Developmental stage-specific epigenetic control of human beta globin gene expression is potentiated in hematopoietic progenitor cells prior to their transcriptional activation

Running head: “Human β-globin gene activation”

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S.B. was supported by the Guy-Bernier Immuno-Oncology fellowship and E.M. is a scholar of the Canadian Institutes of Health Research. This work was supported by grants from the National Cancer Institute of Canada (Terry Fox Foundation) and from the Canadian Cancer Research Society held by E.M.

Abstract word count: 193

Total text word count: 5247

Scientific heading: “Hematopoiesis”
ABSTRACT

To study epigenetic regulation of the human β-globin locus during hematopoiesis, we investigated patterns of histone modification and chromatin accessibility along this locus in hematopoietic progenitor cells (HPC) derived from both humans and transgenic mice. We demonstrate that the developmentally-related activation of human β-like globin genes in humans and transgenic mice HPC is preceded by a wave of gene-specific histone H3 hyperacetylation and K4 dimethylation. In erythroid cells, expression of β-like globin genes is associated with histone hyperacetylation along these genes and, surprisingly, with local deacetylation at active promoters. We also show that endogenous mouse β major and human β-like genes are subject to different epigenetic control mechanisms in HPC. This difference is likely due to intrinsic properties of the human β-globin locus since, in transgenic mice, this locus is epigenetically regulated in the same manner as in human HPC. Our results suggest that a defined pattern of histone H3 acetylation/dimethylation is important for specific activation of human globin promoters during development in human and transgenic HPC. We propose that this transient chromatin acetylation/dimethylation is involved in gene-specific potentiation in HPC, i.e., before extensive chromatin remodelling and transcription take place in erythroid cells.

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INTRODUCTION

Regulation of the “on/off” state of transcription in eukaryotes plays a critical role in embryogenesis and cellular differentiation. This heritable process is linked to epigenetic states involving DNA methylation and changes in chromatin structure, which maintain transcriptional status throughout mitosis and DNA replication. Eukaryotic gene activation results from the interplay of trans-activators and/or repressors with nucleosome-modifying and/or -remodelling factors. Indeed, nucleosome organisation is a key component of epigenetic regulation (and references therein). Observations made in recent years have led to the notion that a combination of histone modifications such as acetylation, phosphorylation and methylation generates a histone code that regulates the use of information from the genetic code. These post-translational modifications of histones are important determinants in nucleosome-nucleosome and nucleosome-DNA interactions and provide precise patterns recognised and bound by specific proteins.

Epigenetic regulation of transcription appears to play an important role during hematopoiesis. For example, abnormal patterns of DNA methylation and chromatin structure are common traits in haematological malignancies, and chromosomal translocations that change the activity of histone acetyltransferases and histone deacetylases (HDACs) are associated with several forms of leukaemia. Hematopoiesis is characterised by a gradual commitment of multipotent hematopoietic progenitors to become bi- or uni-potent progenitors and, eventually, mature blood cells. In HPC, lineage-specific genes are thought to be located in chromatin that is poised for activation, while the chromatin of non-expressed genes would exist as or become increasingly restrictive. The mechanisms responsible for maintenance of an active chromatin state are poorly understood. In HPC, basal expression of genes that eventually become highly transcribed in committed cells might play a role in transcriptional potentiation, although this may not always be a prerequisite.

β-like globin gene expression is tightly regulated during development and hematopoiesis. The human β-globin locus comprises five developmentally regulated genes (ε-γ-α-δ-β) whose high-level expression depend on the Locus Control Region (LCR) which, in turn, consists of five DNaseI hypersensitive sites (HS). The LCR activates β–globin gene transcription through direct interaction with promoter regions and is a major determinant of chromatin structure at the locus. Mice transgenic for the human β-globin locus express the human genes in a developmentally regulated manner. The β-globin genes exhibit a basal level of transcription in hematopoietic progenitor cell lines,
and in HPC of the aorta-gonad-mesonephros region, suggesting that in these cells the β-globin locus is characterised by an "open" chromatin structure. Low-level expression is maintained throughout erythropoiesis and only increases to full expression in differentiated erythroid cells. However, it is not known whether this basal level of expression corresponds to developmental-specific expression of the globin genes in erythroid cells.

The observation that the β-globin locus is uniformly hyperacetylated in chicken erythroid cells suggests that histone acetylation is important for the activation and/or the maintenance of an active globin locus. Additionally, active chicken globin genes are acetylated at lysine 9 of histone H3 (H3 K9) while histones across inactive genes are preferentially H3 K9-methylated. The mouse β major (βmaj) promoter is also acetylated in uninduced MEL (mouse erythroleukemia) cells. Furthermore, the murine β-globin locus is differentially acetylated during development, and active genes as well as the LCR are marked by H3 and H4 acetylation. In murine MEL cells containing a human chromosome 11, the human β-globin locus was found to be acetylated at histones H3 and H4 throughout, whereas peaks of H3 hyperacetylation were characteristic of transcriptionally active genes. However, MEL are proerythroblast-like transformed cells, and the role of histone modifications at the human β-globin locus in vivo at early hematopoietic stages remains to be elucidated.

We have investigated the epigenetic state of the human β-globin locus in human and transgenic HPC and compared this with major epigenetic variations associated with globin activation in erythroid cells in vivo. We demonstrate that histone H3 is hyperacetylated and K4 dimethylated at the human β (huβ) promoter in HPC, and subsequently deacetylated in erythroid cells. The human γ (huγ) promoters are not acetylated in adult HPC as is the case for 11.5 dpc (day post coitus) transgenic mouse-derived fetal liver HPC. Our data therefore provide evidence for a transcriptional potentiation mechanism occurring at the human globin locus in HPC.
METHODS

Bone marrow culture and in vitro colony assays

Mouse bone marrow was cultured in DMEM with 10% characterised fetal bovine serum (HyClone), 3 U/ml Epo, and 300 nM TSA (Sigma) or equal volume of ethanol. Cells were incubated for 8 hr at 37°C in 5% CO₂. For in vitro colony assays, cells were plated onto MethoCult M3434 medium (StemCell Technologies). Single cultures contained 50, 100 or 200 c-Kit⁺/CD31^high/Ly-6C^- cells or 5X10⁵ Ter119^+ cells or 5X10⁴ mouse bone marrow cells. Colony types were determined and scored at day 14 by microscopy and Wright-Giemsa staining.

Cell sorting

Staining with antibodies (PharMingen or Caltag) was carried out on ice for 30 min followed by one wash in PBS-5% heat-inactivated FBS. Cells were analysed using high-speed FACS-Vantage with DIVA option (Becton Dickinson). For c-Kit⁺/CD31^high/Ly-6C^- sorting, cells were incubated with rat anti-Ly-6C antibody (Ab), followed by goat anti-rat PE-conjugated Ab; then biotinylated anti-CD31 Ab, followed by Streptavidin Tri-colour conjugated Ab and anti-c-Kit FITC-conjugated Ab. Otherwise, cells were stained with rat anti-Ter119 Ab and goat anti-rat PE-conjugated Ab. Human mononuclear cells from leukapheresis of healthy donors were separated on Ficoll-Paque (Pharmacia) and stained with mouse anti-CD36 Ab, followed by rat anti-mouse PE-conjugated Ab and anti-CD14 FITC-conjugated Ab. Human CD34^+ cells were stained with anti-CD34 Ab PC5-conjugated. The purity of the sorted populations was evaluated by post-sorting analysis and Wright-Giemsa staining.

DNaseI sensitivity assay

DNaseI sensitivity assay was carried out as previously described²³. About 30000 nuclei were digested with 0 or up to 0.35 U of DNaseI (Roche) for 30 min on ice. Average molecular weight of DNaseI-treated samples was determined by Southern blot.

Chromatin immunoprecipitation (ChIP) assay

Antibodies and ChIP kits were purchased from Upstate Biotechnology. Antibodies were raised against acetylated histones H3 (K9,K14), H4 (K5,K8,K12,K16), phosphorylated histone H3 (S10), dimethylated histone H3 (K4) and non-modified histone H3. Cross-linked chromatin was reduced in size by sonication in order to obtain fragments of 500-bp average size. ChIP assays were carried out as per manufacturer’s instructions.

Duplex PCR and single cell RT-PCR analyses
Quantitative PCR analysis on DNaseI-treated and ChIP samples was performed as previously described\textsuperscript{23}. All primer sequences and amplicons molecular weight are available as supplementary data.

For single cell RT-PCR, single cells were deposited into 96-wells plates. RT-PCR was performed using Qiagen one-step RT-PCR kit.

**S1 nuclease protection assay**

RNA samples were prepared using Trizol (Life Technologies) and the assay was performed as previously described\textsuperscript{23}. 
RESULTS

Purification of hematopoietic progenitor cells (HPC) and erythroid cells

Murine HPC were purified from adult bone marrow by sorting c-Kit⁺/CD31⁺/Ly-6C⁻ cells. These cells comprise about 2% of the nucleated population and are early hematopoietic cells without mature or late-committed properties. Fig.1A shows a typical three-colour flow cytometric analysis. The sorted population is 97% pure and possesses a blast-like phenotype, as revealed by Wright-Giemsa staining (Fig.1B). The hematopoietic potentiality of these cells was ascertained by in vitro colony assays. On average, out of 100 colonies 54 were CFU-GEMM, 21 were CFU-GM, 21 were BFU-E and none were CFU-E. The remaining 4% were large colonies with an undifferentiated morphology (Table 1). Thus, 79% of the HPC population are composed of progenitors with multilineage potential and only 20% of the progenitors already show unilineage commitment. Relative to total bone marrow, the HPC population displays a 95-fold enrichment in CFC (colony forming cell) activity. This cloning efficiency is influenced by cell mortality induced by the long procedure and high-speed sorting.

Murine erythroid cells were isolated from bone marrow according to their Ter119⁺ phenotype, and evaluated by colony assays. Ter119 Ab recognises erythroid cells at different stages of maturation, from early proerythroblasts to mature erythrocytes. As expected, Ter119⁺ cells (Fig.1C) showed no CFC activity in colony assay. After sorting, more than 95% of the cells were Ter119⁺ (data not shown).

To enrich for human HPC, CD34⁺ cells were purified from human leukapheresis samples (hereafter referred to as bone marrow cells). CD34⁺ cells represented 1-2% of total bone marrow (Fig.1D) and displayed 97% purity on average (data not shown).

Human erythroid cells were purified from bone marrow selecting for CD36⁺/CD14⁻ cells. CD36 is a marker of erythroid commitment, which is present on CFU-E, late BFU-E and erythroid cells. Since CD36 is also expressed on monocytes, these were purged using CD14 Ab (Fig.1E) which is expressed at high levels exclusively on monocytes and macrophages. The CD36⁺/14⁻ population displayed 97% purity on average (data not shown).

Single-cell pattern of globin gene expression in HPC

It has been shown that mouse β-globins are expressed at basal levels in HPC and that in transgenic mice LacZ under the control of the huβ promoter is expressed before erythroid commitment. Nevertheless, it is unknown whether human β-like globin genes can be expressed in HPC in vivo. To verify this, single-cell RT-PCR was
performed on bone marrow HPC of a transgenic mouse line carrying a 70kb human β-globin locus (line 2\textsuperscript{14}, hereafter ln2) and on human bone marrow HPC (Fig.2A-C) For RT-PCR of single ln2 c-Kit\textsuperscript{+}/CD31\textsuperscript{high}/Ly-6C\textsuperscript{-} cells or human CD34\textsuperscript{+} cells, we used primers that co-amplify adult human (β and δ) and mouse (β\textsubscript{maj} and β\textsubscript{min}) globin transcripts\textsuperscript{31} or fetal human (γ) and mouse embryonic (βH1) transcripts\textsuperscript{32}. In ln2 HPC, human and mouse transcripts were distinguished by restriction polymorphisms. In ln2 HPC, β\textsubscript{maj}, transgenic huβ (tg-huβ) as well as huγ (tg-huγ) genes are expressed in about half of the cells subjected to RT-PCR (Fig.2D). Interestingly, β and γ gene expression were also detected in about 50% of adult human CD34\textsuperscript{+} cells. The level of huβ gene expression in human CD34\textsuperscript{+} cells was evaluated by S1 nuclease protection assay, and shown to be at least 100-fold lower than in human total bone marrow (data not shown). Thus, the expression of β and γ genes in HPC is not linked to their developmental stage-specific regulation.

**Chromatin accessibility of the β-globin locus during hematopoiesis**

Chromatin conformational changes at the mouse and human β-globin loci during hematopoiesis were investigated by sensitivity to DNaseI digestion. For this purpose, we used ln2 and human bone marrows. Nuclei were treated with different concentrations of DNaseI, and purified DNA was used as template for duplex PCR. One primer set was specific for either the mouse (Fig.3A) or the human (Fig.3B) locus. The second set was specific for genes transcriptionally inactive in hematopoietic cells, namely: ZFP37 (ZFP\textsuperscript{23}) or amylase 2.1y (amy\textsuperscript{22}) for mouse cells, and pax6\textsuperscript{33} or necdin\textsuperscript{21,34} for human cells. PCR reactions were carried out within the linear range of amplification and all primer sets used in duplex PCR were tested to exclude any significant difference in amplification efficiency. Each data point depicted in Fig.3 represents the ratio of the two PCR products normalised to the input, DNaseI-untreated sample. Curves showing a steep drop at the start indicate DNaseI hypersensitivity while the steepness of the curves at later points is a measure of general DNaseI sensitivity. We observed that in ln2 HPC, β\textsubscript{maj} promoter is highly sensitive to DNaseI (Fig.3C), while the tg-huβ promoter is weakly sensitive or insensitive (Fig.3D). To evaluate the degree of DNaseI sensitivity at tg-huβ promoter, a third non-hematopoietic control (the kidney-specific Tamm-Horsfall -THP- gene\textsuperscript{35}) was tested against ZFP. As shown in Fig.3E, DNaseI sensitivity of THP and huβ promoters is comparable. This suggests that chromatin accessibility at the huβ promoter reflects the general situation for promoters of non-expressed genes in hematopoietic cells. Mouse HS3 (mHS3) and the transgenic human HS3 (tgHS3) are both sensitive to DNaseI in ln2 HPC (Fig.3F, G). The same is also found for human HS2 (tgHS2) and HS5 (tgHS5)
In ln2 erythroid (Ter119⁺) cells, DNaseI sensitivity at the LCR and at globin promoters significantly increases, especially at tg-huβ and βmaj promoters (Fig.3C-G).

DNaseI sensitivity was then analysed in human CD34⁺ and CD36⁻/14⁻ cells. As in ln2 HPC, in human CD34⁺ cells the huβ promoter is not hypersensitive to DNaseI digestion (Fig.3H). In contrast, HS3 is open in human HPC just as was observed in ln2 (Fig.3I). Both huβ and HS3 acquire a more accessible chromatin conformation in human erythroid cells (Fig.3H, I).

Taken together, these results indicate that chromatin at the human β-globin LCR is moderately accessible in both human and ln2 HPC whereas the huβ promoter is not; hence chromatin accessibility along the human LCR precedes that at the huβ promoter. On the other hand, unlike its human homologue, the βmaj promoter is already activated and accessible to DNaseI in HPC.

**Histone covalent modifications at the β-globin locus during hematopoiesis**

Patterns of histone covalent modification at the murine and human β-globin loci during hematopoiesis were assessed by ChIP assay using antibodies specific for acetylated histone H3 (AcH3), H4 (AcH4), dimethylated histone H3 or phosphorylated histone H3 (PhH3). ChIP material was used as a template for duplex PCR with one primer set specific for mouse or human globin locus and a second primer set specific for ZFP or amy (ln2 controls), and pax6 or necdin (human controls). PCR reactions were performed under conditions of linear amplification (Fig.4A). Ln2 as well as human controls showed no variation in histone acetylation (data not shown). To establish the relative enrichment or depletion of β-globin sequences for histone modifications, the ratio of the two PCR products (globin and control sequences) was determined in each immunoprecipitated sample and normalised to the input ratio. Both ZFP (Fig.4B) and amy (not shown, but same results) were used as controls for ln2 cells while pax6 (Fig.4C) and necdin (not shown, but same results) were used as controls for human cells. All results were confirmed by two or three independent ChIP assays.

We first analysed the level of AcH3 and AcH4 at human HS3 and HS4 in HPC and erythroid cells derived from ln2 (tgHS3 and tgHS4) and human bone marrow (HS3 and HS4). In ln2 HPC, AcH3 level is higher than the control at tgHS4, but not at tgHS3, whereas histone H4 is acetylated at both tgHS3 and tgHS4 (Fig.4B). In mature erythroid cells AcH3 and AcH4 level increases at both sites (Fig.4B). A similar pattern of acetylation is seen in human HPC and erythroid cells (Fig.4C).
We then examined patterns of histone modification across the βmaj and huβ promoters. At the βmaj promoter only H4 is significantly acetylated in HPC; in erythroid cells AcH3 increases substantially whereas the increase in AcH4 is much smaller (Fig.5A). In contrast to the mouse βmaj promoter, the huβ promoter shows a high level of AcH3 in ln2 HPC and human HPC, which surprisingly decreases in the case of differentiated erythroid cells (Fig.5B and C). The level of AcH4 is very similar to that of the control in HPC as well as erythroid cells. The rather unexpected H3 hyperacetylation at the huβ promoter in HPC and its decrease in erythroid cells appears to be mediated by intrinsic properties of the human β-globin locus since the same patterns of histone acetylation are observed in human and ln2 mouse HPC and erythroid cells.

To exclude the possibility that detectability of AcH3 be lost at the tg-huβ promoter in erythroid cells, we performed ChIP assays with anti-PhH3 and non-modified histone H3 (H3) Ab. Indeed, it has been shown that the anti-AcH3 Ab used for ChIP assays may not recognise AcH3 when also phosphorylated at S1037. As shown in Fig.5B, no significant enrichment for PhH3 was detected at the tg-huβ promoter in erythroid cells. Therefore, the weak acetylation of histone H3 is not the consequence of its phosphorylation. Moreover, ChIP performed with anti-H3 Ab showed that non-modified histone H3 is under-represented at the βmaj promoter in erythroid cells, confirming that histones are mainly acetylated (Fig.5A). Instead, the detection of non-modified histone H3 at the tg-huβ promoter suggests that the promoter is not devoid of nucleosomes in erythroid cells (Fig.5B). Since histone H3 lysine 4 dimethylation (MeK4) has been shown to play an important role during gene activation38, MeK4 was investigated at the tg-huβ promoter. Fig.5B shows that the tg-huβ promoter displays a significant level of MeK4 in HPC, which undergoes a 3-fold enrichment in erythroid cells.

To better define the pattern of acetylation at the tg-huβ gene, AcH3 was investigated at two additional regions situated ~300bp 5’ (huβ5) and ~400bp 3’ (huβ1) of the huβ minimal promoter (Fig.5D). In ln2 and human HPC, no enrichment for AcH3 is detected at huβ5 (Fig.5E), while huβ1 is significantly acetylated. In highly-expressing erythroid cells, huβ5 remains equally hypoacetylated as in HPC; however in ln2 and human erythroid cells the downstream huβ gene (huβ1) is significantly more acetylated than observed in HPC (Fig.5E). As a control, we also analysed histone acetylation at a non-transcribed region of the locus, the intergenic ψβ region. In human and ln2 HPC and erythroid cells, ψβ is not AcH3 (Fig.5F).
Thus, the minimal huβ promoter is epigenetically marked by AcH3 and MeK4 in HPC. In erythroid cells, AcH3 at the minimal promoter decreases while AcH3 across the gene and MeK4 at the tg-huβ promoter further increase. These results suggest that: i) globin gene expression in erythroid cells does not depend on promoter acetylation and ii) AcH3 and MeK4 epigenetically mark the huβ gene and promoter before the establishment of high-level transcription in erythroid cells.

**Globin gene transcription: silencing and induction**

An interesting aspect of the human β-globin locus is the switching process during development and the possibility of using a variety of drugs interfering with acetylation to modify this switch in β-thalassemic patients. We therefore compared histone acetylation at huβ and huγ promoters. γ genes are expressed during embryonic and fetal stages in humans and in ln2 mice. In adult ln2 and human bone marrow cells, human γ gene expression becomes negligible. ChIP analyses revealed that, in contrast to huβ (Fig.5B and C), histone H3 at Gγ/Aγ promoters (Fig.6A) is not acetylated in HPC purified from human or ln2 bone marrow, suggesting that the acetylation mark in HPC would specifically occur at promoters of genes that will become active in erythroid cells. If true this lends to the prediction that huγ promoters should exhibit H3 acetylation in HPC at earlier stages of development when γ genes are expressed.

We therefore purified HPC (c-Kit+/CD31high/Ly-6C- cells) from ln2 11.5 dpc fetal livers for ChIP analysis. At this stage fetal liver HPC will give rise to erythroid cells that express both human γ and β genes. It should be noted however, that embryonic γ- but not β-expressing cells are still in circulation and thus present in the fetal liver erythroid cells. In our hands, the HPC represent 8% of the 11.5 dpc fetal liver, and they possess the same morphology, potential in colony assay, and transcriptional activity as bone marrow derived HPC (Fig.2 and data not shown). As shown in Fig.6B, in ln2 fetal liver HPC, both tg-huγ and tg-huβ promoters are significantly H3 acetylated. Similar to what was observed for the huβ promoter in adult erythroid cells, AcH3 at tg-huγ promoters decreases in 11.5 dpc erythroid cells. Hence H3 deacetylation at tg-huγ again correlates with gene expression. In effect, AcH3 is largely maintained at tg-huβ promoter in erythroid cells, part of which is embryonic non-β expressing cells.

We then investigated whether TSA, a known histone deacetylase inhibitor, would influence the acetylation state of tg-huγ and/or tg-huβ promoters and change their expression levels. Ln2 bone marrow was treated with TSA or with ethanol (TSA solvent) and AcH3 level was evaluated at the tg-huγ and tg-huβ globin promoters in erythroid cells. A 2-3 fold enrichment in AcH3 (TSA- relative to ethanol-treated cells) was seen at both tg-huβ and tg-huγ promoters.
(Fig.6C). This increase does not significantly modify the level or the balance of expression of these genes, as observed by S1 nuclease assay on ethanol- and TSA-treated bone marrow (Fig.6C). Thus a higher level of AcH3 at tg-huβ and tg-huγ promoters in erythroid cells does not modify globin gene transcription.
DISCUSSION

The work presented here assesses, for the first time, variations of chromatin conformation and histone covalent modifications at the human β-globin locus during hematopoietic differentiation in vivo. Our results suggest that a defined pattern of histone H3 acetylation/dimethylation is important for the specific activation of human globin promoters before high-level expression in erythroid cells. We do not know when this gene-specific potentiation is set, but since multipotent progenitors represent the vast majority of the HPC investigated (Table 1), it could be present before erythroid lineage commitment.

Chromatin modifications of the human globin LCR in HPC and erythroid cells

It has been shown that chromatin within lineage-restricted regulatory regions, including the mouse β-globin locus, can be remodelled in HPC. Using transgenic mice and human bone marrow, we demonstrate here that chromatin at the human β-globin LCR is acetylated and accessible (to DNaseI) in HPC in vivo. Previous in vitro investigations using uninduced MEL cells provided some evidence, in this transformed erythroid committed cell line, that the human LCR displays characteristics of chromatin poised for transcriptional activation. We now provide evidence that the human β-globin LCR is activated in a population of HPC, purified from fresh bone marrow and composed mostly of uncommitted progenitors. Accessibility at HS2, HS3 and HS5 increases in mature erythroid cells when compared to HPC, suggesting that the LCR is further remodelled during differentiation to allow high-level β-globin gene expression. The human LCR therefore follows an activation pattern similar to that of other hematopoietic loci. Thus the LCR may already be activated to some extent by partial HS occupancy in HPC, but complete occupancy of HS and extended chromatin remodelling would only be attained in erythroid cells upon additional binding of stage-specific transactivators. The analysis of histone modifications at different HS within the LCR suggests that histone acetylation has already facilitated an activated structure at the human LCR, with the exception of HS3, in both human and ln2 bone marrow HPC. In mature erythroid cells the acetylation is further increased at all sites including HS3. The exceptional behaviour of HS3 is possibly related to its “chromatin opening” ability and/or to the particular chromatin organisation of the HS3 core region.

Chromatin at the human β and mouse β major genes is differently activated during erythropoiesis

The huβ and mouse βmaj promoters show different patterns of histone modifications and DNaseI accessibility during differentiation from HPC to erythroid cells. The mouse βmaj promoter is already largely accessible to DNaseI...
digestion in HPC with a low level of acetylated H3 and a moderate level of acetylated H4. During differentiation, accessibility is further increased (in particular the appearance of the hypersensitive site in the promoter illustrated by the early points in the curve in Fig.3C), H3 acetylation is increased several fold, while H4 acetylation only doubles when compared to controls. In contrast, in human bone marrow the endogenous huβ promoter shows a very different pattern and, importantly, maintains this pattern when the locus is introduced in mice. It is not accessible to DNaseI in HPC and shows a low level of H4 acetylation, but curiously a high level of H3 acetylation. Upon cell differentiation, the huβ promoter becomes more accessible to DNaseI, H4 acetylation hardly changes, but H3 acetylation decreases significantly. The upstream part of the promoter shows a low level of H3 acetylation, which barely changes upon differentiation, while downstream in the gene, histone H3 is already acetylated in HPC and the level increases several folds upon differentiation. Using a cell culture-based system (transformed MEL cells), it has been reported that the huβ promoter is highly acetylated when active\textsuperscript{22}, however we cannot be certain whether our data agree or contradict those results as it is not clear whether the human locus in MEL cells was analysed before (non-expressing) or after induction of differentiation (expressing).

We conclude that the activation of the human and mouse β-globin promoters is different, and that this difference is intrinsic to these loci since the huβ promoter maintains its own activation program when the human β-globin locus is introduced in the mouse. These differences may explain some of the controversies that have arisen with respect to activation of β-globin loci, where data from patients and mice show that expression and DNaseI accessibility at the huβ globin gene are lost when the LCR is deleted (\textsuperscript{13} for review), while expression and sensitivity at the mouse βmaj gene is not lost when the LCR is deleted\textsuperscript{44}. Our data indicate that the mouse βmaj, but not the huβ globin gene, is already accessible to DNaseI digestion in HPC, suggesting that the LCR may indeed not be required to achieve accessibility across the mouse locus. Therefore, even though studies on the mouse β-globin locus have been invaluable tools to understand aspects of globin gene regulation, differences between the epigenetic regulation of the human and the mouse loci should be considered before comparing results obtained with these two β-globin loci.

Interestingly, histone hypoacetylation as we observed at the active huβ promoter is not unique. Indeed, it has been shown that several active genes are as hypoacetylated as the surrounding bulk chromatin\textsuperscript{45,46}. For example histone acetylation, as exemplified at IFN-β\textsuperscript{47} and hormone receptor-dependent genes\textsuperscript{48} in mammals, or at PHO8 gene in \textit{S. cerevisiae}\textsuperscript{49}, is a transient signal which does not engender chromatin accessibility by itself, but rather provides a
mark that facilitates recruitment of remodelling complexes. The synergy between histone acetylation and the activity of SWI/SNF related chromatin-remodelling complex has been reported previously.\textsuperscript{50}

Our results are consistent with a multistage activation model in which the hu\(\beta\) gene and promoter would first be epigenetically marked by histone H3 acetylation and K4 dimethylation in bone marrow HPC, before the onset of high-level transcription. This mark would subsequently be recognised by activators and/or remodelling complexes that in turn would guarantee high-level globin expression in erythroid cells, where indeed chromatin at the hu\(\beta\) promoter is dimethylated and remodelled, but no longer acetylated.

The observed decrease of acetylation at the hu\(\beta\) promoter in erythroid cells can be either the consequence of an active deacetylation mechanism (carried out by HDACs), or of a passive process. In TSA-treated Ter119\(^{+}\) cells the tg-hu\(\beta\) promoter is significantly more acetylated than in non-treated cells, which suggests that the tg-hu\(\beta\) promoter is actively deacetylated by TSA-sensitive HDACs in erythroid cells. The transcription factor EKLF, which is involved in hu\(\beta\) promoter regulation, might play a causal role in hu\(\beta\) promoter deacetylation in erythroid cells since EKLF was found to interact with HDAC1.\textsuperscript{51} However, to date it is still unknown whether EKLF-HDAC1 interaction at the hu\(\beta\) promoter occurs sometime during hematopoiesis.

In summary, we show that the hu\(\beta\) gene and promoter are epigenetically marked in adult HPC, presumably poising the promoter for activation. The mark at the promoter occurs within a very defined region, spanning the promoter TATA, CCAAT and CACCC boxes since no significant H3 acetylation at the upstream hu\(\alpha\) and \(\gamma\beta\) intergenic regions was detected. During differentiation, the hu\(\beta\) promoter undergoes an extensive chromatin remodelling accompanied by histone H3 deacetylation, as revealed by hypersensitivity to DNaseI digestion and histone H3 hypoacetylation in erythroid cells. Such a pattern of modification is not unlike what has been observed for the HNF 4\(\alpha\) gene in differentiating CaCo-2 cells.\textsuperscript{52}

\textit{Human \(\beta\) versus human \(\gamma\) gene epigenetic regulation during development}

The fetal \(\gamma\) globin genes are silenced around birth in humans, but in ln2 the transgenic locus switches from \(\gamma\) to \(\beta\) gene expression in the early fetal liver stage, and only \(\beta\) is expressed in erythroid cells derived from adult bone marrow.\textsuperscript{14} We show that hu\(\gamma\) promoters are not acetylated in ln2 and human bone marrow HPC, while in HPC derived from 11.5 dpc fetal livers these are H3 acetylated. The fact that the hu\(\beta\) promoter is acetylated in both 11.5 dpc and adult HPC suggests that this acetylation is linked to an epigenetic and developmental gene-specific regulatory
mechanism. Using somatic cell hybrids between fetal human or murine transgenic erythroblasts and MEL cells, it was shown that fetal erythroblasts could express the γ genes also in an adult -MEL- environment. This observation together with other cell fusion experiments invoked the suggestion that epigenetic changes taking place sometime during erythroid differentiation might be important for globin gene switching. Here, we have provided evidence that in freshly isolated human and murine HPC, defined epigenetic mark(s) are linked to developmental specific globin gene expression in mature erythroid cells.

Interestingly, TSA-induced H3 acetylation is not sufficient to reactivate tg-huγ gene expression in bone marrow erythroid cells. As previously shown, some HDAC inhibitors are able to induce γ globin gene reactivation in human erythroid cells. From these studies, it is unclear whether or not these HDAC inhibitors directly influence histone acetylation of the human β-globin locus. On the other hand, treatment of bone marrow cells with HDAC inhibitors is not sufficient to modify the pattern of globin gene expression in adult β-YAC transgenic mice carrying the whole human β-globin locus. Thus, epigenetic mechanisms other than histone acetylation might influence huγ gene regulation. As previously suggested, the difference in γ gene reactivation in human and transgenic mouse could also be explained by the absence of a “fetal regulated” globin gene in mice. Then, in human cells, HDAC inhibitors could influence the expression of trans-acting factors which in mice are either missing or not influenced by HDAC inhibitors.

It has been shown that mouse and human globin genes can be expressed at basal levels in adult HPC but it is not known whether this transcription is involved in the maintenance of a local ‘potentiated’ chromatin structure. Our results confirm that huγ and huβ genes are transcribed in bone marrow and fetal liver HPC, and show for the first time that this basal expression is neither linked to the level of promoter acetylation nor to the stage-specific activation of these genes in erythroid cells. Thus, basal globin gene expression in HPC appears to be linked to the human β-globin locus potentiation during hematopoiesis but not to the developmental-specific regulation of globin genes.

Taken together, our results strongly suggest that the pattern of histone acetylation in HPC is important for the transcriptional potentiation of globin genes and, more interestingly, for the developmentally regulated expression of these genes in erythroid cells. We show that the human γ and β globin genes and promoters are epigenetically marked by histone H3 acetylation/dimethylation in HPC and we suggest that this mark can be recognised during differentiation by activators and/or remodelling complexes, such as E-RC1, to allow proper expression in terminally
differentiated erythroid cells.
ACKNOWLEDGMENTS

We thank Silvana Lachance and Sophie Ouellette for technical assistance, Marella de Bruijn for useful comments, Denis-Claude Roy for providing us with leukapheresis samples, Elliot Drobetsky and Marie Trudel for critical review of the manuscript.
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Table 1. Clonogenic ability of c-Kit⁺/CD31<sup>high</sup>/Ly-6C⁻ cells.

Cells were seeded on methylcellulose and colonies were scored at day 14. Percentages shown are the results of three experiments. CFU-E: colony-forming unit-erythroid; mature BFU-E: progenitors that give rise to colonies constituted by 3 up to 8 erythroblast clusters; primitive BFU-E: progenitors that give rise to 9 or more clusters of hemoglobinised erythroblasts; CFU-GM: colony forming unit-granulocyte/macrophage; CFU-GEMM: colony forming unit-granulocyte/erythroid/macrophage/megakaryocyte.
FIGURE LEGENDS

Figure 1. Flow cytometric analysis and Wright-Giemsa staining.
Representative examples of sorting procedures. Log fluorescence distribution of mouse and human stained cells, showing the live gates used for flow cytometry. A) Three-colour flow cytometric analysis of adult mouse bone marrow cells stained with anti-c-Kit, CD31 and Ly-6C Ab. B) Morphology of c-Kit⁺/CD31⁺/Ly-6C⁻ cells (right panel, Progenitors) compared to total bone marrow cells (left panel, Bone marrow) stained with Wright-Giemsa; C) Flow cytometric analysis and sorting window of mouse bone marrow cells stained with Ter119 Ab; D) Sorting profile of human CD34⁺ cells; E) Two-colour flow cytometric analysis and sorting of human mononuclear bone marrow cells tracked by CD36 and CD14 Ab.

Figure 2. Single-cell RT-PCR and S1 nuclease protection assay.
Qualitative analysis of single-cell RT-PCR assays. A) Hu/hu (266bp) as well as βmaj/βmin (343bp) transcripts were amplified and distinguished after EcoRI digestion; wt: wild type mouse total bone marrow RNA; ln2: ln2 total bone marrow RNA; B) 1-6: representative examples of single-cell RT-PCR performed on ln2 c-Kit⁺/CD31⁺/Ly-6C⁻ cells; -ctl: negative control C) 1-9: representative examples of single-cell RT-PCR performed on human CD34⁺ cells; -ctl: negative control; D) Summary of one-step RT-PCR and S1 nuclease protection assays performed on ln2 bone marrow and fetal liver c-Kit⁺/CD31⁺/Ly-6C⁻ cells, and human CD34⁺ cells. The level of human β globin gene expression (determined by S1 nuclease protection assay) in CD34⁺ cells is relative to the expression level in human bone marrow cells. Nd: not done.

Figure 3. DNaseI sensitivity assay of the β-globin locus.
A map of mouse (A) and human (B) β-globin locus; genes are shown as black boxes and the location of LCR HS is indicated by arrows. Amplified regions used for PCR based DNaseI and ChIP assays are indicated by grey boxes; C-I) PCR-based DNaseI analysis. Each point of the curves represents samples of comparable molecular weight (ranging from 12Kb to 0.5Kb). For βmaj/ZFP and tg-huβ/ZFP an additional DNaseI-treated sample of 150bp average size was included. The intensity of the PCR products was quantitated by Phosphorimager and plotted on graphs relative to the input (DNaseI untreated chromatin). Y-axis: relative amount of PCR products (globin relative to ZFP37 or pax6 products); X-axis: increasing DNaseI concentration, ranging from 0 up to 0.35 U (see Materials and Methods). Standard errors of mean (SEM) are indicated by vertical lines and are the results of at least three independent
experiments. The *p* value was obtained using the unpaired Student’s *t* Test. ZFP: ZFP37; βmaj: β major promoter; tg-huβ: human β promoter in transgenic ln2; mHS3: murine HS3; tgHS3: human HS3 in transgenic ln2; huβ: human β promoter in human bone marrow; HS3: human HS3 in human bone marrow.

**Figure 4. ChIP analysis of the human β-globin LCR in ln2 and human bone marrow cells.**

Immunoprecipitated and unbound (input) chromatin samples were subjected to duplex PCR analysis with one primer set specific for the human globin locus LCR and a second primer set specific for ZFP37 (ZFP) or pax6 gene. All PCR reactions were performed in parallel under conditions of linear amplification. Products were quantified by Phosphorimager. The level of enrichment of globin regions relative to the control and input samples is represented by bars, with their correspondent SEM deviations. A value of 1 indicates that no enrichment was detected. **A**) Duplex PCR ran in linear range of amplification. The same template DNA was subject to 29, 31 or 33 cycles of PCR amplification. Bars show the total intensity of the two PCR products and the line indicates the globin/control ratio. In the example, HS3 and pax6 primer sets were used; **B**) ChIP performed with anti-acetylated histone H3 (AcH3) and H4 (AcH4) Ab. Shown is the level of acetylation of HS3 (tgHS3) and HS4 (tgHS4) in ln2 HPC and erythroid cells; **C**) ChIP performed with anti-AcH3 and AcH4 Ab. Shown is the level of acetylation of HS3 and HS4 in human HPC and erythroid cells.

**Figure 5. ChIP analysis of mouse and human β-globin genes and promoters.**

Immunoprecipitated samples were subjected to duplex PCR analysis with one primer set specific for human or mouse β globin regions and a second primer set specific for ZFP37 (ZFP) or pax6 gene. The level of enrichment of globin regions relative to the control regions and input samples is represented by bars, with their correspondent SEM deviations. **A**) and **B**) ChIP analysis of ln2 HPC and erythroid cells. Either βmaj or the transgenic huβ promoter was analysed by PCR-based ChIP assay. On the X-axis: antibodies used for ChIP assays. AcH3: anti-acetylated H3; AcH4: anti-acetylated H4; MeK4: anti-dimethylated H3; PhH3: anti-phosphorylated H3; H3: anti-non-modified histone H3; **C**) ChIP analysis of huβ promoter in human HPC and erythroid cells. For ChIP analysis anti-AcH3 or AcH4 Ab were used; **D**) Schematic representation of the huβ region; huβ exon 1 and 2 are indicated by grey boxes and amplicons are shown by dotted lines. **E**) Huβ5, huβ1 and **F**) human ψβ regions in ln2 (tg-huβ5, tg-huβ1 and tg-psβ) and human (huβ5, huβ1 and psβ) HPC and erythroid cells were investigated by PCR-based ChIP assays performed with anti-AcH3 Ab.
Figure 6. ChIP analysis of human γ and β promoters in bone marrow and 11.5 dpc fetal liver cells.

Chromatin from ln2 and human bone marrow cells was immunoprecipitated with anti-acetylated H3 (AcH3) or H4 (AcH4) Ab. Immunoprecipitated and input samples were subjected to duplex PCR analysis with one primer set specific for huγ or huβ promoters and another specific for ZFP37 (mouse) or pax6 (human) gene A) ChIP analysis of huγ promoters in ln2 and human bone marrow HPC and erythroid cells; B) ChIP analysis of huγ and huβ promoters (tg-huγ and tg-huβ) in ln2 11.5 dpc fetal liver HPC and erythroid cells; C) ChIP and S1 nuclease protection assays of tg-huβ and tg-huγ promoters in ln2 TSA-treated Ter119+ cells. ChIP assays were performed with anti-AcH3 Ab and the level of acetylation of TSA-treated samples (TSA) relative to their respective ethanol-treated (ETOH) controls is represented by bars, with their correspondent SEM deviations. Mouse β-actin transcript was used as internal control for S1 nuclease protection assay; tg-huβ and tg-huγ expression level in TSA-treated cells is relative to the ethanol treated controls.
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Developmental stage-specific epigenetic control of human beta globin gene expression is potentiated in hematopoietic progenitor cells prior to their transcriptional activation

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