MINIREVIEW

Chromatin Structure and Control of β-Like Globin Gene Switching

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The human β-globin locus is a complex genetic system widely used for analysis of eukaryotic gene expression. The locus consists of five functional β-like globin genes, ε, $^{G}\gamma$, $^{A}\gamma$, δ, and β, arrayed on the chromosome in the order that they are expressed during ontogeny. Globin gene expression is regulated, in part, by the locus control region, which physically consists of five DNaseI-hypersensitive sites located 6-22 Kb upstream of the ε-globin gene. During ontogeny two switches occur in β-globin gene expression that reflect the changing oxygen requirements of the fetus. The first switch from embryonic ε- to fetal γ-globin occurs at six weeks of gestation. The second switch from γ- to adult δ- and β-globin occurs shortly after birth. Throughout the locus, *cis*-acting elements exist that are dynamically bound by *trans*-acting proteins, including transcription factors, co-activators, repressors, and chromatin modifiers. Discovery of novel erythroid-specific transcription factors and a role for chromatin structure in gene expression have enhanced our understanding of the mechanism of globin gene switching. However, the hierarchy of events regulating gene expression during development, from extracellular signaling to transcriptional activation or repression, is complex. In this review we attempt to unify the current knowledge regarding the interplay of *cis*-acting elements, transcription factors, and chromatin modifiers into a comprehensive overview of globin gene switching. Exp Biol Med Vol. 227(9):683–700, 2002

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Hemoglobinopathies are a group of inherited disorders characterized by the absence of functional α-like or β-like globin chains (1). Sickle cell diseases (SCDs) and β-thalassemias are two of the most common categories of hematopoietic diseases. SCDs include sickle cell anemia, sickle cell-hemoglobin C disease, and sickle cell-β-thalassemia. Millions worldwide are impacted; one of 400 African Americans, over 70,000 victims, is afflicted. These diseases are major health problems, associated with severe morbidity, lower-than-average life expectancy, and serious, long-term disability. Sickle cell anemia alone is a major hemoglobinopathy, caused by single point mutation in the sixth codon of the β-globin gene that ultimately affects the shape of red blood cells, rendering them ineffective for oxygen transport. β-thalassemias result from an array of mutations in the β-globin locus that lead to severely decreased or absent adult β-globin synthesis, the consequence of which is anemia. Clearly, it is of interest to combat these deadly diseases.

In the circulatory system, erythrocytes (red blood cells) transport oxygen to bodily tissues and carbon dioxide to the lungs for exhalation. Within erythrocytes, this process is mediated by hemoglobin, a molecule that consists of two α-like and two β-like globin chains and four iron-coordinated heme moieties. The human α-like and β-like globin loci, located on chromosomes 16 and 11, respectively, encode these protein chains. During development, different α- and β-globin genes are expressed to produce a developmental stage-specific hemoglobin molecule that meets the oxygen demand of the developing fetus. Naturally occurring mutations within these loci cause the production of abnormal hemoglobins. Mutations in the adult β-globin gene result in SCD and β-thalassemias, whereas mutations in the α-globin genes cause α-thalassemias (1). Individuals with defective adult β-globin genes are phenotypically normal if they carry compensatory mutations that result in in-

creased synthesis of the fetal β-like globin genes ($^{G}\gamma$- and $^{A}\gamma$-globin), a condition called hereditary persistence of fetal hemoglobin [HPFH, (1)]. The observation that increased γ-globin production overcomes SCDs or β-thalassemias led to the proposed use of γ-globin gene constructs in vectors for gene therapy, or γ-globin gene reactivation via targeted drug intervention, for treatment of these diseases. Thus, understanding the molecular mechanisms of globin gene switching is central to the development of these therapeutic modalities for application to the patient population. Ultimately, a cure for these disorders will depend on the replacement of the mutant globin gene by gene therapy.

β-Like Globin Gene Switching. The human β-globin locus consists of five functional β-like globin genes and an upstream regulatory element, the locus control region (LCR), that is physically composed of five DNase I-hypersensitive sites (HSs) [Fig. 1A; (2–6)]. The genes are expressed in a tissue- and developmental-specific fashion and are arranged spatially in the order of their expression during ontogeny, 5′-ε-$^{G}\gamma$-$^{A}\gamma$-δ-β-3′. During development, two switches of globin gene expression and site of hematopoiesis occur (Fig. 1B). The ε-globin gene is expressed during the first six weeks of gestation in primitive, nucleated erythroid cells of the yolk sac, while the γ- and β-globin genes are silent (embryonic or primitive erythropoiesis). During the first switch $^{G}\gamma$- and $^{A}\gamma$-globin gene expression is activated in the definitive hematopoietic cells of the fetal liver (fetal definitive erythropoiesis). The ε-globin gene is concomitantly silenced with γ-globin gene activation. During the second switch shortly after birth, the β-globin gene, and to a lesser extent the δ-globin gene, are activated in the bone marrow and spleen (adult definitive erythropoiesis). When the adult β-globin gene is expressed, the γ-globin genes are reciprocally silenced.

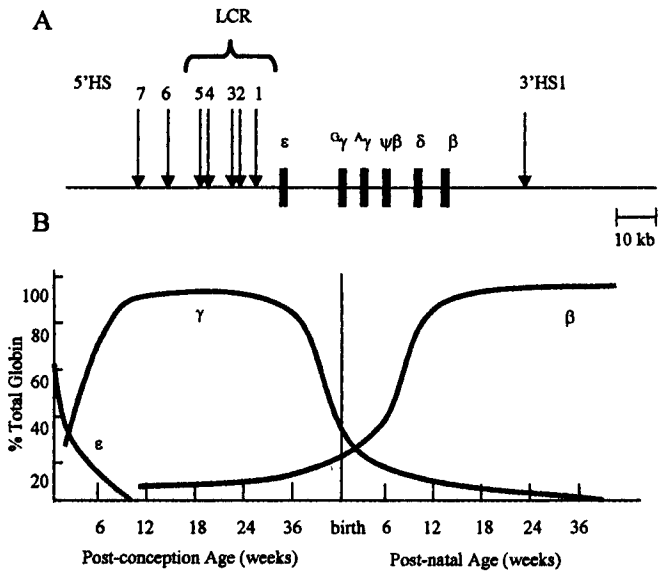

Figure 1. The structure of human β-globin locus and globin gene expression profile during ontogeny. (A) The locus consists of five functional genes and one pseudogene, indicated as colored boxes, which are arrayed in the order of expression during development, 5′-ε-$^{G}\gamma$-$^{A}\gamma$-δ-β-3′. Upstream of the locus are seven DNase I-hypersensitive sites (HSs) indicated by arrows. The first five HSs (5′HS1-5) constitute the locus control region (LCR). Downstream of the locus is a single hypersensitive site, 3′HS1. (B) β-like globin gene expression. The x-axis is the age of the fetus in weeks. The y-axis is the expression of each globin gene as a percentage of total globin gene expression. Time of birth is denoted with a vertical line. The ε-globin expression pattern is shown in blue, γ-globin in green, and β-globin in pink, corresponding to the colors representing the genes in panel A. The embryonic gene is expressed during the first six weeks of gestation. The first switch from ε- to γ-globin occurs shortly after conception, and the second switch from γ- to β-globin occurs shortly after birth.

Studies utilizing transgenic mice produced with a variety of constructs containing portions or the entire human β-globin locus have provided insight into the *cis*-control of globin gene switching. Knockout mice bearing null mutations in a number of genes have identified several trans-acting proteins necessary for globin gene expression. The individual genes of the human β-globin locus are developmentally regulated through their proximal promoters, but expression in transgenic mice is usually low and variable due to position of integration effects. Position effects are overcome by linkage to the LCR and high-level, position-independent expression is observed for the human γ- or β-globin genes (2, 7, 8). However, individual γ- or β-globin genes linked to the LCR display improper temporal regulation that is restored only when the two are linked in tandem to the LCR. These data indicated that the fetal γ-globin to adult β-globin switch is controlled by promoter competition for the LCR (7–9). Unlike the γ- and β-globin genes, when the ε-globin gene is linked alone to the LCR, it is both activated and silenced autonomously (10), although the gene also appears to be regulated competitively during primitive erythropoiesis (11).

Cis-Regulatory Elements. Studies of *cis*-linked regulatory elements that control β-like globin gene expression have revealed a variety of motifs that activate or repress the globin genes at the appropriate stage of development. These elements include promoters, enhancers, silencers, insulators, MARs/SARs, boundary elements and the LCR (Table I). Since promoter and enhancer function is well defined for transcriptional activation of genes, only the unique attributes of the remaining elements will be considered regarding globin gene expression.

Silencers. Silencers bind repressor protein complexes that interfere with promoter activity, thereby downregulating gene expression (12). A silencer located in the distal promoter of the ε-globin gene controls autonomous repression of ε-globin gene expression during the fetal and adult stages of development (10, 13–15). GATA-1 and YY1 proteins constitute at least two of the components of the repressor complex (16). Mutational studies of this silencer indicate that it also encompasses sequences important for the activation of γ-globin transgene expression during the embryonic stage of development in mice (15). Additionally, two direct repeat (DR) elements located in the proximal ε-globin promoter bind a novel protein called direct repeat

Table I. *Cis*-Acting Transcriptional Regulatory Elements

Element/Description
Locus control region (LCR)
• Physically defined by the presence of DNase I-hypersensitive sites that bind tissue-specific and ubiquitous transcription factors and synergistically contribute to overall LCR function
• Opens locus chromatin domains
• Insulates against the effects of surrounding negative chromatin
• Has cell lineage-specific enhancer activity
• Influences timing of replication and origin utilized
• Linked transgenes are expressed in a tissue specific, integration site-independent, copy-number dependent manner; that is, position effect variegation of linked transgenes is ameliorated
Promoter
• Region of DNA where the RNA polymerase complex binds and initiates transcription
Enhancer
• Stimulates transcription from a promoter in a distance- and orientation-independent manner
Silencer
• Element that binds a repressor protein or protein complex resulting in downregulation of gene expression
Insulator
• Creates independent functional domain without enhancement or activation of gene expression by blocking the effects of surrounding chromatin
• Disrupts interaction between a promoter and another regulatory element when placed between them
Matrix attachment region (MAR) or scaffold attachment region (SAR)
• DNA element that promotes binding to the nuclear matrix
• A DNA loop between two MARs may constitute an open chromatin domain by providing a barrier against surrounding chromatin effects and a structural restraint to chromatin remodeling
• May be involved in replication timing and developmental regulation of gene expression
• No enhancer activity
Boundary element
• DNA region that may have unique terminal domain sequence elements and associated DNA binding proteins
• Role in regulating "steady states" between open and closed chromatin environments during development
• Has some properties of insulators

erythroid-definitive binding protein (DRED). Binding of this protein appears to interfere with erythroid Krüppel-like factor (EKLF) binding to the promoter, thus silencing ε-globin gene expression during the adult stage of definitive erythropoiesis.

Insulators. Insulator elements protect against the negative effects of neighboring heterochromatin and may serve as boundary elements that flank or demarcate an open, transcriptionally active chromatin domain (17). Insulator elements may block histone deacetylase activity (18). The human β-globin LCR has insulator properties because linked transgenes are expressed in erythroid cells in a position-independent manner (2). Insulators facilitate the activity of enhancers located within an open chromatin domain (19). 5′HS5 of the LCR may act as an insulator (20).

MARs/SARs. Matrix attachment regions (MARs) or scaffold attachment regions (SARs) are elements that promote binding to the nuclear matrix, resulting in the formation of contiguous DNA sequence loops. These elements may provide a barrier by shielding the locus from the effects of surrounding negative chromatin or provide a structural restraint to chromatin remodeling (19, 21); thus the DNA loop may be a target for transcriptional activation. MARs may protect DNA from the effects of *cis*-acting elements in neighboring loops as chromatin decondenses or may bring the *cis*-acting elements of nearby loops close together (22). MARs may aid in the juxtaposition of distant *cis*-regulatory sequences and gene promoters within the same loop (22). A 2.6 Kb region of the LCR containing 5′HS5 has been identified as having sequences similar to MARs (11, 23), as has the chicken β-globin 5′HS4 (22) and mouse 5′HS6 (24). Although the role of 5′HS5 remains controversial (Zafarana *et al.*, 1995), recent evidence suggests that this region may behave more like a silencer (12) than as an insulator (25).

Boundary Elements. Boundary elements may be located at various positions within a locus and may assume a restrictive role regarding gene expression when associated with binding proteins. Three properties may be characteristic of boundary elements; possible association with insulators, maintenance of a steady state between open and closed chromatin, and presence of terminal domain sequence elements and binding proteins (19). Gribnau *et al.* (26) defined boundary elements as sequences that isolate specific chromatin domains. They contain *cis*-acting elements that have a positive influence within the domain and prevent chromatin influence from outside the domain. Pikaart *et al.* (18) evaluated a 1.2 Kb sequence of the chicken β-globin locus 5′HS4 and showed that it had insulator activity. In addition, it demarcates the DNA boundary for the active chromatin domain in erythroid cells. This 5′ boundary-insulator element protects β-globin transgenes from position-of-integration effects and allows gene expression levels concomitant with the type of enhancer associated with the transgene. Transgenes lacking this element lost DNase I-sensitivity, and were methylated and hypoacetylated, properties normally associated with inactive chromatin. Boundary elements may exist well upstream and downstream of the β-globin locus, defining the β-globin chromatin domain. In addition, they may be found within the locus itself, demarcating developmentally regulated embryonic, fetal and adult globin gene expression chromatin subdomains (26).

LCR. LCRs have been identified in at least 36 mammalian loci of different species, including humans, mice, rats, rabbits, and goats. The human β-globin LCR was functionally defined on the basis of its effect on linked transgenes (2); it was physically defined by the presence of five

HSs (2–6), areas of nucleosome disruption where DNA is susceptible to digestion with DNase I, thereby rendering the region accessible to transcription and chromatin remodeling factors. Four of the HSs (5′HS1–4) are erythroid-specific; one (5′HS5) is ubiquitous (23). Two additional HSs have since been discovered at the 5′ end of the β-globin domain (5′HS6, 7) (27). LCRs confer high-level, position-independent, copy number-dependent, tissue-specific gene expression on transgenes (2, 28).

Individual HSs within the LCR appear to have different roles in chromatin remodeling and control of globin gene switching. The HS properties are summarized in Table II. The LCR contains a general enhancer element, 5′HS2, that functions during all three developmental stages. 5′HS2 contains binding sites for Sp1, NF-E2, GATA-1, and USF. Mutation of individual binding sites within 5′HS2 did not eliminate position-independent expression (29), suggesting that the remaining wild-type binding sites within the mutant 5′HS2 sequences were sufficient to maintain the open chromatin state. Experiments also verified that 5′HS2 could elicit transcription of downstream genes in either orientation, characteristic of enhancer ability (30). Similar to other enhancers, 5′HS2 encodes E-box sequences, which are binding sites for the basic helix-loop-helix family of transcription factor proteins, such as USF and Tal 1 (SCF) (31). Binding of NF-E2 is directly correlated with 5′HS2 enhancer function (32). However, 5′HS2 does not display enhancer activity by itself in single-copy transgenes; it requires the presence of another LCR HS (33). Ellis *et al.* (33) demonstrated that a 1.9 Kb 5′HS3 sequence has chromatin-opening function or chromatin-remodeling activity. Replacement of the 5′HS2 core with the 5′HS3 core restored chromatin-opening activity as measured by DNase I-hypersensitivity, but was unable to restore normal gene activation, allowing γ-globin to be expressed only weakly (32).

Deletion of the HS core element (200–300 bp) from 5′HS2, 3, or 4 interferes with LCR function and disrupts the DNase I-hypersensitivity of all sites (32). The collection of sequence elements that comprise the LCR synergizes to interact, directly or indirectly, with gene-proximal regulatory elements to achieve proper gene regulation (34). Functionality of the LCR as a unit may be orientation dependent (11). When the LCR was inverted relative to the rest of the locus, globin gene expression was reduced throughout development. 5′HS5 has sequence homology to MARs and may function as an insulator (23); thus, when the LCR sequence is inverted, 5′HS5 may insulate the globin genes from interacting with the LCR, thus silencing their expression.

LCR-Globin Gene Interaction. Other than the LCR, most of the *cis*-acting elements described above function locally, over nearby regions of chromatin. In contrast, the LCR functions over long distances to activate globin gene expression and maintain the β-globin chromatin domain in erythrocytes. Initially, the competition hypothesis was proposed by Choi and Engel (9) to account for LCR-globin gene interaction and globin gene switching in chickens. This hypothesis stated that the globin gene promoters competed for interaction with the LCR during development. Applied to human globin gene switching, it was assumed that during the fetal stage of development, the availability of fetal stage-specific factors favors the interaction of the γ-globin gene with the LCR, whereas in the adult stage of erythropoiesis the presence of adult stage-specific factors favors the interaction between the LCR and the β-globin gene. As a result, the β-globin gene is turned off competitively in the fetus, whereas the γ-globin gene is turned off competitively in the adult (7, 8, 28, 35, 36). However, the mechanism by which the LCR interacts with the globin genes has yet to be defined. Four models of LCR function have been proposed (Fig. 2).

The looping model (Fig. 2A) suggests that the LCR 5′HSs fold into a holocomplex, with the HS core elements forming an active site that binds transcription factors and the core-flanking sequences constraining the holocomplex in the proper conformation. This structure loops so that the LCR closely associates with gene-proximal promoter and enhancer elements to deliver bound transcription proteins that interact with the basal transcription apparatus already bound at the promoter, thereby activating globin gene expression (33, 34, 37–43). A variation of this model suggests that initially the LCR is a multiple element receptor that acts as a hub for factor binding that directs chromatin remodeling (44). Once chromatin-remodeling activity has been initiated, the LCR directly interacts with downstream genes to facilitate their expression.

Deletion of the 5′HS2 core abolished expression of the ε-, γ-, and β-globin genes (32). Based on these data, a model was proposed suggesting that the remaining 5′HS2 flanking regions were able interact with the flanking sequences of the other 5′HSs to form the normal holocomplex conformation. Removal of only the 5′HS2 core in effect destroyed the active site of the holocomplex, resulting in a dominant negative mutation that crippled LCR function. However, when the entire 5′HS2 region of conserved sequence similarity (core and flanking sequences) was removed, the ε-, γ- and β-globin genes were expressed in the correct temporal order, although the levels of each were decreased several fold (45). Thus, the remaining 5′HS sites were able to adapt a different holocomplex conformation with a slightly less effective active site comprised of the remaining 5′HS cores and constrained in form by the remaining 5′HS flanking sequences. Similar results were found with 5′HS3 core deletions versus complete deletion of 5′HS3 (45, 46). In addition, the 5′HS3 core could functionally replace the 5′HS4 core, but the 5′HS4 core could not functionally replace the 5′HS3 core, supporting the existence of a LCR holocomplex active site (46).

In the tracking model (Fig. 2B), auxiliary transcription factors and co-factors bind to LCR sequences forming an activation complex that migrates, or tracks, linearly along the DNA helix (47, 48). When this transcription complex

Table II. LCR 5′ Hypersensitive Site (HS) Function in Transgenic or Chimeric Mice

Analysis of individual or multiple HSs linked to reporter gene
5′HS4
• Transcription activation activity
• Position-independent expression
• Copy number-dependent expression (136,137)
5′HS3
• Transcription activation activity
• Position-independent expression when linked to β-globin gene
• Copy number-dependent expression when linked to β-globin gene
• Position-dependent expression when linked to $^{A}\gamma$-globin gene
• Chromatin opening activity in single copy transgenes (33,136–138)
5′HS2
• Enhancer activity
• Position-independent expression
• Copy number-dependent expression
• Binding sites for GATA1, NF-E2, Sp1, Ap-1, USF; all necessary for enhancer function
• Deletion of single GATA1-NF-E2, Sp1, or USF site does not impair position-independent expression
• 373 bp HS core confers position-independent expression
• No enhancer activity, position-dependent expression in single copy transgenes (29,33,136,137,139,140)
5′HS1
• No transcriptional activation activity (137)
Analysis of individual or multiple HS deletions or rearrangements within whole β-globin loci
5′HS5
• Deletion within murine β-globin locus had minimal effect on globin gene expression, not necessary to insulate from surrounding negative chromatin (24)
5′HS4
• 280 bp HS core deletion signficantly altered globin gene expression at all developmental stages in 155 Kb human β-YAC transgenic mice
• Replacement of 5′HS3 core with 5′HS4 core in 155 Kb human β-YAC transgenic mice altered levels of globin gene expression, but not total globin expression
• 875 bp deletion in 70 Kb human β-globin locus cosmid construct had no effect
• 280 bp core deletion in 248 Kb β-YAC mice resulted in position-dependent expression of γ- and β-globin during definitive erythropoiesis (34,46,141)
5′HS3
• 225 bp HS core deletion significantly altered globin gene expression at all developmental stages in 155 Kb human β-YAC transgenic mice
• Replacement of 5′HS4 core with 5′HS3 core in 155 Kb human β-YAC transgenic mice did not affect globin gene expression
• 2.3 Kb deletion in 248 Kb human β-YAC transgenic mice decreased ε-globin gene expression and increased γ-globin gene expression in embryo
• 1.4 Kb deletion in 70 Kb human β-globin locus cosmid resulted in reduction of expression of all globin genes
• 234 bp core deletion in 248 Kb human β-YAC transgenic mice abolished ε-globin gene expression during primitive erythropoiesis, γ-globin gene expression during definitive erythropoiesis, and caused position-dependent β-globin gene expression (34,45,46,142)
5′HS2
• No significant change in expression levels or temporal pattern following deletion within murine β-globin locus
• 1.9 Kb deletion in 248 Kb human β-YAC transgenic mice caused slight decrease in globin gene expression throughout development, but no change in temporal pattern
• 742 bp deletion in 70 Kb human β-globin locus cosmid caused variable gene expression
• 375 bp core deletion in 155 Kb human β-YAC mice significantly decreased ε- and γ-globin expression during primitive erythropoiesis, no γ-globin expression and significantly decreased β-globin expression during definitive erythropoiesis; replacement of 5′HS2 core with 5′HS3 core partially rescued γ-globin gene expression during primitive erythropoiesis (32,34,45,143)
5′HS1
• 1.7 Kb deletion in 70 Kb human β-globin locus cosmid caused variable gene expression (34)

encounters the basal transcription machinery located at the correct developmental stage promoter, the complete transcriptional apparatus is assembled and transcription of that gene ensues. Deacetylases and methylases within the complex may reorganize chromatin after the transcription complex activates transcription, possibly to limit activation to a particular developmental stage.

The facilitated-tracking model (Fig. 2C) combines aspects of the looping and tracking models (48). Transcription factors bind 5′HS sequence motifs of the LCR and this

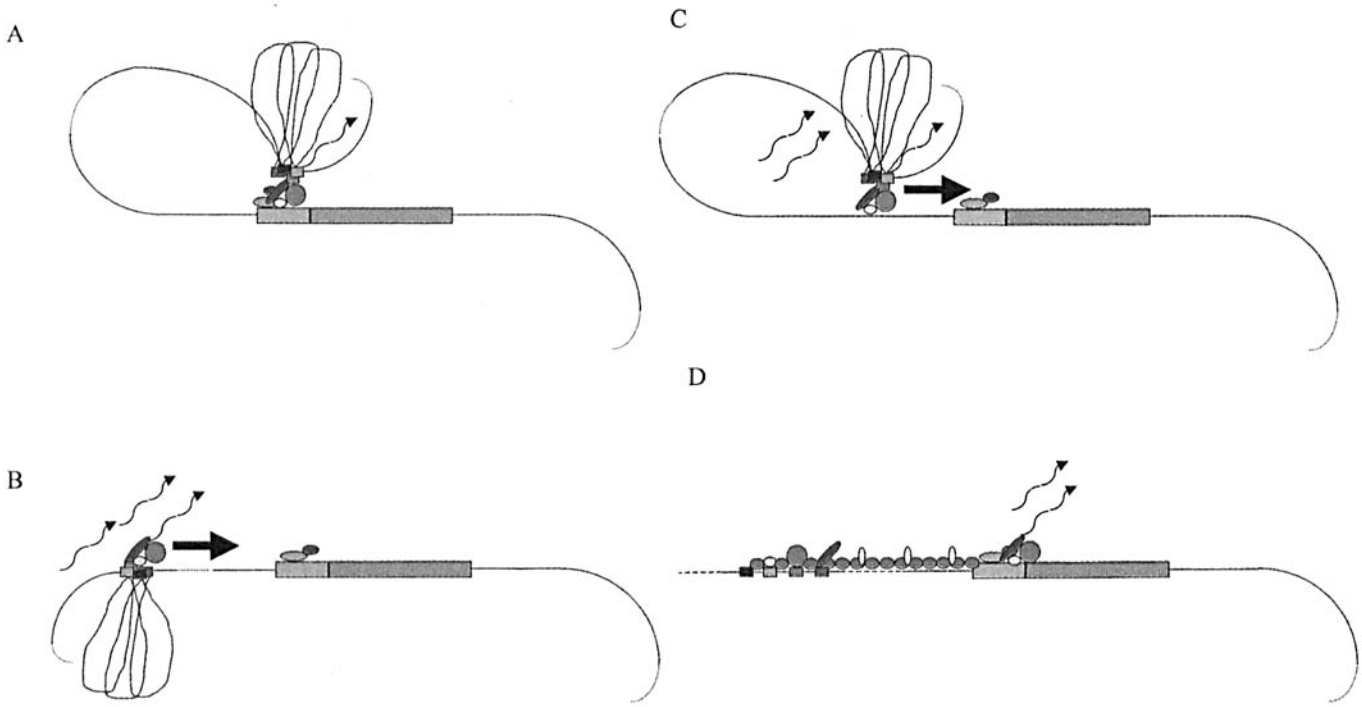

Figure 2. Models of LCR function. A globin gene is denoted as a green rectangular box with the promoter region indicated in a lighter green. Transcription factors are shown as colored ovals and circles. The four erythroid-specific hypersensitive site cores (HSs) are indicated by small colored boxes. The flanking DNA sequences of the HSs are depicted as loops between the HS cores. Transcripts are denoted by wavy arrows. (A) Looping model. Transcription factors bind to the LCR HSs and the gene promoter. The LCR directly interacts with the gene promoter by looping out the intervening DNA, thus forming an active transcription complex at the gene promoter. (B) Tracking model. Sequence-specific transcription factors bind to the LCR forming a complex that tracks down the DNA sequence until encountering transcription factors bound to the appropriate gene promoter, initiating high-level gene expression. (C) Facilitated tracking model. Aspects of both looping and tracking models are combined. Sequence-specific transcription factors bind the LCR, looping then occurs to deliver the bound transcription factors proximal to the gene promoter, followed by tracking until they encounter transcription factors bound to the appropriate gene promoter. (D) Linking model. Sequential binding of transcription factors along the DNA directs changes in chromatin conformation and defines the transcriptional domain. The transcription factors are linked to one another from the LCR to the gene promoter by non–DNA-binding proteins and chromatin modifiers (shown as colored ovals and circles).

complex loops to contact downstream DNA, where the transcription factor complex is released. Subsequently, the activation complex tracks downstream to the appropriate promoter elements with their associated bound proteins and gene expression proceeds.

The linking model (Fig. 2D) suggests that there is a sequential stage-specific binding of transcription factors and chromatin facilitator proteins throughout the locus defining the transcriptional domain (38). Transcription factors bound to gene promoters and hypersensitive sites of a transcriptionally primed locus are tethered to one another by a chain of non–DNA-binding facilitating factors. In the β-globin locus this continuous protein complex may link the LCR to the β-like globin gene to be transcribed (49). A mammalian protein complex homologous to the *Drosophila* Chip protein complex may act as the guiding protein for transcription initiation within the globin locus, forming the bridge between transcription factors bound to the gene promoters and factors bound at the LCR (38). This Chip-like protein complex may allow transcriptional activation of one globin gene at a time, while simultaneously blocking transcription outside of the region. The Chip-like proteins interact with the transcription factors bound to a promoter region at a specific developmental time point targeting that promoter for transcriptional activation through interaction with the LCR. At the appropriate stage the Chip-like chain elongates, moving to the next transcription factor-bound promoter to target that one for LCR interaction. Thus, globin gene switching proceeds.

Chromatin Remodeling Function of the LCR. The mechanism by which chromatin structure controls gene expression has been the focus of recent investigations. Chromatin is an orderly packaging of DNA in nucleic acid-protein complexes called nucleosomes that ultimately aids in the partitioning of DNA to daughter cells during cell division. Chromatin exerts control over gene expression by alternating between so-called "open" and "closed" conformations or states. Open chromatin is generally DNase I-sensitive and hyperacetylated, whereas closed chromatin is DNase I-insensitive and underacetylated. Mechanisms by which chromatin structure controls activation and repression of transcription are not well understood. Proteins that bind and activate transcription may access DNA without disruption of the nucleosome or they may require the assistance of protein cofactors that function to make DNA more accessible by modifying the nucleosome (50). Other less

defined parameters such as the nature of the inherent DNA sequence and the stage of the cell cycle influence the structure of chromatin (51).

Controversial data exist regarding the chromatin-opening function of the LCR. Naturally occurring mutations within the human β-globin locus and experiments utilizing transgenic mice in which β-globin transgenes are located ectopically demonstrate that the LCR has a role in chromatin remodeling. However, data from cell lines or chimeric mouse lines, in which the LCR was deleted, suggest that the LCR does not have chromatin opening function. The role of the LCR in modulating β-globin locus chromatin structure is best exemplified by certain mutations underlying human thalassemias. Hispanic thalassemia is caused by a 35 Kb deletion encompassing the LCR and 22 Kb upstream (6). In these patients the β-globin locus chromatin domain is in a closed, DNase I-resistant, transcriptionally inactive conformation, demonstrating that the LCR functions to open chromatin in addition to its direct role in globin gene activation (52). However, when the β-globin LCR was deleted from the endogenous mouse β-globin locus in embryonic stem (ES) cells and somatic cell lines, β-like globin transcript levels were reduced, whereas the switching pattern during development remained normal and chromatin existed in an open, DNase I-sensitive, conformation. These results suggest that the LCR is not necessary for establishment of the open chromatin locus; it functions primarily as an enhancer for transcriptional activation of the globin genes (53). In addition, experiments using DT40-MEL hybrid cells bearing a human β-globin locus, in which LCR 5'HS2-5 were deleted, demonstrated that the human LCR was necessary for transcriptional activation of gene expression, but not for maintenance of an open chromatin state (20, 49, 54). Therefore, the LCR may participate in transcriptional activation of an open chromatin domain through recruitment of additional transcription factors, through interaction with the already recruited transcriptional complex to fine-tune gene activation, or both (55). The contradictory results with the Hispanic deletion versus the deletions in mice or cell lines may be due to differences in the history of the chromosome or differences in the size of the deletion. Clearly, experiments in transgenic mice demonstrating position-independent expression of LCR-linked genes support a role for the LCR in chromatin remodeling. Finally, Iler *et al.* (56) inserted a DNase I-hypersensitive site-forming element (HSFE), a 920 bp region of 5'HS4 containing NF-E2, Sp1 and GATA-1 binding sites, upstream of a β-globin transgene bearing a 280 bp promoter region and observed a 3-fold activation of β-globin gene expression and concomitant prevention of β-globin gene silencing. DNaseI hypersensitivity assays indicated that incorporation of the HSFE upstream of the minimal β-globin gene promoter increased the extent of open chromatin at the promoter, and the proportion of promoters in an open chromatin configuration. Thus, this LCR element maintains a chromatin state that is conducive for the binding of additional factors that may be involved in further opening of chromatin or activating gene transcription.

Trans-Acting Factors. Many transcription factors controlling β-globin gene expression have been identified and characterized. These factors form an intricate network of protein-protein and protein-DNA interactions with each other, globin gene promoters, LCR HSs, and other *cis*-acting intergenic regions. These data are summarized in Table III. Some erythroid-specific transcription factors are targets of histone acetyltransferases (HATs) and some exist as phosphoproteins, indicating that their activities are regulated by post-translational modification and that they may be involved in chromatin remodeling of the β-globin locus.

NF-E2. NF-E2 was initially identified as an erythroid-specific DNA-binding protein that recognizes an AP-1-like motif in 5'HS2 of the LCR (57–59). This transcription factor is a heterodimer composed of basic leucine zipper proteins (Fig. 3), a 45 kD subunit (p45 NF-E2) and a small, 18 kD subunit (p18 small Maf protein). At least five homologues of p45 NF-E2 have been cloned from various tissues; all are members of the *Drosophila* cap'n'collar (CNC) family of transcription factors (60). Three small Maf proteins are involved in erythropoiesis, Maf G, K, and F. Both the p45 and p18 subunits of NF-E2 are hematopoietic cell lineage-specific, even though initially it was thought that the Maf subunits were expressed ubiquitously (61). In addition to binding p45 NF-E2, Maf proteins can bind Maf recognition elements (MAREs) as homodimers, or as heterodimers with non-erythroid transcription factors such as Fos, which is involved in expression of immediate early genes in various cell lineages (62). The transactivation potential of the NF-E2 complex is modulated via interaction of the p45 subunit with different Maf subunits, resulting in different regulatory specificities. Competition between Fos, NF-E2, and other transcription factors for interaction with specific Maf subunits may be involved in erythroid cell differentiation (62).

Functional studies have established that NF-E2 binding sites and MAREs within the β-globin LCR are important for transcriptional activation and are required for formation of hypersensitive sites within the LCR (58, 63). *In vitro* analyses showed that NF-E2 binding sites are important for chromatin remodeling activity and are necessary for ε-globin expression and formation of 5'HS2 in minichromosomes (64). Mice homozygous for a deletion of the p45 subunit gene die shortly after birth from thrombocytopenia, although globin gene expression is normal, suggesting that another protein can substitute for p45 to activate globin synthesis (65). Two other cap'n'collar proteins, Nrf-1 and Nrf-2, were identified as potential replacements for p45 NF-E2 in mice (29, 66, 67). However, Nrf-1 could not rescue globin gene expression in p45 NF-E2-deficient MEL cells (68). Mouse lines bearing double knockout mutations of p45 NF-E2 and Nrf-2 did not exhibit increased severity

Table III. Erythroid Transcription Factors

Protein	Protein family	DNA/protein interactions	Expression pattern	[a]KO/ Overexpression	Modulation	References
DRED (direct-repeat-erythroid-definitive protein)		Binds the direct repeat element in ε-globin promoter	MEL cells			(98)
EKLF (erythroid Krüppel-like factor)	Krüppel-like zinc finger	Binds to CACCC box element in β-globin promoter, LCR 5′HS3; interacts with GATA-1	Erythroid cell lineages throughout erythropoiesis	KO lethal by 14 dpc due to β-thalassemia	GATA-1 activates expression; acetylated *in vitro* by CPB an α p300; phosphorylated by CKII	(85,87,88,93, 95,124)
FKLF, FKLF-2 (fetal Krüppel-like factor)	EKLF/Ap1; TIEG (FLKF), BTEB1 (FKLF-2) subfamilies; Krüppel-like zinc finger	Binds to CACCC box of γ-globin promoter	Predominantly in erythroid cells, MEL cells	Overexpression of both activates γ- and ε-globin; FKLF-2 activates other erythroid promoters such as GATA-1 and glycophorin B		(96,97)
FOG (Friend-of-GATA)	Zinc finger	Interacts with GATA-1	Coexpressed with GATA-1 during embryonic development in erythroid and megakaryocytic cells	KO lethal during embryonic development due to failure of megakaryopoiesis and arrested erythropoiesis		(83,84)
GATA-1	GATA family	Binds (T/A)GATA(A/G) DNA sequence; interacts with FOG	Vertebrate erythroid, megakaryocyte and mast cell lineages	KO lethal at 10.5-11.5 dpc; overexpression inhibits terminal differentiation of erythroid cell lines	GATA-1/GATA-2 binding competition; acetylation by p300 and CBP causes conformational change and stimulates GATA-1 transcriptional activity *in vitro;* perturbs nucleosomes	(75,76,126, 144-149)
GATA-2	GATA family	GATA-element	Early erythroid cells, mast cells, megakaryocytes, pluripotent hematopoietic stem cells	KO causes lethality *in utero* due to anemia resulting from an early hematopoietic defect in all hematopoietic cell lineages; overexpression blocks hematopoiesis	GATA-1/GATA-2 binding competition; phosphorylated via the MAP kinase pathway	(81,82,150)
NF-E2	Basic leucine-zipper family of transcription factors; small subunits part of the Maf protein family	GCTGA(G/C)TCA (Maf-recognition element or MARE); this sequence includes the core AP-1-binding motif; large p45-NF-E2 subunit interacts with small p18 Maf subunits	p45 and Mafs expressed in the hematopoietic system; in addition, Mafs widely expressed during embryogenesis	p45 KO leads to fatal hemorrhage due to failure of megakaryocyte differentiation (lack of platelets); MafG KO lethal due to impaired megakaryopoiesis; MafK KO has no phenotype. MafG::MafK KO lethal; MafK overexpression catalyzes terminal erythroid differentiation	Phosphorylated via the MAP kinase pathway; acetylated by CBP/p300; chromatin remodeling at 5′HS2 and ε-globin promoter	(64,65,114, 151-157)
COUP-TFII (NF-E3)	Nuclear orphan receptor	Binds to the direct repeat elements in ε- and γ-globin promoters	Erythroid cells; peak of expression coincides with γ- to β-globin switch	KO lethal at 10.5 dpc		(99)
SSP (stage-selector protein)		Binds the stage selector element (SSE) in ε- and γ-globin promoters and LCR 5′HS2 and 3; contains NF-E4	Hematopoietic cells of fetal liver, cord blood, bond marrow; K562 and HEL cell lines	Overexpression in K562 cells induces γ-globin gene expression		(158–161)
YY1 (NF-E1)	GLI-Krüppel-like family	Developmental repressor of the human ε-globin gene together with GATA-1	Ubiquitous			(16,158)

[a] KO: knockout mutation

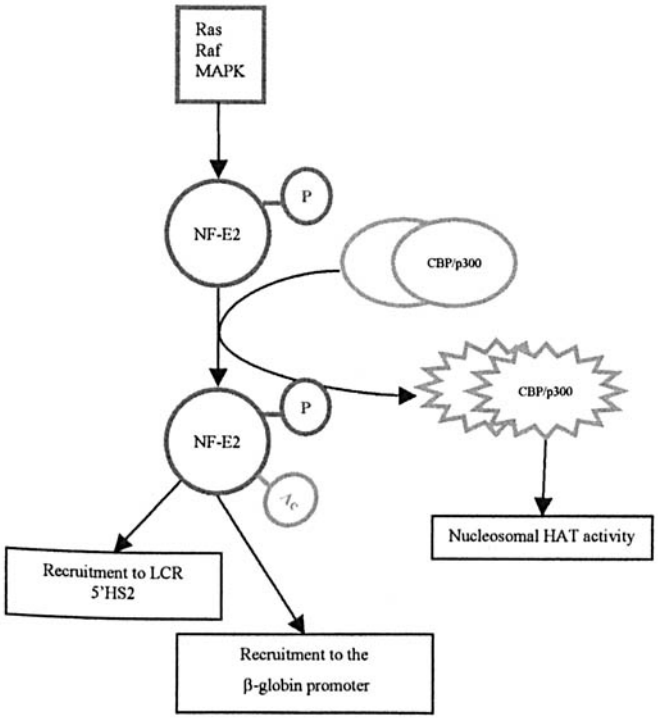

Figure 3. NF-E2 activation and function. The MAP kinase pathway phosphorylates NF-E2 in response to extracellular signals. Phosphorylated NF-E2 is a target of the CBP/p300 complex, which acetylates the protein allowing it to bind to the LCR 5'HS2 and the β-like globin promoters. Interestingly, the interaction of CBP/p300 with NF-E2 increases the CBP/p300 nucleosomal histone acetylase activity.

of hematopoietic defects compared to single p45 NF-E2 knockout mice, demonstrating that Nrf-2 does not compensate for NF-E2 activity *in vivo* (69). The protein product of a third Nrf gene, Nrf-3, is highly expressed in human placenta, B-cells and monocytes (60). Studies *in vitro* indicated that Nrf-3 binds the MARE of the human β-globin enhancer and activates transcription of a luciferase reporter gene in transient transfection assays in fibroblast cell lines. However, the role of Nrf-3 *in vivo* has not been established.

Casteel *et al.* (70) showed that p45 subunit activation was stimulated by cyclic adenosine monophosphate-dependent protein kinase (PKA), a serine/threonine kinase, in erythroid and nonerythroid cells. The cAMP signal transduction pathway has been shown to promote hemoglobin production in erythropoietin-responsive cell lines, and PKA is necessary for erythroid gene expression (71). Activation of p45 by PKA requires only the N-terminal transactivation domain of p45, suggesting that PKA regulates the interaction of p45 with downstream effectors (70). NF-E2 DNA binding and transactivation were shown to be stimulated specifically by the Ras-Raf-MAP kinase signaling pathway, which is essential for erythroid differentiation of MEL cells. In addition, NF-E2 is regulated, in part, by the MAP kinase protein kinase C (PKC), which also influences LCR 5'HS2 enhancer activity, independent of 5'HS2-promoter distance (72). Tandem NF-E2-binding sites in the LCR are important for mediating this signal cascade. Further, NF-E2 may modulate transcription through direct interaction with the basal transcription apparatus component TATA-binding protein-associated factor, $TAF_{II}130$ (73). Together, these data suggest that there may be a direct physical interaction between transcription factors bound to the β-globin LCR and the basal transcription apparatus bound to the individual promoters, mediated, in part, through NF-E2. These data also imply that the function of NF-E2 in both transcriptional activation and the formation of the active β-globin locus chromatin domain may be controlled by various signaling pathways.

Chen *et al.* (74) demonstrated that CREB binding protein CBP/p300 NF-E2 interaction results in increased CBP/p300 nucleosomal HAT activity and acetylation of NF-E2. Thus, the erythroid transcription factor NF-E2 influences the activity of the general chromatin remodeling complex CBP/p300, which in turn modulates the activity of the erythroid protein. NF-E2 may be a general globin gene expression initiator, with a role in LCR and globin promoter chromatin activation, whereas other erythroid factors may have greater developmental and promoter specificity.

GATA-1 and GATA-2. GATA-1 is an erythroid-specific transcription factor required for globin gene switching and erythroid cell maturation (Fig. 4). It belongs to the family of GATA zinc finger transcription factors, which are characterized by their ability to bind the nucleic acid consensus sequence WGATAR (75, 76). GATA-1 binding sites are found in the globin gene promoters and in the hyper-

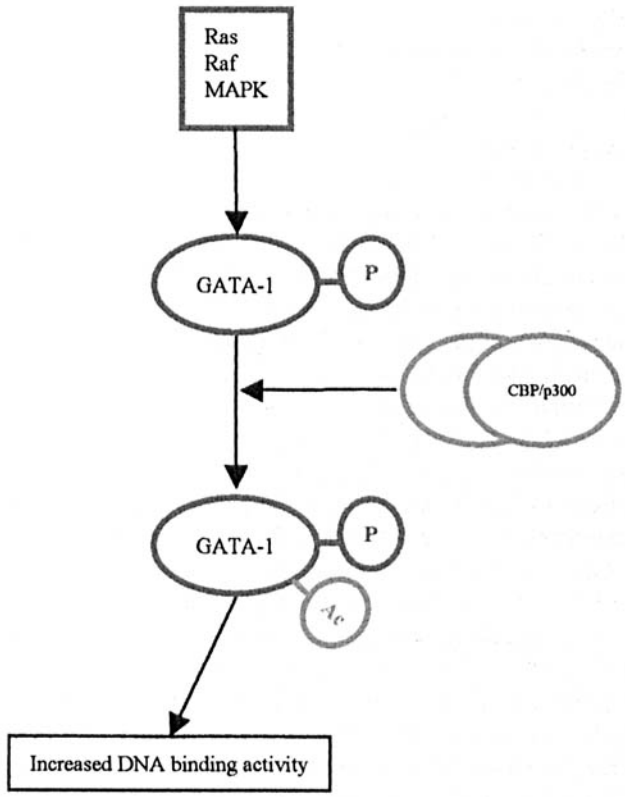

Figure 4. GATA-1 activation and function. The MAP kinase pathway phosphorylates GATA-1 in response to extracellular signals. The CBP/p300 complex acetylates GATA-1 increasing its DNA binding activity. The effect of the phosphorylation has not been determined.

sensitive site cores of LCR 5′HS1-5. GATA-1 functions as either an activator or a repressor of gene expression, depending on the context of the binding sequence and its interaction with other proteins. The protein acts as an activator when bound to the γ-globin gene promoter or 5′HS1–5 (59). Although GATA-1 activates ε-globin gene expression (77), it also functions as a repressor when it binds to the ε-globin gene silencer in the presence of the ubiquitous transcription factor YY1 (16). In addition, GATA-1 homodimerizes (78) and interacts with other transcription factors, such as SP-1 and EKLF (75), further contributing to the complex network of GATA factor interactions.

GATA-2 is essential for embryonic hematopoiesis and is expressed in number of other tissues, including endothelial cells (79). GATA-2 and GATA-1 recognize similar motifs but seem to have discrete roles in β-globin gene regulation (75, 76). In early hematopoietic precursor cells GATA-2 is predominantly expressed, but later in development GATA-1 expression predominates (80). Loss of GATA-2 function causes fatal embryonic anemia due to a deficiency of pluripotent, primitive hematopoietic cells (81). Persons *et al.* (82) maintained high levels of GATA-2 in primitive hematopoietic cells using retroviral GATA-2 expression constructs. Continued GATA-2 expression in these cells blocked both amplification and differentiation of cells. Thus, there appears to be a dose-dependent effect of GATA-2 on red cell differentiation; that is, downregulation of GATA-2 is important for progression of erythroid cell differentiation (82).

FOG. Friend of GATA-1 (FOG) was isolated using the yeast-two-hybrid system to identify proteins that directly interact with GATA-1 (83). FOG has nine zinc-fingers and binds GATA-1 via finger 6 at a minimum; however, it does not bind DNA. The protein is co-expressed with GATA-1 during embryonic development in erythroid and megakaryocytic cells. Mice bearing FOG null mutations die during embryonic development (days E10.5–E12.5) due to severe anemia resulting from arrested erythropoiesis and megakaryopoiesis (84). Analysis of mice and cell lines deficient in FOG demonstrated that primitive and definitive erythropoiesis were defective (83, 84)

EKLF. EKLF is a zinc finger transcription factor that activates the β-globin gene promoter by binding with high affinity to the CACCC element located at –90 relative to the transcription start site (Fig. 5) (85). Point mutations in the CACCC box drastically reduce affinity of EKLF (86). EKLF null mice have a normal globulin (developmental) expression pattern during early embryogenesis with a slight increase in γ-globin production, but they die during fetal definitive erythropoiesis from β-thalassemia (35,87–89). EKLF also has an effect on the chromatin structure of the β-globin locus. Absence of ELKF leads to complete loss of HS formation at the β-globin promoter, and to decreased DNase I-sensitivity at LCR 5′HS3 (35). It also stimulates the formation of 5′HS3, whereas an ubiquitous transcription factor, Sp1, which also binds CACCC boxes, does not have

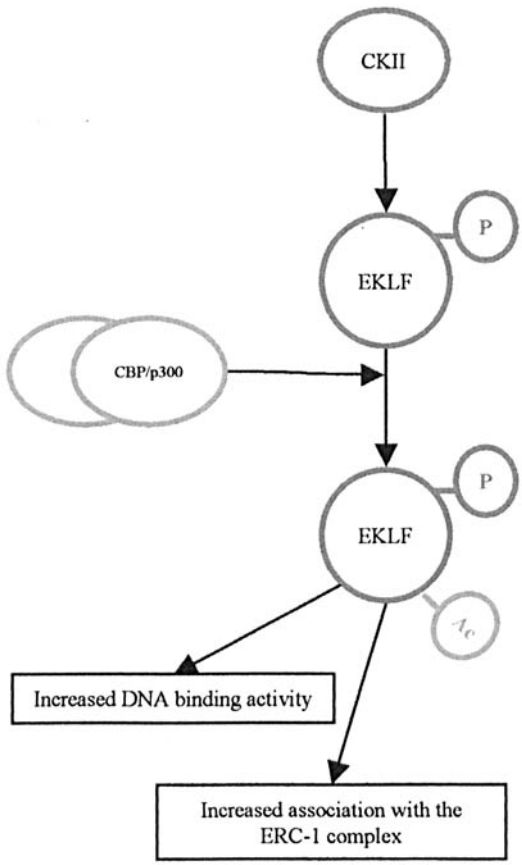

Figure 5. EKLF activation and function. Casein kinase II (CKII) is a ubiquitous kinase that phosphorylates EKLF during development allowing it to activate transcription from target promoters to higher level than the unphosphorylated form of EKLF. EKLF is also a target of the CBP/p300 acetylase complex. Phosphorylated and acetylated EKLF has a higher affinity for DNA and more tightly associates with the ERC-1 chromatin remodeling complex.

an effect on 5′HS3 formation (90). EKLF has been implicated in the human fetal to adult globin gene switch as demonstrated by Donze *et al.* (91), who found that EKLF binds with eight times higher affinity to the adult β-globin CACCC box than to the γ-globin gene promoter, suggesting that EKLF is predominantly involved in adult β-globin promoter activation. Finally, EKLF recruits the repressor complex, mSIN3a/HDAC to the ε-globin region (92), and thus may be involved in remodeling the embryonic chromatin into a repressed state.

CKII and EKLF. EKLF is also expressed in erythroid tissues and cell lines that do not express adult β-globin. Post-translational modifications have been shown to modulate EKLF activity. The transactivation domain of EKLF contains serine and threonine residues that can be phosphorylated by kinases. Specifically, a casein kinase II (CKII) site within the ELKF activation domain is phosphorylated by CKII (93). CKII is an ubiquitously expressed heterotetrameric enzyme that modifies the activity of various proteins, especially the DNA binding affinity of transcription factors such as c-Jun and p53. CKII is also involved in modulating developmental processes, such as the wingless (Wg) signaling pathway in *Drosophila* (94). In erythroid

cell lines, CKII phosphorylation of EKLF increases its transcriptional activity, possibly via modulation of DNA binding or protein-protein interactions (93). In addition to CKII, EKLF interacts with GATA-1 and Sp1 *in vitro* and synergistically *in vivo* to activate reporter genes (95).

FKLF and FKLF2. Another erythroid-specific transcription factor, called FKLF (fetal Krüppel-like factor), activates γ- and ε-globin genes in K562 cells (96). FKLF is a zinc-finger transcription factor with little homology to known transcription factors aside from the zinc finger domain, common to all EKLF-type zinc finger proteins. FKLF activates γ-globin transcription via the CACCC element in the promoter, but not through the CACCC element of LCR 5′HS2, which is involved in γ-globin expression (97). FKLF also activates β-globin gene transcription, but to a lesser degree than EKLF. It remains to be demonstrated whether FKLF is an essential activator of the ε- or γ-globin genes. Additionally, the role of FKLF in fetal globin gene expression *in vivo*, if any, has yet to be established. A second, related fetal transcription factor that activates γ-globin gene expression, FKLF-2, was cloned from murine fetal yolk sac and its human homologue was isolated from fetal liver (97). FKLF-2 activates various erythroid promoters in addition to γ-globin, indicating that it may play a role in erythroid differentiation. However, the *in vivo* significance of this protein also needs to be verified.

DRED, COUP-TFII, SSP, Id2, CBF1 (HS2NF5), and Ubiquitous Transcription Factors. DRED was identified as a repressor of the epsilon globin gene (98). It appears to prevent binding of EKLF to the ε-globin gene promoter and silences epsilon globin expression during definitive erythropoiesis. Initially, GATA-1 and the ubiquitous transcription factor YY1 were implicated as part of the ε-globin repressor complex (16).

COUP-TFII (NF-E3) is an orphan receptor that has both repressor and activator properties and may be involved in globin gene switching by repressing ε-globin expression in fetal erythroid cells (99). In mice, COUP-TFII binds to the same direct repeats of the ε- and γ-globin promoters as DRED, possibly assisting in repression of expression from these genes. Supporting its role in ε-globin gene silencing, the level of COUP-TFII peaks at the time of the switch from embryonic to adult globin gene expression in mice.

The stage selector protein (SSP) regulates γ-globin gene expression as a part of a complex including the ubiquitous transcription factor CP2, and a 40–45 kD protein that has not been identified (100). A basic helix-loop-helix (HLH) protein, Id2, enhances γ-globin gene expression in K562 cells (101). This protein may act downstream of other transcription factors, because it further activates transcription from already active γ-globin promoters in K562 cells, but not from transcriptionally silent ε- and β-globin genes (101).

HS2NF5 was identified in murine cell lines as a factor that binds to LCR 5′HS2 and appears to be involved in regulating activity of the LCR (102, 103). This protein was later identified as CBF1, a mammalian homologue of the *Drosophila* suppressor of hairless, which is part of the Notch signaling pathway. The Notch signaling pathway is important for the development of various organs during neurogenesis and myogenesis (104, 105). Thus, the Notch signaling pathway may regulate hematopoiesis in vertebrates via the HS2NF5/CBF1 transcription factor (103).

Additionally, ubiquitous transcription factors such as Sp1, YY1, and USF are involved in control of β-globin gene expression. These proteins work in concert with the erythroid-specific transcription factors to activate or repress globin gene expression in erythroid cell lineages (16, 29, 31).

Role of Chromatin Remodeling in Control of Globin Gene Expression and Modulation of Erythroid-Specific Transcription Factor Activity. Although *cis*-acting DNA elements and trans-acting proteins have been identified that regulate β-globin gene expression, the link between these modalities of transcriptional control and the role of chromatin structure and remodeling is less well characterized. Chromatin remodeling increasingly has been found to be important in control of transcription. Global changes to chromatin, including acetylation, phosphorylation, and methylation play roles in locus activation. Several nucleosome remodeling factors, which change the structure of DNA wound around histones such as the SWI/SNF-complexes (switch/sucrose non-fermenting), and chromatin remodeling factors such as the HAT complex CBP (CREB-binding protein)/p300, have been shown to interact with the erythroid-specific transcription factors and influence their conformation and activity. The yeast SWI/SNF and *Drosophila* NURF (nucleosome remodeling factor) protein complexes have been implicated in the initiation of chromatin remodeling, making DNA accessible to other chromatin remodeling and transcription factors (50, 106). Chromatin remodeling of the β-globin locus may determine which genes will be targeted for transcriptional activation (26). Analysis of general DNase I-sensitivity and intergenic transcription in mice containing the whole human β-globin locus suggest that there are at least two transcriptionally active chromatin subdomains that are developmentally regulated in addition to the preexisting "open" state of the locus domain in erythroid cells (80). LCR chromatin is open throughout development. An embryonic/fetal ε-/γ-globin domain is open during embryonic/fetal erythropoiesis, but closed during adult erythropoiesis; the converse is true of an adult δ-/β-globin domain.

Acetylation. Cell cycle stage affects chromatin conformation and therefore the degree of gene accessibility to transcription factors. Histone acetylation occurs during chromatin remodeling. In fact, acetylation activity varies at different stages of the cell cycle and thus may link, in part, cell cycle progression and chromatin structure. Lysine residues within histones are acetylated, neutralizing their basic character, thus altering their DNA binding. The disruption of nucleosome-DNA contacts allows transcription factor ac-

cess and the opportunity to activate gene expression (107). Thus, hyperacetylation is associated with transcriptional activation of a locus. Factors that influence histone acetylation of β-globin locus chromatin, such as those that direct acetyltransferase activity or initiate a signal cascade resulting in histone acetylation may be important points of control via chromatin structure. Histone acetylation, particularly at H4, may recruit the general transcription factor TFIID to gene promoters via the $TAF_{II}250$ subunit, allowing formation of a stable transcription preinitiation complex (106). Evidence suggests that recruitment of TFIID to specific β-like globin gene promoters depends upon erythroid transcription factors such as NF-E2, which binds TFIID directly via its $TAF_{II}130$ subunit and allows activation of β-like globin genes (73). Experiments comparing the normal human β-globin locus and the Hispanic thalassemia deletion locus demonstrated that the degree of acetylation of gene sequences and intergenic sequences might influence the association of the locus with heterochromatin (108). The Hispanic allele, which lacks LCR 5′HS2-5 and 22 Kb of upstream sequence, is transcriptionally inactive and the locus chromatin domain is completely closed as measured by DNase I-sensitivity. In addition, the locus was underacetylated and found to be closer to the centromere, whereas the normal allele was acetylated at histones H3 and H4 and was localized further from the centromeric region. Using another construct in which only 5′HS2-5 were deleted, the locus was transcriptionally inactive, but the chromatin domain was open. The locus was acetylated and localized away from the centromere. Histone H3 was less acetylated than H3 in the normal locus. Thus, acetylation may serve as an indicator of a transcriptionally active β-globin locus.

Phosphorylation. Similar to acetylation, phosphorylation of histone H3 disrupts DNA-nucleosome interaction and increases transcription factor accessibility to DNA. Mitogen activated MAP kinase pathways, as well as the stress-activated p38 pathway, activate histone H3 phosphorylation (106). Phosphorylation coincides with the onset of specific "immediate-early" gene expression. The p38 MAPK pathway is induced in response to stress, such as elevated temperature, change in osmolarity, nutrient deficiency, or decreased oxygen tension (109, 110). Studies on p38 knockout mice established a role for the p38 stress pathway in the switch from primitive to definitive erythropoiesis (111). The majority of p38 null mice die *in utero* due to a failure of angiogenesis, those that survive are anemic due to a lack of adult β^{maj}-globin gene expression.

Transcription factor activity is regulated by phosphorylation. Both GATA-1 (112) and NF-E2 (113) are phosphorylated. Although phosphorylation of GATA-1 does not appear to influence its DNA-binding activity, phosphorylation (and acetylation) of NF-E2 p45 via the Ras-Raf-MAPK pathway increases ATP-dependent binding of NF-E2 to both the LCR 5′HS2 and the β-globin gene promoter, suggesting that nucleosome disruption by NF-E2 involves energy-dependent nucleosome remodeling factors (114).

CpG Methylation. CpG methylation may act as a deterrent to formation of the transcription preinitiation complex or transcription factor accessibility and thereby indirectly prevent further chromatin remodeling. Evidence suggests that methylation has no effect on nucleosome formation and its role as a chromatin-remodeling factor in vertebrates remains controversial (115). However, data demonstrate that methylated DNA recruits methyl-binding proteins that interact with histone deacetylases, which do have a role in chromatin state alteration (116). Areas of active chromatin are usually undermethylated, and DNA methylation of CpG islands at promoter regions is associated with a loss of DNase I-hypersensitivity (19). Thus, when methylated, the chromatin of a locus is in an inactive state and is transcriptionally silent. Because the functional human β-globin genes have no CpG islands, methylation may not be a contributing factor affecting chromatin remodeling (117).

SWI/SNF Complexes and EKLF. EKLF interacts with SWI/SNF-like chromatin remodeling factors. SWI/SNF complexes have been implicated in the global regulation of chromatin structure and transcription via assembly and mobilization of nucleosomes by breaking and reestablishing histone-DNA contacts. However, the subunit composition of these complexes varies, indicating specificity in control of different genetic loci (118). *In vitro* studies of EKLF indicate that tissue-specific transcription activity of EKLF requires a coactivator, the EKLF coactivator remodeling complex 1 (E-RC1), to generate a DNase I-hypersensitive, transcriptionally active β-globin promoter on reconstituted chromatin templates (119). The E-RC1 chromatin-remodeling complex was isolated from MEL cells and contains, at a minimum, BRG1, BAF170, BAF155, and INI1 (BAF47) homologues of yeast SWI/SNF subunits.

Another chromatin remodeling complex, the PYR complex, was purified from MEL cells and is involved in the γ- to β-globin gene expression switch (120). PYR specifically binds to a pyrimidine-rich DNA sequence between the γ- and δ-globin genes and binds to the PYR element only in definitive hematopoietic cells. DNA binding is dependent on both the nucleotide sequence as well as length of the region. PYR has similar, but not identical, subunit composition to E-RC1, consisting of BAF57, INI1, BAF60a, and BAF170 homologues. The PYR complex does not contain BRG1 in contrast to E-RC1, which may, in part, account for different specificities of the two complexes. The PYR complex may bind to the ε/γ-δ/β boundary element identified by Gribnau *et al.* (26), influencing the change in chromatin structure of the locus during the γ- to β-globin switch. Additionally, this complex was shown to include a repressor component, a nucleosome-remodeling deacetylase (NuRD), which has both nucleosome remodeling and histone deacetylase functions (120). The DNA binding subunit of PYR *in vitro* is Ikaros, a zinc finger transcription factor involved in normal B- and T-cell development (121–123).

The *in vivo* function of these subunits has not yet been determined.

EKLF, GATA-1, and NF-E2 Acetylation. ELKF is also a target of histone acetyltransferases (124). HATs transfer an acetyl group to specific lysines on proteins, effectively reducing the positive charge on these proteins and impairing or reducing binding activity to negatively charged DNA. Multiple HATs may interact with EKLF *in vivo* to exert a range of effects that could account for some of the properties exhibited by EKLF. EKLF associates with the HATs CBP, p300, and P/CAF *in vivo* (124). However, only CBP and p300 were shown to modulate the transcription of globin genes by enhancing EKLF transactivation in erythroid cells.

GATA-1 interacts with CBP/p300 *in vitro* and is a target of CBP (125). *In vitro* data suggests that GATA-1 binding causes extensive, cooperative breakage of histone-DNA contacts and that the GATA-1-DNA complex formation is one step in the formation of a fully hypersensitive site (126). Acetylation of GATA-1 apparently changes the conformation of the protein and increases its DNA-binding capability (126). This observation was surprising because acetylation of positively charged lysines usually decreases affinity for DNA. In addition, the lysine residues of GATA-1 were shown to be important for hematopoietic differentiation, but the mechanism by which they function is unknown (127).

CBP/p300 also acetylates p45NF-E2, increasing recruitment of this protein to the LCR 5'HS2, as well as to the β-globin promoter (128). Interestingly, interaction of CBP/p300 with p45NF-E2 also increases the histone acetylase activity of the CBP/p300 complex (74).

Chromatin Remodeling as a Global Regulator of Gene Expression. The regulation of globin gene switching is a very complex process requiring the coordination of different cellular pathways and molecular events. The interaction of trans-acting transcription factors and other protein cofactors with *cis*-linked locus regulatory sequences represents the final level of gene activation or repression; that is, the assembly of a complete transcription or repressor complex. Many other levels of control precede these terminal events in the hierarchy of gene regulation, including cell cycle stage, integration of various extracellular signaling and signal transduction pathways, and the physical state of the chromatin domain. Erythroid-specific molecular pathways, as well as ubiquitous cellular processes, play a role. All of these motifs form a network that must be finely orchestrated to achieve developmental changes. Figure 6 summarizes what is known about the

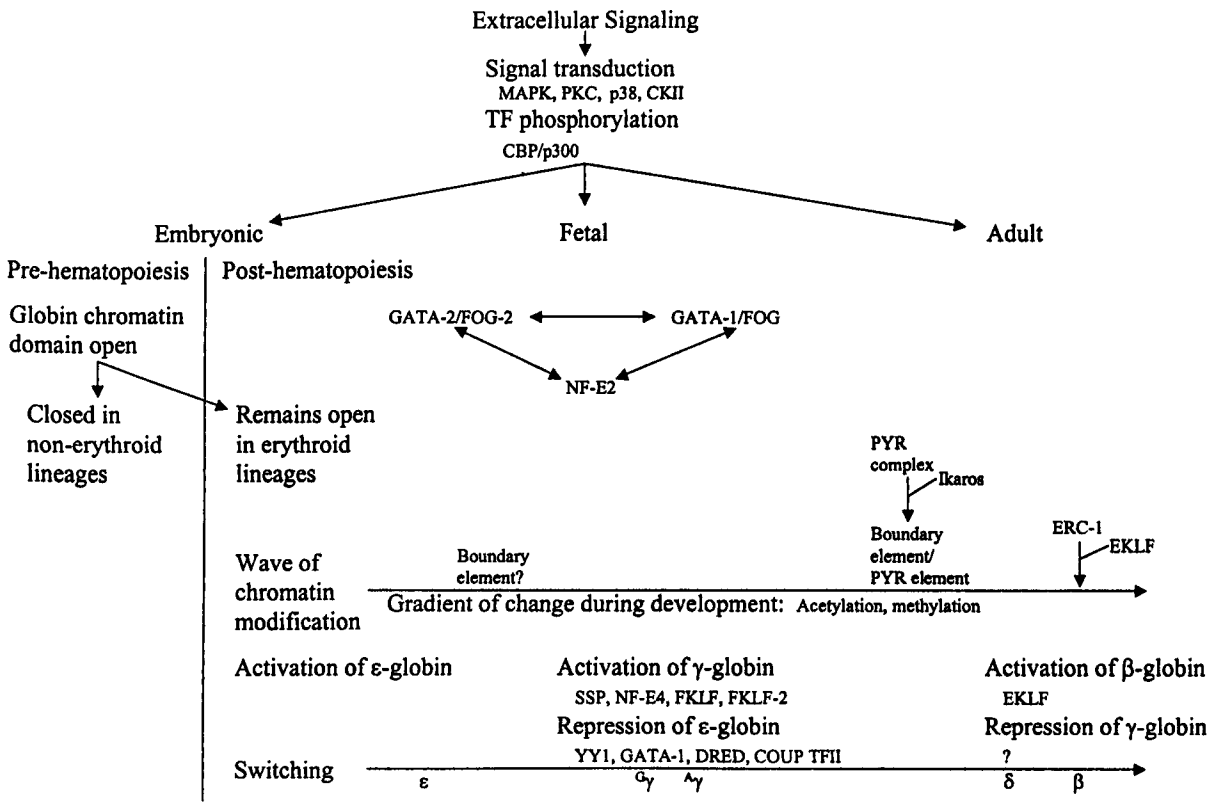

Figure 6. Factors involved in β-globin gene switching. One-headed arrows indicate positive effect. Double-headed arrows indicate interaction between transcription factors. Two cellular stages are depicted, representing the continuum from stem cell to mature erythrocyte pre- and post-hematopoiesis. Three levels of regulation are indicated: (i) extracellular signaling leading to transcription factor activation and modification at different stages of hematopoiesis, (ii) interactions of the hematopoietic transcription factors and chromatin modifiers during ontogeny, and (iii) a wave of chromatin modification throughout the globin locus resulting in appropriate globin gene expression during development. Globin gene switching is regulated by the concerted action of these various molecular events that alter chromatin states and interaction of transcription factors at specific gene promoters/regulatory regions and between each other. See text for a more detailed discussion of this figure.

molecular machinery involved in globin gene switching, but also illuminates the gaps in our knowledge. Clearly, the cascade of events involved initially in triggering hematopoiesis and later in globin gene switching is complex and largely uncharacterized. Although components involved in globin gene expression have been identified at different levels within the regulatory hierarchy, links between these molecular pathways are lacking. While we are not attempting to generate a global model encompassing the interplay of these diverse cellular processes, we are hoping to demonstrate the complex system underlying β-globin gene switching.

At the highest level, extracellular signals or stresses, such as hypoxia, activate intracellular signaling pathways in erythroid cells that eventually lead to remodeling of the β-globin locus chromatin domain and ultimately to changes in globin gene expression. These various intracellular signals are transduced to catalyze differential acetylation and phosphorylation of transcription factors, developmental stage-specific changes in subunit composition of transcription factor complexes (for example, Maf protein composition of NF-E2) or changes in the concentration of these factors (for example, EKLF). Concomitantly, localized changes in β-globin locus chromatin structure may occur; that is, the β-globin locus chromatin may consist of embryonic, fetal, and adult transcription subdomains that open and close as the developmental stage dictates (26). Finally, erythroid-specific and ubiquitous transcription factors interact with chromatin remodeling complexes at both at the LCR, and the gene promoters assemble the transcription apparatus necessary to catalyze developmental stage-appropriate β-like globin mRNA synthesis.

Examples of events at each of these levels affecting chromatin structure and gene expression have been demonstrated in genetic regulatory systems. Signaling pathways have been shown to alter chromatin structure at other loci. Lymphocyte antigen receptor signaling regulates PIP2 (phosphatidyl inositol 4,5-bisphosphate) levels resulting in chromatin remodeling (129). In lymphocytes, PIP2 controls the association of the mammalian SWI/SNF complex with chromatin or components of the nuclear matrix leading to rapid decondensation of the chromosomes. These data demonstrate that a direct link can exist between a signaling pathway and regulation of chromatin structure. Additionally, the CBP/p300 complex responds to T cell signaling pathways (129).

More localized changes in chromatin structure may be influenced by the binding of specific transcription factors to various *cis*-regulatory sites in the β-globin locus; for example, the reversible displacement of histones by GATA-1. These localized changes in chromatin permit the binding of other proteins or protein complexes, such as SWI/SNF-like complexes, that directly interact with globin gene transcription factors or further open chromatin subdomains within the β-globin locus. Studies of the human CD2 locus (hCD2) support this mechanism (130). One of the HSs comprising the hCD2 LCR, HSS3, is a T cell-specific enhancer. HSS3 binds a HMG box containing protein-1 (HBP1). Deletion of the HBP1 binding site in HSS3 resulted in position effect variegation (PEV) of a hCD2 transgene in mice. HBP1 also interacts with the retinoblastoma (RB) family of proteins (131), that, in turn, interact with a SWI/SNF complex and the histone deacetylase, HDAC1 (132). Thus, the initial binding of HBP1 to hCD2 LCR HSS3 may result in the recruitment of RB family proteins and subsequently, SWI/SNF and HDAC1 chromatin-remodeling complexes to the locus to activate transcription (130). Similarly, activation of the granulocyte-specific mim-1 gene requires recruitment of a chromatin-remodeling complex by multiple transcription factors, including CCAAT/enhancer-binding protein beta (C/EBP-β) and the oncogene Myb (133). A chimeric protein composed of the N-terminal activation domain of C/EBP-β fused to Myb resulted in a functional activator that recruited a SWI/SNF complex and induced mim-1 transcription even in the absence of normal C/EBP-β. Thus, cooperation of these transcription factors is essential for recruitment of a chromatin-remodeling complex to this locus. Additionally, C/EBP-β interacts with p300. The histone acetylase activity of this complex may modulate Myb activity by acetylation or directly acetylate the histones of the mim-1 gene, thus making it more accessible to SWI/SNF complex binding (134, 135).

Other molecular events may control globin gene switching more directly. By the onset of primitive embryonic erythropoiesis, the β-globin locus is generally DNase I-sensitive and the ε-globin gene is expressed. An "enhanceosome" consisting of NF-E2, GATA-1, FOG, and EKLF may aid the recruitment of the basal transcription apparatus to the ε-globin gene promoter via binding to gene proximal and LCR sequences.

The first switch, from ε-globin synthesis during primitive erythropoiesis in the embryonic yolk sac to γ-globin expression during definitive erythropoiesis in the fetal liver occurs at approximately six weeks gestation. Silencing of ε-globin gene expression requires formation of a repressor complex bound to the ε-globin promoter that may be composed of YY1, DRED, GATA-1, COUP-TFII, and EKLF. This complex interacts with a mSin3A/HDAC chromatin remodeling complex, thereby preventing ε-globin gene expression.

During the switch from γ-globin synthesis to β-globin expression during definitive erythropoiesis in the bone marrow, E-RC1 and PYR complexes may bind to the LCR and the chromatin boundary element between γ- and δ-globin genes. The PYR complex may actively open β-globin gene regional chromatin to assist in activation of β-globin gene expression, while the mSin3A subunit maintains a closed conformation of upstream chromatin, thus repressing ε- and γ-globin production and establishing stable adult β-globin expression. COUP-TFII also binds to the γ-globin promoters in mice and may be involved in repression of γ-globin gene expression (99). Therefore, silencing of γ-globin syn-

thesis may occur, in part, through an autonomous mechanism similar to repression of ε-globin gene expression, although other repressor complex proteins have not yet been identified.

Major gaps exist in our knowledge about β-globin gene switching. Continued research to fill the gaps in the cascade of events controlling these molecular switches is necessary for the rational design of therapies for a variety of hemoglobinopathies. In addition, studies of gene regulations using β-globin loci remains one of the leading paradigms in which new molecular mechanisms will be discovered and existing ones better characterized.

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