The Journal of Immunology

RESEARCH ARTICLE | JULY 15 2002

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Masafumi Arima; ... et. al

J Immunol (2002) 169 (2): 829–836.
https://doi.org/10.4049/jimmunol.169.2.829

A Putative Silencer Element in the *IL-5* Gene Recognized by Bcl6[1]

Masafumi Arima,*† Hirochika Toyama,* Hirohito Ichii,* Satoko Kojima,* Seiji Okada,* Masahiko Hatano,* Gang Cheng,† Masato Kubo,‡ Takeshi Fukuda,† and Takeshi Tokuhisa[2]*

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 NH_2-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 NH_2-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)[3] 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 hemopoietic 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.

*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,Yamazaki, Noda City, Chiba, Japan

Received for publication November 12, 2001. Accepted for publication May 2, 2002.

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[1] This work was supported in part by Health Sciences Research Grants (Research on Eye and Ear Sciences, Immunology, Allergy, and Organ Transplantation) from the Ministry of Health and Welfare of Japan, Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, special coordination funds for the promotion of science and technology from the Science and Technology Agency of Japan, and Grant-in-Aid from The Tokyo Biochemical Research Foundation. M.A. was a recipient of Health Sciences Research Grants from the Ministry of Health and Welfare of Japan.

[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.

0022-1767/02/$02.00

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. 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 (MS-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 (Corning Glass, Corning, NY) at 37°C for 60 min. CD4$^+$ T cells (1×10^6) or Th clones (1×10^6) were cultured on an anti-CD3 mAb-coated plate in 1 ml of RPMI 1640 supplemented with 10% FCS at 37°C in 5% CO_2. 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, those mice were challenged with an aeroallergen as nebulized OVA (1% in saline) for 30 min twice at an interval of 60 min. The trachea was isolated from the mice by blunt dissection 24 and 72 h after the last challenge. A small caliber tube was inserted into the trachea and secured in the airway. Three successive volumes (0.75 ml) of PBS with 0.1% OVA were then instilled and gently aspirated and pooled. Each bronchoalveolar lavage fluid (BALF) sample was centrifuged, and the supernatants were stored at −70°C until use. The level of each cytokine in the supernatants was determined by ELISA. Lymphocytes in the pellet of BALF were stained with H&E. Eosinophils in the pellet were identified by Luna staining (20).

Isolation of nuclear proteins and Western blot analysis

Nuclear proteins were isolated from Th1 and Th2 clones according to the method as described elsewhere (28), with slight modification. Briefly, Th cells (1×10^7) were resuspended in 400 μl of cold hypotonic buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 100 mM PMSF, and 5 μg/ml aprotinin). Nuclei were collected by centrifugation and disrupted by sonication in 100 μl immunoprecipitation buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 100 mM PMSF, and 5 μg/ml aprotinin) at 4°C. Nuclear extracts were immediately stored at −80°C. The amount of protein was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Bcl6 in nuclear proteins from Th1 and Th2 clones was detected by Western blot (29). Briefly, 20 μg of nuclear proteins were resolved by SDS-PAGE and transferred to a polyvinyl difluoride membrane (Immobilon-P; Millipore, Bedford, MA). Blots were incubated with rabbit anti-Bcl6 Ab (19) followed by HRP-conjugated donkey anti-rabbit Ig (Amersham, Arlington Heights, IL) for 1 h at each step and developed with ECL reagents (Amersham International).

EMSA

Double-stranded oligonucleotides corresponding to a putative Bcl6-binding sequence in the murine *IL-5* gene (5′-AACCTTACTACCCCATGCCAACAGAAAGCATAAAATGGTT-3′; mIL5BS) were synthesized. Binding activity of Bcl6 to the mIL5BS was determined by EMSA (30). Briefly, the mIL5BS was labeled with digoxigenin (DIG) using DIG Oligonucleotide 3′-End-Labeling kits (Roche Molecular Biochemicals, Indianapolis, IN). Binding reactions were performed in the mixture containing purified GST-Bcl6 zinc finger protein (50 ng) or nuclear proteins (3 μg), poly(d(I-C)) (0.5 μg; Amersham Pharmacia Biotech, Piscataway, NJ), and the DIG-labeled probe (15 fM) in 10 μl of reaction buffer (10 mM HEPES (pH 7.8), 50 mM KCl, 1 mM DTT, 50 μg/ml BSA). This mixture was separated by electrophoresis on a 6% nondenaturing polyacrylamide gel and transferred to a nylon membrane (Roche Molecular Biochemicals) using an electroblot (Bio-Rad). The DIG-labeled probe was detected with sheep anti-DIG Ab conjugated with alkaline phosphatase. The Ab detection reaction was performed using an ECL detection system (Roche Molecular Biochemicals). Competitive EMSA was done by adding 10- or 50-fold molar excess of unlabeled double-stranded oligonucleotide to the mixture. Sequences of the mutant oligonucleotide (one base mismatch; underlined) were as follows: Mut1, 5′-AACCTTACTACCCCATGCCAACATAAAG CATAAAATGGTT-3′ and Mut2, 5′- AACCTTACTACCCCATGCCAA CAGCAAGCATAAAATGGTT-3′. Oligonucleotide containing Oct2A-binding sequence (5′-GTACGGAGTATCCAGCTCCGTAGCATGCAA ATCCTCTGG-3′) was used as a nonspecific competitor. To detect Bcl6 in the mixture, anti-Bcl6 mAb (mouse IgG1, kindly provided by Dr. T. Fukuda, 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 mIL5BS.

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 directly to CD4$^+$ T cells from Bcl6$^{-/-}$ and Bcl6$^{+/+}$ mice or to Th clones at a final concentration of 1%. Cross-linking of proteins on chromatin was allowed to occur at room temperature for 10 min, and the cells were lysed by SDS lysis buffer with protease inhibitors. Chromatin in the lysate was sonicated to an average length of 200–500 bp as determined by agarose gel electrophoresis. The suspension was precleared 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 with mild shaking for 1 h at 4°C, washed, and eluted. After precipitation, the supernatant from the "no-Ab" sample was processed to the cross-link reversal step and analyzed as an unfractionated input 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 H_2O. 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′-GGAAAAGAAAAGGGACATCTCCTTG-3′ and 5′-TTCCTGGAGTAA ACTGGGGGAG-3′ (201 bp); murine monocyte chemoattractant protein 1 (MCP-1) promoter including the Bcl6-binding region (31), 5′-GGAAAA GAAAAAGCAGAGCCACTCCATTCACAC-3′ and 5′-TTATTGTA AGCCAGGGGGGTGG-3′ (290 bp).

Luciferase reporter and IL-5 expression constructs

To make the luciferase reporter genes with the mIL5BS, the double-stranded oligonucleotides containing two copies of the mIL5BS (2xIL5BS) and its mutants (underlined) (mu1 (2xIL5BSmu1; 5′-AACCTTACTAC CCCATGCCAACATAAAGCATAAAATGGTT-3′) and mu2 (2xIL5BSmu2; 5′-AACCTTACTACCCCATGCCAACATCAAGCATAAAATGGTT-3′)) with the *Sac*I and *Xho*I site on each flank were synthesized. The *Sac*I-*Xho*I fragment of 2xIL5BS, 2xIL5BSmu1, and 2xIL5BSmu2 was ligated into the *Sac*I- and *Xho*I-digested pGL3 control vector (pGL3C contains the luc reporter gene with SV40 promoter and enhancer; Promega, Madison, WI) to construct pGL3C-2xIL5BS, pGL3C-2xIL5BSmu1, and pGL3C-2xIL5BSmu2, respectively. The double-stranded oligonucleotides containing four copies of the mIL5BS (4xIL5BS) with a *Sac*I site on both flanks were synthesized to make pGL3C-4xIL5BS by ligation into the *Sac*I-digested pGL3 control.

Two *IL-5* expression vectors were constructed as follows. The *Bam*HI fragment carrying the murine *IL-5* cDNA from pSP6K-mTRF23 (Ref. 32; kindly provided by Dr. K. Takatsu, University of Tokyo, Tokyo, Japan) was constructed into the *Bam*HI-digested pGEM-7Z plasmid (pGEM7Z-IL-5). Fragments of the *IL-5* cDNA were obtained by PCR with the sense primer (5′-TGACTTTGAACTCAGTGTGTAGCCAAG-3′ (870–896)) immediately upstream from the *Bst*EII site (+896) of pGEM7Z-IL-5 and the antisense primer with a new *Eco*RI site (underlined) (5′-TTTGAATTCA GAATATTATATACGTTG-3′ (1495–1505)). The *Bst*EII-*Eco*RI fragment of PCR products was subcloned into the *Bst*EII-*Eco*RI-digested pGEM7Z-IL-5 (pGEM7Z-IL-5E). The *IL-5* gene with a deletion of the mIL5BS was constructed as follows. The sense fusion sequence (5′-ATACCTGAA

TAACATGTAAGGTTGTG-3′) and its antisense fusion sequence (5′-CA CAACCTTACATGTTATTCAGGTAT-3′) of the upstream (1316–1326) and the downstream (1362–1376) sequence of mIL5BS (1327–1361) were synthesized as primers. PCRs were done between the sense primer (870–896) and the antisense fusion primer and between the sense fusion primer and the antisense primer (1495–1505). These two PCR products were used as templates for the second PCR, which was performed with the sense primer (870–896) and the antisense primer (1495–1505). The *Bst*EII-*Eco*RI fragment of PCR products was subcloned into the *Bst*EII-*Eco*RI-digested pGEM7Z-IL-5E (pGEM7Z-IL-5(Δ1327–1361)). Each *Bam*HI-*Eco*RI fragment of pGEM7Z-IL-5E or pGEM7Z-IL-5(Δ1327–1361) was subcloned into the *Bgl*II and *Eco*RI digested MSCV/IRES-EGFP (33) to generate M(I-E)IL-5 or M(I-E)IL-5(Δ1327–1361), respectively.

Gene transfection and luciferase assay

K562 (Bcl6 null) cells were transfected with the luciferase reporter gene (4 μg) and Bcl6 expression vector (pcDNA3-Bcl6) or control vector (pcDNA3) (total DNA; 14 μg), or with the IL-5 expression vector (4 μg) and pcDNA3-Bcl6 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 Bcl6$^{-/-}$ and control mice, unless otherwise stated.

Results

Production of IL-5 by CD4$^+$ T cells stimulated with anti-CD3 is strongly regulated by Bcl6

To examine regulation of Th2-type cytokine productions by Bcl6, we used CD4$^+$ T cells in the spleen of Bcl6$^{-/-}$ 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. 1*A*, the amount of IL-5 was strikingly augmented in culture supernatants of Bcl6$^{-/-}$ T cells and the level was 16-fold higher than that of Bcl6$^{+/+}$ T cells. The amount of IL-4 in the culture supernatants of Bcl6$^{-/-}$ T cells was 3-fold higher than that of Bcl6$^{+/+}$ T cells. In contrast, the amount of IFN-γ showed no significant difference between Bcl6$^{-/-}$ and Bcl6$^{+/+}$ 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 Bcl6$^{-/-}$ T cells was the highest among those cytokine productions regardless of the dose of stimulation examined (Fig. 1*B*).

The regulatory effect of Bcl6 on production of Th2 cytokines was confirmed by overexpression of Bcl6 in CD4$^+$ T cells. We have recently established two lines of transgenic mice carrying the *lck-Bcl6* gene. Thymocytes and splenic T cells from those transgenic mice expressed the exogenous *Bcl6* gene and produced larger amounts of Bcl6 protein compared with those of littermate control mice. When splenic CD4$^+$ T cells of lck-Bcl6-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-Bcl6 T cells was reduced (Fig. 2*A*). Since the level of IL-4 and IL-5 produced by lck-Bcl6 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 Bcl6 to expression of the *IL-5* gene was supported by cytokine levels in BALF of lck-Bcl6 mice after challenge with an aeroallergen. The level of IL-4 and IL-5, but not that of IFN-γ and IL-2 in the BALF of lck-Bcl6 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-Bcl6 mice up to 72 h after challenge (Fig. 2*B*). However, the level of IFN-γ and IL-2 in the BALF of lck-Bcl6 mice was not reduced. Furthermore, we examined the number of eosinophils and lymphocytes in the BALF of lck-Bcl6 mice 72 h after challenge. The number of total cells in the BALF of lck-Bcl6 mice was approximately half of that in control mice (Fig. 2*C*). Percentages of eosinophils and lymphocytes in the BALF were 13 and 19% in the lck-Bcl6 mice and 61 and 11% in the control mice, respectively. The percentage and the cell number of eosinophils in the lck-Bcl6 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.

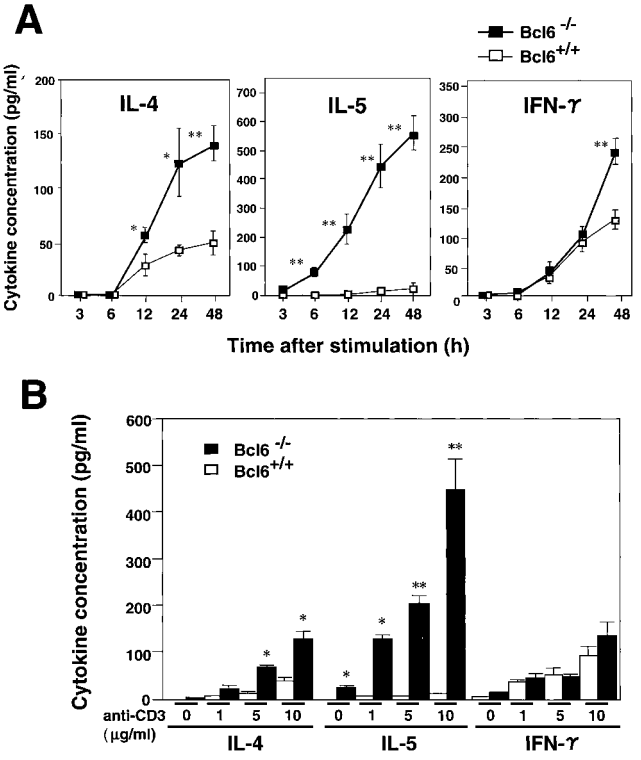

FIGURE 1. Production of IL-5 by Bcl6$^{-/-}$ T cells is strongly augmented. *A*, CD4$^+$ T cells of Bcl6$^{-/-}$ (■) and Bcl6$^{+/+}$ (□) mice were stimulated with anti-CD3 mAb (10 μg/ml) for 48 h. *B*, CD4$^+$ T cells of Bcl6$^{-/-}$ (filled bar) and Bcl6$^{+/+}$ (open bar) mice were stimulated with various doses (0, 1, 5, or 10 μg/ml) of anti-CD3 mAb for 24 h. The amount of IL-4, IL-5, and IFN-γ in the culture supernatants at various time points (*A*) or 24 h (*B*) after stimulation was measured by ELISA. Results represent the mean ± SD of three to six wells per group. These results are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

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. 3*A*, 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 (TTC-(N3–4)-GAA) conserved in the Bcl6-binding sequences of the known target genes (31, 35, 36) is not preserved.

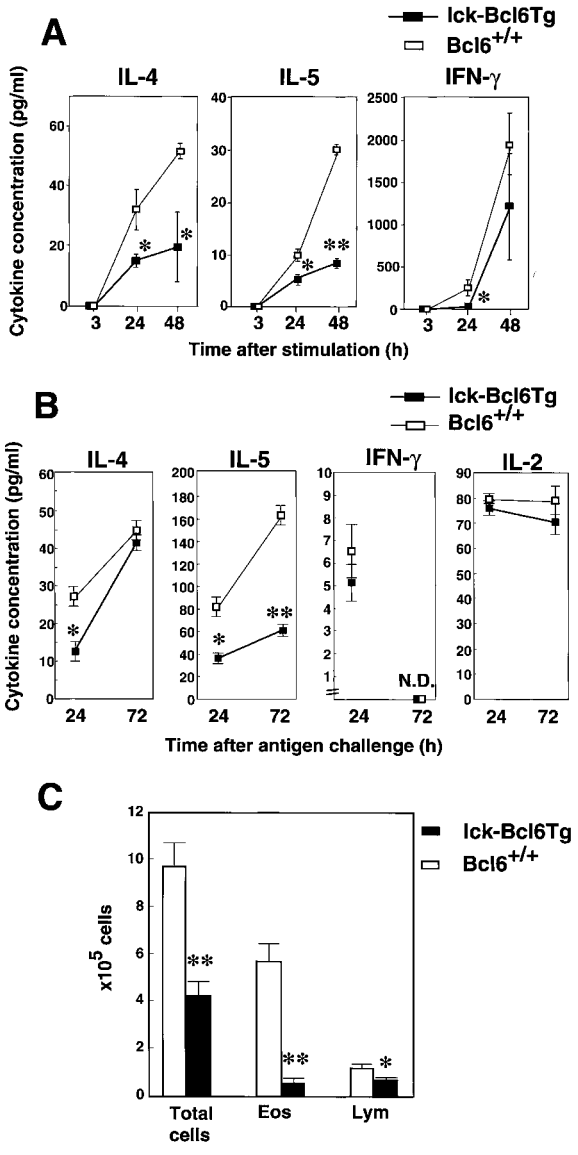

FIGURE 2. Production of IL-5 by lck-Bcl6 T cells is strongly suppressed. *A*, $CD4^+$ T cells of lck-Bcl6 (■) and $Bcl6^{+/+}$ (□) mice were stimulated with anti-CD3 mAb (10 μg/ml) for 48 h. The amount of IL-4, IL-5, and IFN-γ in the culture supernatants at various time points after stimulation was measured 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. *, $p < 0.05$; **, $p < 0.01$.

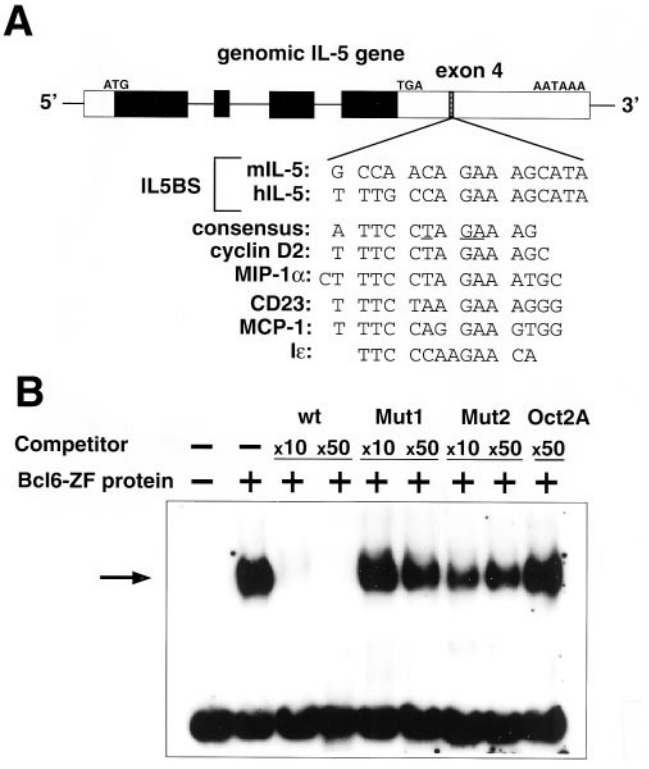

FIGURE 3. Bcl6 binds to the putative Bcl6-binding sequence in the *IL-5* gene. *A*, A genomic map of the human *IL-5* gene. Each box indicates exons. Closed boxes are coding regions of IL-5. ATG, start codon; TGA, stop codon; AATAAA, poly(A) additional sequence. The putative Bcl6-binding sequences (IL5BS) in the human and murine *IL-5* genes (hIL-5 and mIL-5) 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.

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 the DIG-labeled mIL5BS as a probe, gel retardation bands were observed (Fig. 3*B*). The major retarded band was obviously destroyed by a nonlabeled probe with the same sequence (Fig. 3*B*, 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. 4*A*). We then examined binding activity of Bcl6 in those nuclear proteins to mIL5BS by EMSA. As shown in Fig. 4*B*, 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. 4*B*, *lane 8*) but not with Mut1 (Fig. 4*B*, *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. 4*B*, *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. 4*B*, *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

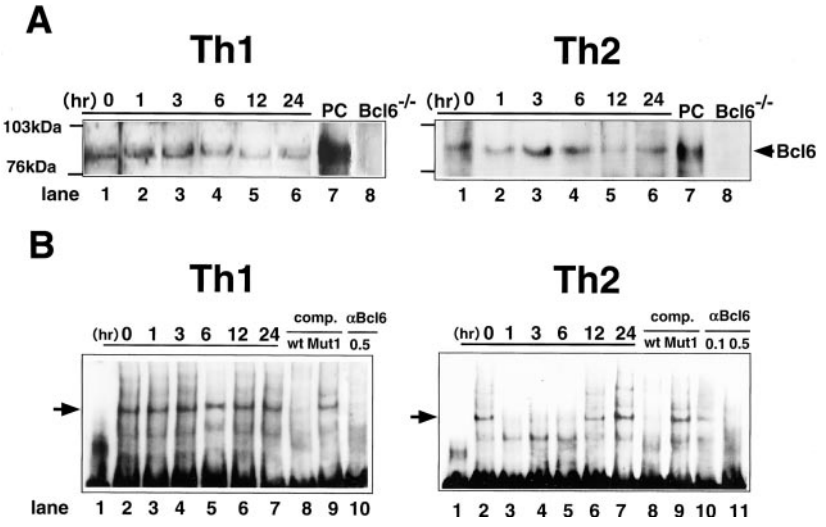

FIGURE 4. Bcl6 in Th2 clones but not in Th1 clones loses its binding activity to IL5BS after anti-CD3 stimulation. *A*, Th1 (28−4) and Th2 (MS-SB) clones were stimulated with anti-CD3 mAb (10 μg/ml) for 24 h. The amount of Bcl6 in nuclear proteins from those clones was measured by Western blot. pc, Cell lysates from transfectants of Bc16 as a positive control. Bcl6$^{-/-}$: nuclear proteins of spleen cells from Bcl6$^{-/-}$ 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 two independent experiments. Arrows indicate the Bcl6-specific retarded band. wt, Wild type.

(Fig. 4*B*, *lanes 3–5*) and returned to almost the same level as that of unstimulated cells at 12 h after stimulation (Fig. 4*B*, *lanes 6* and *7*). The transient diminution of the retarded band after anti-CD3 stimulation was also observed in the OVA-specific Th2 (DO10Th2–3) clone (data not shown).

We further confirmed the binding of Bcl6 to the IL5BS in chromatin of CD4$^+$ T cells or Th clones (28−4 and MS-SB) by the ChIP assay. Bcl6 and chromatin complexes from CD4$^+$ T cells or Th clones were immunoprecipitated with anti-Bcl6 Abs. The PCR products, including the mIL5BS sequence, were detected in the complexes from CD4$^+$ T cells of Bcl6$^{+/+}$ but not in those of Bcl6$^{-/-}$ mice (Fig. 5*A*). We also examined the binding of Bcl6 to the Bcl6-binding sequence in a promoter region of the *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 Bcl6$^{+/+}$ but not in those of Bcl6$^{-/-}$ 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. 5*B*). 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.

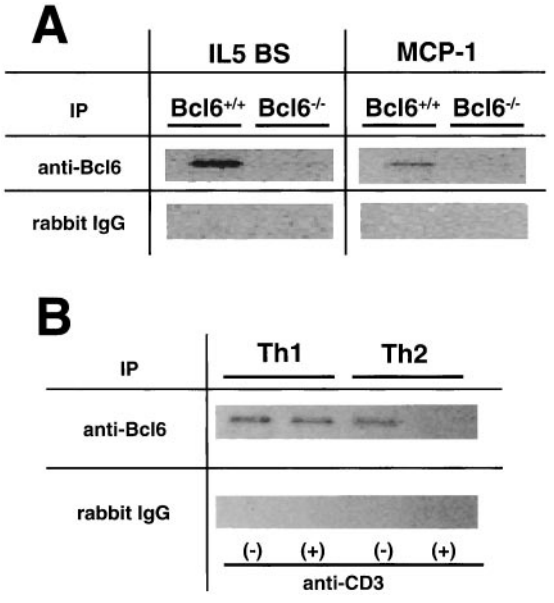

FIGURE 5. Binding of Bcl6 to the mIL5BS in CD4$^+$ T cells or in Th clones is detected by ChIP assay. *A*, Bcl6 on the chromatin in CD4$^+$ T cells was immunoprecipitated (IP) by polyclonal anti-Bcl6 Abs. The Bcl6-binding sequence in the *IL-5* gene (IL5BS) and that in the promoter region of *MCP-1* gene in the precipitated chromatin were detected by PCR. *B*, Th1 (28−4) and Th2 (MS-SB) clones were stimulated with anti-CD3 mAb (10 μg/ml) for 1.5 h. Bcl6 on the chromatin in those Th clones was immunoprecipitated by polyclonal anti-Bcl6 Abs. Rabbit polyclonal IgG was used as a negative control. These results are representative of three independent experiments.

IL5BS is required for Bcl6 to display its repressor activity

We examined a requirement of the IL5BS for Bcl6 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 (Bcl6 null) cells were cotransfected with pGL3C-IL5BS and various doses of pcDNA3-Bcl6, 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-Bcl6 in a dose-dependent manner (Fig. 6*A*). However, luciferase activity in K562 cells transfected with pGL3C was not reduced by pcDNA3-Bcl6. The reducing activity by pcDNA3-Bcl6 was confirmed in K562 cells transfected with pGL3C-2xIL5BS (Fig. 6*B*). 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-Bcl6.

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-Bcl6 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-Bcl6 was similar to that by K562 cells transfected with M(I-E)IL-5 without pcDNA3-Bcl6 (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-Bcl6 were cotransfected with M(I-E)IL-5, IL-5 production was significantly reduced to <1% of the maximum by pcDNA3-Bcl6 in a dose-dependent manner (Fig.

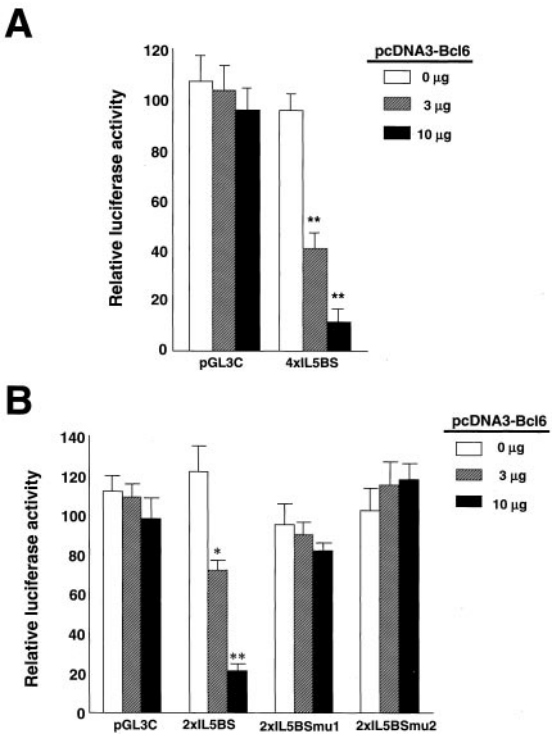

FIGURE 6. Bcl6 can repress expression of the reporter gene with the IL5BS. The luciferase reporter gene (*pGL3C*) or the reporter gene with the IL5BS (pGL3C-4xIL5BS; four copies of the IL5BS (*A*), pGL3C-2xIL5BS; two copies of the IL5BS (*B*)) was cotransfected with various doses (0, 3, or 10 μg) of 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. *, $p < 0.05$; **, $p < 0.01$.

7). 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

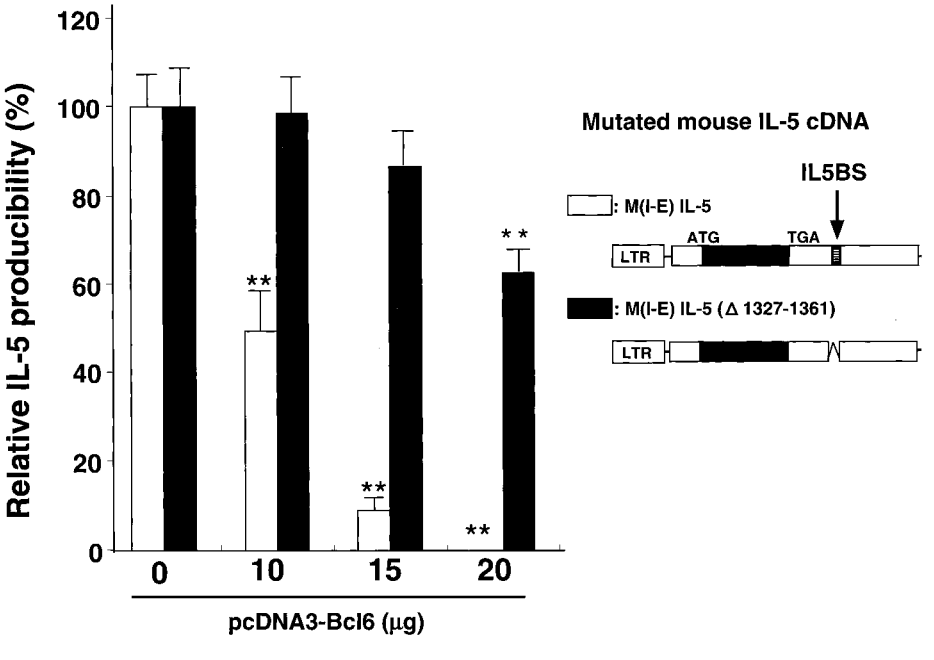

FIGURE 7. The IL5BS is required for Bcl6 to repress expression of the exogenous *IL-5* gene. The murine *IL-5* cDNA expression vector (M(I-E)IL-5, □) or that with deletion of the mIL5BS (M(I-E)IL-5(Δ1327–1361), ■) was cotransfected with various doses (0, 10, 15, or 20 μg) of pcDNA3-Bcl6 into K562 cells. Those transfectants 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′-ATTCCTAGAAAG-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′-ATTCCTAGAAAG-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′-TTC-CTA-GAA-3′), the human and murine IL5BSs do not, confirming less importance of the TTC residues in the CBS (34). Thus, this silencer element may be critical for regulation of murine and human *IL-5* gene expressions.

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 the target DNA sequence by acetylation of lysine residues in the zinc finger domain (48). Additional work is required to elucidate mechanisms of posttranscriptional modifications of Bcl6 to lose 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.

Acknowledgments

We are grateful to Dr. Tetsuya Fukuda for discussion. We also thank Hisae Satake for skillful technical assistance and Keiko Ujiie for secretarial services.

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