# MINIREVIEW

# Genetic Modulation of Sickle Cell Anemia

## (43870A)

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*Abstract.* **Sickle cell anemia, a common disorder associated with reduced life span of the red blood cell and vasoocclusive events, is caused by a mutation in the β-hemoglobin gene. Yet, despite this genetic homogeneity, the phenotype of the disease is heterogeneous. This suggests the modulating influence of associated inherited traits. Some of these may influence the accumulation of fetal hemoglobin, a hemoglobin type that interferes with the polymerization of sickle hemoglobin. Another inherited trait determines the accumulation of α-globin chains. This review focuses on potential genetic regulators of the phenotype of sickle cell anemia.** [P.S.E.B.M. 1995, Vol 209]

Hemoglobin, the oxygen-carrying pigment of the red blood cell, is a complex of iron-containing porphyrin or heme groups and globin protein chains that nestle the heme, protecting it from noxious influences. Mutations in genes that code for the globin subunits of human hemoglobin perhaps are humankind's most common inherited disorders (1). They come in two classes: reduced synthesis of structurally normal globin polypeptides and production of globin chains that have their primary amino acid sequence altered. The former disorders are known as thalassemias and the latter as hemoglobinopathies. In both types of inherited globin disorders, some specific mutations have reached extraordinarily high frequencies in certain populations. The effects of these mutations make the red cell a poor host for the intraerythrocytic parasite, *Plasmodium falciparum*. Sickle cell anemia is one of the hemoglobinopathies, and the frequency of the sickle hemoglobin gene has reached polymorphic frequencies in some regions where the selective pressure of *P. falciparum* malaria confers a survival advantage on heterozygous carriers. While heterozygotes, said to carry the sickle cell trait, are normal in most all respects, the price of a very high heterozygote frequency is a so-called balanced polymorphism, in which the shortened survival of severely affected homozygotes with sickle cell anemia is balanced by the enhanced reproductive capacity of the carrier heterozygote. All homozygotes for the sickle hemoglobin gene are not similarly ill. Characteristically, sickle cell anemia, caused by homozygosity for a single gene that produces an abnormal hemoglobin molecule, is clinically pleiotropic (2).

Human hemoglobin is an interactive tetrameric molecule with two pairs of globin subunits. In the predominant variety of hemoglobin, called hemoglobin (Hb) A, the tetramer is made of two α-chains and two β-chains. A sickle hemoglobin (Hb S) or $\beta^s$-globin gene results when a GAG → GTG mutation occurs in the codon for the sixth amino acid of the β-globin chain and changes the usual glutamic acid residue to a valine (Fig. 1). This mutation seems to have occurred at least five times in the ancient history of equatorial Africa and the Middle East. As malaria flourished in these regions with the rise of agriculture, the affected populations and the prevalence of the Hb S gene expanded. Sickle cell anemia in the new world was established via the infernal slave trade of the 18th century (2).

When incorporated into the hemoglobin tetramer, the $\beta^s$-globin polypeptide chain, whose synthesis is directed by the Hb S gene, causes sickle hemoglobin molecules to polymerize when deoxygenated. Poly-

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0037-9727/95/2091-0001$10.50/0

merization is reversible, so upon reoxygenation sickle hemoglobin returns to solution. Hemoglobin normally acquires oxygen in the lungs and transports it to the body tissues to support metabolism. Sickle hemoglobin does this well. However, as deoxygenated Hb S polymerizes, a complicated train of events is set into motion. The sickle red cell is distorted by polymerized hemoglobin, changes occur in the properties of the red cell membrane, and the life of the cell and rheology of the blood is altered (Fig. 1). Viability of the sickle cell is curtailed by high concentrations of sickle hemoglobin and short-lived cells produce a hemolytic anemia. What is more important, the damaged red cell initiates occlusion of small blood vessels. Vital organs injured by the vasoocclusive process can cause pain and ultimately have their function diminished. The life span of those with the disease is reduced, while their existence can be oppressive and spent seeking medical relief.

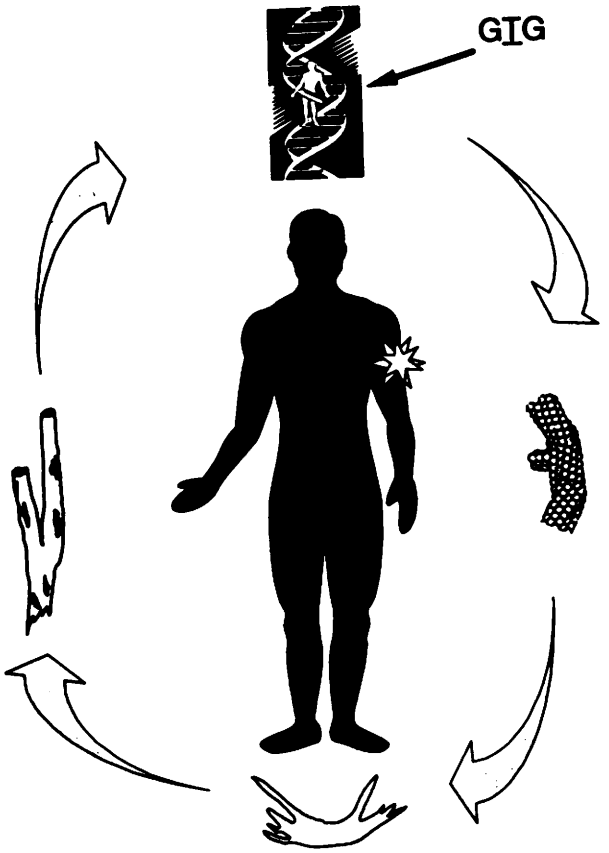


**Figure 1.** The pathophysiology of sickle cell anemia. A mutation of GAG → GTG in the codon for amino acid number six of the β-globin chain causes a glutamic acid residue to be replaced by valine. The combination of abnormal $\beta^S$-globin chains with normal α-globin chains produces sickle hemoglobin (Hb S; $\alpha_2\beta^S_2$). Deoxygenated Hb S polymerizes and distorts the erythrocyte into a variety of "sickled" forms. High intracellular concentrations of Hb S and Hb S polymer injures the membrane and contents of the cell and ultimately causes vasoocclusion. Interaction of sickle cells with vascular endothelium may initiate the vasoocclusive process. Vasoocclusion has the potential for damaging virtually all organs. Painful episodes, typified by severe pain in the abdomen, chest, or extremities, are the most frequent disease-related event for most patients with sickle cell anemia, and frequent episodes of pain are associated with an early mortality.

Curiously, despite genetic identity at the β-hemoglobin locus, not all patients with sickle cell anemia are affected equally. Some are sick all of the time, and some almost never (2). That a genetically homogeneous disease is clinically so heterogeneous has attracted the attention of clinicians and biomedical scientists who have tried to define the causes of this variability. In this review, I will focus on some potential genetic modulators of sickle cell anemia.

## The β-Globin Gene Cluster, Globin Gene Switching, and Fetal Hemoglobin

Located near the end of the short arm of chromosome 11 is the β-globin gene cluster, a constellation of five active globin genes. Each gene is expressed at different times and at different levels during development. The organization and times of expression of these genes from embryonic to adult life are shown in Figure 2. In adults, Hb A ($\alpha_2\beta_2$) is the major hemoglobin type accounting for nearly 97% of all hemoglobin. About 2%–3% Hb $A_2$ ($\alpha_2\delta_2$) and <1% fetal hemoglobin (Hb F; $\alpha_2\gamma_2$) make up the remainder. During most of intrauterine life and in the first months of infancy the γ-globin genes are expressed and Hb F predominates. In

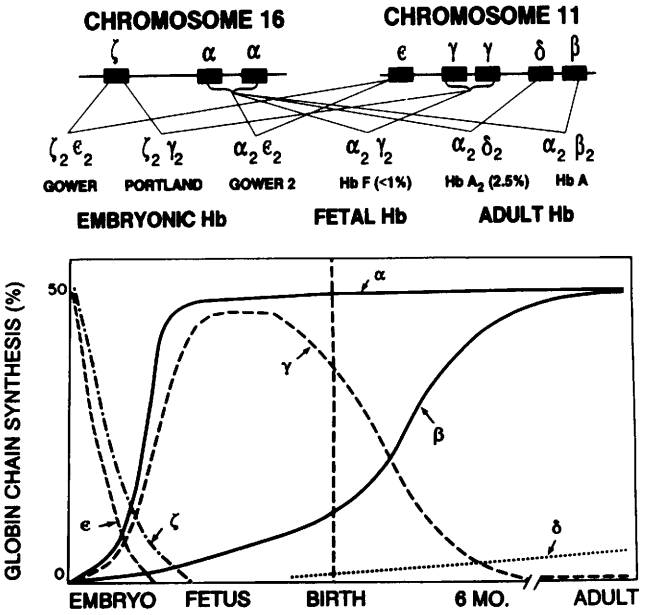


**Figure 2.** The organization of globin genes and the kinetics of hemoglobin switching from embryo to adult. Functional globin genes are shown in the 5′ to 3′ order on their respective chromosomes, and beneath the genes are the hemoglobin molecules formed as heterodimers of α- and non-α-chains. The predominant embryonic, fetal, and adult hemoglobins are shown, along with the temporal switching off and on of different globin genes. While the γ-globin genes of Hb F are almost completely inactivated in adults, a small amount of Hb F continues to be made. The persistence of this trace of Hb F provides the basis for reactivating the γ-globin genes in sickle cell anemia and the β thalassemias.

adults, β-globin gene expression dominates with a trace of the δ-chain of Hb $A_2$ accumulating. Some understanding of the tissue-specific and developmental regulation of globin gene expression is now accruing.

Hemoglobin gene switching is the process of sequential activation and inactivation of globin genes (3–5). Still incompletely understood, the process of globin gene switching involves the complex interactions of stage specific transcription factors, chromosomal gene order, gene proximity to the globin locus control region (LCR), *cis*-acting sequences that act positively and negatively in transcriptional regulation, and erythroid specific and ubiquitous *trans*-acting factors like GATA-1, NF-E2, Sp-1, and YY1 (4) (Fig. 4). A series of four DNase hypersensitive sites, called the LCR, is located 6–18 kb 5′ to the ϵ-globin gene and plays critical roles in the tissue and developmental specific expression of genes within the β-globin gene-related complex (6–8). Each hypersensitive site contains different combinations of conserved binding domains for erythroid specific and ubiquitous DNA-binding proteins that influence gene transcription. The LCR can activate the entire β-globin gene territory. The hypersensitive sites have redundant functions and each may interact preferentially with specific genes of the β-like cluster; for example, hypersensitive (HS)-1 interacts with the embryonic ϵ gene, HS-III with the fetal γ genes, and HS-IV with the adult β gene (8) (Fig. 4). While HS-II does not appear to have specificity for interaction with the γ-globin genes in transgenic animals, as discussed below there is some evidence for its association with changes in Hb F levels in sickle cell anemia. The regions of HS-II that affect globin gene transcription in cell lines and transgenic mice are not completely defined but these regions are likely to be the elements that bind important transcription factors (7, 9–11).

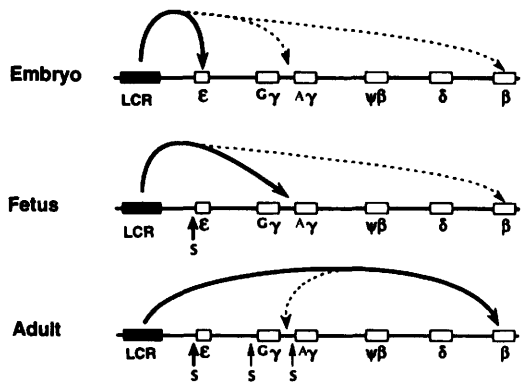


**Figure 3.** A possible scheme of interaction between the β locus control region and globin genes during development (3). Long-range effects over 60 kb of DNA are likely to occur by DNA looping mechanisms that approximate interacting sequences. Positively and negatively acting transcription factors bind the gene promoters and the complexes formed between promoters and the LCR. S denotes silencers that may inactivate embryonic genes in the fetus and fetal genes in the adult. (From Ref. 3, with permission.)

Fetal hemoglobin, the product of the γ-globin genes, functions well in oxygen transport. When Hb F and sickle hemoglobin coexist, Hb F has the interesting property of inhibiting Hb S polymerization (Fig. 5). By preventing deoxy Hb S polymer from forming, Hb F can therefore serve a vital function as an inhibitor of the adverse consequences of high concentrations of Hb S.

The linked γ-globin genes of Hb F differ by a single amino acid codon at position 136 of the γ globin chain: $^{G}\gamma$ (glycine) and $^{A}\gamma$ (alanine). The $^{A}\gamma$ gene has two allelic forms: $^{A}\gamma^{I}$ (isoleucine) and $^{A}\gamma^{T}$ (threonine). Hb F levels in sickle cell anemia can vary over two orders of magnitude. All red cells do not contain this hemoglobin. Hb F is sequestered in a restricted number of cells, called F cells, whose numbers are determined genetically. There are likely to be genetic determinants of Hb F level that are not linked to the β-globin gene cluster (12, 13). One possible F-cell production locus is X chromosome linked and has been localized between DXS143 and DXS16 within Xp22.3–22.2 (14). This locus may account, in part, for the higher Hb F levels in females compared with males, an observation found in both the normal population and in patients with sickle cell anemia (12, 15). Another gene affecting Hb F production may be localized to chromosome 6q (16; Thein SL, personal communication). The Hb F level in sickle cell anemia is set by the number of F cells, the amount of Hb F per F cell, and the differential survival of F cells and non-F cells (17, 18).

Hb F is the most thoroughly studied modulator of

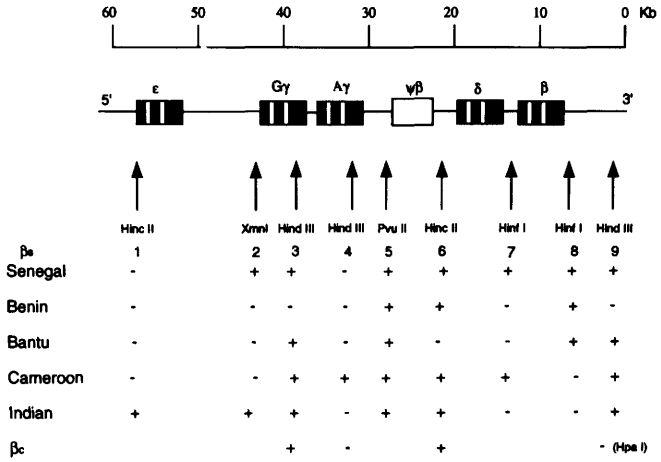


**Figure 4.** The haplotype of the β-globin gene cluster. In the top portion of the figure, the arrangement of genes within the human β globin-like gene cluster and the restriction endonuclease sites that are used to define the haplotype are shown. "Plus" signs signify that a specific polymorphic site is cleaved by a given enzyme, while a "minus" sign indicates the failure to cleave. The pattern of polymorphic restriction sites in the five haplotypes commonly associated with the $\beta^{S}$ gene are shown in the lower portion of the figure. Indian denotes the Arab-India haplotype.

sickle cell anemia. Most genetic determinants that appear to influence the course of sickle cell anemia work via their influence on Hb F production. Great attention has been devoted to means of pharmacologically and genetically increasing the synthesis of Hb F in sickle cell anemia. While at first only very high levels of Hb F were considered capable of influencing the manifestations of sickle cell anemia (19–21), we now realize that any increment of Hb F is clinically important (22, 23). We also have the partial ability to manipulate pharmacologically Hb F levels in sickle cell anemia (24, 25).

## β-Globin Gene Cluster Haplotypes

A haplotype is determined by using genetic markers to distinguish one chromosome or region of a chromosome from another. β-Globin gene cluster haplotype is defined by the nonrandom association of combinations of restriction endonuclease cleavage sites located within and around these genes. These sites are present in limited combinations in the world's population (26, 27) and may serve as one genetic marker of the phenotypic heterogeneity of sickle cell anemia. Three haplotypes, named for their regions of highest frequency in Africa, are found in most patients with sickle cell anemia: the Benin, the Bantu or Central African Republic, and the Senegal type (28, 29). The Cameroon haplotype is less common (30). Many atypical haplotypes are present in African-Americans but are rare in indigenous Africans. In the United States, the $\beta^S$ gene is found with at least 16 different haplotypes. Atypical haplotypes result from genetic admixture of African-Americans and other ethnic groups. This provides the opportunity for recombinational events to occur around the hot spot 5′ to the δ gene when typical African and other non-African haplotypes mix (31, 32). Common haplotypes in sickle cell anemia are shown in Figure 4.

## Haplotype and Hb F in Sickle Cell Anemia

Haplotype may be a marker for the phenotypic heterogeneity of sickle cell anemia (26, 27, 33–51). This possibility was originally raised in African populations with sickle cell anemia and different β-globin haplotypes who had distinct hematological differences. Compared with patients in Africa, the Middle East, and India, where those with sickle cell anemia are usually homozygous for a haplotype, patients in developed lands are commonly heterozygous. This has complicated the interpretation of the potential association of haplotype with phenotype. Initial work on the prevalence and clinical effects of β-globin gene haplotype in sickle cell anemia was done on a small number of African subjects.

Although they may be convenient earmarks for genetic regulators of γ-globin gene expression, most of

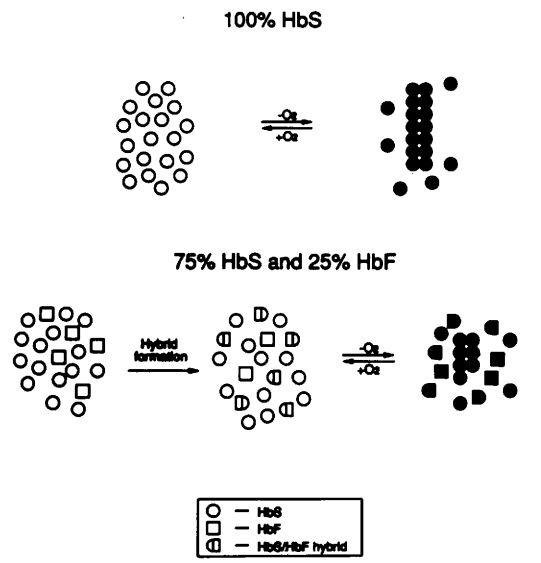


**Figure 5.** Sparing effect of Hb F on the polymerization of Hb S (25). The top panel shows the reversible polymerization of deoxygenated Hb S. In the lower panel, the presence of 25% Hb F in the red cell not only reduces the concentration of Hb S, but further retards sickling because hybrid tetramers with the composition, $\alpha_2\beta^S\gamma$, do not enter the polymer phase. A similar protective effect is seen with Hb $A_2$. (From Ref. 25, with permission.)

the polymorphic restriction sites used to assign a haplotype have no known role in the differential transcription and temporal regulation of these genes. An exception is the Xmn I site that is 5′ to the $^G\gamma$ gene in the Senegal and Arab-India haplotypes. This site is strongly associated with high expression of the $^G\gamma$ gene compared with the $^A\gamma$ gene (29, 52). Also associated with a high $^G\gamma$:$^A\gamma$ ratio but lacking the −158 C → T mutation characteristic of the Senegal and Arab-India haplotypes is the Cameroon haplotype. There are numerous sequence differences in the 5′-flanking portions of both γ-globin genes and in the large introns of these genes that appear to be haplotype-specific (53). While it has been speculated that these polymorphic regions may contribute to the variation of Hb F in sickle cell anemia, there are no functional studies that support this notion. The influence of haplotype on Hb F level is still being clarified.

Considerable variance exists among individuals with the Senegal and Arab-India haplotypes, which have been associated with the highest Hb F levels (29, 36, 38, 45, 54, 55). This suggests that various factors are affecting simultaneously Hb F (33, 47, 56, 57). The Senegal haplotype may provide the genetic environment necessary for high levels of γ-globin gene expression only in the presence of erythroid hyperplasia (58). Haplotype may serve as a marker for the effects of undefined *cis*- and *trans*-acting factors that temper globin gene expression. We found gender- and haplotype-linked effects on Hb F synthesis in sickle cell anemia. In 384 patients, hemoglobin concentrations were higher in males with Benin and Bantu haplo-

types. In the Senegal haplotype, hemoglobin was equal in males and females, but Hb F in females was higher than in males. Hb F in Senegal haplotype carriers was the highest observed. Females with the Senegal haplotype and high Hb F may have less hemolysis and thus higher hemoglobin levels. Therefore, gender- and haplotype-associated factors modulate Hb F in sickle cell anemia. While subject to many exceptions, some clinical features of sickle cell anemia found in patients with the common β-gene haplotypes are shown in Figure 6.

## LCR and Hb F

Each haplotype is associated with characteristic sequence variations in the β-globin gene 5′ HS-II of the LCR (41, 53). These polymorphic variations in HS-II are shown in Table I. Since this region probably has an important role regulating globin gene transcription, it was of interest to see if this area of polymorphic variation was associated with phenotypic variation in sickle cell anemia. In one study, HS-II from a Senegal haplotype homozygote showed several sequence variations from a Benin homozygote in conserved regions of regulatory importance (59). A sickle cell anemia patient homozygous for the Benin haplotype was found to have 21% Hb F and 65% $^{G}\gamma$-chains. In this haplotype, Hb F levels are usually much lower and $^{G}\gamma$-chain forms about 40% of γ chains. The sequence of HS-II was characteristic of the Senegal type but lacked the −158 Xmn I restriction site (Fig. 4) suggesting a crossover 5′ to the $^{G}\gamma$ gene (59). There is little published information beyond this intriguing case to suggest that this portion of HS-II truly influences γ-globin gene expression in sickle cell anemia. Examining additional exceptional patients who display a discordance between haplotype and Hb F levels should help confirm this supposition, and studies of HS-II polymorphisms in transgenic animals would help prove or disprove the functional significance of this association.

Few chromosomes have been sequenced in limited regions of HS-III, and no variation between the major β-gene haplotypes was present (60). Because HS-III may be γ-gene specific during development in embryonic and fetal transgenic mice, future studies might examine all phylogenetically conserved sites within HS-III in a larger number of $\beta^S$ chromosomes.

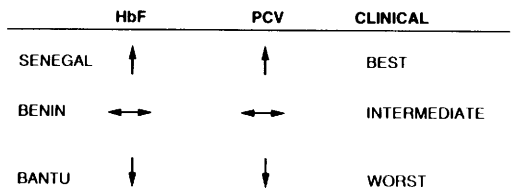


**Figure 6.** The relationships between haplotype and clinical features of sickle cell anemia. PCV connotes packed cell volume. The clinical features include events like osteonecrosis, acute chest syndrome, renal failure, and other common disease complications. These associations of haplotype and phenotype in sickle cell anemia are only generalities, and within each haplotype group there is considerable heterogeneity whose cause is unknown.

## β-Globin Gene Silencer

Located −530 bp 5′ to the β-globin gene is an AT-rich with the core structure, $(AT)_x(T)_y$, that varies polymorphically and is linked to the β-globin gene cluster haplotype. This locus has been proposed to serve as a β-globin gene silencer and may influence the expression of the β-globin gene by binding a putative repressor protein BP1 (Table II). The possibility still exists that this area has no functional significance in the regulation of the β-globin gene (61, 62). Depending upon the $(AT)_x(T)_y$ composition, BP1 is bound with greater or lesser affinity and may inhibit variably β-globin gene transcription. This region has been proposed as a possible cause of the silent carrier β thalassemia phenotype (63, 64). The Arab-India motif has a higher affinity for BP1 than the Bantu haplotype motif. This may be reflected at the protein level by less Hb S in Indian carriers of the $\beta^S$ gene and a normal α-globin genotype, when compared with blacks with sickle cell trait. For example, with the $(AT)_9(T)_5$ motif, Hb S levels in sickle cell trait with four α-globin genes were 33.8% ± 3.3% compared with a mean of ~39% with the $(AT)_8(T)_4$ motif, a reduction of ~13% (62). However, nondeletion α thalassemia, which can reduce the percent Hb S in sickle cell trait, is also prevalent in this population, and its presence may confound this analysis (65). The −530 area may therefore influence the clinical heterogeneity of sickle cell anemia by suppressing $\beta^S$ synthesis. As $\beta^S$ gene transcription declines, a reciprocal increase in γ-globin gene transcription may take place. Recent data show that the $(AT)_9(T)_5$ motif, with the −158 C → T that defines the Xmn I restriction site, may be associated with high Hb F in some homozygous β thalassemia patients (66). In these patients, the $(AT)_9(T)_5$ motif was associated with ~10 g/dl Hb F while the $(AT)_7(T)_7$ was accompanied by ~5 g/dl of Hb F. While preliminary, these clinical observations suggest that this motif may have clinically relevant effects upon β-globin gene transcription and perhaps, by impairing the production of β-globin, increase the synthesis of Hb F. As yet, there are no studies in sickle cell anemia that relate the composition of the −530 area with Hb F levels. If the sequence of the −530 area is always linked to the β-globin gene haplotype, these studies would be redundant. But, if as noted in the example of the Benin chromosome bearing a Senegal-like HS-II, some form of genetic rearrangement alters the sequence of this region, and by doing so affects β- and γ-gene transcription, then learning the composition of the −530 $(AT)_x(T)_y$ might be informative.

**Table I.** Sequence Differences in Portions of HS-II Present in Three Different $\beta^S$ Chromosomes

| Haplotype | −10924 (8580) | −10905 (8598) | −10623–10570 (8883–) | −10390 |
|---|---|---|---|---|
| Reference | T | A | 10TA.2CA.2TA.CG.11TA | A |
| Benin | G | G | 8TA.2CA.2TACGTG.7TA | T |
| Senegal | T | A | 9TA.2CA.2TA.CG.10TA | A |
| Atypical | T | A | 9TA.2CA.2TA.CG.10TA | A |

*Note.* The polymorphic nucleotide positions in four different portions of HS-II of the β LCR are given relative to the mRNA capping site of the ε-globin gene (GenBank coordinates). The atypical haplotype was found in a patient homozygous for the Benin haplotype with an unusually high level of Hb F. Modified from Refs. 59 and 125.

**Table II.** DNA Sequence Surrounding the −530 Region in Different $\beta^S$ Haplotypes

| Haplotype | −551 | −543 | −530–526 | −521 | $(AT)_X(T)_Y$ |
|---|---|---|---|---|---|
| Reference | T | C | AT——TTT | C | $(AT)_7T_7$ |
| Senegal | T | C | ATAT—— | T | $(AT)_8T_4$ |
| Benin | T | T | ATAT—— | C | $(AT)_8T_4$ |
| Bantu | C | C | ——TTTTT | C | $(AT)_6T_9$ |
| Cameroon | T | C | ATAT——T | C | $(AT)_8T_5$ |
| Arab-India | C | C | ATATAT—T | C | $(AT)_9T_5$ |

*Note.* The polymorphic variable nucleotides in the region about 530 bp 5′ to the β-globin gene are shown in five $\beta^S$ chromosome haplotypes as well as a "normal" reference chromosome. The $(AT)_X(T)_Y$ region lies between positions −530 and −526. Data is modified from Ref. 62.

## Four Base Pair Deletion Linked to the $^A\gamma$ Gene

The Cameroon haplotype $\beta^S$ chromosome that invariably contains the $^A\gamma^T$ gene is linked to a deletion of four base pairs (bp) (AGCA) at −222 to −225 5′ to this allele (67). This is the only β-globin gene haplotype associated with the four bp deletion. We found the four bp deletion present in all racial groups and always linked to the $^A\gamma^T$ allele in 89 adults (68). To assess the effect of the four bp deletion on γ-globin synthesis we studied 70 African-American newborns and found that the $^A\gamma^T$-globin chain was always present at a lower level than the $^A\gamma^I$-globin chain in heterozygotes. There was no decrease in the percentage of $^A\gamma$- compared with $^G\gamma$-globin in heterozygotes for the four bp deletion, but there was a significant decrease in total Hb F in newborns with the deletion. The percentage of total $^A\gamma$-globin was not significantly different between the groups with and without the four bp deletion. In $^A\gamma^I$ homozygotes, Hb F averaged 66.5% while in $^A\gamma^I$:$^A\gamma^T$ mixed heterozygotes, Hb F was 50.2% ($P < 0.03$), implying that the four bp deletion is associated with decreased expression of not only the $^A\gamma^T$-globin gene, but also the $^G\gamma$-globin gene in *cis*. The expression of the $^A\gamma^T$ allele was always less than that of the $^A\gamma^I$ allele, as reported previously. In contrast to other reports, we did not see a difference in $^A\gamma$-globin gene expression relative to the $^G\gamma$-globin gene in $^A\gamma^T/^A\gamma^I$ heterozygotes when compared with $^A\gamma^I$ homozygotes. Studies in β thalassemia with the four bp deletion also suggested decreased $^G\gamma$ gene expression in *cis* to the $^A\gamma^T$ allele. Together, these results implicate the region of the four bp deletion as a possible *cis*-acting element that augments the expression of both γ-globin genes when combined with a *trans*-acting factor. If this is true, then the four bp deletion might modulate the course of sickle cell anemia in individuals with the Cameroon haplotype.

There is additional *in vitro* evidence for the functional significance of the four bp deletion. A *trans*-acting factor bound the 3′ enhancer and the promoter region of the $^A\gamma$-globin gene. Using a series of scanning mutations in gel shift competition assays, the locus of binding in the promoter was localized to the GCAGCA sequence that includes the site of the four bp deletion. In a transient expression system, *in vitro* studies of the promoter containing the four bp deletion did not show reduced activity when compared with the control. In this work, the construct used lacked the 3′ enhancer previously found to bind the factor. The site of the four bp deletion may facilitate interaction between the promoter and the 3′ enhancer. There is preliminary evidence that the four bp deletion creates a new site for the binding of a repressor protein. Finally, the protein product of the homeodomain locus, HOX B2, binds the promoter and 3′ enhancer of the γ-globin gene besides sites in the LCR (69, 70).

Unfortunately, the scarcity of patients with sickle cell anemia and the Cameroon haplotype in the United States makes it very difficult to gauge what, if any, influence the presence of this genetic locus has upon Hb F levels.

## $^G\gamma$ Gene 5′ Regulatory Region

In recent work, an area of about 0.5 kb that lies 1.65 to 1.15 kb 5′ to the $^G\gamma$ gene has been proposed as

yet another region that potentially has a regulatory role in γ-globin gene expression (71). This region, designated the pre-$^{G}\gamma$ framework, was found to have four polymorphic variants that, like most other *cis*-acting sequences with possible regulatory roles, are linked to the β-gene cluster haplotype. Interest was drawn to this area because it contained four GATA-1 binding sites as well as an SP 1 and CRE protein-binding domains. By gel retardation assay, strongest protein binding was associated with the Senegal pre-$^{G}\gamma$ framework. Functional studies of the polymorphic pre-$^{G}\gamma$ variants in two erythroid cell lines, measured as the enhancer function of the pre-$^{G}\gamma$ framework by CAT activity, showed that the Benin haplotype-linked pre-$^{G}\gamma$ enhancer activity was 7-fold lower than the Bantu and Senegal type pre-$^{G}\gamma$ framework. Some of these results are difficult to interpret. In the sickle cell anemia patients whose chromosomes formed the basis for this work, the Hb F levels of the haplotype homozygotes are different from most results reported. Perhaps they represent transiently elevated Hb F in young patients rather than stable adult levels. Whether the pre-$^{G}\gamma$ framework is one of the important haplotype-linked regulatory regions awaits further study.

### None-Gene Hereditary Persistence of Hb F

Mutations in proximal and distal promoters of the $^{G}\gamma$ and $^{A}\gamma$ globin genes are likely to be responsible for the phenotype of nondeletional hereditary persistence of fetal hemoglobin (HPFH) (72–85). In these conditions, the Hb F level in heterozygotes is 3% to 20%, and its distribution among erythrocytes is usually uniform (i.e., not localized to a few F cells). Either the $^{G}\gamma$ or $^{A}\gamma$ gene is affected, and the β-globin gene in *cis* is expressed ($\beta^{+}$). These disorders differ from the first described examples HPFH caused by large deletions affecting the δ- and β-globin genes, whose phenotype merges with that of the δβ thalassemia (1). In these disorders, the Hb δ- and β-globin genes are deleted from the affected chromosome. Chromosomes that contain duplicated, triplicated, or quadruplicated γ-globin loci may also cause high Hb F (86, 87). Among the $^{A}\gamma$ HPFH mutations described are the following: C → T at position −202 (82); C → T at −196 (72, 76); T → C at −175 (81); T → C at −198 (77); C → G at −195 (83); C → G at −195 (84); and G → A at position −117 (74, 75). Similar clusters of point mutations have occurred in the $^{G}\gamma$-globin gene promoter and are associated with the $^{G}\gamma$ HPFH (73, 77–79, 85, 88). Mutations causing nondeletion HPFH occur in regions of the gene that were first suspected to play a role in the regulation of globin gene expression because of their conservation during evolution and a resemblance to viral promoters (76). Some of these mutations increase the expression of reporter genes transfected into erythroid cell lines, alter the interaction between the mutant promoter and the major human erythroid DNA binding protein, GATA-1, and bind other transcriptionally-active proteins, suggesting their functional relevance (82, 89–95). Portions of the LCR can activate a construct containing the −117 G → A γ-gene promoter in adult transgenic animals, further emphasizing the functional relevance of this mutation.

Could this class of mutation be responsible for some instances of sickle cell anemia with very high Hb F levels and, therefore, be an additional genetic modulator of disease severity? In a group of 299 adult patients with sickle cell anemia and a spectrum of Hb F concentrations, we examined the γ gene promoters in 30 patients with Hb F levels in the highest decile (mean Hb F 12.4%). No mutations were encountered. These mutations are sufficiently rare to be unlikely to account for the high Hb F phenotype in sickle cell anemia (Lu Z-H, Steinberg MH, unpublished observations).

A special case is the C → T mutation at position −158 5′ to the γ-globin gene that is linked to the Senegal and Arab-India haplotypes of sickle cell anemia (52, 96) (Fig. 4). It is still debated whether this is a true nondeletion HPFH mutation. In the Saudi population, the −158 C → T mutation is strongly, but imperfectly, associated with high levels of Hb F and $^{G}\gamma$ chains in sickle cell anemia (97). Individuals with the sickle cell trait whose $\beta^{S}$ chromosome carries the Arab-India haplotype are not anemic and have normal Hb F levels. *In vitro* culture studies of Hb F production in erythroid colonies derived from precursor cells obtained from carriers of the Arab-India haplotype have higher levels of Hb F synthesis than controls (58, 98). This system may mimic in some respects perturbed erythropoiesis akin to that occurring with hemolysis. It has been hypothesized that the −158 C → T mutation may play a permissive role; it is necessary, but alone is insufficient, to promote increased transcription in the $^{G}\gamma$ gene. For this to take place, expanded erythropoiesis or hemolytic stress must be present. Alternatively, this mutation may represent a polymorphic marker linked to other mutations, for example in HS-II, that are critical for regulating $^{G}\gamma$ gene expression.

There is also debate on the effects of the Arab-India haplotype and the accompanying high Hb F levels on the phenotype of sickle cell anemia. This haplotype accounts for over 90% of the $\beta^{S}$ gene-associated haplotypes in India and is prevalent in the Eastern oasis of Saudi Arabia. The $\beta^{S}$ gene in the Western Province of Saudi Arabia is present on the Benin haplotype chromosome reflecting the flow of this gene from Africa via the Arab slave trade. Sickle cell anemia in this portion of the Arabian peninsula resembles the African disease. In the Eastern Province of this country, a hematologically milder form of sickle cell anemia is present. These patients have high Hb F lev-

els, but they still have vasoocclusive events at a rate similar to their compatriots from the Western province (99). More detailed studies are needed to resolve this inconsistency. Sickle cell anemia in parts of India, and in descendants of these peoples in Malaysia, is akin to the disease in the Eastern Province of Saudi Arabia (100, 101), and these individuals also carry the Arab-India haplotype.

## α Thalassemia

The duplicated α-globin genes are present as two identical coding sequences that sit adjacent to the telomere of the short arm of chromosome 16 (102) (Fig. 2). α thalassemia in blacks is usually a result of the deletion of one or two α-globin genes from a chromosome. Missing even two of the normal complement of four α-globin genes is not clinically important. Since nearly a third of African-Americans carry an α-globin gene deletion, there are frequent instances in which α thalassemia and sickle cell anemia coexist (102, 103). The hematological and clinical consequences of the interactions of these two disorders have been studied intensively. The presence of α thalassemia with sickle cell anemia is associated with less hemolysis, higher hemoglobin concentration, lower mean corpuscular volume (MCV), and lower reticulocyte count, when compared with individuals with normal α-globin gene numbers (104–107) (Table III). α thalassemia does not appear to modify the effect of haplotype on Hb F levels in sickle cell anemia despite an early report to the contrary (15). Therefore, it is unlikely that any clinical benefit α thalassemia confers upon sickle cell anemia is mediated through its effects on Hb F level. α thalassemia is distributed evenly among all β-globin gene haplotypes in African-Americans with sickle cell anemia.

α thalassemia does have an effect upon the phenotype of sickle cell anemia. Characteristic of all forms of thalassemia in which erythrocyte hemoglobin concentration is reduced, α thalassemia produces a red cell with reduced sickle hemoglobin concentration in patients with sickle cell anemia. Hb S polymerization depends on hemoglobin concentration, so concurrent α thalassemia should diminish the polymerization potential of sickle hemoglobin in sickle cell anemia. When these conditions coexist, there is less hemolysis and anemia is less severe. Clinically, the outcome of coincident α thalassemia and sickle cell anemia is paradoxical. Vasoocclusive events appear undiminished in sickle cell anemia with α thalassemia. Fewer dense and poorly deformable cells that are a consequence of α thalassemia raise the PCV, and, since these cells contain Hb S, blood viscosity is increased. Raising the number of sickle cells, as occurs with α thalassemia, seems to promote vasoocclusion. Patients with sickle cell anemia and α thalassemia have more episodes of pain and more bone disease than do patients with sickle cell anemia alone. But, more blood may also have beneficial effects in some organs, and thus skin ulcers of the leg and retinal vascular disease may be less common in carriers of α thalassemia and sickle cell anemia. Some evidence has suggested that the combination of α thalassemia and sickle cell anemia may promote longevity, but this has not been proved.

## Hemoglobin $A_2$

Hb $A_2$, the tetramer of α- and δ-globin chains, impairs the polymerization of Hb S to the same extent as does the γ-globin chain of Hb F (108); δ22 (Ala) and δ87 (Gln) are the residues that inhibit polymerization (108). Hb $A_2$ has the advantage of being evenly distributed in all red cells while Hb F is sequestered in F-cells. The naturally low level of Hb $A_2$ makes it an inconsequential contributor to the total hemoglobin concentration, but there are instances when the level of Hb $A_2$ is increased far beyond its usual values. Hb $A_2$ has a similar oxygen affinity, Bohr effect, and cooperativity as Hb A (109). When Hb $A_2$ and Hb F levels are high, the combination of these two hemoglobins may modulate sickle cell disease and cause a mild phenotype (110).

Normally, Hb $A_2$ is only 2%–3% of the total hemoglobin. In bone marrow, δ-globin chains are synthesized at a reduced rate compared with β-chain; δ-globin is not synthesized in reticulocytes. δ-Globin mRNA half-life was less than one third that of β-globin mRNA (109), but this alone cannot account for the vast reduction of Hb $A_2$ compared to Hb A levels (111, 112). The instability of δ mRNA may depend on the sequence of the 3′ untranslated region. Transcriptional control is another important determinant of δ-globin gene expression. δ-Gene transcription was 50 times

**Table III.** Hematological Features of Sickle Cell Anemia with α Thalassemia

| Genotype | Hemoglobin | MCV | Reticulocytes (%) | Hb $A_2$ (%) |
|---|---|---|---|---|
| αα/αα | 8.0 ± 1.1 | 92 ± 7 | 11 ± 6 | 2.8 ± 0.4 |
| −α/αα | 8.6 ± 1.1 | 83 ± 7 | 9 ± 6 | 3.3 ± 0.6 |
| −α/-α | 9.2 ± 1.3 | 72 ± 4 | 7 ± 5 | 3.8 ± 4 |

*Note.* Genotype indicates the number of α-globin genes present in patients with sickle cell anemia. Hb-hemoglobin concentration in g/dl, MCV-mean corpuscular volume in femtoliters. These data report the average determined from studies of black patients with α thalassemia and sickle cell anemia (105, 106).

less efficient than that of the β gene (113, 114). Transcription of a hybrid βδ-globin gene was equivalent to that of the normal β-globin gene, but a δβ gene was expressed similar to a normal δ-globin gene, suggesting that the δ-globin gene promoter was responsible. While several differences exist between the β- and δ-gene promoters in areas critical for transcription, one interesting change is present in the CCAAT box. In the δ-gene promoter, the conserved CCAAT box is replaced by CCAAC. The second intervening sequence of the δ-globin gene also has been shown to reduce δ gene transcription.

Hb $A_2$ levels are increased above normal in β thalassemia. The mechanism for the "compensatory" increases in δ-globin synthesis is not totally clear but may result from a "competition" among β-globin-like gene promoters for transcription factors and the LCR. Some individuals with β thalassemia trait have Hb $A_2$ concentrations that are significantly higher than the usual 5.0% ± 1.1% (109). These exceptionally high Hb $A_2$ levels are the result of deletions that remove the β-gene promoters (109). Elimination of β-gene promoters by 5′ deletions may increase the likelihood that the LCR interacts with the δ gene in *cis*. Increased binding of transcription factors, such as GATA 1 and NF-E2 (115, 116), to the remaining δ- and γ-gene promoters may enhance the transcription of these genes. Competitive interaction between the LCR and β-like gene promoters seems likely, although other mechanisms may be possible (6, 117, 118).

It is not likely that variation in Hb $A_2$ level affects the phenotype of sickle cell anemia. While Hb $A_2$ is increased in sickle cell anemia with α thalassemia, compared with sickle cell anemia alone (Table III), the increase is small, and, if a benefit is present, it is probably undetectable with our current imprecise means of quantifying phenotypic variation in sickle cell anemia. However, in the rare instances where $\beta^0$ thalassemia is caused by deletions of the 5′ portion of the β-globin gene that cause the Hb $A_2$ level to far exceed the expected, there could be modulation of the mixed heterozygous condition, sickle cell anemia-$\beta^0$ thalassemia. Usually, this disorder closely resembles sickle cell anemia (110).

## Prediction of Phenotype in Sickle Cell Anemia

Can the phenotype of sickle cell anemia be predicted? A reliable method of foretelling disease severity early in life, or perhaps antenatally, may be important as new treatments, like bone marrow transplantation, chemotherapeutic manipulation of Hb F level, or even gene therapy, that all have potentially serious complications approach clinical use (119–121). Since we now know that Hb F concentration influences the occurrence of painful episodes and other vasoocclusive events and that the frequency of painful episodes may portend mortality in adults, there are compelling reasons to attempt to raise Hb F levels in sickle cell anemia (22, 122). At this point in time, establishing a prognosis using one or two genetic markers, like the presence of α thalassemia or the nature of the β-globin gene cluster haplotype, is unlikely to be sufficient to assess severity in an individual. Employing additional informative genetic markers will most likely be required for this purpose. Diagnostic means that not only detect the presence of sickle cell anemia, but also determine its prognosis, would be of considerable help when hazardous treatments are considered.

It may be simplistic to expect that even if we could predict with extreme precision the rate of fall of Hb F level in childhood and its steady-state level in adults we would be able to foretell the consequences of the disease that beset an individual. Some patients survive many years with few problems even when Hb F levels are quite low and expected to presage an early and painful demise (123). The distribution of Hb F in F cells may be vitally important and not reflected in the measurement of Hb F levels. For example, 10% F cells each having nearly 100% Hb F is not likely to be as advantageous as 20% F cells each having 50% Hb F. Clearly, there are factors unrelated to the hemoglobin genes, like vascular reactivity, erythrocyte membrane structure and function, leukocyte biology, and endothelial cell integrity, that may be integral components of the pathogenesis of sickle cell anemia (2, 124). When the roles of these determinants are delineated and consolidated into a comprehensive model of pathogenesis, and environmental influences are controlled, it may be then possible to establish an accurate prognosis.

This work was supported in part by Research Funds of the Department of Veterans Affairs.

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