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# Inhibition of β protein 1 expression enhances β-globin promoter activity and β-globin mRNA levels in the human erythroleukemia (K562) cell line

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***Objective*. In this paper, we report new observations related to the mechanism of the negative regulation of the important adult β-globin gene in the erythroid cells at the embryonic-fetal stage of their development. We focused on the role of the silencer II region located upstream of the β-globin gene, which along with its cognate binding protein BP1, negatively regulates β-globin transcription.**

***Materials and Methods*. We prepared plasmid constructs containing the wild-type silencer II sequence, a mutated silencer II sequence, or a mutated control sequence in the β-globin promoter 690-bp insert, which in turn was linked to an enhanced green fluorescent protein (EGFP) reporter gene. A human erythroleukemia cell line (K562) with embryonic-fetal phenotype was transfected with these EGFP constructs.**

***Results*. Flow cytometry and fluorescence digital imaging showed about threefold increase in the β-globin promoter activity of the mutated silencer II construct. Introduction of a small interfering RNA (siRNA) complementary to BP1 into the cells caused a 75% decrease in BP1 expression and a simultaneous ~40% elevation of β-globin promoter activity as well as an increase in β-globin mRNA levels, as compared with controls. We detected no changes in the mRNA levels of positive regulators of hemoglobin transcription such as EKLF and GATA-1.**

***Conclusion*. Our results support the involvement of BP1 in the mechanism of the negative regulation of β-globin transcription. A better understanding of this mechanism may lay the groundwork for novel gene therapy approaches to inhibit the expression of abnormal structural variants of adult β globin, such as sickle hemoglobin.** **© 2004 International Society for Experimental Hematology. Published by Elsevier Inc.**

Expression of globin genes within the β-globin cluster is regulated to produce different hemoglobin β chains during the embryonic (ε), fetal (γ), and adult (β) stages of erythroid cell development. Each distinct hemoglobin tetramer exhibits a different affinity for oxygen, resulting in optimal $O_2$/$CO_2$ exchange between the developing fetus and mother. Thus, developmentally appropriate transcriptional activation of each different β chain is essential. This is achieved by the binding of transcriptional activator proteins to the successive β-chain promoters; the locus control region (LCR), consisting of five DNase I–hypersensitive sites (HS1-5) located from 8 to 22 kbp 5′ to the ε-globin gene, also participates in transcriptional activation [1–4]. Analysis of LCR function at its endogenous location in cell lines suggests that its function is limited to globin gene transcription activation [5,6], whereas transgenic mice experiments suggest that the LCR is also responsible for the open chromatin structure of the β-globin locus.

Accurate developmental expression of globin genes is achieved through a precise balance of negative and positive regulation. Positive regulation is provided by transcription factors such as GATA, CACCC binding protein(s), β-DRE, and stage-selecting factor [7–11]. These proteins bind to sites in the β-like globin promoters and exert lineage-specific and/or gene-specific activation. Negative regulation occurs by competition between genes for interaction with the LCR [12,13] and by autonomous gene silencing, involving sequences located in the proximal and distal promoters. Several silencers have been identified in the β-globin cluster, particularly upstream of the embryonic globin gene [14–17]. The

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adult β-globin gene includes two upstream silencer regions, and a common effector, termed β protein 1 (BP1), binds to both of these regions [18]. BP1 binds within silencer I at −530 bp and within silencer II at −300 bp relative to the cap site (+1). At both silencers, architectural chromatin proteins of the high mobility group (HMG) bind to and bend the DNA, possibly providing access for BP1 and other repressor proteins. HMG-I (Y) bends DNA at or near the BP1 binding site in silencer I but not in silencer II [19]; HMG1 bends DNA near the silencer II [20].

BP1, the repressor protein that binds both β-globin silencers, is found in vivo in the fetal liver during erythropoiesis; in vitro, it is gradually extinguished during erythroid differentiation of the human cell line MB-02 [21]. The BP1 gene belongs to the Distal-less (DLX) family of homeobox genes, which are expressed during early development. This gene shares extensive identity with DNA sequence of human DLX4 and appears to be a splice variant of DLX4. BP1 maps near a cluster of other homeobox genes on human chromosome 17q21-22 [22].

In our earlier studies of BP1 function in K562 cells, an erythroleukemia cell line with an embryonic-fetal phenotype, we have shown that introduction of multiple mutations in two BP1 binding sites (silencer I and silencer II) results in enhanced β-globin promoter activity [23]; thus, BP1 downregulates β-globin transcription when the silencers are intact. In the present study we explored the mechanisms by which BP1 negatively regulates β-globin gene expression. We investigated whether multiple mutations near but not at silencer II enhanced the activity of the β-globin promoter, as do mutations in the silencer II BP1 binding site. We also probed the role of the BP1 protein itself: we suppressed BP1 protein synthesis in K562 cells by transfecting cells with a small RNA duplex corresponding to 21 bases of the BP1 mRNA, and then examined its effect on β-globin promoter activity, β-globin mRNA levels, and expression of GATA-1 and EKLF, which are positive regulators of β-globin transcription.

## Materials and methods

*Cell culture*

K562 cells (ATCC, Manassas, VA, USA) were grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were regularly subcultured to maintain exponential growth.

*Plasmid construction*

Three DNA clones of a 690-bp fragment of the adult human β-globin regulatory region, from −640 to +50 relative to the cap site (+1), were amplified by the polymerase chain reaction (PCR) from a 35-kb cosmid containing the β-globin gene locus. The 690-bp PCR product, containing the β-globin promoter and an upstream regulatory region including silencers I and II, was coupled to the *Hind*III/*BamH*I cloning sites of the multisite promoterless vector pEGFP-1 (Clontech, Palo Alto, CA, USA) to drive transcription of an enhanced green fluorescent protein (EGFP) reporter gene. Mutations in the previously determined BP1 binding site (silencer II, positions −294 to −302) were introduced as described earlier [20]. The wild-type silencer II sequence, 5′-TTCAATATG-3′, was mutated to 5′- TTGCTCGAC-3′. To form the mutant control construct, the sequence 5′-TCAAT-3′ (nucleotide positions −340 to −345, near but not within silencer II) was mutated to 5′-GTCGA-3′ by the "megaprimer" method of site-directed mutagenesis [24]. All mutations were confirmed by automated DNA sequencing. The experimental constructs are diagrammed in Figure 1.

*Transfection and RNA interference experiments*

Transfection of K562 cells was performed using an electroporation apparatus (Nucleofector System, Amaxa, Koeln, Germany). K562 cells ($2 \times 10^6$) were transiently transfected with 5 μg of plasmid DNA (EGFP reporter constructs) and/or 1 μg of double-stranded RNA (dsRNA) (Xeragon, Qiagen, Germantown, MD, USA) using the manufacturer's recommended Nucleofector kit (Amaxa). The small interfering RNA (siRNA) oligos used were as follows: effective BP1 siRNA, sense GCUCCUGAAGCAGAAUUCUdTdT, anti-sense AGAAUUCUGCUUCAGGAGCdTdT; ineffective BP1 specific siRNA, sense CAGUUAGAGGAGAAAAAGGdTdT, anti-sense CCUUUUUCUCCUCUAACUGdTdT. The EGFP siRNA and control (nonsilencing) siRNA were obtained from Xeragon, Qiagen. Control siRNA, which is also referred in the text as nonspecific siRNA, did not have any matches with mRNAs sequences existing in Gene Bank.

*Fluorescence-activated cell sorting (FACS) analysis*

Transfected K562 cells (~$1 \times 10^6$) were washed once in phosphate-buffered saline (PBS) and resuspended in 1.5 mL of PBS. EGFP expression in K562 cells was detected by a FACScan (Becton-Dickinson, San Jose, CA, USA) flow cytometer equipped with an argon laser emitting at 488 nm, and analyzed using CellQuest

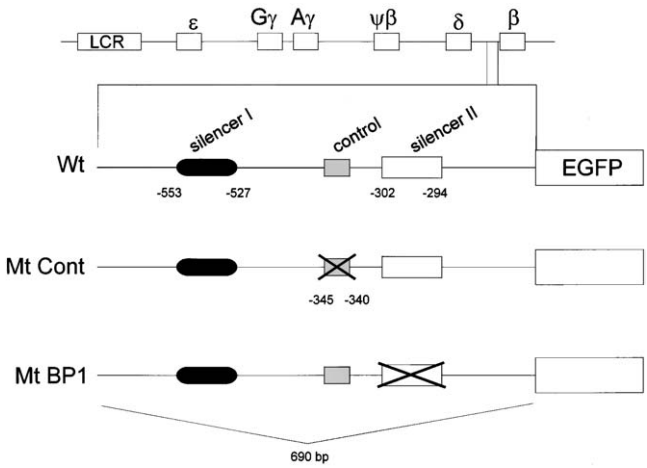


**Figure 1.** EGFP reporter constructs used in this study. Part of the human β-globin gene cluster map is expanded to show the location of the β-globin promoter and the upstream promoter element (690-bp fragment) used to drive expression of the EGFP reporter gene. Within this 690-bp region (from −640 to +50 relative to the cap site at +1), the constructs contain one of the following: a wild-type silencer II sequence (Wt); its mutated version (MtBP1); or a mutated control sequence (Mt Cont). LCR, locus control region.

(Becton-Dickinson) software. In each experiment, at least 10,000 cells were analyzed using the FL1 emission channel to monitor GFP fluorescence.

*RT-PCR and quantitative real-time PCR assay*

Total RNA was isolated using TRIzol (Life Technologies, Rockville, MD, USA), according to the manufacturer's protocol and quantitated by absorbance at 260 nm. About 200 μg of total RNA was digested with 10 U RNase-free DNase I (Ambion, Austin, TX, USA) for 30 minutes at 37°C, extracted with phenol and chloroform, and stored at −80°C until needed for reverse transcriptase (RT)-PCR. Each 50-μL RT reaction (Ready-to Go RT-PCR beads, Amersham Biosciences, Piscataway, NJ, USA) contained 1 μg total RNA, 250 ng oligo-dT, 200 mM concentrations of each dNTP, 200 U of Reverse Transcriptase and Ribonuclease Inhibitor, and 2.0 U of *Taq* DNA polymerase. The reaction mixture was incubated at 42°C for 30 minutes, then at 95°C for 5 minutes. The reverse-transcription reaction mixture then was subjected to PCR conditions, after adding 100 ng of a gene-specific primer set to each tube. Primers used (and resultant product size) were as follows. BP1 forward primer, 5′-GCTGAA AGAGGCTCAGAGAGA-3′; BP1 reverse primer, 5′-AGGTCTGGGAAGACAGCTTTG-3′ (224 bp); β-globin forward primer, 5′-CTTTAGTGATGGCCTGGCTC-3′; β-globin reverse primer, 5′-GGCAGAATCCAGATGCTCAAG-3′ (338 bp); γ-globin forward primer, 5′-GAACGTCTGAGATTATCAAT-3′; γ-globin reverse primer, 5′-GAAATGGATTGC CAAAACGG-3′ (397 bp); GATA1 forward primer, 5′-CCATTGCTCAACTGTATGGAG-3′; GATA1 reverse primer, 5′-ACTATTGGGGACAGGGAGTGATG-3′ (249 bp); EKLF forward primer, 5′-GCAAGAGCTACACCAAGAGC-3′; EKLF reverse primer, 5′-GCTGTC TATGGGTCCGTGTT-3′ (410 bp); β-actin forward, 5′-CCTCGCCTTTGCCGATCC-3′; β-actin reverse primer, 5′-GGATCTTCATGAGGTAGTCAGTC-3′ (626 bp). β-actin was an internal control for all samples. PCR conditions were as follows: 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute; 19 cycles were used for GATA-1, γ-globin, and β-actin, and 28 cycles for other genes. The amplified fragments were separated by 1% agarose gel electrophoresis along with molecular weight markers and stained with ethidium bromide. Band intensities were assessed using Image-Pro Plus software, version 4.1 (Media Cybernetics, Des Moines, IA, USA). Alternatively, the Agilent 2100 bioanalyzer (DNA1000 LabChip) was used for the analysis of RT-PCR products. The LabChip (Caliper Technologies, Mountain View, CA, USA) separates nucleic acid fragments by capillary electrophoresis in a chip with microfabricated channels and automates the detection as well as on-line data evaluation.

Quantitative real-time PCR assays of transcripts were carried out with the use of gene-specific double-labeled fluorescent probes in a 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA, USA) as previously reported [25]. We used 6-carboxyfluorescein (FAM) as the 5′ fluorescent reporter; we added tetramethylrhodamine (TAMRA) to the 3′ end as a quencher. The following primer and probe sequences were used: β-globin forward primer, 5′-CT CATGGCAAGAAAGTGCTCG-3′; β-globin reverse primer, 5′-AAT TCTTTGCCAAAGTGATGGG-3′; β-globin probe, 5′-FAM-CGT GGATCCTGAGAACTTCAGGCTCCT-TAMRA-3′; γ-globin forward primer, 5′-GGCAACCTGTCCTCTGCCTC-3′; γ-globin reverse primer, 5′-GAAATGGATTGCCAAAAC-GG-3′; γ-globin probe, 5′-FAM-CAAGCTCCTGGGAAATGTGCTGGTG-TAMRA-3′. All probes were designed to span exon junctions in the fully processed message. Standard curves were based on accurately deter mined dilutions of plasmids containing γ- or β-globin cDNA as a template. Plasmid dilutions covered a dynamic range of 5 logarithmic orders or greater.

*Fluorescence intensity analysis of digital images*

Fluorescence was measured by capturing digital fluorescent images (1200 × 1600 pixels) from transfected K562 cells growing in 12-well plates, using a digital RT-Slider Spot camera (Diagnostic Instruments, Sterling Heights, MI, USA) mounted on an inverted fluorescence microscope (Zeiss Axiovert S100TV; Zeiss, Oberkochen, Germany) equipped with a 100-W mercury lamp. Off-line image analysis was performed using Image-Pro Plus software, version 4.1 (Media Cybernetics, Des Moines, IA, USA). Mean pixel intensity (8-bit pixel depth) was calculated from each cell; background fluorescence was subtracted from all values. Statistical significance was determined by Student's *t*-test or ANOVA with post hoc comparisons of means using Bonferroni's *t*-procedure.

## Results

*EGFP reporter constructs*

To determine whether multiple mutations near but not at silencer II of the β-globin sequence enhanced the activity of the β-globin promoter as effectively as mutations in silencer II itself, we analyzed β-globin promoter activity in three EGFP-reporter constructs: one contained a wild-type silencer II sequence (Wt); the second contained a mutated silencer II sequence (MtBP1); the third, a mutated control sequence (Mt Cont) bearing multiple mutations slightly upstream of silencer II. The region chosen for the mutant control (Fig. 1) is a CAAT box with sequence similarity to the binding core element of BP1 but residing outside the silencer II element; it does not interfere with HMG or other known protein binding sites.

We transfected K562 erythroleukemia cells with these three EGFP-reporter constructs and then evaluated EGFP expression using digital fluorescence microscopy (Figs. 2 and 3). K562 cells normally express BP1 at a high level and β-globin mRNA at a low level. Quantitative GFP-based fluorescence intensity analyses showed that mean cell fluorescence values 24 hours after transfection were similarly low in cells transfected with the wild-type construct (Fig. 2A–D and Fig. 3) and the mutant control construct (Fig. 2E–H and Fig. 3). However, mean cell fluorescence increased threefold in K562 cells transfected with the construct mutated in the BP1 site (Fig. 2I–L and Fig. 3).

*RNA interference*

We hypothesized that inhibition of BP1 expression may enhance β-globin promoter activity. To test this hypothesis we used RNA interference. We used 21-nucleotide duplexes of siRNAs targeting two different sequences of the BP1 coding region. In K562 cells cotransfected with these siRNAs and our EGFP constructs, we measured the effects of RNA interference on inhibition of BP1 expression, first by EGFP reporter assays and then by quantitative PCR. siRNAs were

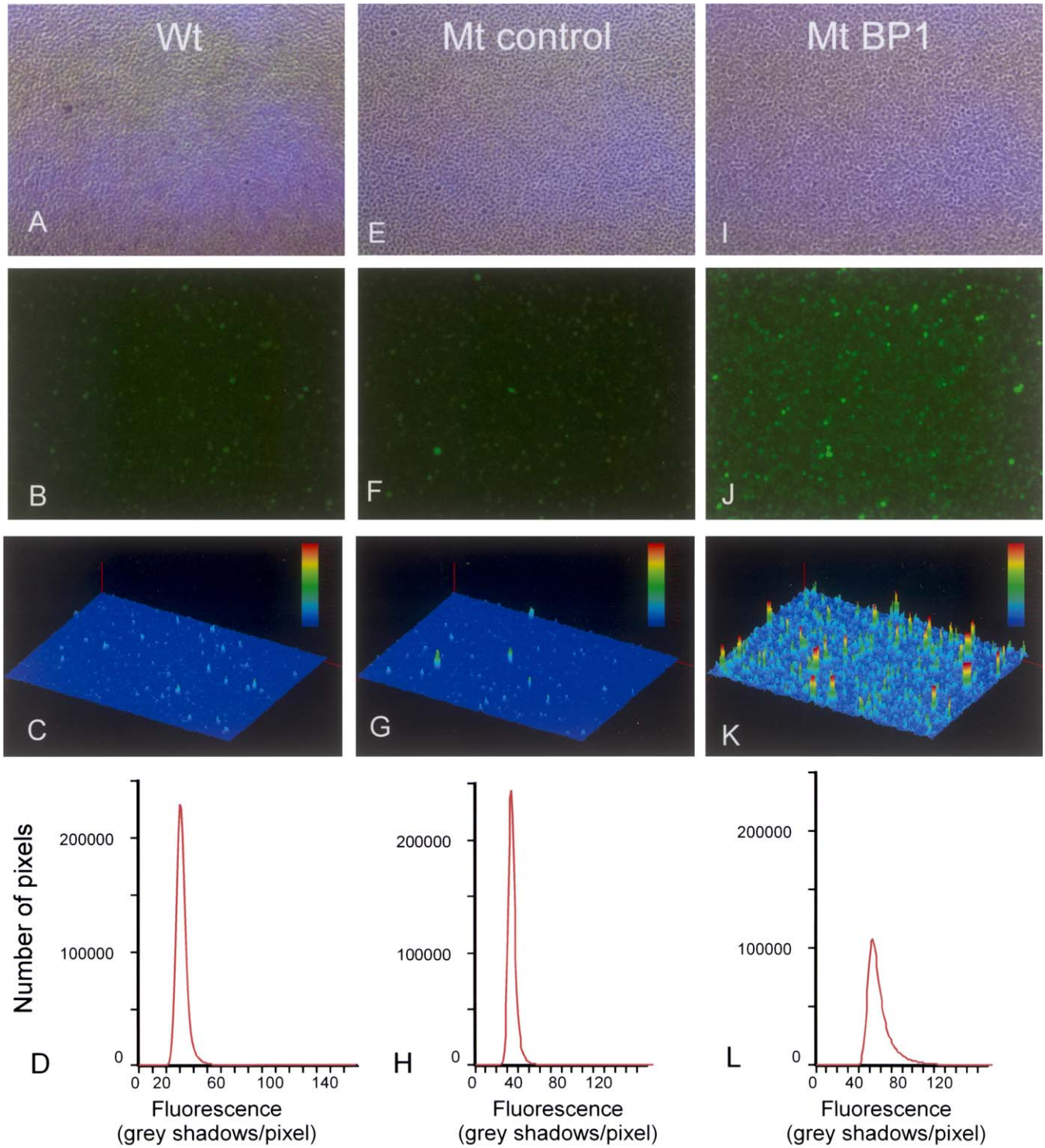


**Figure 2.** Digital fluorescence microscopy analysis of EGFP expression driven by the three β-globin promoters used in this study from one representative experiment. Quantitative fluorescence intensity analyses of digital images of K562 cells transfected with the wild-type construct **(A–D)**, with the mutant control construct **(E–H)**, and with the mutated BP1 binding site construct **(I–L)**. A, E, I: phase contrast images; B, F, J: fluorescent images; C, G, K: surface plots of fluorescence intensity in images shown in B, F, J, respectively. Note higher fluorescence intensity in cells transfected with MtBP1 construct (K) as compared with cells transfected with Wt (C) or Mt Control (G). D, H, L: fluorescence histograms of images shown in B, F, J, respectively. Note pixel intensity redistribution with increase in the number of pixel with higher mean fluorescence value displayed by the MtBP1 (L).

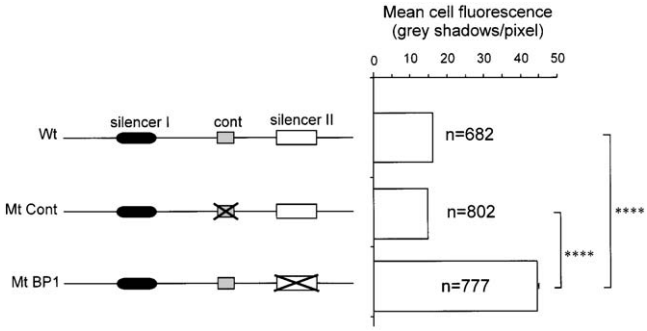


**Figure 3.** Statistical analysis of β-globin promoter activity data shown in Figure 2. The means and standard deviations of GFP-based fluorescence are shown. Statistical significance was determined by Student's *t*-test or ANOVA with post hoc comparisons of means using Bonferroni's *t*-procedure. ****, $p < 0.0001$. Four culture wells were imaged to make each measurement. The average number of measured cells per image is indicated beside each bar.

introduced into the cells with the Nucleofector device (Amaxa, Koeln, Germany).

*Efficacy of siRNA-mediated silencing*

To establish the efficacy of RNA interference in our experimental paradigm, we evaluated RNA interference in cells transfected with a siRNA directed against the GFP coding sequence. In these experiments we used an EGFP reporter plasmid (pEGFP-C1, Clontech, Palo Alto, CA, USA). The expression of EGFP in this plasmid is driven by a cytomegalovirus promoter. Two groups of K562 cells were cotransfected with EGFP plasmid along with nonspecific (nonsilencing) siRNA or with GFP-specific siRNA. The third group of cells (control) was transfected with plasmid alone, without siRNAs. Quantitative fluorescence intensity analysis of digital images detected only a minor (about 10%) decrease in EGFP expression 24 hours later in cells transfected with nonspecific siRNA, as compared with control cells (25.7 ± 0.8 grey shadows/pixel , n = 467 vs 22.9 ± 0.8 grey shadows/pixel, n = 443, $p < 0.05$). In contrast, cell transfection with EGFP-siRNA resulted in up to 70% decrease in mean cell fluorescence (25.7 ± 0.8 grey shadows/pixel, n = 467 vs 9.6 ± 0.3 grey shadows/pixel, n = 444, $p < 0.0001$). Altogether, our data indicated that using this method, EGFP siRNA was effectively inserted into the cells, where it exerted specific suppression of EGFP synthesis.

*siRNA-mediated BP1 silencing*

We selected a specific and effective siRNA targeted against BP1 by using RT-PCR. For this purpose, two groups of K562 cells were transfected with siRNAs targeting two different sequences from the BP1 coding region. We isolated total RNA from these cells 24 hours later. Total RNA was reverse transcribed into cDNA and amplified by PCR using BP1-specific primers. PCR products from five independent experiments were quantitated by digital image analysis. One siRNA (referred to as non-siRNA) did not change the BP1 mRNA level as compared with the controls used: cells transfected with nonspecific siRNA, and cells transfected with buffer alone (data not shown). In the cells transfected with the effective siRNA, BP1 expression as measured by integral fluorescence decreased from 75 to 90% as compared with the control (Fig. 4).

*Analysis the activities of β-globin promoters using EGFP reporter assays in combination with FACS*

We used flow cytometry to monitor the effect of siRNA-mediated BP1 suppression on the activity of wild-type and mutated β-globin promoters in EGFP reporter constructs. To perform reporter-gene assay experiments, we transfected siRNAs and EGFP constructs into three groups of K562 cells by electroporation. Each group of cells was cotransfected with one of three EGFP constructs (wild-type; mutant control; or mutant silencer II) together with one of three types of siRNAs: nonspecific siRNA; a BP1-specific but ineffective siRNA; or a siRNA effective against BP1. We analyzed GFP fluorescence by FACS 24 hours after transfection. Electroporation resulted in 70 to 75% of the cell population expressing GFP from the transfected reporter constructs.

When cells were cotransfected with nonspecific (control) siRNA or ineffective BP1 siRNA and one of the three β-EGFP-reporter constructs, GFP fluorescence did not change significantly from levels seen in the absence of any siRNA (data not shown). The differences in fluorescence intensity previously observed among cells transfected with the three

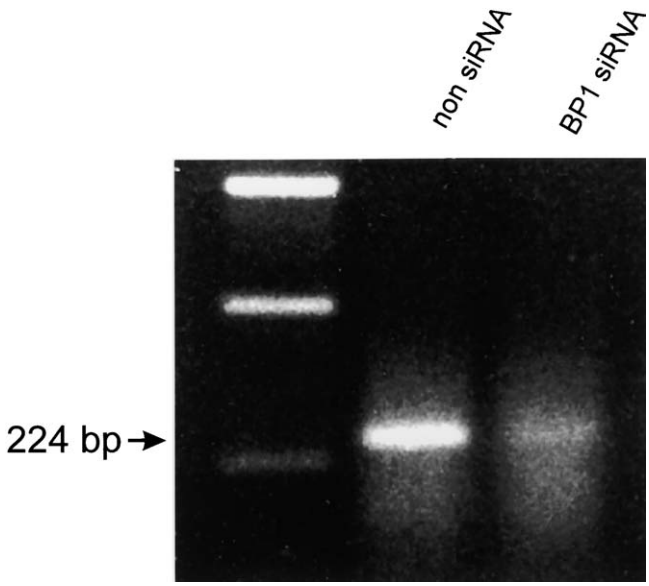


**Figure 4.** Selection of an effective siRNA directed against BP1, using RT-PCR. Conditions of RT-PCR reaction described in Materials and methods. BP1 PCR products (224 bp) from one representative experiment (we performed five independent experiments) were separated by 1% agarose gel electrophoresis along with molecular weight markers.

EGFP-reporter constructs themselves persisted in the presence of these inactive siRNAs. EGFP expression driven by the β-globin promoter with mutated BP1 binding site was up to 2.8-fold higher than that seen with wild-type or mutant control constructs (Fig. 5). Importantly, these data were comparable with the results of digital image analysis (Figs. 2 and 3).

Cotransfection of cells with an siRNA effective against BP1 and one of the three β-EGFP-reporter constructs increased the GFP fluorescence driven by each β-globin promoter construct. In the presence of a specific and effective BP1 siRNA, the wild-type β-globin promoter increased GFP fluorescence 40% over controls (Fig. 5A); the mutant control construct increased fluorescence by 45% (Fig. 5B); the construct with mutated silencer II BP1 binding site increased fluorescence by 75% (Fig. 5C).

*β-globin mRNA levels after suppression of BP1 by siRNA*

In our study, we used two different methods to analyze the levels of β-globin transcripts in K562 cells treated with siRNAs. Firstly, total RNA from three independent RNAi experiments was subjected to quantitative chip-based RT-PCR assays. We revealed significant changes in β-globin transcript synthesis 24 hours after cell transfection with BP1 siRNA (Table 1 and Fig. 6). Specifically, the mean levels of β-globin transcript in K562 cells were 37% higher than in controls. Analyses of RT-PCR products showed that BP1 suppression had no effect on the levels of expression of γ-globin, GATA-1, or EKLF (Fig. 6).

To further investigate changes in β-globin transcript synthesis in siRNA experiments we also used TaqMan PCR. As shown in Table 1 and Figure 7, β-globin mean transcript levels value (expressed in attomoles, $10^{-18}$ mol) was up to 40% higher in the cells transfected with BP1siRNA as compared with the control cells. Real-time PCR did not find significant changes in γ-globin mRNA levels in K562 cells transfected with BP1siRNA (data not shown).

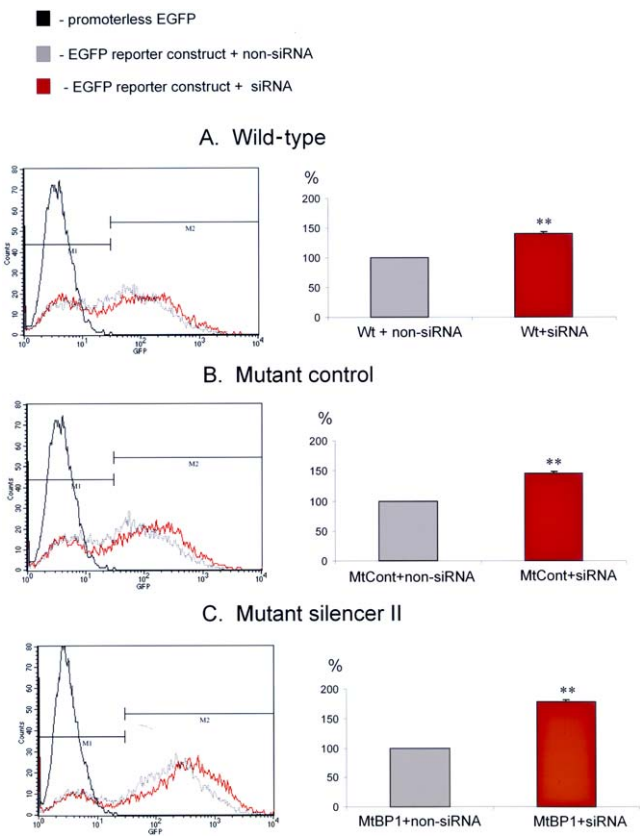


**Figure 5.** Analysis of β-globin promoter activity in K562 cells using EGFP reporter assays in combination with fluorescence-activated cell sorting (FACS). Positive GFP fluorescence (M2, left panels of Fig. 5) in cells was defined as fluorescence with intensity greater than that in 99% of control cells transfected with EGFP promoterless plasmid (M1, black line histogram). Data were expressed as mean fluorescence intensity (MFI) of the gated viable cells. Cotransfection of cells with a siRNA effective against BP1 together with one of the three β-EGFP-reporter constructs increased the GFP fluorescence driven by each β-globin promoter. As shown in **A** (left panel), in the presence of a specific and effective BP1 siRNA (red line histogram) the wild-type β-globin promoter increased GFP fluorescence over control (cotransfection experiments using ineffective siRNA against BP1, gray line histogram). The data show one representative experiment of 3 performed. We detected 40% increase of MFI of EGFP construct with wild-type β-globin promoter (A; right panel) when MFI values received for this construct in the experiments using effecive siRNA (red bar) were normalized to those in control (gray bar). **(B)**: FACS analysis data received after transfection of K562 cells with another EGFP construct containing β-globin promoter referred to as mutant control (Mt Cont). The right panel shows 45% increase of MFI of K562 cells cotransfected with this EGFP construct and effective siRNA (red bar) compare with control (gray bar). **(C)**: FACS analysis data received after transfection of K562 cells with EGFP construct containing a mutant BP1 binding site in β-globin promoter (Mt BP1). Right panel shows 75% increase of MFI of cells cotransfected with this EGFP construct and effective siRNA (red bar) compare with control (gray bar). $^{**}p < 0.001$.

## Discussion

The major finding of this study is that the inhibition of BP1 expression by siRNA-mediated knockdown enhances β-globin promoter activity and increases β-globin mRNA levels in K562 cells. Specifically, we have made three observations. First, mutating the DNA sequence of silencer II (in the MtBP1 construct, Fig. 1) significantly enhanced expression of the EGFP reporter gene (up to threefold). Multiple mutations in a control region near but not in silencer II (the MtCont construct, Fig. 1) did not change promoter activity. Second, inhibition of BP1 expression by a specific, effective siRNA resulted in 40% and 75% enhancement of the β-globin promoter activity of wild-type and mutant silencer II constructs, respectively. To our surprise, siRNA suppression of BP1 did not invoke the same degree of promoter activity as we detected for EGFP-reporter construct containing mutated sequence of silencer II. Levels of GATA-1 and EKLF expression were not affected by BP1 suppression.

Previous experiments from our and other laboratories have shown that BP1 represses the β-globin gene [20,22,23].

**Table 1.** Quantifications of β-globin transcripts in K562 cells transfected with BP1 siRNA using Agilent 2100 Bioanalyzer (DNA1000 LabChip) and TaqMan PCR

| siRNA | DNA1000 LabChip cDNA (ng/μL) mean ± SEM | TaqMan PCR amol globin mRNA/μg total RNA mean ± SEM |
|---|---|---|
| Control siRNA | 1.17 ± 0.03 (n = 3) | 56296 ± 1924 (n = 4) |
| BP1 siRNA | 1.60 ± 0.06 (n = 3)* | 78075 ± 4350 (n = 6)* |

*$p < 0.005$ as compared with cells transfected with control siRNA.

The BP1 protein binding site in both silencers has been determined by DNase I footprint analysis [18], and its consensus sequence, (A/T)T(A/C)(A/T)ATATG, has been deduced [21]. BP1 binds within silencer I at −530 bp and within silencer II at −300 bp relative to the cap site (+1). Since the sequence of silencer II does not interfere with the HMG binding sites (as does silencer I), we asked whether multiple mutations near but not in silencer II would enhance the activity of the β-globin promoter as silencer II mutations do. We introduced multiple mutations in the TCAAT (nucleotide coordinates −340 to −345) sequence of the β-globin promoter. The CAAT binding motif was chosen because it

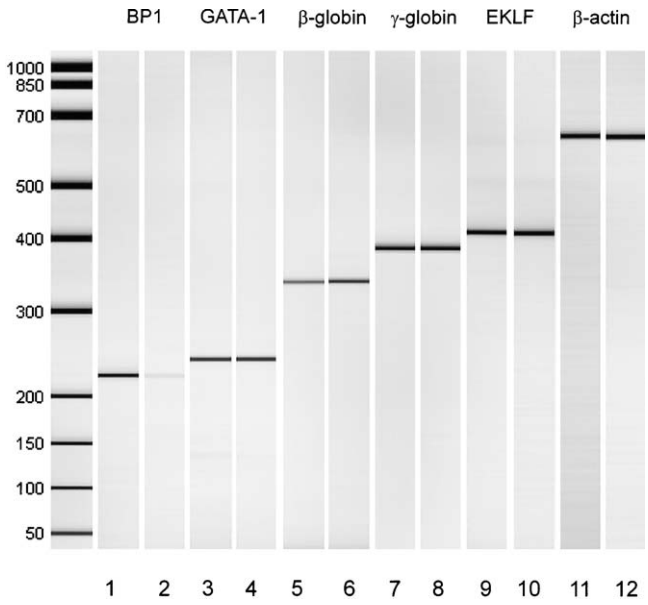


**Figure 6.** RT-PCR analysis of expression of BP1 (224 bp), GATA-1 (249 bp), β-globin (338 bp), γ-globin (397 bp), EKLF (410 bp) and β-actin (626 bp) in K562 cells after BP1 suppression by siRNA, determined with Agilent 2100 bioanalyzer software. Total RNA was isolated 24 hours after transfection of non-siRNA (control, odd numbers on the bottom of the image) or siRNA against BP1 (even numbers on the bottom) and subjected to RT-PCR assays as described in Materials and methods. The conditions for PCR were as follows: 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute; 19 cycles for GATA-1, γ-globin, and β-actin; 28 cycles for other genes.

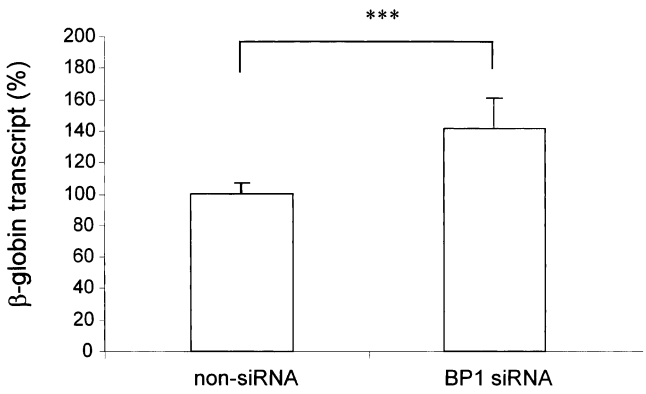


**Figure 7.** Real-time PCR analysis of β-globin mRNA levels in K562 cells after BP1 suppression by siRNA. Total RNA was isolated 24 hours after transfection of cells with non-siRNA (control) or siRNA against BP1 and subjected to quantitative real-time PCR assays of β-globin transcripts as described in Materials and methods. β-globin mean transcript level values (expressed in attomoles) from BP1siRNA-treated cells were normalized to those in control. Twenty-four hours after transfection of cells with siRNA effective against BP1, β-globin mean transcript levels were ~40% higher than in controls. ***$p < 0.005$.

is present in the binding sites of DLX4, Dlx2 and Dlx5 [26,27] as well as in the silencer II sequence [18]. Using EGFP-reporter assays quantitated by digital fluorescence microscopy, we found that the promoter activities of the wild-type and mutant-control constructs were similarly very low. However, digital image analysis showed a threefold increase in the mean fluorescence of K562 cells transfected with the construct mutated in the silencer II BP1 binding site. Thus, the silencer II BP1 binding sequence is required for repression of β-globin promoter activity.

We hypothesized that inhibition of BP1 synthesis may de-repress β-globin promoter activity. To test this hypothesis, we used RNA interference. It has been shown that introduction of dsRNA into an organism causes specific interference with gene expression [28,29]. This interference results from specific targeting of mRNA for degradation by incompletely characterized cellular machinery present in plant, invertebrate, and mammalian cells [30–32].

We used RT-PCR to identify a siRNA that was effective against BP1, then demonstrated the consequences of BP1 suppression by flow cytometry, RT-PCR, and real-time PCR. As predicted by our earlier studies [20,23], inhibition of BP1 expression de-repressed the β-globin promoter activity of EGFP-reporter constructs. Surprisingly, inhibition of BP1 mRNA in cells with an intact silencer II did not elicit the same degree of promoter activity as did mutations in the silencer II sequence. If BP1 is one of two transcript variants of the DLX4 gene, the other transcript variant may exert some inhibitory activity at silencer II when BP1 expression is suppressed.

BP1 is a member of the *Distal-less* homeobox gene family. *Distal-less* is the earliest known gene specifically

expressed in developing insect limbs; its expression is maintained throughout limb development. Many vertebrate genes have homeoboxes similar in sequence to that of the *Drosophila distal-less* gene [26]; the Distal-less (Dlx) family comprises at least six different members, DLX1–DLX6. The DLX proteins are postulated to play a role in forebrain and craniofacial development. Recent work implicates the Dlx genes of vertebrates in a variety of other developmental processes ranging from neurogenesis to hematopoiesis [26].

Several of the Dlx genes produce multiple transcripts, due either to alternative transcription initiation (e.g., Dlx1) [33] or to alternative splicing (e.g., Dlx5) [34]. Human DLX4 and two other isoforms of this gene, DLX7 and BP1, have been identified [21,22,27]. BP1 and DLX7 [35] share almost complete sequence identity from nucleotides 565 to 1251 of BP1 (including their homeoboxes), and they map to the same chromosomal region, 17q21-22 [22]. A consensus binding site for DLX7 has not yet been determined; the DLX7 protein does bind to silencer I DNA, although it lacks repressor function [22]. BP1 and DLX7 isoforms exhibit similar binding specificities in vitro because they share the same homeodomains, but they have different functions, possibly due to spliced transcripts encoding different activation or repression domains, or because of binding to different partner proteins [22].

DLX7 activates both GATA-1 and MYC in hematopoietic cells [36]. Ectopic DLX7 also can inhibit apoptosis by upregulating expression of intercellular adhesion molecule 1 [37]. However, it is not known whether DLX7 activates GATA-1, MYC, or intercellular adhesion molecule 1 directly.

Among the transcription factors known to act on globin genes, EKLF and GATA-1 have been suggested to preferentially activate β-globin transcription [10,38]. The EKLF-DNA binding site consensus sequence 5′-NCNCNCCCN-3′ corresponds to a functionally important motif within the adult β-globin gene promoter. Some patients with β-thalassemia have mutations of this sequence [39]. Targeted disruption of the EKLF gene in mice also results in lethal β-thalassemia [40]. EKLF knockout mice die of severe anemia at the fetal liver stage, due to failure of adult β-globin gene activation. $EKLF^{-/-}$ mice bearing a complete human β-globin locus transgene have reduced levels of β-globin but elevated levels of γ-globin expression, compared with wild-type mice bearing the same transgene [41,42]. Our RT-PCR experiments showed that EKLF is expressed in K562 cells, but no significant difference in EKLF expression was found between cells transfected with non-siRNA and siRNA against BP1.

GATA-1, a zinc finger transcription factor, plays a central role in erythroid development. It was first identified by its ability to bind functionally important DNA regulatory sequences found in globin genes [43,44]. Several proteins have been reported to interact physically with GATA-1, including EKLF/Sp1 [45], LMO-2 [46], FOG-1 [47], p300/CBP [48], and PU.1 [49–51]. We did not find any association between GATA-1 expression and BP1 inhibition in K562 cells.

The construct with a mutated BP1 site not only had increased β-globin promoter activity by itself, but showed still greater promoter activity in cells where BP1 expression was suppressed by RNA interference. It is possible that a decrease in cellular BP1 levels reduced BP1 binding to the intact silencer I (in addition to the mutation preventing binding to silencer II); thus, the promoter would lose two sources of negative regulation. The observed increase in promoter activity fits well with our previously described combinatorial model of adult β-globin gene repression by BP1 [20]. In this model, BP1 binding to both silencer regions induces HMG-mediated DNA bending, which brings together the BP1 molecules to form a stable silencing complex that impairs the binding of positive regulators such as EKLF and GATA-1. Our current results support this hypothesis and indicate that an inhibition of BP1 and an increase in endogenous β-globin is not directly associated with upregulation of EKLF or GATA-1.

Previous studies, e.g. [20,23], assessed effect of the mutated silencer II region in β-globin promoter by analyzing lysed cells. To our knowledge, the current study is the first one to analyze this effect in live cells. For this purpose we used combination of fluorescent digital imaging and flow cytometry. Using live cells in our experiments was important because we later introduced the β-globin promoter with mutated silencer II sequence into the β-globin locus of 35 kb cosmid construct. Then this construct was used to produce transgenic mice. Currently, we are analyzing the mechanism of regulation of β-globin expression by BP1 (mouse Dlx4) in vivo.

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## References

1. Tuan D, Solomon W, Li Q, London IM. The"β-like globin" gene domain in human erythroid cells. Proc Natl Acad Sci U S A. 1985;82:6384–6388.
2. Forrester WC, Takegawa S, Papayannopoulou T, Stamatoyannopoulos G, Groudine M. Evidence for a locus activation region: the formation of developmentally stable hypersensitive sites in globin-expressing hybrids. Nucleic Acids Res. 1987;15:10159–10177.
3. Grosveld F, van Assendelft GB, Greaves DR, Kollias G. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell. 1987;51:975–985.
4. Engel JD, Tanimoto K. Looping, linking, and chromatin activity: new insights into β-globin locus regulation. Cell. 2000;100:499–502.
5. Epner E, Reik A, Cimbora D, et al. The β-globin LCR is not necessary for an open chromatin structure or developmentally regulated transcription of the native mouse β-globin locus. Mol Cell. 1998;2:447–455.
6. Reik A, Telling A, Zitnik G, Cimbora D, Epner E, Groudine M. The locus control region is necessary for gene expression in the human β-globin locus but not the maintenance of an open chromatin structure in erythroid cells. Mol Cell Biol. 1998;18:5992–6000.

7. Miller IJ, Bieker JJ. A novel, erythroid cell–specific murine transcription factor that binds to the CACCC element and is related to the Kruppel family of nuclear proteins. Mol Cell Biol. 1993;13:2776–2786.
8. Amrolia PJ, Cunningham JM, Ney P, Nienhuis AW, Jane SM. Identification of two novel regulatory elements within the 5′-untranslated region of the human A γ-globin gene. J Biol Chem. 1995;270:12892–12898.
9. Bottardi S, Santoro C. CTF/NF-1 binds the stage selector element of the human γ-globin gene promoter. Biochem Biophys Res Commun. 1995;215:874–880.
10. Weiss MJ, Orkin SH. GATA transcription factors: key regulators of hematopoiesis. Exp Hematol. 1995;23:99–107.
11. Asano H, Stamatoyannopoulos G. Activation of β-globin promoter by erythroid Kruppel-like factor. Mol Cell Biol. 1998;18:102–109.
12. Li Q, Peterson KR, Stamatoyannopoulos G. Developmental control of ε- and γ-globin genes. Ann N Y Acad Sci. 1998;850:10–17.
13. Li Q, Peterson KR, Fang X, Stamatoyannopoulos G. Locus control regions. Blood. 2002;100:3077–3086.
14. Gumucio DL, Heilstedt-Williamson H, Gray TA, et al. Phylogenetic footprinting reveals a nuclear protein which binds to silencer sequences in the human γ and ε globin genes. Mol Cell Biol. 1992;12:4919–4929.
15. Hardison R, Chao KM, Adamkiewicz M, et al. Positive and negative regulatory elements of the rabbit embryonic ε-globin gene revealed by an improved multiple alignment program and functional analysis. DNA Seq. 1993;4:163–176.
16. Raich N, Clegg CH, Grofti J, Romeo PH, Stamatoyannopoulos G. GATA1 and YY1 are developmental repressors of the human ε-globin gene. EMBO J. 1995;14:801–809.
17. Li J, Noguchi CT, Miller W, Hardison R, Schechter AN. Multiple regulatory elements in the 5′-flanking sequence of the human ε-globin gene. J Biol Chem. 1998;273:10202–10209.
18. Berg PE, Williams DM, Qian RL, et al. A common protein binds to two silencers 5′ to the human β-globin gene. Nucleic Acids Res. 1989;17:8833–8852.
19. Chase MB, Haga SB, Hankins WD, et al. Binding of HMG-I(Y) elicits structural changes in a silencer of the human β-globin gene. Am J Hematol. 1999;60:27–35.
20. Drew L, Tang DC, Berg PE, Rodgers GP. The role of trans-acting factors and DNA-bending in the silencing of human β-globin gene expression. Nucleic Acids Res. 2000;28:2823–2830.
21. Chase MB, Fu S, Haga SB, et al. BP1, a homeodomain-containing isoform of DLX4, represses the β-globin gene. Mol Cell Biol. 2002;22:2505–2514.
22. Fu S, Stevenson H, Strovel JW, et al. Distinct functions of two isoforms of a homeobox gene, BP1 and DLX7, in the regulation of the β-globin gene. Gene. 2001;278:131–139.
23. Ebb D, Tang DC, Drew L, Chin K, Berg PE, Rodgers GP. Identification of regulatory elements that repress adult β-like globin genes. Blood Cells Mol Dis. 1998;24:356–369.
24. Sarkar G, Sommer SS. The "megaprimer" method of site-directed mutagenesis. Biotechniques. 1990;8:404–407.
25. Smith RD, Li J, Noguchi CT, Schechter AN. Quantitative PCR analysis of HbF inducers in primary human adult erythroid cells. Blood. 2000;95:863–869.
26. Panganiban G, Rubenstein JLR. Developmental functions of the *Distal-less*/Dlx homeobox genes. Development. 2002;129:4371–4386.
27. Quinn LM, Johnson BV, Nicholl J, Sutherland GR, Kalionis B. Isolation and identification of homeobox genes from the human placenta including a novel member of Distal-less family, DLX4. Gene. 1997;187:55–61.
28. Fire A. RNA-triggered gene silencing. Trends Genet. 1999;15:358–363.
29. Hannon GJ. RNA interference. Nature. 2002;418:244–251.
30. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila*-cells. Nature. 2000;404:293–296.
31. Lee NS, Dohjima T, Bauer G, et al. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nat Biotechnol. 2002;20:500–505.
32. McManus MT, Haines BB, Dillon CP, et al. Small interfering RNA-mediated gene silencing in T lymphocytes. J Immunol. 2002;169:5754–5760.
33. McGuinness T, Porteus MH, Smiga S, et al. Sequence, organization, and transcription of the Dlx-1 and Dlx-2 locus. Genomics. 1996;35:473–485.
34. Liu JK, Ghattas I, Liu S, Chen S, Rubenstein JL. Dlx genes encode DNA-binding proteins that are expressed in an overlapping and sequential pattern during basal ganglia differentiation. Dev Dyn. 1997;210:498–512.
35. Nakamura S, Stock DW, Wydner KL, et al. Genomic analysis of a new mammalian Distal-less gene: Dlx-7. Genomics. 1996;38:314–324.
36. Shimamoto T, Nakamura S, Bollekens J, Ruddle FH, Takeshita K. Inhibition of DLX-7 homeobox gene causes decreased expression of GATA-1 and c-myc genes and apoptosis. Proc Natl Acad Sci U S A. 1997;94:3245–3249.
37. Shimamoto T, Ohyashiki K, Takeshita K. Overexpression of the homeobox gene DLX-7 inhibits apoptosis by induced expression of intercellular adhesion molecule-1. Exp Hematol. 2000;28:433–441.
38. Donze D, Townes TM, Bieker JJ. Role of erythroid Kruppel-like factor in human γ- to β-globin gene switching. J Biol Chem. 1995;270:1955–1959.
39. Orkin SH, Kazazian HH Jr, Antonarakis SE, et al. Linkage of β-thalassaemia mutations and β-globin gene polymorphisms with DNA polymorphisms in human β-globin gene cluster. Nature. 1982;296:627–631.
40. Perkins AC, Sharpe AH, Orkin SH. Lethal β-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. Nature. 1995;375:318–322.
41. Perkins AC, Gaensler KM, Orkin SH. Silencing of human fetal globin expression is impaired in the absence of the adult β-globin gene activator protein EKLF. Proc Natl Acad Sci U S A. 1996;93:12267–12271.
42. Wijgerde M, Gribnau J, Trimborn T, et al. The role of EKLF in human β-globin gene competition. Genes Dev. 1996;10:2894–2902.
43. Evans T, Felsenfeld G. The erythroid-specific transcription factor Eryf1: a new finger protein. Cell. 1989;58:877–885.
44. Tsai SF, Marting DI, Zon LI, D'andrea AD, Wong GG, Orkin SH. Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature. 1989;339:446–451.
45. Merika M, Orkin SH. Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Kruppel family proteins Sp1 and EKLF. Mol Cell Biol. 1995;15:2437–2447.
46. Osada H, Grutz G, Axelson H, Forster A, Rabbitts TH. Association of erythroid transcription factors: complexes involving the LIM protein RBTN2 and the zinc-finger protein GATA1. Proc Natl Acad Sci U S A. 1995;92:9585–9589.
47. Tsang AP, Visvader JE, Turner CA, et al. FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. Cell. 1997;90:109–119.
48. Blobel GA, Nakajima T, Eckner R, Montminy M, Orkin SH. CREB-binding protein cooperates with transcription factor GATA-1 and is required for erythroid differentiation. Proc Natl Acad Sci U S A. 1998;95:2061–2066.
49. Nerlov C, Querfurth E, Kulessa H, Graf T. GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. Blood. 2000;95:2543–2551.
50. Rekhtman N, Radparvar F, Evans T, Skoultchi AI. Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. Genes Dev. 1999;13:1398–1411.
51. Zhang P, Behre G, Pan J, et al. Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. Proc Natl Acad Sci U S A. 1999;96:8705–8710.