PU.1 Can Recruit BCL6 to DNA To Repress Gene Expression in Germinal Center B Cells[▽]

Fang Wei,¹ Kristina Zaprazna,¹ Junwen Wang,² and Michael L. Atchison^{1*}

Department of Animal Biology and Mari Lowe Center for Comparative Oncology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, Pennsylvania 19104,¹ and Department of Biochemistry, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong, China²

Received 20 February 2009/Returned for modification 2 April 2009/Accepted 22 June 2009

BCL6 is a transcriptional repressor crucial for germinal center formation. BCL6 represses transcription by a variety of mechanisms by binding to specific DNA sequences or by recruitment to DNA by protein interactions. We found that BCL6 can inhibit activities of the immunoglobulin kappa (Igκ) intron and 3' enhancers. At the Igκ 3' enhancer, BCL6 repressed enhancer activity through the PU.1 binding site. We found that BCL6 physically interacted with PU.1 in vivo and in vitro, and the results of sequential chromatin immunoprecipitation assays and transient-expression assays suggested that BCL6 recruitment to the Igκ and Igλ 3' enhancers occurred via PU.1 interaction. By computational studies, we identified genes that are repressed in germinal center cells and whose promoters contain conserved PU.1 binding sites in mouse and human. We found that many of these promoters bound to both PU.1 and BCL6 in vivo. In addition, BCL6 knockdown resulted in increased expression of a subset of these genes, demonstrating that BCL6 is involved in their repression. The recruitment of BCL6 to promoter regions by PU.1 represents a new regulatory mechanism that expands the number of genes regulated by this important transcriptional repressor.

The B-cell lymphoma 6 (BCL6) gene was identified on the basis of its location at chromosomal breakpoints in non-Hodgkin's disease B-cell lymphomas (7, 55). About 30% of diffuse large cell lymphoma cases contain translocations between the BCL6 locus at chromosome 3q27 and other genes (7, 11, 55). BCL6 belongs to the BTB-POZ zinc finger family of transcription factors and contains Kruppel-type zinc finger motifs at the carboxyl terminus and a POZ motif at the amino terminus. The six BCL6 zinc fingers bind to the consensus DNA sequence TTCCT(A/C)GAA (9, 39), and the BCL6 POZ domain physically interacts with corepressor proteins, including nuclear receptor corepressor (N-CoR), BCL-6-interacting corepressor (B-CoR), SMRT (silencing mediator of retinoid acid and thyroid hormone receptor)/mSIN3A (mammalian SIN3A), Mi-2/NURD (nucleosome remodeling and histone deacetylation), and histone deacetylase complexes to mediate its potent transrepressor activity (1, 12, 13, 18, 21, 52, 57).

BCL6 plays crucial roles in germinal center biology. Knock-out studies revealed that *bcl6*^{-/-} mice fail to develop germinal centers, lack affinity maturation, and mount reduced levels of antigen-specific antibody responses (10, 14, 54). In addition, inhibition of BCL6 function in B-cell lines initiates changes characteristic of plasma cell differentiation (40). BCL6 is thought to exert its effect on germinal center development by modulating the transcription of genes involved in cell cycle regulation, proliferation, activation, class switch recombination, and differentiation. BCL6 can repress a variety of genes, including Blimp-1, ATR (ataxia-telangiectasia and Rad3-related), CD80, p53, programmed cell death2, monocyte chemo-

tactic peptide 1 (MCP-1), MCP-3, multidrug resistance-associated protein 1 (MRP1), and interleukin 18 (6, 22, 27, 30, 36, 37, 40, 43, 45). While BCL6 represses the above genes by binding directly to their promoter DNA sequences, the assortment of genes regulated by BCL6 is expanded by the work of Phan et al. (31) who showed that BCL6 can repress CDKN1A (cyclin-dependent kinase inhibitor 1A) gene expression by physical interaction with the transcriptional activator Miz-1 bound to the CDKN1A promoter. Thus, gene regulation mediated by BCL6 is complex, and BCL6 can control gene expression by multiple mechanisms.

Although BCL6 mRNA can be detected in many tissues (3), its protein expression in B cells is exquisitely regulated following antigen encounter. Nascent pregerminal center B cells up-regulate BCL-6 protein, migrate to the follicular area, and initiate germinal center formation (14). Within the germinal center, B cells proliferate and undergo affinity maturation. B cells in the germinal center dark zone (centroblasts) and light zone (centrocytes) are able to migrate between both areas of the germinal center, and both cell types express reduced levels of surface immunoglobulin (Ig) compared to naïve B cells (2, 38). B cells turn over their original surface Ig and replace those molecules with newly mutated versions that are then subjected to positive selection for affinity maturation against antigen (2). The mechanism that reduces surface Ig expression on germinal center B cells is presently unclear. While Ig transcript level changes in germinal center B cells have not been detected in microarray studies of centroblasts and centrocytes (20), transient changes in Ig expression in specific germinal center subcompartments might go undetected due to lack of pure populations of the appropriate cell types.

Within the germinal center, BCL6 expression inversely correlates with surface Ig expression. In mouse studies, nearly all germinal center B cells express smaller amounts of surface Ig

* Corresponding author. Mailing address: School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104. Phone: (215) 898-6428. Fax: (215) 573-5189. E-mail: atchison@vet.upenn.edu.

[▽] Published ahead of print on 29 June 2009.

than naïve B cells do, as measured by flow cytometry (42, 51). Expression of surface Ig protein in germinal center cells is low, perhaps to clear the surface of preexisting Ig molecules in order to select for Ig proteins that have undergone somatic hypermutation (SHM) (16). Given the inverse relationship between BCL6 and Ig expression in germinal center B cells, we were curious as to the impact of BCL6 on Ig gene enhancer activity and gene expression. Interestingly, we found that BCL6 expression can repress both immunoglobulin kappa (Ig κ) 3' enhancer and intron enhancer activities. In addition, we found that the mechanism of BCL6 repression at the Ig κ 3' enhancer represents a novel repression mechanism that expands the genes regulated by BCL6 to those that contain PU.1 binding sites, but no discernible BCL6 site. Here we report that BCL6 repression at the Ig κ 3' enhancer occurred through the PU.1 binding site, and repression also occurred at the Ig λ 3' enhancer, which contains a similar PU.1 binding motif. We found that BCL6 can physically interact with PU.1 in vivo and that BCL6 can be recruited by PU.1 to PU.1 DNA binding sites. To explore the generality of this regulatory mechanism, we further identified a number of promoters with PU.1 binding sites that are repressed in germinal center cells and that also bound to BCL6 in vivo. BCL6 knockdown experiments showed that several of these promoters are repressed by BCL6. Thus, PU.1 can recruit BCL6 to a subset of promoters in germinal center B cells, resulting in their transcriptional repression. This represents a novel repression mechanism that broadens the number of genes regulated by BCL6 in germinal center cells.

MATERIALS AND METHODS

DNA constructs. Reporter plasmids containing the 3'Ek and E1k enhancers, the PU.1 motif, and the c-Jun/c-Fos motif were described previously (32–35). The rPL32 reporter plasmid was described (4). CMV-PU.1 (CMV stands for cytomegalovirus), CMV-PU.1 Δ 242–272(Δ ETS), GST-PU.1, and plasmids for in vitro translation of wild-type PU.1, PU.1 Δ 7–33, and PU.1 Δ 118–168 were described previously (26, 34, 35). CMVPU.1ETS was prepared by insertion of an EcoRI fragment containing the appropriate PU.1 sequences in pcDNA3.1. GST-PU.1ETS was prepared by inserting a fragment expressing sequence from positions 160 to 272 at the EcoRI site of plasmid GST-5X-1. Flag-BCL6, GST-Bcl6, GST-Bcl6 ZN finger, Bcl6, and Bcl6 Δ ZF were gifts from Riccardo Dalla-Favera (Columbia University Medical Center). Full-length and zinc finger-deleted BCL6 cDNA flanked by SalI-BamHI or EcoRI sites, respectively, were blunted with Klenow polymerase and subcloned into the blunted HpaII site of the MigR1 bicistronic retrovirus.

Antibodies and immunoprecipitation. Antibodies to PU.1 (T21; sc-352), BCL6 (N3; sc-858), interferon regulatory factor 4 (IRF4) (M17; sc-6059), and IRF8 (C19; sc-6058) were purchased from Santa Cruz Biotechnology. For coimmunoprecipitation, whole-cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40) supplemented with 1 mM phenylmethylsulfonyl fluoride, and 1 $\times$ mammalian protease inhibitor cocktail (Sigma). For spleen experiments, spleens were harvested from either control untreated mice or 16 days after injection with *Toxoplasma gondii*, and 1 mg of spleen cell lysate was used per coimmunoprecipitation. Cell lysates (250 to 1,000 μ g) were immunoprecipitated with 40 μ l EZview red Anti-FLAG M2 affinity gel (catalog no. F2426; Sigma) or with anti-PU.1 or anti-BCL6 antibodies followed by incubation with 40 μ l agarose A beads (Invitrogen) according to the manufacturer's instructions. Immunoreactive proteins were visualized using the ECL kit (Amersham Biosciences) following the manufacturer's protocol.

Cell culture, transfection, and infection. Mouse plasmacytoma cell line S194 was cultured in Dulbecco modified Eagle medium with 10% horse serum. Human cell lines RAMOS, RAJI, and BJAB were maintained in RPMI 1640 medium with 10% fetal bovine serum. The cell line 293, virus packaging cell line BOSC cells, and fibroblast cell line NIH 3T3 were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. For retroviral packaging, subconfluent BOSC cells were cotransfected with retroviral DNA, Gag-

Pol, and envelope plasmids using Lipofectamine 2000 (Invitrogen). After 48 to 72 h, conditioned medium was harvested, supplemented with Polybrene (4 μ g/ml) (Sigma-Aldrich), and used for infection of B cells. NIH 3T3 cells were transfected by the calcium phosphate coprecipitation method. S194 cells were transfected by the DEAE-dextran procedure, and chloramphenicol acetyltransferase (CAT) assays were performed as previously described (28, 32).

ChIP assays. Chromatin immunoprecipitation (ChIP) was carried out as previously described (5). For all ChIP experiments, quantitative PCR analyses with primers shown in Table 1 were performed by real-time PCR using a Roche Lightcycler 1.5 and repeated at least three times. Relative increases were calculated on the basis of preimmune ChIP sample quantitative PCR values. Relative enrichments for each region are presented as the percentage of input. For sequential ChIP, a second immunoprecipitation was performed using chromatin samples eluted from the agarose beads of the first ChIP by two incubations in 10 mM dithiothreitol at 37°C for 30 min.

BCL6 knockdown. The puromycin-resistant pSM2 retroviral vectors targeting the BCL6 transcript (RHS1764-9694981) or vector control (RHS1707) were purchased from Open Biosystems. Forty-eight hours after infection, the cells were selected with puromycin (2.5 μ g/ml) for 2 days before harvesting to isolate RNA and proteins.

GST pulldown. Glutathione *S*-transferase (GST) fusion proteins were prepared essentially as described previously (29). Equivalent counts of ³⁵S-labeled proteins translated in rabbit reticulocyte lysate (Promega, Madison, WI) or 1.5 mg of spleen cell lysate isolated from mice injected with *T. gondii* were incubated with approximately equivalent amounts (as judged by Coomassie blue staining) of GST or GST fusion proteins bound to glutathione-agarose beads overnight at 4°C in NETN (100 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% Nonidet P-40) with 1 mg/ml bovine serum albumin. Beads were washed six to eight times in NETN, and bound proteins were eluted in 1 $\times$ sodium dodecyl sulfate loading dye and resolved on 10% sodium dodecyl sulfate polyacrylamide gels.

RNA isolation, RT-PCR, and quantitative PCR reactions. RNA was isolated using Trizol reagent (Sigma-Aldrich). Reverse transcription reactions were performed using the SuperScript first-strand synthesis system for reverse transcription-PCR (RT-PCR) (Gibco BRL, Rockville, MD), and PCR was performed with the primers shown in Table 1.

Computational analysis. We used the transcription start sites annotated in the DBTSS database (version 5.2.0) (53), with 30,929 human and 18,883 mouse entries. We used known PU.1 binding sites from the TRANSFAC database (23) and constructed a propensity model (49) to capture the interdependency among the individual binding site positions. We then scanned the putative PU.1 binding sites in bp –500 to +100 promoter regions around each transcription start site in human and mouse (50). We used a sliding window of size 8 (the length of PU.1 binding site) to scan along the 600-bp promoter sequence and recorded the *P* value for each window by the computational model. If the *P* value of a window was less than a cutoff value of 10^{–4}, this window was regarded a hit. If there were hits in both the homologous human and mouse promoters, the gene was selected as a putative PU.1 target. Using this method, we selected a total of 3,705 putative PU.1 target genes.

RESULTS

BCL6 can repress the Ig κ 3' enhancer through the PU.1 DNA binding region. To test the impact of BCL6 expression on Ig enhancer activity, we transfected S194 plasmacytoma cells (which lack BCL6) with reporter plasmids containing either the Ig κ 3' enhancer or the Ig κ intron enhancer, linked to the thymidine kinase promoter driving expression of the chloramphenicol acetyltransferase gene. Transfections were performed in the presence or absence of CMV-BCL6. Interestingly, BCL6 expression resulted in a 14-fold repression of Ig κ 3' enhancer activity compared to the empty vector control (7% activity compared to the value in the absence of BCL6) (Fig. 1A, lanes 1 and 2). BCL6 also reduced Ig κ intron enhancer activity approximately fourfold to 25% of the level in the absence of BCL6 (Fig. 1A, lanes 3 and 4). In contrast, BCL6 did not reduce activity of the housekeeping ribosomal protein L32 promoter (Fig. 1A, lanes 5 and 6). Thus, BCL6 can repress both the Ig κ 3' and intron enhancers, but repression of the 3'

TABLE 1. Primers used in this study

Primer ^a	Primer sequence	
	5' Oligonucleotide	3' Oligonucleotide
ChIP primers		
hIgκ enhancer	ATGCGCTTTCCAGCTCTG	GCAGATTCTCTGGGTGAAGGG
hIgλ enhancer	ACTCAGGCCCAAACATGCC	CAGCCCTGTTGGAGGAGGAT
mIgκ enhancer	GCACAGAGTACCCACCCATATCTC	CTTGAAAGGGTGTGGAGTGCACCA
hACTB	GCCACGGCTGCTTCCA	GAACCGTCTATTGCCAATG
hp53A	TCAAGTGATCTGCACACCTC	GAAGAGAGACTAACTAGAGG
hp53C	TGATAAGGGTTGTGAAGGAG	ATATGAAGGGTGAAGGAAG
EPB41L2	GCAGCCTCTGGAAGCAGTAA	GACTCAGAAGCACACTGGCTG
CCND2	CAACAGCTACCTGGGCTCTCA	GGTTGGGCTCCAGACCACTT
CD72	GATGCATGGAGAGGAAGCACA	TGCACGGCTTAGCAATTGG
CCR7	TCAGGATCCTATGACCAGCGA	CATGAAGAGGCTCACTCACCC
DUSP6	TTATGTGCCTGCTCCTCCT	GGATACATTCTCCGGGCCA
FTH1	GCCTCCCTGTAGATCGTCACA	CTGAAGGAAGATTTCGGCCAC
KLK1	CCTCCGTCTTCTGTTCTGTC	ACCCAGATGTTGGCCAAAG
STIM	TTGTGCTAGGAGGAGTGGGC	CACGTGGAAGGTGCCAAAG
TNFRSF14	GCAGAAGCTGAGATGGGAACA	CAGGAGCTGTCCAGGCAAA
FCGR1	CGGCAGACACCCTCAAAGTAA	GCTGCTCCCTGACTCCAAGA
CCR6	CAACCCATCTTCTTCCTCCA	TGATGACCACAGATCCTGGG
FUT7	AGTGGGTGATACTGCCGTGG	AGCCAGGGCAATATGACGC
TUBA2	GCCGTGAAGGCCTCTCAAA	GAAGGCAGCAGGCTCAGAGA
IRF9	TCAGGTGGGCACTGTGG	GCAGCATCTGAGCATCTC
IL-24	AGCATGAATAGGGCA	GGCTCTACCACCTGGCTGT
LBK5	CCACCACCCCGCCGCTTAT	CCTCCACACCTCCCCCTGAACCT
RT-PCR primers		
mIgκ constant region	TGATGGCAGTGAACGACAAAATGG	AGGAAAGGGAGGAGGAGGAGAAGG
mGAPDH	CTGAGGACCAGGTTGTCTCC	GCCTCTCTTGCTCAGTGTCC
hIgκ constant region	TCTGTCTTCATCTTCCCGCCATCT	CCCCTGTTGAAGCTCTTTGTGACG
hACTB	GCCACGGCTGCTTCCA	GAACCGTCTATTGCCAATG
ATR	ACGCATCAGCTCATTTGTAA	CTTCGCAATAAGAACGGCTG
hbcl6	TTGTTGTGAGCCGTGAGCAGTT	CCCGCAAATTGAGCCGAGAT
KLK1	CCCGATTCACTCCCGATT	CCAGGAGGCTCATGTTGAAGC
IRF9	GTGCTCCAGGACTCCCTCAA	TGTGGCTCAGGGCTGCTGCTG

^a hIgκ, human Igκ; hIgλ, human Igλ; mIgκ, mouse Igκ; hACTB, human ACTB; hp53A, human p53A; hp53C, human p53C; mGAPDH, mouse glyceraldehyde-3-phosphate dehydrogenase; hbcl6, human BCL6.

enhancer is most dramatic. Igκ 3' enhancer activity is dependent upon function of the ETS (erythroblast transformation-specific) domain transcription factor, PU.1 (32, 34). We tested whether BCL6 could reduce activity of a reporter containing a multimerized PU.1 binding site from the Igκ 3' enhancer. Indeed, activity of this reporter was reduced 10-fold in the presence of BCL6 (Fig. 1A, lanes 7 and 8). Western blot analysis showed efficient expression of transiently transfected CMV-BCL6 (Fig. 1A, right panel).

We previously showed that Igκ 3' enhancer activity can be reconstituted in fibroblasts with transcription factors PU.1, IRF4 (PIP), c-Fos, and c-Jun (33). Therefore, we tested whether BCL6 could repress reporters dependent upon multimerized c-Jun/c-Fos or PU.1 binding sites derived from the Igκ 3' enhancer. We cotransfected NIH 3T3 cells with reporters containing multimerized c-Jun/c-Fos or PU.1 sites with either CMV-jun and CMV-fos or CMV-PU.1, respectively, in the absence or presence of CMV-BCL6. While BCL6 expression had little impact on c-Fos/c-Jun activity (Fig. 1B, left panel), BCL6 strongly repressed PU.1 transactivation (Fig. 1B, right panel). Thus, we conclude that BCL6 can repress Igκ 3' enhancer activity via the PU.1 binding region. Western blot data showed that transfected plasmids were efficiently ex-

pressed (Fig. 1B, right panel), although endogenous levels of c-Jun masked the level of transfected c-Jun.

BCL6 physically interacts with PU.1. We visually scanned the Igκ 3' enhancer DNA sequence and observed no obvious BCL6 binding sequence. We also performed electrophoretic mobility shift assays to test whether BCL6 bound directly to the 3' enhancer region but failed to detect BCL6 DNA binding. This suggested that BCL6 might be recruited to the enhancer by protein interactions. As BCL6 could repress PU.1 transcriptional activity, we hypothesized that BCL6 might physically interact with PU.1.

To test this idea, we performed coimmunoprecipitation studies of human germinal center-like BJAB lymphoma cells, which express endogenous PU.1 and BCL6 proteins. Immunoprecipitation with anti-PU.1 antibody, but not preimmune antibody, brought down BCL6 (Fig. 2A). PU.1 can physically interact with IRF4 and IRF8 (24, 25), and BJAB cells express IRF8 (Fig. 2A, bottom panel). Despite the presence of IRF8, which could potentially compete with BCL6 for interaction with PU.1, we still observed PU.1-BCL6 interaction (Fig. 2A, top panel). We also transfected 293 cells with CMV-PU.1 expression vector in the absence or presence of CMV-FlagBCL6, and cell lysates were prepared. Coimmunoprecipitation with

FIG. 1. BCL6 can repress Igk enhancer activity. (A) Various CAT reporter plasmids (shown above the lanes) were transfected into S194 plasmacytoma cells with (+) or without (–) CMV-BCL6 (BCL6). Activity without BCL6 is defined as 100%. The levels of CAT activity (as a percentage) are shown below each lane. The Western blot in the right panel of cell lysates normalized to β-galactosidase levels from cotransfected CMV-βGAL shows efficient expression of BCL6 from transfected CMV-BCL6. (B) Multimerized (four copies) Jun-Fos, or PU.1 DNA binding site CAT reporters were transfected into NIH 3T3 cells with the expression plasmids listed above each lane. For each combination, activity without BCL6 is defined as 100%. The Western blots in the right panel show expression of c-Fos, PU.1, and BCL6 in the appropriate samples. Endogenous levels of c-Jun obscured the levels of protein expressed from CMV-Jun. Samples were normalized to cotransfected CMV-βGAL.

anti-Flag affinity beads followed by Western blotting with anti-PU.1 antibody showed that PU.1 interacted with BCL6 in vivo (Fig. 2B). We then performed GST pulldown assays with various PU.1 and BCL6 proteins (Fig. 2C). Interaction between in vitro-translated ³⁵S-labeled PU.1 and BCL6 fused to GST (GST-BCL6) was easily detected, but no interaction was observed with GST alone (Fig. 2D, lanes 1 to 3). To determine the PU.1 and BCL6 domains important for interaction, we performed additional GST pulldown assays using various PU.1 deletion mutants (Fig. 2D, lanes 4 to 12) or BCL6 deletion mutants (Fig. 2E, lanes 1 to 9). Deletions within the PU.1 activation domain (deletion of positions 7 to 33 [Δ7-33]) or PEST domain (Δ118-168) had no impact on PU.1-BCL6 interaction (Fig. 2E, lanes 4 to 9), suggesting that the PU.1 ETS domain is necessary for interaction with BCL6. Pulldown with a GST fusion protein containing only the PU.1 ETS domain

showed that this sequence was sufficient for interaction with BCL6 (Fig. 2D, lanes 10 to 12). Evaluation of BCL6 deletion mutants showed that deletion of the BCL6 zinc fingers ablated PU.1 interaction, while the zinc finger domain alone was able to interact with PU.1 (Fig. 2E, lanes 4 to 9). Thus, the BCL6 zinc finger domain is necessary and sufficient for interaction with PU.1. As the PU.1-related protein Spi-B is also expressed in germinal center B cells, we tested the ability of this protein to physically interact with BCL6. GST pulldown experiments showed that Spi-B also interacted with BCL6 (Fig. 2F).

To ensure that the interactions we observed also occur in primary germinal center cells, we performed coimmunoprecipitation and GST pulldown experiments with splenic lysates isolated from control mice or mice infected with the parasitic protozoan *T. gondii*. Immunoprecipitation with anti-PU.1 antibody followed by Western blotting with anti-BCL6 antibody indicated that PU.1 and BCL6 physically interacted in primary germinal center B cells isolated from infected animals (Fig. 2G, lanes 4 and 5), but not in control splenic B cells, which express very low levels of BCL6 (Fig. 2G, lanes 2 and 3). Again, this interaction occurred even in the presence of endogenous IRF4 and IRF8 (Fig. 2G, bottom panels). As expected, incubation of splenic lysates with GST-BCL6 resulted in pulldown of PU.1 (Fig. 2H, lanes 1 and 2), whereas incubation with GST-PU.1ETS domain resulted in pulldown of BCL6 (Fig. 2H, lanes 3 and 4). In total, our results clearly indicate that BCL6 and PU.1 can physically interact in vivo.

BCL6 is recruited to the Igκ and Igλ 3' enhancers through PU.1. The Igκ and Igλ 3' enhancers both contain PU.1 binding sites (Fig. 3A). As the Igκ 3' enhancer region does not contain BCL6 binding sites, we investigated whether BCL6 might be recruited to this region by PU.1 in vivo. ChIP assays using BJAB germinal center-like cells demonstrated that PU.1 and BCL6 bound to the Igκ 3' enhancer in vivo (Fig. 3B). Since RAMOS germinal center-like cells express Igλ, and there is a PU.1-IRF4 motif in the Igλ enhancer similar to the Igκ 3' enhancer (Fig. 3A), we performed ChIP assays with RAMOS cells and assayed binding to the Igλ 3' enhancer. Interestingly, PU.1 and BCL6 bound to the Igλ 3' enhancer as well (Fig. 3C). Quantitative PCR verified that both PU.1 and BCL6 bound in vivo to both enhancers, while neither protein was detected at the β-actin gene (Fig. 3B and C, bottom panels). Similar levels of signal were observed at the p53c region (which binds BCL6 in vivo) compared to the Igλ 3' enhancer (Fig. 3D). Therefore, the level of BCL6 binding to the Igλ 3' enhancer was similar to that observed at a bona fide BCL6 DNA binding site (p53c) in vivo. No signal was observed at the negative-control p53a region (Fig. 3D).

To determine whether PU.1 recruited BCL6 to both Igκ 3' and Igλ 3' enhancer regions in vivo, we performed sequential ChIP experiments. Chromatin extracts from BJAB and RAMOS cells were first immunoprecipitated with anti-PU.1 antibody or preimmune antibody and then subjected to a second round of immunoprecipitation using either preimmune or BCL6 antibody. As predicted, BCL6 was observed at both enhancers only when it was first immunoprecipitated with PU.1 rather than preimmune antibody (Fig. 3E and F). This strongly suggested that PU.1 recruits BCL6 to DNA in vivo. To confirm this, we initially attempted to knock down PU.1 by RNA interference approaches to determine whether this resulted in

lost BCL6 binding. However, we failed to sufficiently reduce PU.1 protein levels. As an alternative approach, we transfected our PU.1 binding site reporter plasmid with plasmids expressing either PU.1 alone, BCL6 alone, or BCL6 plus PU.1, and evaluated protein-DNA interactions by ChIP assays. While BCL6 alone bound poorly to the PU.1-dependent reporter plasmid, binding was increased twofold in the presence of PU.1 (Fig. 3G, PU.1, BCL6, and PU.1 BCL6 bars). Deletion of the PU.1 ETS domain needed for interaction with BCL6 resulted in lost BCL6 recruitment, as did deletion of the BCL6 zinc finger domain needed for interaction with PU.1 (Fig. 3G). Therefore, PU.1 can recruit BCL6 to DNA.

BCL6 can repress Ig light-chain expression. To determine whether BCL6 recruitment to the Ig κ locus resulted in altered Ig κ expression, we performed BCL6 overexpression studies of S194 cells (which do not express endogenous BCL6) and BCL6 knockdown studies of BJAB cells (which do express endogenous BCL6). BCL6 cDNA was inserted into the bicistronic MigR1 retrovirus carrying the green fluorescent protein gene, and RNA and cell lysates were prepared from transduced S194 cells. This retroviral approach enabled us to express BCL6 in essentially all cells in the culture as evidenced by nearly 100% green fluorescent protein-positive cells (data not shown), but expression levels of BCL6 were very low compared to endogenous BCL6 in RAMOS, RAJI, and BJAB germinal center-like cells (Fig. 4A). Nonetheless, real-time RT-PCR for Ig κ transcripts showed that endogenous Ig κ expression was repressed 76% by BCL6 compared to the control (Fig. 4B). Since BCL6 cannot interact with PU.1 when the zinc finger domain is deleted, we also tested the BCL6- Δ ZF construct. No repression of Ig κ gene expression was observed in S194 cells transduced with the BCL6- Δ ZF construct (Fig. 4B). In contrast, depletion of BCL6 in germinal center-like BJAB cells by short hairpin RNA (shRNA) knockdown (Fig. 4C) resulted in an increase in Ig κ gene expression that was even greater than that observed with the positive-control gene, ATR (36) (Fig. 4D). In total, our data indicate that BCL6 can repress endogenous Ig κ gene expression in vivo.

BCL6 binds to multiple PU.1 binding regions. BCL6 is crucial for germinal center development and regulates a number of genes (6, 22, 27, 30, 36, 37, 40, 43, 45). Recruitment of BCL6 to DNA by PU.1 would represent a new mechanism of BCL repression that would increase the number of genes that could be regulated by BCL6. To explore the generality of this mechanism, we questioned whether BCL6 could be recruited to other PU.1 binding sites in genes downregulated in germinal

center cells. We performed a computational analysis of the -500- to +100-bp promoter regions in the mouse and human genomes. This analysis showed that the PU.1 consensus site (AGAGGAAG) lies within 3,705 gene promoters that are conserved in mouse and human genomes. A comparison of the promoters identified above with the published literature of genes downregulated in germinal center cells (20) revealed 14 genes common to both lists. These 14 genes were chosen for further study to test whether PU.1 and BCL6 both bound to these promoters. ChIP assays were performed in BJAB and RAMOS germinal center-like cells with primers to the 14 genes identified above. Nearly all of the promoters showed strong PU.1 and BCL6 binding in both RAMOS and BJAB cells (Fig. 5A). Quantitative RT-PCR data showed both PU.1 and BCL6 enriched at all 14 promoters with the change in enrichment ranging from 2.5-fold to 24-fold (Fig. 5B). To determine whether BCL6 is responsible for repressing expression of these genes, we performed BCL6 shRNA knockdown experiments in BJAB cells and evaluated transcript levels for the genes identified above. BCL6 knockdown resulted in >90% reduction in BCL6 expression (Fig. 5C). Interestingly, two genes in addition to Ig κ (Kallikrein 1 [KLK1] and IRF9) showed increased levels of expression after BCL6 knockdown, indicating that BCL6 is needed for transcriptional repression of these genes (Fig. 5C). Lack of increased expression by the other genes analyzed suggests that factors in addition to BCL6 must repress expression of these genes. Our results with the KLK1 and IRF9 genes indicate that the PU.1-BCL6 interaction mechanism that we detected at the Ig κ and Ig λ loci is a general mechanism for regulating gene expression.

DISCUSSION

PU.1 recruitment of BCL6 to DNA represents a novel mechanism of BCL6 transcriptional repression, but BCL6 can be recruited to DNA by other proteins as well. As mentioned earlier, Phan et al. in the Dalla-Favera laboratory showed that BCL6 can be recruited to DNA by interaction with the Miz-1 transcription factor, and this recruitment leads to repression of the cell cycle regulatory gene p21 (30). BCL6 can also inhibit AP-1 transactivation function by interaction with the c-Jun leucine zipper region (48). Thus, BCL6 can regulate gene expression by a variety of mechanisms, including direct DNA binding, recruitment to DNA by Miz-1, recruitment by PU.1, and physical interaction with c-Jun.

Our data clearly show that PU.1 can recruit BCL6 to DNA

FIG. 2. PU.1 and BCL6 physically interact in vivo. (A) Cell lysates from BJAB cells were immunoprecipitated (IP) with either preimmune or anti-PU.1 antibody and then immunoblotted with anti-BCL6 antibody. The levels of IRF4 and IRF8 in BJAB cells compared to cell lines A20 and S194 are shown in the Western blots in the bottom panel. α IRF4, anti-IRF4 antibody. (B) CMV-PU.1 and CMVFlag-BCL6 were cotransfected into 293 cells. Cell lysates were immunoprecipitated with anti-FLAG affinity beads and immunoblotted with anti-PU.1 antibody. CoIP, coimmunoprecipitation. (C) Maps of PU.1 and BCL6 proteins used for GST pulldown studies. (D) PU.1 can directly interact with BCL6 via the PU.1 ETS domain. GST, GST-BCL6, or GST-ETS proteins were incubated with 35 S-labeled PU.1 or BCL6 proteins shown above each lane. Wild-type (WT) PU.1 and PU.1 protein with various deletions were tested. (E) The BCL6 zinc fingers are necessary and sufficient for interaction with PU.1. GST or GST-PU.1 proteins were incubated with the 35 S-labeled proteins shown above each lane. (F) Spi-B can interact with BCL6. GST-BCL6 was incubated with 35 S-labeled Spi-B and evaluated by GST pulldown. (G) PU.1 and BCL6 interact in primary splenic germinal center cells. Control or infected spleen lysates were incubated with preimmune or anti-PU.1 antibody followed by Western blotting (WB) with anti-BCL6 antibody (α -BCL6). Short and long exposures are shown for lanes 4 and 5. The input signal of BCL6 from infected spleen lysate is shown in lane 1, and the presence of IRF4 and IRF8 in spleen is shown in the two blots at the bottom of the panel. (H) Infected spleen lysates were incubated with either GST-BCL6 or GST-ETS and then subjected to Western blotting with anti-PU.1 or anti-BCL6, respectively.

FIG. 3. BCL6 binds to the endogenous Igκ and Igλ 3' enhancers in BJAB and RAMOS germinal center-like cells. (A) Schematic representations of the Igκ 3' and Igλ₂₋₄ enhancers are shown with specific transcription factors represented as circles. (B) ChIP was performed with PU.1 and BCL6 antibodies using BJAB chromatin and primers for the Igκ 3' enhancer (IgκE) and β-actin (ACTB) gene. (C) ChIP was performed with PU.1 and BCL6 antibodies using RAMOS cell chromatin followed by PCR with Igλ 3' enhancer and β-actin primers. The bottom panels in panels B and C show quantitative PCR data of the change in enrichment of Igκ 3' or Igλ 3' enhancer DNA relative to the β-actin gene. (D) BCL6 binding to the Igλ 3' enhancer is comparable to binding to the p53 gene promoter. ChIP with anti-BCL6 antibody was evaluated by quantitative PCR for binding to the Igλ 3' enhancer compared to negative-control (p53a) and positive-control (p53c) samples. (E and F) BCL6 is recruited to DNA by PU.1. Sequential ChIPs with anti-PU.1 antibody followed by anti-BCL6 antibody were performed using BJAB (E) and RAMOS (F) chromatin followed by quantitative PCR for Igκ and Igλ 3' enhancers, respectively. The antibodies used are shown below each bar. Error bars show standard deviations of the means. (G) PU.1 recruitment of BCL6 to DNA requires the PU.1 ETS domain and the BCL6 zinc finger domain. NIH 3T3 cells were transfected with the multimerized PU.1 binding site reporter plasmid and expression plasmids encoding either PU.1, BCL6, PU.1ΔETS, or BCL6ΔZn finger in the combinations shown below each panel. ChIP assays were performed with preimmune, anti-PU.1, and anti-BCL6 antibodies and QPCR was performed with primers that amplify the PU.1 binding site region.

as evidenced by the following: (i) their cooccupancy at PU.1 binding sites as detected by ChIP assay, (ii) the presence of BCL6 at PU.1 sites after sequential ChIP with PU.1 and BCL6 antibodies, (iii) recruitment of BCL6 to PU.1 binding sites only in the presence of PU.1, (iv) lost BCL6 recruitment by a PU.1 mutant lacking the BCL6 interaction domain, and (v) lost BCL6 recruitment of a BCL6 mutant lacking the PU.1 inter-

action domain. While PU.1 can clearly recruit BCL6 to DNA, we cannot rigorously exclude the possibility that other factors at the Igκ 3' enhancer are involved in this recruitment. Mutation of the PU.1 binding site within the Igκ 3' enhancer unfortunately is not helpful in resolving this issue. PU.1 serves as a nucleating protein in the Igκ 3' enhancer, and mutation of the PU.1 binding site results in loss of transcription factor

FIG. 4. BCL6 can repress expression of the endogenous Ig κ gene. (A) BCL6 is expressed at low levels in transduced S194 cells. The level of BCL6 in control (lane 1) or transduced S194 cells (lane 2) is compared to the level of endogenous BCL6 in germinal center-like RAMOS, RAJI, and BJAB cells. α BCL6, anti-BCL6 antibody. (B) S194 plasmacytoma cells were infected with empty retroviral vector or vectors expressing either the intact BCL6 cDNA or BCL6 Δ ZnF retrovirus. Quantitative RT-PCR was performed to reveal the level of mouse Ig κ transcripts. (C) BCL6 shRNA knockdown reduces BCL6 protein levels. BJAB cells were infected with vectors expressing either BCL6 shRNA (Bcl6i) or scrambled control shRNA (noni), and loss of BCL6 protein was shown by Western blotting. (D) BCL6 knockdown increases Ig κ gene expression. BCL6 knockdown resulted in a 2.5- to 3-fold induction of Ig κ gene expression, which was even more dramatic than the change for the ATR gene positive control. hKc, human Ig κ constant region.

assembly on the enhancer and lost enhancer activity. Thus, we cannot test whether BCL6 represses enhancer activity of a PU.1 binding site mutant enhancer, since enhancer activity is already ablated. While we cannot rigorously exclude the function of flanking Ig κ 3' enhancer factors in BCL6 recruitment, our transfection assays with multimerized enhancer binding sites strongly indicate that PU.1 is a key factor in BCL6 recruitment and repression at the Ig κ 3' enhancer.

We also identified several additional genes that can be regulated by PU.1 recruitment of BCL6 to DNA. We performed genome-wide analyses of PU.1 binding sites in all human and mouse promoters and identified 14 gene promoters that are downregulated in germinal center cells compared to naïve B cells. These promoters contain conserved PU.1 binding sites, but no discernible BCL6 site. We found that BCL6 bound to these promoters apparently via recruitment by PU.1. Our BCL6 knockdown studies indicate that BCL6 is required for transcriptional repression of at least 2 of the 14 genes evaluated, IRF9 and KLK1. Thus, the novel mechanism we identified here expands the number of genes important for germinal center biology that are regulated by BCL6.

KLK1 belongs to a family of serine proteases, and its importance for germinal center function is unclear at this time (56). However, IRF9 is part of the interferon (IFN) stimulated gene factor 3 (ISGF3) heterotrimeric complex along with STAT1 (signal transducer and activator of transcription factor 1) and STAT2 (44). This complex regulates gene expression in

response to IFN signaling. IRF9 is also involved in the germinal center reaction and plays a role in class switch recombination (44). Interestingly, IRF9 is required for immunoglobulin G (IgG) autoantibody production in an experimental model for systemic lupus erythematosus (44). *Irif9*^{-/-} mice produce IgM autoantibodies but fail to produce switched IgG autoantibodies. In contrast, production of IgG antibodies to nonauto-immune antigens is normal. Thus, downregulation of IRF9 expression by PU.1 and BCL6 may provide a protective role in the immune response involving autoantibodies. *Irif9*^{-/-} mice also fail to activate IFN-inducible genes in response to type I IFN (19). Thus, downregulation of IRF9 expression by PU.1 recruitment of BCL6 could play a regulatory role in the mechanism of downregulation of the IFN response.

PU.1 is upregulated in centroblasts within the germinal center where BCL6 is also highly expressed (46). B cells within the germinal center proliferate very rapidly in response to T-cell-dependent antigens, and these cells undergo immunoglobulin gene class switch recombination and SHM. SHM requires function of the Ig κ enhancers (8, 15, 41, 47), and PU.1 is one of four proteins (PU.1, IRF4, c-Fos, and c-Jun) that can reconstitute Ig κ 3' enhancer activity in fibroblasts (33). PU.1 is crucial for Ig κ 3' enhancer activity (33–35), which suggests that modulation of its function could have substantial impact on enhancer function. While PU.1 is generally considered a transcriptional activator, the PU.1 binding site in the Ig κ 3' enhancer has also been implicated in negative regulation (17).

We found that PU.1 can interact with transcriptional repressor BCL6 and this interaction targets BCL6 to the Ig κ and λ light-chain 3' enhancers. In the germinal center, B cells turn over their original surface Ig and replace these molecules with newly mutated versions that are then subject to positive selection for affinity maturation against antigen. Downregulation of Ig expression may be important at a specific stage of the SHM mechanism or may enable replacement of unmutated Ig molecules with newly mutated molecules. This clearance of original unmutated Ig may assist in the efficient identification and selection of cells expressing Ig molecules with increased affinity for antigen. Thus, the clearance of surface immunoglobulin with germinal center centroblasts and centrocytes may be partly due to the repression of immunoglobulin gene expression by BCL6, although transcript profiling has thus far not detected large differences in Ig transcription in various germinal center cell types (2). However, small differences in transcription coupled with additional posttranscriptional mechanisms may combine to result in reduced surface Ig expressed in centroblasts and centrocytes. Alternatively, the repression mechanism we identified here may impact expression of Ig genes in specific germinal center cell types not evaluated as pure populations in microarray studies, or this repression mechanism may be temporally regulated during the germinal center reaction.

The PU.1 site in the 3' enhancer is crucial for enhancer activity, and PU.1 DNA binding leads to the recruitment of IRF4 or IRF8 to an adjacent DNA binding site (24, 32–34). IRF4 is generally downregulated in germinal center centroblasts and then upregulated as cells mature into memory cells and plasma cells (20). IRF8 expression, however, is high within the germinal center, and IRF8 can synergize with PU.1 in DNA binding and transcriptional activation (24). We previ-

FIG. 5. (A) BCL6 coexists with PU.1 at multiple PU.1 binding sites in BJAB and RAMOS cells. ChIP experiments were performed in RAMOS and BJAB cells using PU.1 and BCL6 antibodies and the primers for the promoters shown on the left of each panel. (B) ChIP DNA was evaluated by quantitative PCR for each promoter. Representative data from one of two experiments are shown. (C) BCL6 knockdown (Bcl6i) versus control vector (noni) results in elevated expression of several of the identified genes. Knockdown of BCL6 resulted in dramatic reduction of BCL6 transcripts, and increased transcripts for the Igk, IRF9, and KLK1 genes. hKc, human Igk constant region.

ously showed that IRF4 can physically interact with PU.1 via the PU.1 ETS domain, similar to BCL6 (29), and therefore, IRF8 might compete with BCL6 for physical interaction with PU.1 and subsequent DNA recruitment. Thus, the interplay

between BCL6 and IRF8 for interaction with PU.1 could control the status of PU.1 as an activator or repressor. At later stages of B-cell development, downregulation of BCL6 and upregulation of IRF4 would result in conversion of PU.1 from

a repressor to its more typically understood role as an activator. It will be interesting to more thoroughly explore this potential mechanism in the future.

In summary, we have identified a new mechanism of BCL6-mediated repression. Multiple genes utilize this mechanism in germinal center B cells, and this mechanism may contribute to clearing Ig molecules from the surface of the cell prior to SHM. Further exploration of this pathway will determine the full assortment of genes regulated by this mechanism and the mechanistic details of competitive protein interactions.

ACKNOWLEDGMENTS

We thank Riccardo Dalla-Favera for supplying various BCL6 constructs and Chris Hunter for supplying mice injected with *T. gondii*. We also thank John Pehrson and Michael May for critically reading the manuscript.

This work was supported by NIH grant GM071830 (M.L.A.) and by grant A.E./M-04/04 from the University Grants Committee of Hong Kong (J.W.).

REFERENCES

- Ahmad, K. F., A. Melnick, S. Lax, D. Bouchard, J. Liu, C.-L. Kiang, S. Mayer, S. Takahashi, J. D. Licht, and G. G. Prove. 2003. Mechanism of SMRT corepressor recruitment by the BCL6 BTB domain. *Mol. Cell* **12**: 1551–1564.
- Allen, C. D. C., T. Okada, and J. G. Cyster. 2007. Germinal-center organization and cellular dynamics. *Immunity* **27**:190–202.
- Allman, D., A. Jain, A. L. Dent, R. R. Maile, T. Selvaggi, R. Kehry, and L. M. Staudt. 1996. BCL-6 expression during B-cell activation. *Blood* **87**:5257–5268.
- Atchison, M. L., O. Meyuhas, and R. P. Perry. 1989. Localization of transcriptional regulatory elements and nuclear factor binding sites in mouse ribosomal protein gene rpl32. *Mol. Cell. Biol.* **9**:2067–2074.
- Bai, Y., L. Srinivasan, L. Perkins, and M. L. Atchison. 2005. Protein acetylation regulates both PU.1 transactivation and Igk 3' enhancer activity. *J. Immunol.* **175**:5160–5169.
- Baron, B. W., J. Anastasi, M. J. Thirman, Y. Furukawa, S. Fears, D. C. Kim, F. Simone, M. Birkenbach, A. Montag, A. Sadhu, N. Zeleznik-Le, and T. W. McKeithan. 2002. The human programmed cell death-2 (PDCD2) gene is a target of BCL6 repression: implications for a role of BCL6 in the down-regulation of apoptosis. *Proc. Natl. Acad. Sci. USA* **99**:2860–2865.
- Baron, B. W., G. Nucifora, N. McCabe, R. I. Espinosa, M. M. Le Beau, and T. W. McKeithan. 1993. Identification of the gene associated with the recurring chromosomal translocations t(3;14)(q27;q32) and t(3;22)(q27;q11) in B-cell lymphomas. *Proc. Natl. Acad. Sci. USA* **90**:5262–5266.
- Betz, A. G., C. Milstein, A. Gonzales-Fernandez, R. Pannell, T. Larson, and M. S. Neuberger. 1994. Elements regulating somatic hypermutation of an Igk gene: critical role of the intron enhancer/matrix attachment region. *Cell* **77**:239–248.
- Chang, C. C., B. H. Ye, R. S. Chaganti, and R. Dalla-Favera. 1996. BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. *Proc. Natl. Acad. Sci. USA* **93**:6947–6952.
- 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**:589–592.
- Dent, A. L., F. H. Vasanwala, and L. M. Toney. 2002. Regulation of gene expression by the proto-oncogene BCL-6. *Crit. Rev. Oncol. Hematol.* **41**:1–9.
- Dhordain, P., O. Albagli, R. J. Lin, S. Ansieau, S. Quief, A. Leutz, J.-P. Kerckaert, R. M. Evans, and D. Leprince. 1997. Corepressor SMRT binds the BTB/POZ repressing domain of the LAZ3/BCL6 oncoprotein. *Proc. Natl. Acad. Sci. USA* **94**:10762–10767.
- Fujita, N., D. L. Jaye, C. Geigerman, A. Akyildiz, M. R. Mooney, J. M. Boss, and P. A. Wade. 2004. MTA3 and the Mi-2/NuRD complex regulate cell fate during B lymphocyte differentiation. *Cell* **119**:75–86.
- Fukuda, B. T., T. Yoshida, S. Okada, M. Hatano, T. Miki, K. Ishibashi, S. Okabe, H. Koseki, S. Hirotsawa, M. Taniguchi, N. Miyasaka, and T. Toku-hisa. 1997. Disruption of the Bcl6 gene results in an impaired germinal center formation. *J. Exp. Med.* **186**:439–448.
- Goyenechea, B., N. Klix, J. Yelamos, G. T. Williams, A. Riddell, M. S. Neuberger, and C. Milstein. 1997. Cells strongly expressing Igk transgenes show clonal recruitment and hypermutation: a role for both MAR and the enhancers. *EMBO J.* **16**:3987–3994.
- Henderson, A., and K. Calame. 1998. Transcriptional regulation during B cell development. *Annu. Rev. Immunol.* **16**:163–200.
- Hiramatsu, R., K. Akagi, M. Matsuoka, K. Sakumi, H. Nakamura, L. Kingsbury, C. David, R. R. Hardy, K.-I. Yamamura, and H. Sakano. 1995. The 3' enhancer region determines the B/T specificity and pro-B/pre-B specificity of immunoglobulin V κ -J κ joining. *Cell* **83**:1113–1123.
- Huynh, K. D., W. Fischle, E. Verdin, and V. J. Bardwell. 2000. BCoR, a novel corepressor involved in BCL-6 repression. *Genes Dev.* **14**:1810–1823.
- Kimura, T., Y. Kadokawa, H. Harada, M. Matsumoto, M. Sato, Y. Kashiwazaki, M. Tarutani, R. Sok-Pin Tan, T. Takasugi, T. Matsuyama, T. W. Mak, S. Noguchi, and T. Taniguchi. 1996. Essential and non-redundant roles of p48 (ISGF3 γ) and IRF-1 in both type I and type II interferon responses, as revealed by gene targeting studies. *Genes Cells* **1**:115–124.
- Klein, U., Y. Tu, G. A. Stolovitzky, J. L. Keller, J. J. Haddad, V. Miljkovic, G. Cattoretti, A. Califano, and R. Dalla-Favera. 2003. Transcriptional analysis of the B cell germinal center reaction. *Proc. Natl. Acad. Sci. USA* **100**:2639–2644.
- Lemerrier, C., M.-P. Brocard, F. Puvion-Dutilleul, H.-Y. Kao, O. Albagli, and S. Khochbin. 2002. Class II histone deacetylases are directly recruited by BCL6 transcriptional repressor. *J. Biol. Chem.* **277**:22045–22052.
- Lin, K.-L., C. Tunyaplin, and K. Calame. 2003. Transcriptional regulatory cascades controlling plasma cell differentiation. *Immunol. Rev.* **194**:19–28.
- Matys, V., O. V. Kel-Margoulis, E. Fricke, I. Liebich, S. Land, A. Barre-Dirrie, I. Reuter, D. Chekmenev, M. Krull, K. Hornischer, N. Voss, P. Stegmaier, B. Lewicki-Potapov, H. Saxel, A. E. Kel, and E. Wingender. 2006. TRANSFAC and its module TRANSCOMP: transcriptional gene regulation in eukaryotes. *Nucleic Acids Res.* **34**:D108–D110.
- Nagulapalli, S., and M. L. Atchison. 1998. Transcription factor Pip can enhance DNA binding by E47, leading to transcriptional synergy involving multiple protein domains. *Mol. Cell. Biol.* **18**:4639–4650.
- Nagulapalli, S., A. Goheer, L. Pitt, L. P. McIntosh, and M. L. Atchison. 2002. Mechanism of E47-Pip interaction on DNA resulting in transcriptional synergy and activation of immunoglobulin germ line sterile transcripts. *Mol. Cell. Biol.* **22**:7337–7350.
- Nagulapalli, S., J. M. R. Pongubala, and M. L. Atchison. 1995. Multiple proteins physically interact with PU.1. *J. Immunol.* **155**:4330–4338.
- 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**:211–221.
- Park, K., and M. L. Atchison. 1991. Isolation of a candidate repressor/activator, NF-E1 (YY-1, δ), that binds to the immunoglobulin κ 3' enhancer and the immunoglobulin heavy-chain μ E1 site. *Proc. Natl. Acad. Sci. USA* **88**:9804–9808.
- Perkel, J. M., and M. L. Atchison. 1998. A two-step mechanism for recruitment of Pip by PU.1. *J. Immunol.* **160**:241–252.
- Phan, R. T., and R. Dalla-Favera. 2004. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* **432**:635–639.
- Phan, R. T., M. Saito, K. Basso, H. Niu, and R. Dalla-Favera. 2005. BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. *Nat. Immunol.* **6**:1054–1060.
- Pongubala, J. M. R., and M. L. Atchison. 1991. Functional characterization of the developmentally controlled immunoglobulin kappa 3' enhancer: regulation by Id, a repressor of helix-loop-helix transcription factors. *Mol. Cell. Biol.* **11**:1040–1047.
- Pongubala, J. M. R., and M. L. Atchison. 1997. PU.1 can participate in an active enhancer complex without its transcriptional activation domain. *Proc. Natl. Acad. Sci. USA* **94**:127–132.
- Pongubala, J. M. R., S. Nagulapalli, M. J. Klemsz, S. R. McKercher, R. A. Maki, and M. L. Atchison. 1992. PU.1 recruits a second nuclear factor to a site important for immunoglobulin κ 3' enhancer activity. *Mol. Cell. Biol.* **12**:368–378.
- Pongubala, J. M. R., C. Van Beveren, S. Nagulapalli, M. J. Klemsz, S. R. McKercher, R. A. Maki, and M. L. Atchison. 1993. Effect of PU.1 phosphorylation on interaction with NF-EM5 and transcriptional activation. *Science* **259**:1622–1625.
- Ranuncolo, S. M., J. M. Polo, J. Dierov, M. Singer, T. Kuo, G. Greally, R. Green, M. Carroll, and A. Melnick. 2007. Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. *Nat. Immunol.* **8**:705–714.
- 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**:1841–1847.
- Rossbacher, J., A. M. Haberman, S. Neschen, A. Khalil, and M. J. Schlomchik. 2006. Antibody-independent B cell-intrinsic and -extrinsic roles for CD21/35. *Eur. J. Immunol.* **36**:2384–2393.
- Seyfert, V. L., D. Allman, Y. He, and L. M. Staudt. 1996. Transcriptional repression by the proto-oncogene BCL6. *Oncogene* **12**:2331–2342.
- 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**:199–212.
- Sharpe, M. J., C. Milstein, J. M. Jarvis, and M. S. Neuberger. 1991. Somatic hypermutation of immunoglobulin κ may depend on sequences 3' of C κ and occurs on passenger transgenes. *EMBO J.* **10**:2139–2145.
- Shinall, S. M., M. Gonzalez-Fernandez, R. J. Noelle, and T. J. Waldschmidt. 2000. Identification of murine germinal center B cell subsets defined by the

- expression of surface isotypes and differentiation antigens. *J. Immunol.* **164**: 5729–5738.
43. 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**:426–431.
 44. Thibault, D. L., A. D. Chu, K. L. Graham, I. Balboni, L. Y. Lee, C. Kohlmoos, A. Landrigan, J. P. Higgins, T. Tibshirani, and P. J. Utz. 2008. IRF9 and STAT1 are required for IgG autoantibody production and B cell expression of TLR7 in mice. *J. Clin. Invest.* **118**:1417–1426.
 45. Toney, L. M., G. Cattoretti, T. Graf, T. Merghoub, P.-P. Pandolfi, R. Dalla-Favera, B. H. Ye, and A. L. Dent. 2000. BCL-6 regulates chemokine gene transcription in macrophages. *Nat. Immunol.* **1**:214–220.
 46. Torlakovic, E., A. Malecka, J. H. Myklebust, A. Tierens, H.-C. Aasheim, J. M. Nesland, E. Smeland, S. Kvaloy, and J. Delabie. 2005. PU.1 protein expression has positive linear association with protein expression in germinal center B cell genes including BCL-6, CD10, CD20, and CD22: identification of PU.1 putative binding sites in the BCL-6 promoter. *J. Pathol.* **206**:312–319.
 47. van der Stoep, N., J. R. Gorman, and F. W. Alt. 1998. Reevaluation of 3' E κ function in stage- and lineage-specific rearrangement and somatic hypermutation. *Immunity* **8**:743–750.
 48. 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**:1922–1929.
 49. Wang, J., and S. Hannenhalli. 2006. A mammalian promoter model links cis elements to genetic networks. *Biochem. Biophys. Res. Commun.* **347**:166–177.
 50. Wang, J., S. Zhang, R. M. Schultz, and H. Tseng. 2006. Search for basophil target genes. *Biochem. Biophys. Res. Commun.* **348**:1261–1271.
 51. Wolniak, K. L., R. J. Noelle, and T. J. Waldschmidt. 2006. Characterization of (4-hydroxy-3-nitrophenyl)acetyl (NP)-specific germinal center B cells and antigen-binding B220⁺ cells after primary NP challenge in mice. *J. Immunol.* **177**:2072–2079.
 52. Wong, C. W., and M. L. Privalsky. 1998. Components of the SMRT corepressor complex exhibit distinctive interactions with the POZ domain oncoproteins PLZF, PLZF-RAR α , and BCL-6. *J. Biol. Chem.* **273**:27695–27702.
 53. Yamashita, R., Y. Suzuki, H. Wakaguri, K. Tsuritani, K. Nakai, and S. Sugano. 2006. DBTSS: DataBase of Human Transcription Start Sites, progress report 2006. *Nucleic Acids Res.* **34**:D86–D89.
 54. Ye, B. H., G. Cattoretti, Q. Shen, J. Zhang, N. Hawe, R. de Waard, C. Leung, M. Nouri-Shirazi, A. Orazi, R. S. K. Chaganti, P. Rothman, A. M. Stall, P.-P. Pandolfi, and R. Dalla-Favera. 1997. The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. *Nat. Genet.* **16**:161–170.
 55. Ye, B. H., P. H. Rao, R. S. K. Chaganti, and R. Dalla-Favera. 1993. Cloning of bcl-6, the locus involved in chromosome translocations affecting band 3q27 in B-cell lymphoma. *Cancer Res.* **53**:2732–2735.
 56. Yoon, H., G. Laxmikanthan, J. Lee, S. I. Blaber, A. Rodriguez, J. M. Kogot, I. A. Scarisbrick, and M. Blaber. 2007. Activation profiles and regulatory cascades of the human kallikrein-related peptidases. *J. Biol. Chem.* **282**: 31852–31864.
 57. Zhang, H., S. Okada, M. Hatano, S. Okabe, and T. Tokuhisa. 2001. A new functional domain of Bcl6 family that recruits histone deacetylases. *Biochim. Biophys. Acta* **1540**:188–200.