Histone Deacetylase 9 Activates γ-Globin Gene Expression in Primary Erythroid Cells*

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Strategies to induce fetal hemoglobin (HbF) synthesis for the treatment of β-hemoglobinopathies probably involve protein modifications by histone deacetylases (HDACs) that mediate γ-globin gene regulation. However, the role of individual HDACs in globin gene expression is not very well understood; thus, the focus of our study was to identify HDACs involved in γ-globin activation. K562 erythroleukemia cells treated with the HbF inducers hemin, trichostatin A, and sodium butyrate had significantly reduced mRNA levels of HDAC9 and its splice variant histone deacetylase-related protein. Subsequently, HDAC9 gene knockdown produced dose-dependent γ-globin gene silencing over an 80–320 nm range. Enforced expression with the pTarget-HDAC9 vector produced a dose-dependent 2.5-fold increase in γ-globin mRNA (p < 0.05). Furthermore, ChIP assays showed HDAC9 binding in vivo in the upstream Gγ-globin gene promoter region. To determine the physiological relevance of these findings, human primary erythroid progenitors were treated with HDAC9 siRNA; we observed 40 and 60% γ-globin gene silencing in day 11 (early) and day 28 (late) progenitors. Moreover, enforced HDAC9 expression increased γ-globin mRNA levels by 2.5-fold with a simultaneous 7-fold increase in HbF. Collectively, these data support a positive role for HDAC9 in γ-globin gene regulation.

Hemoglobin switching from fetal γ-globin to adult β-globin gene expression occurs shortly before birth and is usually completed by the first 6–12 months of life. During adult stage development, fetal hemoglobin (HbF) α2γ2 production reaches a basal level of less than 2% of total hemoglobin (1). Understanding the molecular events involved in γ-globin gene reactivation has been the focus of intense investigation for more than 2 decades, with a potential application for the treatment of sickle cell disease and β-thalassemia. Molecular events known to promote γ-globin expression include binding of developmental stage-specific transcription factors, such as fetal Kruppel-like factor, to the γ-globin promoter CACCC box element (2, 3). Moreover, epigenetic modifications during erythroid maturation that allows interactions between the β-globin locus control region and the γ-globin gene promoters (4, 5) are involved as well.

Various pharmacological agents, such as butyrate, decitabine, and hydroxyurea, have been shown to induce HbF synthesis in vitro and in vivo (6–8); however, hydroxyurea is the only drug approved for clinical use in sickle cell patients (9). Our laboratory has shown that histone deacetylase inhibitors, including sodium butyrate (NaB) and trichostatin A (TSA), induce γ-globin gene expression via the p38 mitogen-activated protein kinase signaling cascade (10, 11). Generalized acetylation of histones to confer chromatin accessibility is considered the main mechanism by which γ-globin gene activation is accomplished by HDAC inhibitors; however, other HbF inducers worked independently of histone hyperacetylation (12–14). A better understanding of the role of chromatin-modifying proteins would be useful for the development of more potent HbF inducers for therapeutic purposes.

Currently, 18 mammalian HDAC genes have been identified that have been classified into four groups based on sequence homologies (15). Class I HDAC genes (HDAC1, -2, -3, and -8) are nuclear proteins with ubiquitous expression. Previous investigations in the globin field have primarily focused on understanding the role of Class I HDAC genes in γ-globin gene regulation. Interaction of HDAC1 with NE-F4 minimizes its activation potential at the γ-promoter in fetal erythroid cells (16). During γ- to β-globin switching, HDAC1 and the chromatin remodeling protein Mi-2 contribute to γ-globin silencing (17). More recently, it was shown that the short chain fatty acid RB7 mediated displacement of HDAC3 and its adapter protein, NcoR (nuclear receptor co-repressor) from the γ-globin promoter to stimulate transcription (18). However, limited investigations have been performed to determine the role of Class II HDAC genes (HDAC4, -5, -6, -7, -9, and -10) in γ-globin gene regulation. These proteins display tissue-specific expression and have the ability to shuttle between the nucleus and cytoplasm of cells.

HDACs remove the acetyl group from histones and are associated with a variety of repressor proteins; thus, HDACs are generally viewed as transcriptional co-repressors (19). In addition to histones, HDACs deacetylate non-histone proteins, including a variety of transcription factors and many regulators involved in cell signalling or metabolism (15, 20). Contrasting studies have also shown the association of HDACs with gene activation (21) and deacetylation of certain proteins

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‡ The abbreviations used are: HbF, fetal hemoglobin; HDAC, histone deacetylase; HDRP, histone deacetylase-related protein; NaB, sodium butyrate; TSA, trichostatin A; RT-qPCR, reverse transcription-quantitative PCR; qPCR, quantitative PCR; PI, propidium iodine; pTarT, pTarget; ac-H3, acetylated histone H3.
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required for transcription activation signals (22). These observations support the dynamic role of HDACs as co-repressors and co-activators in their overall control of gene expression (23).

The objective of our study was to identify Class II HDAC genes involved in γ-globin gene regulation. We first screened for changes in expression of Class II HDAC subtypes in response to the HbF inducers NaB, TSA, and hemin. Interestingly, the expression of HDAC9 and histone deacetylase-related protein (HDDR), a spliced variant of HDAC9 lacking the catalytic domain, were significantly decreased by all three drugs in K562 cells. These data provided indirect evidence that they might be involved in the γ-globin gene regulation. Subsequent data generated using siRNA knockdown and enforced HDAC9 expression mediated a positive regulatory effect on γ-globin gene expression. Chromatin immunoprecipitation (ChIP) assays demonstrated in vivo HDAC9 and HDAC1 binding in the upstream Gγ-globin promoter. Studies in primary erythroid progenitors confirmed the ability of HDAC9 to activate γ-globin gene expression in early and late erythroid progenitors. The implications of these findings in γ-globin gene regulation are discussed.

EXPERIMENTAL PROCEDURES

Tissue Culture—K562 erythroleukemia cells were cultured in Iscove’s modified Dulbecco’s medium (Invitrogen) containing 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen) at 37 °C and 5% CO₂. For drug induction studies, 3 million cells were treated for 48 h with 50 μM hemin, 2 mM NaB, or 0.5 μM TSA. Cell counts and viability were measured using 0.4% trypan blue stain. Drugs and antibiotics were purchased from Sigma.

Reverse Transcription–Quantitative PCR (RT-qPCR) Analysis—To quantify gene expression levels, total RNA was isolated using RNA Stat-60™ (TEL-TEST “B” Inc., Friendswood, TX) and used for RT-qPCR analysis as described previously (11). Briefly, cDNA was generated from total RNA using the Improm-II reverse transcriptase system and oligo(dT) 15 primers (Promega, Madison, WI). qPCR was performed on an iCycler (Bio-Rad) using SYBR Green iQ Supermix (Bio-Rad) and 10 pm gene-specific primers. Levels of γ-globin, β-globin, and the internal control GAPDH mRNA were quantified as follows. Standard curves were generated using serial 10-fold dilutions of Topo7 base plasmids carrying the γ-globin cDNA sequences (Topo7-γ-globin), Topo7-β-globin, and Topo7-GAPDH plasmids. For expression profiling of Class II HDAC genes, standard curves were generated with 10-fold dilutions of genomic DNA (from 500 ng to 0.5 ng) and HDAC gene-specific primers (Table 1). All gene mRNA levels were calculated as a ratio of GAPDH.

Propidium Iodine (PI) Cell Cycle Analysis—To test the effects of drug treatments on cell cycle progression, we performed PI staining after NaB and TSA treatments. One million K562 cells were washed in phosphate-buffered saline and then fixed in 70% ethanol at 4 °C for 45 min. The cells were pelleted and resuspended in 500 μl of PI staining solution composed of 50 μg/ml PI, 0.1 mg/ml RNase A, and 0.05% Triton X-100 on ice for 1 h in the dark. PI-stained cells were analyzed by flow cytometry using a FACSCalibur instrument with CellQuest analysis software (BD Biosciences). For each sample, 10,000 cells were analyzed, and a histogram was plotted with FL2-W on the x axis and cell count on the y axis. The fluorescence levels were collected using the FL-2 filter at 470 V. Appropriate gates were set to quantify the distribution of cells in the different stages of the cell cycle.

siRNA-mediated Gene Silencing—For gene silencing studies, K562 cells were transfected with SMARTpool siRNAs for HDAC7, HDAC9, HDAC10, and HDRP (M-series, Dharmacon) by Oligofectamine™ (Invitrogen) per the manufacturer’s instructions. The siRNA for HDAC9 (siHDAC9, M-005241-03, Dharmacon) targeting exons 3, 7, 9, and 10 was used at different concentrations of 80, 160, and 320 nM. For the 80 nM reaction, 1 μl of a 20 μM stock solution of siHDAC9 was diluted with 40 μl of Opti-MEM I reduced serum medium, and then 1 μl of Oligofectamine diluted in 8 μl of Opti-MEM was added and incubated for 5 min at room temperature. Control reactions included scrambled siRNAs (80 nM) or Oligofectamine containing only Opti-Medium (mock). To complete the transfections, 50,000 cells were suspended in 250 μl of Opti-MEM I, and then the siRNA-Oligofectamine was mixed in 24-well plates. After 4 h of incubation, Iscove’s modified Dulbecco’s medium with 30% fetal bovine serum was added, and cells were incubated at 37 °C for 48 h.

Transient Transfection Assay—Transient enforced gene expression studies were performed as described previously (24) using 10 million K562 cells and 10, 20, 30, and 40 μg of empty vector control pTarget (pTarT) or pTarget-HDAC9 (pTarT-HDAC9) expression vector carrying a FLAG tag. Cells were transfected using a GenePulser machine (Bio-Rad) at 260 V and 975 microfarads and then incubated at 37 °C for 48 h. Total RNA and protein were harvested to perform RT-qPCR or Western blot analysis, respectively.

siHDAC9/HDAC9 Rescue Studies—For combined knockdown and rescue experiments, K562 cells were transfected simultaneously with SMARTpool siHDAC9 and a pTarget-HDAC9, pCDNA3.1-HDAC1, pCDNA3.1-HDAC7, or pMT3-HDAC10 expression vector; the respective empty vector controls were also tested, and γ-globin expression levels were normalized by subtracting empty control values. Transfections were performed using a Nucleofector device and a K562-Nucleofector kit (Promega, Gaithersburg, MD) per the manufacturer’s instructions. Briefly, 100 μl of Nucleofector solution was mixed with 5 million K562 cells and then siHDAC9 (160 nM) and 15 μg of pTarget, pTarget-HDAC9, pMT3, or pMT3-HDAC10 expression vector. Control experiments included mock (no DNA) and scrambled siRNA (160 nM) reactions. Cells were electroporated using the T-016 Amaxa program and incubated for 48 h, and then RNA was isolated for RT-qPCR analysis of γ-globin, HDAC9, HDAC10, and actin gene expression.

Western Blot Analysis—K562 cells from the different experimental conditions were mixed with lysis buffer (Promega) to isolate total protein that was quantified by Bradford assay (Bio-Rad). For Western blot analysis, 150 μg of protein was separated by 10% SDS-PAGE and transferred to nitrocellulose
membranes. Mouse monoclonal anti-FLAG M2 (F-1804, Sigma) primary antibody (1:1000) was used to quantify FLAG-HDAC9 levels for enforced expression studies. Goat polyclonal HDAC9 (1:500 dilution) or mouse monoclonal HDAC7 (1:5000) primary antibody (from Santa Cruz Biotechnology (sc-10408) and Abcam (ab50212), respectively) was used to measure endogenous HDAC9 and HDAC7 mRNA levels in siRNA studies. Membranes were blocked in 5% bovine serum albumin and then incubated with primary antibodies overnight at 4 °C. Actin antibody (Chemicon Millipore, Billerica, MA) (MAB1501) was used as a loading control. Horseradish peroxidase-conjugated anti-goat and anti-mouse secondary antibodies were purchased from Pierce (catalog nos. 31400 and 31430, respectively), the ECL system (Amersham Biosciences) was used to visualize proteins, and band intensities were quantified using the ChemiDoc System (Bio-Rad).

**ChIP Assay**—ChIP assays were performed in untreated K562 cells and after NaB (2 mM) and TSA (0.5 μM) drug treatments as described previously (11, 24), using the Upstate Biotechnology, Inc. (Lake Placid, NY) protocol. Briefly, 40 million cells from different conditions were cross-linked with 1% formaldehyde. After cell lysis, nuclei were sonicated using a Sonicator 3000 (Misonix, Farmingdale, NY) for 3–5 pulses at 10 W and output level 5 on ice to generate about 250–500-bp DNA fragments. Immunoprecipitation reactions were performed with HDAC1 (sc-7872) or HDAC9 (sc-10408) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). TFII D (sc-204X), IgG (Sigma), and no antibody reactions were set up as controls. After reverse cross-linking at 65 °C, chromatin was purified by ethanol precipitation and used for qPCR analysis with primers covering approximately 2000 bases of the Gγ and Aγ-globin gene promoters (Table 2). Acetylated histone H3 (ac-H3) levels in the γ-globin gene promoters were used as a positive control.

**Two-phase Liquid Culture System**—Primary erythroid progenitors were grown from human peripheral blood mononuclear cells purchased from Carter BloodCare (Fort Worth, TX) in accordance with guidelines of the Institutional Review Board at the University of Texas at Dallas. Erythroid progenitors were generated using the Fibach method (25). During phase 1, cells were grown in Iscove’s modified Dulbecco’s medium supplemented with 30% fetal bovine serum and 50 ng/ml each of granulocyte-monocyte colony-stimulating factor, interleukin-3 and -6, and stem cell factor. To initiate phase 2 on day 7, the medium was changed, and erythropoietin (3 units/ml) and stem cell factor (50 ng/ml) were added. Cells were harvested on days 7, 14, 21, and 28 for cytospin preparations stained with Giemsa and RT-qPCR analysis to measure γ-globin, β-globin, and GAPDH levels. The γ/γ + β and β/γ + β levels were calculated after each gene mRNA level was normalized by GAPDH.

For siRNA experiments, erythroid cells were transfected on day 11 and day 28 using a Nucleofector device and the CD34+ Nucleofector kit (Amaxa). Briefly, 1 million cells were mixed with 100 μl of Nucleofector solution and siHDAC9 at 80 and 320 nM concentrations in triplicates. Cells were electroporated on the U-08 setting for erythroid progenitors and incubated for 72 h. Similar experimental settings were used to perform enforced expression studies with 5 million primary cells. Cells were electroporated with 5, 10, and 15 μg of pTarT or pTarT-HDAC9 expression vector or 2 μg of the control pMaxGFP reporter (Amaxa) to monitor transfection efficiency. The percentage of GFP-positive cells determined at 24 h by flow cytometry (FACSCalibur, BD Biosciences) was used to normalize gene expression levels quantified by RT-qPCR analysis.

**Fluorescent Immunocytochemistry**—Cytospin cell preparations were fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 min and then permeabilized with 0.3% Triton X-100 solution. Cells were then blocked in a 5% bovine serum albumin solution for 1 h at room temperature. Immunostaining was performed at 4 °C overnight with anti-HbF fluorosecin isothiocyanate (FITC)-conjugated antibody (Bethyl Laboratories, Inc., Montgomery, TX). Cells were stained with mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotechnology, Inc.). Erythroid cells were photographed with an Olympus BX 51 phase-contrast epifluorescent microscope equipped with Hoffman Modulation optics. Phase-contrast images were recorded with a CCD camera (1/100-s exposure), and fluorescence images were photographed through 485/20-nm emission and 540/35-nm excitation filters.

**Enzyme-linked Immunoassay (ELISA)**—Total hemoglobin was quantified using 20 μl of protein extract isolated from one million primary erythroid cells mixed with 5 ml of Drabkin’s reagent (Sigma), and then cyanmethemoglobin was measured at 540 nm. HbF levels were measured using the human Hemoglobin F ELISA Quantitation Kit (Bethyl Laboratories, Inc.). Briefly, 96-well plates were coated with sheep anti-human HbF antibody (1 mg/ml) blocked with 1% bovine serum albumin, and then horseradish peroxidase-conjugated secondary antibody (1 mg/ml) was added. Raw data were analyzed in GraphPad PRISM (GraphPad Software, Inc., La Jolla, CA), and HbF levels were calculated as a ratio of total hemoglobin corrected for total protein levels for each sample (HbF/total Hb/total protein).

**Statistical Analysis**—The data are reported as the means ± S.E. from at least five data points generated from independent drug treatments or transfections. Data were analyzed by a two-tailed Student’s t test, and values of p < 0.05 were considered statistically significant. Statistical analyses were performed using Microsoft Excel (Redmond, WA).

**RESULTS**

**HbF Drug Inducers Alter Transcription Levels of Class II HDACs in K562 Cells**—Our initial screening studies were aimed at determining the possible involvement of Class II HDAC members in γ-globin regulation. HDAC gene-specific PCR primers were designed (Table 1) that produced amplification products from 300 to 500 bp. Changes in HDAC expression levels were quantified in K562 cells after treatment with the HbF inducers hemin (50 μM), TSA (0.5 μM), and NaB (2 mM) (26) for 48 h; γ-globin mRNA levels were monitored to ensure that adequate induction was achieved (Fig. 1A). We observed 4.2-, 2.8-, and 2.6-fold γ-gene induction by hemin, TSA, and NaB, respectively. The effects of HDAC inhibitors
on cell cycle kinetics were monitored by PI stain and flow cytometry. NaB increased the number of cells in G0/G1 phase with a simultaneous decrease in G2/M cell counts (Fig. 1B). By contrast, TSA did not have a significant effect on cell cycle kinetics. In the same samples, steady state Class II HDAC mRNA levels were measured (Fig. 1C), which showed that hemin decreased HDAC6 (50%), HDAC7 (54%), HDAC9 (39%), and HDRP (95%) significantly \( (p < 0.05) \); hemin also increased HDAC4 expression 1.6-fold \( (p < 0.05) \). A similar analysis was performed for TSA, where we observed decreased HDAC9 and HDRP expression and a 2.4-fold increase in HDAC7. Finally, studies with NaB demonstrated its ability to repress HDAC4, -7, -9, and HDRP by 50–80% (Fig. 1B). By contrast, NaB significantly increased HDAC5 (1.6-fold) and HDAC10 (1.8-fold) gene expression. These studies demonstrated the diverse indirect effects of HbF inducers on HDAC gene expression and their potential role in \( \gamma \)-globin gene regulation. The most dramatic effects were observed for HDAC7, HDAC9, HDAC10, and HDRP; therefore, we performed siRNA studies to determine which protein might be directly involved in \( \gamma \)-globin expression.

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**FIGURE 1. Treatment of K562 cells with known HbF inducers alters Class II HDAC levels.** Cells were treated for 48 h with hemin \( (50 \mu M) \), NaB \( (2 \text{ mM}) \), and TSA \( (0.5 \mu M) \) and analyzed by RT-qPCR (see “Experimental Procedures”). A, the level of \( \gamma \)-globin mRNA synthesis was calculated as a ratio to GAPDH, and untreated levels were normalized to 1. Data were calculated as the means ± S.E. (error bars). *, the level of mRNA for treated cells was significantly different from levels obtained in untreated cells \( (UT) \) \( (p < 0.05) \). B, K562 cells were treated with the drug inducers and then were stained with propidium iodide (see “Experimental Procedures”). Ten thousand cells were analyzed at 470 V (FL-2) to quantify DNA content. S, synthesis; G2/M, G2 and mitosis. C, RT-qPCR analysis was performed to determine the relative mRNA levels of Class II HDAC genes in the absence (−) or presence (+) of drug treatments.

**TABLE 1**

| HDAC gene | Gene ID   | Primer sequence                                                      | PCR product |
|-----------|-----------|---------------------------------------------------------------------|-------------|
| HDAC4     | NM_006037 | 5′-GTTCCAGAGGACGACGGAG-3′                                            | 267 bp      |
| HDAC4     | NM_005474 | 5′-GGAGGGGGAAGTCGCTC-3′                                              | 229 bp      |
| HDAC5     | NM_006044 | 5′-GCCAGACCTGAGTAAGAGC-3′                                            | 418 bp      |
| HDAC6     | NM_015401 | 5′-AGGGCTCGACCCACACCTTCTCTCTC-3′                                    | 547 bp      |
| HDAC7     | NM_178423 | 5′-CCGACCGCAGGCTGTGCTACTA-3′                                         | 300 bp      |
| HDAC9     | NM_032019 | 5′-AGGCCTGCACTCCACACCAC-3′                                           | 202 bp      |
| HDAC10    | NM_006037 | 5′-AACTTGAAGTGGCGGTCCA-3′                                            | 1008 bp     |
| HDRP      | NM_04707  | 5′-TTACAAATCCCTGAGGCTTAAT-3′                                         |             |

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to determine if γ-globin gene transcription was affected by HDAC gene knockdown at the 80, 160, and 320 nM concentrations. We observed a 35% decrease in γ-globin expression at the 320 nM concentration of siRNA for HDAC7 (Fig. 2B). Although knockdown of HDAC9 produced a dose-dependent decrease in γ-globin mRNA levels of 20–50% (Fig. 2B), HDAC10 and HDRP knockdown did not alter γ-globin expression significantly. We then confirmed HDAC7 and HDAC9 knockdown by Western blot analysis. There was a dose-dependent 80% decrease in HDAC9 protein at the 320 nM concentration (Fig. 2C), whereas HDAC7 protein was silenced by 50% at the amounts tested (Fig. 2D). We also completed analyses to evaluate the potential off-target effects of siHDAC9 treatments. There were no significant changes in the mRNA levels of HDAC1 (0.9–1.2-fold change), HDAC7 (0.86–1.20-fold change), or HDAC10 (1.09–1.15-fold change) compared with scrambled siRNA in K562 cells treated with 320 nM siHDAC9. These results suggest that HDAC9 exerts a specific positive regulatory effect on γ-globin gene expression in K562 cells.

**HDAC9 Enforced Expression Activates γ-Globin Gene Expression**—To determine the direct regulatory effects of HDAC9 on γ-globin gene transcription, enforced expression studies were completed with the constitutively active pTarT-HDAC9 vector. K562 cells were transfected with 10, 20, 30, and 40 μg of pTarT-HDAC9 for 48 h, and then HDAC9, γ-globin, and GAPDH mRNA levels were quantified by RT-qPCR (see “Experimental Procedures”). A 30-fold increase in HDAC9 mRNA levels was produced at the 20-μg concentration (Fig. 3A). All HDAC9 mRNA values were normalized by GAPDH, and the effects of the empty vector (pTarT) were subtracted at each amount tested. Western blot performed with a FLAG antibody showed an increase in HDAC9 protein (Fig. 3A) starting at 10 μg. Interestingly, at the highest amounts of pTarT-HDAC9, a larger band appeared that might be due to protein modifications, such as phosphorylation or nonspecific antibody cross-reaction. To ascertain whether HDAC9 directly activates γ-globin transcription, mRNA levels were measured after enforced expression (Fig. 3B). We observed a maximal 3-fold increase in γ-globin mRNA levels at the 20- and 30-μg amounts of pTarT-HDAC9, confirming the ability of HDAC9 to stimulate γ-globin expression.

To gain direct evidence for the ability of HDAC9 to exert a positive effect on γ-globin, we performed knockdown/rescue experiments in which K562 cells were transfected with siHDAC9 and pTarT-HDAC9 simultaneously for 48 h (see “Experimental Procedures”). The results of these experiments are summarized in Fig. 3C. In the presence of 84% γ-globin gene silencing produced by 160 nM siHDAC9, we observed 5.3-fold (p = 0.0033) trans-activation by pTarT-HDAC9 compared with no rescue of γ-globin expression by HDAC1, HDAC7, or HDAC10. We observed a highly significant increase in target HDAC mRNA synthesis for all expression vectors after enforced expression (data not shown).

**HDAC9 and HDAC1 Bind in the Gγ-Globin Upstream Promoter Region in Vivo**—We next asked the question whether HDAC9 interacts in the γ-globin promoter to produce its effects. ChIP assays were performed in K562 cells, to determine whether HDAC9 binds the γ-globin promoter in vivo. Eight overlapping primer sets (Table 2) were designed for RT-qPCR to measure chromatin enrichment for the region between −2000 and −191 relative to the γ-globin gene cap site (Fig. 4A). In silico TESS (Transcription Element Search System) analysis identified a potential HDAC1 binding site at −1376 bp in the Gγ-globin promoter. Because HDAC1 is a binding partner of HDAC9 (27, 28), in vivo binding of both proteins
ChIP assays were performed in the increased ac-H3 levels, ranging from 8.6- to 11-fold. Additional proteins tested.

**TABLE 2**

| Globin gene | Primer position | Primer sequence | PCR product |
|-------------|-----------------|-----------------|-------------|
| G/A-1F      | ~467 to ~447    | 5’-TCAAAATCCTGAGCCTATG-3’ | 290         |
| G/A-1R      | ~182 to ~191    | 5’-TTGAGATGTTGAGGGAAGG-3’ | 390         |
| G/A-2F      | ~825 to ~807    | 5’-AAAACCCCTGGTCACTAGC-3’ | 305         |
| G/A-3F      | ~434 to ~457    | 5’-TGTCACATGGTTTAGGATAGG-3’ | 268         |
| G/A-3R      | ~1091 to ~1070  | 5’-GCAATATGTGCAGAAACAGC-3’ | 305         |
| G/A-4F      | ~801 to ~819    | 5’-CTGACAGGAGGCTTTTG-3’ | 268         |
| G/A-4R      | ~1360 to ~1337  | 5’-GAGGACATATAAGCCTTACAG-3’ | 305         |
| G/5F        | ~1070 to ~1092  | 5’-GAGCAGATATAAGCCTTACAG-3’ | 268         |
| A-5F        | ~1714 to ~1690  | 5’-GCAATATGTGCAGAAACAGC-3’ | 305         |
| A-5R        | ~1717 to ~1695  | 5’-TCAAAATCCTGAGCCTATG-3’ | 290         |
| A-6F        | ~1305 to ~1287  | 5’-GCAATATGTGCAGAAACAGC-3’ | 305         |
| A-6R        | ~1707 to ~1728  | 5’-GCAATATGTGCAGAAACAGC-3’ | 305         |
| G-7F        | ~2015 to ~1997  | 5’-GCAATATGTGCAGAAACAGC-3’ | 305         |
| G-7R        | ~1706 to ~1731  | 5’-GCAATATGTGCAGAAACAGC-3’ | 305         |

**Figure 3. Enforced HDAC9 expression activates γ-globin gene transcription.** Experiments were completed to determine the ability of HDAC9 to augments γ-globin gene transcription after transient transfection with the pTarT-HDAC9 vector for 48 h (see “Experimental Procedures”). A, levels of mRNA were calculated as a ratio to GAPDH after subtracting HDAC9 levels observed for transfections with the pTarT empty vector at each amount tested. **,** p < 0.01. Shown is the immunoblot analysis with anti-FLAG antibody for different amounts of the pTarT-HDAC9 vector. B, the effect of enforced HDAC9 expression on γ-globin gene transcription was measured by RT-qPCR. C, knockdown/rescue experiments were performed by the simultaneous addition of siHDAC9 and the different expression vectors shown (see “Experimental Procedures”). For each condition, the presence (+) or absence (−) of the various reagents is shown. Transfections with the respective empty vector control were performed, and the data shown represent γ-globin levels after subtracting values obtained for the empty vector controls. Only enforced HDAC9 expression rescued the silenced γ-globin gene compared with the other HDAC proteins tested. Error bars, S.E.

was tested. Control reactions were performed with no antibody and with ac-H3 and IgG antibodies.

Compared with IgG levels, HDAC1 and HDAC9 antibody produced 3.5- and 4.3-fold chromatin enrichments, respectively (p < 0.05), in the −1714/−1331 Gγ-globin promoter region (Fig. 4B, G–G5); however, no enrichment was observed in the analogous Aγ-globin promoter (A–A5). Moreover, enrichment was not observed in the other promoter regions studied (Fig. 4B) or with no antibody controls (data not shown). Across both γ-globin promoters, we observed increased ac-H3 levels, ranging from 8.6- to 11-fold. Additional ChIP assays were performed in the Gγ-globin G5 region after drug inductions (Fig. 4C). Both NaB and TSA mediated enhanced HDAC9 and HDAC1 binding in vivo. By contrast, there was no change in HDAC7 binding in this region. NaB and TSA produced a simultaneous increase in acetylated histone H3 levels (Fig. 4C). Western blot analysis for HDAC1, HDAC7, and HDAC9 after drug treatments show no change in protein levels (data not shown), suggesting the ability of these drugs to facilitate enhanced protein binding at the chromatin level. We propose a schematic to describe in vivo HDAC9/HDAC1 binding in the γ-globin promoter. It is not clear whether additional factors are recruited to the promoter to mediate gene activation (Fig. 4D).

**HDAC9 Is an Activator of γ-Globin Expression during Early Erythroid Maturation**—To confirm its physiological relevance, we tested the ability of HDAC9 to stimulate γ-globin in human primary erythroid progenitors derived from peripheral blood mononuclear cells grown in the two-phase liquid culture system (see “Experimental Procedures”). Cells were harvested on days 7, 14, 21, and 28 for cell morphology and γ-globin and β-globin gene profiling. By day 21, we observed...
orthochromatophilic erythroblast (1%), which increased further to 38% at day 28 (Fig. 5A). On day 7, we observed maximal $\gamma$-HbF gene mRNA followed by a switch by day 21 (Fig. 5B); in late erythroid progenitors (day 28), the $\gamma$-HbF genes were silenced. These data established the two-phase culture as a good system to study the effects of HDAC9 on $\gamma$-HbF gene expression.

In the first set of experiments, we performed HDAC9 gene silencing in early progenitors, transfected for 72 h with 80 and 320 nM siHDAC9 on day 11 in culture. The pMAxGFP vector was used to monitor transfection efficiency which remained at 40% at 24 h for all transfections. RNA was harvested for $\gamma$-HbF, $\beta$-HbF, and GAPDH RT-qPCR analysis, and the $\gamma$/$\beta$ ratio was calculated after normalization by GAPDH and GFP efficiency. After siHDAC9 treatment, we observed 50% target gene silencing and abolishment of HDAC9 protein synthesis (Fig. 6A). HDAC9 silencing also repressed the $\gamma$/$\beta$ ratio by 60% at the 320 nM concentration (Fig. 6B) compared with scrambled controls. Complementary studies with over pTarT-HDAC9 expression (5, 10, and 15 ng) produced a dose-dependent 1.2–5.4-fold increase in $\gamma$/$\beta$ mRNA ratio (Fig. 6C).

We next corroborated the increase in $\gamma$-globin expression at the protein level using FITC-conjugated anti-HbF antibody studies. DAPI staining was also performed as a control for cell count and viability. Shown in Fig. 6D are the fluorescent images after enforced HDAC9 expression; note the increase in HbF-positive cells (44%) at the 15-ng amount (Fig. 6E). The low variation in GFP levels suggests that the increase in HbF-positive cells was not due to a difference in transfection efficiency. Direct quantification of HbF protein by ELISA showed a 2-fold increase ($p < 0.05$) at the same amount (Fig. 6F). To normalize the levels of HbF, we measured total hemoglobin using Drabkin’s reagent (see “Experimental Procedures”), which allowed us to indirectly assess whether HDAC9 stimulated hemoglobin synthesis in general and/or specifically targeted $\gamma$-globin. On day 11, we did not observe a significant change in total hemoglobin at the different HDAC9 levels; however, the level of HbF increased 2-fold, suggesting that Hba protein would be decreased. These data generated by siRNA silencing and enforced HDAC9 expression in early progenitors are consistent with findings in K562 cells, suggesting that HDAC9 has the ability to enhance expression of an actively transcribed $\gamma$-globin gene.

**HDAC9 Can Reactivate Silenced $\gamma$-Globin Genes during Late Erythroid Maturation**—In the final set of experiments, we performed similar experiments in day 28 late erythroid progenitors, where the $\gamma$-to-$\beta$-globin switch has occurred and
HDAC9 Activates γ-Globin Expression

DISCUSSION

Understanding molecular mechanisms for γ-globin gene activation is essential to the development of new treatment options for β-hemoglobinopathy patients. Our laboratory and others have shown that the HDAC inhibitors, including NaB and TSA, increase HbF via the p38 mitogen-activated protein kinase signaling pathway (11, 29–31). In other cell types, these drugs also alter Class I and Class II HDAC proteins that are dependent on zinc ion for enzymatic activity (32, 33). However, in erythroid cells, the magnitude of HDAC inhibition does not directly correlate with the magnitude of γ-globin induction by these compounds (13, 34), suggesting that HDAC subtypes or site-specific acetylation may be an important mechanism involved in γ-globin gene activation.

HDACs are chromatin-modifying enzymes that remove the acetyl group from histone tails and act as transcriptional co-repressors (19). They also play an important role in the regulation of many biological processes, such as the cell cycle regulation, cell differentiation, and survival, and HDAC inhibition leads to growth arrest or apoptosis of tumor cell lines, making them promising targets for cancer therapy (35, 36). We began our investigations by screening Class II HDAC family members because they are highly tissue-specific in expression and have nuclear localization and import signals required for cell differentiation and proliferation (37, 38). NaB, TSA, and hemin significantly altered the mRNA levels of HDAC7, -9, and -10 and HDRP while simultaneously producing γ-globin activation. This effect could be due to loss of HDAC activity at the promoter or to secondary effects involving changes in the expression of other genes. Direct evidence was obtained through siHDAC9 studies in K562 cells, where a dose-dependent decrease in γ-globin gene expression was produced, and enforced HDAC9 expression reversed the silencing effect in γ-globin rescue studies. These data provide the first evidence for a positive regulatory role of HDAC9 in γ-globin regulation.

FIGURE 5. The γ- to β-globin switch is recapitulated in vitro. A, peripheral blood mononuclear cells were grown in a two-phase liquid culture system to produce erythroid progenitors (see “Experimental Procedures”). Cytospin cell preparations were made at the days shown and stained with Giemsa. Note the appearance of orthochromatophilic erythroblast (black arrows) by day 21. B, shown is the RT-qPCR data for the γ/β mRNA levels after each globin gene mRNA level was normalized by GAPDH. Error bars, S.E.
downstream of the cap site. HDAC binding along with deacetylation of non-histone proteins are critical determinants in c-Jun activation (42). Our data demonstrate for the first time that HDAC9 mediates γ-globin gene activation in contrast to HDAC3, which acts as a repressor (16, 18).

To confirm a physiological role, we performed HDAC9 studies in primary erythroid progenitors. In the well-established two-phase liquid culture system, γ- to β-globin switching is recapitulated around day 21; therefore, we performed studies on day 11 (early) and day 28 (late) of erythroid maturation to determine the effects of HDAC9 on γ-globin expression before and after γ-globin silencing. HDAC9 knockdown was sufficient to reduce the γ/β-globin ratio on day 11, suggesting that HDAC9 interacts in a γ-globin promoter in an active chromatin domain. Furthermore, we observed in vivo HDAC9 binding in the silenced γ-globin gene on day 28, suggesting that the effect of HDAC9 is maintained throughout erythroid maturation. Additional evidence supporting a key role of HDAC9 was obtained from enforced expression studies; HDAC9 overexpression was sufficient to increase γ-globin gene transcription and induce HbF synthesis during erythroid differentiation.

To elucidate molecular mechanisms, we investigated the in vivo occupancy of HDAC9 at the γ-globin promoter by a ChIP assay. Previous findings from our laboratory demonstrated chromatin enrichment in the −1376 region after the HDAC1 ChIP assay (11). Because HDAC1 interacts with HDAC9 (27, 32), we expanded our ChIP assay, which revealed in vivo occupancy by HDAC1 and HDAC9 in the −1714 to −1331 G-5, γ-globin region only; furthermore, HDAC7 was not bound, supporting the specificity of HDAC protein binding in the G-5 region. By contrast, there was no chromatin enrichment in the Aγ-globin promoter. We propose a model by which HDAC9 might regulate γ-globin expression (Fig. 4C). HDAC9 and HDAC1 either alone or combined with other transcription factors bind the −1376 open chromatin region of the Gγ promoter. Whereas drug treatments with NaB and TSA decreased mRNA levels for HDAC9, we observed enhanced HDAC9 binding to this region where increased ac-h3 levels occurred, suggesting that HDAC enzyme substrate specificity of our drug inducers may not correlate directly with cellular mRNA levels; it is known that NaB preferentially inhibits Class I HDAC enzymes (43). Studies by Bradner et al. (43) demonstrated a negative role for HDAC1 and HDAC2 in γ-globin expression, suggesting in our model that HDAC9 mediates trans-activation. HDAC9 has at least six different proteins produced by alternative splicing, which gives rise to proteins with different functions (44). Both

FIGURE 6. HDAC9 activates γ-globin gene expression in early erythroid progenitors. Peripheral blood mononuclear cells were grown in the two-phase liquid culture system for 28 days (see “Experimental Procedures”). Studies were performed on day 11, and then cells were harvested after 72 h for mRNA and protein analysis. A, siHDAC9 treatments were performed by Amaxa transfection and target gene silencing confirmed by RT-qPCR (graph) and Western blot analysis (lower gel). B, progenitors were transfected with different concentrations of siHDAC9, and the levels of γ-globin, β-globin, and GAPDH gene expression were measured by RT-qPCR. Shown is the ratio of γ/β-globin mRNA after the level for each gene was divided by GAPDH. C, the γ/β-globin ratios obtained after pTarT-HDAC9 transfection were compared with the empty vector (pTarT) normalized to 1. **, values obtained for pTarT-HDAC9 were significantly different from those for pTarT at the p < 0.01 level. D, HbF-positive cells were visualized by immunostaining with FITC-conjugated anti-γ-globin antibody (FITC-globin) after enforced HDAC9 expression. DAPI staining was performed to visualize cell nuclei and to determine cell counts. Images were photographed at ×40. E, the images generated in C were used to count 500 DAPI- and FITC-positive cells in the same field to calculate the percentage of HbF-positive cells. F, an ELISA was performed to quantify HbF protein levels after enforced HDAC9 expression. HbF levels obtained by ELISA were divided by total hemoglobin and total protein (HbF/Hb/Protein), and levels in untreated cells were normalized to 1. Error bars, S.E.
HDAC6 and HDAC9 possess a unique C-terminal domain, which may produce special biological effects distinct from other deacetylases (45). Because HDACs lack the intrinsic DNA binding activity and are recruited to their target genes by other proteins (46), our ChIP data suggest that HDAC9 forms a multiprotein complex via association with transcriptional proteins in vivo; however, it is not clear whether additional factors are recruited to this region to mediate gene activation. Sequential ChIP studies will be performed to ascertain whether HDAC9 and other factors co-localize to further define its mechanism of γ-globin gene activation.

Class II members, such as HDAC9, are recruited by the well known transcriptional activator MEF2 (myocyte enhancer factor 2), which could be a candidate protein involved in γ-globin regulation (47). In silico analysis using TESS identified multiple consensus MEF2 binding sites in the γ-globin promoters, including the G-5 (−1717 to −1331) region (data not shown), which are targets for future investigation. Recent studies of genetic ablation of BCL11A demonstrated increased γ-globin expression in vitro and in vivo (48, 49), supporting its role in globin gene switching during development. BCL11A also interacts with COUP-TF1 (50) and HDAC1 by co-immunoprecipitation (43); however, BCL11A does not bind the upstream γ-globin G-5 region bound by HDAC1 and HDAC9 (48) in our study. Our data provide novel insights to unravel the complexity of γ-globin regulation and will aid efforts to identify molecular events critical for HbF induction. This information will also aid efforts to develop HDAC9 as a therapeutics target for treatments of β-hemoglobinopathy patients.

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