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A Putative Silencer Element in the *IL-5* Gene Recognized by Bcl6

Masafumi Arima,*† Hirochika Toyama,* Hirohito Ichii,* Satoko Kojima,* Seiji Okada,* Masahiko Hatano,* Gang Cheng,† Masato Kubo,‡ Takeshi Fukuda,† and Takeshi Tokuhisa2*

The *Bcl6* gene is ubiquitously expressed in adult murine tissues and its product functions as a sequence-specific transcriptional repressor. *Bcl6*-deficient mice displayed eosinophilic inflammation caused by overproduction of Th2 cytokines. The regulatory mechanism of those cytokine productions by *Bcl6* is controversial. When CD4+ T cells from *Bcl6*-deficient and lck-*Bcl6*-transgenic mice were stimulated with anti-CD3 Abs, production of IL-5 among Th2 type cytokines was preferentially affected by the amount of *Bcl6* in the T cells. We found a putative *Bcl6*-binding sequence (IL5BS) on the 3′ untranslated region in the murine and human IL-5 genes, and specific binding of *Bcl6* protein to the sequence was confirmed by gel retardation assay and chromatin immunoprecipitation assay. The binding activity of endogenous *Bcl6* was transiently diminished in Th2 but not in Th1 clones after anti-CD3 stimulation. The exogenous *Bcl6* repressed expression of the reporter gene with the IL5BS in K562 cells and the repressor activity was lost by a point mutation of the IL5BS. Furthermore, the IL5BS was required for *Bcl6* to repress expression of the IL-5 cDNA. Thus, the IL5BS may act as a silencer element for *Bcl6* to repress expression of the IL-5 gene. The *Journal of Immunology*, 2002, 169: 829–836.

Chromosomal translocations involving 3q27 were detected in some non-Hodgkin’s lymphomas, particularly in diffuse large B cell lymphomas (1, 2). The human protooncogene *BCL6* has been identified from chromosomal breakpoints (3–5). The *BCL6* gene encodes a 92- to 98-kDa nuclear phosphoprotein (6, 7) that contains the BTB/POZ domain in the NH2-terminal region and Krüppel-type zinc finger motifs in the COOH-terminal region (3–5, 8). The *Bcl6* gene is well conserved between human and murine, with a 100% identity of the zinc finger motifs at the amino acid level (8). This gene is ubiquitously expressed in various tissues including lymphatic organs and the expression is predominant in germinal center B cells (6, 7). Furthermore, expression of this gene is induced in activated lymphocytes as an immediate early gene (8). Since the NH2-terminal half of the protein contains repressor domains (9–12) and the zinc finger motifs bind to specific DNA sequences (13, 14), *BCL6* can function as a sequence-specific transcriptional repressor. Indeed, the BTB/POZ domain of *BCL6* can bind to silencing mediator of retinoid and thyroid receptor protein (SMRT) and recruit the SMRT-histone deacetylase complex to silencer regions of target genes to repress expression of those genes (15, 16).

To observe physiological functions of *Bcl6*, this gene was disrupted in the mouse germline (17–19). All of the hematopoietic lineages, including mature lymphocytes, did develop in *Bcl6*-deficient (*Bcl6−/−*) mice. However, germinal center formation was impaired in *Bcl6−/−* mice due to the abnormality of B cells but not T cells of *Bcl6−/−* mice (19). In addition, *Bcl6−/−* mice displayed inflammatory responses in multiple organs, especially the heart and lung, characterized by infiltration of eosinophils at a young adult age (17, 18, 20). Many factors are involved in generation of tissue eosinophilia and IL-5 is an important cytokine involved in controlling the growth, differentiation, and activation of eosinophils (21, 22). Production of Th2 cytokines including IL-5 by *Bcl6−/−* T cells was augmented (17, 18). Thus, mechanisms of this eosinophilic inflammation could be partly explained by a functional dominance of Th2 cells in *Bcl6−/−* mice.

Since *Bcl6*-binding DNA sequences resembled the sequence motif bound by the STAT factors and IL-4 induces differentiation of Th0 cells to Th2 cells (23), *Bcl6* might repress IL-4-induced transcription by competitive binding to DNA sites recognized by the IL-4-responsive STAT factor, STAT6 (17). However, STAT6 and *Bcl6* double-deficient mice could display inflammatory responses with infiltration of eosinophils in multiple organs (24), indicating that overproduction of Th2 cytokines by *Bcl6−/−* T cells cannot be explained by loss of competitive inhibition of STAT6 activity. In this study, we identified a *Bcl6*-binding DNA sequence in the 3′ untranslated (3′UT) region of murine and human IL-5 cDNA. We discuss this DNA sequence as a putative silencer element in the IL-5 gene.

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*Department of Developmental Genetics (H2), Graduate School of Medicine, Chiba University, Chiba, Japan; †Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Tochigi, Japan; and ‡Division of Immunobiology, Research Institute for Biological Science, Science University of Tokyo, Noda City, Chiba, Japan

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2 Address correspondence and reprint requests to Dr. Takeshi Tokuhisa, Department of Developmental Genetics (H2), Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan. E-mail address: tokuhisa@med.m.chiba-u.ac.jp

3 Abbreviations used in this paper: SMRT, silencing mediator of retinoid and thyroid receptor protein; DIG, digoxigenin; IL5BS, a putative *Bcl6*-binding sequence in the *IL-5* gene; m, murine; h, human; BALF, bronchoalveolar lavage fluid; ChIP, chromatin immunoprecipitation; CBS, consensus binding sequence; UT, untranslated region; MCP-1, monocyte chemoattractant protein 1.
Materials and Methods

Animals

C57BL/6 and BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). Bcl6−/− mice (20) and transgenic mice carrying the human Bcl6 cDNA under the control of the murine lck-proximal promoter (lck-Bcl6; H. Ichii and T. Tokuhisa, manuscript in preparation) were described elsewhere (25). The transgenic mice derived from C57BL/6 mice were backcrossed with BALB/c mice for one generation and used for this study. Those mice were maintained under specific pathogen-free conditions in the animal center of Graduate School of Medicine, Chiba University (Chiba, Japan).

Stimulation of splenic CD4+ T cells and Th clones with anti-CD3 mAb

Spleen cells were incubated with anti-CD8 (53-6.72) at 4°C for 30 min and cultured for 1 h on the plate coated with antimouse Ig to eliminate B and CD8+ T cells. Purity of CD4+ T cells was >90% in viable cells. A keyhole limpet hemocyanin-specific Th1 clone (28-4) and an autoreactive Th2 clone (M5-SB) have been established as described elsewhere (25). Two OVA-specific clones (Th1, DO10Th1-3; Th2, DO10Th2-3) were established from DO10-transgenic mice (26) according to a method described elsewhere (27). Those Th cells were stimulated every 4 wk with specific Ags (keyhole limpet hemocyanin, 100 μg/ml; OVA peptide 323–339, 1 mM) and irradiated splenocytes (30 Gy) from syngeneic mice and maintained with IL-2 (10 U/ml) or IL-4 (60 U/ml) for Th1 or Th2 clones, respectively. Monoclonal anti-CD3 (145-2C11) Ab (1–10 μg/ml) was coated on 24-well culture plates (Coming Glass, Corning, NY) at 37°C for 60 min. CD4+ T cells (1 × 106) or Th clones (1 × 105) were cultured on an anti-CD3 mAb-coated plate in 1 ml of RPMI 1640 supplemented with 10% FCS at 37°C in 5% CO2. The amount of IL-4, IL-5, and IFN-γ in the culture supernatants was measured by ELISA (BD Pharmingen, San Diego, CA).

Induction of allergic airways inflammation

Lck-Bcl6-transgenic mice were sensitized by i.p. injection with 8 μg of OVA in alum twice at an interval of 5 days. Twelve days after sensitization, animals were challenged by i.p. injection with 8 μg of OVA in alum twice at an interval of 5 days. After the second challenge, the trachea was opened, and the BALF was collected by cannulation into the trachea and secured in the airway. Three successive volumes (0.75 ml) of PBS with 0.1% OVA were instilled into the trachea and washed out with PBS. The trachea was removed, and the airway was opened. PBS with 0.1% OVA was added directly to the trachea to wash the airway. The tracheas were fixed in 1% in saline for 30 min twice at an interval of 60 min. The trachea was cleared with salmon sperm DNA/protein A/agarose-50% slurry for 30 min at 4°C and incubated with 2 μg of Bcl6-specific rabbit polyclonal Abs (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit IgG (Santa Cruz Biotechnology), or no Ab overnight at 4°C with mild shaking. The immune complexes were incubated with salmon sperm DNA/protein A agarose-50% slurry for 30 min at 4°C and washed with PBS containing 0.5% Tween 20. After centrifugation, the supernatants from the "no-Ab" sample was used to probe to filter, which was washed as described elsewhere (27). Chromatin cross-links were reversed by 0.5 M NaCl. After proteinase K digestion, DNA in samples was phenol extracted, ethanol precipitated, and resuspended in 50 μl of H2O. Two microliters of DNA solution was used for 27 cycles of PCR amplification. PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) and was then conducted according to the manufacturer’s recommendations. Briefly, formaldehyde solution (37%; Fisher Scientific, Pittsburgh, PA) was added to the "no-Ab" sample to a final concentration of 1% in 1% formaldehyde (F/A). Cells were incubated with 1% formaldehyde for 10 min, followed by incubation at 37°C for 10 min. The cross-links were reversed by 0.5 M NaCl. After proteinase K digestion, DNA in samples was phenol extracted, ethanol precipitated, and resuspended in 50 μl of H2O. Two microliters of DNA solution was used for 27 cycles of PCR amplification. PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

The following primers were used in the ChIP assays: mIL5BS, 5′-AACCTTACTACCCCATGCCAACAATAAGCATAAAATGTGTT-3′ and Mut2, 5′-AACCTTACTACCCCATGGCACAAGCATAAAATGTGTT-3′. Oligonucleotide containing Oct2A-binding sequence (5′-GTACGGAGTGCTGCTGAGTACCAGAATCTCTGG-3′) was used as a nonspecific competitor. To detect Bcl6 in the mixture, anti-Bcl6 mAb (monoclonal antibody kindly provided by Dr. T. Fukushima, Tokyo Medical and Dental University, Tokyo, Japan) was preincubated with nuclear proteins for 30 min at 4°C, followed by incubation with the DIG-labeled mlILSBS.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) and was then conducted according to the manufacturer’s recommendations. Briefly, formaldehyde solution (37%; Fisher Scientific, Pittsburgh, PA) was added to the "no-Ab" sample to a final concentration of 1% in 1% formaldehyde (F/A). Cells were incubated with 1% formaldehyde for 10 min, followed by incubation at 37°C for 10 min. The cross-links were reversed by 0.5 M NaCl. After proteinase K digestion, DNA in samples was phenol extracted, ethanol precipitated, and resuspended in 50 μl of H2O. Two microliters of DNA solution was used for 27 cycles of PCR amplification. PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

The following primers were used in the ChIP assays: mIL5BS, 5′-GGGAAAGAAGGGGACATCTCTGGT-3′ and Mut2, 5′-TTCTCTGGATGACTGCGGGGAG-3′ (201 bp); murine monocyte chemotactic protein 1 (MCP-1) promoter including the Bcl6-binding region (31), 5′-GAGAACGAAATCAGCCCTCACTATAC-3′ and 5′-TATTTGTGAGACCCAGGGGTTG-3′ (290 bp).

 Luciferase reporter and IL-5 expression constructs

To make the luciferase reporter gene with the mlILBS, the double-stranded oligonucleotides containing two copies of the mlILBS (2xILBS) and its mutants (underlined) (mut1 (2xILBSmut1; 5′-AACCTTACTACCCCATGCCAACAATAAGCATAAAATGTGTT-3′ and mut2 (2xILBSmut2; 5′-AACCTTACTACCCCATGGCACAAGCATAAAATGTGTT-3′) with the SacI and XhoI site on each flank were synthesized. The SacI-XhoI fragment of 2xILBS, 2xILBSmut1, and 2xILBSmut2 was ligated into the SacI and XhoI-digested plgL control vector (pGL3; Luc reporter gene) by using T4 DNA ligase. The resulting vector was named as plgLmut1 and plgLmut2. The double-stranded oligonucleotides containing four copies of the mlILBS (4xILBS) with the SacI site on both flanks were synthesized to make pGL4xILBS by ligation into the SacI-digested plgL control.

Two IL-5 expression vectors were constructed as follows. The BamHI fragment carrying the murine IL-5 cDNA from pSP65-mTrF23 (25; kindly provided by Dr. K. Takatsu, University of Tokyo, Tokyo, Japan) was inserted into the BamHI-digested pGEM-7zilasmid (pGEMTzIL-5). Fragments of the IL-5 cDNA were obtained by PCR with the sense primer (5′-TGACTTTGGAATCTGATGTGGAGACAG-3′ (870–896) immediately upstream from the BsrEI site (+896) of pGEMTzIL-5 and the antisense primer with a new EcoRI site (underlined) (5′-TCTGAATCTAATTATATATGTTGGAATGT-3′ (1495–1505). The BsrEI-EcoRI fragment of PCR products was subcloned into the BsrEI-EcoRI-digested pGEMTzIL-5 (pGEMTzIL-5E). The IL-5 gene with a deletion of the mlILBS was constructed as follows. The sense sequence (5′-ATACCTGAA
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TAACATGTAAGGTTGTTG-3') and its antisense fusion sequence (5'-CAACACTTACAGTTATTCAAGTTAT-3') of the upstream (1316–1326) and the downstream (1362–1376) sequence of mIL-5BS (1327–1361) were synthesized as primers. PCRs were done between the sense primer (870–896) and the antisense primer (1348–1358) of Bcl6 cDNA. The amount of IL-5 was strikingly augmented in culture supernatants of Bcl6 T cells. We showed no inhibition of IL-5 production but not that of IL-4 was continued in 24 h after stimulation. The number of total cells in the BALF of lck-Bcl6 mice 72 h after challenge. The number of total cells in the BALF of lck-Bcsl6 mice was approximately half of that in control mice (Fig. 2B). However, the level of IFN-γ and IL-2 in the BALF of lck-Bcsl6 mice was not reduced. Furthermore, we examined the number of eosinophils and lymphocytes in the BALF of lck-Bcsl6 mice 72 h after challenge. The number of total cells in the BALF of lck-Bcsl6 mice was approximately half of that in control mice (Fig. 2C). Percentages of eosinophils and lymphocytes in the BALF were 13 and 19% in the lck-Bcsl6 mice and 61 and 11% in the control mice, respectively. The percentage and cell number of eosinophils in the lck-Bcsl6 mice were approximately one-fifth and one-seventh of those of the control mice, respectively, suggesting the reduction of IL-5 production. These results suggested the IL-5 gene as a target gene of the sequence specific transcriptional repressor, Bcl6.

Bcl6 binds to a DNA sequence in the IL-5 gene

We tried to identify a DNA sequence similar to Bcl6-binding sequences (13, 14) in the genomic IL-5 gene by computer analysis. As shown in Fig. 3A, a similar sequence was found in exon 4 of the murine and human IL-5 genes as the 3' UT region of IL-5 cDNA. The most important residue (GA in GAAAG) of the Bcl6-binding sequence (34) is conserved in both sequences, whereas the STAT-binding GAS motif (TTTC-N3-4-GAA) conserved in the Bcsl6-binding sequences of the known target genes (31, 35, 36) is not preserved.

Gene transfection and luciferase assay

K562 (Bcsl6 null) cells were transfected with the luciferase reporter gene (4 µg) and Bcsl6 expression vector (pcDNA3-Bcsl6) or control vector (pcDNA3) (total DNA; 14 µg), or with the IL-5 expression vector (4 µg) and pcDNA3-Bcsl6 or pcDNA3 (total DNA 24 µg). For all transfections, pRL-tk vector (1 µg) was cotransfected as an internal control for transfection efficiency. Electroporation was conducted using a Gene Pulser (Bio-Rad) at 0.22 kV and 960 microfarads. Luciferase activity in cell extracts was determined using the Luciferase Assay kit (Promega) and standardized using luciferase activity by pRL-tk vector. The amount of IL-5 in culture supernatants was measured by ELISA. IL-5 productivity was calculated as a ratio between IL-5 concentration and luciferase activity by pRL-tk vector and expressed as a percentage of the ratio from cells transfected with the IL-5 expression vector and pcDNA3.

Statistical analysis

All data are expressed as mean ± SD. The Student’s t test was used for the comparison of data between Bcsl6−/− and control mice, unless otherwise stated.

Results

Production of IL-5 by CD4+ T cells stimulated with anti-CD3 is strongly regulated by Bcsl6

To examine regulation of Th2-type cytokine productions by Bcsl6, we used CD4+ T cells in the spleen of Bcsl6−/− mice at 4 wk of age without eosinophilic inflammation. CD4+ T cells were stimulated with anti-CD3 mAb (10 µg/ml) for 48 h and the amount of IFN-γ, IL-4, and IL-5 in culture supernatants was measured by ELISA. As shown in Fig. 1A, the amount of IL-5 was strikingly augmented in culture supernatants of Bcsl6−/− T cells and the level was 16-fold higher than that of Bcsl6+/+ T cells. The amount of IL-4 in the culture supernatants of Bcsl6−/− T cells was 3-fold higher than that of Bcsl6+/+ T cells. In contrast, the amount of IFN-γ showed no significant difference between Bcsl6−/− and Bcsl6+/+ T cells until 24 h after stimulation. When CD4+ T cells were stimulated with various doses (0–10 µg/ml) of anti-CD3 for 24 h, augmentation of IL-5 production by Bcsl6−/− T cells was the highest among those cytokine productions regardless of the dose of stimulation examined (Fig. 1B).

The regulatory effect of Bcsl6 on production of Th2 cytokines was confirmed by overexpression of Bcsl6 in CD4+ T cells. We have recently established two lines of transgenic mice carrying the lck-Bcsl6 gene. Thymocytes and splenic T cells from these transgenic mice expressed the exogenous Bcsl6 gene and produced larger amounts of Bcsl6 protein compared with those of littermate control mice. When splenic CD4+ T cells of lck-Bcsl6-transgenic mice were stimulated with anti-CD3 (10 µg/ml) for 48 h, the amount of IL-4 and IL-5, but not that of IFN-γ, in culture supernatants of lck-Bcsl6 T cells was reduced (Fig. 2A). Since the level of IL-4 and IL-5 produced by lck-Bcsl6 T cells was 1/2.5 and 1/4 of that by control T cells, respectively, reduction of IL-5 production was more significant than that of IL-4.

The preferential regulation of Bcsl6 to expression of the IL-5 gene was supported by cytokine levels in BALF of lck-Bcsl6 mice after challenge with an allergen. The level of IL-4 and IL-5, but not that of IFN-γ and IL-2 in the BALF of lck-Bcsl6 mice was reduced to half of those of the control 24 h after challenge. The inhibition of IL-5 production but not that of IL-4 was continued in the BALF of lck-Bcsl6 mice up to 72 h after challenge (Fig. 2B). However, the level of IFN-γ and IL-2 in the BALF of lck-Bcsl6 mice was not reduced. Furthermore, we examined the number of eosinophils and lymphocytes in the BALF of lck-Bcsl6 mice 72 h after challenge. The number of total cells in the BALF of lck-Bcsl6 mice was approximately half of that in control mice (Fig. 2C). Percentages of eosinophils and lymphocytes in the BALF were 13 and 19% in the lck-Bcsl6 mice and 61 and 11% in the control mice, respectively. The percentage and cell number of eosinophils in the lck-Bcsl6 mice were approximately one-fifth and one-seventh of those of the control mice, respectively, suggesting the reduction of IL-5 production. These results suggested the IL-5 gene as a target gene of the sequence specific transcriptional repressor, Bcsl6.

Bcsl6 binds to a DNA sequence in the IL-5 gene

We tried to identify a DNA sequence similar to Bcsl6-binding sequences (13, 14) in the genomic IL-5 gene by computer analysis. As shown in Fig. 3A, a similar sequence was found in exon 4 of the murine and human IL-5 genes as the 3' UT region of IL-5 cDNA. The most important residue (GA in GAAAG) of the Bcsl6-binding sequence (34) is conserved in both sequences, whereas the STAT-binding GAS motif (TTTC-N3-4-GAA) conserved in the Bcsl6-binding sequences of the known target genes (31, 35, 36) is not preserved.
EMSA was performed to examine binding activity of Bcl6 to the putative Bcl6-binding sequence in the murine IL-5 gene (mIL5BS). When GST-Bcl6 zinc finger protein was incubated with DIG-labeled mIL5BS as a probe, gel retardation bands were observed (Fig. 3B). The major retarded band was obviously destroyed by a nonlabeled probe with the same sequence (Fig. 3B, wild type (wt)) as a cold competitor. In contrast, mutated cold probes (one base mismatch; underlined) substituted from (GAAAG) to (TAAAG) (Mut1) or to (GCAAG) (Mut2) and a nonspecific cold probe (Oct2A-binding sequence) as a cold competitor did not inhibit formation of the gel retardation band, indicating sequence specific binding of Bcl6 to the mIL5BS. GST-Bcl6 zinc finger protein also specifically bound to the similar sequence in the human IL-5 gene (hIL5BS; data not shown).

We then investigated binding activity of Bcl6 in nuclear proteins from Th1 (28–4) and Th2 (MS-SB) clones to mIL5BS by EMSA. We examined the amount of Bcl6 in nuclear proteins from those Th clones after anti-CD3 stimulation by Western blot. The similar amount of Bcl6 protein was detected in both Th1 and Th2 clones before stimulation, and the amount of Bcl6 in both Th1 and Th2 clones did not change after stimulation (Fig. 4A). We then examined binding activity of Bcl6 in those nuclear proteins to mIL5BS by EMSA. As shown in Fig. 4B, one major band was detected in nuclear proteins from an unstimulated Th1 clone (lane 2). This band was specifically destroyed by cold competition with 50-fold molar excess of wild-type oligonucleotides (Fig. 4B, lane 8) but not with Mut1 (Fig. 4B, lane 9) as a competitor. Furthermore, the band was also destroyed by the addition of anti-Bcl6 mAb in the mixture of nuclear proteins and mIL5BS probe (Fig. 4B, lane 10), indicating that Bcl6 in nuclear proteins from the Th1 clone binds to mIL5BS. The band did not disappear in the Th1 clone after stimulation with anti-CD3 (Fig. 4B, lanes 3–7). A similar Bcl6-binding profile was observed in the OVA-specific Th1 (DO10Th1–3) clone (data not shown). One major IL5BS-specific band was also detected in nuclear proteins from Th2 clones before stimulation. When the Th2 clone was stimulated with anti-CD3, binding activity of Bcl6 disappeared within 1 h after stimulation.

FIGURE 2. Production of IL-5 by lck-Bcl6 T cells is strongly suppressed. A, CD4+ T cells of lck-Bcl6 (■) and Bcl6+/+ (□) mice were stimulated with anti-CD3 mAb (10 μg/ml) for 48 h. The amount of IL-4, IL-5, and IFN-γ in the culture supernatants at various time points after stimulation was measured by ELISA. B and C, lck-Bcl6 mice immunized with OVA were challenged with aerosolized OVA. B, The amount of IL-4, IL-5, IFN-γ, and IL-2 in BALF of lck-Bcl6 mice 24 and 72 h after challenge was measured by ELISA. ND, Not detected. C, The number of eosinophils (Eos) and lymphocytes (Lym) in the BALF of lck-Bcl6 mice 72 h after challenge. Results represent the mean ± SD of three to six wells per group. These results are representative of three independent experiments. *, p < 0.05; **, p < 0.01.

FIGURE 3. Bcl6 binds to the putative Bcl6-binding sequence in the IL-5 gene. A, A genomic map of the human IL-5 gene. Each box indicates exons. Closed boxes are coding regions of IL-5. ATG, start codon; TGA, stop codon; AATAAA, poly(A) additional sequence. The putative Bcl6-binding sequences (IL5BS) in the human and murine IL-5 genes (hIL5 and mIL5) are compared with the consensus Bcl6-binding sequence and the Bcl6-binding sequences of the known target genes. B, GST-Bcl6 zinc finger protein was incubated with DIG-labeled mIL5BS, and the retardation band was detected by EMSA. Mut1, Mut2, and Oct2A-binding sequence as a cold competitor did not inhibit formation of the gel retardation band. An arrow indicates the retarded band.
FIGURE 4. Bcl6 in Th2 clones but not in Th1 clones loses its binding activity to IL5BS after anti-CD3 stimulation. A, Th1 (28–4) and Th2 (MS-SB) clones were stimulated with anti-CD3 mAb (10 μg/ml) for 24 h. The amount of Bcl6 in nuclear proteins from those clones was measured by Western blot. pc, Cell lysates from transfectants of Bc16 as a positive control. Bcl6−/−: nuclear proteins of spleen cells from Bc16−/− mice as a negative control. An arrow indicates Bcl6 band. B, Nuclear proteins from those clones were incubated with DIG-labeled mIL5BS, and the retardation band by Bcl6 was detected by EMSA. Mut1 as a cold competitor did not inhibit formation of the gel retardation band. These results are representative of three independent experiments. Arrows indicate the Bcl6-specific retarded band. wt, Wild type.

FIGURE 5. Binding of Bcl6 to the mIL5BS in CD4+ T cells or in Th clones is detected by ChIP assay. A, Bcl6 on the chromatin in CD4+ T cells was immunoprecipitated (IP) by polyclonal anti-Bcl6 Abs. The Bcl6-binding sequence in the IL-5 gene (IL5BS) and that in the promoter region of MCP-1 gene (31) as a positive control. The PCR products, including the Bcl6-binding sequence, were observed in the same complexes from CD4+ T cells of Bc16−/+ but not in those of Bc16−/− mice. The chromatin precipitation from CD4+ T cells with rabbit polyclonal IgG did not show any significant PCR products. The mIL5BS PCR products were also detected in the complexes from Th1 and Th2 clones without stimulation (Fig. 5B). The products were still observed in those from Th1 clones but not in those from Th2 clones 1.5 h after anti-CD3 stimulation. These results indicate that Bcl6 binds to the IL5BS in T cells.

IL5BS is required for Bcl6 to display its repressor activity

We examined a requirement of the IL5BS for Bc16 to display its repressor activity using the reporter gene containing two or four repeats of the mIL5BS (pGL3C-IL5BS) between the virus promoter and the luciferase reporter sequence (pGL3C). K562 (Bc16 null) cells were cotransfected with pGL3C-IL5BS and various doses of pcDNA3-Bc16, and luciferase activity in K562 cells was measured 48 h after transfection. Luciferase activity in K562 cells transfected with pGL3C-4xIL5BS was reduced by pcDNA3-Bc16 in a dose-dependent manner (Fig. 6A). However, luciferase activity in K562 cells transfected with pGL3C was not reduced by pcDNA3-Bc16. The reducing activity by pcDNA3-Bc16 was confirmed in K562 cells transfected with pGL3C-2xIL5BS (Fig. 6B). When we introduced mutations in the IL5BS (GAAAG) (pGL3C-2xIL5BSmu1(TAAAG) and pGL3C-2xIL5BSmu2 (TCAAG)), the activity from those mutated pGL3C-2xIL5BSs was not suppressed by pcDNA3-Bc16.

We further investigated a role of the IL5BS in Bcl6-mediated repression for the IL-5 gene expression by cotransfection of the murine IL-5 cDNA under the control of a retrovirus promoter (M(I-E)IL-5) and various doses of pcDNA3-Bc16 into K562 cells or NIH3T3 cells. A construct of M(I-E)IL-5 deleted with the mIL5BS, M(I-E)IL-5(Δ1327–1361), was used to confirm a critical role of the mIL5BS in Bcl6-mediated repression. The amount of IL-5 in culture supernatants of K562 cells 24 h after transfection was measured by ELISA. The amount of IL-5 produced by K562 cells transfected with the deletion construct without pcDNA3-Bc16 was similar to that by K562 cells transfected with M(I-E)IL-5 without pcDNA3-Bc16 (data not shown). The absolute amount of IL-5 in each culture supernatant varied from 100 to 400 pg/ml. When various doses of pcDNA3-Bc16 were cotransfected with M(I-E)IL-5, IL-5 production was significantly reduced to <1% of the maximum by pcDNA3-Bc16 in a dose-dependent manner (Fig. 7A).
In contrast, there was no significant change in IL-5 production by K562 cells cotransfected with M(I-E)IL-5(Δ1327–1361) and pcDNA3-Bcl6 up to 15 μg. Similar results were obtained using NIH3T3 cells (data not shown). These results suggest that Bcl6 binds onto the mIL5BS in the IL-5 gene to repress its expression.

**Discussion**

A functional dominance of Th2 cells in Bcl6−/− mice has already been reported by other groups (17, 18). They showed that anti-CD3 activation of T cells from Bcl6−/− mice produced higher levels of Th2-type cytokines at mRNA and protein levels. Since they used lymph node cells and infiltrating cells in the lung of Bcl6−/− mice with Th2 dominant inflammation, we expected that those augmented cytokine productions were derived from activated Th2 cells in inflammatory lesions. Thus, we isolated CD4+ T cells from young Bcl6−/− mice without eosinophilic inflammation and stimulated those Bcl6−/− T cells with anti-CD3. Production of IL-4 and IL-5 by those Bcl6−/− T cells was clearly augmented. However, augmentation of IL-5 production was much higher than that of IL-4 production regardless of the dose (1–10 μg/ml) of stimulation examined. Thus, augmentation of IL-4 and IL-5 productions by Bcl6−/− T cells cannot be explained only by a functional dominance of Th2 cells in Bcl6−/− T cells. Furthermore, both IL-4 and IL-5 productions by splenic CD4+ T cells from lck-Bcl6 mice and in BALF of immunized lck-Bcl6 mice were reduced, and the reduction of IL-5 production was higher than that of IL-4. These results suggest that expression of the IL-5 gene is specifically regulated by Bcl6.

Th2 cells often coordinately produce IL-4 and IL-5. However, it is unclear whether similar molecular mechanisms underlie transcription of the two genes. Although the transcription factor GATA-3 (37–39) was shown to be sufficient for expression of the IL-4 and IL-5 genes (37), the antisense GATA-3 RNA inhibits IL-5 but not IL-4 promoter activation (40). These results suggested that Bcl6 might regulate expression of GATA-3 to modulate IL-5 expression in Bcl6−/− and lck-Bcl6 T cells. However, no report indicates that GATA-3 is the direct target gene of Bcl6. The 5′ flanking region (1.2 kb) of the IL-5 gene directs its expression in Th2 clones but not in Th1 clones (41–43). Transient transfection assays with a series of deletion constructs of the 1.2-kb region indicated that negatively acting elements map to the most 5′ side of the region (44, 45). However, any transcriptional repressor that binds

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**FIGURE 6.** Bcl6 can repress expression of the reporter gene with the IL5BS. The luciferase reporter gene (pGL3C) or the reporter gene with the IL5BS (pGL3C-4xIL5BS; four copies of the IL5BS (A), pGL3C-2xIL5BS; two copies of the IL5BS (B)) was cotransfected with various doses (0, 3, or 10 μg) of pcDNA3-Bcl6 into K562 cells. Luciferase activity in K562 cells was measured 48 h after transfection. pGL3C-2xIL5BSmu1 and pGL3C-2xIL5BSmu2 have mutations in the IL5BS. Results represent the mean ± SD of triplicate cultures per group. These results are representative of two independent experiments. *, p < 0.05; **, p < 0.01.

**FIGURE 7.** The IL5BS is required for Bcl6 to repress expression of the exogenous IL-5 gene. The murine IL-5 cDNA expression vector (M(I-E)IL-5, □) or that with deletion of the mIL5BS (M(I-E)IL-5(Δ1327–1361), ■) was cotransfected with various doses (0, 10, 15, or 20 μg) of pcDNA3-Bcl6 into K562 cells. Those transfecants were cultured for 24 h. The amount of IL-5 in culture supernatants was measured by ELISA. Results represent the mean ± SD of triplicate cultures per group. These results are representative of three independent experiments. ***, p < 0.01.
to the elements is not known and Bcl6-binding sequences were not found in the elements, suggesting that the promoter region does not contain the binding region of Bcl6.

We found a Bcl6-binding DNA region (IL5BS) in exon 4 of the murine and human IL-5 genes, and the binding was confirmed by the ChIP assay and the EMSA. This IL5BS is required for Bcl6-mediated repression of the exogenous reporter gene and the IL-5 cDNA in K562 cells (Fig. 7) and NIH3T3 cells (data not shown) by transient transfection assay. Therefore, the IL5BS is a novel silencer element in the IL-5 gene. This silencer region was supported by previous reports using the human and murine IL-5 genes with deletion of the 3′ UT including this element (32, 46). However, the IL5BS is not the same as the consensus binding sequence (CBS; 5′-ATTCTCTAGAAG-3′) of Bcl6 (13, 14). We have determined the important residues of CBS (34). Three nucleotides of T, G, and A in the CBS (5′-ATTCTCTAGAAG-3′) are important nucleotides for Bcl6-binding and the GA is the most important one. The GA residues are conserved in the IL5BS like in the other known Bcl6-binding sequences (31, 35, 36). However, the residue T is not conserved in the IL5BS and also in some of those known Bcl6 target genes (MCP-1 and CD23) (31), suggesting that the T residue is not essential for the binding. Indeed, we confirmed the binding of Bcl6 to the Bcl6-binding sequence of MCP-1 by the ChIP assay. Although the CBS and the other Bcl6-binding sequences of the known Bcl6 target genes contain the STAT-binding GAS motif (5′-TTCCTAGAAG-3′), the human and murine IL5BS do not, confirming less importance of the TTC residues in the elements is not known and Bcl6-binding sequences were not found in the elements, suggesting that the promoter region does not contain the binding region of Bcl6.

The binding activity of Bcl6 to mIL5BS was transiently diminished in Th2 clones but not in Th1 clones after stimulation (Figs. 4 and 5), although both Th1 and Th2 clones have a similar amount of Bcl6 protein in nucleus even after stimulation. These results may be explained by a functional modification of Bcl6 in Th2 cells but not in Th1 cells after stimulation. Bcl6 in Th2 cells may be post-transcriptionally modified to lose its binding activity to mIL5BS after stimulation. Transcriptional activity of several factors is regulated by posttranscriptional modifications (47–51). A zinc finger-type transcription factor, GATA-1, increases its binding activity to IL5BS in activated Th2 cells. Binding activity of Bcl6 to mIL5BS did not disappear in Th1 clones after stimulation, suggesting one possible silencing mechanism of IL-5 gene expression in Th1 cells. Since Bcl6 may repress transcription through mechanisms involving SMRT recruitment and histone deacetylation, Bcl6 that binds to mIL5BS may deacetylate histones of the promoter region of the IL-5 gene to close the chromatin structure. This chromatin remodeling may inhibit binding of other important transcriptional activators to the promoter region.

Bcl6 also regulates expression of the IL-4 gene. Previous reports have demonstrated that the silencer region of the IL-4 gene contains two STAT6 binding sites (25) and that recombinant Bcl6 apparently binds to this region (35). Differentiation of naive CD4+ T cells into mature Th2 cells is associated with chromatin remodeling of cytokine gene loci (52). Those Th2-type cytokine (IL-4 and IL-5) genes make a gene cluster within a 150-kb region of the human 5q23–31 chromosomal region (53), which is syntenic with the corresponding region of murine chromosome 11. DNase I assay indicated that structural changes in chromatin during Th1 differentiation occurred in the IL-4 silencer region (52). Thus, Bcl6 binds to the IL-4 silencer region in Th1 cells and may deacetylate histones of the chromosomal region to repress expression of those cytokine genes by recruiting the SMRT and histone deacetylase complex. Although we cannot identify other putative Bcl6-binding sequences in the IL-4 gene by computer analysis, Bcl6 binds to various putative Bcl6-binding sequences in the gene cluster including the IL5BS and the IL-4 silencer region and may play a role in regulating expression of Th2-type cytokine genes in the gene cluster.

In summary, we identify the putative silencer region in the IL-5 gene and Bcl6 binds to the region.

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References

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