The Journal of Immunology

# Direct Repression of *prdm1* by Bcl-6 Inhibits Plasmacytic Differentiation

Chainarong Tunyaplin, A. L. Shaffer, Cristina D. Angelin-Duclos, Xin Yu, Louis M. Staudt and Kathryn L. Calame



*J Immunol* 2004; 173:1158-1165; ;
doi: 10.4049/jimmunol.173.2.1158
http://www.jimmunol.org/content/173/2/1158



*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

# Direct Repression of *prdm1* by Bcl-6 Inhibits Plasmacytic Differentiation[1]

**Chainarong Tunyaplin,* A. L. Shaffer,† Cristina D. Angelin-Duclos,[2]* Xin Yu,† Louis M. Staudt,† and Kathryn L. Calame[3]***

**We have identified two intronic regions of mouse *prdm1*, the gene encoding B lymphocyte-induced maturation protein-1 (Blimp-1), which confer transcriptional repression in response to Bcl-6. The Bcl-6 response element in intron 5, which is conserved between mice and humans, was studied in detail. It binds Bcl-6 in vitro and was shown by chromatin immunoprecipitation to be occupied by Bcl-6 in vivo. Neither Bcl-6 response element functions as a STAT3-response element, showing that STAT3 does not compete with Bcl-6 at these sites. Bcl-6$^{-/-}$ mice confirm the biological importance of Bcl-6-dependent repression of *prdm1*. These mice have elevated Ab response, increased Ig-secreting cells, and increased Blimp-1$^{+}$ cells in spleen following immunization and their splenic B cells show accelerated plasmacytic development in vitro.** ***The Journal of Immunology*****, 2004, 173: 1158–1165.**

B lymphocyte-induced maturation protein-1 (Blimp-1)[4] is a transcriptional repressor that plays a central role in the terminal differentiation of B cells to plasma cells (1). Ectopic expression of Blimp-1 in lymphoma cell lines or in primary murine splenic B cells is sufficient to induce differentiation to Ig-secreting plasma cells (2, 3). Recently, studies in mice lacking Blimp-1 in mature B cells showed severe defects in pre-plasma memory cells, plasma cells, and Ig secretion (4). Therefore, Blimp-1 is considered the "master regulator" of plasmacytic differentiation. It has been suggested (5–7) that repression of Blimp-1 in germinal center (GC) B cells is important to delay plasmacytic differentiation and to allow affinity maturation and class switch recombination (CSR).

Several B cell targets of Blimp-1 have been identified. Blimp-1 represses c-*myc* via a binding site located 290 bp from P1 (8). Repression of c-*myc* and cessation of cell cycle is required but not sufficient for plasmacytic differentiation (9). Blimp-1 also represses *CIITA* promoter III (3) and the *PAX-5* promoter (10). More recently, microarray studies in B cells have revealed several gene expression programs that are altered in response to Blimp-1 including: repression of proliferation genes, repression of genes required for GC or activated B cells, and induction of genes necessary for Ig secretion (11).

Although Blimp-1 is found in all plasma cells–those resulting from both T-independent and T-dependent Ags, in both primary and secondary responses–it is not detected in early bone marrow B cells, memory B cells in spleen, and most GC B cells (12). However, a small subset of GC B cells, possibly committed to a plasmacytic fate, express Blimp-1. Little is known about regulation of *prdm1* except that developmental stage-specific expression in B cells is determined at the transcriptional level (13). Recent studies have shown that Bcl-6 can repress Blimp-1 expression (5–7). The transcriptional repressor Bcl-6 is abundant in GC B cells and is required for GC formation, affinity maturation, and CSR (14, 15). Ectopic expression of Bcl-6 inhibited induction of Blimp-1 mRNA and plasmacytic differentiation in Bcl-1 lymphoma cells and primary splenocytes (6). A blocking form of Bcl-6 induced Blimp-1 mRNA in the presence of cycloheximide, suggesting direct repression by Bcl-6 (5). Because IL-6 is important for plasma cell differentiation, it was suggested that STAT3, activated by IL-6, might compete with Bcl-6 for binding to sites in *prdm1*, similar to competition between STAT6 and Bcl-6 on the Iε promoter (16). A recent study by Vasanwala et al. (17) showed that Bcl-6 can repress the human *PRDM1* gene by interfering with the AP-1 activator.

In this study, we identify two intronic regions in mouse *prdm1* that confer Bcl-6-dependent repression. One of the two Bcl-6 response elements (BREs) was found to be conserved between mice and humans. Bcl-6 binds to this site both in vitro and in vivo. Neither of the two regions that confer Bcl-6-dependent repression functions as a STAT3-response element. These data establish a molecular mechanism for direct repression of *prdm1* by Bcl-6. In addition, *Bcl-6*$^{-/-}$ mice have elevated Blimp-1$^{+}$ plasma cells in vivo and in vitro, establishing the functional importance of this repression.

## Materials and Methods

### *Plasmids*

A reporter containing the Blimp-1 basal promoter was described (13). A series of Blimp-1 reporters was generated by cloning genomic regions (fragments 1–5 in Fig. 1) 5′ of the basal promoter. The 570-bp fragment containing BRE1 was generated by PCR from fragment 3 using primers 5′-TGGAGATGTCAACTGGAATT-3′ and 5′-CCCAGCACGGCACCATCCATGC-3′. The 330-bp fragment containing BRE2 was generated by PCR from fragment 4 using primers 5′-CCGGTACCTAGAAATCAGTTGACC-3′ and 5′-CCGAGCTCAGCTGACTAATCACAGGAG-3′.

*Departments of Microbiology and Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, New York, NY 10032; and †Metabolism Branch, Division of Clinical Sciences, National Cancer Institute, Bethesda, MD 20892

Received for publication December 29, 2003. Accepted for publication May 10, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants RO1-AI43576 and RO1-50659 (to K.L.C.).

[2] Current address: Laboratoire de Biologie Moleculaire et Cellulaire Ecole Normale Supérieure de Lyon, Lyon, France.

[3] Address correspondence and reprint requests to Dr. Kathryn L. Calame, Departments of Microbiology and Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, 701 West 168th Street, Hammer Health Science Center 1202, New York, NY 10032. E-mail address: klc1@columbia.edu

[4] Abbreviations used in this paper: Blimp-1, B lymphocyte-induced maturation protein-1; GC, germinal center; CSR, class switch recombination; BRE, Bcl-6 response element; ChIP, chromatin immunoprecipitation; ko, knockout; NP-KLH, nitrophenyl-keyhole limpet hemocyanin; AP, alkaline phosphatase; 4-OHT, 4-hydroxytamoxifen.



0022-1767/04/$02.00

PCR products were sequence-verified and cloned upstream of the Blimp-1 basal promoter. The expression vectors for Bcl-6 (pMT2-Bcl-6) and the control vector (pMT2) were described (18). All transfections have been performed with at least two independent preparations of plasmid.

### *Site-directed mutagenesis*

Site-directed mutagenesis of the BRE1 was performed as described (19). BRE1 deletion in the context of 330-bp *Xba*I/*Pvu*II was generated by using primers 5′-CCGGTACCTAGAAATCAGTTGACC-3′, 5′-CCCCTAAGAC TACAAGCTGCAGTAGGCAAAACAA-3′ and 5′-CCGAGCTCAGCT GACTAATCACAGGAG-3′. The mutated BRE1 was then cloned into the Blimp-1 basal promoter reporter. BRE1 deletion in the context of 5-kb *Sac*I was generated by using primers 5′-CTGCTTTCTCGGTTTCAGTTGA GCTCGAAAATGTGAGTCGGCATA-3′, 5′-AGCAGCTGACTAATCAC AGG-3′ and 5′-GTGGGTAGGTACCTGCCCAA-3′. Then, a 700-bp *Avr*II/ EcoR5 from the 5-kb *Sac*I was replaced with the same fragment from the site-directed mutated PCR product.

### *Cell lines and transfection*

Plasmacytoma cell line, P3X, was used for Bcl-6 cotransfection experiments. Cells were maintained in RPMI 1640 supplemented with 10% FCS, 50 $\mu$M 2-ME, and 10 $\mu$g/ml gentamicin. Cells ($3 \times 10^6$) were pulsed in 300 $\mu$l of complete media at 960 $\mu$F and 240 V (Bio-Rad, Richmond, CA). The molarity of different reporters was kept constant at 1.3 pmol. Bcl-6 expression vector (pMT2-Bcl-6) or control expression vector (pMT2) were cotransfected at a constant amount of 0.3 pmol. The total amount of DNA transfected was adjusted to 30 $\mu$g with pMT2. After transfection, cells were resuspended in 5 ml of complete media and harvested 16–18 h later. To correct for transfection efficiency, 0.3 $\mu$g of RL-tk (Promega, Madison, WI) was included in each transfection. After the incubation period, cells were harvested in 1× passive lysis buffer (Promega). Firefly luciferase activity was measured using standard conditions (25 mM Gly-Gly, 15 mM $MgSO_4$, 4 mM EGTA, 10.7 mM $KPO_4$, 1 mM DTT, 1.43 mM ATP, and 0.057 mM luciferin) in a Berthold LB9501 luminometer. The firefly luciferase activity was quenched by the addition of NaCl to 0.5 M. Then, 25 pmol of coelenterazine were added to the reaction and the Renilla luciferase activity was measured in the luminometer. WI-L2-Bcl-6 cells have been described (5). Cells were maintained in Phenol Red-free RPMI 1640 supplemented with 10% charcoal/dextran-treated FCS (HyClone, Logan, UT) and 10 $\mu$g/ml gentamicin. Cells are transfected as described (14) with slight modification. Immediately after transfection, cells were divided in halves. One half was fed with complete media while the other half was fed with complete media and 50 nM 4-hydroxytamoxifen. Cells were harvested at 20 h posttransfection and luciferase activity was determined as described above.

HepG2 cells were used in STAT3 activation experiments. The cells were maintained in DMEM supplemented with 10% FCS, 1× nonessential amino acid, 10 mM sodium pyruvate and 10 $\mu$g/ml gentamicin. Cells ($2.5 \times 10^5$) were transfected with 0.3 pmol of luciferase reporters using the calcium phosphate method. Cells were transfected for 8 h before the medium was changed. After 24 h, cells were treated with 10 ng/ml IL-6 (a gift from Dr. C. W. Schindler, Columbia University, New York, NY) to activate STAT3 and harvested 8 h later. In the DN-STAT3 experiment, 3 $\mu$g of YF-STAT3 expression vector was cotransfected. One microgram of RL-null (Promega) was cotransfected in each transfection to correct for transfection efficiency. The luciferase activity was determined as described above.

### *DNaseI footprinting*

A 332-bp radioactive plus strand probe was generated by PCR from the 5-kb *Sac*I using primers 5′-CCATCTAGAATCAGTTGACC-3′ and 5′-CAGCTGACTAATCACAGGA-3′, of which the former was radiolabeled by standard kinase reaction. DNaseI footprinting: the footprinting was performed as described in SureTrack instruction (Pharmacia, San Diego, CA) with the following modifications. A $2 \times 10^4$ cpm probe was used. For each probe, 60, 80, or 100 ng of GST-Bcl-6 or 100 ng of GST were incubated with the probes in a 10-$\mu$l reaction (16) at room temperature for 20 min. Then, 40 $\mu$l of 1× binding buffer was added before being subjected to digestion by 0.006 or 0.012 U of DNaseI (Worthington Biochemical, Lakewood, NJ). A GA ladder was generated with a $4 \times 10^4$ cpm probe as described in the instructions (Pharmacia).

### *Chromatin immunoprecipitation (ChIP) analysis*

The WI-L2 B cell lymphoma cell line expressing FLAG-tagged Bcl-6 fused to the estrogen receptor ligand binding domain has been described (5). Twenty million WI-L2 cells were incubated with 1 $\mu$M 4-hydroxytamoxifen or left untreated for 2.5 h to induce nuclear translocation of Bcl-6. After the induction, ChIP were performed essentially as described (10) except that the formaldehyde cross-linking was performed at 37°C for 30 min. The chromatin was immunoprecipitated in duplicate to control for the variability within the experiment. The conditions for amplifying BRE1 are 94°C, 30 s; 55°C, 30 s; and 72°C, 30 s for 35 cycles. The conditions for amplifying CSF-1 and *PRDM1* exon 7 are 94°C, 30 s; 55°C, 30 s; and 72°C, 30 s for 38 cycles. Primers for BRE1 PCR are BRE1-up: 5′-TGCTTTCTCAGTT TCAGTTG-3′ and BRE1-down: 5′-TTAAATGGCTGTAGGCGGAC-3′. Primers for *PRDM1* exon 7 are 5′-GCCAAGTTCACCCAGTTTGT-3′ and 5′-GATTCGGGTCAGATCTTCCA-3′. Primers for CSF-1 PCR have been described (10). The PCR products were separated on 1.5% agarose in 1× Tris-phosphate EDTA blotted, and probed with either BRE1-down, exon 7 internal probe (5′-CAAGGTTCACCTGAAAGGGA-3′) or CSF-1-up primers.

### *Comparison of* prdm1 *genes*

Genomic sequences for human and mouse *prdm1* were retrieved from www.ensembl.org and compared by bl2seq (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) using the default parameter.

### *Bcl-6 knockout (ko) mice and immunization*

Bcl-6 ko mice were described previously (14). Three- to 4-wk-old Bcl-6 heterozygous (control) or ko littermates were injected sterilely i.p. with 100 $\mu$g of nitrophenyl-keyhole limpet hemocyanin (NP-KLH) in alum. After the times indicated, mice were sacrificed, their spleens removed, fixed, and stained as described below.

### *Immunohistochemistry*

Syndecan-1 and λ single staining: splenic sections were incubated with anti-syndecan-1 (BD Pharmingen, San Diego, CA) or anti-λ (Southern Biotechnology Associates, Birmingham, AL) overnight at room temperature. The primary Abs were detected with alkaline phosphatase (AP)-conjugated secondary Abs and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate. Blimp-1 and sydecan-1 double-staining: splenic sections were incubated with rabbit anti-Blimp-1 overnight at room temperature. The anti-Blimp-1 Ab was detected with an AP-conjugated secondary Ab and NBT/BCIP. After color development, the AP was inactivated by boiling in 10 mM EDTA. Slides were then incubated with anti-syndecan-1 (BD Pharmingen) which was detected with AP-conjugated secondary Ab and Fast Red substrate.

### *Bcl-6 heterozygous and ko B cell in vitro culture*

Splenic B cells from 3- to 4-wk-old Bcl-6 heterozygous and ko mice (11) were purified using anti-CD19 magnetic beads (Miltenyi Biotec, Auburn, CA) (>98% purity, our unpublished data). Cells ($5 \times 10^6$/ml) were cultured in RPMI 1640 + 10% FCS, 2-ME, L-glutamine, penicillin and streptomycin, and 25 $\mu$g/ml LPS (Sigma-Aldrich, St. Louis, MO). After 24 h, aliquots of cells were analyzed by FACS as described (5) using anti-mouse CD19 and anti-mouse syndecan-1 (BD Pharmingen). After 72 h, supernatants were collected and the amount of Igs was quantitated using an isotyping CBA kit (BD Pharmingen).

### *Semiquantitative PCR for Blimp-1, cyclophilin A, and $\beta_2$-microglobulin*

Bcl-$6^{+/-}$ and Bcl-$6^{-/-}$ splenocytes were stimulated with LPS for 24 h, after which the RNA was purified using the TRIzol method (Invitrogen Life Technologies, Carlsbad CA). The cDNA was generated using the random-priming method and the cDNA was used in the semiquantitative PCR. The primers for Blimp-1 amplification have been described (11). The primers for cyclophilin A are 5′-CACCGTGTTCTTCGACATCA-3′ and 5′-ACCCTG GCACATGAATCCT-3′. The primers for $\beta_2$-microglobulin are 5′-AGAC TGATACATACGCCTGCAG-3′ and 5′-GCAGGTTCAAATGAATCT TCAG-3′.

## Results

### *Identification of Bcl-6 response elements in* prdm1

Vasanwala et al. (17) reported that Bcl-6 repressed Blimp-1 by associating with AP-1 and interfering with its function in the human *PRDM1* promoter. Of the two AP-1 binding sites in the human promoter, only one, located 1748 bp 5′ of the transcriptional start site, is conserved in the mouse promoter. However, a luciferase reporter containing the *prdm1* transcription initiation sites and the putative AP-1 site (−4086 to +212) did not show

Bcl-6-dependent repression (data not shown). This suggests that in the mouse *prdm1* gene, Bcl-6 may use a different mechanism to repress transcription.

Therefore, we sought to map functional BRE(s) in mouse *prdm1* by generating a series of luciferase reporters containing the *prdm1* promoter (−901 to +212; Ref. 13) and other portions of the gene starting from nucleotide +213 to ~6 kb 3′ to the poly(A) site. (Fig. 1*a*). Reporters were transiently cotransfected into P3X (a Blimp-1$^{+}$Bcl-6$^{-}$ plasmacytoma) with an expression plasmid encoding Bcl-6 or a control plasmid. Reporters containing either the 6.5-kb *Sac*I fragment (fragment 3) or the 5-kb *Sac*I fragment (fragment 4) were repressed by Bcl-6 (Fig. 1*b*), suggesting the presence of at least two BREs in mouse *prdm1*. No other region tested conferred a response to Bcl-6 (Fig. 1*b*). The location of these BREs was further mapped by additional deletion and cotransfection experiments to a 570-bp fragment in intron 3 and a 330-bp *Xba*I/*Pvu*II fragment in intron 5 (Fig. 2*a*). We compared the sequence of these two intronic regions between the mouse and human genes. Only the 330-bp *Xba*I/*Pvu*II fragment in intron 5 is conserved between mouse and human *prdm1* genes (Fig. 2*a*). No conserved region was found for the 570-bp fragment in intron 3 (Fig. 2*a*). Therefore, we focused our further studies on the conserved BRE in intron 5, which we call BRE1.

A second system was used to confirm the activity of BRE1. WI-L2-Bcl-6 is a human B cell lymphoma cell line engineered to express, constitutively, a Bcl-6 fusion protein containing the estrogen receptor ligand binding domain (Bcl-6-ERD) (5). Nuclear

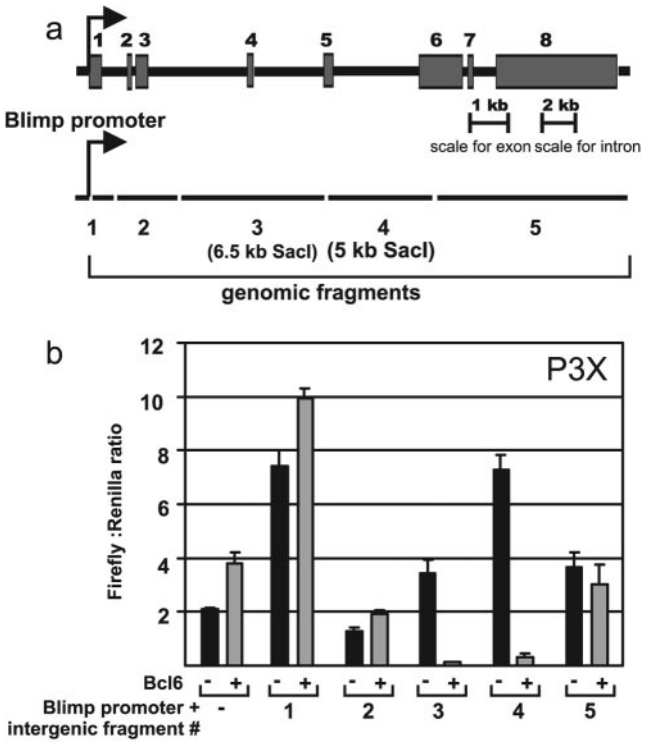


**FIGURE 1.** Mapping BREs in *prdm1*. *a*, Schematic representation of the locus. The arrow denotes transcriptional start site and gray boxes denote the eight exons (13). The scales for exons and introns are indicated. The five genomic fragments tested by transfection are indicated below the diagram. *b*, Bcl-6 repression by cotransfection. P3X cells were transiently cotransfected with *prdm1*-based luciferase reporters and a Bcl-6 expression vector (+) or control vector (−). The numbers below the graph denote the genomic fragments in the reporters. The data were corrected for transfection efficiency using Renilla luciferase. The mean ± SD of triplicate data points is shown.

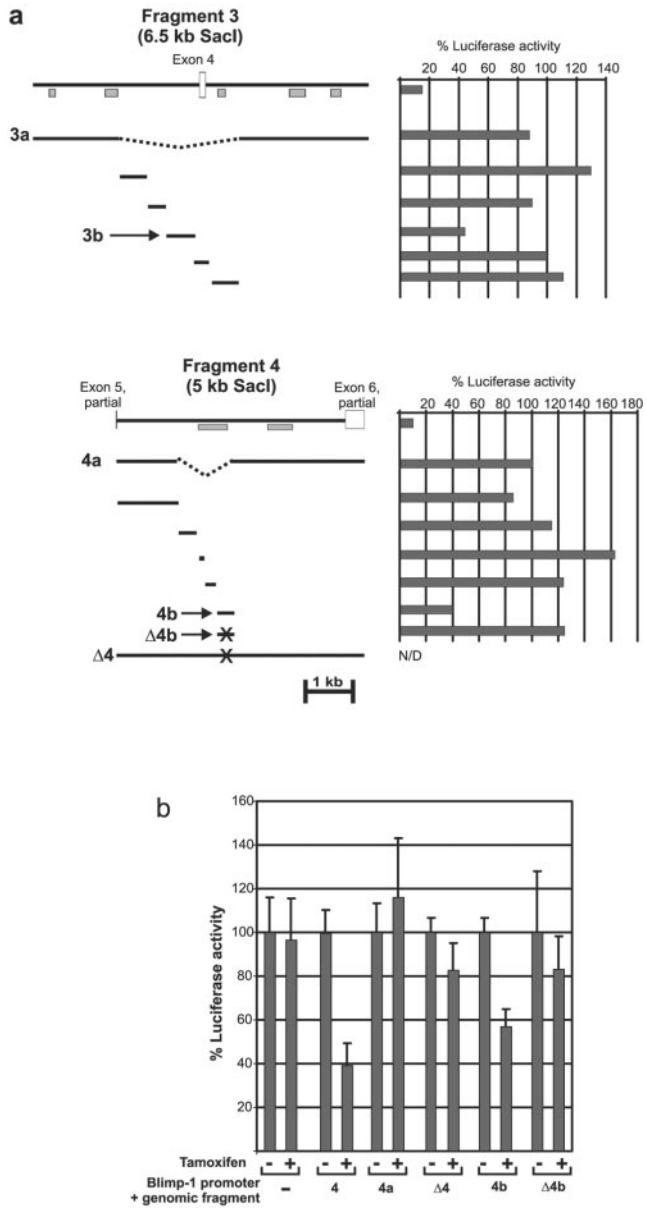


**FIGURE 2.** Mapping BREs. *a*, Schematic representation showing genomic fragment 3 (6.5-kb *Sac*I) and fragment 4 (5-kb *Sac*I), various deletion mutants, and Bcl-6-dependent repression as measured by transient cotransfection in P3X. Open boxes are exons. Bars under the gene indicate regions conserved between mouse and human (longer than 200 bp). Fragment 3b is a 570 bp generated by PCR located in intron 3. Fragment 3a is fragment 3 in which a 2.2-kb internal region had been deleted. Fragment 4a is fragment 4 in which the 770-bp internal region had been deleted. Fragment 4b is a 330-bp *Xba*I/*Pvu*II fragment from intron 5. Fragments Δ4b and Δ4 are fragments 4b and 4 in which a BRE1 footprint had been deleted. The activity of the reporters (after the normalization with Renilla luciferase) in the absence of Bcl-6 cotransfection is adjusted to 100% (not shown in the figure). The activity of the reporters in the presence of Bcl-6 cotransfection is normalized to the percentage of the activity in the absence of Bcl-6. *b*, Transient transfection of the *prdm1*-based luciferase reporters containing the Blimp-1 basal promoter and the wild-type or mutated BREs in WI-L2-Bcl-6 cells (see *Results*). The activity of the reporters (after the normalization with Renilla luciferase) in the absence of Bcl-6 activation with tamoxifen is shown as 100%. The activity of the reporters in the presence of Bcl-6 (+tamoxifen) is normalized to the percentage of the activity in the absence of Bcl-6. The data shown represent the mean ± SD of three to five transfection experiments.

translocation and activation of Bcl-6 can be induced in these cells by the addition of 4-hydroxytamoxifen (4-OHT). Transfection of *prdm1*-based reporters into WI-L2-Bcl-6 cells and subsequent treatment with 4-OHT removes experimental variability derived from cotransfection of a Bcl-6 expression plasmid. As shown in Fig. 2*b*, the *prdm1* reporter containing the 5-kb *Sac*I fragment with BRE1, is repressed when 4-OHT is added to the culture. 4-OHT addition did not repress a similar reporter containing an internal deletion in the 5-kb *Sac*I fragment that removed the 770-bp *Xba*I/*Pvu*II fragment containing BRE1 (fragment 4a; Fig. 2). Taken together, cotransfections in P3X and transfections in WI-L2-Bcl-6 are consistent with a BRE located within the 330-bp *Xba*I/*Pvu*II fragment in intron 5.

DNaseI footprinting, using rBcl-6, was used to identify the exact location of BRE1. Within the 330-bp *Xba*I/*Pvu*II fragment, one Bcl-6 footprint was observed in a 16-bp sequence located ~2100 bp 3′ of exon 5 (Fig. 3). This binding site has homology to the

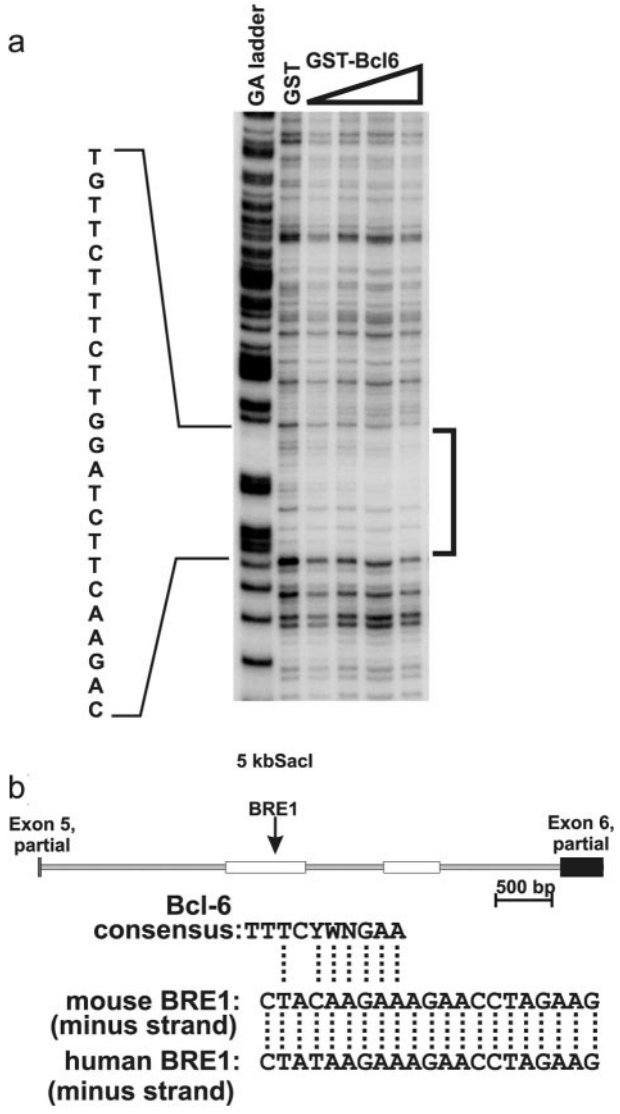


**FIGURE 3.** DNaseI footprinting of BRE1. *a*, DNaseI ladder of the BRE1 in the presence of either GST or GST-Bcl-6. The GA ladder of the probes was used to locate the footprints. *b*, The sequences of mouse and human BRE1 compared with consensus Bcl-6 binding site. The regions of homology (longer than 200 bp, opened boxes) between mouse and human *prdm1* are shown in the diagram. The 5-kb *Sac*I contains two regions of homology. The BRE1 is located in one conserved region in the intron 5.

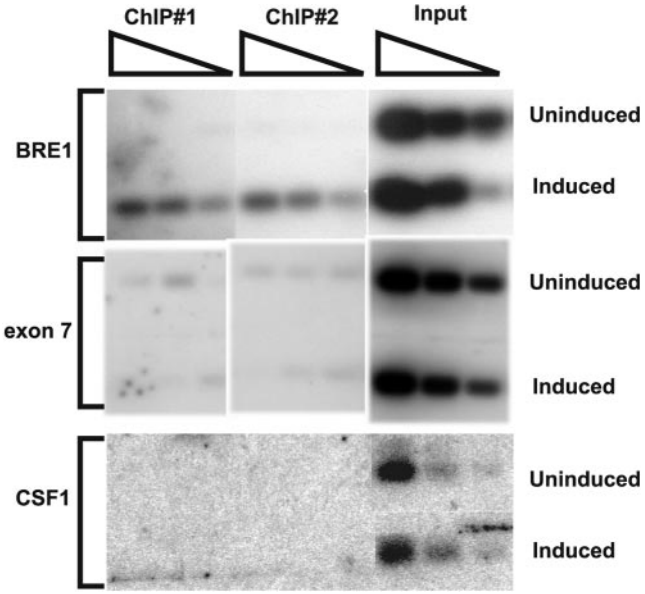


**FIGURE 4.** In vivo binding of Bcl-6 to BRE1. A ChIP experiment was performed on chromatin prepared from uninduced and tamoxifen-induced WI-IL-2-Bcl-6 cells using anti-FLAG (Bcl-6 is FLAG-tagged). Duplicate immunoprecipitations are shown (ChIP #1 and #2). The PCR product shown was amplified from 4-fold serial dilutions of input DNA and 2-fold serial dilutions of IP DNA. PCR for exon 7 of *prdm1* and the CSF-1 gene were used as negative control.

Bcl-6 binding consensus sequence (Fig. 3*b*) and a homologous sequence is present in intron 4 of human *PRDM1* (the equivalent of mouse intron 5). The binding site was altered using site-directed mutagenesis and tested for its ability to confer Bcl-6-dependent repression. In both P3X and WI-L2-Bcl-6 cells, the 330-bp *Xba*I/*Pvu*II fragment conferred Bcl-6-dependent repression but a mutated form of the 330-bp *Xba*I/*Pvu*II fragment containing site-directed deletion in the footprinted region did not confer Bcl-6-dependent repression (Fig. 2*b*). Further, the 5-kb *Sac*I fragment

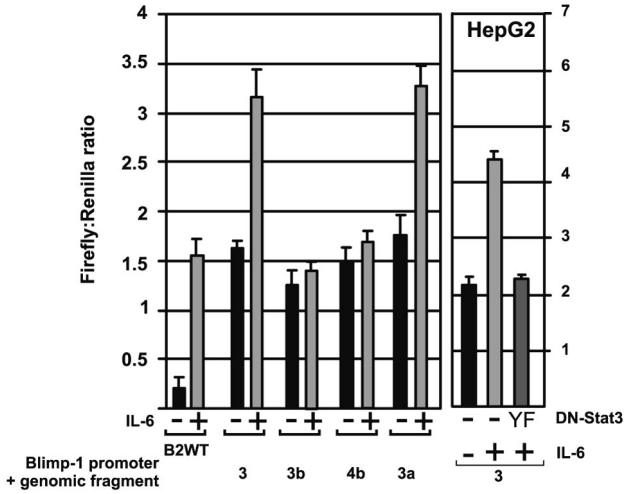


**FIGURE 5.** Lack of STAT3-dependent response of the BREs. Transient transfection of the *prdm1*-based luciferase reporters into HepG2. IL-6 was used to activate STAT3 in HepG2 cells. The reporters used are described in Fig. 2*a*. B2WT is a positive control luciferase reporter containing a hexamer of the consensus STAT3 binding site. YF denotes the DN-STAT3 that can no longer be phosphorylated and was used in a cotransfection experiment to interfere with STAT3 activation. The data were corrected for transfection efficiency using Renilla luciferase. The mean ± SD of triplicate data points is shown.

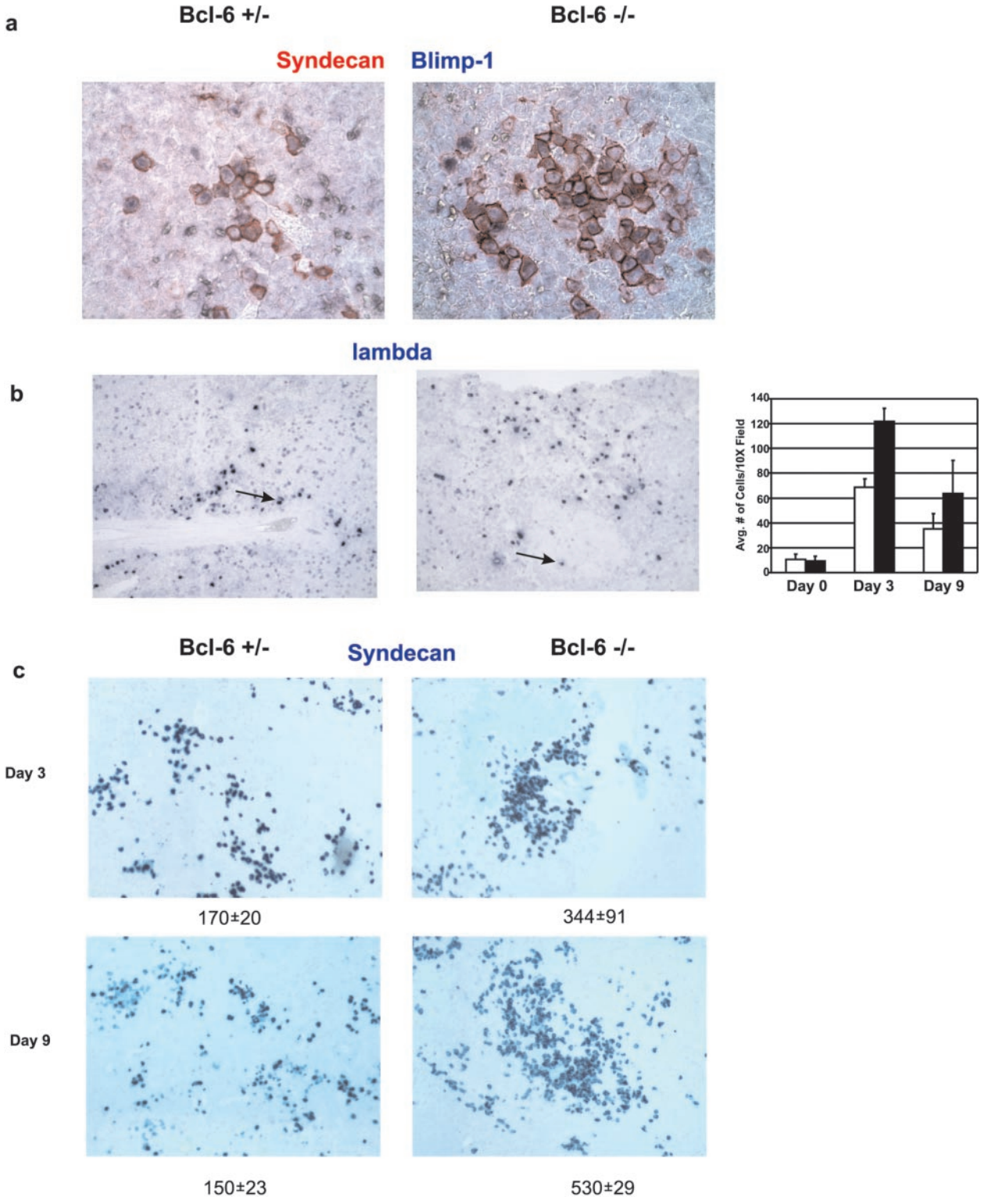


**FIGURE 6.** *(continued)*

with a deletion of BRE1 was not repressed by Bcl-6 (Fig. 2*b*). Taken together, these results establish BRE1 as a functional response element mediating direct Bcl-6-dependent repression of *prdm1*.

*Bcl-6 binds to BRE1 in vivo*

To test whether BRE1 functions in vivo, we performed ChIP assays. When chromatin from WI-L2-Bcl-6 cells was immunoprecipitated using an anti-FLAG (Bcl-6-ERD is FLAG-tagged), BRE1 was specifically coimmunoprecipitated in cells treated with 4-OHT but not from untreated control cells (Fig. 4). A control gene, CSF-1 and exon 7 of *PRDM1*, was not coimmunoprecipitated with anti-FLAG in induced cells. Thus, we conclude that Bcl-6 binds to BRE1 in vivo, providing strong evidence that BRE1 participates in Bcl-6-dependent transcriptional repression of *prdm1*.

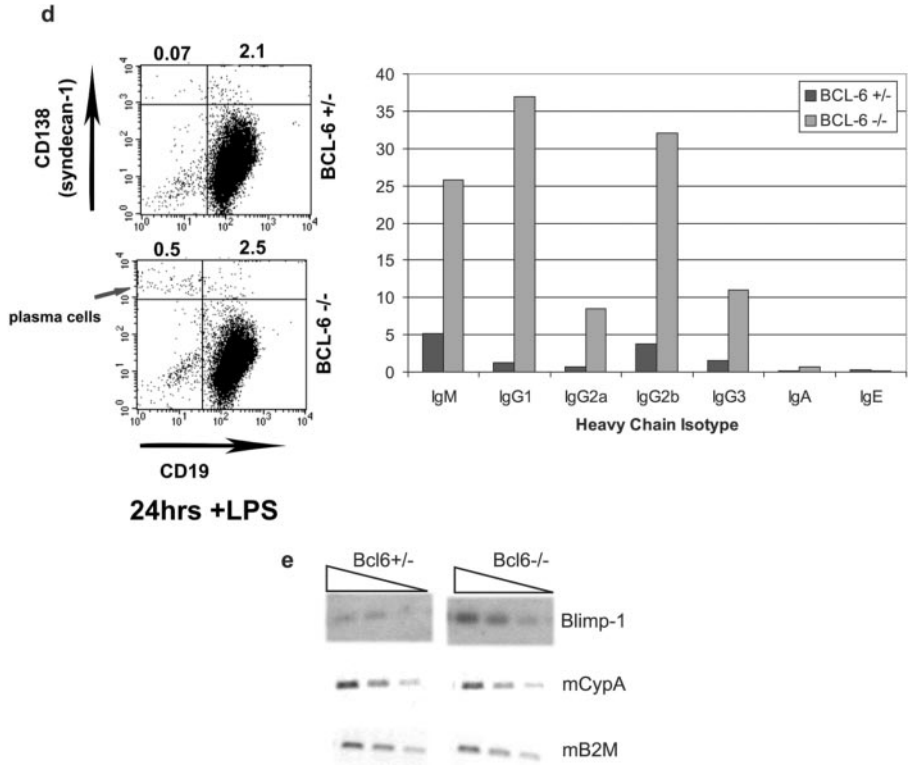


**FIGURE 6.** Enhanced plasmacytic differentiation in Bcl-6$^{-/-}$ mice. *a*, Representative spleen sections stained for Blimp-1 (blue) and syndecan-1 (red) 9 days postimmunization. *b*, Spleen sections stained for Ig λ (blue) day 3 postimmunization. Cells which stain faintly have surface expression of λ and those that stain darkly (one such cell is indicated by an arrow in each panel) have cytoplasmic λ, indicating that they are Ig-secreting cells. Ten to 20 microscopic fields were counted and the average number and the SD of cytoplasmic λ$^+$ cells per ×10 microscopic field is shown in the graph. *c*, Spleens from Bcl-6$^{-/-}$ and Bcl-6$^{+/-}$ littermates were collected at days 3 and 9 after immunization with NP-KLH and were stained for syndecan-1 (blue). The number of syndecan-1$^+$ cells in three random ×20 microscopic fields were counted and expressed as average cells per ×20 microscopic field ± SD. The slides were counterstained lightly with methyl green. *d*, Ability of Bcl-6$^{-/-}$ and Bcl-6$^{+/-}$ B cells to differentiate into plasma cells. Equal numbers of CD19$^+$ splenic B cells from heterozygous and Bcl-6 ko mice were stimulated with LPS. The number of CD19$^-$, syndecan-1$^+$ plasma cells was measured by FACS 24 h poststimulation. The level of secreted Ig was quantified from culture supernatants using isotyping CBA kit (BD Pharmingen) 72 h poststimulation. *e*, Blimp-1 mRNA in Bcl-6$^{-/-}$ splenocytes. Semiquantitative RT-PCR was performed for Blimp-1 (2-fold serial dilution), cyclophilin A (mCypA, 4-fold serial dilution) and $\beta_2$-microglobulin (mB2M, 4-fold serial dilution). The signal for Blimp-1 was detected by hybridization. The signal for mCypA and mB2M was detected by EtBr.

### *STAT3 does not compete with Bcl-6 binding at the identified BREs*

Interfering with STAT3 activity in Bcl-1 cells inhibits induction of Blimp-1 mRNA and plasmacytic differentiation (6). Because the consensus binding sites for Bcl-6 and STAT3 are similar, it was suggested that STAT3 may compete with Bcl-6 for binding and induce Blimp-1 by relieving Bcl-6-dependent repression. We tested this notion by transfecting reporters containing BRE1 on the 5-kb *Sac*I fragment or in the 330-bp *Xba*I/*Pvu*II fragment, into HepG2 cells, where STAT3 can be activated by IL-6 (20, 21). Neither the reporter with the 5-kb *Sac*I fragment nor the reporter with the 330-bp *Xba*I/*Pvu*II fragment (Fig. 5 and data not shown) was activated by IL-6. Furthermore, protein from nuclear extracts of HepG2 cells treated with IL-6 to activate STAT3 did not bind to an oligonucleotide corresponding to BRE1 although there was binding to an IFN-γ activation site control oligonucleotide (data not shown). We also tested the 6.5-kb *Sac*I fragment, which contains the BRE not conserved in the human gene. Although this fragment conferred activation in response to IL-6, the activity was present on mutant reporters lacking BRE activity. Thus, we conclude that the BRE elements identified in this study are unlikely to confer IL-6-dependent activation via competitive binding by STAT3.

### *Repression of* prdm1 *by Bcl-6 in vivo*

When human tonsils were stained for Bcl-6 and Blimp-1, nearly mutually exclusive expression was observed in GC B cells (data not shown), consistent with previous microarray data (5) and providing evidence that Bcl-6 represses Blimp-1 expression in vivo. To explore Bcl-6-dependent repression of murine *prdm1* in vivo, we investigated *Bcl-6*$^{-/-}$ mice. B cells from *Bcl-6*$^{-/-}$ mice do not form GCs and the mice ultimately succumb to a Th2-type inflammatory disease (14, 15). However, their ability to form plasma cells has not been thoroughly examined. We hypothesized that if Bcl-6-dependent repression of *prdm1* is important in vivo, activated B cells in *Bcl-6*$^{-/-}$ mice might display exaggerated and/or accelerated plasmacytic differentiation.

*Bcl-6*$^{-/-}$ mice survive poorly, limiting the numbers available for study; however, we were able to immunize a limited number of mice with the T-dependent immunogen NP-KLH. Mice were sacrificed 3 and 9 days postimmunization. Plasma cells in the spleen were analyzed using immunohistochemistry (Fig. 6) for surface syndecan-1 (red), nuclear Blimp-1 (blue), and for cytoplasmic λ (blue). Cells with cytoplasmic λ, used as a measure of Ig-secreting cells formed in response to NP-KLH, increased markedly on day 3 and were still significantly elevated on day 9 (Fig. 6*b*). At both times, there were 2-fold more Ig-secreting cells in the *Bcl-6*$^{-/-}$

mice than in *Bcl-6*$^{+/-}$ littermate controls. Similar increases were observed for syndecan$^{+}$ cells, which were also Blimp-1$^{+}$ (Fig. 6, *a* and *c*). We measured anti-NP IgM and IgG1 in the sera 3 days postimmunization in two independent experiments. The IgM anti-NP titer in the Bcl-6$^{-/-}$ mice was consistently ~2-fold higher than that of Bcl-6$^{+/-}$ littermate control in both experiments. Thus, a reproducible 2-fold increase in secreting cells and serum Ig was observed in *Bcl-6*$^{-/-}$ mice following immunization with NP-KLH.

The ability of purified splenic B cells to differentiate ex vivo in response to LPS was also analyzed. After CD19$^{+}$ splenic B cells were treated with LPS for 24 h, there were ~7-fold more CD19$^{-}$syndecan1$^{+}$ plasma cells in cultures from *Bcl-6*$^{-/-}$ mice compared with controls (Fig. 6*d*). This rapid plasmacytic differentiation of Bcl-6$^{-/-}$ B cells correlates with ~4-fold higher levels of Blimp-1 mRNA in LPS-stimulated *Bcl-6*$^{-/-}$ splenocytes (Fig. 6*e*). The amount of Ig secreted in cultures from *Bcl-6*$^{-/-}$ B cells was also significantly elevated, relative to controls, for all isotypes, except IgA and IgE (Fig. 6*d*). These data are consistent with the in vivo results and show accelerated Blimp-1 mRNA induction and plasmacytic differentiation in the absence of Bcl-6.

## Discussion

### *The mouse* prdm1 *gene is a direct target of Bcl-6-mediated transcriptional repression*

These studies demonstrate that the *prdm1* gene, encoding Blimp-1, is a direct target of the Bcl-6 transcriptional repressor. We have identified two regions, located in introns 3 and 5 of mouse *prdm1*, that confer Bcl-6-dependent repression. The Bcl-6 response element in intron 5 (BRE1) is conserved in the human gene and was shown to be bound by Bcl-6 both in vitro and in vivo. Our study adds *prdm1* to the list of IL-5, IL-18, B7-1, and Bcl-6 itself that are direct targets of Bcl-6 (22–26). Our results are consistent with previous microarray studies which identified *PRDM1* as a direct target of Bcl-6 repression (5).

We have provided strong evidence that Bcl-6 directly represses murine *prdm1* by binding to BRE1 located in intron 5. Based on the strong conservation of BRE1 in human *PRDM1*, the data suggest, but do not prove, that Bcl-6 may also bind and directly repress the human gene. Bcl-6 was previously shown to interfere with AP-1 activation in the human gene (17). Although we have no evidence that this also occurs in the murine gene, it is certainly possible that, in addition to the direct repression we have demonstrated, an AP-1-dependent mechanism or another mechanism may also be involved in Bcl-6-dependent repression of *prdm1*. Given the physiological importance of Bcl-6-dependent repression of Blimp-1 expression, to ensure complete affinity maturation, selection, and CSR in the GC, it is not surprising that multiple mechanisms may be involved.

Recently, an isoform of human and mouse Blimp-1 (Blimp-1$\beta$) that is functionally impaired was described (27). This truncated form is transcribed from an alternate promoter located in intron 4 of mouse *prdm1* resulting in a truncated protein that lacks the N-terminal acidic domain and part of the PRDI-BFI and RIZ homology domain (PR) domain. We do not know whether this promoter is also regulated by Bcl-6. However, the Blimp-1 antiserum we used in the Blimp-1/Bcl-6 double immunofluorescent staining (data not shown) was raised against a portion of Blimp-1 that is common to both full-length Blimp-1 (Blimp-1$\alpha$) and Blimp-1$\beta$ (1) and should recognize both isoforms, yet we do not see overlapping expression of Blimp-1 and Bcl-6 (data not shown). Therefore, it seems likely that Bcl-6 represses the expression of both isoforms of Blimp-1.

Dominant-negative STAT3 can functionally reverse IL-2/IL-5-dependent Blimp-1 induction and plasmacytic differentiation in the Bcl-1 cell line, indicating a possible role for STAT3 in the regulation of *prdm1* (6). Because the binding sites for all STAT proteins are similar (28) and Bcl-6 was shown to compete with STAT6 for binding and function of CD23b and I$\epsilon$ genes (14, 16), it was proposed that STAT3 might compete with binding of Bcl-6 to *prdm1*. This hypothesis is appealing because IL-6 and IL-10, which are important for differentiation of plasma cells, activate STAT3 (29, 30) and STAT3 activation in naive B cells in response to BCR cross-linking is mediated through IL-6 and IL-10 (31). However, we show that neither of the BREs identified in this study are STAT3 response elements in our assay system and STAT3 does not compete for binding to the identified BRE1 on *prdm1*. Thus, although STAT3 is likely to induce *prdm1*, induction does not appear to be mediated by the Bcl-6 response elements identified in this study.

### *Derepression of Bcl-6 as a trigger for plasmacytic differentiation*

Bcl-6-mediated repression appears to play a critical role in regulating Blimp-1 expression. Shaffer et al. (5) showed that expression of a blocking form of Bcl-6 was sufficient to induce Blimp-1 in Burkitt lymphoma cell lines. Further, treatment with a histone deacetylase inhibitor, which negates Bcl-6-mediated repression (32), induces Blimp-1 and terminal differentiation of the mature B cell line, L10A, and primary splenic B cells (33). By repressing *prdm1*, Bcl-6 plays a major role in preventing premature plasmacytic differentiation and ensuring the GC reaction is complete.

Ex vivo, purified splenic B cells from *Bcl-6*$^{-/-}$ mice showed enhanced plasmacytic differentiation and elevated Ig secretion (Fig. 6), suggesting that low levels of Bcl-6 in naive B cells may play a role in regulating plasmacytic differentiation. *Bcl-6*$^{-/-}$ mice also had more syndecan$^{+}$Blimp-1$^{+}$ cells (Fig. 5) in spleen after immunization with NP-KLH. We also found increased number of IgM anti-NP secreting cells (data not shown) in the *Bcl-6*$^{-/-}$ mice. These data provide strong support for the concept that one important function of Bcl-6 in GC B cells is to inhibit plasmacytic differentiation via repression of *prdm1* (5–7). Our data extend an earlier report that also showed increased plasmacytoid cells in spleens of *Bcl-6*$^{-/-}$ mice (15). Our data are also consistent with the finding of Toyama et al. (34) who showed an increase of Ag-specific IgM-secreting plasma cells in the bone marrow of the recipient mice that had received Bcl-6$^{-/-}$ B cells.

The extent of plasmacytic differentiation in vivo could have been underestimated in these studies due to effects of homeostatic mechanisms, including the limited capacity of the spleen to support plasma cells (35), that may limit Ig-secreting cells. Indeed, limited splenic capacity and/or consequences of hyperinflammation probably account for the lack of elevated serum Ig in *Bcl-6*$^{-/-}$ mice despite observed increases in plasmacytic development in spleen both in vivo and ex vivo.

What signals in normal B cells finally relieve Bcl-6-dependent repression of *prdm1* and allow plasma cells to develop? BCR cross-linking activates Erk-1, which phosphorylates Bcl-6 and targets it for proteasome-dependent degradation (36, 37). Perhaps acquisition of a high affinity BCR, via somatic hypermutation and selection, causes sufficient Bcl-6 degradation to derepress *prdm1* and allow Blimp-1-dependent plasmacytic differentiation. Another possible mechanism is by acetylation of Bcl-6 because Bcl-6 was shown to be acetylated, resulting in inactivation (32). Regardless of mechanism, once Bcl-6-dependent repression of *prdm1* is relieved, plasma cell fate can be maintained by a feedback loop in which Blimp-1 represses Bcl-6 expression (11).

## Acknowledgments

We thank Drs. Y. R. Zou and C. Schindler for critically reading the manuscript, Drs. R. Dalla-Favera and C. Schindler for plasmids and cell lines, Dr. G. Siu for assistance on DNaseI footprinting, and the members of Calame laboratory for helpful discussion.

## References

1. Turner, C. A., Jr., D. H. Mack, and M. M. Davis. 1994. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell 77:297*.
2. Schliephake, D. E., and A. Schimpl. 1996. Blimp-1 overcomes the block in IgM secretion in lipopolysaccharide/anti-$\mu$ $F(ab')_2$-co-stimulated B lymphocytes. *Eur. J. Immunol. 26:268*.
3. Piskurich, J. F., K. I. Lin, Y. Lin, Y. Wang, J. P. Ting, and K. Calame. 2000. BLIMP-I mediates extinction of major histocompatibility class II transactivator expression in plasma cells. *Nat. Immunol. 1:526*.
4. Shapiro-Shelef, M., K. I. Lin, L. J. McHeyzer-Williams, J. Liao, M. G. McHeyzer-Williams, and K. Calame. 2003. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity 19:607*.
5. Shaffer, A. L., X. Yu, Y. He, J. Boldrick, E. P. Chan, and L. M. Staudt. 2000. BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. *Immunity 13:199*.
6. Reljic, R., S. D. Wagner, L. J. Peakman, and D. T. Fearon. 2000. Suppression of signal transducer and activator of transcription 3-dependent B lymphocyte terminal differentiation by BCL-6. *J. Exp. Med. 192:1841*.
7. Fearon, D. T., P. Manders, and S. D. Wagner. 2001. Arrested differentiation, the self-renewing memory lymphocyte, and vaccination. *Science 293:248*.
8. Lin, Y., K. Wong, and K. Calame. 1997. Repression of c-*myc* transcription by Blimp-1, an inducer of terminal B cell differentiation. *Science 276:596*.
9. Lin, K. I., Y. Lin, and K. Calame. 2000. Repression of c-*myc* is necessary but not sufficient for terminal differentiation of B lymphocytes in vitro. *Mol. Cell. Biol. 20:8684*.
10. Lin, K. I., C. Angelin-Duclos, T. C. Kuo, and K. Calame. 2002. Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. *Mol. Cell. Biol. 22:4771*.
11. Shaffer, A. L., K. I. Lin, T. C. Kuo, X. Yu, E. M. Hurt, A. Rosenwald, J. M. Giltnane, L. Yang, H. Zhao, K. Calame, and L. M. Staudt. 2002. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity 17:51*.
12. Angelin-Duclos, C., G. Cattoretti, K. I. Lin, and K. Calame. 2000. Commitment of B lymphocytes to a plasma cell fate is associated with blimp-1 expression in vivo. *J. Immunol. 165:5462*.
13. Tunyaplin, C., M. A. Shapiro, and K. L. Calame. 2000. Characterization of the B lymphocyte-induced maturation protein-1 (Blimp-1) gene, mRNA isoforms and basal promoter. *Nucleic Acids Res. 28:4846*.
14. Dent, A. L., A. L. Shaffer, X. Yu, D. Allman, and L. M. Staudt. 1997. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science 276:589*.
15. Ye, B. H., G. Cattoretti, Q. Shen, J. Zhang, N. Hawe, R. de Waard, C. Leung, M. Nouri-Shirazi, A. Orazi, R. S. Chaganti, et al. 1997. The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. *Nat. Genet. 16:161*.
16. Harris, M. B., C. C. Chang, M. T. Berton, N. N. Danial, J. Zhang, D. Kuehner, B. H. Ye, M. Kvatyuk, P. P. Pandolfi, G. Cattoretti, et al. 1999. Transcriptional repression of Stat6-dependent interleukin-4-induced genes by BCL-6: specific regulation of iε transcription and immunoglobulin E switching. *Mol. Cell. Biol. 19:7264*.
17. Vasanwala, F. H., S. Kusam, L. M. Toney, and A. L. Dent. 2002. Repression of AP-1 function: a mechanism for the regulation of Blimp-1 expression and B lymphocyte differentiation by the B cell lymphoma-6 protooncogene. *J. Immunol. 169:1922*.
18. Cattoretti, G., C. C. Chang, K. Cechova, J. Zhang, B. H. Ye, B. Falini, D. C. Louie, K. Offit, R. S. Chaganti, and R. Dalla-Favera. 1995. BCL-6 protein is expressed in germinal-center B cells. *Blood 86:45*.
19. Smith, A. M., and K. P. Klugman. 1997. "Megaprimer" method of PCR-based mutagenesis: the concentration of megaprimer is a critical factor. *BioTechniques 22:438*.
20. Nakajima, K., Y. Yamanaka, K. Nakae, H. Kojima, M. Ichiba, N. Kiuchi, T. Kitaoka, T. Fukada, M. Hibi, and T. Hirano. 1996. A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells. *EMBO J. 15:3651*.
21. Bromberg, J. F., M. H. Wrzeszczynska, G. Devgan, Y. Zhao, R. G. Pestell, C. Albanese, and J. E. Darnell, Jr. 1999. Stat3 as an oncogene. *Cell 98:295*.
22. Arima, M., H. Toyama, H. Ichii, S. Kojima, S. Okada, M. Hatano, G. Cheng, M. Kubo, T. Fukuda, and T. Tokuhisa. 2002. A putative silencer element in the IL-5 gene recognized by Bcl6. *J. Immunol. 169:829*.
23. Takeda, N., M. Arima, N. Tsuruoka, S. Okada, M. Hatano, A. Sakamoto, Y. Kohno, and T. Tokuhisa. 2003. Bcl6 is a transcriptional repressor for the IL-18 gene. *J. Immunol. 171:426*.
24. Niu, H., G. Cattoretti, and R. Dalla-Favera. 2003. BCL6 controls the expression of the B7-1/CD80 costimulatory receptor in germinal center B cells. *J. Exp. Med. 198:211*.
25. Wang, X., Z. Li, A. Naganuma, and B. H. Ye. 2002. Negative autoregulation of BCL-6 is bypassed by genetic alterations in diffuse large B cell lymphomas. *Proc. Natl. Acad. Sci. USA 99:15018*.
26. Pasqualucci, L., A. Migliazza, K. Basso, J. Houldsworth, R. S. Chaganti, and R. Dalla-Favera. 2003. Mutations of the BCL6 proto-oncogene disrupt its negative autoregulation in diffuse large B-cell lymphoma. *Blood 101:2914*.
27. Gyory, I., G. Fejer, N. Ghosh, E. Seto, and K. L. Wright. 2003. Identification of a functionally impaired positive regulatory domain I binding factor 1 transcription repressor in myeloma cell lines. *J. Immunol. 170:3125*.
28. Leonard, W. J., and J. J. O'Shea. 1998. Jaks and STATs: biological implications. *Annu. Rev. Immunol. 16:293*.
29. Choe, J., and Y. S. Choi. 1998. IL-10 interrupts memory B cell expansion in the germinal center by inducing differentiation into plasma cells. *Eur. J. Immunol. 28:508*.
30. Lai, C. F., J. Ripperger, K. K. Morella, J. Jurlander, T. S. Hawley, W. E. Carson, T. Kordula, M. A. Caligiuri, R. G. Hawley, G. H. Fey, and H. Baumann. 1996. Receptors for interleukin (IL)-10 and IL-6-type cytokines use similar signaling mechanisms for inducing transcription through IL-6 response elements. *J. Biol. Chem. 271:13968*.
31. Fan, H., and T. L. Rothstein. 2001. Lymphokine dependence of STAT3 activation produced by surface immunoglobulin cross-linking and by phorbol ester plus calcium ionophore treatment in B cells. *Eur. J. Immunol. 31:665*.
32. Bereshchenko, O. R., W. Gu, and R. Dalla-Favera. 2002. Acetylation inactivates the transcriptional repressor BCL6. *Nat. Genet. 32:606*.
33. Lee, S. C., A. Bottaro, and R. A. Insel. 2003. Activation of terminal B cell differentiation by inhibition of histone deacetylation. *Mol. Immunol. 39:923*.
34. Toyama, H., S. Okada, M. Hatano, Y. Takahashi, N. Takeda, H. Ichii, T. Takemori, Y. Kuroda, and T. Tokuhisa. 2002. Memory B cells without somatic hypermutation are generated from Bcl6-deficient B cells. *Immunity 17:329*.
35. Sze, D. M., K. M. Toellner, C. Garcia de Vinuesa, D. R. Taylor, and I. C. MacLennan. 2000. Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival. *J. Exp. Med. 192:813*.
36. Moriyama, M., T. Yamochi, K. Semba, T. Akiyama, and S. Mori. 1997. BCL-6 is phosphorylated at multiple sites in its serine- and proline-clustered region by mitogen-activated protein kinase (MAPK) in vivo. *Oncogene 14:2465*.
37. Niu, H., B. H. Ye, and R. Dalla-Favera. 1998. Antigen receptor signaling induces MAP kinase-mediated phosphorylation and degradation of the BCL-6 transcription factor. *Genes Dev. 12:1953*.