Regulation of human fetal hemoglobin: new players, new complexities

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The human globin genes are among the most extensively characterized in the human genome, yet the details of the molecular events regulating normal human hemoglobin switching and the potential reactivation of fetal hemoglobin in adult hematopoietic cells remain elusive. Recent discoveries demonstrate physical interactions between the β locus control region and the downstream structural γ- and β-globin genes, and with transcription factors and chromatin remodeling complexes. These interactions all play roles in globin gene expression and globin switching at the human β-globin locus. If the molecular events in hemoglobin switching were better understood and fetal hemoglobin could be more fully reactivated in adult cells, the insights obtained might lead to new approaches to the therapy of sickle cell disease and β thalassemia by identifying specific new targets for molecular therapies. (Blood. 2006;107:435-443)

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Introduction

An understanding of the regulation of human fetal hemoglobin expression is required to rationally approach the problem of hemoglobin switching and the reactivation of fetal hemoglobin in adult hematopoietic cells. The human globin loci are among the best characterized in the human genome at the gene and protein levels.1-2 The human β-globin locus on chromosome 11 contains a powerful set of enhancer elements, termed the β locus control region (βLCR), upstream (5′) of the β locus structural genes (Figure 1).3-5 The ε-globin gene is the most 5′ globin structural gene and is active in early fetal life (Figure 1). The γ-globin genes (γA and γ2) are the major β-like genes expressed in most of fetal life; the δ- and β-globin genes are activated late in fetal life, with the β-globin gene being most highly expressed in erythroid cells after birth and in adults. At the other major globin locus, the α-globin locus on chromosome 16, by contrast, α-globin gene expression begins in early fetal life and is predominant throughout fetal and adult life.6 Regulation at the α-globin locus differs greatly from that of the β-globin locus and is beyond the scope of this review.7-10

Human globin gene transcription leads to the expression of mRNA precursors in the nucleus that are subsequently processed into mature globin mRNAs in the cytoplasm.1,2 Here, the translational machinery of the cell, including enzymes and ribosomes, result in the production of globins that combine with heme to form the normal human hemoglobins. The major hemoglobin in fetal life is fetal hemoglobin (HbF, α2γ2), whereas the major hemoglobin in adult life is hemoglobin A (HbA, α2β2). Hemoglobin A2 (HbA2, α2δ2) is a minor hemoglobin, whose expression is usually less than 2% of the total hemoglobin.

The evolution of the human γ-globin genes and HbF production in primates is relatively recent; mice have no γ-globin genes. The development of γ-globin genes is most likely related to the increased oxygen affinity of HbF over HbA, which favors oxygen delivery to the fetus in the placental circulation. The switch from human γ- to β-globin gene expression in late fetal life is a major event in hemoglobin biology. The consequences of this switch lead to diseases in humans: the hemoglobinopathies such as sickle cell anemia (SS disease) and β thalassemia.1,2 Although the production of HbF during fetal life is normal and optimal in patients with these disorders, the switch to predominantly HbA synthesis around birth causes pathology. In SS disease, the normal switch to β-globin gene expression results in the accumulation of β2, which forms hemoglobin S (HbS, α2β2), and leads to HbS aggregation, hemolytic anemia, and clogging of small vessels.11,12 In human β thalassemia, the switch results in decreased or absent normal human β-globin production and HbA. This leads to excess α-globin accumulation and precipitation in early erythroid cells and causes apoptosis and ineffective erythropoiesis.1,2,6,13

In both SS and homozygous β thalassemia (Cooley anemia), insufficient HbF is produced in adult hematopoietic cells after the switch to compensate for the lack of normal β-globin production. Treatments with hydroxyurea and butyrate compounds have resulted in increased levels of HbF and clinical benefit in some patients with sickle cell disease and β thalassemia.14-18 If the molecular events in hemoglobin switching were better understood and HbF could be more fully reactivated in adult cells, the insights gained might lead to a cure for these disorders.

The players at the β-globin locus

At the murine and human β-globin loci, gene transcription is controlled by the complex interactions between (1) cis-acting sequences: the βLCR and downstream globin gene sequences embedded with histones in nucleosomes, in chromatin (Figure 1), and (2) trans-acting factors: transcription factors and chromatin remodeling activities.19-22

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Supported by US Public Health Service grants DK-56635, HL-59887, and DK-07373 from the National Institutes of Health.

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The β-globin LCR

The β-globin LCR upstream (5′) of the globin structural genes is the major structural component of the murine and human β-globin loci and is required for normal high-level globin gene transcription (Figure 1). The murine and human βLCRs contain 5 critical DNAase 1 hypersensitive (HS) sites, HS1-5, that are formed in regions devoid of nucleosomes and that are sites more accessible than other regions of chromatin to interactions with transcription factors and downstream gene sequences.31,32,33 Another HS site, 3′ HS1, is 3′ to the β-globin structural globin gene, and olfactory receptor (OR) sequences are at both the 5′ and 3′ borders of the β-globin locus (Figure 1).31,32,33

Erythroid cell-specific transcription factors

Several important transcription factors play critical roles in murine globin gene expression. GATA-1, which binds to the promoters of many erythroid cell-specific genes and to βLCR sequences, is required for normal erythroid cell differentiation.26-29 Mice deficient in GATA-1 cannot produce mature erythroid cells30-32 and erythropoiesis can be normalized by restoring GATA-1 function.31,32 Erythroid Krupple-like factor (EKLF) is necessary for activation of the adult β-globin gene and also has important interactions with the βLCR.33,34 Mice with EKLF deficiency die in utero with a disease resembling severe β thalassemia.35 The transcription factor NF-E237 is also important in its function at the human β-globin locus although mice deficient in NF-E2 have only mild erythroid defects.38,39 NF-E4 is a critical transcription factor in chicken globin switching.40,42 More recently, a human homologue of chicken NF-E4, NF-E4p22, has been shown to be active in human fetal globin gene activation.41,44

Chromatin remodeling activities

Acetylation of histones in chromatin leads to increased expression of specific genes at structural gene loci, including the β-globin locus, by making gene sequences more available to transcription factors.21,45-52 Specific acetylations of histones H1-H8 in chromatin can be achieved either by histone acetyltransferases (HATs) alone or by adenosine triphosphate (ATP)-driven molecular machines providing this function, such as SWI/SNF complexes.53-56 SWI/SNF complexes have been shown to be active at the murine and human β-globin loci.57-60 By contrast, deacetylation of histones in chromatin by histone deacetylases (HDACs), acting either as enzymes that are HDACs or as part of chromatin remodeling complexes with HDACs, can repress and silence specific gene function at many loci and may be involved in repressing human γ-globin gene expression.51,60-70 Enzymatic complexes can modify histone N-terminal tails by methylation, phosphorylation, and ubiquitination as well.71

The complexities: interactions of the players at the β-globin locus

Many new details about the complex interactions between βLCR elements and downstream globin gene sequences in chromatin have been elucidated recently in mouse erythroleukemia (MEL) cells, and in mice at both the murine and human β-globin loci, the latter using human β locus-containing sequences in transgenic mice.20-23 These recent experiments show that the HS sites at the βLCR are required to form a unique 3-dimensional structure at the chromosomal location of the β-globin locus on chromosome 11, with “looping out” of the locus in a unique spatial configuration, an active chromatin loop at the β-globin locus, termed an active chromatin hub (ACH).23 The ACH includes the βLCR sequences interacting with downstream globin genes and intergenic sequences, transcriptional factors, and chromatin remodeling elements necessary for globin gene expression and hemoglobin switching in a specific compact configuration present uniquely in erythroid cells (Figure 1).20-23 The ACH structure is not found in nonerythroid cells.23

The technology

Studies defining long-range physical contacts between the βLCR and specific downstream gene sequences in the ACH specific to erythroid cells have used 3 related techniques that have in common: (1) cross-linking of proteins and DNA in intact cells in their native chromatin configuration by treatment of the cells with formaldehyde; (2) lysis of the cells, fragmentation of the chromatin, and removal of protein-DNA cross-links; and (3) identification of specific β locus DNA sequences interacting with each other by the use of polymerase chain reaction (PCR) probes representing specific small defined regions of the locus from 5′ of the LCR to 3′ of the structural β-globin genes. In chromatin immunoprecipitation (ChIP) analyses, after chromatin fragmentation, antibodies to specific proteins are used to isolate the DNA sequences associated with these proteins in vivo, for example, antibodies to acetylated histones or specific transcription factors.24,45,48,52,72-79 In so-called chromosome conformation capture (3C) experiments, specific DNA sequences in contact with each other in vivo are identified by restriction digestion and controlled religation of DNA fragments after chromatin cross-linking.20-23,80 In another procedure, tagging and recovery of associated proteins (TRAP) technology, biotin-labeled probes are used to uniquely extract newly synthesized RNA associated with protein sequences after cross-linking.81,82

Interactions between the βLCR and downstream gene sequences

Although mice lack a true γ-globin gene homologue, mouse yolk sac (YS) cells express mouse embryonic globin genes that function analogously to human γ-globin genes. In mice transgenic for cosmids or yeast artificial chromosomes (YACs) containing human β-globin locus sequences, YS cells at embryonic (E) days 9 to 11 express the human γ-globin gene, whereas mouse fetal liver (FL) cells at E11

Figure 1. The human β-globin locus. (A) The β-globin locus on chromosome 11 embedded in chromatin is shown. (B) A linear map with the globin LCR and its hypersensitive (HS) sites is indicated by the vertical arrows. The structural α-, γ-, β-, and β-globin genes as well as the locations of the olfactory receptor (OR) genes are shown.
to E15 express the human β-globin gene, recapitulating human γ-to β-globin switching to varying extents.20,21,35,59,83-85 Using 3C technology, recent cross-linking studies have shown physical interactions specifically between 5' HS sites in the murine βLKR with HS sequences 3' to the β structural genes in FL at E14.5 (adult-type cells), whereas no such interactions are seen in brain tissue from the same embryos, nor between sequences between the 5' and 3' HS sites.21 In uninduced MEL cells, committed to the erythroid lineage but not induced to erythroid differentiation, a βACH structure exists that is different from that in brain cells even when there is only low-level globin synthesis. In these uninduced MEL cells, however, there are only limited interactions between 5' HS sequences and the 3' HS1.20 On induction of MEL differentiation and the accompanied dramatic increase in globin synthesis, the interactions between βLCR elements and 3' sequences become much more extensive.20

To further define the elements critical for formation of the active βACH in human erythroid cells, transgenic mice with P1-derived artificial chromosomes (PACs) carrying a 185-kb fragment of human β-globin locus DNA, the effect of specific deletions at the locus were studied.33 When the human structural β-globin gene promoter is deleted, there continue to be significant βACH interactions between the βLKR elements upstream, the 3' HS1 and the human structural genes.21 In mice with deletions of the human β-globin promoter and with additional deletions of either human βLKR HS2 or HS3, the HS3 deletion disrupts βACH formation, whereas the HS2 deletion has little additional effect.21 These studies show that interactions between HS3 and the human β-globin promoter are critical for βACH formation.

**Interactions of transcription factors with globin sequences in chromatin**

The mouse β-globin gene LCR, essential for the activated transcription of genes in the cluster, contains multiple binding sites for transcription factors. ChIP experiments show that in uninduced MEL cells, the LCR is occupied by small Maf proteins, and on erythroid maturation, the NF-E2 complex is recruited to the LCR and the active β-globin promoters. The presence of the NF-E2 complex is associated with a more than 100-fold increase in murine β-globin transcription.48 GATA-1 is associated with murine HS2 in ChIP experiments as well.76

Other experiments using 3C technology show that there are specific physical interactions between the transcription factors GATA-1, FOG-1, and EKLF and the βLKR and downstream globin sequences, again demonstrating the close contact and multiple interactions of these 2 important regions of the βACH.52,53 In one study, EKLF-deficient mice were unable to form the βACH, indicating its critical role in the chromatin configuration in adult erythroid cells.53 In another, GATA-1-induced loop formation correlates with the onset of β-globin transcription and occurs independently of new protein synthesis.22 GATA-1 occupies the murine β-globin promoter even in FL erythroblasts of mice lacking the LCR, suggesting that GATA-1 binding to the promoter and LCR are independent events that occur prior to loop formation.22 The results show that GATA-1 and FOG-1 are essential for the formation of the βACH.22

**Acetylation of chromatin at the β-globin locus**

Early ChIP experiments using MEL cells and mouse FL demonstrated that the murine β-globin locus is more acetylated when the β-globin genes are active than when they are not.45 These studies show hyperacetylation only at the βLKR and the β structural genes and not of sequences between these regions and were among the first consistent with looping of chromatin fibers, physical association of these sequences, and formation of the βACH.45 In YS cells, the murine βLKR and both active and inactive promoters are hyperacetylated.45 The specific inhibition of HDACs in these studies selectively increased acetylation at a hypacetylated promoter in FL, suggesting that active deacetylation contributes to silencing of promoters.45

ChIP studies also show methylation of an individual lysine residue, H3-meK79, at the active murine β-globin gene in adult erythroid cells that is dependent on the presence of p45/NF-E2.50 In addition, acetylated H3, and H4 and H3-meK4 are enriched at both the β-globin structural gene and the βLKR. An HDAC-dependent surveillance mechanism may counteract constitutive histone acetyltransferase (HAT) access at chromatin sites.51 There are clearly many other complex relationships between histone acetylation on one hand and deacetylation and methylation on the other at the β-globin locus.51,52,86-89

RNA polymerase II activity is also associated with active chromatin remodeling at the β-globin locus.75,77,79,82,90 Although most transcription is of globin structural gene sequences activated by their association with the βLKR, there is also transcription of intergenic sequences as well.52,90-93 Most intriguing are the unique developmental stage-specific intergenic transcripts from the region between the ε and γ genes in embryonic-fetal cells and from between the γ- and δ-globin genes in adult cells.93

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**Human γ-to β-globin switching**

The details of the interactions between the cis-acting elements and trans-acting factors and their relationships in regulating the human γ-to β-globin switch remain elusive. Presumably, in human fetal life, interactions between transcription factors, chromatin remodeling complexes, and structural gene sequences in chromatin favor expression of human γ genes, and, subsequently in adult-type hematopoietic cells, β-globin gene expression is preferred.

**Studies in transgenic mice**

Changes in the interactions between the βLKR and the downstream embryonic-fetal and adult globin genes during development and globin switching have been elucidated recently by 3C studies.20 Cells that express embryonic globin genes have extensive interactions between the structural embryonic gene sequences and HS2 and HS3 sites in the βLKR.20 By contrast, cells expressing adult β-globins have specific interactions between the adult β-globin genes and HS2 and HS3.20 Transgenic mice carrying the entire human β-globin locus were used in similar studies and, again, show a switch in the association of HS βLKR from contacts with embryonic and fetal genes in YS cells to contacts with the adult human β-globin gene in adult-type E14.5 FL cells.20 The same interactions between the adult human β-globin gene and 5' HS elements were obtained by C3 analysis using fresh adult erythroid cells from human bone marrow as with the E14.5 FL cells, providing evidence that the same conformational changes occur between the human βLKR and the downstream structural genes at the native adult human β locus in normal human erythroid cells as in PACs in transgenic mice.20

To further define the interactions required for switching, the human β-globin promoter and either human HS2 or HS3 were
deleted in transgenic mice.\textsuperscript{21} YS and FL cells doubly mutant for the \(\beta\) promoter and HS2 (\(\Delta\beta/\Delta\mathrm{HS2}\)) are relatively unaffected.\textsuperscript{21} However, whereas \(\Delta\beta/\Delta\mathrm{HS3}\) mice express human \(\gamma\) genes in \(\gamma\) cells, there is no formation of the \(\beta\)-ACH in adult FL.\textsuperscript{21} These results suggest that the deletion of HS3 completely destablizes normal chromatin structure at the \(\beta\)-globin locus in adult-type cells.

Transgenic mice containing cosmids or YACs with deletions of \(\beta\) locus sequences have also been used to study the \(\text{cis}\)-acting elements controlling human \(\gamma\)- to \(\beta\)-globin switching.\textsuperscript{59,83,85,94} The results in mice with these deletions have been interesting but inconsistent. Some studies have shown that deletion of intergenic \(\gamma\)-\(\delta\) sequences leads to delayed switching,\textsuperscript{59,83} whereas others show either no effects or competing positive and negative effects.\textsuperscript{83,85,94}

### Fetal globin expression in human mutants

Natural mutations at the human \(\gamma\)-globin locus in patients with increased HbF have shed important light on the mechanisms regulating human HbF. Studies of patients with these mutations are clearly more related to the biology of human HbF regulation than those in transgenic mice or using cell lines. Transgenic mouse experiments, even with very large human \(\beta\)-globin locus-containing YACs or PACs, are potentially flawed by the random position of these sequences and the fact that mice do not have \(\gamma\)-globin genes. Data have been reported on many individuals with point mutations in the human \(\gamma\) promoter that increase HbF in adult-type erythroid cells.\textsuperscript{2,95} These patients are referred to as having the non-deletion form of hereditary persistence of fetal hemoglobin (HPFH), as compared to those with deletion-type HPFH. Mutations have been described in the promoters of both the \(\mathrm{Gy}\) and \(\mathrm{A}^\gamma\) genes in these patients, and in most cases, the point mutations are between \(-114\) and \(-202\) upstream of the human \(\gamma\)-globin gene transcriptional initiation sites.\textsuperscript{2,95} Several of these mutations modify binding sites for GATA-1 or NF-E4 or are at the distal CAAT box upstream. The level of HbF in heterozygotes for these mutations varies between 10\% and 30\% with mutations in the \(\mathrm{Gy}\) promoter and between 4\% and 20\% in those with \(\mathrm{A}^\gamma\) mutations.\textsuperscript{2,95} A deletion upstream of the \(\mathrm{A}^\gamma\) gene between \(-114\) to \(-102\) also leads to about 30\% HbF in heterozygotes.\textsuperscript{86}

It is difficult to quantify the effects on \(\gamma\)-globin transcription and posttranscriptional events in heterozygotes for these and other mutations without using the sophisticated techniques described recently to measure the output of single mutated alleles described in the primary erythroid cells of patients.\textsuperscript{97} As these recent studies reveal, the levels of HbF in heterozygotes with the same affected \(\gamma\) allele are not constant and depend on the effects of the other allele.\textsuperscript{97} Thus, only homozygous mutations affecting human \(\gamma\)-globin expression are truly informative.

**Figure 2. Mutations and the extent of deletion in thalassemias and HPFH.** A \(\times\) indicates point mutation. Dotted lines indicate deletions. There is more deletion of \(\gamma\)-\(\delta\) intergenic sequences in HPFH than in \(\delta\)-\(\beta\) thalassemia, and the 3’ extent of the deletion is greater in HPFH.

### Table 1. Hemoglobin defects in homozygotes for thalassemia and related disorders

<table>
<thead>
<tr>
<th>State</th>
<th>Anemia</th>
<th>HbA</th>
<th>HbA(_2)</th>
<th>HbF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>None</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>(\beta^+) thalassemia</td>
<td>Severe</td>
<td>Decreased</td>
<td>Normal</td>
<td>Increased*</td>
</tr>
<tr>
<td>(\beta^-) thalassemia</td>
<td>Severe</td>
<td>Absent</td>
<td>Normal</td>
<td>Increased*</td>
</tr>
<tr>
<td>(\delta-\beta) thalassemia</td>
<td>Mild</td>
<td>Absent</td>
<td>Absent</td>
<td>Increased†</td>
</tr>
<tr>
<td>HPFH</td>
<td>None</td>
<td>Absent</td>
<td>Absent</td>
<td>Increased‡</td>
</tr>
<tr>
<td>Corfu</td>
<td>Mild</td>
<td>Decreased</td>
<td>Absent</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Severe anemia is usually defined as having hemoglobin levels below 70 g/L and requires frequent transfusions.

*Slightly increased over normal (5%-10% of total normal Hb levels).
†Moderately increased over normal (10%-30% of total normal Hb).
‡Markedly increased, that is, almost completely compensatory (85%-100% of total normal Hb).

Fortunately, homozygotes for some naturally occurring mutations that affect the human \(\gamma\)-globin locus are available. Some African homozygotes with an uncommon, benign disorder, deletion HPFH, express \(\gamma\)-globin genes fully in adult life, have 100\% HbF, and have no anemia.\textsuperscript{2,95} If human patients with sickle cell disease or \(\beta\) thalassemia could reactivate their HbF production to that of HPFH patients, they would be cured. In fact, Saudi patients doubly heterozygous for \(\beta^+\) and HPFH, expressing 30\% HbF in most red blood cells, are largely asymptomatic.\textsuperscript{98,99}

In the African form of deletion HPFH, large deletions begin just 3’ to the \(\mathrm{A}^\gamma\) gene and extend far 3’ of the structural \(\beta\)-globin gene; the deletion includes intergenic \(\gamma\)-\(\delta\) sequences and the entire structural \(\delta\) and \(\beta\) genes (Figure 2; Table 1). The mechanism leading to the increased level of HbF in HPFH has been shown to be due, at least in part, to enhancer activity provided by the DNA sequences from 3’ to the deletion brought into proximity of the structural \(\gamma\)-globin genes. These human enhancer sequences up-regulate human \(\gamma\) globin expression in transgenic mice.\textsuperscript{84,95,100,101}

Other deletions at the human \(\beta\)-globin locus beginning further 3’ to the \(\gamma\)-globin gene than the HPFH deletion, in the \(\gamma\)-\(\delta\) intergenic region, and extending 3’ to delete the \(\delta\) and \(\beta\)-globin genes, result in a lesser increase in \(\gamma\)-globin than in HPFH in a syndrome known as \(\delta\)-\(\beta\) thalassemia (Figure 2; Table 1).\textsuperscript{1,2,6} Clinically, patients with \(\delta\)-\(\beta\) thalassemia have increased \(\gamma\)-globin and HbF, and some anemia and are intermediate in severity between those with homozygous \(\beta\) thalassemia and HPFH (Table 1). Patients homozygous for \(\beta\) thalassemia, usually due to point mutations in the \(\beta\)-globin gene leading to either reduced (\(\beta^+\)) or absent (\(\beta^-\)) \(\beta\)-globin production have much less \(\gamma\)-globin compensation, more excess \(\alpha\)-globin accumulation, and more severe anemia requiring transfusions than patients homozygous for \(\delta\)-\(\beta\) thalassemia (Table 1).\textsuperscript{1,6,13} Patients with homozygous \(\delta\)-\(\beta\) thalassemia often do not require blood transfusions and are referred to as patients with thalassemia intermedia. Homozygotes with hemoglobin Lepore have high levels of HbF as well and are of interest.\textsuperscript{5} However, the decreased expression of the Lepore (fusion \(\delta\)-\(\beta\)) gene, and the unknown effects of the loss of intergenic sequences between the normal \(\delta\) and \(\beta\) genes in this condition, make it difficult to interpret the cause of the increased HbF in these cases.

The differences between the clinical syndromes of \(\beta\) thalassemia on the one hand, and of \(\delta\)-\(\beta\) thalassemia and HPFH on the other, associated with different levels of \(\gamma\)-globin and HbF compensation as well as differences in the extent of the deletions in these latter conditions (Figure 2), has long suggested a role for DNA sequences between the human \(\gamma\) and \(\delta\)-\(\beta\) globin genes (intergenic \(\gamma\)-\(\delta\) sequences) in \(\gamma\)-globin gene expression in these disorders and in normal human globin switching.\textsuperscript{1,102} However,
because of the lack of data from suitable homozygotes, and the negative results of some transgenic mouse experiments, such a role for these intergenic γ-δ sequences has been in question.\textsuperscript{2,85} In addition, the enhancer activity from sequences 3’ to the HPFH deletion alluded to earlier provided an alternative explanation for the increased HbF in HPFH.\textsuperscript{2}

The Corfu deletion

The recent finding that 2 patients homozygous for the Corfu deletion (Figure 2) have 88% and 90% HbF, only mild anemia, and no transfusion requirement provide the first strong evidence in humans that intergenic γ-δ sequences alone can regulate the activity of HbF in adult-type cells, and perhaps play a role in normal human hemoglobin switching as well.\textsuperscript{97} The 7.2-kb Corfu deletion extends from the γ-δ region upstream of the δ gene to involve the 5’ end of the structural δ-globin gene (Figure 2).

Using erythroid cell cultures from patients homozygous for the Corfu deletion, the authors show that nascent γ-globin gene transcripts are greatly increased at each γ locus, as compared to similar cultures from healthy subjects.\textsuperscript{97} In addition, γ-globin mRNA and γ-globin expression are also extremely high in the Corfu homozygotes. These results indicate that the Corfu deletion alone can almost completely reactivate γ-globin expression in adult-type cells.\textsuperscript{97} It is unclear whether the persistence of human γ-globin and HbF in patients with the Corfu, δ-β thalassemia, or HPFH deletions require the affected cells to undergo normal developmental stage-specific switching in late fetal life, and whether the same deletions occurring later in adult-type cells have the same effects. Early chromosomal events at the human β-globin locus in late fetal life may normally silence normal γ-globin gene expression and limit the extent to which γ-globin reactivation can occur in adult human hematopoietic stem cells and their progeny. Single adult erythroid cells produce both γ- and β-globins, and adult erythroid cells continue to express some γ-globin.\textsuperscript{2} However, the data from patients with HPFH, δ-β thalassemia, and Corfu deletion indicate that developmental γ silencing, to the extent that it occurs, can be overcome by the deletion of the sequences included in these syndromes.

These data also suggest that the intergenic γ-δ sequences are among the critical determinants of the process of human γ-globin gene reactivation, and perhaps of normal human γ to β-globin switching as well. Although the human βLCR and structural gene sequences are required for high-level globin expression of both the human γ- and β-globin genes at the β-globin locus, changes in these sequences are not required for continued high-level γ-globin gene expression or γ-globin reactivation in adult-type erythroid cells.

It is possible that the γ-δ intergenic region deleted in Corfu patients contains sequences that both repress and can potentially activate γ-globin gene transcription. The presence of both positive-acting and negative-acting sequences and their interactions in chromatin might explain the differences in γ-globin gene expression with different extents of deletion of this region, for example, in different patients with δ-β thalassemia and in transgenic animals.\textsuperscript{83,100} The previously described and characterized unique RNA transcripts from the intergenic γ-δ region sequences postulated to play a role in regulating normal human γ-globin gene expression,\textsuperscript{93} are disrupted in patients with the Corfu mutation.\textsuperscript{82} The molecular mechanisms by which loss of these sequences might affect the output of the γ-globin genes is unknown.

**TRANSCONTIONAL REGULATION OF GLOBIN EXPRESSION**

**Transacting factors in transcriptional regulation of γ-globin gene expression**

Although interactions between the βLCR and downstream globin genes mediated by transcription factors such as GATA-1, FOG-1, and EKLF have been documented to occur at the β-globin locus,\textsuperscript{22,35} these interactions by themselves do not provide clues to human γ- to β-globin switching because all of these components are present in both embryonic-fetal and adult cells.\textsuperscript{34} Normal human γ- to β-globin switching probably requires developmental stage-specific changes in transcription factors or chromatin remodeling activities (or both) that lead to either repression of γ-globin gene expression or activation of β-globin genes (or both).

Whereas GATA-1, FOG-1, and NF-E2 are not differentially active in fetal versus adult human cells, EKLF is known to be active predominantly in adult-stage cells, and specifically enhances human β-globin and not γ-globin gene expression.\textsuperscript{44} EKLF-deficient transgenic mice do not form the βACH or activate human β-globin gene expression.\textsuperscript{35} In addition, although EKLF interacts with CBP/p300 and SWI/SNF proteins presumably to activate murine β-globin gene transcription in adult-stage MEL cells,\textsuperscript{57,104} it also interacts with Sin3A and HDAC1 corepressors via its zinc finger domain in K562 embryonic-fetal cells.\textsuperscript{70} A key lysine that is both a substrate for CBP acetylation and required for Sin3A interaction has been identified in these studies.\textsuperscript{70} Although these results remain to be confirmed in vivo, the acetylation status of EKLF may determine its effects at different stages of human globin switching.\textsuperscript{70} Fetal Kruppel-like factors, FKLF and FKLF-2, have been described.\textsuperscript{105,106} FKLF preferentially increases human embryonic-fetal globin gene expression in the human embryonic fetal cell line, K562.\textsuperscript{105}

Two protein complexes have been described that interact with γ-globin promoter elements. The direct repeat erythroid-definitive (DRED) complex contains the nuclear orphan receptors TR2 and TR4 and binds with high affinity to DR1 (direct repeat) sites in the human e- and γ-globin promoters.\textsuperscript{107,108} The human adult β-globin gene has no DR1-binding sites, and this suggests that γ-globin repression in adult cells may be the major function of DRED. This repression by DRED is supported by the fact that a point mutation at −117 of the Aγ promoter associated with high HbF disrupts a DR1-binding site.

Another protein complex binding to the human γ-globin promoter region is the stage selector protein (SSP) complex.\textsuperscript{109,110} This complex has recently been shown to have a unique human p22NF-E4 subunit as its erythroid-specific component\textsuperscript{111} and to have a role in globin switching.\textsuperscript{43,44} Enforced expression of human p22NF-E4 increases human γ-globin expression in K562 cells and delays human γ- to β-globin switching in transgenic mice with a human β-globin YAC.\textsuperscript{44} In addition, site-specific acetylation of p22NF-E4 prevents its ubiquitination and reduces the interaction of p22NF-E4 with HDAC1.\textsuperscript{43}

**PYR complex: a possible switch complex**

Our group has described a chromatin remodeling complex called PYR complex found only in adult hematopoietic cells whose presence suggests the importance of human intergenic γ-δ- globin sequences in human γ to β switching (Figure 3).\textsuperscript{112} PYR complex binds to a 250-bp polypyrimidine (PYR)–rich DNA sequence 1 kb upstream of the human δ-globin gene, and the PYR complex DNA-binding site is included in the Corfu deletion (Figure 2).\textsuperscript{112}

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Deletion of 511 bp of DNA including the PYR binding site upstream of the human δ-globin gene delays human γ to β switching in mice transgenic for a human β-globin locus-containing cosmId.59,113

More recently, we have shown that the transcription factor Ikaros, predominantly expressed in adult hematopoietic cells, is the DNA-binding subunit of the PYR complex.60 Mice that do not express Ikaros (Ik−/−) have no PYR complex binding activity. Ik−/− mice with a transgene carrying human γ- to β-globin sequences have delayed human γ- to β-globin switching as well.114 Taken together, the data suggest that PYR complex has a role in normal human γ to β switching, and that perhaps inhibition of PYR complex action, for example, by siRNA inhibition of Ikaros activity, may be an additional therapeutic approach to reactivation of γ-globin in adult erythroid cells.

The PYR complex has been isolated from MEL cells and characterized biochemically.59,60 It is a single complex with Ikaros as its DNA-binding subunit and contains both positive-acting protein subunits of the SWI/SNF complex that activate gene transcription and repressive protein subunits of the NURD complex, an HDAC-containing complex that can repress transcription (Figure 3).60

Butyrate compounds, known inhibitors of HDAC action,61,69,115,116 increase HbF in patients with SS disease and β thalassemia.17,117,118 Butyrate and other HDAC inhibitors may function to increase HbF production in humans by preventing the HDACs in the PYR complex from repressing γ-globin gene expression (Figure 3). This hypothesis remains to be directly studied and confirmed.

A model of human γ to β-globin switching

As indicated earlier, a unique chromosomal domain, the BACH, is present in all erythroid cells at the human β-globin locus establishing a loop-stem structure in the region (Figure 3).20,21 In fetal life, the γ-globin genes in chromatin associate with the βLCR, ubiquitous, and erythroid-specific and fetal stage-specific transcription factors, and with putative embryonic-fetal stage-specific chromatin remodeling complexes or activities including the potential effects of SSP and FKLF, and favor γ-globin transcription (Figure 3).20,21 In adult-type erythroid cells, the BACH configuration is changed so that the βLCR now preferentially associates with and activates human β-globin gene expression (Figure 3).20,21 One event in this process in vivo for inducing conformational changes at the β-globin locus in adult-type cells may be the adult stage-specific formation and action of the PYR complex, by binding to the intergenic γ-δ sequences at the PYR-binding site and repressing γ-globin transcription by its HDACs (Figure 3). The SWI/SNF subunits of PYR complex may be involved as well. All of these proposed roles for PYR complex must await confirmation by further studies of the role of Ikaros and the PYR complex similar to those recently described.20,21 In addition, DRED and other currently unknown interactions of ubiquitous and adult stage-specific transcription factors and chromatin remodeling complexes are most likely involved in this process, as well as GATA-I, EKLF, and NF-E2.

Therapeutic approaches to reactivation of γ-globin and HbF in adult erythroid cells

Expression of HbF can be controlled at the cellular level by the increased production of cells synthesizing large amounts of HbF by erythropoietin, other cytokines, changes in cell cycle kinetics, and other unknown factors.2,119,120 In situations of “stress erythropoiesis” HbF is produced preferentially by early erythroid progenitors and this can lead to increased HbF levels.2,119,120 However, even with severe stress, the number of HbF-producing clones and their output is limited and does not compensate for the deficit or lack of HbA-producing cells in disorders such as human homozygous β thalassemia.2,119,120

Intracellularly, human γ-globin gene expression during development and in reactivation in adult-type cells can be controlled at the levels of posttranscriptional processing of γ mRNA transcripts, mRNA stability, translation, and posttranslational events, as well as at the level of transcription. In transgenic mice, the relative half-lives of human γ- and β-globin gene transcripts are similar.19

Agents that increase human HbF in patients may work at one or more levels.115,118,121 For example, hydroxyurea and 5-azacytidine kill dividing cells preferentially and may increase γ-globin expression indirectly through this effect.115,118,122 5-Azacytidine is also a demethylating agent and may reactivate γ expression by this mechanism as well.118,122 Butyrate may work both by HDAC inhibition and by increasing γ-globin translation on ribosomes.121 The combination of erythropoietin and hydroxyurea has synergistic effects in increasing HbF in some patients.115,118,123

Several important signal transduction pathways have been shown to be associated with increases in γ-globin in K562 cells and

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Figure 3. The proposed role of the PYR complex in human hemoglobin switching. The circles indicate unspecified chromatin remodeling complexes and transcription factors. The details of these interactions in chromatin between the βLCR elements, globin structural genes, erythroid transcription factors, and these chromatin remodeling complexes are unknown. In fetal-embryonic cells, the human γLCR is associated with the γ-globin gene loci downstream. The blue circles include the potential activities of FKLF and SSP in this process at the γ-globin promoter. In adult-type cells, the βLCR associates with and activates β-globin gene expression. New interactions leading to repression of γ-globin gene expression occur, and PYR complex binding and its HDACs may contribute to this process. The SWI/SNF complex subunits, the NURD subunits, and the DNA-binding subunit Ikaros of the PYR complex are shown.

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Several important signal transduction pathways have been shown to be associated with increases in γ-globin in K562 cells and
primary human CD34+ cells, and modified by hydroxyurea and butyrate therapy.124-128 This signaling includes (1) modulation of soluble guanylate cyclase and of cyclic AMP and GMP124-128; (2) increased nitric oxide production;26; (3) changes in protein kinases modulated by stem cell factor127; and (4) inhibition of Stat3 phosphorylation.125 New therapies to increase HbF levels in humans may result from exploiting these observations.

Inhibition of DRED complex activity, or, as mentioned earlier, of Ikars and PYR complex action by siRNAs or other small molecules in adult erythroid cells may add to the therapeutic regimen for increasing HbF. Similarly, increasing the expression of proteins such as NF-E4, and Id2 and of other proteins shown to be up-regulated with increased HbF may be useful as well.44,129

Last, there appears to be an inverse relationship between human γ- and β-globin accumulation in adult erythroid cells.97 In patients doubly heterozygous for Corfu and β thalassemia genes or heterozygotes for Corfu alone when there is more β-globin and HbA present, there are less than expected levels of γ-globin and HbF accumulation.97 In contrast, this inverse relationship is not seen when measuring primary globin transcripts in the erythroid cells of these patients.97 Primary transcripts from a single Corfu locus in patients doubly heterozygous for Corfu and β thalassemia genes or Corfu heterozygotes (with one Corfu locus) are shown to be expressed, as expected, at about half the level of γ transcripts of the Corfu homozygotes (with 2 Corfu loci). These data indicate that posttranscriptional events may determine the limits to which γ-globin production and HbF can increase in adult cells producing β-globin and HbA.97 This effect may be due to the preferential stabilization of human β mRNA over γ mRNA mediated by differential binding to limiting amounts of ribonucleoprotein complexes.97,130 Thus, down-regulating human β-globin expression may be a useful additional approach to optimizing HbF production in patients being treated with HbF-inducing agents.

**Summary**

Although many of the events controlling the activity of the β-globin locus are known, the details of those regulating normal human hemoglobin switching and reactivation of HbF in adult hematopoietic cells remain to be elucidated. If the molecular events in hemoglobin switching or γ-globin gene reactivation were better understood and HbF could be fully reactivated in adult cells, the insights obtained might lead to a cure for these disorders. An active chromatin configuration with interactions between the βLCR and downstream genes by looping of chromatin fibers has been demonstrated in all erythroid cells studied. These interactions are modulated by transcription factors and chromatin remodeling activities. In human fetal cells, the γ-globin gene is preferentially activated by these interactions, and γ-globin and HbF predominate. In adult-stage cells, these interactions result in the βLCR enhancing human β-globin gene expression by associating specifically with sequences at the β-globin gene instead of the γ-globin gene. The potential role of intergenic γ-sequences in this process has recently been strengthened by studies in patients homozygous for the Corfu deletion who have very high HbF levels, only mild anemia, and no transfusion requirements. In addition, an adult stage-specific hematopoietic cell-specific chromatin remodeling complex, PYR complex, may be involved in switching, and suggests new approaches to reactivating HbF such as preventing Ikars action by siRNAs in adult erythroid cells. Similarly, increased activity of FKLF and SSP, and repression of DRED complex, may increase γ-globin and HbF in adult erythroid cells as well. Identification and characterization of other chromatin remodeling complexes and their interactions with transcription factors, the LCR, and downstream sequences in chromatin should provide further details of the dynamic processes of γ-globin gene regulation and human hemoglobin switching and will hopefully lead to new combination therapies to enhance fetal hemoglobin expression in adult erythroid cells.

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