Generation of a genomic reporter assay system for analysis of γ- and β-globin gene regulation

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ABSTRACT **A greater understanding of the regulatory mechanisms that govern γ-globin expression in humans, especially the switching from γ- to β-globin, which occurs after birth, would help to identify new therapeutic targets for patients with β-hemoglobinopathy. To further elucidate the mechanisms involved in γ-globin expression, a novel fluorescent-based cellular reporter assay system was developed. Using homologous recombination, two reporter genes, DsRed and EGFP, were inserted into a 183-kb intact human β-globin locus under the control of ^{G}γ- or ^{A}γ-globin promoter and β-globin promoter, respectively. The modified constructs were stably transfected into adult murine erythroleukaemic (MEL) cells and human embryonic or fetal erythroleukemic (K562) cells, allowing for rapid and simultaneous analysis of fetal and adult globin gene expression according to their developmental stage-specific expression. To demonstrate the utility of this system, we performed RNA interference (RNAi)-mediated knockdown of BCL11A in the presence or absence of known fetal hemoglobin inducers and demonstrated functional derepression of a γ-globin-linked reporter in an adult erythroid environment. Our results demonstrate that the cellular assay system represents a promising approach to perform genetic and functional genomic studies to identify and evaluate key factors associated with γ-globin gene suppression.—Chan, K. S. K., Xu, J., Wardan, H., McColl, B., Orkin, S., Vadolas, J. Generation of a genomic reporter assay system for analysis of γ- and β-globin gene regulation. *FASEB J.* 26, 1736–1744 (2012). www.fasebj.org**

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β-Thalassemia and sickle cell disease (SCD) are the commonest single-gene disorders worldwide. The clinical presentations of these disorders appear after birth when the fetal γ-globin gene is progressively silenced and replaced by the defective adult β-globin gene. Currently, the available treatment options are limited to regular blood transfusions for life, which is associated with iron toxicity, requiring patients to undergo intense chelation therapy to minimize iron-related complications.

At present, hydroxyurea (HU) remains the only agent approved for treatment of SCD. It can ameliorate the clinical symptoms and dramatically reduce the frequency of pain crisis. In addition, recent clinical trials have identified HU to be beneficial in some patients with β-thalassemia by increasing fetal hemoglobin (HbF) and reducing the frequency of blood transfusion (1). Mechanistically, the direct effect of HU on HbF induction is likely mediated through the nitric oxide-dependent activation of soluble guanylyl cyclase (2, 3). Other agents, such as 5-azacytidine (5-aza) and 5-aza-2′-deoxycytidine (decitabine) have also been demonstrated to increase HbF by inhibiting DNA methyltransferase (4, 5). Moreover, a separate group of compounds under evaluation are short-chain fatty acids (SCFAs), such as butyrate and its derivatives (6). Butyrate has been shown to inhibit histone deacetylase, thus affecting nucleosome stability and chromatin accessibility. These studies have led to the appreciation of the role of epigenetic modifications, such as DNA methylation and histone acetylation, in globin gene expression and regulation (7). As a result, many investigations have focused on the pharmacological induction of HbF using epigenetic-specific agents (8–10).

Abbreviations: BAC, bacterial artificial chromosome; BCL11A, B-cell lymphoma/leukemia 11A; decitabine, 5-azacytidine-2′-deoxycytidine; DMSO, dimethyl sulfoxide; DRED, direct repeat erythroid-definitive; DsRed, red fluorescent protein; EGFP, enhanced green fluorescent protein; KLF1, Krüpple-like factor 1; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FOP, friend of PRMT1; Hb, hemoglobin; HbF, fetal hemoglobin; HDAC, histone deacetylase; HU, hydroxyurea; LCR, locus control region; MEL, murine erythroleukemia; MPF, median peak fluorescence; mRNA, messenger ribonucleic acid; RNAi, RNA interference; SCD, sickle cell disease; SCFA, short-chain fatty acid; sh1, short hairpin RNA 1; sh2, short hairpin RNA 2; shRNA, short hairpin RNA; YAC, yeast artificial chromosome.

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Much of what has been learned over the past 3 decades about the transcriptional regulation network of the globin genes is ultimately relevant to γ-globin switching. However, a better understanding of the molecular mechanism responsible for γ-globin silencing could pave the way for the development of targeted therapeutic strategies for patients with β-hemoglobinopathy that could be used in combination with other potential therapies (11). Recent insight into γ-globin switching has come from the results of genome-wide association studies. These led to the identification of critical transacting factors, such as B-cell lymphoma/leukemia 11A (BCL11A; refs. 12–14) and Krüpple-like factor 1 (KLF1; ref. 15), as well as the region between the HBS1 gene *HBS1L* and the oncogene *MYB* (16, 17). In addition, several other factors have been identified as having important roles in γ-globin gene expression, including SOX6 (12), TR2/TR4 direct repeat erythroid-definitive (DRED) complex (18), COUP-TFII (19), friend of PRMT1 (FOP; ref. 20), and NF-E4 (21). The search for a single switching mechanism has given way to the realization that γ-globin switching may involve many factors, including several with undefined roles.

To further elucidate the genetic modulation of HbF and evaluate potential therapeutic targets, we have focused on the creation of cellular assay systems that recapitulate the expression of globin genes according to their developmental stage-specific expression patterns. Previously, we reported the generation of a human erythroleukemic cell line (K562) stably transfected with bacterial artificial chromosome (BAC) carrying the entire human β-globin locus, modified to express the enhanced green fluorescent protein (EGFP) under the control of ^{G}γ or ^{A}γ-globin promoter (22, 23). Since K562 is embryonic and fetal in origin, its utility is restricted to the evaluation of erythroid differentiation, hemoglobinization, and γ-globin gene expression.

In this study, we extended our investigations to create the next-generation cellular assay system suitable for evaluating γ-globin gene silencing. We report the construction and characterization of adult murine erythroleukemic (MEL) cells stably transfected with the entire human β-globin locus, modified to express red fluorescent protein (DsRed) and EGFP under the endogenous ^{G}γ/^{A}γ-globin and β-globin promoters, respectively. To assess the utility of this system, we performed RNA interference (RNAi)-mediated knockdown of BCL11A in the presence or absence of known fetal hemoglobin inducers. Our results demonstrate derepression of the γ-globin linked reporter in an adult erythroid environment following BCL11A depletion. Thus, this dual-reporter cellular assay represents a unique system that can be used to identify key regulators of γ-globin silencing.

MATERIALS AND METHODS

Preparation of genomic dual-reporter constructs

The generation of the genomic dual-reporter constructs, pEBAC^{A}γRedβEGFP and pEBAC^{G}γRedβEGFP, was previously described (22). These constructs contained a 183-kb genomic fragment encompassing the entire β-globin locus, with either the ^{G}γ- or ^{A}γ-globin gene replaced by DsRed and the β-globin gene replaced by EGFP. These constructs were propagated in the *Escherichia coli* strain DH10B (Invitrogen, Chadsworth, CA, USA) cultured in Luria broth supplemented with 12.5 μg/ml chloramphenicol.

Cell culture

The MEL cell lines were maintained in continuous culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine. Cells were incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2.

Establishment of the MEL fluorescent reporter cell lines

To generate the stable cell lines, pEBAC^{G}γRedβEGFP and pEBAC^{A}γRedβEGFP were purified using CsCl-ethidium bromide density gradient centrifugation and linearized with restriction endonuclease *Srf*I (Roche, Mannheim, Germany), which recognizes a site at the extreme 3′ end of the β-globin locus. MEL cells (2×10^7 cells/ml) were washed with OptiMEM (Invitrogen) and electroporated with 10 μg of linearized BAC DNA under the following conditions: 280 V, 1000 μF, ∞ Ω. The transfected cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Due to the large size of the dual-reporter constructs, transfection efficiencies were <1% based on the percentage of EGFP-expressing cells, as assessed by flow cytometry. At 2 d post-transfection, individual EGFP-positive cells were isolated by fluorescence-activated cell sorting (FACS) using a MoFlo cell sorter (Beckman Coulter, Fullerton, CA, USA). Selected cells were clonally expanded in DMEM culture medium at 37°C in a humidified incubator supplemented with 5% CO_2. Clones were maintained in continuous culture for 8 mo, and their expression profile was monitored by flow cytometry.

Analysis of DsRed and EGFP expression

The DsRed and EGFP expression of the stably transfected clones was monitored by flow cytometry using a LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The percentage of cells positively expressing the reporter genes and their level of expression were analyzed using BD FACSDiva software (Becton Dickinson).

Treatment of cells with inducers of globin gene expression

The responsiveness of the dual-reporter assay to previously described HbF inducers was tested using butyrate, decitabine, and HU. In brief, butyrate (0–10,000 μM), decitabine (0–10 μM), and HU (0–1000 μM) were added to 2×10^5 MEL^{A}γRedβEGFP or MEL^{G}γRedβEGFP reporter cells in 6-well plates. Cells were incubated for 7 d in the presence of 2% dimethyl sulfoxide (DMSO) prior to flow cytometric analysis. The increase in median fluorescent index in response to various inducers was then determined by flow cytometric analysis, while relative messenger RNA (mRNA) levels were measured by real-time quantitative RT-PCR (qRT-PCR).

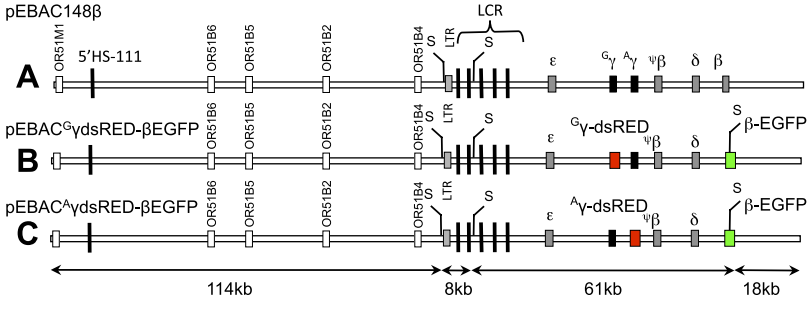

Figure 1. Schematic diagram of the dual-reporter-modified β-globin locus used in this study. *A*) Diagram of the unmodified β-globin locus. *B*) Diagram of the β-globin locus containing the EGFP-modified β-globin gene and the DsRed-modified ^{G}γ-globin gene. *C*) Diagram of the β-globin locus containing the EGFP-modified β-globin gene and the DsRed-modified ^{A}γ-globin gene. Locations of *Sfi* I sites (S) are highlighted; expected sizes of fragments are indicated by arrows.

Southern blot analysis

The integrity of the human β-globin BAC in MEL cells was examined by Southern blot analysis following digestion of chromosomal DNA with the restriction endonuclease *Sfi* I. To avoid shearing of genomic DNA, cells were suspended at a concentration of 5×10^6 cells/ml in 0.8% InCert agarose (FMC BioProducts, Rockland, ME, USA). The agarose plugs were incubated overnight in proteinase K buffer (100 mM EDTA at pH 8.0, 0.2% sodium deoxycholate, and 1% sodium lauryl sarcosine with 0.4 mg/ml of proteinase K) at 50°C. The plugs were then washed 3 times with 2 mM phenylmethylsulfonylfluoride and stored in Tris-EDTA (TE) buffer at 4°C. The DNA plugs were digested overnight with *Sfi* I and loaded into the wells of a 0.8% agarose gel. The genomic DNA was then separated in a CHEF-DRII pulsed-field gel electrophoresis apparatus (Bio-Rad, Richmond, CA, USA). The gel was subsequently incubated in depurination buffer (0.25 M HCl) for 10 min, followed by 30 min incubation in denaturation buffer (1.5 M NaCl and 0.5 M NaOH). After rinsing in dH_2O, the gel was incubated in neutralization buffer (0.5 M Tris and 1.5 M NaCl) for 30 min. The DNA was then blotted onto a HybondN+ membrane (Amersham, Piscataway, NJ, USA) by capillary transfer in 20× SSC. A 598-bp probe specific for the EGFP gene was generated by PCR (using primers 5′-CTACCGGACTCAGATGATCCA-3′ and 5′-TACTTGTACAGCTCGTCCATG-3′) and labeled with ^{32}P-labeled using the Radprime DNA labeling system (Invitrogen) according to the manufacturer's recommendations. Hybridization was performed at 65°C overnight in Church buffer (0.5 M Na_2HPO_4, 1 mM EDTA, 7% (w/v) SDS, and 1% bovine serum albumin) using ^{32}P-labeled probes at a concentration of 3×10^6 cpm/ml.

RNA isolation and RT–PCR

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad). Real-time quantitative RT-PCR was performed using the iQ SYBR Green Supermix (Bio-Rad). Relative expression was quantified using the $\Delta\Delta C_t$ method as described previously (12). Sequences of primers used in this study are listed in Supplemental Table S1.

Lentiviral RNAi

Lentiviral short hairpin RNA (shRNA) constructs in the pLKO vector were obtained from a large collection of shRNAs, as described previously (13, 24). shRNA1 (sh1) targeting mouse BCL11A gene contains the sequence CCGGGCATAGACGATGGCACTGTTACTCGAGTAACAGTGCCATCGTCTATGCTTTTTG, and shRNA2 (sh2) targeting mouse BCL11A gene contains the sequence CCGGCCAGAGGATGACGATTGTTTACTCGAGTAAACAATCGTCATCCTCTGGTTTTTG. shRNA against GFP was used to produce control lentiviruses. Lentivirus preparation and transduction of cells were carried out as described previously (24). The cells were washed 3 times with PBS and seeded in fresh medium containing puromycine (1 μg/ml) at 24 h post-transduction. At 7 to 10 d following selection, cells were seeded into differentiation medium containing 2% DMSO. DsRed and EGFP expressing cells were analyzed by flow cytometry.

Western blot method antibodies and reagents

Expression of BCL11A was assessed using antibody 15E3AC11 (Abcam Inc., Cambridge, MA, USA), as described previously (12). β-Actin (MAB1501R; Millipore, Billerica, MA, USA) was analyzed as a loading control.

RESULTS

Generation of dual-reporter cell lines

The previously described BAC clone pEBAC148βG contains the entire human β-globin locus as a 183-kb genomic insert and the EGFP reporter gene in place of the β-globin gene (23). Using homologous recombination in DY380 bacterial cells (25), we replaced the ^{G}γ-globin or ^{A}γ-globin genes with the DsRed reporter gene to generate two dual-reporter BAC clones, pEBAC^{G}γRedβEGFP and pEBAC^{A}γRedβEGFP (**Fig. 1**). The desired clones were confirmed by sequencing and fine-resolution mapping using *Eco*RI restriction enzyme digestion (**Fig. 2**). As predicted from sequence analysis, the ^{G}γ-globin modification in the pEBAC^{G}γRedβEGFP construct removed a 1581-bp fragment, whereas modification of ^{A}γ-globin in pEBAC^{A}γRedβEGFP removed a 2642-bp fragment. Both constructs contain an extra 2239-bp fragment for the EGFP reporter gene (Fig. 2*A*). In addition, the banding patterns of the *Eco*RI digestion confirmed that the two dual-reporter constructs contained the correct modifications in the absence of any rearrangements (Fig. 2*B*).

To functionally evaluate the dual-reporter BACs, MEL cells containing the dual-reporter BAC transgene pEBAC^{A}γRedβEGFP or pEBAC^{G}γRedβEGFP were created by stable transfection. MEL cells are a well-characterized adult erythroleukemic cell line derived from mice infected with Friend virus. They represent erythroid precursors that can be induced to express adult globins (26). To select for stable transfected cells, flow cytometric single-cell sorting of

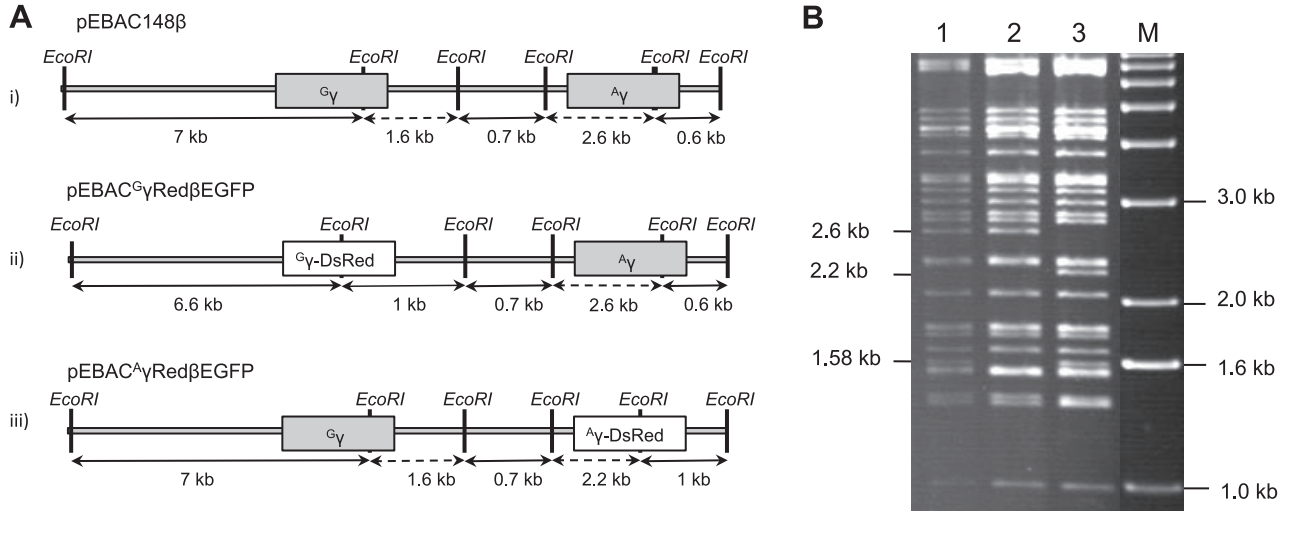

Figure 2. Analysis of dual-reporter β-globin BACs following *Eco*RI digestion. *A*) Schematic representation showing the locations of the *Eco*RI recognition sites neighboring γ-globin genes in pEBAC148β (*i*), pEBAC^{G}γRedβEGFP (*ii*),and pEBAC^{A}γRedβEGFP (*iii*). Arrows indicate fragments used to differentiate the constructs. *B*) *Eco*RI restriction enzyme digested modified β-globin BACs were separated on 0.7% Agarose MP gel at 70 V overnight. Lane 1, pEBAC148βG; lane 2, pEBAC^{G}γRedβEGFP; lane 3, pEBAC^{A}γRedβEGFP; lane 4, molecular marker (X).

EGFP-positive clones was performed at 2 d post-transfection. A total of 96 individual cells was collected for each MEL dual-reporter line, and 24 selected clones were expanded for further analysis. The expression of EGFP driven by the β-globin promoter was similar between the two dual reporters for most of the clones (**Fig. 3**). However, the expression of DsRed driven by either ^{G}γ- or ^{A}γ-globin promoter was undetectable. Based on the percentage of EGFP-positive cells and median peak fluorescence (MPF), one clone from each of the constructs was chosen (clone 22 from pEBAC^{G}γRedβEGFP and clone 7 from pEBAC^{A}γRedβEGFP). More than 90% of the cells in these clones expressed relatively high levels of EGFP. The stability of expression was assessed by measuring MPF over a period of 8 mo in the

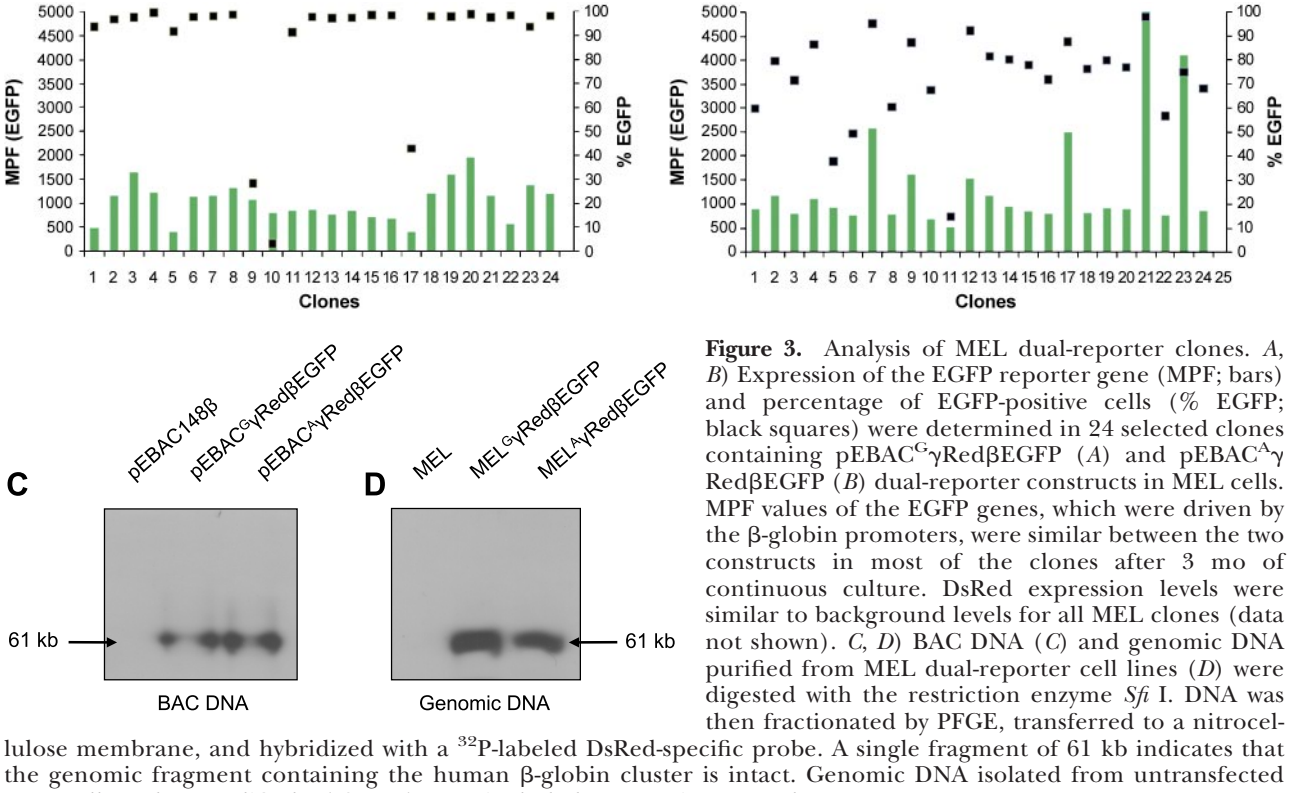

Figure 3. Analysis of MEL dual-reporter clones. *A*, *B*) Expression of the EGFP reporter gene (MPF; bars) and percentage of EGFP-positive cells (% EGFP; black squares) were determined in 24 selected clones containing pEBAC^{G}γRedβEGFP (*A*) and pEBAC^{A}γ RedβEGFP (*B*) dual-reporter constructs in MEL cells. MPF values of the EGFP genes, which were driven by the β-globin promoters, were similar between the two constructs in most of the clones after 3 mo of continuous culture. DsRed expression levels were similar to background levels for all MEL clones (data not shown). *C*, *D*) BAC DNA (*C*) and genomic DNA purified from MEL dual-reporter cell lines (*D*) were digested with the restriction enzyme *Sfi* I. DNA was then fractionated by PFGE, transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labeled DsRed-specific probe. A single fragment of 61 kb indicates that the genomic fragment containing the human β-globin cluster is intact. Genomic DNA isolated from untransfected MEL cells and unmodified BAC DNA were included as negative controls.

absence of antibiotic selection. Both reporter cell lines displayed uniform EGFP expression and stable levels of EGFP-positive cells during the analysis period (data not shown). Based on their stability and reproducibility in EGFP expression, clones 7 and 22 were selected for further analysis. They were designated MEL^{G}γRedβEGFP and MEL^{A}γRedβEGFP, respectively (Fig. 3).

Characterization of BAC transgene

The integrity of the transgenes in the MEL dual-reporter cell lines was investigated by Southern blot analysis. BAC DNA (pEBAC148β, pEBAC^{G}γRedβEGFP, and pEBAC^{A}γRedβEGFP), as well as genomic DNA isolated from the two MEL dual-reporter cell lines and untransfected MEL cells, was digested with restriction endonuclease *Sfi* I and probed with a PCR-amplified DsRed-specific probe. A single band was observed for the dual-reporter MEL cell lines, corresponding to the 61-kb DNA fragment that was also present in BAC DNA digested with *Sfi* I. This analysis suggested that the DNA sequence between the locus control region (LCR) and the β-globin gene was intact in the stably transfected MEL^{G}γRedβEGFP and MEL^{A}γRedβEGFP dual-reporter cell lines (Fig. 3).

In addition, the MEL cell lines containing the dual-reporter human β-globin locus were further characterized by PCR analysis. Primer pairs corresponding to the 5′-end, HS111, LTR-5′, LTR-3′, and 5′HS5 regions of the human β-globin locus were used to confirm the presence of human β-globin upstream elements (Supplemental Table S2). Both MEL^{A}γRedβEGFP and MEL^{G}γRedβEGFP reporter cell lines were positive for all PCR products, which suggests that this region remained intact (Supplemental Fig. S1).

Reporter gene expression

To assess developmental stage-specific expression of the γ-globin-linked DsRed reporter gene, the two dual-reporter BAC constructs were introduced into the human erythroid leukemic K562 cell line. The K562 cell line was derived from a patient with chronic myeloid leukemia in acute phase and predominantly expresses embryonic/fetal hemoglobins [Hb Gower 1 ($\zeta_2\varepsilon_2$), Hb Portland ($\zeta_2\gamma_2$), and Hb Gower 2 ($\alpha_2\varepsilon_2$); ref. 27]. Following stable transfection of K562 cells with the two dual-reporter constructs, the majority of the isolated clones expressed DsRed, whereas a small fraction of the total population expressed EGFP (**Fig. 4**). Notably, the differences in the level of reporter gene expression between MEL and K562 cells demonstrated that the cellular reporter assay systems contained the necessary elements for developmental-stage and tissue-specific expression of the reporter genes.

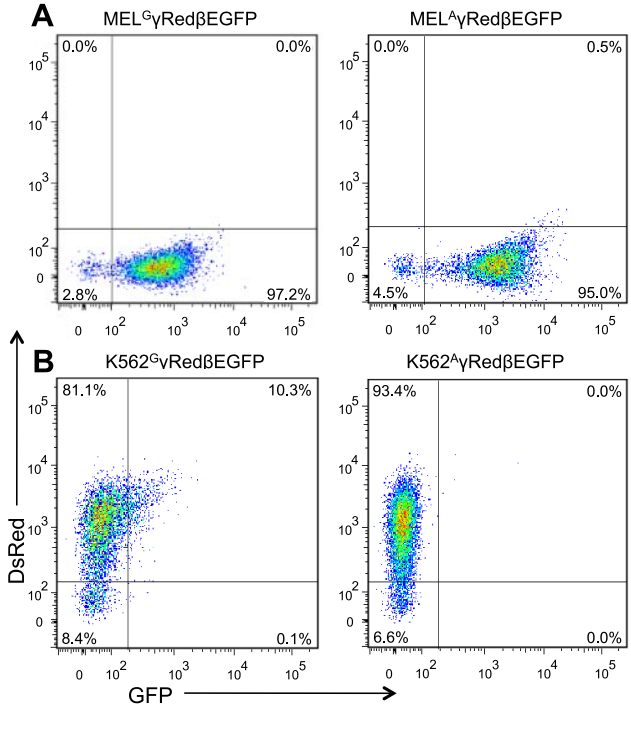

Figure 4. Expression of DsRed and EGFP in the dual-reporter cells. *A*) MEL cells stably transfected with pEBAC^{G}γRedβEGFP and pEBAC^{A}γRedβEGFP. *B*) K562 cells stably transfected with pEBAC^{G}γRedβEGFP and pEBAC^{A}γRedβEGFP. Transfected cells were maintained in continuous culture without antibiotic selection for >240 d. Percentage of DsRed- or EGFP-expressing cells remained stable during the time course. Flow cytometric profiles of stably transfected cells were set using untransfected MEL or K562 cells transfected with single-reporter plasmids expressing either EGFP or DsRed.

Dual-reporter assay system as a tool for studying γ-globin silencing

With recent genetic and functional studies revealing BCL11A as a critical mediator of γ-globin silencing in humans and transgenic mice carrying the human β-globin locus (12, 13, 28), we asked whether the dual-reporter assay system could be used to assess γ-globin gene silencing. In MEL cells, the endogenous mouse adult β-globin genes ($\beta^{major}/\beta^{minor}$-globins) were predominately expressed following erythroid differentiation. The embryonic εy-globin and βh1-globin genes were expressed at very low levels, which paralleled the expression of human fetal (γ-) globin gene and γ-globin-linked DsRed reporter gene (**Figs. 5*A*** and **6*B***). Notably, the very low level expression of γ-globin genes in MEL reporter cells mirrored the level of γ-globin expression in yeast artificial chromosome (YAC) or BAC transgenic mice carrying the native human β-globin locus (29, 30). Therefore, in MEL dual-reporter cells, the γ-globin gene and γ-linked DsRed reporter gene are regulated in a developmental stage-specific manner.

We next examined whether depletion of BCL11A affected expression of reporter genes controlled by the

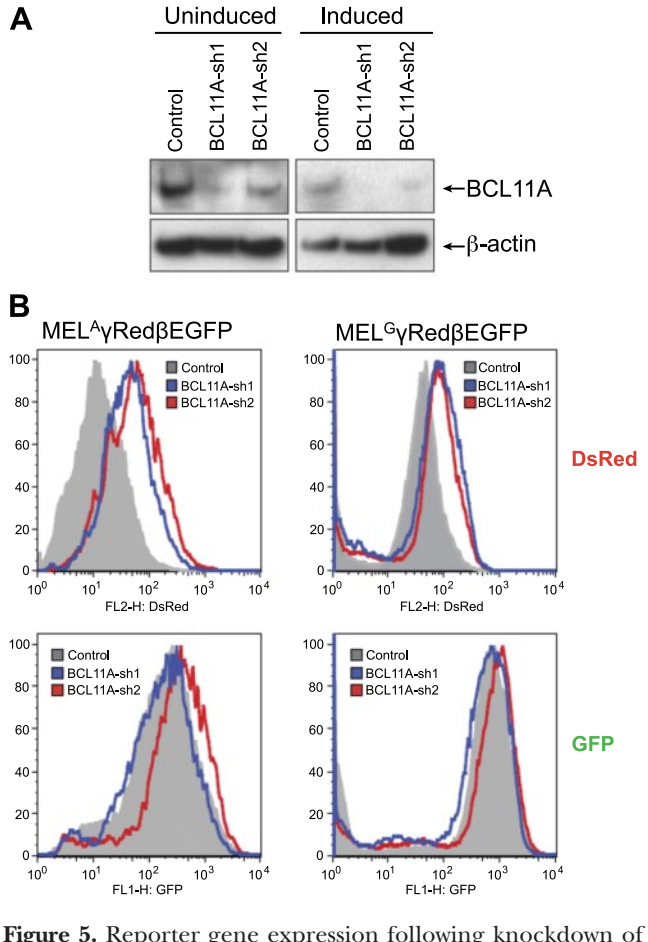

Figure 5. Reporter gene expression following knockdown of BCL11A. *A*) Transduction of MEL^{A}γRedβEGFP reporter cells with lentiviruses containing BCL11A-specific shRNAs (sh1 and sh2) resulted in a robust knockdown of BCL11A proteins. shRNA against GFP was used as control. Cells were harvested 7 d post-transduction, and whole-cell lysates were prepared and analyzed by Western blot using antibody for BCL11A. β-Actin was analyzed as a loading control. *B*) Comparisons of DsRed (top panels) and EGFP expression profiles (bottom panels) in two MEL reporter lines without (shaded) and with BCL11A knockdown (unshaded).

γ-globin promoter in the MEL dual-reporter cell lines. MEL reporter cells were transduced with lentiviruses expressing control shRNA or two shRNAs against BCL11A (containing a puromycin-selectable cassette). Puromycin selection was imposed 1 d after transduction for a period of 7 to 10 d. As shown in Figs. 6*A* and **7**, transduction of BCL11A-specific shRNA lentiviruses led to strong down-regulation of BCL11A protein and mRNA in both uninduced and DMSO induced cells. To determine the consequences of BCL11A knockdown, we examined the mRNA expression of εγ-globin, βh1-globin, and β^{major}/β^{minor}-globins by quantitative RT-PCR. Knockdown of BCL11A led to a substantial increase of murine εγ- and, to a lesser extent, βh1-globin mRNA, while the expression of β^{major}/β^{minor}-globins remained mostly unaffected (Fig. 6). These results are consistent with previous findings in unmodified MEL cells (12).

We next assessed the expression of γ-linked DsRed and β-linked EGFP reporter genes in both dual-reporter cell lines on knockdown of BCL11A (Fig. 6). Depletion of BCL11A led to an increase in γ-globin-linked DsRed expression in the MEL^{A}γRedβEGFP reporter line and, to a lesser extent, the MEL^{G}γRedβEGFP line (Fig. 5*B*). Notably, EGFP expression remained largely unchanged in both lines on BCL11A knockdown. Since the DsRed modified β-globin locus replaced only one of two γ-globin genes (^{G}γ or ^{A}γ), we measured γ-globin gene expression from the unmodified ^{G}γ or ^{A}γ-globin gene. As anticipated, knockdown of BCL11A led to a significant increase in γ-globin mRNA (HBG) expression in both dual-reporter lines with or without DMSO induction (Fig. 6). Moreover, knockdown of BCL11A led to an increase in human ε-globin gene (HBE1) expressed from the BAC transgenes, which mirrored derepression of murine embryonic β-like globin genes.

To further evaluate the utility of the dual-reporter cell lines, we assessed the responsiveness of the reporter genes to known HbF inducers. The MEL^{A}γRedβEGFP reporter cells were treated with butyrate, HU, or decitabine. Treatment of the dual-reporter cell line with these compounds alone did not induce DsRed expression (Fig. 7). However, combination of BCL11A depletion and decitabine or HU treatment led to a strong synergistic effect, resulting in a much stronger derepression of γ-linked DsRed reporter in a dose-dependent manner (Fig. 7). Overall, decitabine was more effective at inducing DsRed reporter in MEL cells, but only when BCL11A was depleted.

DISCUSSION

A better understanding of the mechanisms of fetal hemoglobin gene regulation is of clinical importance for the development of targeted therapeutic strategies for the major β-hemoglobinopathies, β-thalassemia and SCD. To study the regulatory mechanisms underlying this clinically important developmental switch, a number of studies have utilized cultured human primary erythroid cells to identify and evaluate γ-globin gene regulators. Key limitations have been noted, particularly with high-throughput analysis. Alternatively, several studies have reported the creation of various erythroid cell lines carrying small constructs composed of a micro-LCR linked to reporter genes driven by the γ- and β-globin gene promoters (31, 32). However, the removal of the globin genes from their native chromatin environment, followed by random chromosomal integration, often leads to poor and variegated transgene expression followed by transcriptional silencing. It has proven difficult to design artificial DNA constructs that utilize native promoters and regulatory elements to reproduce natural physiological control mechanisms, especially when many of the complex mechanisms involved in the regulation of gene expression have yet to be fully elucidated. A conceptually simpler alternative is to introduce the globin gene in its natural genomic

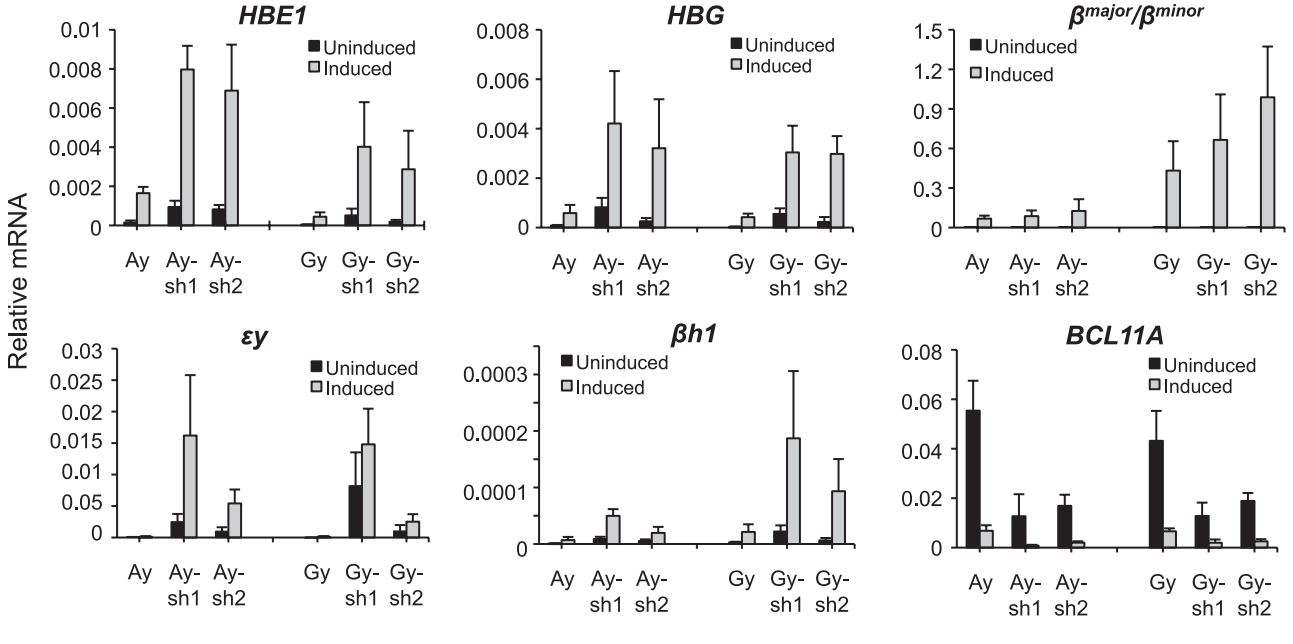

Figure 6. Analysis of murine and human β-like globin expression following knockdown of BCL11A. Expression of murine and human β-like globin transcripts was measured by qRT-PCR in dual-reporter MEL cell lines that were uninduced or induced in 2% DMSO for 7 d. Aγ, MEL^{A}γRedβEGFP reporter line; Gγ, MEL^{G}γRedβEGFP line. Lentiviral shRNA-mediated knockdown of BCL11A (sh1 and sh2) resulted in elevations of murine embryonic β-like globin genes (εy and βh1), as well as human ε-globin (HBE1) and γ-globin (HBG) mRNA levels. Transcript levels were normalized against mouse *Gapd* transcript levels. All results represent means ± SD of ≥3 independent experiments.

context. This approach allows the inclusion of all short- and long-range regulatory elements that are essential for controlling gene expression, and more likely preserves the physiologically relevant responses.

Here we report the construction of a dual-fluorescence-reporter cell line containing a modified human β-globin locus whereby the γ-globin gene (^{G}γ or ^{A}γ) is replaced by a DsRed reporter gene and the β-globin gene is replaced by the EGFP gene in the context of the intact β-globin locus. Following stable integration in MEL cells, we noted a remarkable level of constancy and uniformity of reporter gene expression in the absence of antibiotic selective pressure, suggesting that the modified β-globin locus contains the regulatory elements necessary for developmental-stage and tissue-specific expression of reporter genes. The distinctive high-level expression of EGFP in the absence of DsRed expression makes this dual-reporter cellular assay system a unique model to identify key regulators of γ-globin silencing.

We next investigated whether the dual-reporter cellular assay could be used in genetic and functional studies. In previous studies, BCL11A was shown to be a critical mediator of fetal to adult hemoglobin switch. BCL11A binds the upstream locus control region, particularly the

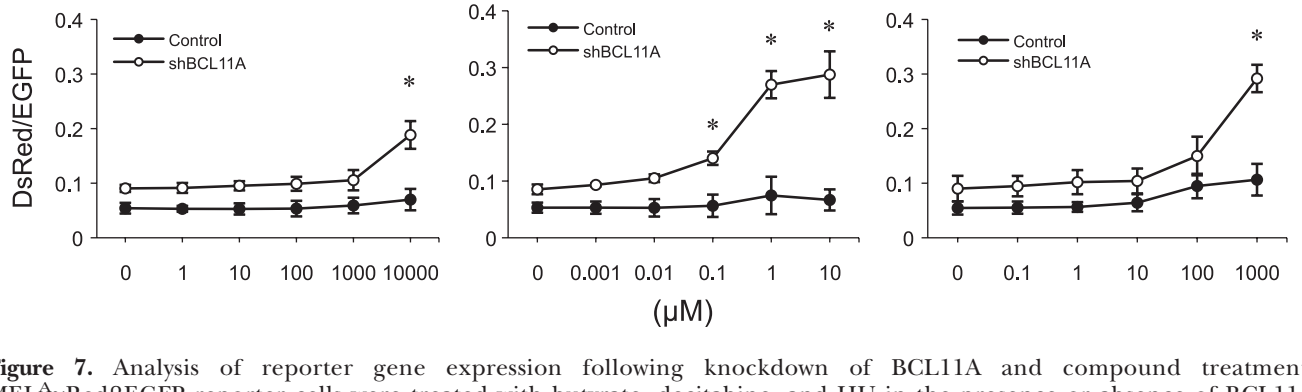

Figure 7. Analysis of reporter gene expression following knockdown of BCL11A and compound treatment. MEL^{A}γRedβEGFP reporter cells were treated with butyrate, decitabine, and HU in the presence or absence of BCL11A knockdown. Expression of DsRed and EGFP reporter gene was analyzed by flow cytometry. Panels show comparisons of DsRed expression relative to EGFP (DsRed/EGFP) in MEL^{A}γRedβEGFP reporter cells that were transduced with lentiviruses expressing control shRNA (control) or BCL11A-specific shRNA (shBCL11A). Knockdown of BCL11A was performed using lentiviral construct containing BCL11A shRNA sequence (sh1, Fig. 5*A*). Data represent means ± SD of 3 independent experiments. *$P < 0.05$.

HS3 site, and the intergenic regions between $^{A}\gamma$-globin and δ-globin genes to reconfigure the β-globin locus (12). To test the functional role of BCL11A on γ-linked DsRed expression in the dual-fluorescence-reporter MEL cell lines, we depleted BCL11A expression by RNAi. Loss of BCL11A led to an increase in the expression of γ-globin-linked DsRed reporter gene, while β-globin-linked EGFP expression remained largely unchanged. Moreover, knockdown of BCL11A was accompanied by an increase in mouse embryonic β-like globins and reactivation of human γ-globin in MEL reporter cells.

With the recent interest in epigenetic mechanisms regulating globin gene expression, awareness has increased regarding the use of epigenetic drugs to reactivate γ-globin gene expression. We further evaluated the utility of the dual-reporter MEL cell lines by treatment with known HbF inducers butyrate, decitabine, or HU. Interestingly, treatment with these compounds alone did not induce DsRed expression. However, combination of compound treatment and BCL11A depletion led to a strong synergistic effect in derepression of DsRed expression in a dose-dependent manner (Fig. 7). Notably, decitabine was more effective at inducing the DsRed reporter in MEL cells, particularly at low levels (0.1–1μM), but only when BCL11A was depleted. Decitabine is a DNA methyltransferase inhibitor that induces γ-globin gene expression in primary erythroid cells with concomitant demethylation of the γ-globin promoter (33). Moreover, depletion of DNA methyltransferase 1 (DNMT1) by RNAi in primary erythroid progenitors derived from $CD34^{+}$ baboon bone marrow cells reverses hypermethylation of the embryonic ε- and γ-globin promoters and increases the expression of both genes (34).

HU is currently the only pharmacologic agent widely used to ameliorate the symptoms of SCD with well-appreciated variable responsiveness to HbF induction and treatment. HU is primarily thought to inhibit DNA replication and cause cell cycle arrest due to inhibition of ribonucleotide reductase (35). Another potentially important effect of HU is that metabolism of the drug results in the production of nitric oxide and activation of guanylate cyclase. On the one hand, the precise mechanisms by which HU exerts its therapeutic actions are not well understood (1). On the other hand, the SCFA butyrate did not have a significant effect on DsRed expression in our assay. However, this finding does not preclude the possibility of using more selective and potent SCFAs as potential histone deacetylase (HDAC) inhibitors, as have been identified in recent chemical and genetic screens (8).

In this study, we report a system that recapitulates the expression of different globin genes according to the developmental stage of the originating source. We show that the dual-reporter cell line is responsive to knockdown of BCL11A. More important, we demonstrate that the dual-reporter assay system can recapitulate the synergistic effect between HbF inducers and loss of BCL11A, as recently demonstrated *in vivo* using normal β-YAC transgenic mice (36). Based on these findings, we propose that modulation of the MEL cell-based dual-reporter assay system using RNAi may allow for the discovery of additive or synergistic regimens that increase responsiveness to otherwise weak or ineffective drug candidates. As seen with decitabine and HU, the true potential of these agents was observed only when BCL11A was reduced. Mechanistically, loss of BCL11A in primary adult erythroid cells has been shown to reconfigure the human β-globin locus, resulting in robust induction of fetal hemoglobin and decreased chromatin occupancy of its partner protein HDAC1 and the repressive H3K27me3 mark found in the region (12, 37). It is plausible that the altered regional chromatin conformation of the human β-globin cluster on knockdown of BCL11A may become amenable to γ-globin inducers. While BCL11A is critical for γ-globin gene silencing, we cannot exclude the possible contribution of other regulatory factors or other multiprotein complexes such as DRED or Polycomb-group proteins, which may act independently or in combination with BCL11A to repress γ-globin transcription (18). Our study highlights the utility of the MEL cell-based dual-reporter assay to gain an insight into a clinically significant problem that would otherwise be difficult to investigate using other lines of investigation. FJ

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