2013

RETRACTED ARTICLE: The BCL11A transcription factor directly activates RAG gene expression and V(D)J recombination

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Retraction for Lee et al., “The BCL11A Transcription Factor Directly Activates RAG Gene Expression and V(D)J Recombination”

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Volume 33, no. 9, p. 1768–1781, 2013, https://doi.org/10.1128/MCB.00987-12. Analysis of our data indicated duplicate bands in Fig. 6E and 7A. It is unclear whether the source of these errors was accidental or purposeful. However, these errors did not change the conclusions of the paper. Nonetheless, we thank the journal for recognizing these errors, and we hereby retract the paper.

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The BCL11A Transcription Factor Directly Activates RAG Gene Expression and V(D)J Recombination


Published Ahead of Print 25 February 2013.

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Recombination-activating gene 1 protein (RAG1) and RAG2 are critical enzymes for initiating variable-diversity-joining (VDJ) segment recombination, an essential process for antigen receptor expression and lymphocyte development. The transcription factor BCL11A is required for B cell development, but its molecular function(s) in B cell fate specification and commitment is unknown. We show here that the major B cell isoform, BCL11A-XL, binds the RAG1 promoter and Erag enhancer to activate RAG1 and RAG2 transcription in pre-B cells. We employed BCL11A overexpression with recombination substrates in a cultured pre-B cell line as well as Cre recombinase-mediated Bcl11alox/lox deletion in explanted murine pre-B cells to demonstrate direct consequences of BCL11A/RAG modulation on V(D)J recombination. We conclude that BCL11A is a critical component of a transcriptional network that regulates B cell fate by controlling V(D)J recombination.

RAG expression occurs at two distinct points during B cell development: the first phase results in the assembly of the immunoglobulin heavy chain (IgH) in pro-B cells, whereas the second catalyzes Ig light (L) chain assembly in pre-B cells. RAG expression is tightly regulated at both posttranscriptional and transcriptional levels (6). In addition to the individual promoters for RAG1 and RAG2, the RAG locus encodes at least 5 distal enhancer elements: the RAG enhancer (Erag), the proximal enhancer (Ep), the distal enhancer (Ed), and two recently discovered regions termed Irag1 and Irag2 (7–17) (Fig. 1A). Erag is the strongest enhancer, as demonstrated by a 5- to 10-fold reduction in RAG expression and a partial block at the pro-B-to-pre-B transition following targeted deletion of Erag in mice (10). A number of transcription factors bind to their corresponding DNA motifs within single or multiple regions within the RAG locus (Fig. 1A), and several of these interactions have been shown to activate RAG transcription (7, 8, 11, 14, 18, 19).

Originally discovered as the gene disrupted by t(2;14)(p13; q32) translocation in unusually aggressive cases of B cell chronic lymphocytic leukemia (20–23), the B cell lymphoma/leukemia 11A gene (BCL11A) was subsequently identified as an Kruppel-like zinc finger oncogene in numerous B cell malignancies (24–29). Among the five BCL11A isoforms previously identified (23, 30), extra long (BCL11A-XL) (NCBI accession number AJ404611) is expressed far more abundantly in hematopoietic lineages (23, 30, 31) (Fig. 1B). All isoforms share a conserved N terminus and an atypical C×HC zinc finger, which define a “super-family” of 5 genes essential to myeloid/lymphoid (EHZF), megakaryocytic (FOG1 and FOG2), T lymphoid (BCL11B), and B lymphoid (BCL11A) development (30) (Fig. 1B). Targeted deletion of Bcl11a in the mouse indicated that Bcl11a is selectively required for progression at the earliest stage (pre-pro-B) of B cell progenitor commitment prior to the RAG-dependent formation of pro-B cells (25). However, a recent study (32) employing a tamoxifen-inducible global Bcl11a knockout approach reported the loss of all common lymphoid progenitor (CLP) lineages, including T and NK cells, while sparing myeloid lineages. While resolution of this conflict remains, it is clear from these and other studies (33; G. C. Ippolito et al., submitted for publication) that BCL11A expression commences prior to that of the RAG transactivators shown in Fig. 1A, whose expression is lost or highly reduced in Bcl11a knockouts (25, 32). Not clear is whether their loss is an indirect result of the strong progenitor block in Bcl11a-deficient CLP or whether these factors are direct transcriptional targets of Bcl11a.

Here we identify RAG1 as a direct target of BCL11A-XL. BCL11A-XL binds within the RAG1 promoter and Erag enhancer to activate RAG1 and RAG2 transcription in pre-B cells while repressing RAG1 promoter activity in epithelial and fibroblast-derived cell lines. Overexpression of BCL11A-XL in a V(D)J recombination-competent pre-B cell line induces RAG expression and V(D)J recombination. Cre-mediated deletion of a lox-bearing Bcl11a locus in cultured pro/pre-B cells abolishes RAG expression and V(D)J recombination. We show that BCL11A-XL either directly or indirectly regulates additional RAG activators as well as
activators of \( V\kappa-J\kappa \) locus accessibility. We propose that, in addition to its earlier hematopoietic progenitor role, BCL11A is essential for the pro-B-to-pre-B transition, at least in part, from direct loss of V(D)J recombination.

**MATERIALS AND METHODS**

**Mice.** C57BL/6 mice with loxP sites flanking exons 1 and 2 of \( Bcl11a \) were generated and genotyped as previously described (29, 34, 35; Ippolito et al., submitted). Mice were bred and housed in the University of Texas animal research facility. All experiments were approved by the IACUC. Four- to six-month-old mice were used for bone marrow (BM) cultures.

**Cell culture.** Human 293T, human Phoenix, mouse NIH 3T3, and human HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL) containing 10% fetal bovine serum (FBS) (HyClone), 100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin. Human NALM6 pre-B cell and mouse A70 pre-B cell lines were maintained in RPMI 1640 (Gibco BRL) containing 10% FBS (HyClone), 50 \( \mu \)M mercaptoethanol, 100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin. Pre-B cells were cultured as described previously (36). B220\(^+\) cells were isolated from mouse BM using B220-labeled magnetic beads (Miltenyi Biotech). Purified cells were overlaid onto an irradiated S17 stromal cell layer. The cells were maintained in pre-B cell medium (Opti-MEM medium [Gibco] containing 15% FBS, 50 \( \mu \)M \( \beta \)-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 \( \mu \)g/ml streptomycin, and 5 ng/ml interleukin-7 [IL-7] [R&D Systems]). Cells were passaged every 3 days onto a new S17 stromal cell layer.

**Retroviruses and transductions.** Human BCL11A-XL cDNA was subcloned into the pXY-ires-puro (pXY-puro) vector, which was a kind gift from Louis Staudt, National Cancer Institute (37). Mouse stem cell-based retroviruses (MIT [mouse stem cell virus–internal ribosomal entry site–Thy-1.1]) encoding the Cre recombinase (MIT-Cre) and the parental vector (MIT) (8) were gifts of Robert Rickert, Burnham Institute. Mouse stem cell virus encoding the antiapoptotic factor Bcl-xL (MSCV–Bcl-xL-Puro) and the stem cell virus encoding the antiapoptotic factor Bcl-xL were gifts from Robert Rickert, Burnham Institute. Viral DNAs were transfected into the Phoenix-eco cell packaging line by using Fugene (Roche) to produce retroviral particles. Viral particles were incubated with 10 \( \mu \)g/ml of DOTAP liposomal transfection reagent \({N\}[-1-(2,3-dioleoyloxy)propyl]_N,N,N\text{-trimethylammonium methyl-sulfate}\) (Roche) for 10 min. Various human pre-B and B cell lines were transduced in 24-well culture plates at 23°C by spin inoculation at 1,000 \( \times \) g for 90 min at 30°C. Forty-eight hours after transduction, cells were selected in the presence of 0.5 to 2 \( \mu \)g/ml of puromycin (Sigma).

**FIG 1** Schematic representation of the RAG locus and BCL11A superfamily. (A) Transcriptional regulators and binding regions. The murine RAG locus is shown to scale with positions of previously described enhancers (blue arrows), promoters (blue boxes), and exons (black boxes); transcriptional polarity of \( Rag1 \) and \( Rag2 \) is indicated with black arrows. Positions of DNA binding sites for transcription factors determined previously (7, 8, 11, 12, 14, 18, 19, 65) or here (BCL11A-XL) to bind within these regions are indicated by short vertical lines. (B) BCL11A superfamily of transcription factors involved in hematological malignancy. Each member has a highly conserved N terminus, MSRRK (blue), shown in this study to be essential for BCL11A-XL transcriptional activity. This is followed by a single, canonical C2HC zinc finger (red), which is followed by one or more single, double, or triple zinc fingers of the C2H2 type (yellow). BCL11A and BCL11B, as well as early hematopoietic zinc finger (EHZF) and the friend-of-GATA hematopoietic transcription regulators FOG1 and FOG2, encode zinc finger proteins with these conserved features, and several have been implicated in malignancy (23, 26, 30, 45, 58).
MIT viruses were preincubated with Polybrene (8 μg/ml) and then diluted 2-fold with Opti-MEM. B220⁺ BM pre-B cells, following culture (for fewer than 5 passages) as described above, were plated at a density of ~1 × 10⁶ cells/ml and 1 day later were transduced with MSCV–Bcl-x, by spin inoculation as described above. Following incubation for 1 h at 37°C, cells were collected, resuspended in pre-B cell medium, and then cultured for an additional 48 h. Cells were then infected at a high multiplicity of infection (MOI) with MIT or MIT-Cre. Culture was continued with IL-7 for 3 days and then collected for PCR and reverse transcription-PCR (RT-PCR) experiments.

Inducible shRNA silencing of BCL11A. Two complementary short hairpin RNA (shRNA) template oligonucleotides targeting exon 2 of BCL11A were synthesized and cloned into the pRSMX-PG vector (5) using HindIII and BglII sites. pRSMX-PG-BCL11A-shRNA retrovirus was produced in Phoenix cells as described above and used to infect a Tet-on human B cell line, BJAB (38), which expresses the Tet repressor. Transduced BJAB cells were selected in the presence of puromycin for 6 days. BCL11A-specific shRNA expression was induced with different concentrations of doxycycline, and samples were tested at regular intervals for 48 h. Levels of BCL11A expression were measured by RT-PCR and Western blot analysis. The following oligomers were used for the BCL11A shRNA template: 5'-GATCACCCACGACATTAAACCAATCAGGATTGCGTTAGGCCTGAGGTTTTTGGAAA-3' (forward) and 5'-AATTTTGCCACCAATTGCACATGGAAAGATTGCGTTAGGTTTTTGGAAA-3' (reverse). BglII and HindIII sites were used for cloning.

Plasmid constructions. pCMV10–BCL11A-XL and pCMV10–BCL11A-S were constructed as follows: BCL11A-XL and BCL11A-S were amplified by PCR with the same forward primer, 5'-GGCAAGCTTATGCTGGGGTG-3', and different reverse primers, 5'-CCGACGCTTATCCTTACCAAAATTTTGATAAATTATAATGCGGATGCT-C3' for the BCL11A-XL isoform and 5'-CCGACGCTTATCCTTACCAAAATTTTGATAAATTATAATGCGGATGC-T3' for the S isoform. The PCR products were digested with BamHI and HindIII and cloned into pCMV10 (Sigma), which contains a sequence of 3× Flag tag. The pX-Y-BCL11A-XL plasmid was constructed by using pCMV10–BCL11A-XL as a template and the following PCR primers: 5'-CGGATCCATCGAATCGACAACCAAGCTGAC-3' (forward) and 5'-GGCGTCGACAGCGAAC-3' (reverse). The PCR products were digested with BamHI and NotI and cloned into the pX-Y-ires-puro plasmid using BglII and NotI sites. pEGFC1–BCL11A and BCL11A-XL-K5N were generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit and protocol from Stratagene. The following primers were used: 5'-GCCGCTCCGGCACAACCGAGAAGGTCAAC-3' (forward) and 5'-GTCTCGGCCGCAAGCAAGGC-3' (reverse). The deletion mutant pEGFC1–BCL11A and pX-Y-BCL11A-XL-K5N was constructed by using PCR with the following primers: 5'-GCCGCTACAGCGACATCGGAC-3' (forward) and 5'-GGCGTCGACAGCGAACGTTCTGAGGTTCGACACGAGACTCAGG-3' (reverse). The PCR products were digested with BamHI and NotI and cloned into the plasmid vector pEGFC1. The final clones were verified by sequencing.

ChiP and EMSA. Chromatin immunoprecipitation (ChiP) was performed according to instructions provided with the Upstate ChiP kit. Human pre-B and B cell lines (Nalm6 and Raji) were cross-linked by incubation at room temperature for 8 min in a final concentration of 1% formaldehyde. Fixed cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris [pH 8.1]). Lysetes were sonicated for 0.5 min six times to a modal distribution of ~1,000 bp. Sonicated chromatin was diluted 10-fold in a dilution buffer (0.01% SDS, 1,1% Triton X-100, 1.2 mM EDTA, 167 mM Tris-HCl [pH 8.1], 167 mM NaCl) and then immunoprecipitated with purified rabbit IgG or with an anti-BCL11A-XL specific rabbit polyclonal antibody (BL1797; Bethyl). Antibody–chromatin complexes were washed with a low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), a high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), an LiCl wash buffer (0.25 mM LiCl, 1% IGEPAL CA-630 [octylphenoxypolyethoxyethanol], 1% deoxycholic acid [sodium salt], 1 mM EDTA, 10 mM Tris [pH 8.1]), and a TE wash buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). These complexes were eluted in elution buffer (1% SDS, 0.1 M NaHCO₃) and reverse cross-linked at 65°C for 6 to 12 h in the presence of 0.2 M NaCl. After proteinase K treatment for 1 h at 45°C, DNA was recovered by using PCR purification columns (Invitrogen) and then used for PCR analysis. The RAG1 and RAG2 promoters and the Erg enhancer region were detected by using the following primers: RAG1 forward primer 5'-CATTTCAGGGAGGGAGACCT-3', RAG1 reverse primer 5'-GGAGGCTCACCCAAAAATGA-3', RAG2 forward primer 5'-GGGTCTCTGTCAGGACA-3', RAG2 reverse primer 5'-AGGACAAATGGCGAGACACAT-3', Erg forward primer 5'-GGCAGTGGAAATGGCTGAAAC-3', and Erg reverse primer 5'-GAGGACAGGGAGGCTTAA CATTG-3'. ChiP-PCR of RAG1, RAG2, and Erg was produced 161-bp, 180-bp, and 196-bp products, respectively. Thermal cycling conditions were 95°C for 5 min and then 35 cycles at 95°C for 10 s, 55°C for 15 s, 72°C for 10 s, and 72°C for 7 min. PCR conditions used for the Erg enhancer were 95°C for 5 min and then 35 cycles at 95°C for 1 min, 57°C for 1 min, 72°C for 1 min, and 72°C for 7 min.

To construct electrophoretic mobility shift assay (EMSA) probes, the proximal promoter region (243 bp upstream of the transcriptional start site [TSS] [-243] to 123 bp downstream of the TSS [+123]) of the mouse RAG1 gene (39) was amplified by PCR using RAG1 forward primer 5'-CATTTCAGGGAGGGAGACCT-3' and RAG1 reverse primer 5'-GGGCAAGTTGCTTCTGTGTCAGA-3' from plasmid template RLP-Luc. The Erg enhancer (10) probe was a 196-bp fragment extending from the KpnI site to the end of the region A (11). The probe for the distal promoter region of Erg (positions -713 to -563) was generated by KpnI-HindIII digestion of plasmid -713-RIP-Luc. Probes were end labeled with [32P] by using polynucleotide kinase and then purified by using Bio-Spin columns (Bio-Rad) and 6% native gels. Reaction mixtures consisted of 5 to 10 μg of nuclear extract prepared from various B cell lines, probes, and a binding buffer (20 mM HEPES [pH 7.9], 40 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol [DTT], 0.1% NP-40, 3 mg/ml bovine serum albumin, 10% glycerol, 2% Ficoll, 50 μg/ml of sonicated salmon sperm DNA, and protease inhibitor cocktail). The binding mixture was incubated at room temperature for 30 min. For the antibody binding competition assays, 100 ng of BCL11A polyclonal antibody (BL1797) or of monoclonal antibody (MAB) mAb123 (40) or an equal amount of a control rabbit IgG antibody or an irrelevant tubulin MAB was added at the initiation of each binding reaction. Cold competitors were carried out with increasing molar ratios of wild-type (5'-GGTCAGTCCGGTCCACACTCACCTCCACGAG-3') and mutant (5'-GGTCAGTCCGGTCCACACTCACCTCCACGAG-3') duplexed, gel-purified oligonucleotides spanning the putative BCL11A binding site at Erg nucleotide (nt) positions 121 to 131. (Mixtures were fractionated on 6.0 to 7.5% non-denaturing acrylamide. The gels were dried and analyzed by using a PhosphorImage [Molecular Dynamics].)

Luciferase assays. Pre-B cells (A70, A70-BCL11A-XL, and A70-BCL11A-XL-K5/A) and non-B cells (293T, COS-7, and NIH 3T3) were plated at ~1 × 10⁶ cells per well in 12-well plates. Twenty-four hours later, pre-B cells were transiently transfected with 1 μg of firefly luciferase reporters (detailed in Fig. 6), 5 ng of Renilla luciferase, and, in some cases, 1 μg of pFLAG-MA1 using the mouse B cell Nucleofector kit (VPA-1010, program Z-01) and cell line kit (VCA-1003, program M13) according to the manufacturer's recommended protocol. The transfection efficiency (determined by pEG-GFP transfection in parallel assays) achieved in pre-B cells was 40 to 50%. Empty vector plasmid DNA was used to keep the total amount of transfected DNA equal under each experimental condition. Non-B cells were transiently transfected with 1 μg of the firefly luciferase reporter constructs (detailed in Fig. 6), 5 ng of Renilla luciferase, and either wild-type BCL11A-XL or BCL11A-XL-K5/A expression vectors over a concentration range of 0.1 to 1.5 ng. An empty pCMV10 construct was used as a control.
was then added to each transfection mix to ensure that final DNA concentrations were equal.

Forty-eight hours after transfection, cells were harvested for dual-luciferase reporter assays (Promega), using cell lysis and luciferase measurements as described in the manufacturer’s manual. Values were normalized by determining the ratio of firefly to Renilla luciferase for each transfection. To determine fold expression, the firefly/Renilla ratio in experimental samples (i.e., those with BCL11A-XL overexpression, the Renilla vector, and its respective firefly luciferase RAG reporter) was compared to the firefly/Renilla ratio from basal luciferase expression samples (i.e., those without BCL11A-XL overexpression). Western blotting confirmed that BCL11A-XL protein lysate levels corresponded to the concentration of input DNA.

Microarray. Briefly, 3 μg of mRNA of various human pre-B and B cell lines was reverse transcribed by using aminomethyl UTP/dexoyxynucleotide triphosphates (dNTPs). Synthesized cDNA was hydrolyzed and column purified (Invitrogen). Each cDNA sample was coupled with Cy3 or Cy5 dye, mixed, and hybridized to full genome human array slides. Microarray slides were scanned by using a GenePix 4000a microarray scanner.

Flow cytometry. A70-INV and A70-INV-BCL11A-XL cells were incubated in the absence or in the presence of 3 μM ST571 (Novartis) for 3 days. The harvested cells were washed two times with ice-cold PBS and resuspended in PBS at a concentration of 1–10^7 cells/ml. A minimum of 5,000 cells per sample was used for each analysis. Flow cytometry (BD FACSCalibur flow cytometry system) was used to analyze the samples for green fluorescent protein (GFP)-positive signals. Cell debris and dead cells were excluded from the analysis by forward- and side-scatter analysis. The parental A70-INV cells were used for GFP gating. Data were analyzed by using Cell Quest software. Bcl11aL/L cultured NALT and NALM6 pre-B cells and checked for Flag (Sigma), 1:2,000 for BCL11A-mAB123 (prepared in our laboratory). The following dilutions were used for each antibody: 1:5,000 for Flag (Sigma), 1:2,000 for RAG1, and 1:3,000 for BCL11A-XL cDNA. The primers for endogenous rearrangements (44). Cytoplasmic contamination into the nuclear fraction will result in contamination within the soluble NP fraction (2). Lamin B signals were used to evaluate contamination of nuclear fractions into the cytoplasmic fractions (44). Cytoplasmic contamination was also assessed by anti-cytochrome c Western analysis.

RESULTS

BCL11A-XL modulates RAG expression. As an initial approach, we used microarrays to identify genes that are deregulated by BCL11A-XL overexpression in mature B (Raji, Ramos, OCI-LY7, and BJAB) and pre-B (NALM6) human cell lines. B cell lines were transduced with pXy-puro (mock control) or with the same virus containing an N-terminally Flag-tagged, full-length BCL11A-XL cDNA. Among the 17,856 clones tested, 43 clones, which represent 39 genes, showed alterations of at least 2-fold in all four cell lines. As expected from previous studies of BCL11A function, most of the transcripts were downregulated (data not shown). Among the 11 upregulated genes, RAG1 was consistently observed, even though the basal transcript levels in the mature lines were near baseline (Fig. 2A). We created a stable BCL11A-XL-overexpressing line of NALM6 pre-B cells and checked RAG1 and RAG2 expression by semiquantitative RT-PCR. As shown in Fig. 2B, both RAG genes were upregulated.
BCL11A-XL is recruited either directly or indirectly to the proximal antibody (Fig. 3A, data not shown). This indicated that enriched in pulldowns with anti-BCL11A but not with control antibodies (Fig. 3B, lanes 3 and 5) but not with control antibodies (Fig. 3B, lanes 4 and 6). Specific double-stranded-oligonucleotide competition (Fig. 3B, compare lanes 8 to 10 to lanes 11 and 12) identified a BCL11A binding site that closely matched (8/10) the BCL11A consensus determined by EMSAs were performed to validate and extend the results shown in Fig. 3A. A 32P-labeled 196-bp probe extending from the promoter and within the ChIP region shown in Fig. 3A. Further consistent with our ChIP-PCR results, no binding to Erag-RAG2 (Fig. 1A, lanes 2 to 7) and 0.4 for PU.1, an essential myeloid/lymphoid transactivator, which was shown to bind to the Ep element (Fig. 1A) (16). Accordingly, we observed partial ablation of the BCL11A complex with anti-Pu.1 (Fig. 3B, lane 7), suggesting that these factors might act in concert or in opposition at this apparent composite site. Using a similar approach with nuclear extracts prepared from pre-B (NALM6) (Fig. 3C, lanes 2 to 7), pro-B (HAPTL1) (Fig. 3C, lanes 8 to 13, and D, lanes 2 to 10), and mature B (Raji) (Fig. 3C, lanes 14 to 19, and E, lanes 2 to 4) cell lines, we validated two similarly migrating BCL11A complexes within the distal (nt −426 to −416) and proximal (nt −207 to −197) regions of the RAG1 promoter (Fig. 3C to E). Ablation as opposed to supershifting of complexes suggested that both anti-BCL11A antibodies interfere with DNA binding, leading to dissociation of BCL11A-DNA complexes. Both proximal and distal BCL11A-XL binding sites are well within the ChIP region shown in Fig. 3A. Further consistent with our ChIP-PCR results, no binding to Rag2 promoter probes was observed (data not shown), nor did we detect specific binding when recombinant, in vitro-translated BCL11A-XL, -L, or -X was employed in the EMSAs (data not shown). This suggested that BCL11A-XL binding requires a complex provided by another factor(s), potentially PU.1, present in nuclear extracts in pro-B, pre-B, and mature B cells.

BCL11A-XL transactivates RAG1 and Erag-RAG2 transcription in pre-B cells. Transformation of murine fetal liver or bone marrow B cell progenitors by the Abelson murine leukemia virus arrests cells at the early (large) pre-B cell stage with low to negligible Rag1 and Rag2 expression levels (6). One such line, A70 (39), expresses negligible levels of BCL11A (Fig. 4A) and was employed for functional evaluation of Rag activation. Stable BCL11A-XL overexpression, achieved by retroviral transduction, induced high levels of endogenous Rag1 and Rag2 (Fig. 4A). These cells (termed A70-BCL11A-XL) were compared to the parental BCL11A-XL-negative A70 line following transient transfection with luciferase reporter construct and under the control of a modified H1 promoter containing binding sites for the tetracycline repressor (38). Significant downregulation of BCL11A-XL protein (left) and transcript (right) with concomitant downregulation of RAG1 and RAG2 was observed 24 h following addition of the tetracycline analogue doxycycline (Dox).

5. FIG 2 BCL11A-XL regulates RAG expression. (A) Microarray identification of RAG1 upregulation. The indicated human B cell lines were stably transduced with a BCL11A-XL (XL) retrovirus or with the pXy-puro empty vector. Expression profiling was performed in multiple (n > 4) independent experiments tracked by multiple probe elements spotted per microarray. Shown are the 11 transcripts upregulated by minimally 2-fold relative to the vector control in all cell lines. (B) Overexpression of BCL11A-XL upregulates RAG. Human NALM6 pre-B cells were stably transduced with BCL11A-XL or the pXy-puro virus control (V). (Left) Western blot in which Flag-BCL11A indicates anti-Flag detection of viral BCL11A-XL expression. Total BCL11A, anti-BCL11A-XL MAb detection; Tubβ, an anti-β-tubulin control. (Right) RT-PCR demonstrates upregulation of RAG1 and RAG2. Serial template dilution (3-fold) (indicated by triangles) confirmed that RT-PCR was semiquantitative. GAPDH was used as a loading control. (C) Inducible shRNA knockdown of BCL11A-XL reduces RAG expression. BJAB B cells were stably transduced first with a retrovirus expressing the bacterial tetracycline repressor (38). Significant downregulation of BCL11A-XL protein (left) and transcript (right) with concomitant downregulation of RAG1 and RAG2 was observed 24 h following addition of the tetracycline analogue doxycycline (Dox).
The reporter constructs that were previously shown to recapitulate authentic activities of the proximal Rag1 (−243-R1p-Luc) and Rag2 (−279-R1p-Luc) promoters in the absence or presence of the full 2.3-kb Erag enhancer. A70-BCL11A-XL firefly luciferase activities were normalized relative to cotransfected Renilla luciferase activities and plotted as fold activation over a promoterless luciferase control.

As shown in Fig. 4B, BCL11A-XL only modestly increased Rag1 proximal promoter-driven transcription levels (average, 1.6-fold). Appending Erag to the reporter provided an ~4-fold en-
hancement in A70, which was doubled in the A70-BCL11A-XL transductants. When the RAG1 promoter was extended to include the more distal BCL11A-XL binding region located at position 640 (719-R1p-Luc), we observed an 3-fold increase above the levels observed for the proximal Rag1 promoter alone and an 12-fold increase in the presence of Erag. Consistent with the EMSA and ChIP data, no effect of BCL11A-XL overexpression on Rag2 proximal promoter-luciferase activity alone was observed. However, inclusion of Erag on the construct resulted in an 3-fold increase in the absence of BCL11A-XL and an 14-fold increase in Rag2 reporter activity in the presence of BCL11A-XL overexpression (Fig. 4B).

The N termini of several members of the BCL11A superfamily (30) constitute the transactivation domain, and K5 was shown to be essential in several cases (26, 45) (Fig. 1B). Activation of all reporter activities by BCL11A-XL was abrogated by the K5/A substitution (Fig. 4B). Collectively, the data indicate that BCL11A-XL acts at the Rag1 promoter, primarily through a distal binding site, whereas BCL11A-XL activates Rag2 transcription exclusively via Erag binding.

Enforced expression of BCL11A in non-B cells represses RAG1-mediated transcription. The above-described results, along with the PU.1 DNA binding cooccupancy implications of Fig. 3 and previous observations that RAG1 expression is not lymphoid restricted (9, 12), prompted us to test the cell type specificity of BCL11A-mediated RAG transactivation. Contrary to what was observed for pre-B cells, BCL11A-XL overexpression in 293T cells led to strong repression (~5- to 6-fold) of both proximal (~243-R1p) and distal (~719-R1p) Rag1 promoter-driven activities, whereas Rag2 and Erag-RAG2 luciferase activities were unaffected (Fig. 4B). This indicated that the more proximal BCL11A binding site within the Rag1 promoter is sufficient for targeting transcription.
maximal repression. Similar results were observed for additional non-B cell lines (NIH 3T3, COS-7, and HeLa) and were confirmed by careful dose-response curves following transient cotransfection (data not shown).

BCL11B, the highly similar parologue of BCL11A (23), was shown to interact with the nucleosome-remodeling and histone deacetylase (NuRD) complex with enhanced repression of model substrates (46). One of the NuRD components, metastasis tumor antigen 1 (MTA1), was shown to interact with and enhance BCL11B repression synergistically, while MTA2 had no effect (46). We observed significant augmentation of BCL11A-XL recombination following cotransfection with MTA1 (Fig. 4B) but not with MTA2 (data not shown). Conversely, enforced overexpression of BCL11A-XL with MTA1 in A70 pre-B cell lines had no effect on activation of any of the Rag1 or Rag2 constructs (data not shown).

We considered, as an alternative explanation for the opposite effects in B versus non-B cells (other than B-lineage-specific co-factors), a differential subcellular localization of BCL11A-XL. In B cells, exogenous or ectopically overexpressed BCL11A-XL accumulates primarily within the nuclear matrix of paraspeckles (30). We observed localization indistinguishable from that following ectopic delivery of GFP–BCL11A-XL or GFP–BCL11A-XL-K5/A into 293T cells (Fig. 4C, top). Biochemical subcellular fractionations (Fig. 4C, bottom) indicated that, as in B cells (30), wild-type and mutant BCL11A-XL accumulate preferentially within the nuclear matrix.

The results suggest that BCL11A-XL acts in a context-dependent, cell-type-specific mode to activate or repress RAG1 transcription. The data further suggest that repressive BCL11A-XL complexes differ from activation complexes by the inclusion/exclusion of the MTA1 corepressor but are indistinguishable in their subcellular localization.

BCL11A-XL induces ectopic V(D)J recombination in A70-INN pre-B cells. The A70-INN Ab1-transformed pre-B cell line retains the properties of the parental A70 cell line, but it has been engineered for inducible V(D)J recombination analysis by integration of a recombination substrate, pMX-INN, which, upon inversion, activates expression of GFP (39). A70 and A70-INN also constitutively express a Bcl2-Ep transgene, enabling them to survive apoptotic signaling for several days (39). We transduced A70-INN cells with empty virus (pXY-puro) or with pXY-BCL11A-XL and then puromycin selected bulk (uncloned) cells. As with A70 parental cells, BCL11A protein was barely detectable in untransduced or mock-transduced A70-INN cells (Fig. 5A). Transduced cells expressed significant levels of BCL11A-XL, resulting in robust induction of Rag1 and Rag2 (Fig. 5B).

The strategy used to detect the inversion of pMX-INN and V-J recombination of the endogenous κ light chain locus was described previously (39) (Fig. 5C). As a positive control, strong induction of recombination is achieved by treatment of A70-INN cells with the Ab1 kinase inhibitor STI571 (47). STI571 causes differentiation of early pre-B cells to a late-pre-B-cell-like state (47). Strong Rag1 and Rag2 expression ensued, followed by Vx-Jk recombination and, in A70-INN cells, inversion of the integrated pMX-INN via cleavage at the flanking recombination signal sequences (RSSs) (Fig. 5C) (39). As shown in Fig. 5D, mock viral transduction produced 0.7% and 18.1% GFP-positive cells without or with STI571 treatment, respectively (Fig. 5D and B). This level of background GFP expression is similar to previously reported data (39). BCL11A-XL-infected cells produced 7% and 34.5% GFP-positive cells without or with STI571 treatment, respectively (Fig. 5DC and DD).

An ~10-fold increase in recombination substrate inversion by BCL11A-XL in the absence of STI571 treatment suggested that BCL11A-XL acts independently. Consistent with this interpretation, the level of substrate inversion by BCL11A-XL in the presence of STI571 was additive. Unlike the G1/S arrest induced by STI571 treatment, BCL11A-XL transduction was accompanied by no alteration in cell cycling (as measured by DNA content of propidium iodide-stained cultures [data not shown]).

To verify that GFP expression resulted from inversion of pMX-INN, we carried out semiquantitative PCR on genomic DNA using a 3′ primer complementary to hCD4, a component of the pMX-INN vector which can be used to detect inverted GFP. pMX-INN inversion allows successful PCR product formation by providing the reverse primer with the correct orientation of GFP cDNA (Fig. 5C). As shown in Fig. 5E, an increase in the intensities of inverted GFP-hCD4 PCR products was observed in STI571-treated or BCL11A-XL-transduced cells compared to STI571-un-treated or mock virus-transduced cells.

Modulation of BCL11A expression in transformed or normal murine pre-B cells modulates endogenous V(D)J recombination. Although integrated as a single copy, the pMX-INN reporter locus may not display the equivalent heterochromatin structure as the endogenous light chain loci in pre-B cells. To assess whether the endogenous locus can also be induced to rearrange by BCL11A-XL overexpression, genomic DNA was analyzed by PCR using a “universal” Vκ forward primer and Jκ-2 reverse primer (Fig. 6A, top). Efficient PCR can occur only if Vκ rearrangement deletes the intervening sequence between Vκ and Jκ elements. Vκ rearrangement was evaluated by Southern blotting with a Jκ-2 oligonucleotide probe (Fig. 6A). As shown in Fig. 6A (bottom), increased levels of Jκ rearrangement were observed in both STI571-treated (lanes 4 to 6) and BCL11A-XL-transduced (lanes 10 to 12) cells compared to control cells.

To address the issue in normal developing B cells, we employed mice with loxp (L) sites flanking exon1 of the Bell11 gene (Bell11aLoLo) (29, 35; Ippolito et al., submitted). Bone marrow cultures were established as previously described (8, 36), by expanding bead-purified B220+ cells in culture with IL-7. In an effort to delete Bell11a, the cells were infected with either an empty vector (MIT [mouse stem cell virus–internal ribosomal entry site–Thy-1.1]) or the equivalent retrovirus which expresses Cre recombinase (MIT-Cre). Within 3 days, the MIT-Cre-infected cells underwent a proliferative block and apoptosis and died within 3 days (data not shown). To overcome this and allow for sufficient numbers of viable cells to be harvested, we first transduced the cells with a retrovirus (MSCV–Bcl-xL) that encodes the antiapoptotic Bcl-xL factor and cultured these cells for 2 days prior to superinfection with either MIT or MIT-Cre. Proliferation was rescued in the MIT-Cre cultures, and cells were collected 3 days after secondary infection for analysis.

It was essential for quantitative comparisons to determine the percentage of B220+ cells infected under both conditions. Taking advantage of the Thy-1.1 marker encoded in both vectors, we observed by flow cytometry that control and Cre-deleted B220+ B cell progenitors showed comparable transduction efficiencies (>75%) (Fig. 6B). Since MIT-Cre-infected cells required Bcl-xL expression to bypass apoptosis, it was crucial to check their pro-
liferative status, particularly since both V(D)J recombination and RAG2 expression are regulated at the G1 phase of the cell cycle (48). As shown in Fig. 6C, comparable cell cycle progression within normal limits for diploid cells was observed for control and Cre-deleted cultures.

To quantify the extent of Cre-mediated Bcl11aL/L deletion and VJ/H9260 reduction at the DNA level, we employed 5-fold dilutions of genomic DNA analyzed by both semiquantitative and real-time PCR by using the scheme shown in Fig. 6A. Figure 6D shows the normalized, real-time fold reduction values, indicated under each corresponding ethidium bromide-stained, semiquantitative PCR band for the MIT-Cre condition (calculation is further detailed in the legend). VJ/H9260 rearrangement was strongly reduced (average of 15-fold) in direct correlation with Bcl11a deletion (122-fold). This was paralleled by a dramatic reduction (13- to 19-fold) in Rag1, Rag2, and Bcl11a transcript levels (Fig. 6E).

BCL11A-XL overexpression upregulates additional genes implicated in V(D)J recombination and pre-Pro-B cell progression. In addition to RAGs, transcription of several genes previously shown to encode proteins involved in V(D)J recombination were modulated following BCL11A-XL overexpression in A70 pre-B cells (Fig. 7). Both interferon-regulatory factor 4 (IRF4) and IRF8 were strongly upregulated by BCL11A-XL overexpression. Upregulation of these transcription factors has been reported to be essential for Ig light chain recombination (49). We also observed consistent upregulation of Foxp1 and FoxO1, factors previously shown to activate RAG via binding to Erag (8, 11). Conversely, the Erag binding activators E2a and FoxO1 remained unchanged. Ig germ line transcription, often observed as a consequence of chromatin accessibility of the J/H9261 locus (7), and the 5 chain of the pre-B cell receptor were unaffected, whereas its partner subunit, Vpre-B, was strongly upregulated by BCL11A-XL. Inspection of the ENCODE human B cell GM12878 ChIPseq data for BCL11A (28) indicated strong binding peaks within 2 kb upstream or downstream of the transcriptional start sites of VpreB, FoxO1, IRF8, and IRF4, suggesting that these loci are also directly targeted by BCL11A.

Transcript modulation following STI571 treatment correlated only modestly with that induced by BCL11A-XL overexpression, further emphasizing the different mechanisms of these two RAG activators. For example, and as observed for other Abelson pre-B cell lines (6), STI571 induced expression of Spi-B and Ig germ...
line transcription (Fig. 7). However, in contrast to data from a previous report (47), we observed robust STI571 repression of IRF4 and IRF8 in A70 cells. Perhaps, the difference lies in the cell lines employed. The previous results (47) were obtained with the Abelson pre-B 200-8 line, which in our hands undergoes massive cell death within 24 h of STI571 treatment (data not shown). The constitutive expression of the Bcl2 transgene in A70 cells (39) allowed us to analyze events even 4 to 6 days following STI571 treatment without cell death.

**DISCUSSION**

**BCL11A** was identified as a proto-oncogene implicated in multiple B cell malignancies (26, 28, 29) and represents one of ~200 “ultraconserved” genes in the human genome (19). Initially thought to be exclusive to B cells, BCL11A is the first genetically and functionally validated transcriptional regulator of both developmental control of globin switching and silencing of -globin expression in humans (41, 50). While targeted knockout (25, 32) identified Bcl11a as being essential for normal B cell development in the mouse, the transcriptional mechanism and direct targets of its action are incompletely understood.

We initially identified RAG1 as a potential target gene of the major BCL11A isoform (BCL11A-XL) by microarray analysis (Fig. 2A). Both it and RAG2 were confirmed by a combination of BCL11A-XL overexpression and shRNA knockdown (Fig. 2B and C). In principle, BCL11A-XL might regulate RAG transcription.
within the lines that BCL11A was recruited to discrete regions of chromatin and by possibilities, we observed both by BCL11A chromatin occupancy mately impact RAG expression. To discriminate between these 1778 mcb.asm.org Molecular and Cellular Biology drive consistent with the binding studies, BCL11A-XL failed to the either directly by interacting with cis-regulatory elements within the RAG locus or indirectly by affecting other pathways that ultimately impact RAG expression. To discriminate between these possibilities, we observed both by BCL11A chromatin occupancy and by in vitro DNA binding using pro-B, pre-B, and mature B cell lines that BCL11A was recruited to discrete regions of chromatin within the RAG1 promoter and the Erag enhancer but not within the RAG2 promoter (Fig. 1A and 3). Transcriptional reporter studies in mouse pre-B cells suggested that two BCL11A binding sites within the RAG1 promoter synergize to activate transcription (Fig. 4B). The more promoter-distal BCL11A binding site conveyed much stronger (>4-fold) Erag enhancement (Fig. 4B). Further consistent with the binding studies, BCL11A-XL failed to drive Rag2 transcription unless Erag was appended to the construct (Fig. 4B). High-resolution, genome-wide ChIPseq, derived publically from the human B cell lymphoma GM12878 (28; http://www.factorbook.org/mediawiki/index.php/BCL11A) and from the human pre-B ALL REH (our unpublished data), identified BCL11A binding sites corresponding to those identified here (Fig. 3F) as well as several others scattered across the RAG locus (data not shown). This, along with the finding (29, 35) that within the expansive βγ-globin locus, BCL11A tends to associate with distal control elements rather than proximal promoters, prompts caution as to overly simplified conclusions of the relative importance of any single BCL11A binding site within this complex regulatory locus. The genome-wide ChIP data (28) further indicate that the 500 strongest BCL11A sites within the genome overlap PU.1 sites. Similarly, equivalent genome-wide ENCODE data for PU.1 ChIPseq (28; http://www.factorbook.org/mediawiki/index.php /PU.1) indicated with high statistical significance ($P < 1.0^{-11}$) that a large proportion of PU.1 peaks are in close vicinity to BCL11A peaks. Taken with our EMSA data shown in Fig. 3B, it is highly probable that these factors broadly collaborate or antagonize transcription via closely spaced or even overlapping composite sites.

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These findings, along with the observation that recombinant BCL11A-XL failed to bind established target motifs in vitro, prompted us to test the transcriptional consequences of enforced BCL11A expression in non-B cells. Unexpectedly, we observed strong repression of RAG1 promoter-driven transcription in epithelial and fibroblast-derived cell lines, with the more promoter-proximal BCL11A-XL binding site being sufficient to target the activity (Fig. 4B and data not shown). In addition to suggesting that a lineage-restricted cofactor(s) is required for BCL11A-XL to transactivate RAG in lymphoid cells, potential physiologic relevance is provided by the observations that the RAG1 promoter is not lymphoid restricted (9, 12) and that RAG expression is observed in several nonlymphoid malignant cell types (35, 48, 51–54). Additional relevance may be drawn from the observation that Bcl11a expression within T lineages is limited to double-negative-2 (DN2) stage thymocytes, in which V(D)J recombination of the TcRβ locus initiates (55). At DN3, Bcl11a is extinguished, and expression of its highly similar paralogue, Bcl11b, ensues (56). Although Bcl11b has not previously been implicated in Rag control, Bcl11b<sup>−/−</sup> thymocytes are impaired in Vβ-Dβ recombination (57).

The domain responsible for both non-B cell repression and pre-B cell activation of BCL11A-XL maps to the N terminus, as deletion of the first 80 amino acids or a single-point mutation (K5/A) within the highly conserved MSRRK motif (Fig. 1B) abrogated both transcriptional activities while retaining normal BCL11A-XL subnuclear localization (Fig. 4B and C and data not shown). The N-terminal motif defines a superfamily of transcription factors crucial to the development, differentiation, and malignancy of several hematopoietic lineages (Fig. 1B) (23, 26, 30, 45, 58). The N terminus, when extended to include the equally conserved C<sub>2</sub>HC zinc finger (Fig. 1B), is required for BCL11A-XL to dimerize with itself, with all other BCL11A isoforms (30), and with several corepressor complexes, including NuRD, LSD1/CoREST, NCoR/SMRT, and SIN3 (46, 51; data not shown). Consistent with
this, we observed enhanced BCL11A-XL repression of RAG1 following coexpression with MTA1, a single member of the 12
membered NuRD complex, which we suspect to be limiting under the conditions which we employed (Fig. 4B and data not shown).
Collectively, the results suggest that BCL11A’s N-terminal do
main is employed in a context-dependent fashion for assembly of both well-characterized corepressor complexes as well as coactiva
tion complexes whose members remain to be defined.

As anticipated, we observed modulation of V(D)J recombi
nation proportional to BCL11A-XL overexpression in cultured A70
pre-B cells (Fig. 5 and 6A) or following Cre-mediated knockdown in cultured explants of Bcl11a<sup>L/L</sup> bone marrow-derived pre-B cells (Fig. 6B and D). We suggest that the robustness of the effect, particularly in normal pre-B cells that dominate the IL-7 cultures, is derived from both direct transcriptional activation by BCL11A-XL of the RAG locus and upregulation of additional transcription factors that either perform the redundant function or act to promote chromatin accessibility of the V(D)J locus (Fig. 7). Validated examples of the first group include FOXP1, which transactivates RAG directly via binding to Erag (Fig. 1A and 3) (8, 11). Elevation and concerted action of this and similar factors that upregulate RAG may be sufficient for the V<sub>k</sub>-J<sub>k</sub> inversion of the exogenous recombination target pMX-IN (Fig. 5C and D). Ex
amples of the second category, which have been shown to be re
quired for chromatin accessibility of the endogenous V(D)J locus, include IRF4 and IRF8 (Fig. 7). V(D)J recombination and B cell development are blocked in IRF4/IRF8 double knockout mice at the pre-B cell stage (49). Accessibility and recombination of V<sub>k</sub>-J<sub>k</sub> has been correlated with selected posttranslational modifications of nucleosomal histone (H) tails (59–61). Ectropic expression of IRF4 in IRF4/8<sup>−/−</sup> pre-B cells was shown to increase acetylation of H3K9 and H4K14 as well as trimethylation of H3K4, marks associated with active chromatin (49). However, induction of J<sub>c</sub>-C<sub>g</sub> germ line transcription, a second feature typically but not always (62) associated with locus accessibility, was not observed follow
ing BCL11A-XL overexpression (Fig. 7). Perhaps, the concomi
tant lack of BCL11A-XL upregulation of Spi-B (Fig. 7), which, along with IRF4, has been reported to be sufficient for inducing Ig<sub>k</sub> germ line transcripts (36), is rate limiting under these condi
tions. Resolution will require a careful assessment of BCL11A
mediated germ line transcription and histone epigenetic altera
tions across this locus.

Consistent with a recent report by Yu et al. (32), we observed significantly reduced proliferation and increased apoptosis rela
tive to controls in floxed cultures of Bcl11a<sup>L/L</sup> B220<sup>+</sup> bone marrow pre-B cells (Fig. 6 and data not shown). In further agreement with that report (32), prior infection of Bcl11a<sup>L/L</sup> cells with a Bcl-x<sub>L</sub>-encoding retrovirus bypassed Cre-mediated apoptosis and prolifer
ation. Bcl11a<sup>−/−</sup> fetal liver hematopoietic progenitors suffer a severe pre-pro-B cell block (57), typically as a consequence of a prolifera
tive defect in IL-7R signaling (63, 64) or a defect in V(D)J recombi
nation (6). The weight of data assembled here, along with the observations (25, 32, 50, 55) that Bcl11a expression in mouse hematopoiesis initiates at the hematopoietic stem cell stage, sug
ests that the initial D-J recombination step of antigen receptor assembly will be impaired by conditional Bcl11a deficiency. It may be informative that Foxp1, a putative target of BCL11A-XL (Fig. 7), activates this earliest RAG-mediated step of V(D)J joining (11). Further in vivo studies in the context of potential collaborat
ing transcription factors such as Foxp1 and PU.1 are required to
eucidate whether Bcl11a is, indeed, at the top of the hierarchy of V(D)J transcriptional control.

ACKNOWLEDGMENTS
We thank Chhaya Das, Maya Ghosh, and June Harriss for excellent tech
nical support. We thank Mark Schlissel for sharing luciferase constructs. We thank members of our laboratory for critically reading the manuscript and Paul Das for its preparation.

G.C.I. acknowledges support from NIH grant F32-CA110624, and P.W.T. acknowledges support from NIH grant R01-CA13534, the Cancer Prevention Research Institute (grant CPRIT RP100612), and the Marie Betzner Morrow Endowment.


(EHZF), the human homolog to mouse Evi3, is highly expressed in primitive human hematopoietic cells. Blood 103:2062–2070.


