Erythroid-Specific Expression of β-Globin by the *Sleeping Beauty* Transposon for Sickle Cell Disease†

Jianhui Zhu,‡ Betsy T. Kren,‡ Chang Won Park,‡ Rasim Bilgim,‡ Phillip Y.-P. Wong,‡ and Clifford J. Steer*,‡,§

Department of Medicine and Department of Genetics, Cell Biology, and Development, University of Minnesota Medical School, Minneapolis, Minnesota 55455

Received November 28, 2006; Revised Manuscript Received February 22, 2007

ABSTRACT: Sickle cell disease (SCD) results predominately from a single monogenic mutation that affects thousands of individuals worldwide. Gene therapy approaches have focused on using viral vectors to transfer wild-type β- or γ-globin transgenes into hematopoietic stem cells for long-term expression of the recombinant globins. In this study, we investigated the use of a novel nonviral vector system, the *Sleeping Beauty* (*SB*) transposon (Tn) to insert a wild-type β-globin expression cassette into the human genome for sustained expression of β-globin. We initially constructed a β-globin expression vector composed of the hybrid cytomegalovirus (CMV) enhancer chicken β-actin promoter (CAGGS) and full-length β-globin cDNA, as well as truncated forms lacking either the 3′ or 3′ and 5′ untranslated regions (UTRs), to optimize expression of β-globin. β-Globin with its 5′ UTR was efficiently expressed from its cDNA in K-562 cells induced with hemin. However, expression was constitutive and not erythroid-specific. We then constructed *cis SB*-Tn-β-globin plasmids using a minimal β-globin gene driven by hybrid promoter IHK (human *ALAS2* intron 8 erythroid-specific enhancer, HS40 core element from human αLCR, *ankyrin-1* promoter), IHβp (human *ALAS2* intron 8 erythroid-specific enhancer, HS40 core element from human αLCR, β-globin promoter), or HS3βp (HS3 core element from human βLCR, β-globin promoter) to establish erythroid-specific expression of β-globin. Stable genomic insertion of the minimal gene and expression of the β-globin transgene for >5 months at a level comparable to that of the endogenous γ-globin gene were achieved using a *SB*-Tn β-globin *cis* construct. Interestingly, erythroid-specific expression of β-globin driven by IHK was regulated primarily at the translational level, in contrast to post-transcriptional regulation in non-erythroid cells. The *SB*-Tn system is a promising nonviral vector for efficient genomic insertion conferring stable, persistent erythroid-specific expression of β-globin.

Sickle cell disease (SCD),[1] an inherited monogenic disorder, affects thousands of people worldwide. Ex vivo gene transfer into pluripotent hematopoietic stem cells (HSCs) remains a potential therapeutic approach for SCD. Viral vectors have been the major vehicle for delivery of transgenes in correcting recessive monogenic disorders (*1*), in part because of their natural ability to infect cells. Specific viral vectors have been engineered to deliver transgenes into HSC ex vivo. Introduction of the transduced stem cells into the transgenic sickle mice has resulted in sufficient engraftment and therapeutic levels of normal red blood cells (*2*).

Both host defense strategies against viruses and potential risks associated with viral vectors have posed a significant barrier to success with any viral vector-based gene therapy protocol. These include host immune response, cellular inhibitory factors, methylation-associated proviral and transgene silencing, chromatin positional effects, interference of viral promoters with internal transgene regulatory elements, and tumorigenesis from activation of flanking genomic genes (*3*−*6*). In addition, systemic instability and the high costs and biosafety issues for production are still major concerns for viral vector-mediated gene therapy. The most fundamental problems associated with nonviral vector systems have been the low frequency of transgene integration and poor transfection efficiency of the target cells. One potential candidate for efficiently inserting a β-globin transgene into

† Support for this study was provided in part by grants from the National Institutes of Health (P01 HL55552) and the Alexander and Margaret Stewart Trust (C.J.S.).

* To whom correspondence should be addressed: Department of Medicine, University of Minnesota Medical School, Mayo Mail Code 36, Mayo Building, Room A536, 420 Delaware Street SE, Minneapolis, MN 55455. Telephone: (612) 624-6648. Fax: (612) 625-5620. E-mail: steer001@umn.edu.

‡ Department of Medicine.

§ Department of Genetics, Cell Biology, and Development.

[1] Abbreviations: αLCR, α-globin gene locus control region; B, backward; β, full-length β-globin cDNA; β3′Δ, β-globin cDNA with the 3′ untranslated region deleted; βCDS, β-globin coding sequence; βLCR, β-globin gene locus control region; βp, β-globin promoter; CAGGS, cytomegalovirus enhancer chicken β-actin hybrid promoter; CDS, coding sequence; CMV, cytomegalovirus; DsRed2, *Discosoma* sp. red fluorescent protein; DMSO, dimethyl sulfoxide; eIF, mouse initiation factor 4A1 promoter; F, forward; FBS, fetal bovine serum; HSC, hematopoietic stem cell; HSB3, hyperactive *Sleeping Beauty* transposase version 3; IHK, human *ALAS2* enhancer (i8), HS-40 element, and *ankyrin-1* hybrid promoter; IHβp, human *ALAS2* enhancer (i8), HS-40 element, and β-globin hybrid promoter; HS3βp, human HS3B element and β-globin hybrid promoter; IR/DR, inverted repeat/direct repeat; LCR, locus control region; poly(A), polyadenylation; pT2, *Sleeping Beauty* transposon version 2; RT, reverse transcription; SCD, sickle cell disease; *SB*, *Sleeping Beauty*; SB10, *Sleeping Beauty* transposase version 10; Tn, transposon; UTR, untranslated region.

10.1021/bi6024484 CCC: $37.00
Published on Web 05/18/2007

the host genome for long-term stable expression is the *Sleeping Beauty* (*SB*) transposon system (*SB*-Tn).

The *SB*-Tn system was reconstructed by correcting key mutations within fish *Tc*l-like transposons (*7*). Since then, it has been used successfully to generate transgenic mice (*8*) and as a gene therapy vector capable of delivering genes to mouse liver for somatic integration and long-term transgene expression (*9*). *Sleeping Beauty* has unique features as a gene therapy vector for a variety of cell targets, including human primary T cells (*10*). It integrates transgenes randomly into TA-rich regions of the host genome at a frequency comparable with that of viral vectors in both quiescent and replicating cells (*9*, *11*). It does not appear to be immunogenic in rodent models, can be produced easily in mass quantity, and maintained pathogen-free in any laboratory at minimal cost. *SB*-Tn works by a cut-and-paste mechanism as either a two-plasmid (*trans*) system or a single plasmid in *cis* containing both the IR/DR-flanked transgene and transposase cassette external to the Tn (*12*, *13*).

In this study, we constructed *cis SB*-Tn//β-globin transgenes as a more convenient and efficient method for therapeutic application. Since transgene or transposon and plasmid size are important for transfection and transposition efficiency (*11*), we initially expressed β-globin from its cDNA and a CAGGS promoter. We then tested a series of novel minimal gene constructs, devoid of the β-globin locus control region (LCR), for erythroid-specific expression. Our results showed that a hybrid IHK promoter (*5*, *14*) resulted in the most efficient erythroid-specific expression of β-globin from a minimal 1.54 kb gene. We were able to achieve stable genomic insertion and long-term expression of the β-globin transgene in hematopoietic K-562 cells. Interestingly, erythroid-specific expression of β-globin driven by IHK occurred at the translational level, independent of the proteasomal degradation mechanism observed with the universal CAGGS promoter. Our results suggest that the nonviral *SB*-Tn system has the capacity to provide stable gene transfer and efficient position-independent expression of β-globin as a potential gene therapy approach for SCD.

MATERIALS AND METHODS

Plasmids and Sleeping Beauty Transposon Vectors. A human β-globin full-length cDNA clone pDNR-LIB (MGC, 14540; IMAGE, 4292125) was purchased from ATCC (Manassas, VA). The plasmid was digested into fragments containing either the entire β-globin cDNA (SfiI), the cDNA without the 3′ untranslated region (UTR) (SfiI and MseI), or only the β-globin coding sequence (CDS) (NcoI and MseI). Following agarose gel electrophoresis, excision, and isolation of the β-globin cDNA fragments, the ends were modified with Klenow polymerase (New England Biolabs, Tozier, MA) and subcloned into the pCAGGS vector (*15*) at position 1724 by blunt-end ligation to make pCAGGS/β, pCAGGS/β3′Δ, and pCAGGS/βCDS. These β-globin transgenes were driven by the strong hybrid CAGGS promoter and utilized the rabbit globin poly(A) signal.

pT2/β-globin transposon vectors were constructed by digesting pCAGGS/β3′Δ with SalI and HindIII and subcloning the transgene into the *cis* vector pT2//eIF-SB10, creating pT2-pCAAGS-β3′Δ//eIF-SB10. The *cis* pT2 vectors contained the SB10 transposase gene driven by mouse initiation factor promoter 4A1 (eIF-SB10) (InvivoGen, San Diego, CA). To construct the erythroid-specific hybrid promoters, IHK, IHβp, and HS3βp, human *ALAS2* intron 8 (nucleotides 2595698−2595940 of GenBank accession number NT_011630.14), HS-40 (nucleotides 103493−103849 of GenBank accession number NT_037887.4), and HS3 (nucleotides 12459−13097 of GenBank accession number NG_000007) enhancer elements, *ankyrin-1* (nucleotides 11975446−11975833 of GenBank accession number NT_007995.14), and β-globin promoter (nucleotides 867297−867910 of GenBank accession number NW_925006.1) were amplified by PCR using primer sets *ALAS2* i8-F (SpeI)/*ALAS2* i8-B (NheI), HS-40-F (NheI)/HS-40-B (SphI), and HS3-F (SpeI)/HS3-B (SphI), *ankyrin*-1-F (NsiI)/*ankyrin*-1-B (EagI), and βp-F (SnaBI)/βp-B (EagI), respectively (Table 1). The enhancer and promoter fragments were inserted into pT2/β-3′Δ//eIF-SB10 by multiple cloning steps, which resulted in the replacement of the CAGGS promoter with the hybrid IHK, IHβp, or HS3βp.

Table 1: Primers Used for PCR and RT-PCR[a]

primer name	sequence
ALAS2-F	ACTAGTCACTGCAGAGTTGATGAG
ALAS2-B	GCTAGCATTCTGCTGCTTTGAGAT
HS-40-F	GCTAGCTCGACCCTCTGGAACCTA
HS-40-B	GCATGCGAATTTCTTCAGCTCCAG
HS3-F	ACTAGTACTGAGCTCAGAAGAGTCAAG
HS3-B	GCATGCAATAACCCTATGAGATAGACA
ankyrin-1-F	CATGCATGCGACTAAACCGGACTCCCTTTC
ankyrin-1-B	ACGGCCGCCGGTCTTTCAGCAGGGGCCC
βp-F	TACGTATCTATTTTTAGACATAATTTA
βp-B	ACGGCCGAAGCAATAGATGGCTCTG
LP1	GTGTCATGCACAAAGTAGATGTCC
RP1	CTAGGATTAAATGTCAGGAATTGTG
LP2	ACTGACTTGCCAAAACTATTGTTTG
RP2	GRGAGTTTAAATGTATTTGGCTAAG
β-actin-F	CCAAGGCCAACCGCGAGAAGATGAC
β-actin-B	AGGGTACATGGTGGTGCCGCCAGAC
β-CAGGS-F	CTGCTAACCATGTTCATGCC
β-globin-F	TCACCTGGACAACCTCAAGG
β-globin-B	GGACAGCAAGAAAGCGAGC
GAPDH-F	CCCTTCATTGACCTCAAC
GAPDH-B	TTCACACCCATCACAAAC
β-gene-F	ACGGCCGACATTTGCTTCTGACACAAC
β-gene-B	GAGATCTAGTAGTTGGACTTAGGGA

[a] All primer sequences are written in a 5′ to 3′ direction and grouped according to pairs with the exception of those used to determine β-globin transcript levels from the CAGGS, HS3βp, IHβp, and IHK promoters (β-globin-F), which utilized the same backward primer in the β-globin CDS. F, forward primer; B, backward primer. That portion of the primers used for amplification of the genomic DNA for the promoters and the 1.54 kb region of the β-globin allele that represents the restriction endonuclease sites in the F and B primers are underlined.

PCR using β-gene-F and -B primers (Table 1) was used to amplify the region of globin loci (GenBank accession number U01317) from nucleotide 62137 to 63671 and replace the β-globin cDNA in pT2/IHKβ-3′Δ//eIF-SB10 with the 1.54 kb portion of the β-globin gene. This region starts at the transcript initiation site and ends in the third exon, 61 nucleotides 3′ to the CDS stop codon. The initiation site of the sequence is identical to that of the 5′ UTR inclusive cDNA but also includes 61 nucleotides of the 3′ UTR which is absent in the 3′ truncated cDNA sequence. The amplified genomic sequence was inserted into pT2/IHKβ-3′Δ//eIF-SB10 using the 5′ EagI and 3′ BglII sites that flank the β-3′Δ sequence. Both the genomic and cDNA IHK-driven tran-

scripts utilized the rabbit globin poly(A) signal that was present in the original CAGGS-driven pT2/β-3′Δ//eIF-SB10 vector.

pT2/IHK-β-gene//eIF-HSB3 was constructed by transferring a SpeI to BglII fragment containing the IHK-β-gene from pT2/IHK-β-gene//eIF-SB10 to an EcoRV site of pKT2//eIF-HSB3 (*16*).

Cell Culture and Transfections. The K-562 (ATCC catalog number CCL-243) and MEL (ATCC catalog number CCL-745) cell lines were maintained in RPMI 1640 (formula 03-0078DJ, Invitrogen Corp., Carlsbad, CA) supplemented with 10% FBS (Omega Scientific, Inc., Tarzana, CA) and 100 units/mL penicillin, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B. HuH-7 (*17*), U-87 (ATCC catalog number HTB-14), Daoy (ATCC catalog number HTB-186), HCT 116 (ATCC catalog number CCL-247), and MCF7 (ATCC catalog number HTB-22) cell lines were grown in Dulbecco's modified Eagle's medium (Invitrogen Corp.) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA) and 100 units/mL penicillin, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B.

Lipofectamine 2000 (Invitrogen Corp.) was used to transfect cell lines. Eighteen hours prior to transfection, 1×10^6 cells were plated on 100 mm dishes. For each transfection, 5 μg of DNA plasmid and 14 μL of lipofectamine 2000 were each diluted into 350 μL of OPTI-MEM I (Invitrogen Corp.). The two solutions were mixed to form the DNA−lipofectamine complexes, which were then added to the 5.5 mL of medium in the 100 mm dish. After 18 h, the cells were split into two or more 100 mm dishes and maintained for an additional 18−24 h. The transfected cells were then treated for 48 h with hemin (Fluka, Sigma-Aldrich, St. Louis, MO) at a final concentration of 20 μM to induce β-globin synthesis or MG132 (Sigma-Aldrich) at a final concentration of 0.1, 1, 5, or 20 μM for 24 h to inhibit β-globin degradation. MG132 at a final concentration of 5 μM was used to compare the CAGGS and IHK *SB*-Tns. The parallel cultures transfected with the DsRed2-expressing reporter plasmid, pT2-CAGGS-DsRed2, were imaged using a spot camera coupled to an Olympus IX-70 phase contrast/fluorescent microscope 48 h post-transfection.

Ten micrograms of plasmid pT2/IHKβ-gene//eIF-SB10 and 25 μL of lipofectamine 2000 were each diluted in 500 μL of OPTI-MEM I, mixed, and added to the 5 mL of medium in the dish. Five hours after transfection, the medium was changed and the cells were maintained until 18 h post-transfection. At this time, the transfected cells were split into two dishes. One K-562 dish was treated with hemin and one HuH-7 dish treated with MG132 as described above.

For long-term passage experiments, 10 μg of plasmid pT2/IHKβ-gene, pT2/IHKβ-gene//eIF-SB10, pT2/IHKβ-gene//eIF-*HSB3*, or the reporter plasmid pT2-CAGGS-DsRed2 was transfected into K-562 cells. Following a medium change 5 h post-transfection, the cells were maintained until 24 h when 20 μM hemin was added to induce globin synthesis. At 72 h post-transfection, the cells were passaged by replating 10% of the cells and harvesting the remaining 90% for Western blot analysis. The cells were passaged every 3 days, using 10% of the cells for the plating. For the time points that were analyzed, hemin (20 μM) was added 48 h prior to the cells being harvested. No antibiotic selection or continuous hemin induction was used during the long passage experiments.

For MEL induction, dimethyl sulfoxide (DMSO) was added to the cells 48 h prior to transfection at a final concentration of 1.5% to induce differentation from proerythroid to adult erythroid cells (*18*). The cells were split for transfection into 100 mm dishes as described above and maintained in medium supplemented with 1.5% DMSO. For transfection, 5 μg of pT2/HS3βp-gene//eIF-SB10, pT2/IHβp-gene//eIF-SB10, or pT2/IHKβ-gene//eIF-SB10 was diluted into 175 μL of OPTI-MEM I medium and mixed with 175 μL of OPTI-MEM I medium containing 2.5 μg of protamine sulfate (Sigma-Aldrich) and incubated at room temperature for 10 min. The protamine−plasmid complex was then mixed with 350 μL of OPTI-MEM I medium containing 14 μL of lipofectamine 2000 and added to the MEL cultures. When the medium was changed 5 h later, the induced cells were maintained in complete medium containing 1.5% DMSO. Hemin induction and processing of the induced and noninduced MEL transfected cells for analysis were carried out as described for the K-562 cells.

Western Blot Analyses. At the indicated times post-transfection, the cells were washed twice with PBS, harvested by scraping, and recovered by centrifugation at 1200 rpm for 14 s. Following lysis of the cells in 1× SDS−PAGE sample buffer [50 mM Tris-HCl (pH 6.7) containing 2% (w/v) SDS, 100 mM β-mercaptoethanol, and 10% (w/v) glycerol], 50 μg of total protein was separated by 13.5% SDS−PAGE and eletrophoretically transferred to nitrocellulose membranes. The immunoblots were processed as previously described (*12*). The primary mouse monoclonal anti-β-globin 37-8 or anti-γ-globin 51-7 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:500. The monoclonal anti-β-actin AC-15 antibody (Sigma-Aldrich) was used for loading control at a dilution of 1:10000. The secondary horseradish peroxidase-conjugated antibody, goat anti-mouse, was obtained from Pierce Biotechnology, Inc. (Rockford, IL). The proteins were detected with ECL (enhanced chemiluminescence), using the Supersignal West Pico Chemiluminescent substrate from Pierce Biotechnology.

Inverted, Nested PCR Analysis. Genomic DNA was purified from the harvested cells using the DNeasy tissue kit from Qiagen, Inc. (Valencia, CA), according to the manufacturer's recommendation. Two micrograms of the genomic DNA samples was digested using either KpnI or PvuI and PacI in final volume of 100 μL, at 37 °C overnight. The products were purified using the QIAquick PCR purification kit from Qiagen, as specified by the manufacturer. The DNA was eluted in 50 μL of buffer and ligated using 1200 units of T4 DNA ligase (Promega, Madison, WI) in 600 μL of 1× T4 ligase buffer at room temperature for 5−6 h. The ligation products were purified using the QIAquick PCR purification kit, and the DNA was eluted in 50 μL of elution buffer. Using 10 μL of the eluted DNA as a template, the first inverted PCR amplification was performed in a reaction volume of 25 μL with right IR/DR primer 1 (RP1) and left IR/DR primer 1 (LP1) (Table 1) using Expand Hi-Fidelity polymerase (Roche Molecular Diagnostics, Indianapolis, IN), dNTP, and enzyme concentrations recommended by the manufacturer. Following denaturation for 3 min at 94 °C, the DNA was amplified for

21 cycles of 93 °C for 30 s, 58 °C for 30 s, and 68 °C for 5 s with a final extension for 7 min at 72 °C. From this initial PCR mixture, 0.5 μL was removed to serve as a template for a second PCR using nested primers [right IR/DR primer 2 (RP2) and left IR/DR primer 2 (LP2) (Table 1)] in 50 μL, under standard conditions for Expand Hi-Fidelity polymerase. Following the initial 3 min denaturation step, the PCR was performed for 40 cycles using the same cycle parameters. The products from the PCRs were separated on 1.0% agarose gel, stained with ethidium bromide, and visualized by UV light. The relevant DNA fragments were excised from the gel, purified using the Qiagen gel isolation kit according to the manufacturer's protocol, and sequenced directly using the RP2 or LP2 primer.

RT-PCR Analysis. The cells were harvested 72 h post-transfection, and total RNA was isolated using TRIzol Reagent (Invitrogen Corp.) and the manufacturer's recommended protocol. RNA (0.5 μg) was treated with RQI RNase-Free DNase (Promega) in 10 μL to eliminate any remaining episomal DNA vectors. After 30 min at 37 °C, the DNase was inactivated by incubation of the reaction mixtures at 65 °C for 15 min. The DNase-treated total RNA (20 ng) was used as the template to perform RT-PCR using the Titan One Tube RT-PCR system (Roche Molecular Diagnostics). Primers for the CAGGS-driven β-globin cDNA are indicated as CAGGS-F and β-globin-B (Table 1). The RT-PCR included the primer, dNTP, and enzyme conditions recommended by the manufacturer. The PCR amplification was performed using a RT step at 50 °C for 30 min followed by denaturation at 94 °C for 2 min and 33 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 30 s with a final extension at 68 °C for 6 min. For erythroid-specific promoters, the indicated β-globin-F and -B primers were used for PCR amplification (Table 1). The RT step at 45 °C for 30 min was followed by denaturation for 2 min at 94 °C and 25, 33, or 35 cycles at 94 °C for 15 s, 55 °C for 20 s, and 72 °C for 30 s with a final extension at 72 °C for 5 min. These primers spanned the exon 2−exon 3 junction and thus ensured that only spliced transcripts were amplified by the RT-PCR.

For the control β-actin, parallel RT-PCRs were performed with the β-actin-F and -B primers using a RT step at 55 °C, for 30 min followed by denaturation at 94 °C for 2 min and 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 45 s with a final extension at 68 °C for 6 min. For the GAPDH controls, GAPDH-F and GAPDH-B primers were used and the reactions were performed using 30 min at 45 °C for RT, followed by denaturation at 94 °C for 2 min and 35 cycles at 94 °C for 30 s, 52 °C for 20 s, and 72 °C for 30 s with final extension at 72 °C for 5 min. The products from the PCRs were separated on a 1.0% agarose gel, stained with ethidium bromide, and visualized with UV light, and digital images were acquired using a Kodak (Rochester, NY) digital imaging system.

Densitometry and Statistical Analysis. The Western blots and digital gel images were analyzed using ImageJ version 1.37 for OS X calibrated using a Stouffer sensitivity guide (Stouffer Graphic Arts Equipment Co., South Bend, IN). Statistical analysis was performed using GraphPad InStat version 3.5 for OS X (GraphPad Software, San Diego, CA) for the unpaired *t* tests or ANOVA and Turkey−Kramer multiple comparison tests depending on the number of groups being compared. P values of <0.05 were considered significant. The means ± one standard deviation (SD) were derived from three or more independent experiments.

RESULTS

Expression of β-Globin from cDNA Driven by the pCAGGS Promoter. We initially used either the full-length or subsections of the β-globin cDNA (≤640 bp) to optimize protein expression and significantly reduce the size of the transgene in *SB*-Tn. The full-length (β), 3′ untranslated region (UTR)-deleted (β3′Δ), or 5′ and 3′ UTR-deleted (βCDS) cDNAs were placed under control of the hybrid CAGGS promoter, together with the rabbit globin poly(A) sequence (Figure 1A). Since the CAGGS promoter functions ubiquitously (*15*), we tested expression of the three β-globin expression vectors by Western blot analysis in both erythroid and non-erythroid cell lines (Figure 1B). To monitor transfection efficiency, the cell lines were transfected in parallel with a plasmid expressing the humanized red fluorescent protein, DsRed2, under control of the CAGGS promoter (*12*) (Figure 1C). In K-562 cells, both pCAGGS-β and pCAGGS-β3′Δ expressed similar levels in media supplemented with 20 μM hemin to induce translation (*19*) (Figure 1B, lanes 2 and 4). Interestingly, pCAGGS-βCDS exhibited a dramatic decrease in β-globin levels (Figure 1B, lane 6), suggesting that the 5′ UTR of the β-globin cDNA might be essential for efficient transcription or translation. Although similar levels of transfection were observed in both cell lines using the DsRed2 reporter (Figure 1C), no detectable β-globin expression was observed in HuH-7 cells (Figure 1B, lanes 7−12).

To determine whether β-globin protein expression was controlled at transcriptional and/or post-transcriptional levels, we performed RT-PCR analysis using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization. The results (Figure 1D) indicated no significant difference in levels of β-globin transcripts among the different constructs with or without hemin treatment (1.33 ± 0.19 vs 1.21 ± 0.21 units and 3.38 ± 0.37 vs 3.43 ± 0.38 units) for K-562 and HuH-7 cells, respectively. However, the steady-state transcript levels produced by the CAGGS promoter were 3.4 ± 0.3-fold ($P < 0.001$) higher in HuH-7 cells. Taken together, the data indicated that regulation of β-globin protein expression was occurring at the post-transcriptional level and the 5′ UTR of the transcript was required for efficient translation in K-562 cells.

β-Globin Undergoes Proteasomal Degradation in Non-Erythroid Cells. We also transfected U87 glioblastoma, HCT116 colon carcinoma, Daoy cerebellar meduloblastoma, and MCF7 breast cancer-derived cell lines with the pCAGGS-β3′Δ or pCAGGS-DsRed2 plasmid. Interestingly, the same pCAGGS-β3′Δ construct was expressed constitutively in the U87 and HCT116 cell lines (Figure 2A, lanes 1, 2, 5, and 6), yet no detectable expression in either Daoy or MCF7 cell lines (Figure 2A, lanes 3, 4, 7, and 8) was observed. Again, the difference was independent of transfection efficiency as determined by DsRed2 expression (Figure 2B), suggesting post-transcriptional regulation of β-globin expression in the non-erythroid cell lines.

Previous studies have shown that α-globin levels are regulated, in part, by the ubiquitin pathway in erythroid cells (*20*). We tested whether β-globin expression is regulated by

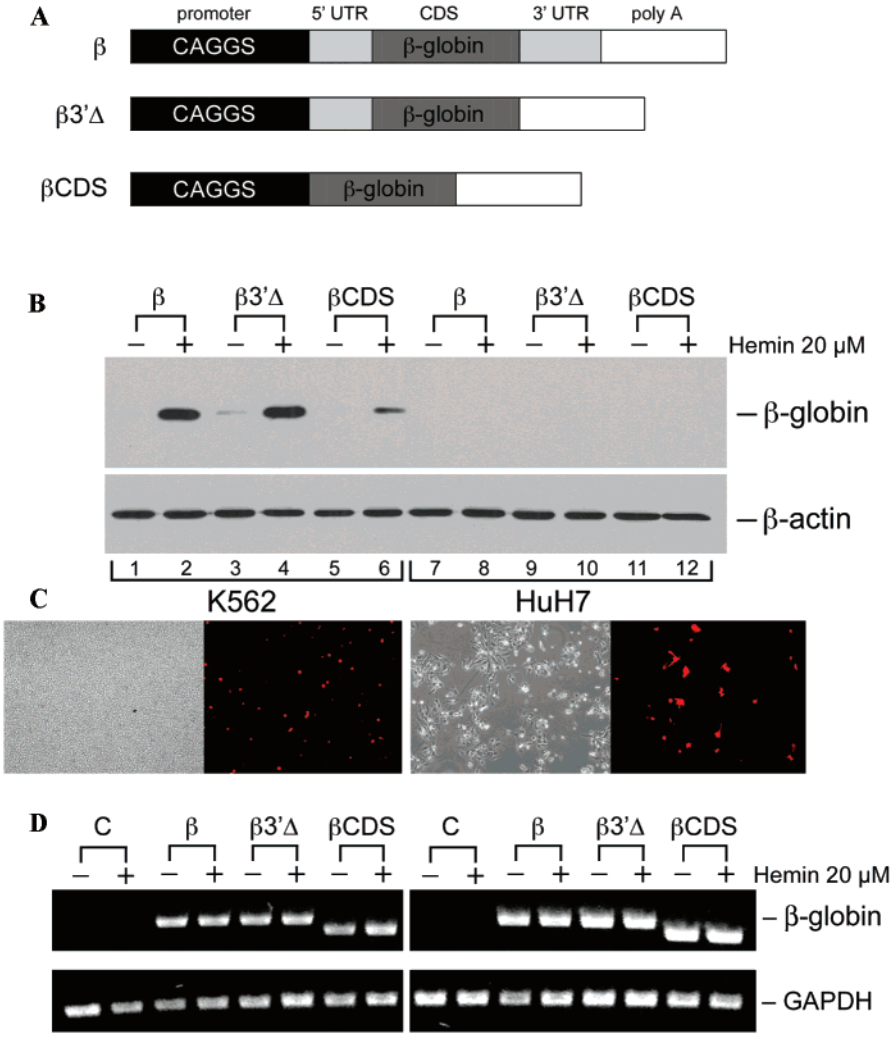

FIGURE 1: Expression of β-globin from cDNA in K-562 and HuH-7 cells. (A) Structure of the β-globin cDNA expression cassettes. Abbreviations: CAGGS, cytomegalovirus (CMV) enhancer:chicken β-actin promoter; 5′ UTR, 5′ untranslated region; CDS, β-globin coding region; 3′ UTR, 3′ untranslated region; poly A, rabbit globin poly(A) signal from the pCAGGS vector. (B) Western blot analysis of cell extracts from K-562 and HuH-7 cells transiently transfected with the pCAGGS-β, pCAGGS-β3′Δ, or pCAGGS-βCDS construct and induced to express β-globin with hemin (20 μM). The cells were transfected as described in Materials and Methods. After 36 h, hemin was added to the culture medium, and the cells were harvested following incubation for an additional 48 h. Lane loading was normalized to 50 μg of protein using β-actin as an internal control. The addition of hemin (+) is indicated for each construct at the top. Abbreviations: β, pCAGGS-β-globin; β3′Δ, pCAGGS-β3′Δ; βCDS, pCAGGS-β-CDS. (C) Transfection efficiency with pCAGGS-DsRed2 plasmid in K-562 and HuH-7 cells. The cells were transfected in parallel with the β-globin constructs described in panel A. The live cell cultures were photographed 48 h post-transfection. The phase contrast panels are shown at the left for each pair, and the same field using red fluorescent microscopy is shown at the right. (D) Reverse-transcribed PCR analysis of β-globin mRNA levels from pCAGGS-β, pCAGGS-β3′Δ, and pCAGGS-βCDS in the presence or absence of hemin in K-562 (left) and HuH-7 (right) cells. The construct and addition of hemin (+) to the cultures are indicated at the top, and glyceraldehyde-3-phosphate dehydogenase (GAPDH) transcript levels were used as an internal standard to verify RNA quality and lane loading. The smaller size of the amplicon observed using the pCAGGS-βCDS construct reflects the predicted size due to the deletion of the 51-nucleotide 5′ UTR.

the same proteolytic pathway in non-erythroid cells using MG132, a well-known proteasome inhibitor (*21*). Western blot analysis indicated that β-globin protein levels increased proportionally with higher concentrations of MG132 (Figure 2C, left panel, lanes 5, 6, 9, and 10). At the highest dose, the observed decrease in the β-globin level (Figure 2C, left panel, lanes 7 and 11) was associated with substantial apoptosis (*22*). Similarly, the amount of β-globin protein also increased in both Daoy and MCF7 cells incubated with MG132 (Figure 2C, right panel, lanes 2 and 4). RT-PCR analysis of steady-state transcript levels (Figure 2D) confirmed that the transcript was present in all cases at levels greater than or equal to that observed in K-562 cells. In addition, hemin did not significantly affect transcript abundance in any of cell lines that were examined ($P < 0.001$). Taken together, these data demonstrated that β-globin can be expressed from its cDNA containing the 5′ UTR, and its expression in non-erythroid cells is regulated post-translationally by proteasomal degradation.

Expression of β-Globin from a Minimum Gene Construct Driven by IHK and IHβp Hybrid Promoter. Since β-globin expression driven by the pCAGGS promoter lacks tight erythroid-specific control, we elected to use small enhancer elements (i8 from human *ALAS2* intron 8; HS40 from human α-globin LCR) linked to the human *ankyrin-1* promoter to drive β-globin expression in *SB*-Tn. To analyze the relative strength of this erythroid-specific IHK promoter, we also constructed i8-HS40 linked to β-globin promoter (βp,

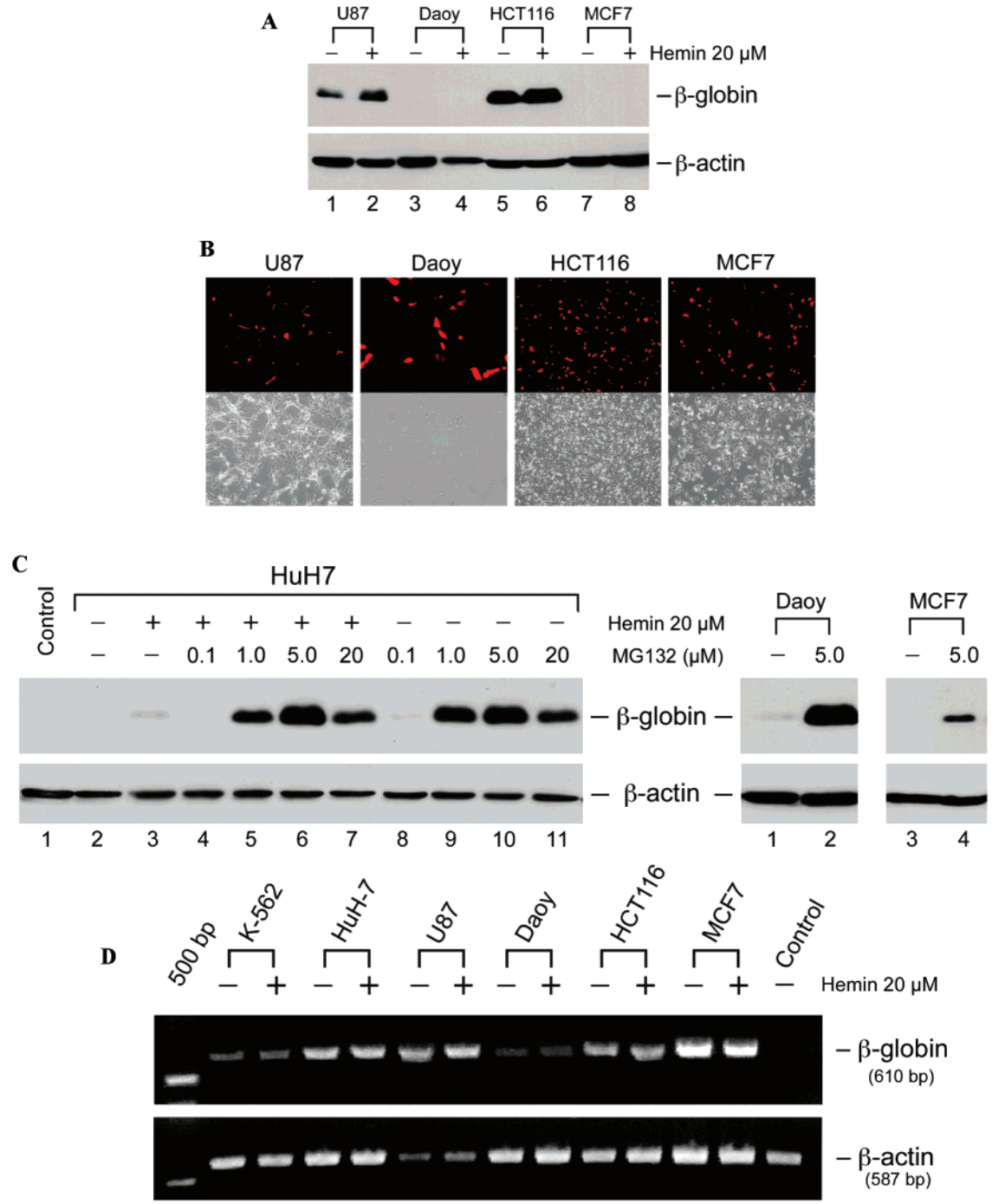

FIGURE 2: Expression of β-globin by the CAGGS promoter in non-erythroid cells. (A) Western blot analysis of cell extracts from U87, Daoy, HCT116, and MCF7 cells transiently transfected with the pCAGGS-β3′Δ plasmid. The cells transfected with the β-globin construct were collected 48 h after the addition of hemin (20 μM), and Western blot analysis was performed as described in Materials and Methods. β-Actin was used to control for lane loading. (B) The transfection efficiency in U87, Daoy, HCT117, and MCF7 cells was determined by parallel transfection with the pCAGGS-DsRed2 plasmid. (C) The β-globin protein undergoes proteasomal degradation in non-erythroid cells. Western blot analysis of HuH-7 (left) or Daoy and MCF7 cells (right) transiently transfected with the pCAGGS-β3′Δ plasmid. Twenty-four hours post-transfection, the cells were first induced with hemin (20 μM), and then MG132 was added at the indicated concentrations (top) after an additional 24 h. The cells were harvested and processed for Western blot analysis 72 h after transfection. Control, no transfection control for HuH-7. (D) β-Globin transcript levels in erythroid and non-erythroid cell lines. Reverse-transcribed PCR analysis of β-globin mRNA levels from pCAGGS-β3′Δ in the presence or absence of hemin. The cell line is indicated at the top, and β-actin transcript levels were used as an internal standard to confirm RNA quality. The 500 bp DNA maker is shown at the left with the transcript and its respective size at the right.

nucleotides −611 to −1) and the HS3 core element from the βLCR linked to βp to form IHβp and HS3βp, respectively. However, our preliminary results with pT2-IHK-β3′Δ//eIF-SB10 showed that the level of β-globin protein expression was dramatically decreased compared to that of the CAGGS promoter (data not shown). RT-PCR analysis indicated that the steady-state β-globin transcript levels from the IHK promoter had decreased 51.5 ± 2.4% ($P < 0.001$) relative to that of the CAGGS promoter. Since β-globin introns are critical for stabilization and cytoplasmic trans-

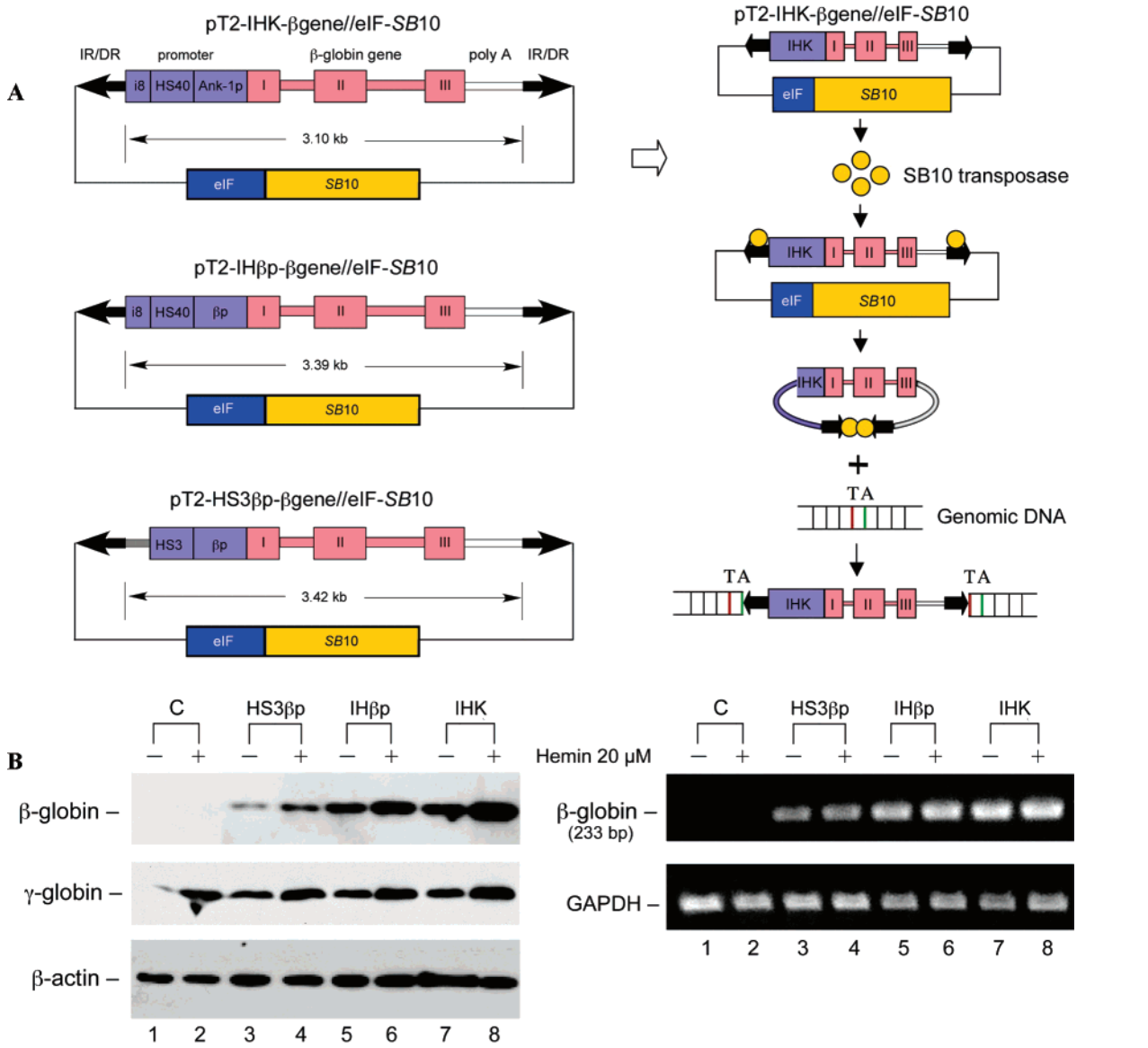

FIGURE 3: Expression of *β*-globin from different *cis* pT2-*β*-globin//eIF-SB10 constructs in K-562 cells. (A) Structure of *cis* pT2-promoter-*β*-gene//eIF-SB10 constructs (left) and schematic of the cut-and-paste transposition mechanism used by the *SB*-Tns (right). The obligate SB transposase is expressed from the transgene located in the vector backbone, external to the IR/DRs flanking the Tn IHK-*β*gene cargo. Following binding of the transposase to the IR/DRs, the Tn is cut from the plasmid vector and pasted into a TA dinucleotide in the host genomic DNA which is duplicated during the insertion process. Abbreviations: i8, human *ALAS2* intron 8 erythroid-specific enhancer element; HS40, core element of αLCR; HS3, core element of *β*LCR; *β*p, human *β-globin* promoter; Ank-1p, human *ankyrin-1* promoter; I, II, and III, *β*-globin exons; poly A, rabbit globin poly(A) signal from the pCAGGS vector; IR/DR, transposon inverted repeat/direct repeat; eIF, mouse initiation factor 4A1 promoter; SB10, *Sleeping Beauty* transposase gene version 10. (B) Western blot (left) and RT-PCR (right) analysis of *β*-globin protein and transcript levels in K-562 cells transfected with pT2-IHK-*β*-gene//eIF-SB10 (lanes 7 and 8), pT2-IH*β*p-*β*-gene//eIF-SB10 (lanes 5 and 6), or pT2-HS3*β*p-*β*-gene//eIF-SB10 (lanes 3 and 4). Lanes 1 and 2 contained nontransfected controls. Twenty-four hours after transfection, hemin (20 *μ*M) was added to induce *β*-globin expression. The cells were harvested for Western blot and RT-PCR analysis after incubation for an additional 48 h. The proteins and transcripts are indicated at the left. The hybrid promoter used to drive the *β*-gene in the construct and addition (+) of hemin to the cultures is shown at the top. Abbreviations: HS3*β*p, HS3 core element of *β*LCR and human *β-globin* promoter; IH*β*p, human *ALAS2* intron 8 erythroid-specific enhancer element, HS40 core element of αLCR, and human *β-globin* promoter; IHK, human *ALAS2* intron 8 erythroid-specific enhancer element, HS40 core element of αLCR, and human *ankyrin-1* promoter. *β*-Actin and GAPDH served as normalization controls.

location of the mRNA, we amplified the minimum *β*-globin gene (1.5 kb) from the transcription start site to 61 nucleotides beyond the translational stop codon. This sequence was cloned downstream of IHK, IH*β*p, and HS3*β*p in *cis SB*-Tn plasmids (Figure 3A, left).

The *β*-globin transcripts utilize the rabbit globin poly(A) signal from the pCAGGS vector. Since the three *cis SB*-Tn plasmids are similar in structure and size (8.36, 8.58, and 8.61 kb, respectively), the relative activity of the three promoters can be judged by the level of *β*-globin expression. We transfected K-562 cells with equal amounts of the three vectors. The Western blot analysis of the cell extracts showed that the IHK hybrid promoter was most efficient in driving *β*-globin expression with or without hemin induction (Figure 3B, lanes 7 and 8). Moreover, the amount of *β*-globin produced with hemin was greater than that achieved by the endogenous *γ*-globin gene (Figure 3B, lane 8). The IH*β*p promoter, using the same enhancer elements as IHK, is less

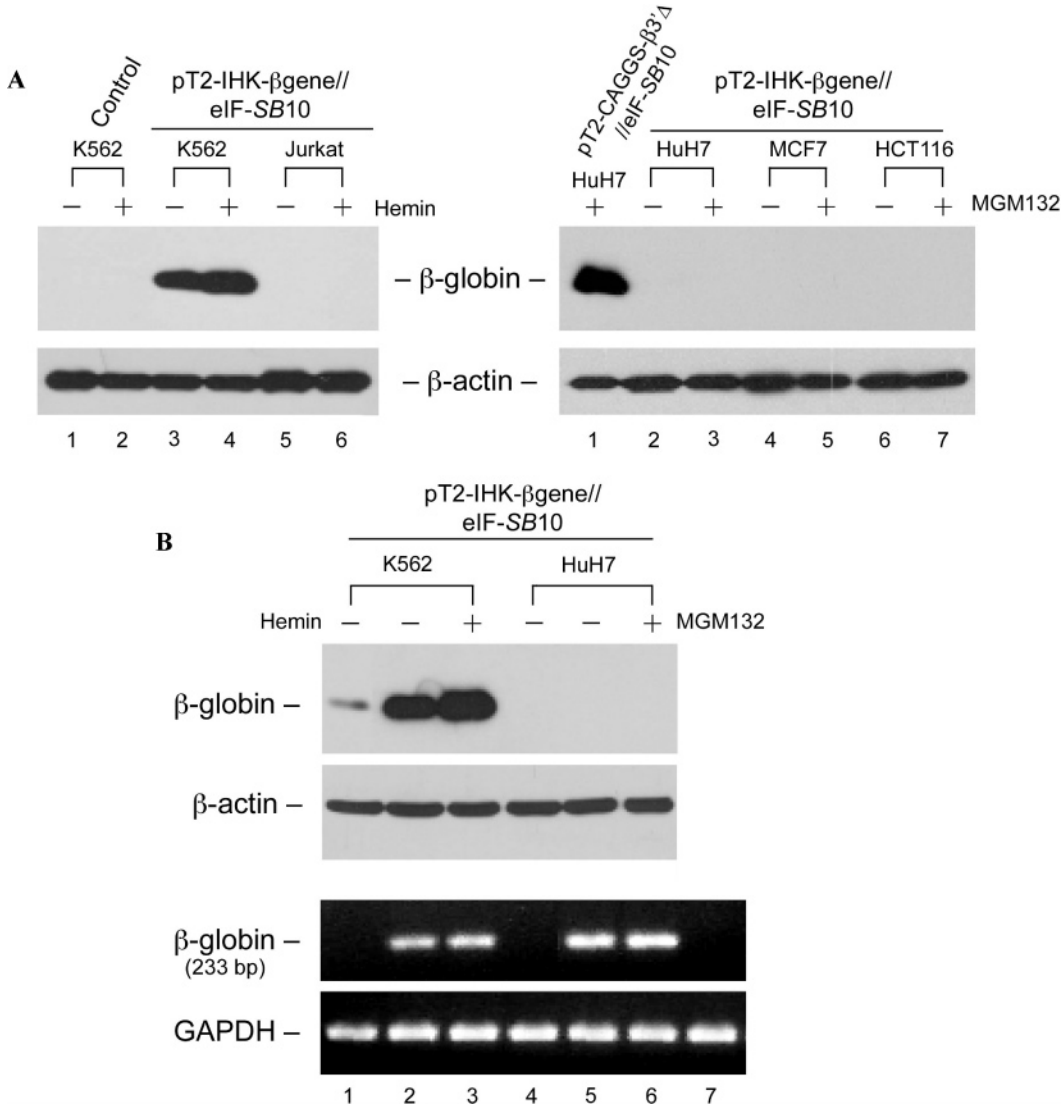

FIGURE 4: Erythroid-specific expression of β-globin by the hybrid IHK promoter. (A) Western blot analysis of cell extracts from blood origin cells, K-562 and Jurkat, transfected with pT2-IHK-β-gene//eIF-SB10, induced to express β-globin with 20 μM hemin (left), and from non-erythrocyte HuH-7, MCF7, and HCT116 cells transfected with pT2-IHK-β-gene//eIF-SB10 (lanes 2−7) or pT2-pCAGGS-β3′Δ// eIF-SB10 (lane 1) (right). MG132 (5 μM) was added 48 h post-transfection to inhibit proteasome activity, and the non-erythrocyte cells were incubated for an additional 18 h before being harvested. Total protein extracts (50 μg) from the collected cells were subjected to Western blot analysis using β-actin as an internal control for equal lane loading. The proteins that were detected are indicated in the middle, and the addition (+) of hemin or MG132 is shown above the blots. (B) Parallel Western blot (top) and RT-PCR analysis (bottom) of cell samples for K-562 and HuH-7 cells transfected with pT2-IHK-β-gene//eIF-SB10. Hemin (20 μM) was added to induce β-globin synthesis in K-562 cells and MG132 (5 μM) to inhibit proteasome activity in HuH-7 cells. Lanes 1 and 4 are nontransfected controls for K-562 and HuH-7, respectively. A RT-PCR negative control without reverse transcriptase using RNA sample from lane 6 is included (lane 7). Ten microliters of the RT-PCR (25 cycles) products was analyzed by agarose gel electrophoresis. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was amplified in parallel and served to normalize RNA sample loading. The cell line and the addition of hemin (+) or MG132 (+) to the cultures are indicated at the top.

efficient in driving β-globin expression but still comparable to the endogenous γ-globin (Figure 3B, lanes 5, 6, and 8). Surprisingly, when the enhancer elements were replaced with HS3, the most active region in producing high-level expression of β-type globin proteins (*23*), the level of β-globin expression decreased dramatically (Figure 3B, lanes 3 and 4). RT-PCR analysis (Figure 3B, right panel) indicated that the level of protein expression was determined by the transcriptional activity of the promoter. Specifically, the IHK promoter produced ~3-fold and the IHβp promoter 2.3-fold more transcripts than HS3βp ($P < 0.001$). In addition, there was no significant transcriptional activation with the addition of hemin for any of the promoters. Our data demonstrated that the *ankyrin-1* promoter is more active than the *β-globin* promoter, whereas i8, HS40 enhancer elements are stronger than HS3 when combined with the β-globin promoter in K-562 cells.

The IHK Promoter Drives Erythroid-Specific Expression of β-Globin. To further test the specificity of the IHK promoter, we transfected erythroid K-562 and non-erythroid Jurkat cells, originally derived from a T-cell lymphoma, with the *cis* pT2-IHK-β-gene *SB*-Tn. The Western blot analysis showed efficient expression of the β-globin transgene in K-562 cells as predicted (Figure 4A, left panel, lanes 3 and 4), whereas expression was undetectable in Jurkat cells (Figure 4A, left panel, lanes 5 and 6). We also transfected other non-erythroid cancer cell lines, including HuH-7, MCF7, and HCT116, with the same *cis SB*-Tn. Unlike

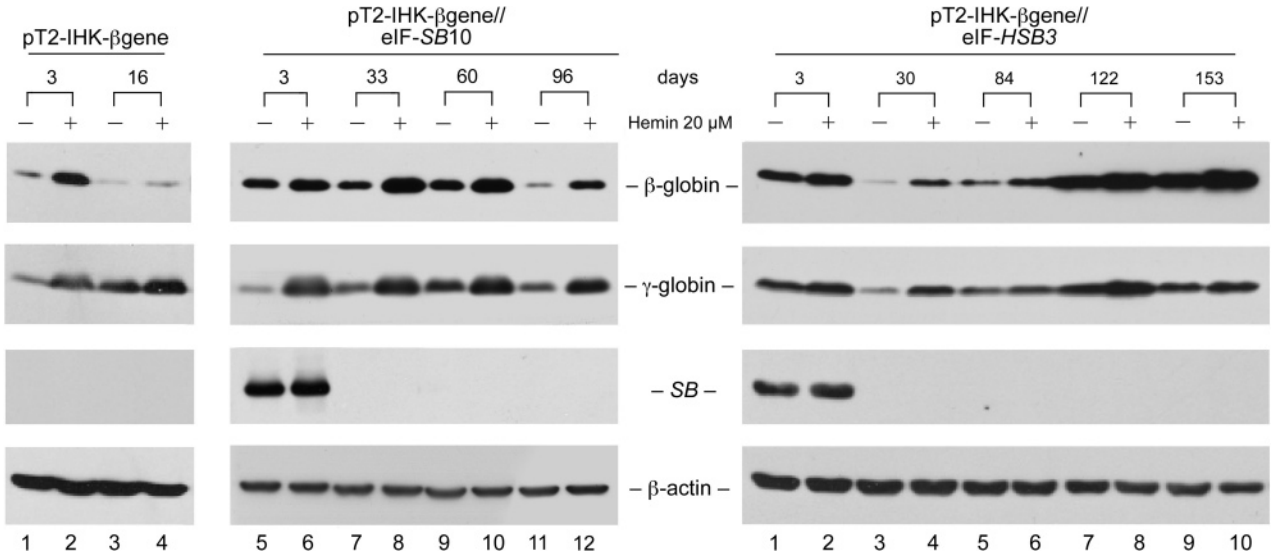

FIGURE 5: Long-term expression of β-globin mediated by SB10 and *HSB*3. Western blot analysis of β-globin expression in K-562 cells transfected with pT2-IHK-β-gene//eIF-*HSB3* (right) and pT2-IHK-β-gene//eIF-SB10 or pT2-IHK-β-gene (left). The cells were passaged every 3 days by plating 10% of cells from the previous passage. Hemin (20 μM) was included in the medium 48 h prior to the cells being harvested at each time point. Fifty micrograms of total protein was subjected to Western blot analysis, and β-actin was used to normalize sample loading. The time (days) to harvest after transfection is indicated at the top. Cell extracts from pT2-IHK-β-globin (left, lanes 1−4), pT2-IHK-β-globin//eIF-SB10 transfected cells (left, lanes 5−12), and pT2-IHK-β-globin//eIF-*HSB3* (right) are shown. The protein levels determined by Western blot analysis are shown in the middle, and the addition (+) of hemin to the culture is indicated above the panels.

β-globin expression driven by the CAGGS promoter (Figure 2A,C; Figure 4A, right panel, lane 1), none of the non-erythroid cell lines produced detectable β-globin (Figure 4, right panel, lanes 2−7), even with the proteasome inhibitor, MG132 (Figure 4, right panel, lanes 3, 5, and 7). The parallel transfection of each cell line with pT2-CAGGS-DsRed2 exhibited a similar transfection efficiency (data not shown). Interestingly, RT-PCR showed that HuH-7 cells transfected with pT2-IHK-β-gene//eIF-SB10 resulted in higher levels of β-globin transcripts than K-562 cells with or without MG132 (Figure 4B, bottom panel, lanes 2, 3, 5, and 6), although the β-globin protein was undetectable (Figure 4B, top panel, lanes 5 and 6). Thus, IHK promoter activity in the non-erythroid cells was regulated post-transcriptionally with the following order of activity: HuH-7 > HCT116 > MCF7 (data not shown).

Long-Term Expression of β-Globin with SB-Tn Transposition in K-562 Cells. We determined the effect of *SB* on long-term stable expression of β-globin in K-562 cells that are capable of differentiating into erythroid cells when induced by hemin, pyruvate, or DMSO (*24*). After transfection with the different *SB*-Tn-β-globin gene constructs, cells were passaged every 3 days by plating only 10% of the prior culture. At specific passages, hemin was added to half of the cultures 48 h before cell harvesting to induce β- or endogenous γ-globin expression. Total protein extracts from induced and non-induced cells transfected with pT2-IHK-β-gene//eIF-SB10 or pT2-IHK-β-gene were analyzed by Western blotting. The results indicated that β-globin expression was comparable to that of endogenous γ-globin 2 months after transfection with the *cis* IHK-β-gene SB10-Tn; by 3 months, it had declined to ~50% of the γ-globin levels (Figure 5, left panel, lanes 9−12). In contrast, at 2 weeks post-transfection, only a trace of β-globin was detected in cells transfected with the SB10 transposase-deleted pT2-IHK-β-globin construct (Figure 5, left panel, lane 4). Moreover, because the transposase expression cassette was located outside the IR/DRs and predicted not to integrate into the genome, no SB10 was detectable 3 days post-transfection.

Transfection with the pT2-IHK-β-gene//eIF-HSB3 plasmid containing a hyperactive transposase (*16*) increased the sustained level of expression of β-globin (Figure 5, right panel, lanes 7−10). Even with the improved transposition, *HSB*3 transposase was undetectable 3 days post-transfection (Figure 5, right panel, lanes 1 and 2). Although the latter steady-state levels were at times lower than those observed 3 days post-transfection, there was no change when normalized to the amount of endogenous γ-globin produced in the pT2-IHK-βgene//eIF-HSB3 and pT2-IHK-βgene//eIF-SB10 transfected cells. In all cases, hemin induction of the β-globin protein occurred only if the γ-globin protein was induced in parallel. The β-globin levels produced in the HSB3 and SB10 *cis* Tn transfected cells averaged 105 ± 12 and 101 ± 10%, respectively, of the level of endogenous γ-globin expression.

It is worth noting that the average transfection efficiency for K-562 cells using lipofectamine 2000 was 25−35% based on the number of positive pT2-CAGGS-DsRed2 cells in parallel transfected cultures (data not shown). The results indicate that transposition by HSB3 of the IHK-β-gene Tn in 25−35% of the cells can produce levels of β-globin comparable to or higher than that observed for the endogenous γ-globin gene in 100% of the cells. Furthermore, the >5 month expression of the integrated β-globin from the pT2-IHK-β-gene suggests that the IR/DR regions of Tn and hybrid IHK promoter do not inhibit cell growth or promote silencing of β-gene expression.

Identification of SB-β-Globin Tn Transposition Sites in the Genome of K-562 Cells. Studies aimed at confirming that the stable expression of β-globin in K-562 cells transfected with *cis* pT2-IHK-β-gene//eIF-SB10 resulted from SB10-mediated transposition were conducted. Genomic DNA was purified 4 weeks post-transfection and used as the template for inverted, nested PCR using primer pairs

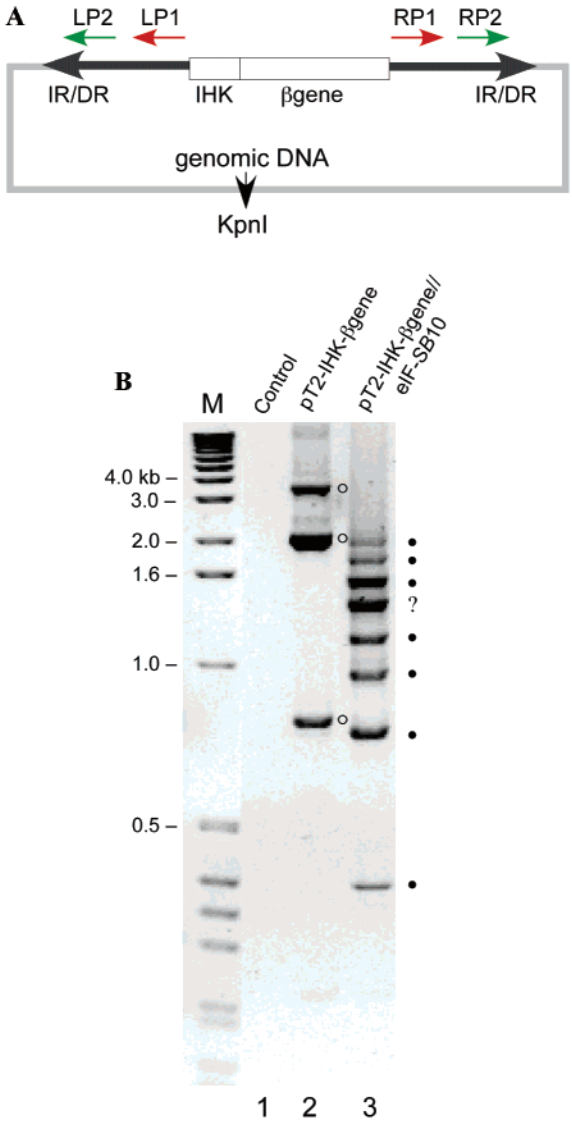

FIGURE 6: SB10-mediated β-globin transposition and identification of the insertion sites in K-562 cells transfected with pT2-IHK-β-gene//eIF-SB10 or pT2-IHK-β-gene. (A) Schematic design for inverted, nested PCR analysis. The genomic DNA samples were digested by KpnI and subjected to self-ligation. The ligated products were used as a template for the initial inverted PCR amplification with the LP1/RP1 primer pair. The second inverted PCR amplification step was performed using an LP2/RP2 internal nested primer pair. The consequent PCR products were analyzed by agarose gel electrophoresis and visualized by UV light after ethidium bromide staining. (B) Inverted, nested PCR analysis of the genomic DNA samples purified from K-562 cells 4 weeks post-transfection with an unrelated plasmid (control), pT2-IHK-β-gene, and pT2-IHK-β-gene//eIF-SB10. Amplified PCR bands from either episomal plasmid or random integration are denoted with empty circles and those from canonical SB insertion footprints with filled circles. The question mark indicates the amplified band of unknown identity. Lane M contained DNA markers with the size of the bands indicated in kilobases at the left.

derived from the Tn IR/DR region (Figure 6A). PCR products analyzed by agarose gel electrophoresis indicated that eight distinct amplicons were present with genomic DNA from pT2-IHK-β-gene//eIF-SB10 transfected cells (Figure 6B). Three PCR bands were also detected when genomic DNA from pT2-IHK-β-globin transfected cells was used as the template. No products were produced with DNA from mock-transfected cells.

To determine the identity of the PCR products, the individual bands were excised from the gel, purified, and sequenced. Analysis of sequence data from the PCR products revealed that seven of eight bands from pT2-IHK-β-gene//eIF-SB10 transfected cells [Figure 6B (●)] had the canonical SB insertional footprint consisting of the Tn IR/DR, duplicated TA, and flanking host genomic DNA (*7*). One band [Figure 6B (?)] did not match any sequence in the human genome or in the nonredundant nucleotide sequence database of GenBank (NCBI). In contrast, the three bands from pT2-IHK-β-gene transfected cells were derived from either episomal fragments of the plasmid or random integration [Figure 6B (○)]. A total of 20 flanking genomic DNA sequences were recovered from two independent transfections, and the insertion sites were distributed over 15 chromosomes, with 13 transpositions in regions not associated with identified genes and seven intronic insertions (Figure 7). Alignment of the 200 bp of flanking sequences immediately adjacent to the insertion site indicated no significant similarity other than it being TA-rich, consistent with the previous reports of the random nature of SB-mediated transposition (*7*, *25*).

Determination of the Specificity of the IHK-β-Globin-Tn Construct in Adult Erythroid Cells. To determine the expression pattern of the IHK-β-gene Tn in an adult erythroid cell, we used the murine erythroid leukemia (MEL) CLL-745 cell line. In this well-established model, differentiation of the proerythroid MEL cells to adult erythroid cells is accomplished by addition of DMSO to the medium (*18*). We examined the specificity of three different promoter constructs for the β-globin gene in both the proerythroid and adult erythroid states of the MEL cells (Figure 8). Only the induced MEL cells expressed β-globin protein, and in contrast to K-562 cells, each of the three promoters functioned efficiently and produced equivalent steady-state levels (Figure 8, top right panel). RT-PCR analysis indicated that the three different promoters resulted in equivalent levels of the β-globin transcript in the induced cells (Figure 8, bottom right panels) and gave a 1.8 ± 0.06-fold increase over their respective levels in the non-induced cells ($P < 0.001$). In contrast, analysis of the non-induced MEL cells by RT-PCR indicated a significantly ($P < 0.001$) decreased level of transcripts with HS3βp. The absolute level of β-globin transcript, however, when normalized with GAPDH in the non-induced MEL cells from the IHβp and IHK constructs did not differ significantly from that of the same promoters in K-562 cells (Figure 3B, right panel), but without detectable protein. Taken together, the data indicated that β-globin protein expression is controlled, in part, at the translational level during differentiation. However, the significant up-regulation in the induced MEL cells suggests that factors affecting erythroid promoter function also modulate β-globin gene expression in adult erythroid cells.

DISCUSSION

There have been several reports on the success of mini-LCRs combined with β-globin promoter in achieving high-level β-globin expression in viral vector-based systems, both in vitro and in vivo (*5*, *26*). This is in contrast to two decades of studies indicating that β-globin can be efficiently expressed only from its full-length gene (*27*). However, even a 6.5 kb β-globin expression cassette for viral vectors adopted

Flanking sequences at insertion sites	Insertion site
IR/DR(L)	
ACTTCAACTGTACATTCTATTGATTGATTGCTACAAAGTAAACTATCCCA	6q21
ACTTCAACTGTAAAAGAGTATTTTTCATTATTTCCACTAAATATTGAAAG	17q22 *
ACTTCAACTGTATATTCTTACAAGAGCCTTCTGGAGACTGAATACTTTTT	22q12.3
ACTTCAACTGTAGGTGACAAGGACGTCTTAGAGCAATGGACTGTCTGCTC	10p15.3 *
ACTTCAACTGTAAGTGTTGTTGTTAACCTTAAGTTCCATTTAATTATTTG	7q34
ACTTCAACTGTAACTACTGAATGCTTGAAGTGTGGCTACTGTAATGGAGG	4p14
ACTTCAACTGTATATATTTCATAATATCACAGCTCCCCTCACCTGGGGGA	2p13
ACTTCAAGTGTATGTAAACTTCCGACTTCAACTGTAAATGGGAGCTAAATA	5q23.1
IR/DR(R)	
ACTTCAACTGTAAATATGGTACATTTGTCGCAATCAATGAATCAATAATG	1q25
ACTTCAACTGTATTTCATAACACTAGTGTGAGGATTAAATAACATAAAAA	3q22.3 *
ACTTCAACTGTACTCTGAATAATCTTCATTTACAGCACTTTTATTAACAG	20q13
ACTTCAACTGTAAATTAATTAAGAGAAGAATTCAGGGAGAAATAGAATGT	6p23 *
ACTTCAACTGTATCTAATCCCCATATCTACTATTGTAAAATTTTTTTCTG	Xp22
ACTTCAACTGTAGAAGGTACCCTC ACCTCAGCTACAGCAAGTGAGTCTG	7P11.2 *
ACTTCAACTGTAATTTGTATTGAAATGCTGCAAAAACCTTCATAGCAACAT	15q13.1 *
ACTTCAACTGTATGTGACAGAAAACTTTTATCATAAACATGTTCAAAAAATC	1p35.2
ACTTCAACTGTACCTATCCGACAAAGATCTAATATCCAGAATCTACAAGGA	4p14
ACTTCAACTGTATGTTTATCAGGGATTTTGGTCTGTAGTTTTCTTTTTTGGTT	6p14.1
ACTTCAACTGTAGTGCTATAAATTTCCCTCTACACACTGCTTTAAATGTGTC	9q32
ACTTCAACTGTATAATTCCAGCTATTTGGGAGGCTGAGGCAGGAGAATCGC	9q34.12 *

FIGURE 7: Identification of the insertion sites in K-562 cells transfected with pT2-IHK-β-gene//eIF-SB10. The amplicons resulting from the second inverted PCRs were isolated from the agarose gel and sequenced directly using the RP2 or LP2 primer as described in Materials and Methods. The identified flanking genomic sequences of the insertion sites are displayed. The regions in the ID/DR of the transposon and the requisite duplicated TA are shown in bold letters. The chromosomal location established by BLAST analysis is shown at the right, and the intronic insertion sites are marked with asterisks.

by the *SB*-Tn system would result in a significantly reduced transposition efficiency (*28*). Thus, our study was designed to construct an efficient erythroid-specific β-globin expression cassette with minimal size for high-level SB delivery and transposition into cells.

We initially tested the possibility of using β-globin cDNA (640 bp) to express the protein. Only one previous study demonstrated the expression of β-globin transcripts in HEK293 cells using a plasmid system (*29*). Moreover, it has been reported that the β-globin gene introns are critical for transcript stabilization and cytoplasmic translocation of the mRNA (*30*). Our results demonstrated that β-globin mRNA transcribed from its cDNA by the strong hybrid CAGGS promoter is, in fact, functional in both erythroid and non-erythroid cell lines. In fact, the CAGGS promoter (*15*) includes an intron derived from fusion of the first chicken β-actin intron and a second intron from rabbit globin, thus perhaps fulfilling a mRNA processing requirement necessary for the accumulation of the β-globin transcript. Our results also demonstrated that the 5′ UTR of the β-globin cDNA is essential for efficient high-level expression of the protein. This observation is consistent with previous studies on β-thalassemia, in which a single-base deletion at position +10 in the β-globin 5′ UTR resulted in a translational defect (*31*). Interestingly, hemin increased the β-globin levels in both erythroid K-562 and non-erythroid U87 and HCT116 cells. This suggests that even with the additional 5′ UTR sequences from β-actin exon 1, translational activity of the hybrid transcript is regulated by hemin in a manner similar to that of endogenously produced β-globin transcripts in erythrocytes (*32*). The expression of β-globin protein in HuH-7, MCF7, and Daoy cells was detected only with the proteasome inhibitor, MG132, suggesting a significant role for post-translational regulation of tissue specificity for globin synthesis. This minimal reported 1.6 kb CAGGS-β3′Δ β-globin cassette was predicted to result in the most efficient transposition with SB (*11*).

Previous studies demonstrated that the βLCR HS core elements synergize to confer high-level and position-independent gene expression. Thus, the HS2, HS3, and HS4 core elements have been used frequently as enhancers in conventional viral vector design. However, the complex role that these regulatory elements play in on−off switching of β-like globin gene expression during development suggests they could also silence the transgene based on chromatin structure. In fact, the human β-globin mini-LCR (HS4−HS3−HS2 core elements) silences promoter expression when viral vector insertion is in a nonpermissive orientation (*33*) while simultaneously inducing transcription in flanking regions of the genome. However, a larger mini-LCR confers greater stability and stronger β-globin transgene expression as the addition of a HS3−HS2 flanking region increased activity by 200−300% over HS3−HS2 core elements alone (*34*).

To mitigate the potential side effects from mini-LCR as well as minimize the size of the transposon, we constructed and tested IHK, IHβp, and HS3βp as alternative erythroid-specific hybrid promoters. *ALAS*2 is a major regulatory enzyme in erythrocytes and is active at the first step of the heme biosynthetic pathway (*35*). Thus, we chose the ALAS2 intron 8 erythroid-specific enhancer element (i8, 250 bp) so that β-globin expression from the integrated transgene was responsive to cellular environmental change (*36*). The αLCR HS40 core element was selected because it is capable of enhancing the activity of a heterologous promoter such as the viral SV40 promoter or γ-globin promoter in an erythroid-specific manner (*37, 38*). The minimum 271 bp *ankyrin-1* promoter contains all of the sequences necessary and

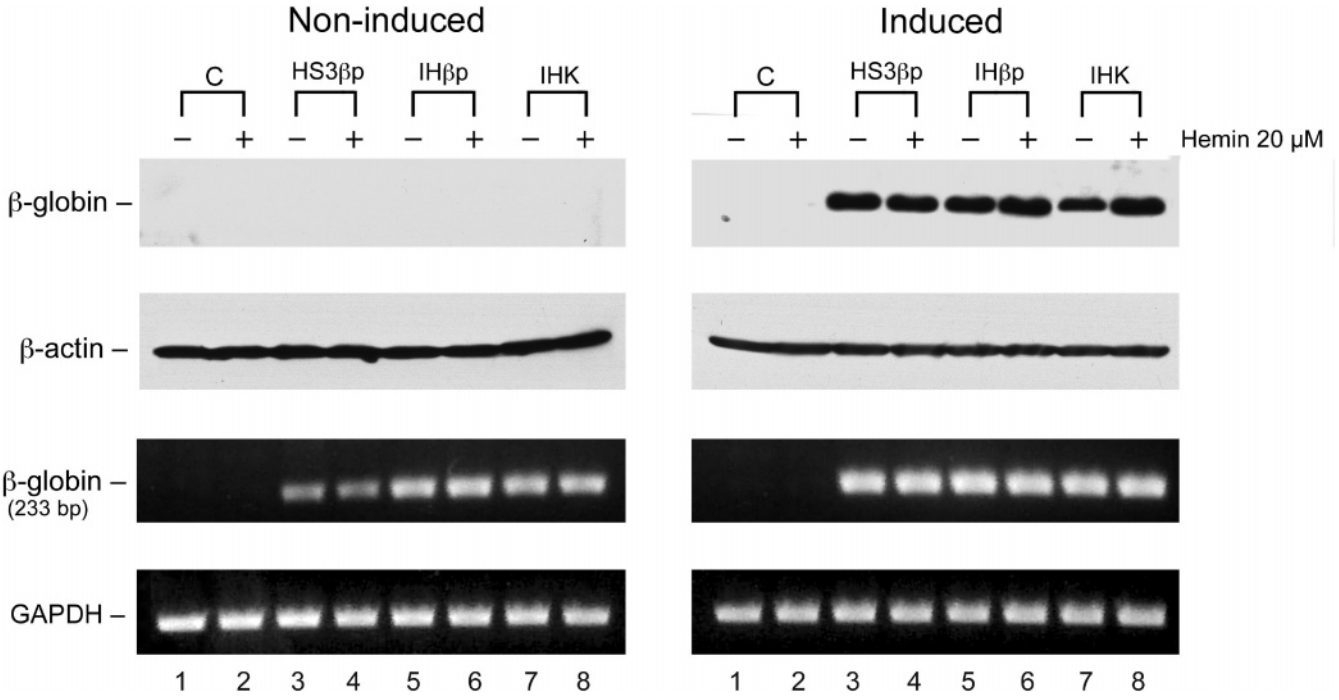

FIGURE 8: Expression of the different *cis* β-gene//eIF-SB10 constructs in adult erythroid cells. Proerythroid MEL cells were differentiated to adult erythroid cells by addition of DMSO to the medium 56 h prior to transfection. After induction with hemin 24 h post-transfection, the cells were harvested 48 h later and processed for Western blot and RT-PCR analysis as described in Materials and Methods. Noninduced "proerythroid" MEL cell (left) Western blots of β-globin and β-actin are shown in the top two panels, while the agarose gels of RT-PCR analysis of β-globin and GAPDH transcript levels are shown below. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was amplified in parallel and served to normalize RNA sample loading. β-Actin was used as a lane loading control for the Western blots. The expression levels in the adult erythroid cells of the same constructs by Western blot (top right panels) and RT-PCR analysis (bottom right panels) demonstrated robust expression by all three promoter constructs. The erythroid promoter used to drive the β-gene and addition (+) of hemin to the cultures is indicated at the top. The protein and transcript are shown at the right with the predicted size of the β-globin amplicon given in parentheses. Abbreviations: HS3βp, HS3 core element of βLCR and human β-globin promoter; IHβp, human ALAS2 intron 8 erythroid-specific enhancer element, HS40 core element of αLCR, and human β-globin promoter; IHK, human ALAS2 intron 8 erythroid-specific enhancer element, HS40 core element of αLCR, and human ankyrin-1 promoter.

sufficient for position-independent, copy number-dependent erythroid-specific expression of a linked transgene (*39*). However, when i8 and HS40 core elements were linked with the *ankryn-1* minimal promoter, transcriptional activity was greatly increased (*5*). Our data showed that the level of β-globin expression driven by the hybrid promoters increased in the following order in all the cell types that were examined but adult erythroid cells: IHK > IHβp > HS3βp. As the other regions of the vectors were identical, it indicated that the *ankyrin-1* minimum promoter is stronger than β-globin's, and the IH enhancer is significantly more powerful than HS3. Interestingly, the IHK promoter drives synthesis of β-globin mRNA in the non-erythroid HuH-7 cells and to a smaller extent in HCT116 cells (data not shown). This may result from certain transcription factors such as GATA-1 and BKLF, which activate minimal *ankyrin-1* promoter (*14*) and are expressed in hepatoma cells (*40*). Moreover, in transgenic mice, the minimal *ankyrin-1* promoter produced low levels of transcripts in both liver and lung (*26*). However, despite the robust mRNA levels, there was no detectable β-globin protein expression in the non-erythroid cells, consistent with previous reports that IHK is erythroid-specific in both cell culture and in vivo (*5*).

Unlike K-562 cells, β-globin protein expression was detected only in DMSO-induced MEL cells, suggesting that differentiated erythroid cells alone have the necessary factors for translation of β-globin transcripts. Moreover, in the adult erythroid cells, each of the three hybrid promoters was robustly activated at the transcriptional level and expressed equivalent steady-state levels of both β-globin transcript and protein, suggesting their potential for sustained expression following SB transposition.

It was somewhat surprising that β-globin transcripts expressed by the constitutive CAGGS promoter engaged the translational machinery in non-erythroid cells while those produced by the IHK promoter in the same cells did not. The regulatory role of hemin-induced translation and post-translational mechanisms, such as proteasomal degradation for erythroid-specific expression of globin, is known (*19, 20*). However, our results also suggest a potential role of the promoter in regulating the translational machinery of the cell independent of the transgene.

Typical β-globin gene constructs containing a 3′ enhancer region are 3.5−4.5 kb in size (*34, 41*). However, it has been reported that the 3′ enhancer is not required for high-level expression of β-globin from a mini-LCR/β-globin gene construct (*30*). Thus, we used only 1.54 kb of the β-globin gene from its canonical transcription start site to 61 nucleotides beyond the translational stop codon utilizing the poly(A) signal from the original CAGGS-β3′Δ expression vector. The size of the entire IHK-β-globin transgene is only 3.1 kb, which is the smallest structure reported for sustained high-level erythroid-specific expression of β-globin. It is worth noting that with viral vector expression systems the function of the β-globin promoter absolutely requires the βLCR core elements to overcome silencing from both viral

elements and chromosomal position effects. Our results clearly established that the β-globin promoter copes well with non-βLCR enhancer elements in the *SB*-Tn system for stable efficient expression of β-globin, suggesting i8 in conjunction with HS40 confers position-independent gene expresssion. This also suggests that IR/DR regions of *SB*-Tn have no apparent silencing effect on transgene expression following transposition into the genome (data not shown).

Any DNA element that integrates into the host cell genome has the potential for insertional mutagenesis. Recent studies have shown insertional oncogenesis in *SB*-Tn (T2/Onc) transgenic mice (*42*, *43*). However, this was accomplished with a *SB*-Tn specifically designed for this purpose carrying a splice acceptor/poly(A) signal in both orientations at each end flanking the strong MSCV promoter from the 5′ long terminal repeat of the murine stem cell virus coupled to a splice donor. This construct was intended to truncate the protein expression in both orientations following Tn transposition into an intronic region of a gene or activate expression of a downstream gene if the integration was intergenic, therefore tripling the mutagenesis potential following genomic insertion. In addition, Arf-/- mice, animals deficient for the p53 pathway regulator and tumor suppressor p19Arf, or Rosa26-SB10 transgenic mice were required to produce mice carrying tumors. However, the natural occurrence of adverse events leading to tumor genesis mediated by *SB*-Tn transposition appears to be very low. A significant advantage of *SB* transposase-mediated genomic insertion over vectors derived from viruses is that it shows no substantial DNA sequence preference for transcribed genomic regions (*7*, *25*). Biased insertion of viral vectors into transcriptionally active regions of the mammalian genome is a major concern in gene therapy (*44*−*46*). In particular, oncogenesis is associated with both retro- and lentiviral vectors administered in utero and neonatally (*47*). Moreover, the leukemia observed in hematopoietic stem cells (HSCs), the proposed target cell type for SCD gene therapy (*4*, *48*), is correlated with the apparent integration preference by viral vectors for specific genomic regions. In contrast, the β-globin Tns were located in AT-rich regions, with the genomic insertion sites exhibiting the expected distribution of the gene (∼33%) and nongene (∼66%) chromosomal segments (*8*, *25*). The preference of SB for AT-rich regions may direct the insertions in the actively transcribed regions to the introns, preventing exon disruption (*49*, *50*). In fact, others have reported a similar dramatic bias (96%) in *SB*-Tn transposition events into introns rather than exons in the region of the genome transcribed in humans (*25*). Moreover, the essential IR/DRs of *SB*-Tn do not activate undesirable transcription of flanking genes (data not shown), which has been observed for terminal repeats of viral vectors (*4*).

It is estimated that at least 15−20% of HSCs are required to take up and stably incorporate the wild-type β-globin gene to reach therapeutic levels for gene therapy of SCD. For this purpose, the *SB*-Tn system offers an efficient nonviral replicatively stable method of genomic transgene insertion. Our results demonstrated that an *SB*-β-globin-expressing transgene could be introduced and precisely inserted into random TA dinucleotides in the genome of an erythroid cell line. The ability to express β-globin for more than 50 passages over a 5 month period fulfills an essential requirement for any HSC-targeted gene therapy, inheritance and expression of the transgene by the daughter cells. However, the efficiency for delivering nonviral plasmids into HSCs or directly into animals is much lower than with engineered viral vectors. This difficulty was recently reported with the *Sleeping Beauty* system which enabled stable gene transfer in HSCs, albeit with an exceedingly low overall frequency and an associated high level of cell death (*51*). Various strategies, including expression of wild-type γ-globin and a β/γ globin hybrid β-globin T87Q (*52*−*54*), have been investigated as alternatives for gene therapy of SCD to compensate for low transfection efficiencies of HSCs. These alternate globins exhibit a significantly increased affinity for α-globin compared to that of the wild-type protein in forming functional hemoglobin as well as inhibiting the polymerization of sickle hemoglobin more effectively than the β-globin chain itself. On the basis of clinical studies, it has been calculated that γ-globin gene expression at ∼10−20% of the adult β-globin level would significantly reduce the symptoms associated with SCD (*55*). The long-term efficient expression of β-globin, at levels comparable to that of endogenous γ-globin with only ∼30% transfection efficiency, suggests that the use of IHK-driven γ- or β-globin T87Q-expressing transgenes coupled with optimized ex vivo transfection of HSCs with *cis HSB3*-expressing *SB*-Tn's may provide levels of "normal" hemoglobin sufficient to mitigate the clinical manifestations of SCD.

ACKNOWLEDGMENT

We are grateful to Wenxuan Yin and Xiaoming Ma for technical assistance and thank Carol L. Bruzzone and Brian Gibbens for critical reading of the manuscript.

REFERENCES

1. Thomas, C. E., Ehrhardt, A., and Kay, M. A. (2003) Progress and problems with the use of viral vectors for gene therapy, *Nat. Rev. Genet. 4*, 346−358.
2. Pawliuk, R., Westerman, K. A., Fabry, M. E., Payen, E., Tighe, R., Bouhassira, E. E., Acharya, S. A., Ellis, J., London, I. M., Eaves, C. J., Humphries, R. K., Beuzard, Y., Nagel, R. L., and Leboulch, P. (2001) Correction of sickle cell disease in transgenic mouse models by gene therapy, *Science 294*, 2368−2371.
3. Mohamedali, A., Moreau-Gaudry, F., Richard, E., Xia, P., Nolta, J., and Malik, P. (2004) Self-inactivating lentiviral vectors resist proviral methylation but do not confer position-independent expression in hematopoietic stem cells, *Mol. Ther. 10*, 249−259.
4. Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M. P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C. S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J. I., de Saint Basile, G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet, D., Romana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L. E., Wissler, M., Prinz, C., Rabbitts, T. H., Le Deist, F., Fischer, A., and Cavazzana-Calvo, M. (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1, *Science 302*, 415−419.
5. Moreau-Gaudry, F., Xia, P., Jiang, G., Perelman, N. P., Bauer, G., Ellis, J., Surinya, K. H., Mavilio, F., Shen, C. K., and Malik, P. (2001) High-level erythroid-specific gene expression in primary human and murine hematopoietic cells with self-inactivating lentiviral vectors, *Blood 98*, 2664−2672.
6. Qing, K., Hansen, J., Weigel-Kelley, K. A., Tan, M., Zhou, S., and Srivastava, A. (2001) Adeno-associated virus type 2-mediated gene transfer: Role of cellular FKBP52 protein in transgene expression, *J. Virol. 75*, 8968−8976.
7. Ivics, Z., Hackett, P. B., Plasterk, R. H., and Izsvák, Z. (1997) Molecular reconstruction of *Sleeping Beauty*, a *Tc1*-like transposon from fish, and its transposition in human cells, *Cell 91*, 501−510.

8. Dupuy, A. J., Clark, K., Carlson, C. M., Fritz, S., Davidson, A. E., Markley, K. M., Finley, K., Fletcher, C. F., Ekker, S. C., Hackett, P. B., Horn, S., and Largaespada, D. A. (2002) Mammalian germ-line transgenesis by transposition, *Proc. Natl. Acad. Sci. U.S.A. 99*, 4495−4499.
9. Yant, S. R., Meuse, L., Chiu, W., Ivics, Z., Izsvák, Z., and Kay, M. A. (2000) Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system, *Nat. Genet. 25*, 35−41.
10. Huang, X., Wilber, A. C., Bao, L., Tuong, D., Tolar, J., Orchard, P. J., Levine, B. L., June, C. H., McIvor, R. S., Blazar, B. R., and Zhou, X. (2006) Stable gene transfer and expression in human primary T cells by the *Sleeping Beauty* transposon system, *Blood 107*, 483−491.
11. Geurts, A. M., Yang, Y., Clark, K. J., Liu, G., Cui, Z., Dupuy, A. J., Bell, J. B., Largaespada, D. A., and Hackett, P. B. (2003) Gene transfer into genomes of human cells by the *Sleeping Beauty* transposon system, *Mol. Ther. 8*, 108−117.
12. Kren, B. T., Gosh, S. S., Linehan, C. L., Roy-Chowdhury, N., Hackett, P. B., Roy-Chowdhury, J., and Steer, C. J. (2003) Hepatocyte-targeted delivery of *Sleeping Beauty* mediates efficient transposition *in vivo*, *Gene Ther. Mol. Biol. 7*, 231−240.
13. Mikkelsen, J. G., Yant, S. R., Meuse, L., Huang, Z., Xu, H., and Kay, M. A. (2003) Helper-Independent *Sleeping Beauty* transposon-transposase vectors for efficient nonviral gene delivery and persistent gene expression *in vivo*, *Mol. Ther. 8*, 654−665.
14. Gallagher, P. G., Romana, M., Tse, W. T., Lux, S. E., and Forget, B. G. (2000) The human ankyrin-1 gene is selectively transcribed in erythroid cell lines despite the presence of a housekeeping-like promoter, *Blood 96*, 1136−1143.
15. Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. (1997) 'Green mice' as a source of ubiquitous green cells, *FEBS Lett. 407*, 313−319.
16. Yant, S. R., Park, J., Huang, Y., Mikkelsen, J. G., and Kay, M. A. (2004) Mutational analysis of the N-terminal DNA-binding domain of *Sleeping Beauty* transposase: Critical residues for DNA binding and hyperactivity in mammalian cells, *Mol. Cell. Biol. 24*, 9239−9247.
17. Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T., and Sato, J. (1982) Growth of human hepatoma cells lines with differentiated functions in chemically defined medium, *Cancer Res. 42*, 3858−3863.
18. Glauber, J. G., Wandersee, N. J., Little, J. A., and Ginder, G. D. (1991) 5′-Flanking sequences mediate butyrate stimulation of embryonic globin gene expression in adult erythroid cells, *Mol. Cell. Biol. 11*, 4690−4697.
19. Kollia, P., Noguchi, C. T., Fibach, E., Loukopoulos, D., and Schechter, A. N. (1997) Modulation of globin gene expression in cultured erythroid precursors derived from normal individuals: Transcriptional and posttranscriptional regulation by hemin, *Proc. Assoc. Am. Physicians 109*, 420−428.
20. Shaeffer, J. R., and Cohen, R. E. (1997) Ubiquitin aldehyde increases adenosine triphosphate-dependent proteolysis of hemoglobin α-subunits in β-thalassemic hemolysates, *Blood 90*, 1300−1308.
21. Tawa, N. E., Jr., Odessey, R., and Goldberg, A. L. (1997) Inhibitors of the proteasome reduce the accelerated proteolysis in atrophying rat skeletal muscles, *J. Clin. Invest. 100*, 197−203.
22. Wagenknecht, B., Hermisson, M., Groscurth, P., Liston, P., Krammer, P. H., and Weller, M. (2000) Proteasome inhibitor-induced apoptosis of glioma cells involves the processing of multiple caspases and cytochrome c release, *J. Neurochem. 75*, 2288−2297.
23. Brittain, T. (2002) Molecular aspects of embryonic hemoglobin function, *Mol. Aspects Med. 23*, 293−342.
24. Lozzio, B. B., Lozzio, C. B., Bamberger, E. G., and Feliu, A. S. (1981) A multipotential leukemia cell line (K-562) of human origin, *Proc. Soc. Exp. Biol. Med. 166*, 546−550.
25. Vigdal, T. J., Kaufman, C. D., Izsvák, Z., Voytas, D. F., and Ivics, Z. (2002) Common physical properties of DNA affecting target site selection of *Sleeping Beauty* and other Tc1/mariner transposable elements, *J. Mol. Biol. 323*, 441−452.
26. Sabatino, D. E., Wong, C., Cline, A. P., Pyle, L., Garrett, L. J., Gallagher, P. G., and Bodine, D. M. (2000) A minimal ankyrin promoter linked to a human γ-globin gene demonstrates erythroid specific copy number dependent expression with minimal position or enhancer dependence in transgenic mice, *J. Biol. Chem. 275*, 28549−28554.
27. Buchman, A. R., and Berg, P. (1988) Comparison of intron-dependent and intron-independent gene expression, *Mol. Cell. Biol. 8*, 4395−4405.
28. Izsvák, Z., Ivics, Z., and Plasterk, R. H. (2000) *Sleeping Beauty*, a wide host-range transposon vector for genetic transformation in vertebrates, *J. Mol. Biol. 302*, 93−102.
29. Dixit, M., Webb, M. S., Smart, W. C., and Ohi, S. (1991) Construction and expression of a recombinant adeno-associated virus that harbors a human β-globin-encoding cDNA, *Gene 104*, 253−257.
30. Collis, P., Antoniou, M., and Grosveld, F. (1990) Definition of the minimal requirements within the human β-globin gene and the dominant control region for high level expression, *EMBO J. 9*, 233−240.
31. Ho, P. J., Sloane-Stanley, J., Athanassiadou, A., Wood, W. G., and Thein, S. L. (1999) An in vitro system for expression analysis of mutations of the β-globin gene: Validation and application to two mutations in the 5′ UTR, *Br. J. Haematol. 106*, 938−947.
32. Kollia, P., Fibach, E., Politou, M., Noguchi, C. T., Schechter, A. N., and Loukopoulos, D. (1998) Hydroxyurea and hemin affect both the transcriptional and post-transcriptional mechanisms of some globin genes in human adult erythroid cells, *Ann. N.Y. Acad. Sci. 850*, 449−451.
33. Feng, Y. Q., Warin, R., Li, T., Olivier, E., Besse, A., Lobell, A., Fu, H., Lin, C. M., Aladjem, M. I., and Bouhassira, E. E. (2005) The human β-globin locus control region can silence as well as activate gene expression, *Mol. Cell. Biol. 25*, 3864−3874.
34. Kang, S. H., Levings, P. P., Andersen, F., Laipis, P. J., Berns, K. I., Zori, R. T., and Bungert, J. (2004) Locus control region elements HS2 and HS3 in combination with chromatin boundaries confer high-level expression of a human β-globin transgene in a centromeric region, *Genes Cells 9*, 1043−1053.
35. May, B. K., Dogra, S. C., Sadlon, T. J., Bhasker, C. R., Cox, T. C., and Bottomley, S. S. (1995) Molecular regulation of heme biosynthesis in higher vertebrates, *Prog. Nucleic Acid Res. Mol. Biol. 51*, 1−51.
36. Bottomley, S. S., May, B. K., Cox, T. C., Cotter, P. D., and Bishop, D. F. (1995) Molecular defects of erythroid 5-aminolevulinate synthase in X-linked sideroblastic anemia, *J. Bioenerg. Biomembr. 27*, 161−168.
37. Ren, S., Luo, X. N., and Atweh, G. F. (1993) The major regulatory element upstream of the α-globin gene has classical and inducible enhancer activity, *Blood 81*, 1058−1066.
38. Ren, S., Wong, B. Y., Li, J., Luo, X. N., Wong, P. M., and Atweh, G. F. (1996) Production of genetically stable high-titer retroviral vectors that carry a human γ-globin gene under the control of the α-globin locus control region, *Blood 87*, 2518−2524.
39. Sabatino, D. E., Seidel, N. E., Aviles-Mendoza, G. J., Cline, A. P., Anderson, S. M., Gallagher, P. G., and Bodine, D. M. (2000) Long-term expression of γ-globin mRNA in mouse erythrocytes from retrovirus vectors containing the human γ-globin gene fused to the ankyrin-1 promoter, *Proc. Natl. Acad. Sci. U.S.A. 97*, 13294−13299.
40. Subrata, L. S., Lowes, K. N., Olynyk, J. K., Yeoh, G. C., Quail, E. A., and Abraham, L. J. (2005) Hepatic expression of the tumor necrosis factor family member lymphotoxin-β is regulated by interleukin (IL)-6 and IL-1β: Transcriptional control mechanisms in oval cells and hepatoma cell lines, *Liver Int. 25*, 633−646.
41. Jackson, D. A., McDowell, J. C., and Dean, A. (2003) β-Globin locus control region HS2 and HS3 interact structurally and functionally, *Nucleic Acids Res. 31*, 1180−1190.
42. Collier, L. S., Carlson, C. M., Ravimohan, S., Dupuy, A. J., and Largaespada, D. A. (2005) Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse, *Nature 436*, 272−276.
43. Dupuy, A. J., Akagi, K., Largaespada, D. A., Copeland, N. G., and Jenkins, N. A. (2005) Mammalian mutagenesis using a highly mobile somatic *Sleeping Beauty* transposon system, *Nature 436*, 221−226.
44. Mitchell, R. S., Beitzel, B. F., Schroder, A. R., Shinn, P., Chen, H., Berry, C. C., Ecker, J. R., and Bushman, F. D. (2004) Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences, *PLoS Biol. 2*, E234.
45. Nakai, H., Montini, E., Fuess, S., Storm, T. A., Grompe, M., and Kay, M. A. (2003) AAV serotype 2 vectors preferentially integrate into active genes in mice, *Nat. Genet. 34*, 297−302.
46. Heller, H., Kammer, C., Wilgenbus, P., and Doerfler, W. (1995) Chromosomal insertion of foreign (adenovirus type 12, plasmid,

or bacteriophage λ) DNA is associated with enhanced methylation of cellular DNA segments, *Proc. Natl. Acad. Sci. U.S.A. 92*, 5515−5519.
47. Themis, M., Waddington, S. N., Schmidt, M., von Kalle, C., Wang, Y., Al-Allaf, F., Gregory, L. G., Nivsarkar, M., Themis, M., Holder, M. V., Buckley, S. M., Dighe, N., Ruthe, A. T., Mistry, A., Bigger, B., Rahim, A., Nguyen, T. H., Trono, D., Thrasher, A. J., and Coutelle, C. (2005) Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice, *Mol. Ther. 12*, 763−771.
48. Imren, S., Fabry, M. E., Westerman, K. A., Pawliuk, R., Tang, P., Rosten, P. M., Nagel, R. L., Leboulch, P., Eaves, C. J., and Humphries, R. K. (2004) High-level β-globin expression and preferred intragenic integration after lentiviral transduction of human cord blood stem cells, *J. Clin. Invest. 114*, 953−962.
49. Louie, E., Ott, J., and Majewski, J. (2003) Nucleotide frequency variation across human genes, *Genome Res. 13*, 2594−2601.
50. Winnard, P., Sidell, B. D., and Vayda, M. E. (2002) Teleost introns are characterized by a high A+T content, *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 133*, 155−161.
51. Hollis, R. P., Nightingale, S. J., Wang, X., Pepper, K. A., Yu, X.-J., Barsky, L., Crooks, G. M., and Kohn, D. B. (2006) Stable gene transfer to human CD34$^+$ hematopoietic cells using the *Sleeping Beauty* transposon, *Exp. Hematol. 34*, 1333−1343.
52. Persons, D. A., Hargrove, P. W., Allay, E. R., Hanawa, H., and Nienhuis, A. W. (2003) The degree of phenotypic correction of murine β-thalassemia intermedia following lentiviral-mediated transfer of a human γ-globin gene is influenced by chromosomal position effects and vector copy number, *Blood 101*, 2175−2183.
53. Tisdale, J., and Sadelain, M. (2001) Toward gene therapy for disorders of globin synthesis, *Semin. Hematol. 38*, 382−392.
54. Miller, J. L., Donahue, R. E., Sellers, S. E., Samulski, R. J., Young, N. S., and Nienhuis, A. W. (1994) Recombinant adeno-associated virus (rAAV)-mediated expression of a human γ-globin gene in human progenitor-derived erythroid cells, *Proc. Natl. Acad. Sci. U.S.A. 91*, 10183−10187.
55. Noguchi, C. T., Rodgers, G. P., Serjeant, G., and Schechter, A. N. (1988) Levels of fetal hemoglobin necessary for treatment of sickle cell disease, *N. Engl. J. Med. 318*, 96−99.

BI6024484