chimeras derived from injection of heterozygous mutant ES cells had normal levels of serum IgG2b, but their splenic B cells showed a partial decrease in ability to switch to γ2b. Strikingly, B lymphocytes from chimeras derived by injection of homozygous mutant ES cells were deficient in IgG2b production both *in vivo* and *in vitro*, but normal with respect to production of other Ig heavy chain isotypes. Additional studies demonstrated that lack of ability to produce IgG2b by the mutant B cells correlated with lack of germline transcription and resulted from a specific defect in class-switch recombination to Sγ2b. Together, these studies demonstrate that the I region is an important regulatory element for control of class-switch recombination.**

*Key words:* class switching/gene targeting/germline transcription/Iγ2b/Rag-2-deficient blastocyst complementation

## Introduction

The variable regions of immunoglobulin (Ig) heavy and light chains mediate specific antigen binding and are encoded by germline V, (D) and J gene segments that are assembled during early B cell differentiation by the VDJ recombination process (for review, see Blackwell and Alt, 1989). Differentiating B lymphocytes first produce μ heavy chains which associate with Ig light chains to form an IgM surface receptor. Subsequently, a B cell can change the constant ($C_H$) portion of its expressed heavy chain through a process termed heavy chain class-switch recombination, permitting a clonal lineage of B cells to produce antibodies that retain variable region specificity in association with a different $C_H$ effector function (for review see Lutzker and Alt, 1988a; Esser and Radbruch, 1990). Class-switch recombination juxtaposes a downstream $C_H$ gene such as γ, ε or α to the expressed V(D)J variable region gene and results in deletion of intervening sequences including the $C_\mu$ gene (Cory *et al.*, 1980; Rabbitts *et al.*, 1980; Iwasato *et al.*, 1990; Matsuoka *et al.*, 1990; Schwedler *et al.*, 1990). Unlike VDJ recombination, class-switch recombination does not take place at a specific site, but instead occurs in regions composed of tandem repetitive sequences (termed S regions) located 5′ of each $C_H$ gene except for Cδ (Davis *et al.*, 1980; Dunnick *et al.*, 1980; Kataoka *et al.*, 1980, 1981; Nikaido *et al.*, 1982).

Exposure of B lymphocytes to certain mitogens and cytokines can influence the outcome of the class-switch recombination process. For example, stimulation of murine splenic B cells with bacterial lipopolysaccharide (LPS) leads to the generation of activated B cells that express IgG2b or IgG3 antibodies, whereas simultaneous treatment of splenic B cells with LPS and interleukin-4 (IL-4) suppresses IgG2b and IgG3 expression but leads to production of IgG1 and IgE (Kearney *et al.*, 1976; Layton *et al.*, 1984; Coffman *et al.*, 1986; Snapper and Paul, 1987). The same factors that induce or suppress switching to a particular $C_H$ gene also induce or suppress germline transcription of the corresponding gene prior to the class-switch process. For example, LPS treatment of splenic B cells induces expression of germline γ2b and γ3 transcripts while LPS plus IL-4 suppresses transcription of the germline γ2b and γ3 genes but induces transcription of γ1 and ε transcripts (Lutzker *et al.*, 1988; Berton *et al.*, 1989; Esser and Radbruch, 1989; Gerondakis, 1990; Rothman *et al.*, 1990a,b). Other cytokines similarly influence transcription of and switching to other $C_H$ genes (Stavnezer and Sirlin, 1986; Snapper *et al.*, 1988; Coffman *et al.*, 1989; Lebman *et al.*, 1990; Radcliffe *et al.*, 1990). Observations that expression of germline $C_H$ transcripts generally occurs prior to class switching to a given $C_H$ gene has led to the proposal that germline transcription of a $C_H$ locus is linked to targeting it for class-switch recombination (Yancopoulos *et al.*, 1986; Lutzker and Alt, 1988a; Stavnezer *et al.*, 1988; Rothman *et al.*, 1989).

Primary germline $C_H$ transcripts initiate upstream of a given S region, run through the S region, and terminate at the normal sites downstream of the constant region gene (Lutzker and Alt, 1988b; for review see Rothman *et al.*, 1989). These primary transcripts are processed to juxtapose a small exon (I exon) ~100–500 bp in length to the downstream $C_H$ exons. The precise role of germline transcription and/or transcripts in class-switch recombination is unknown. Germline $C_H$ transcripts appear incapable of encoding proteins due to the presence of multiple stop codons

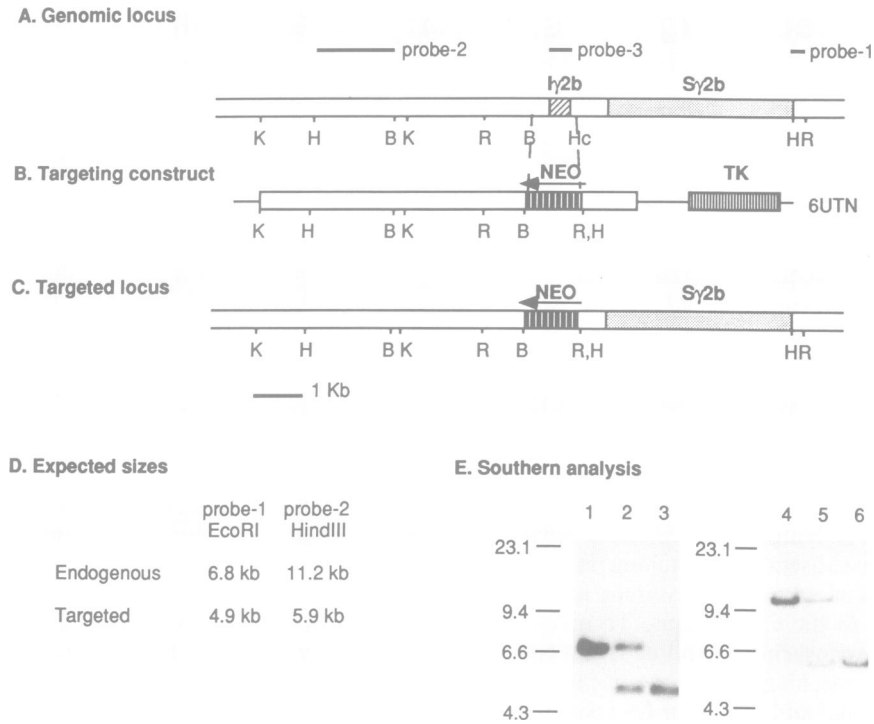


**Fig. 1.** Targeted mutation in the Iγ2b locus. (**A**) Partial restriction map of the γ2b locus. Probes used in Southern blot analyses are shown above the genomic configuration: probe-1, an *Eco*RI−*Hin*dIII fragment; probe-2, a *Hin*dIII−*Bam*HI fragment; probe-3, a PCR fragment containing the complete Iγ2b exon. Restriction enzymes: K, *Kpn*I; H, *Hin*dIII; B, *Bam*HI; R, *Eco*RI; Hc, *Hin*cII. (**B**) Design of the gene targeting construct (6UTN). Thin lines respresent plasmid sequences of SKII pBluescript. (**C**) Partial restriction map of the resulting targeted locus. (**D**) The expected sizes of restriction fragments detected by probe-1 and probe-2 are indicated. (**E**) Southern blot analyses of the Iγ2b mutation in CCE cells. Lanes 1 and 4, wild-type CCE cells; lanes 2 and 5, heterozygous Iγ2b mutant CCE cells ($I\gamma2b^{+/-}$); lanes 3 and 6, homozygous Iγ2b mutant CCE cells ($I\gamma2b^{-/-}$). DNAs in lanes 1−3 were digested by *Eco*RI and probed with probe-1. DNAs in lanes 4−6 were digested by *Hin*dIII and probed with probe-2.

in the I exon; therefore it is unlikely that they function in this manner. A possible function of transcription through the S region may be to open this segment of DNA to facilitate the switch recombination process. Another, not mutually exclusive, possibility is that the primary or processed germline transcript itself may have some functional role(s) in the switching process. This notion is supported by the finding that all characterized $C_H$ gene (except δ) of both mice and humans (Sideras *et al.*, 1989; Gauchat *et al.*, 1990; Nilsson *et al.*, 1991) have a similarly organized germline transcription unit that generates processed germline transcripts with a similar overall structure. If the processed transcripts play a functional role, one might predict that integrity of the I exon would be necessary for efficient switching to the downstream S region.

To test directly for the requirement of an intact I region for Ig heavy chain class switching, we have used gene targeting techniques in embryonic stem (ES) cells to replace the Iγ2b promoter and Iγ2b exon with a bacterial neomycin resistance gene. We then used a novel RAG-2-deficient blastocyst complementation method to create somatic chimeric mice in which all mature lymphocytes derive from the mutant ES cells. Mice derived from the homozygous mutant ES cells showed a selective IgG2b deficiency in their sera and in splenic B cell cultures that could be attributed to a specific defect in class-switch recombination to the γ2b gene.

## Results

### *Generation of $I\gamma2b^{-/-}$ ES cells and somatic chimeric mice*

Results from promoter analysis have shown that sequences from −124 and −53 5′ to the germline transcription initiation sites are essential for Iγ2b promoter activity (J.Zhang and F.W.Alt, unpublished data). Therefore, a gene-targeting construct (6UTN) was made by replacing sequences from approximately −500 bp (a *Bam*HI site) to +470 bp (a *Hin*cII site) with a pMCNEO gene (Stratagene); the replaced sequences include the complete Iγ2b exon and the splice donor site but are ~700 bp upstream from the 5′ end of the Sγ2b region (Figure 1A and B). The positive−negative selection technique (Mansour *et al.*, 1988) was used to select for CCE ES cell clones in which one copy of the endogenous Iγ2b locus was replaced ($I\gamma2b^{+/-}$ mutants). *Eco*RI digested genomic DNA from the wild-type allele should give a 6.6 kb band when hybridized with a 3′ Sγ2b probe (probe-1; Figure 1A and D), whereas the specifically mutated allele should give a 4.9 kb band (Figure 1C and D). The DNA of one of 226 G418 and gancyclovir double resistant clones (#404) gave a band of the expected size from a mutated allele by *Eco*RI digestion (Figure 1E, lane 2). This homologous recombinant was further confirmed by digestion with *Hin*dIII followed by assaying for hybridization to a 5′ Iγ2b probe (probe-2; Figure 1A, D, and E, lane 5).

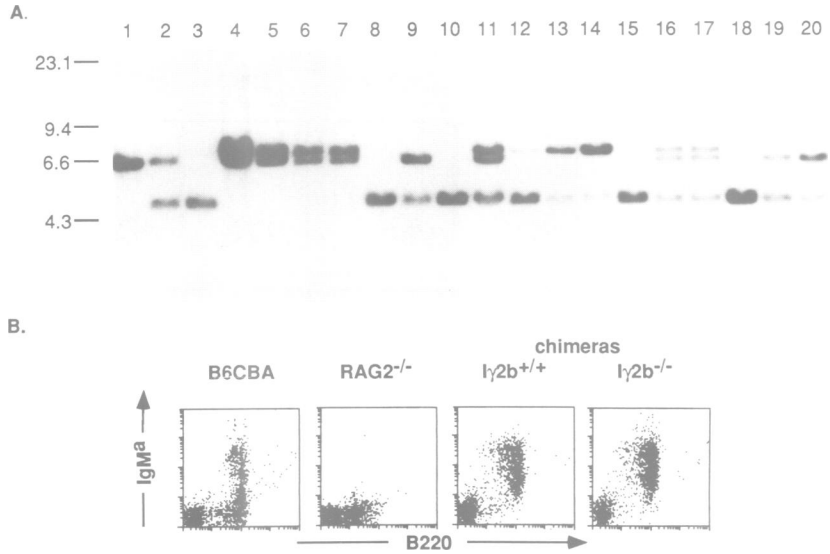


**Fig. 2.** Generation of $I\gamma2b^{-/-}$ somatic chimeric mice using RAG-2-deficient blastocysts. (**A**) Southern blot analysis was performed on genomic DNAs from cell lines and tissues digested by *Eco*RI and probed with probe-1 (Figure 1A). Lane 1, wild-type CCE; lane 2, $I\gamma2b^{+/-}$ CCE; lane 3, $I\gamma2b^{-/-}$ CCE; lane 4, B6/CBA thymus; lane 5, B6/CBA kidney; lane 6, RAG-$2^{-/-}$ thymus; lane 7, RAG-$2^{-/-}$ kidney; lanes 8–20 are tissue DNAs from five different $I\gamma2b^{-/-}$ chimeras (G1, G2, G3, I5, I6): G2, lane 8–9; I6, lanes 10–11; G1, lanes 12–14; G3, lanes 15–17; I5, lanes 18–20. Lanes 8, 10, 12, 15 and 18, thymus; lanes 9, 11, 13, 16 and 19, kidney; lanes 14, 17 and 20, heart. Due to polymorphism in this region, a and b-allotypes give two different sized germline fragments with probe-1 (b-allotype fragment is slightly larger than a-allotype fragment). The RAG-2-deficient blastocysts can have a/a, a/b or b/b genotype because of the F1 self-cross breeding (129Sv and C57BL/6). CCE cell line is of a/a genotype and B6/CBA of a/b genotype. (**B**) Surface staining with $^{PE}$anti-$IgM^a$ and $^{fl}$anti-B220 in fresh splenic cells from 1 month old mice. Shown here are results from one B6/CBA mouse, one RAG-2-deficient mouse and somatic chimeric mice from $I\gamma2b^{+/+}$ CCE cells and $I\gamma2b^{-/-}$ CCE cells.

ES cells in which both copies of the Iγ2b region were replaced by a NEO gene ($I\gamma2b^{-/-}$ mutants) were obtained by selecting the $I\gamma2b^{+/-}$ mutant cells for growth in increased concentrations of G418 (Mortensen *et al.*, 1992; see Materials and methods). Multiple independent homozygous mutant clones were obtained; one (U16) was subcloned to ensure homogeneity and used for further analysis (Figure 1E, lanes 3 and 6). A DNA fragment consisting of the Iγ2b exon (probe-3, Figure 1A) was used to confirm the absence of the Iγ2b exon sequence in homozygous mutant cells (data not shown).

### *Contribution of $I\gamma2b^{-/-}$ cells to lymphoid and non-lymphoid tissues*

Three independent $I\gamma2b^{-/-}$ subclones (U16F, U16G, U16I), one $I\gamma2b^{+/-}$ clone (#404) and two $I\gamma2b^{+/+}$ subclones (all clones are in CCE ES cell line from 129 strain) were injected into blastocysts derived from RAG-$2^{-/-}$ mice (mixed background of 129Sv and C57BL/6), and the injected blastocysts were implanted into foster mothers (B6/CBA F1 hybrids) to derive somatic chimeric mice. Because the RAG-$2^{-/-}$ cells cannot generate mature lymphocytes (Shinkai *et al.*, 1992), all mature B and T cells in the resulting somatic chimeras must derive from the injected ES cells (Chen *et al.*, 1993). To confirm the successful contribution of $I\gamma2b^{-/-}$ ES cells to the somatic chimera, we first assayed *Eco*RI digested DNA from thymus, kidney and heart from various mice for hybridization to probe-1 (Figure 1A). We observed only two polymorphic germline bands of ~6.6 kb in DNA from normal or RAG-$2^{-/-}$ animals (Figure 2A, lanes 4–7). However, with DNA from five independent $I\gamma2b^{-/-}$ ES cell-complemented RAG-$2^{-/-}$ chimeras, we observed ~10–30% contribution of the mutated band (4.9 kb) in kidney or heart DNA but nearly 100% contribution of the mutated band in thymic DNA (Figure 2A, lanes 8–20). The latter finding is consistent with the observation that all mature lymphocytes derive from the injected ES cells under these conditions and confirm the complete contribution of the injected ES cells to the T cell compartment. The $I\gamma2b^{+/-}$ and $I\gamma2b^{+/+}$ ES cells gave similar patterns of contribution to chimeras (data not shown).

Quantitative analysis of contribution to splenic lymphocytes by the Southern blotting method is not possible because of the presence of many non-lymphoid cells in spleen. To confirm the ES cell contribution to splenic B cells, we carried out FACS analyses on spleens of mice derived from the complemented RAG-2-deficient blastocysts for the presence of splenic B lymphocytes. Spleens from normal B6/CBA mice have two populations of B cells, one is $B220^+$ and $IgM^{a+}$, the other is $B220^+$ and $IgM^{a-}$ (Figure 2B; the latter population being cells which express the $IgM^b$ heavy chain allele). RAG-$2^{-/-}$ mice have no $IgM^+$ cells in the spleen (Figure 2B). However, somatic chimeras generated by injection of $I\gamma2b^{+/+}$, $I\gamma2b^{+/-}$ and $I\gamma2b^{-/-}$ ES cells into RAG-$2^{-/-}$ blastocysts had relatively normal numbers of splenic B cells which were all $IgM^{a+}$ confirming that they all derive from the injected 129 strain ES cells (Figure 2B). Together, these results demonstrate the ability of the $I\gamma2b^{-/-}$ ES cells to generate both B and T lymphocytes in the RAG-2-deficient blastocyst complementation system.

### *$I\gamma2b^{-/-}$ B cells do not produce IgG2b in response to LPS stimulation*

Splenic lymphocytes from normal mice (B6/CBA) or from chimeric mice generated with $I\gamma2b^{+/+}$, $I\gamma2b^{+/-}$ and $I\gamma2b^{-/-}$ ES cells were polyclonally activated by culturing

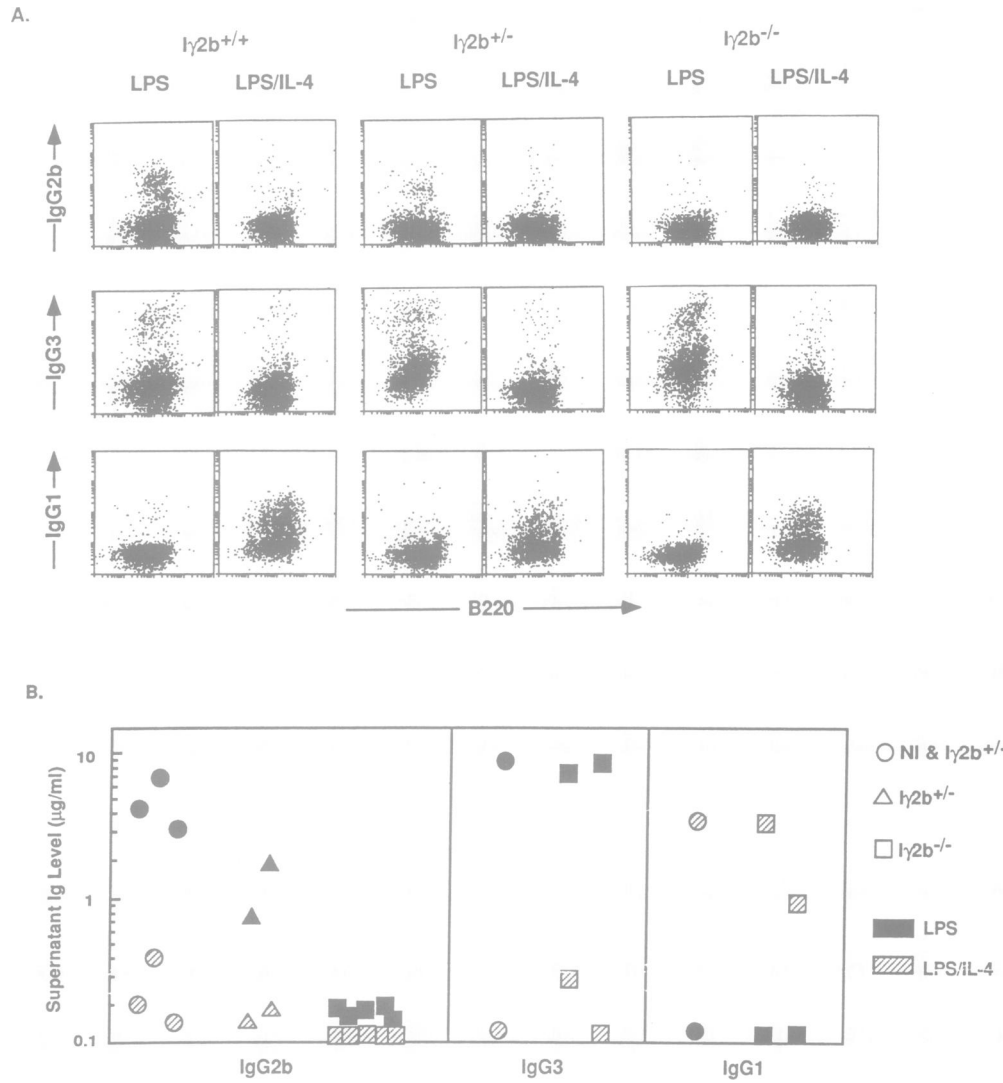


**Fig. 3.** Lack of IgG2b production in splenic B cell cultures from $I\gamma2b^{-/-}$ mice. (**A**) Splenic B cells cultured with LPS or LPS plus IL-4 for 5 days at $5 \times 10^5$ cells/ml were stained with $^{fl}$anti-B220, and $^{bi}$anti-IgG2b, $^{bi}$anti-IgG3; $^{PE}$anti-B220 and $^{fl}$anti-IgG1 to analyze surface expression. Biotin-conjugated antibodies were revealed by PE−streptavidin. Three B6/CBA mice, three chimeric mice from $I\gamma2b^{+/+}$ CCE cells, two chimeric mice from $I\gamma2b^{+/-}$ CCE cells and seven chimeric mice from $I\gamma2b^{-/-}$ CCE cells were analyzed and gave consistent results. Results from one $I\gamma2b^{+/+}$ chimeric mouse, one $I\gamma2b^{+/-}$ chimeric mouse and one $I\gamma2b^{-/-}$ chimeric mouse are shown. (**B**) Supernatants from the same cultures as used in (A) were analyzed by ELISA to evaluate secretion of differnt Ig isotypes. For IgG2b secretion, results from three $I\gamma2b^{+/+}$ chimera, two $I\gamma2b^{+/-}$ chimeras and five $I\gamma2b^{-/-}$ chimeras are shown. For IgG1 and IgG3 secretion, results from one B6/CBA and two $I\gamma2b^{-/-}$ chimeras are shown. NI stands for normal.

with LPS or LPS plus IL-4 for 5 days and then analyzed for surface expression of the various Ig heavy chain isotypes. Significant numbers of spleen cells from normal mice and $I\gamma2b^{+/+}$ chimeras expressed surface IgG2b and IgG3 when cultured in the presence of LPS for 5 days (~12−15% $sIgG2b^+$ and 15−20% $sIgG3^+$), whereas few cells expressed either of these two isotypes on their surfaces in the LPS plus IL-4 treated cultures (Figure 3A). Conversely, IgG1 surface-positive cells in spleens from normal mice and $I\gamma2b^{+/+}$ chimeras were markedly increased in the LPS plus IL-4 cultures (25−30% $sIgG1^+$) compared with the cultures treated with LPS alone (Figure 3A). Strikingly, the profile of isotype expression of the $I\gamma2b^{+/-}$ and $I\gamma2b^{-/-}$ spleen cells following these two treatments was identical to that found with control mice except for the expression of IgG2b. In the LPS-treated splenic cultures from $I\gamma2b^{+/-}$ chimeras, $sIgG2b^+$ cells were also induced, but their number (~7% $sIgG2b^+$, Figure 3A) was approximately half of that from $I\gamma2b^{+/+}$ chimeras. In splenic cultures from $I\gamma2b^{-/-}$ chimeras, there was no IgG2b expressing cells above background under either set of conditions (Figure 3A). This finding indicates that the homozygous replacement of the Iγ2b region specifically blocks the accumulation of sIgG2b-positive splenic lymphocytes following LPS treatment. The results from the $I\gamma2b^{+/-}$ chimeras are most consistent with the possibility that the normal allele in the heterozygous mutant can switch to γ2b while the mutant allele remains inactive.

To analyze further the expression and secretion of different isotypes, ELISAs were done on the supernatants from the same splenic B cell cultures that were used for surface staining. Supernatants from LPS-treated cultures of $I\gamma2b^{+/+}$ and $I\gamma2b^{+/-}$ splenic B cells contained readily detectable levels of both IgG2b and IgG3 isotypes; however, supernatants from the $I\gamma2b^{-/-}$ splenic B cell cultures contained normal levels of IgG3 but very little IgG2b protein

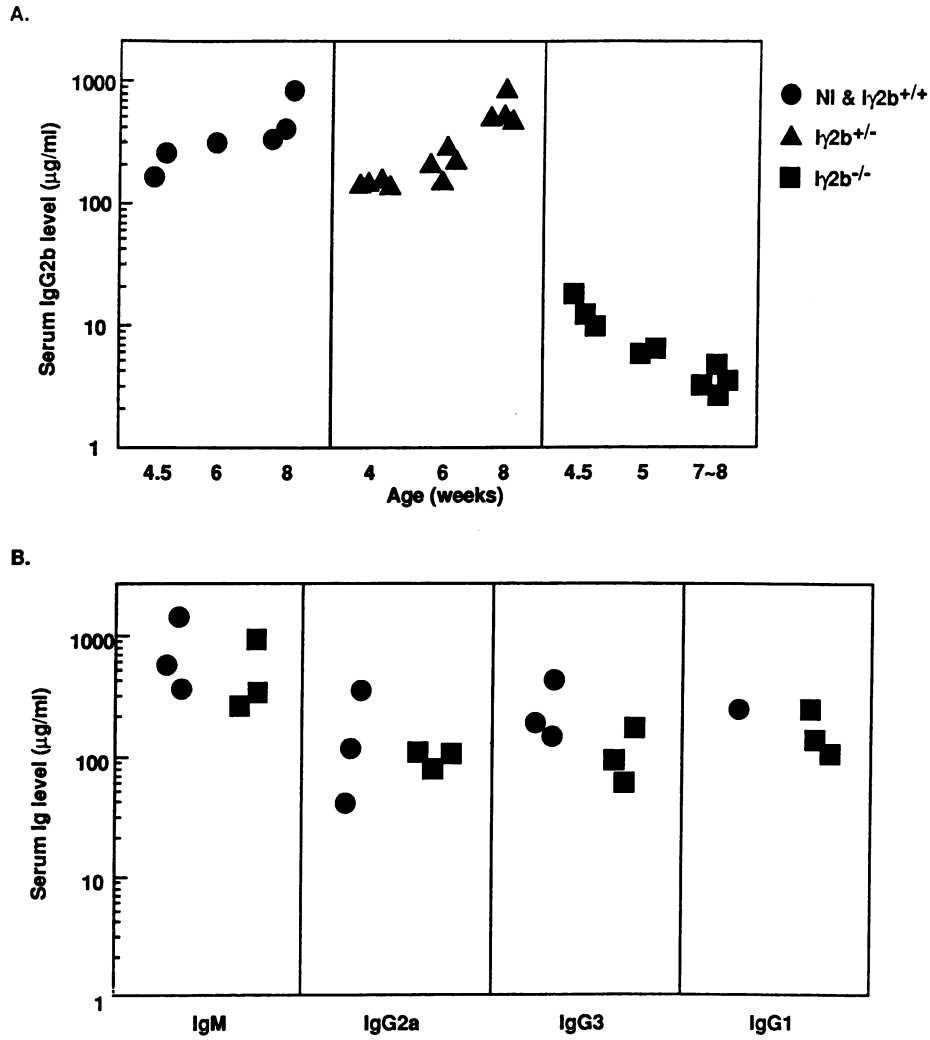


**Fig. 4.** $I\gamma2b^{-/-}$ chimeric mice are selectively deficient for serum IgG2b. (**A**) Serum IgG2b levels were evaluated by ELISA at different ages. Serum samples are from two B6/CBA mice (4.5 weeks), one 129 mouse (6 weeks), three $I\gamma2b^{+/+}$ chimeras (8 weeks), four $I\gamma2b^{+/-}$ chimeras, six $I\gamma2b^{-/-}$ chimeras (three at 4.5 weeks; two at 5 weeks and 7 weeks; and one at 5 weeks, 7 weeks and 8 weeks). NI stands for normal. (**B**) Serum Ig levels in 4½ week old mice were analyzed by ELISA for other Ig isotypes. Results from two B6/CBA mice, one 129 mouse and three $I\gamma2b^{-/-}$ chimeras are shown.

(Figure 3B). In the LPS plus IL-4 cultures, IgG1 secretion was induced in those from both normal mice and $I\gamma2b^{-/-}$ chimeras (Figure 3B). Therefore, the $I\gamma2b^{-/-}$ B cells are specifically deficient in IgG2b production since they can secrete normal levels of other isotypes.

### *$I\gamma2b^{-/-}$ somatic chimeric mice have very low levels of serum IgG2b*

Serum IgG2b levels were evaluated by ELISA on samples from normal mice (B6/CBA, 129Sv), chimeras generated from $I\gamma2b^{+/+}$, $I\gamma2b^{+/-}$ ES cells, and $I\gamma2b^{-/-}$ ES cells (Figure 4A). In normal mice and chimeric mice from $I\gamma2b^{+/+}$ or $I\gamma2b^{+/-}$ ES cells, the level of IgG2b gradually increased with age (Figure 4A). However, in the $I\gamma2b^{-/-}$ chimeras, the serum level of IgG2b decreased with age, particularly from 4½ weeks to ~7 weeks at which stage maternal immunoglobulins are mostly gone (Figure 4A). In 7–8 week old $I\gamma2b^{-/-}$ chimeras, the levels of IgG2b in the serum had declined to values that were 1–2% of those of control mice (Figure 4A). However, similar levels of IgM, IgG2a, IgG3 and IgG1 were detected in $I\gamma2b^{-/-}$ chimeras and normal mice (Figure 4B). These results demonstrate that the integrity of the Iγ2b promoter/exon region is required for natural IgG2b production, but not for the normal production of other heavy chain isotypes.

### *$I\gamma2b$ germline transcripts are abolished in the $I\gamma2b^{-/-}$ B cells*

We employed S1 nuclease protection assays to examine the expression of transcripts from the γ2b, γ3 and ε genes (Figure 5A) in normal or chimeric spleen cells following treatment with LPS or LPS plus IL-4. In both normal, $I\gamma2b^{+/+}$ and $I\gamma2b^{-/-}$ spleen cells, LPS treatment induced Iγ3 and Cγ3 transcripts but not germline ε or Cε transcripts. Conversely, LPS plus IL-4 induced germline ε and Cε transcripts and inhibited expression of germline and Cγ3 transcripts (Figure 5B; Rothman *et al.*, 1990a,b). Expression of Iγ2b and Cγ2b transcripts also were induced by LPS and inhibited by LPS plus IL-4 in normal and $I\gamma2b^{+/+}$ mice. As expected, no Iγ2b transcripts were detected in the $I\gamma2b^{-/-}$ mice; however, a low, but significant level of Cγ2b-containing transcripts were detected in the LPS-treated B cells from $I\gamma2b^{-/-}$ chimeras (<5% the level found in RNA from normal LPS-treated B cells; Figure 5B).

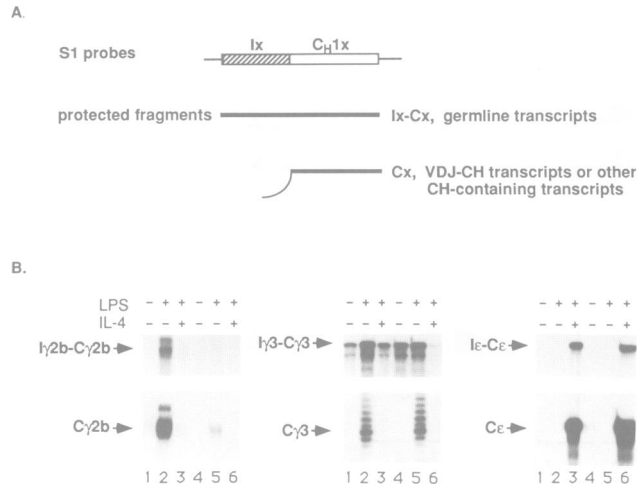


**Fig. 5.** Iγ2b germline transcripts are abolished in the Iγ2b$^{-/-}$ chimeras. Total RNA was isolated from untreated splenocytes and splenic B cells cultured with LPS or LPS plus IL-4 for 5 days. S1 nuclease protection analyses were performed with ssDNA probes for expression of germline and VDJ-γ2b, γ3 and ε transcripts. (**A**) Schematic view of the S1 probes. x strands for γ2b, γ3 or ε. (**B**) Probes for γ2b and γ3 were hybridized to the same RNA samples simultaneously to analyze expression of γ2b and γ3 transcripts. Analysis for ε expression was done separately, but to the same RNA samples. Three B6/CBA mice, two Iγ2b$^{+/+}$ chimeras and five Iγ2b$^{-/-}$ chimeras were analyzed. Lanes 1–3, B6/CBA; lanes 4–6, Iγ2b$^{-/-}$ chimera; lanes 1 and 4, untreated; lanes 2 and 5, treated with LPS; lanes 3 and 6, treated with LPS plus IL-4.

Based on Northern analyses, the LPS-induced Cγ2b-containing transcripts in the Iγ2b$^{-/-}$ cells were similar in size to that of the secreted form of normal VDJ-Cγ2b transcripts (data not shown). In addition, analyses with various probes from the Cγ2b gene showed specific hybridization or protection, indicating that the detected transcripts did not derive from cross-hybridization to transcripts from another γ constant region gene (data not shown). Therefore, the replacement of the Iγ2b promoter and exon greatly reduced, but did not completely eliminate, the LPS induced accumulation of transcripts that include the Cγ2b gene and that probably derive, at least in part, from a very low level of switching to γ2b by the mutant cells (see below).

We also assayed for expression of the *neo*$^r$ gene that replaced the Iγ2b promoter and exon. Little expression of the *neo*$^r$ gene was observed in untreated spleen cells. However, expression of this gene was strikingly increased in LPS-stimulated populations and this increase was inhibited by the addition of IL-4 along with LPS (data not shown). Therefore, expression of the integrated *neo*$^r$ sequences appeared to be regulated similarly to the normal Iγ2b exon that it replaced with respect to LPS or LPS plus IL-4 treatment, suggesting that the elements responsive to these treatments are distal to the Iγ2b promoter *per se*.

### *Switching to γ2b is drastically reduced in Iγ2b$^{-/-}$ spleen cells*

To examine class-switch recombination events directly at the Sγ2b locus in the homozygous mutant, we have taken advantage of the observation that class-switch recombination events to a particular S region places the Iμ exon (Lenon and Perry, 1985) just upstream of the corresponding $C_H$ gene and leads to the generation of 'hybrid germline transcripts' in which the Iμ exon is fused by RNA splicing to the downstream $C_H$ exon (Li *et al.*, in preparation; Figure 6A). To assay for switching by this method, RNA samples from unstimulated, LPS-stimulated, or LPS plus IL-4-stimulated spleen cells from Iγ2b$^{+/+}$ or Iγ2b$^{-/-}$ chimeras were assayed for the ability to protect an Iμ–Cγ2b probe in an S1 nuclease protection assay. Because the Cγ2b portion of the probe cross-hybridizes to portions of the Cγ1 and Cγ3 $C_H$1 exons, this assay permits detection of Iμ–Cγ2b transcripts (which are generated by switching to Sγ2b), Cγ2b-containing transcripts (from germline or VDJ-Cγ2b transcripts), Iμ–Cγ1 transcripts (which result from switching to Sγ1), Cγ1-containing transcripts (from germline or VDJ-Cγ1 transcripts), Iμ transcripts (which represent predominantly Iμ–Cμ transcripts), and Cγ3-containing transcripts (from germline or VDJ-Cγ3 transcripts) (Figure 6A).

In Iγ2b$^{+/+}$ spleen cells, LPS treatment induced Iμ–Cγ2b transcripts, Iμ transcripts, Cγ2b- and Cγ3-containing transcripts (Figure 6B), whereas LPS plus IL-4 treatment substantially blocked induction of these transcripts, but lead to accumulation of Iμ–Cγ1 and Cγ1 transcripts (Figure 6B). In the Iγ2b$^{-/-}$ spleen cells, the results for expression of Iμ–Cγ1, Cγ1, Iμ and Cγ3 transcripts were identical with respect to the various treatments to those obtained with spleen cells from Iγ2b$^{+/+}$ mice (Figure 6B). However, in the LPS-stimulated cells, Iμ–Cγ2b transcripts were barely detectable (<5% the level in the corresponding sample from Iγ2b$^{+/+}$ mice, Figure 6C) and, in agreement with the results described above, Cγ2b transcripts were present but at greatly decreased levels (Figure 6B). These results clearly demonstrate that the lack of IgG2b production by Iγ2b$^{-/-}$ B cells correlates with a dramatic decrease in ability to switch to Sγ2b. However, the generation of a low level of Iμ–Cγ2b transcripts indicates that a very low level of switching still occurs in the mutant animals, probably generating the normal sized γ2b transcripts detected on Northern blots and the IgG2b proteins detected by ELISA.

## Discussion

### *Deletion of the Iγ2b region results in a specific defect in switching to γ2b*

We have used gene targeted mutation of the Iγ2b exon to determine whether an intact Iγ2b region is required for normal class-switch recombination to Cγ2b. Previous studies of cell lines and splenic lymphocytes demonstrated that induction of transcription from the Iγ2b promoter precedes class-switch recombination to the γ2b gene (Lutzker *et al.*, 1988). Subsequent analyses of switching to other $C_H$ genes have also shown similarly organized germline transcription units that are expressed prior to the corresponding class-switch recombination events (Stavnezer *et al.*, 1988; Berton *et al.*, 1989; Esser and Radbruch, 1989; Gerondakis, 1990; Lebman *et al.*, 1990; Rothman *et al.*, 1990a,b). The conservation of these transcription units with respect to all $C_H$ genes that undergo the class-switch process suggested a role for germline transcription and/or transcripts in this recombination event. Our current studies clearly indicate that homozygous mutation of the Iγ2b promoter and Iγ2b exon results in a selective defect in class-switching to γ2b, unequivocally demonstrating the importance of the integrity

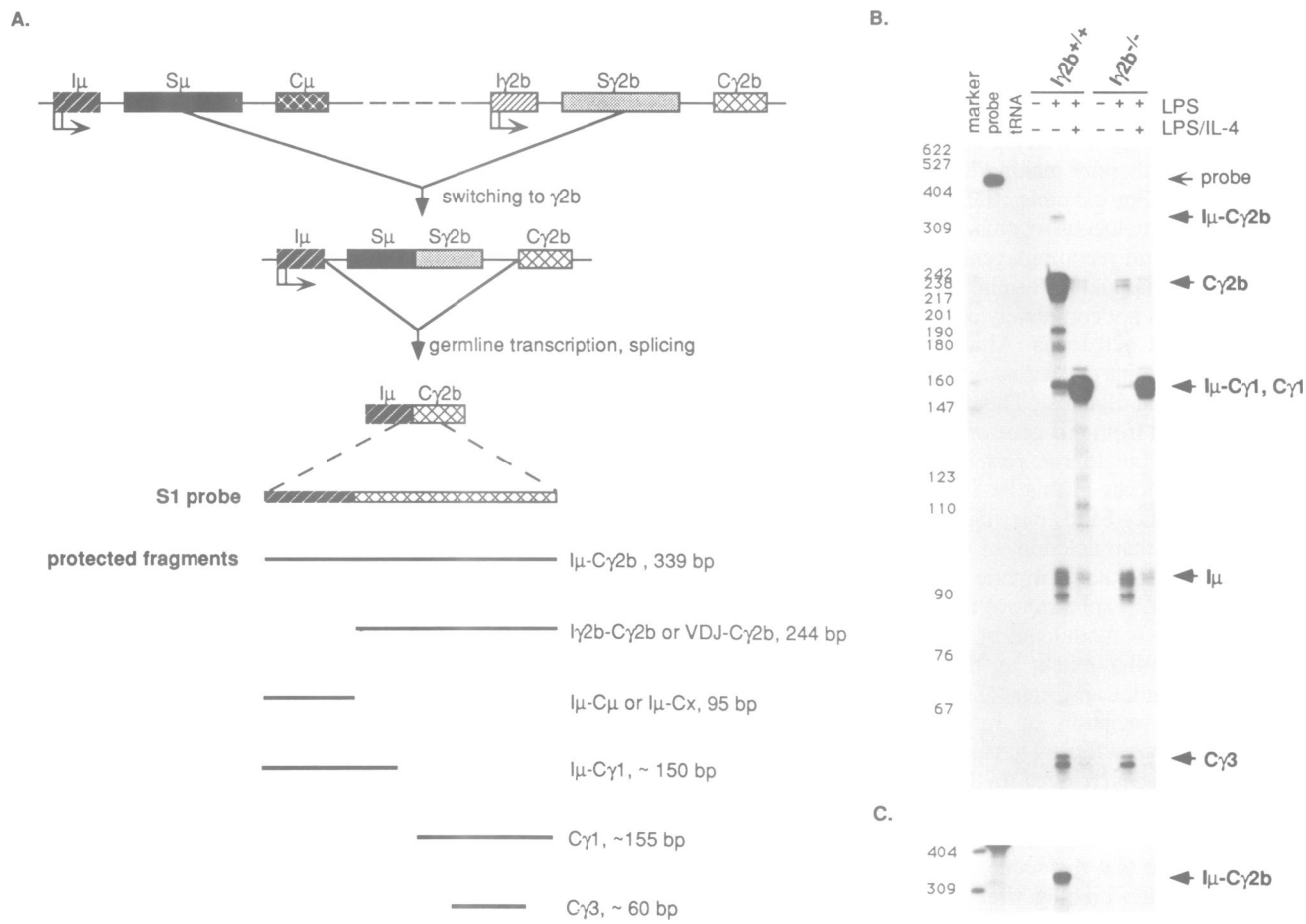


**Fig. 6.** Switching to γ2b is specifically blocked in $I\gamma2b^{-/-}$ B cells. (**A**) Schematic view of the Iμ-Cγ2b S1 probe. The various fragments protected by this probe are as indicated and discussed in the text. Open arrows indicate the direction of germline transcription in the I regions. (**B**) Total RNA was isolated from untreated splenocytes and splenic B cells treated with LPS or LPS plus IL-4 for 5 days. Two $I\gamma2b^{+/+}$ and two $I\gamma2b^{-/-}$ chimeras were analyzed with consistent results. The results from one of each are shown. (**C**) Longer exposure of part of gel between 309 and 404 bp.

of this region for class switching to Cγ2b. In a concurrent study, another group has come to similar conclusions regarding the function of the Iγ1 region; they demonstrated that replacement of a 1.5 kb segment of DNA which included sequences from the Iγ1 region through the first 100 bp of the γ1 switch region resulted in greatly diminished switching to the γ1 gene (Jung *et al.*, 1993).

The mutation that we have introduced replaces the Iγ2b promoter region, Iγ2b exon, and 3′ splice donor site with a *neo*$^r$ gene. However, the sequences replaced in our study lie nearly 1 kb 5′ of the Sγ2b sequences that are involved in class-switch recombination and ~6 kb 5′ of the Cγ2b gene which also remains unaltered. Furthermore, the region of DNA that we have mutated would be deleted by a normal class-switch recombination event and, therefore is not required for or in any way involved in the expression of a switched γ2b gene. Thus, all of the sequences that participate in the actual switch recombination event and the expression of the switched γ2b gene remain completely intact in the $I\gamma2b^{-/-}$ B cells. Correspondingly, we find that the effect of the introduced mutation is to drastically diminish class-switch recombination to the intact Sγ2b region, as determined by the extremely low level Iμ−Cγ2b transcripts in LPS-treated $I\gamma2b^{-/-}$ B cells. However, the mutant cells retained normal ability to switch to and express other upstream and downstream $C_H$ genes both *in vitro* and *in vivo*, indicating that the integrity of the Iγ2b region or switching to γ2b is not a prerequisite for these events. We conclude from these findings that the Iγ2b region is a controlling element that is specifically involved in regulating Ig heavy chain class switching to the Sγ2b region. Purified IgG1-producing B cells heterozygous for the Iγ1 deletion only switched on their normal allele, indicating that the mutation was *cis*-acting (Jung *et al.*, 1993). Our finding that splenic B cells heterozygous for the Iγ2b mutation switch to γ2b at levels ~50% that of wild-type is also consistent with the interpretation that this mutation is *cis*-acting.

### *Function of germline transcription and/or transcripts in targeting switching*

In the $I\gamma2b^{-/-}$ chimeras, we detected expression of IgG2b proteins but the levels were nearly several orders of magnitude below those of control mice. Likewise, we detected a low level of apparently mature Cγ2b transcripts by S1 and Northern blotting assays in LPS-treated splenic lymphocytes from $I\gamma2b^{-/-}$ cells. Finally, we also detected very low levels of Iμ−Cγ2b transcripts in homozygously mutated splenic lymphocytes following LPS treatment. Together, these findings indicate that the $I\gamma2b^{-/-}$ cells retain the potential to switch to γ2b in response to LPS treatment, but that, due to the mutation, this ability is greatly diminished. These findings have certain implications for the

function of the I region in controlling class-switch recombination.

In has been suggested that transcription from the I region promoter somehow predisposes the S region for recombination, perhaps by conferring an 'open' configuration to this region and, thereby making it accessible to recombination machinery. For example, transcription has been shown to stimulate homologous recombination events in yeast possibly by introducing recombinagenic single-strand breaks in the DNA (Thomas and Rothstein, 1989). The Iγ2b replacement mutation has not completely eliminated transcription in and around the Cγ2b locus. Although it faces in the opposite direction, the introduced *neo*$^r$ gene clearly can be transcribed in splenic B cells. Therefore, it seems possible that transcription from this gene or from other cryptic promoters may contribute to the residual level switching that still appears to occur at this locus.

On the other hand, our findings are also consistent with the notion that deletion of the Iγ2b exon *per se* may contribute to the dramatic decrease in switching. In particular, the expression of the *neo*$^r$ gene that replaces the Iγ2b region is modulated by LPS or LPS plus IL-4 treatment to similar extent as that of the endogenous Iγ2b promoter and transcripts (Zhang *et al.*, in preparation). Therefore, retention of functional LPS/IL-4-responsive elements is not sufficient to confer high level switching ability to the mutated γ2b locus. In this regard, it seems likely that the primary or processed Iγ2b transcripts may also be involved in regulating switch recombination process. It is also possible that the sequences deleted in this study may include certain binding site(s) for protein factors that are required for switching. More subtle mutations will allow these various possibilities, none of which are mutually exclusive, to be further distinguished.

### *Use of the RAG-2-deficient blastocyst system to analyze regulation of switch recombination*

We have utilized the RAG-2-deficient blastocyst complementation procedure to generate somatic chimeras with homozygous mutant ES cells (Chen *et al.*, 1993). This system offers many advantages for studying the function of specific genes or regulatory sequences in lymphocytes. In particular, the method obviates the necessity of obtaining germline transmission and instead requires only the generation of homozygous mutant ES cells, which can be obtained quite readily by the increased G418 selection method (Mortensen *et al.*, 1992) or by sequential targeting strategies (Fields *et al.*, 1992). All of the mature lymphocytes in the resulting chimeras are derived from the mutant ES cells and the phenotypes observed in lymphoid tissues can be attributed solely to the mutation in the injected ES cells. Furthermore, the highly reproducible complementation in the resulting chimeras will allow analysis of many mutations in a short time period. Therefore, we can now introduce numerous mutations into the Iγ2b region to define how this controlling element functions by precisely testing the role of transcription versus transcripts in targeting class-switch recombination to the Cγ2b gene.

## Materials and methods

### *Vector construction*

A 10 kb genomic clone that contained the γ2b region (Figure 1A) was isolated from a λ phage library constructed from genomic DNA from CCE cells and subcloned into SKII pBluescript (Stratagene). A *neo* resistance gene (pMCNeo) (Stratagene) was inserted into a *Bam*HI and a *Hinc*II site replacing the entire Iγ2b exon, the splice donor site and 500 bp of sequences upstream of Iγ2b. A 6 kb upstream fragment was further subcloned into the *Bam*HI site to lengthen the 5′ homologous region in the construct. The resulting 9 kb fragment including the *neo*$^r$ gene was then cloned into a pGK-HSVtk vector (Shinkai *et al.*, 1992) to generate the final targeting construct (6UTN) (Figure 1B). The construct was linearized at a *Not*I site before transfection into ES cells.

### *Transfection and screening of Iγ2b mutant ES cell lines*

Transfection and screening for the heterozygous mutants were done as previously described (Shinkai *et al.*, 1992). One Iγ2b$^{+/-}$ clone (#404) was obtained and then plated at $10^3$ and $10^4$ cells/plate and selected in 4.8 mg/ml G418 to screen for homozygous mutants by Southern analyses. Four Iγ2b$^{-/-}$ clones were obtained and one clone (U16) was further subcloned to ensure homogeneity. Three of the subclones (U16F, U16G, U16I) were used for injection. The Iγ2b$^{+/+}$ CCE clones have one copy of the terminal-deoxynucleotidyl-transferase (TdT) mutated and chimeras generated from these clones have normal N regions in all the joints so far examined in both B and T cells (Komori *et al.*, 1993).

### *Generation of chimeric mice*

Three subcloned Iγ2b$^{-/-}$ ES cell lines, one Iγ2b$^{+/-}$ and two Iγ2b$^{+/+}$ ES cell lines (CCE from 129 strain) were injected into blastocysts from the RAG-2-deficient mice (mixed background of 129Sv and C57BL/6) and transplanted into foster mothers (B6/CBA) as described (Chen *et al.*, 1993).

### *Flow cytometry analysis*

Single cell suspensions from the spleens of tested mice were prepared as previously described (Parks *et al.*, 1986) and stained for IgM and B220 surface expression (Figure 2B). Four normal mice (B6/CBA), three chimeric mice from Iγ2b$^{+/+}$ ES cells, two chimeras from Iγ2b$^{+/-}$ ES cells, seven chimeras from Iγ2b$^{-/-}$ ES cells were used for the analysis. Splenic B cells were cultured with 10 μg/ml LPS or 10 μg/ml LPS plus 50 ng/ml rIL-4 for 5 days at $5\times10^5$ cells/ml (Lutzker *et al.*, 1988). Cells from 5 day old culture were washed in PBS/5% FCS twice and stained with various antibodies conjugated with fluorescein (B220, IgG1), phycoerythrin (IgM) and biotin (IgG2b, IgG3, IgE) (PharMingen). Biotin conjugates were revealed by PE−streptavidin (PharMingen). All staining was done using 0.25 μg antibodies for $5\times10^5$ cells. Live cells were gated and the data were analyzed by FACSCAN and LYSYS software and are presented as dot plots.

### *ELISA assays*

Supernatants from LPS or LPS plus IL-4 cultures that were used for FACS analyses above and sera from normal mice (B6/CBA, 129Sv) and chimeric mice (from Iγ2b$^{-/-}$, Iγ2b$^{+/-}$ and Iγ2b$^{+/+}$ ES clones) were analyzed for expression of different Ig isotypes by ELISA as described (Burstein *et al.*, 1991). Serum samples were diluted at 1:200, 1:1000, 1:5000 and supernatant samples were diluted at 1:5 and 1:10. For detection of IgG2b proteins in the Iγ2b$^{-/-}$ samples, serum samples were diluted at 1:5, 1:25, 1:125, and supernatant samples were undiluted. For supernatant ELISA, the medium used for culture (RPMI with 10% FCS and supplemented with L-glutamine) was used as negative control; for serum ELISA, serum samples from RAG-2-deficient mice were used as negative control. Lower limit of detection is 80 ng/ml. All data are presented after subtraction of the negative control. Various monoclonal antibodies used in the assay were from Southern Biotechnology Associate Inc., Zymed and PharMingen.

### *S1 nuclease protection assays*

M13 ssDNA probes for Iγ2b−Cγ2b, Iγ3−Cγ3, Iε−Cε and riboprobe for Iμ−Cγ2b were prepared as described (Lutzker and Alt, 1988b; Rothman *et al.*, 1990a, b; Li *et al.*, in preparation). Hybridization, S1 digestion and analysis on 6% polyacrylamide−7 M urea gels were performed as previously described (Kohl *et al.*, 1986). Quantifications were done on a PhosphorImager (molecular Dynamics).

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