The induction of Bcl-xL is critical for the survival of late proerythroblasts. The erythroid-specific transcriptional network that regulates Bcl-xL expression in erythropoiesis remains unclear. The activation of the central erythropoietic transcriptional factor, GATA-1, leads to the early, transient induction of a transcription repressor, Gfi-1B, followed by the late induction of Bcl-xL during erythroid maturation in G1ER cells. Chromatin immunoprecipitation assays demonstrated that a constant level of GATA-1 binds to the Bcl-xL promoter throughout the entire induction period, while Gfi-1B is transiently associated with the promoter in the early phase. The sustained expression of Gfi-1B abolished GATA-1-induced Bcl-xL expression. Here, we present evidence that GATA-1 binds to the noncanonical GATT motif of the Bcl-xL promoter for trans-activation. Gfi-1B expressed at increased levels is recruited to the Bcl-xL promoter through its association with GATA-1, suppressing Bcl-xL transcription. Therefore, the down-regulation of Gfi-1B in the late phase of erythroid maturation is necessary for Bcl-xL induction. Furthermore, we show that the inhibition of Bcr-Abl kinase by treatment with imatinib caused the up-regulation of Gfi-1B in K562 cells, where Gfi-1B also cooperated with GATA-1 to repress Bcl-xL transcription. Gfi-1B knockdown by RNA interference diminished imatinib-induced apoptosis, while the overexpression of Gfi-1B sensitized K562 cells to arsenic-induced death. These findings illuminate the role of Gfi-1B in GATA-1-mediated transcription in the survival aspect of erythroid cells.

During erythrocyte development, it is essential that committed precursors be protected from cell death. It has been well documented that the increased expression of Bcl-xL during terminal erythroid maturation provides the antiapoptotic function for maintaining the viability of mature, definitive erythrocytes (1, 9, 32). GATA-1, an erythroid-specific transcription activator, has been shown to be an upstream activator of Bcl-xL expression, because the restoration of GATA-1 function in immature G1E erythroblasts derived from GATA-1-deficient embryonic stem cells (38) triggers erythroid maturation through Bcl-xL induction (9, 39). Despite the presence of a typical GATA sequence in the Bcl-xL promoter, it was previously reported that mutations in the proximal GATA-1 binding motif of the Bcl-xL promoter have little effect on the promoter activity (9). Since the expression of Bcl-xL mRNA displayed delayed kinetics after GATA-1 induction, it has been proposed that GATA-1 acts as an indirect activator of Bcl-xL transcription (9). The details of the Bcl-xL transcriptional control by the GATA-1-regulated transcriptional network remain undefined.

Gfi-1B (growth factor independence 1B) is a Gfi family transcriptional repressor that contains a SNAG domain to mediate transcriptional repression and a zinc finger domain at its carboxyl terminus for DNA binding to the TAAATGC(A/T)GCA recognition sequence (the binding site motif is highlighted in bold) (11, 35, 41). Only a small number of Gfi-1B-regulated genes are known, including p21, Socs1, Socs3, and the Gfi-1B gene’s own promoter (14, 16, 35). Gene targeting experiments have shown that Gfi-1B gene disruption results in embryonic lethality due to a loss of red blood cell formation, indicating the necessary role of Gfi-1B in erythropoiesis (25).

In a previous study, the enforced expression of Gfi-1B in early erythroid progenitor cells induced a drastic expansion of erythroblast colonies, independent of erythropoietin. These Gfi-1B-transduced cells exhibited massive apoptosis after 7 to 10 days of culture, with a significant reduction of Bcl-xL expression (22). Because the canonical consensus sequence recognized by Gfi-1B was not found in the Bcl-xL promoter region, it was proposed that Gfi-1B does not exert its transcriptional repression function directly on the Bcl-xL promoter (7, 22).

By searching the microarray database (39) for profiles of gene expression after early and late GATA-1 activation in G1ER cells, we found that Gfi-1B is an early gene induced by GATA-1 activation and that its expression level declines before the rise of the Bcl-xL transcript level in the late phase. Our recent study has demonstrated that Gfi-1B interacts with GATA-1 via the zinc finger domain of Gfi-1B. When Gfi-1B expression is elevated, Gfi-1B forms a complex with GATA-1 which binds to the AATC motifs. These clues compelled us to investigate whether both GATA-1 and Gfi-1B participate directly in the regulation of Bcl-xL expression. Here, our data demonstrate that GATA-1 binds to the Bcl-xL promoter at the GATT promoter region, it was previously proposed that GATA-1 acts as an indirect activator of Bcl-xL transcription (9). The details of the Bcl-xL transcriptional control by the GATA-1-regulated transcriptional network remain undefined.

Gfi-1B (growth factor independence 1B) is a Gfi family transcriptional repressor that contains a SNAG domain to mediate transcriptional repression and a zinc finger domain at its carboxyl terminus for DNA binding to the TAAATGC(A/T)GCA recognition sequence (the binding site motif is highlighted in bold) (11, 35, 41). Only a small number of Gfi-1B-regulated genes are known, including p21, Socs1, Socs3, and the Gfi-1B gene’s own promoter (14, 16, 35). Gene targeting experiments have shown that Gfi-1B gene disruption results in embryonic lethality due to a loss of red blood cell formation, indicating the necessary role of Gfi-1B in erythropoiesis (25).

In a previous study, the enforced expression of Gfi-1B in early erythroid progenitor cells induced a drastic expansion of erythroblast colonies, independent of erythropoietin. These Gfi-1B-transduced cells exhibited massive apoptosis after 7 to 10 days of culture, with a significant reduction of Bcl-xL expression (22). Because the canonical consensus sequence recognized by Gfi-1B was not found in the Bcl-xL promoter region, it was proposed that Gfi-1B does not exert its transcriptional repression function directly on the Bcl-xL promoter (7, 22).

By searching the microarray database (39) for profiles of gene expression after early and late GATA-1 activation in G1ER cells, we found that Gfi-1B is an early gene induced by GATA-1 activation and that its expression level declines before the rise of the Bcl-xL transcript level in the late phase. Our recent study has demonstrated that Gfi-1B interacts with GATA-1 via the zinc finger domain of Gfi-1B. When Gfi-1B expression is elevated, Gfi-1B forms a complex with GATA-1 which binds to the AATC motif of the Gfi-1B promoter to repress transcription, conferring a negative feedback loop (14). Interestingly, the Bcl-xL promoter also contains several conserved AATC motifs. These clues compelled us to investigate whether both GATA-1 and Gfi-1B participate directly in the regulation of Bcl-xL expression. Here, our data demonstrate that GATA-1 binds to the Bcl-xL promoter at the GATT (AATC for the reverse strand) site for trans-activation and that the enforced expression of Gfi-1B represses the Bcl-xL promoter in a GATA-1-dependent manner. Thus, Gfi-1B converts GATA-1-mediated activation to repression. These results suggest that the up-regulation of Gfi-1B is involved in suppressing Bcl-xL transcription in the early phase of GATA-1 activation in G1ER cells, while the decrease of Gfi-1B expression in the late phase allows GATA-1-mediated Bcl-xL transcription.
In this study, we used K562 cells to investigate the regulation of Gfi-1B expression in apoptotic induction. K562 is an erythropoietic cell line established by erythroblasts isolated from a patient with chronic myelogenous leukemia (CML), which is a myeloproliferative disorder associated with t(9;22) translocation, which produces a BCR-ABL oncogene hybrid that encodes a chimeric protein, p210, with a hyperactive tyrosine kinase (3, 8). The p210BCR-ABL protein activates several pathways, including the Ras/MAPK/extracellular signal-regulated kinase 1 and 2/phosphatidylinositol 3-kinase/Akt (26, 33), NF-κB (19, 23), and signal transducer and activator of transcription (STAT) protein pathways (2, 13, 30, 31). Imatinib mesylate, an inhibitor of Bcr-Abl tyrosine kinase, is now widely used in CML treatment (4, 5, 17, 27, 34). The action of imatinib in killing cells expressing Bcr-Abl has been attributed to the inhibition of Bcl-x<sub>L</sub> transcription by blocking Bcr-Abl/STAT5 signaling (6, 13, 21, 36). Here, we found that the treatment of K562 cells with imatinib increased Gfi-1B expression, resulting in Gfi-1B occupancy of the Bcl-x promoter through the formation of a complex of Gfi-1B and GATA-1. As a result, Bcl-x<sub>L</sub> transcription was suppressed. Finally, we demonstrated that the depletion of Gfi-1B expression in K562 cells diminished imatinib-induced apoptosis and that the overexpression of Gfi-1B increased the susceptibility to arsenic-induced apoptosis. These results indicate the role of Gfi-1B in apoptosis and emphasize the importance of GATA-1–Gfi-1B interaction in transcriptional control for erythroid development.

**MATERIALS AND METHODS**

**Materials.** Imatinib mesylate (STI571; Gleevec) was provided by Novartis Pharmaceuticals (Basel, Switzerland). Bcl-x<sub>L</sub> (54H6), STAT5, and phospho-STAT5 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Gfi-1B (B7) and GATA-1 (C-20 and N6) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-tubulin, and anti-Flag were from Sigma (St. Louis, MO).

**Plasmid construction.** The Gfi-1B cDNA was obtained by reverse transcription-PCR (RT-PCR) from K562 mRNA and cloned into frame-in the BamHI site of pCS2-MT. The gene expressing a nonfunctional mutant form of Gfi-1B, expressed by pCS2-P2A-Gfi-1B, was derived from pCS2-Gfi-1B by using the QuikChange XL site-directed mutagenesis kit (Stratagene). The pGL2-3.2 vector containing 3.2 kb of the murine Bcl-x promoter region fused to the promotorless pGL2 firefly luciferase reporter (10) was a generous gift of Gabriel Núñez. The pGL2-3.2 plasmid was digested with HindIII to generate a 0.6-kb fragment of the Bcl-x promoter region, which was subcloned into the pGL2 vector, producing pGL2-0.6. Mutant versions of pGL2-0.6 (pGL2-0.6mut1 and pGL2-0.6mut2) were generated using the QuikChange site-directed mutagenesis kit with a specific mutated primer that converts GATT (positions 404 to 401 relative to the translation start site) to GATT (positions 404 to 401). pCMV2-GATA-1 and the Gfi-1B promoter constructs pGL2-GFI (415 to 19) and pCMV2-Gfi-1B were described previously (14, 15). The MIGR1 and MIGR-Cflag-Gfi-1B plasmids were from Dominique Dume¨nil. The pRK5-STAT5A and pRK5-STAT5C plasmids were described previously (14, 15). The MIGR1 and MIGR-CFlag-Gfi-1B were generated using the QuikChange site-directed mutagenesis kit producing pGL2-0.6. Mutant versions of pGL2-0.6 (pGL2-0.6mut1 and pGL2-0.6mut2) were generated using the QuikChange site-directed mutagenesis kit with a specific mutated primer that converts GATT (positions 404 to 401 relative to the translation start site) to GATT (positions 404 to 401).

**Transfection and luciferase assays.** NIH 3T3 cells were transfected using a DNA mixture containing Lipofectamine (Invitrogen Life Technologies). After transfection for 24 h, cells were analyzed for reporter activity.

K562 cells (5 × 10<sup>5</sup>) were suspended in 0.4 ml of RPMI 1640 medium in the presence of DNA mixtures for electroporation by Gene Pulser (Bio-Rad) at 300 V and 960 μF. Transfected cells were assayed for both firefly and Renilla luciferase activities using the dual luciferase assay system (Promega).

**Preparation of nuclear extracts and gel shift analysis.** The preparation of nuclear extracts from K562 cells or gel shift reactions was as described previously (15). A competitive DNA oligomer or GATA-1 (C-20; Santa Cruz, CA) antibody was added to the nuclear extracts for 30 min on ice prior to the DNA binding reaction. After the DNA binding reaction proceeded at room temperature for 25 min, samples were analyzed by electrophoresis at 150 V for 1.5 h through nondenaturing 4% polyacrylamide gels. Gels were then dried for autoradiography.

**ChIP and re-ChIP assays.** Chromatin immunoprecipitation (ChIP) was performed as described previously (14). In brief, cells (10<sup>6</sup>) were treated with formaldehyde at a final concentration of 1% for 10 min at room temperature. Glycine was added at a final concentration of 125 mM to quench cross-linking. Cell lysates were sonicated to generate DNA fragments averaging <1 kb. After preclearance, cell extracts were incubated with 2 μg of antibody at 4°C overnight, followed by precipitation with protein A-Sepharose beads. After washing, elution, deproteination, and heating, immunoprecipitated DNA was extracted and ethanol precipitated. DNA was resuspended in 50 μl of Tris-EDTA buffer for chromatin reimmunoprecipitation (re-ChIP), chromatin cross-linked to the immunocomplex was eluted by incubation with 50 μl of dithiothreitol (10 mM) at 37°C for 30 min. After centrifugation, the supernatant was diluted with a 20× volume of buffer (20 mM Tris-HCl [pH 8.1], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) and subjected to the ChIP procedure as described above. PCR was applied to the immunoprecipitated DNA with the following thermal cycling program: 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C (30 cycles for the first ChIP and 35 cycles for the re-ChIP), followed by a 5-min extension step at 72°C. PCR products were analyzed on 1.5% agarose gel and visualized by ethidium bromide staining.

**Immunoprecipitation.** Cells lysed in 50 mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 5 mM NaF, and proteinase inhibitors (Sigma) were centrifuged at 10,000 × g for 5 min and preclarified by incubation with normal mouse immunoglobulin G (IgG) and protein A beads for 30 min at 4°C. The supernatants were immunoprecipitated using GATA-1 antibody and protein A beads. The beads were then washed four times with the lysis buffer, and the proteins on the beads were separated by SDS–10% polyacrylamide gel electrophoresis.

**Biotinylated-oligonucleotide pulldown assay.** Nuclear extracts from K562 cells (5 × 10<sup>7</sup>) were prepared as described previously (15). Biotinylated double-stranded oligonucleotides (wild type, 5′-AGGGGATTGTTAGATG-3′, and mutant, 5′-AGGGGATTGTTAATG-3′) (the mutated and corresponding wild-type sequences are underlined) coupled to streptavidin-agarose beads were incubated with 50 μg of the extracts at 30°C for 10 min, followed by six washings with the binding buffer containing 150 mM NaCl prior to separation on SDS-polyacrylamide gel for immunoblot analysis. For competition experiments, the extracts were incubated with the wild-type-oligonucleotide-coupled beads in the presence of a 50-fold molar excess of either the wild-type [5′-CAGCTGACAGAGAAATGTCGACTCT-3′] or mutant [5′-CAGCTGACAGAGAAATGTCGACTCT-3′] oligonucleotide.
RESULTS

Enforced expression of Gfi-1B in GIER cells repressed GATA-1-induced Bcl-xL expression. As an initial attempt to know whether Gfi-1B is involved in the regulation of Bcl-xL expression in erythroid cells, we tested the effect of the enforced expression of Gfi-1B in GIER cells on Bcl-xL expression. GIER cells that stably express GATA-1 fused to the ligand binding domain of the human estrogen receptor (9, 38, 40) were exposed to β-estradiol to activate GATA-1, by which synchronous erythroid maturation was triggered. We infected GIER cells with retrovirus generated from full-length Flag-Gfi-1B or the empty MIGR retroviral vector and measured Bcl-xL transcript levels in response to GATA-1 activation by the semi-RT-PCR method. At 6 h after the β-estradiol treatment of cells infected with the control virus, the Gfi-1B RNA level was markedly elevated, and it declined to the basal level at the time point of 16 h, while the Bcl-xL RNA level did not increase until 16 h after GATA-1 activation and remained elevated at 24 h (Fig. 1A and B, left panel). In general, patterns of Gfi-1B and Bcl-xL RNA expression in GIER cells infected with the control retrovirus were similar to the data deposited in the G1EDb microarray database (data not shown) (39). The early induction of Gfi-1B after GATA-1 activation is consistent with our previous finding that GATA-1 is a direct activator of the Gfi-1B promoter (15). Interestingly, the infection of GIER cells with Flag-Gfi-1B-carrying retrovirus abolished the GATA-1-induced expression of Bcl-xL, indicating that the sustained expression of Gfi-1B negatively modulates Bcl-xL transcription during erythroid maturation. Infection with Flag-Gfi-1B-carrying virus also caused low levels of endogenous Gfi-1B mRNA throughout the 24-h period of GATA-1 activation (Fig. 1A and B, right panel), which was consistent with our previous observation showing negative autoregulation of the Gfi-1B gene.

Gfi-1B and GATA-1 occupancy of the Bcl-x promoter in the early phase of erythroid maturation. Next, we performed chromatin cross-linking and ChIP to know whether Gfi-1B is directly involved in regulating the Bcl-x promoter during GATA-1 activation in GIER cells. Primers specific to the proximal murine Bcl-x promoter were used for the PCR. The result showed that Gfi-1B was associated with the Bcl-x promoter and that the degree of
occupancy was inversely correlated with the level of \textit{Bcl-x} expression while the extent of the binding of GATA-1 to the \textit{Bcl-x} promoter remained constant throughout the period of GATA-1 induction (Fig. 2). PCR using mouse TK1 promoter primers was performed as a negative control. These results suggest that both GATA-1 and Gfi-1B interact with the \textit{Bcl-x} promoter during erythroid maturation and that Gfi-1B acts as a transcriptional repressor of the \textit{Bcl-x} promoter.

**Repression of the \textit{Bcl-x} promoter by the enforced expression of Gfi-1B in K562 cells.** Similar to that of G1ER cells, the
infection of K562 cells by transduction with retroviral Flag-Gfi-1B decreased Bcl-xL expression. Because STAT5 is a direct activator of the Bcl-x gene in hematopoietic cells (31), we then examined the effect of the overexpression of Gfi-1B on STAT5 activation by measuring the phosphorylation status of STAT5. It appeared that the elevation of Gfi-1B expression had no effect on the intensity of STAT5 phosphorylation (Fig. 3A).

To study the functional effect of Gfi-1B on the Bcl-x promoter, we analyzed the sequence within the proximal promoter region of the human Bcl-x gene (10), which revealed several occurrences of the Gfi-1B binding core sequence, the AATC motif, located at −1429, −1024, −924, and −408 upstream of the translation start site. As shown here, the GATT (AATC for the reverse strand) motif at −1429 and −408 is conserved in the murine promoter (10) (Fig. 3B). It was noted that the sequences surrounding these two sites do not match the consensus Gfi-1B binding sequence. To understand whether the enforced expression of Gfi-1B can directly repress the Bcl-x promoter activity, we constructed two reporter plasmids in which 3.2- and 0.6-kb fragments of the murine Bcl-x promoter were linked to the luciferase gene (Fig. 3C). K562 cells were transfected with these two constructed Bcl-x reporter plasmids, along with the Gfi-1B expression vector (pCS2-Gfi-1B), the plasmid expressing a nonfunctional mutant Gfi-1B (pCS2-P2A-Gfi-1B), or an empty vector as a control. The mutant form of Gfi-1B expressed by pCS2-P2A-Gfi-1B carries a mutation in the SNAG domain that disrupts the Gfi-1B transcriptional repressor of the minimal Bcl-x promoter (Fig. 3D). These data clearly show Gfi-1B as a repressor of the minimal Bcl-x promoter.

GATA-1 mediates the Gfi-1B repression of the Bcl-x promoter through binding at the AATC site. We then introduced a mutation at the −408 GATT (the AATC reverse strand) site of the murine minimal Bcl-x promoter, which corresponds to the −408 GATT site of the human Bcl-x promoter. However, the mutation converting Gfi-1B to GATC or AATT decreased the Bcl-x promoter activity, an indication that this site is a positive cis element for trans-activation (Fig. 4A). To test whether GATA-1 may also mediate the trans-activation of the Bcl-x promoter through this site, we expressed GATA-1 in NIH 3T3 cells, a nonerythroid line, to measure its effect on the Bcl-x promoter activity (Fig. 4D). Based on these data, we conclude that GATA-1 is a direct activator of the Bcl-x promoter and mediates the recruitment of Gfi-1B to the promoter for transcriptional repression.

To examine whether GATA-1 can bind to the −408 GATT site of the human Bcl-x promoter, we performed a gel shift assay using a radiolabeled DNA fragment covering the region from −396 to −415 of the Bcl-x promoter sequence. The incubation of this probe with nuclear extracts from 293T cells ectopically expressing GATA-1 gave rise to DNA-protein complexes I and II (Fig. 4E). Complex I was abolished by an excess amount of the unlabeled oligomer but not by the oligomer carrying a mutation in the GATT site. An excess amount of the unlabeled GATA consensus oligonucleotide or the specific GATA-1 antibody also abolished the formation of complex I. No amount of the competitor had an effect on complex II formation, indicating that complex II is a nonspecific DNA-protein complex. Accordingly, we conclude that GATA-1 binds to the −408 GATT site of the human Bcl-x promoter.

Up-regulation of Gfi-1B participates in the imatinib-induced repression of Bcl-xL expression. Since it is well established that the inhibition of Bcl-xL expression is responsible for imatinib-induced apoptosis in CML (6, 13, 18, 21, 36), we then used this system to consider the question of whether Gfi-1B is involved in the imatinib-induced down-regulation of Bcl-xL expression. To address this question, we first examined the effect of imatinib treatment on levels of Gfi-1B and Bcl-xL mRNA expression in K562 cells by semiquantitative RT-PCR. Following the imatinib treatment, the level of Gfi-1B mRNA was markedly increased, with a concurrent decrease in Bcl-xL mRNA expression (Fig. 5, left panels). A similar relationship was reflected in the levels of Gfi-1B and Bcl-xL protein expression as determined by Western blot analysis (Fig. 5, right panels).

Given that the imatinib treatment did not change the
FIG. 4. GATA-1 activates the Bcl-x promoter and mediates Gfi-1B repression. (A) K562 cells were transfected with the Bcl-x reporter constructs pGL2-0.6 and pGL2-0.6mut1 (mut1; carrying a mutation at −401 in the GATT sequence) and pGL2-0.6mut2 (mut2; carrying a mutation at −404 in the GATT sequence), and luciferase activities were determined and expressed as described in the legend to Fig. 3D, with the activity in cells transfected with full-length pGL2-3.2 and the control vector set at 1. The error bars represent standard deviations. wt, wild type. (B) NIH 3T3 cells were transfected with the minimal Bcl-x reporter construct pGL2-0.6 or pGL2-0.6mut2 along with pCMV2-GATA-1 in combination with different amounts of pCS2-Gfi-1B as indicated. The luciferase activities were normalized and expressed relative to that in cells transfected with pGL2-0.6 and control vectors, which was set arbitrarily at 1. Values are the averages of three independent determinations. The error bars represent standard deviations. (C) K562 cells with pS2-Gfi-1B-Flag retroviral integration were electroporated with GATA-1 siRNA or control siRNA for 48 h. Whole-cell lysates were subjected to Western blot analysis using anti-GATA-1, Gfi-1B, Bcl-xL, and β-tubulin antibodies (Ab). (D) Cells were treated with formaldehyde for ChIP assays using anti-Gfi-1B antibody or normal mouse IgG. DNA isolated from immunoprecipitates was amplified by PCR with primers specific for the Bcl-x (G-I [−445 to −196] and G-II [−1745 to −1536]), Gfi-1B, or c-jun promoter. +, present. (E) Results from gel shift assays of the human Bcl-x promoter region from −396 to −415. The 32P-labeled double-stranded oligonucleotide containing the sequence from −396 to −415 of the Bcl-x promoter was used for the assays in the absence or presence of an unlabeled competitor (at a 10-fold excess) with 2.5 μg of nuclear extracts from 293T cells overexpressing Flag-GATA-1, as indicated. The sequence of each competitor is shown below. Mutated and corresponding wild-type sequences are underlined. Control IgG and GATA-1(C-20) antibodies were added to the reaction mixtures where indicated. mut, mutant.
GATA-1 expression level, we then investigated the extent of GATA-1–Gfi-1B complex formation by immunoprecipitation from imatinib-treated cells. As shown in Fig. 6A, endogenous Gfi-1B was coimmunoprecipitated with endogenous GATA-1 by anti-GATA-1 antibody, and imatinib treatment increased the amount of Gfi-B in the GATA-1 complex. To verify that GATA-1 binds to the GATT motif of the human Bcl-x promoter, we performed the streptavidin pulldown assay with biotin-labeled oligonucleotides covering the region from −399 to −413 of the wild-type human Bcl-x promoter sequence and the corresponding sequence mutated at the −408 GATT site. The biotin-labeled oligonucleotides were incubated with nuclear extracts from K562 cells treated with or without imatinib. Western blot analysis of the pulldown proteins showed the association of both GATA-1 and Gfi-1B with the wild-type oligonucleotide but not the one mutated at the −408 GATT site (Fig. 6B). Consistently, the imatinib treatment enhanced the binding of Gfi-1B to the beads containing the wild-type Bcl-x promoter sequence without affecting the amount of GATA-1 binding to DNA. An excess amount of an oligonucleotide containing the typical GATA-1 binding sequence but not the oligonucleotide carrying the GATA-to-CTTA conversion, was capable of abolishing the binding of GATA-1 to the wild-type sequence and diminishing the imatinib-induced increased association with Gfi-1B. These results support the notion that GATA-1 binds to the GATT motif of the Bcl-x promoter and that elevated amounts of Gfi-1B in imatinib-treated cells are brought to the promoter through GATA-1.

Although Gfi-1B in K562 nuclear extracts could still bind to this GATT site in the presence of the GATA-1 binding competitor, an in vivo ChIP analysis showed a lack of Gfi-1B occupancy of the Bcl-x promoter in GATA-1-depleted cells (Fig. 4D), indicating that the in vivo recruitment of Gfi-1B to the promoter is highly dependent on GATA-1. The discrepancy may be that under the in vitro binding conditions, Gfi-1B is capable of binding to the oligonucleotide covering the GATT site of the Bcl-x promoter but that this binding capability is too weak to be detected within the in vivo chromatin environment.

Imatinib treatment increases the cooccupancy of the Bcl-x promoter by Gfi-1B and GATA-1. Using ChIP analysis, we also tested the effect of imatinib treatment on the in vivo occupancy of the Bcl-x promoter by Gfi-1B and GATA-1. The results showed an imatinib-responsive increase in the amount of Gfi-1B bound to the proximal Bcl-x promoter region in vivo. The chromatins covering the distal region of the Bcl-x pro-

FIG. 5. Inverse correlation between Gfi-1B and Bcl-xL expression in response to imatinib treatment. (A) K562 cells treated with imatinib for the indicated periods were processed for RT-PCR analysis (left panel) using primers specific for Gfi-1B, Bcl-xL, and the GAPDH gene and for Western blotting (right panel) with anti-Gfi-1B, anti-Bcl-xL, and β-tubulin antibody. (B) Densitometric analysis of Gfi-1B and Bcl-xL at the RNA (left panel) and protein (right panel) levels, with results normalized by the GAPDH mRNA level and the β-tubulin level, respectively, in each sample.
FIG. 6. Imatinib-induced Gfi-1B formed a complex with GATA-1 on the Bcl-x promoter. (A) K562 cells were treated with or without imatinib (1 μM). After 24 h, whole-cell lysates were prepared and immunoprecipitated with anti-GATA-1 antibody (αGATA-1) or control IgG. The immunoprecipitates (IP) were subjected to immunoblot analysis with anti-Gfi-1B antibody (Ab), followed by reprobing with anti-GATA-1 antibody to confirm that GATA-1 was successfully immunoprecipitated. −, absent; +, present. (B) Pulldown assay using biotin-labeled oligonucleotides containing the Gfi-1B binding sequence (−408 to −405) on the human Bcl-x promoter. Nuclear extracts prepared from untreated or imatinib-treated K562 cells were incubated with biotin-labeled double-stranded oligonucleotides containing the wild-type (WT) or mutated (mut) Gfi-1B binding site. The bound proteins were pulled down with streptavidin-agarose and were analyzed by immunoblotting using antibody against Gfi-1B or GATA-1. Competitor oligonucleotides (oligo) specific for typical and mutated GATA-1 canonical consensus sequences were each included in the reaction mixture as indicated (lanes 3 and 4). Relative amounts of GATA-1 and Gfi-1B protein in lanes 1 to 4 were evaluated by densitometric determination and are shown in the lower panel. (C) In vivo cross-linked protein-DNA complexes isolated from untreated and imatinib-treated K562 cells were subjected to immunoprecipitation with IgG (control) or Gfi-1B antibody (α-Gfi-1B) as indicated. The coimmunoprecipitated DNA was amplified by PCR using the Bcl-x primers G-I (−445 to −196) and G-II (−1745 to −1536), the primer pair for c-jun, or the primer pair specific for the Gfi-1B promoter. Putative Gfi-1 and Gfi-1B binding sites are shown by boxes. The PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. (D) Following ChIP with Gfi-1B antibody, the complexes were subjected to a second immunoprecipitation (re-ChIP) with either anti-GATA-1 antibody (α-GATA-1) or control IgG. The coimmunoprecipitated DNA was amplified by PCR using the indicated primers: G-I, G-II, the primer pair specific for the Gfi-1B promoter, or the c-jun primer pair as a control. (E) K562 cells were transfected with the Bcl-x promoter construct, pGL2-3.2, together with pRL-TK. After 24 h, cells were treated with or without 1 μM imatinib for an additional 24 h. Cells were then harvested for the luciferase assay. Values are the averages of three independent determinations. The error bars represent standard deviations. *, P < 0.05.
moter or the c-jun promoter were not precipitated in this ChIP assay (Fig. 6C). To know whether Gfi-1B and GATA-1 simultaneously cooccupy the Bcl-x promoter in vivo, we performed a re-ChIP analysis. First, ChIP was performed using Gfi-1B antibody. The precipitated chromatin fragments were eluted and subjected to reprecipitation using GATA-1 antibody. The same DNA fragment with Gfi-1B and GATA-1 simultaneously bound would be amplified with the subsequent PCR. As shown in Fig. 6D, re-ChIP assays showed that the imatinib treatment increased the amounts of GATA-1 and Gfi-1B bound on the Bcl-x and Gfi-1B promoter regions in K562 cells. In parallel, we observed that the imatinib treatment caused a 41% reduction in luciferase activity expressed from the full-length Bcl-x reporter plasmid (pGL2-3.2) in K562 cells (Fig. 6E). Collectively, these results indicate that the in vivo association of Gfi-1B with the Bcl-x promoter correlates with the increase of Gfi-1B complexed with GATA-1 and the reduction of Bcl-xL transcription in imatinib-treated cells.

Role of Gfi-1B in apoptosis. Next, we questioned whether the up-regulation of Gfi-1B is necessary for the imatinib-induced repression of Bcl-xL, since there is a possibility that the lack of phopho-STAT5-dependent transcription in imatinib-treated cells is by itself sufficient to cause a reduction of Bcl-xL transcription. To address this question, we tested the effect of Gfi-1B knockdown on the imatinib-induced repression of Bcl-xL and apoptosis. Without imatinib treatment, K562 cells transfected with Gfi-1B siRNA showed reduced Gfi-1B expression at both the mRNA and protein levels (Fig. 7A). The

FIG. 7. Gfi-1B is required for imatinib-induced apoptosis in K562 cells. K562 cells were transfected with Gfi-1B siRNA or control siRNA. After 24 h, cells were treated with or without 1 μM imatinib for an additional 48 h. (A) Western blot analysis with anti-Gfi-1B, anti-Bcl-xL, or β-tubulin antibody (Ab; left panel) and semiquantitative RT-PCR assay using primers specific for Gfi-1B, Bcl-xL, and the GAPDH gene (right panel). −, absent; +, present. (B) The effect of Gfi-1B siRNA transfection on the imatinib-induced up-regulation of Gfi-1B (left panel) and down-regulation of Bcl-xL (right panel) was evaluated by densitometric determination of the protein expression levels, which were normalized by the β-tubulin level in the corresponding sample. Values are the averages of results from three independent experiments. The error bars represent standard deviations. (C) Cells were also harvested and analyzed by annexin V-FITC staining. Values are the averages of three independent determinations. The error bars represent standard deviations. *, P < 0.05.
induction of Gfi-1B by imatinib treatment was diminished in cells transfected with Gfi-1B siRNA, with a concurrent increase in Bcl-xL mRNA and protein levels (Fig. 7B). In parallel, cells were harvested and analyzed by flow cytometry with annexin V staining as an apoptotic indicator. As expected, cells transfected with control siRNA displayed a marked increase in the apoptosis-positive population induced by imatinib treatment. In contrast, transfection with Gfi-1B siRNA effectively abolished imatinib-induced apoptosis (Fig. 7C). Thus, an elevation of Gfi-1B expression is required for imatinib-induced apoptosis.

Finally, we addressed whether the overexpression of Gfi-1B can sensitize K562 cells to death induced by other drugs. It has been shown previously that As2O3 treatment induces K562 cell apoptosis without decreasing Bcl-xL expression, and the 50% inhibitory concentration was determined to be 10 μM (29). Herein, we tested whether the overexpression of Gfi-1B can make K562 cells susceptible to As2O3-induced apoptosis. K562 cells were transfected with pCMV2-Flag-Gfi-1B or an empty vector and were treated with or without As2O3 (0.5 μM) as indicated for an additional 48 h. For comparison, a parallel set of cells transfected with an empty vector was treated with imatinib (1 μM) in the presence or absence of As2O3 (0.5 μM). Cells were then harvested for annexin V-FITC and propidium iodide staining prior to flow cytometric analysis.

![Diagram](image.png)

**FIG. 8.** Overexpression of Gfi-1B renders K562 cells susceptible to As2O3-induced apoptosis. K562 cells were transfected with pCMV2-Flag-Gfi-1B or an empty vector. (A) After 24 h, total lysates were subjected to Western blot analysis using anti-Gfi-1B and β-tubulin antibodies (Ab). –, absent; +, present. (B) Transfected cells were also treated with or without As2O3 (0.5 μM) as indicated for an additional 48 h. For comparison, a parallel set of cells transfected with an empty vector was treated with imatinib (1 μM) in the presence or absence of As2O3 (0.5 μM). Cells were then harvested for annexin V-FITC and propidium iodide staining prior to flow cytometric analysis.

As shown in Fig. 8B, treatment with As2O3 at a low dosage slightly increased the apoptosis-positive population among cells transfected with the control vector. In contrast, the population of cells overexpressing Gfi-1B showed a significant increase in apoptotic cells in response to As2O3 treatment, as seen among the control cells cotreated with imatinib and As2O3. Thus, the overexpression of Gfi-1B in K562 cells, like cotreatment with imatinib, enhances susceptibility to As2O3-induced apoptosis.

**DISCUSSION**

In this study, we demonstrated that GATA-1 is a direct activator of Bcl-xL transcription through binding to the GATT motif. The elevation of Gfi-1B expression in the early stage of erythroid maturation and in imatinib-treated K562 cells suppresses Bcl-xL transcription via the association of Gfi-1B with GATA-1 on the Bcl-x promoter. Thus, Bcl-x is a gene target of the GATA-1–Gfi-1B repression complex. Our results suggest that the down-regulation of Gfi-1B relieves repression, allowing GATA-1 to activate Bcl-xL transcription in the late phase of GATA-1-activated erythroid cells. Thus, GATA-1 and Gfi-1B...
interplay to control the timing of Bcl-xL expression during erythroid development. Moreover, in this report we present the first evidence that Gfi-1B contributes to apoptotic induction in imatinib-treated K562 cells via its repressor role in Bcl-xL transcription.

**Induction of Gfi-1B by inhibiting Bcr-Abl/STAT5 signaling in K562 cells.** The overexpression of Gfi-1B in G1ER cells did not lead to apoptosis because G1ER cells have been transformed by Bcl-2 for the maintenance of a culture of these stem cells as an immortalized cell line (38). In this study, we used K562 cells to induce apoptosis by blocking Bcr-Abl signaling with imatinib treatment. Very interestingly, the inhibition of Bcr-Abl/STAT5 signaling in K562 cells resulted in the up-regulation of Gfi-1B (Fig. 5 and 7A). Given that the depletion of GATA-1 abolished the in vivo binding of Gfi-1B to the Bcl-xL promoter while the depletion of Gfi-1B expression diminished imatinib-induced apoptosis (Fig. 4D and 7C), we conclude that the induction of Gfi-1B in imatinib-treated K562 cells contributes to the inhibition of Bcl-xL transcription in a GATA-1-dependent manner.

We also transfected K562 cells with the Gfi-1B promoter reporter plasmid pGL2-hG (−145 to +19) to test whether the **trans-activation** of Gfi-1B is stimulated by imatinib treatment. We found that imatinib treatment increased the Gfi-1B promoter activity (data not shown). Since the transfection of cells with increasing amounts of a STAT5 (dominant negative) expression vector decreased the Gfi-1B promoter activity stimulated by the imatinib treatment (data not shown), it is conceivable that Gfi-1B expression is negatively modulated by STAT5 signaling in K562 cells. As STAT5 is also a transcriptional activator of Bcl-xL, it is likely that blocking STAT5 signaling not only eliminates the activation machinery but also adds a repressor to suppress Bcl-xL expression.

**Relationship between GATA-1 and Gfi-1B in binding to the Bcl-xL promoter.** It is noted that the Bcl-xL promoter contains characteristic motifs for the binding of several transcription factors, including Ets-1, activator protein 4, nuclear factor-erythroid 2, Evi, GATA-1, and activator protein 1 (10), suggesting that Bcl-xL may be induced by different transcription mechanisms dependent on the cellular context and stimulated signals. As mentioned earlier, the role of GATA-1 in Bcl-xL expression has been obscure because mutations of the typical GATA binding site did not affect GATA-1-activated Bcl-xL transcription (9). Although the conserved GATT motif (AATC for the reverse strand), the core of a Gfi-1B binding consensus sequence, is present in the minimal Bcl-xL promoter, three different experimental findings obtained from this study indicated that GATA-1 binds to the GATT motif in vivo, suggesting that the level of Gfi-1B expression is controlled within a proper range in erythroid cells. The results shown here exemplify a specific case in which the limitation of Gfi-1B expression is important in minimizing its function of repression of the survival gene Bcl-x.

Experimental results from two independent studies, by Osawa et al. and Garcon et al., suggested that Gfi-1B may have distinct effects at the various stages during erythroid differentiation (7, 22). In immature erythroid progenitors, through the zinc finger domains, Gfi-1B would trans-activate target genes implicated in cell proliferation for erythroblast expansion. At the onset of differentiation, Gfi-1B would regulate genes that have to be repressed for differentiation induction. In this study, we add new information that GATA-1 and Gfi-1B interplay to regulate gene expression in the survival aspect of terminal erythroid differentiation.

**ACKNOWLEDGMENTS**

We are grateful to Dave Turner (University of Michigan, Ann Arbor, MI), Gabriel Nunez (University of Michigan, Ann Arbor, MI), and Dominique Duménil (Institut Gustave Roussy, Paris, France) for kindly providing pCS2-MT, pGL-2-5,2, and MIGR-Flag-Gfi-1B plasmids. We thank James Ihle (St. Jude Children’s Research Hospital, Memphis, TN) and Stephen Nimer (Memorial Sloan-Kettering Cancer Center, New York, NY) for providing pRK5-STAT5A, pRK5-STAT5AΔ713, pEQ-PAM3(-E), and pSV-A-MLV-env plasmids. We...
also thank Stuart H. Orkin (Children’s Hospital and Dana Farber Cancer Institute, Howard Hughes Medical Institute, Boston, MA) and Novartis Pharmaceuticals (Basel, Switzerland) for the generous gift of the G1ER cell line and imatinib.

This study was supported by grants NSC95-3112-B-002-006, NSC95-2752-B-002-006-PAE, and NSC94-2320-B-002-018 from the National Science Council, Taiwan, Republic of China.

REFERENCES

1. Benito, A. M., Silva, D., Grillot, G. Nunez, and J. L. Fernandez-Luna. 1996. Apoptosis induced by erythroid differentiation of human leukemia cell lines is inhibited by interleukin-2 withdrawal. Mol. Cell. Biol. 16:2637–2642.

2. Grillot, D. A., M. Gonzalez-Garcia, D. Ekhterae, L. Duan, N. Inohara, S. Ohta, M. F. Seldin, and G. Nunez. 1997. Genome organization, promoter region analysis, and chromosomal localization of the mouse Gfi-1b gene. J. Immunol. 158:4750–4757.

3. Grimes, H. L., T. O. Chan, P. A. Zweidler-Mckay, B. Yong, and P. N. Tsichlis. 1996. The Gfi-1 proto-oncogene contains a novel transcriptional repressor domain, SNAG, and inhibits G1 arrest induced by interleukin-2 withdrawal. Mol. Cell. Biol. 16:623–627.

4. Grosveld, F., P. Rodriguez, N. Meier, S. Kripic, F. Pourfarzad, P. Papadooulos, K. Kolodziej, G. P. Patrinos, A. Hostert, and J. Strouboulis. 2005. Isolation and characterization of hematopoietic transcription factor complexes by in vivo biotinylation tagging and mass spectrometry. Ann. N. Y. Acad. Sci. 1054:55–67.

5. Horita, M., E. J. Andreu, A. Benito, C. Arbona, C. Sanz, I. Benet, F. Prosper, and J. L. Fernandez-Luna. 2000. Blockade of the Bcr-abl kinase activity induces apoptosis and prevents myelogenous leukemia cell cultures by suppressing signal transducer and activator of transcription 5-dependent expression of p38 MAP kinase. J. Biochem. Mol. Biol. 33:1023–1030.

6. Huang, D. Y., Y. Y. Kuo, and Z. F. Chang. 2005. GATA-1 mediates auto-regulation of Gfi-1B transcription in K562 cells. Nucleic Acids Res. 33:5311–5322.

7. Huang, D. Y., Y. Kuo, S. Lai, Y. Suzuki, S. Sugano, and Z. F. Chang. 2004. GATA-1 and NF-Y cooperate to mediate erythroid-specific transcription of Gfi-1B gene. Nucleic Acids Res. 32:3935–3946.

8. Jegalian, A. G., and H. Wu. 2002. Regulation of Sox gene expression by the proto-oncogene GFI-1B: two routes for STAT5 target gene induction by erythropoietin. J. Biol. Chem. 277:2345–2352.

9. Kantarjian, H., C. Sawyers, A. Hochhaus, F. Guilhot, C. Schiffer, C. Gambacorti-Passerini, D. Niederwieser, D. Resta, R. Capdeville, U. Zoellner, A. Saleque, S. Cameron, and S. H. Orkin. 2002. The zinc-finger proto-oncogene Gfi-1b is essential for development of the erythroid and megakaryocytic lineages. Genes Dev. 16:301–306.

10. Mullary, J. C., J. Cammenga, K. L. Mackenzie, F. J. Berguido, M. A. Moore, and S. D. Nimer. 2002. The AML1-EETO fusion protein promotes the expansion of human hematopoietic stem cells. Blood 99:15–23.

11. Ortel, C., T. Jonuleit, A. Gots, H. van der Kuij, H. Michels, J. Duyster, M. Hallen, and W. E. Alling. 1999. The tyrosine kinase inhibitor GCP 5714 (ST1) induces apoptosis in BCR-ABL-positive cells by down-regulating BCR-Abl-mediated transformation. Genes Dev. 13:968–981.

12. Osawa, M., T. Yamaguchi, Y. Nakamura, S. Kaneko, M. Onodera, K. Sawada, A. Jegalian, H. Wu, H. Nakachi, and A. Iwama. 2002. Erythroid expansion mediated by the Gfi-1B zinc finger protein: role in normal hematopoiesis. Blood 100:2769–2777.