Analysis of Hemoglobin F Production in Saudi Arabian Families With Sickle Cell Anemia

By Barbara A. Miller, Mohammed Salameh, Mohammed Ahmed, Nancy Olivieri, Giovanna Antognetti, Stuart H. Orkin, Titus H.J. Huisman, and David G. Nathan

Erythrocytes and progenitor-derived erythroblasts of sickle cell anemia patients from the Eastern Province of Saudi Arabia contain increased fetal hemoglobin and $^{G}\gamma$ globin. A distinctive DNA polymorphism haplotype in the β globin gene cluster (+ + − + + + + −), tightly coupled to a C → T substitution at position −158 5′ to the cap site of the $^{G}\gamma$ globin gene, is strongly associated with sickle cell disease in this region. To determine whether the increased fetal hemoglobin production and/or elevated $^{G}\gamma$ globin content are tightly linked to this haplotype, we studied 55 members of five Saudi families in which sickle cell disease is present. The results did not suggest a tight linkage of the haplotype to increased fetal hemoglobin production. On the other hand, several sickle trait family members heterozygous for the haplotype had normal fetal hemoglobin production in culture but elevated $^{G}\gamma$ to $^{A}\gamma$ ratios in peripheral blood. This observation suggests that in this genetic background increased expression of the $^{G}\gamma$ globin gene may occur without a measurable increase in total fetal hemoglobin production. The family studies also clearly demonstrate that increased fetal hemoglobin production by erythroid progenitors is dependent on zygosity for the sickle gene in this population. These findings strongly suggest that other factors, such as the products of genes stimulated by hemolytic stress or other genetic determinants associated with the Saudi β^{S} chromosome, may interact with the −158 C → T substitution and influence γ globin gene expression in this population.

SICKLE CELL anemia (SCA) patients from the Eastern Province of Saudi Arabia have mean increased circulating fetal hemoglobin levels of 16% ± 7.4%, largely $^{G}\gamma$, whereas their sickle trait (AS) parents have mean levels in the normal adult range (1.1% ± 1.0%).[1] Measurement of fetal hemoglobin in BFU-E–derived erythroblasts[2] in SCA patients and their AS parents provides strong evidence that the ability to produce increased HBF is inherited.[1,3] Most Saudis with SCA are homozygous for a particular DNA polymorphism haplotype that characterizes the β globin gene cluster (+ + − + + + + −).[3] The γ-globin gene region of such a haplotype has been isolated by molecular cloning. Only one nucleotide substitution, a C → T at −158 5′ to the $^{G}\gamma$ cap site recognized by the restriction endonuclease *Xmn*I, was identified.[3] The precise relevance of this substitution to the elevated HBF production in Saudi SCA is, however, uncertain since Saudi normals (AA) who may also have this substitution do not demonstrate increased levels of fetal hemoglobin, either in circulating erythrocytes or in progenitor-derived erythroblasts.[3]

In an effort to examine the association of the −158 C → T substitution and high HbF expression in Saudis, we studied 55 members of five families with sickle cell disease. The results suggest that the −158 C → T substitution, although perhaps a necessary component of the elevated HBF program, is certainly not sufficient. Other factors, particularly those associated with homozygosity for β^{S}, appear at least equally important.

MATERIALS AND METHODS

Blood samples. Blood samples were obtained from Saudi Arabians who are employees of the Arabian American Oil Company (ARAMCO) and from their parents and siblings and children. Families were asked to volunteer blood samples of all available members after demonstration of elevated fetal hemoglobin in the blood of at least one member with SCA. SCA patients were not studied if they had undergone a blood transfusion in the past 6 weeks, had experienced a sickle cell crisis or active infection in the 7 days before assay, or were aged <3 years. The samples from Saudi Arabia were drawn in 10% acid-citrate-dextrose and shipped at 4°C. All assays and cultures were performed within 72 hours.

CBC and reticulocyte counts, hemoglobin electrophoresis on citrate agar and cellulose acetate, and alkali denaturation to determine percentage of fetal hemoglobin were performed on all samples. The ratio of $^{G}\gamma/^{A}\gamma$ in peripheral blood hemoglobin F was determined by high-performance liquid chromatography (HPLC)[4,5] in samples in which a sufficient amount of hemoglobin F permitted this analysis.

Analysis of hemoglobin F in BFU-E–derived erythroblasts. Blood mononuclear cells were prepared and cultured in semisolid media with erythropoietin as previously described.[1] The culture plates were incubated in humidified 4% CO_2 at 37°C for 14 days, and erythroid colonies were then counted. These colonies were "plucked," and the average number of erythroid cells per colony was determined. A radioligand assay of total or fetal hemoglobin per BFU-E–derived cell was performed.[1,6] Calculations of hemoglobin content per BFU-E–derived cell were based on the assumption that all such cells are potential F cells. Percentage of HbF was calculated from picogram of fetal hemoglobin and total hemoglobin content per cell. Reproducibility of this assay has been discussed previously but,

From the Division of Hematology/Oncology, The Children's Hospital Medical Center, and the Dana-Farber Cancer Institute, The Department of Pediatrics, Harvard Medical School and the Howard Hughes Medical Institute at the Children's Hospital, Boston, the Division of Pediatric Hematology/Oncology, The Milton S. Hershey Medical Center, Hershey, PA; Dhahran Health Center, Dhahran, Saudi Arabia; and the Department of Cell and Molecular Biology, Medical College of Georgia, Augusta.

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Address reprint requests to Barbara A. Miller, MD, Department of Pediatrics, Division of Hematology/Oncology, The Milton S. Hershey Medical Center, PO Box 850 Hershey, PA 17033.

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in general, duplicate dishes or multiple assays performed on a sample from one individual yielded results within 10% of the mean.[2] Two Saudi sickle cell patients studied repetitively over 3 years in two different laboratories had mean percentages of HbF ± 1 SD in BFU-E–derived cells of 29.7% ± 3.8% and 22.1% ± 3.2%.

Restriction endonuclease analysis of DNA and determination of the haplotype of the β globin gene cluster. Procedures for DNA isolation, blot hybridization, and probe preparations were as previously described.[4,7] Restriction endonuclease site polymorphisms studied here included: an *Xmn*I site 5′ to the cap site of the $^{G}\gamma$ globin gene, which recognizes the C → T substitution at position −158, an *Ava*II site in the IVS-2 of the β globin gene, and *Hpa*I and *Bam*HI sites 3′ to the β globin gene. Digestion at the −158 *Xmn*I site in Saudi sickle cell disease is always associated with the + + − + + 5′ haplotype, representing polymorphisms at a potential *Hinc*II site 5′ to the ε gene, a *Hind*III site in the IVS-2 of the $^{G}\gamma$ globin gene, a *Hind*III site in the IVS-2 of the $^{A}\gamma$ globin gene, and two *Hinc*II sites, one in the ψβI gene and another 3′ to it.[3] In this article, the presence of the "classic" 5′ haplotype of the Saudi β globin gene complex (+ + − + +) was determined by digestion with *Xmn*I at −158 C → T substitution and is denoted simply as +. The 3′ haplotype described by the *Ava* II site in the IVS-2 of the β globin gene, the *Hpa*I and *Bam*HI sites 3′ to the β globin gene, and the hemoglobin A or hemoglobin S genotype are fully noted. Thus, the complete classic Saudi β^{S} haplotype is described here as + + + − S.

RESULTS

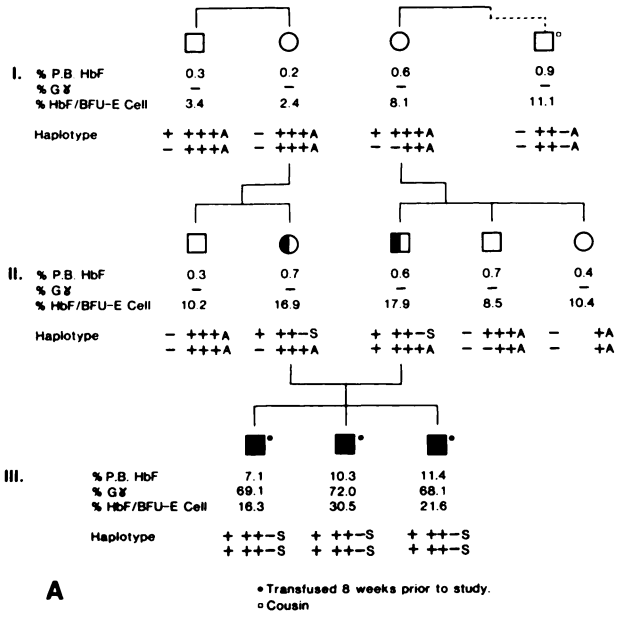
Hemoglobin F production in five Saudi Arabian sickle cell families. Five Saudi Arabian families with sickle cell disease were studied to resolve whether determinants responsible for the increased HBF production observed in Saudis might be tightly linked to the β globin gene complex. Results of these studies are shown in Fig 1, families 1 through 5.

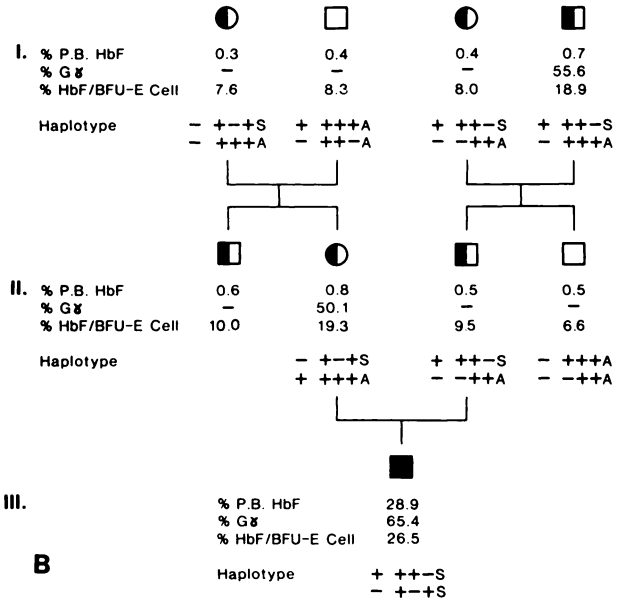
In family 1, all three children with SCA were homozygous for the + + + − S haplotype, which is frequently associated with Saudi sickle cell disease. They also had high $^{G}\gamma/^{A}\gamma$ ratios and increased, albeit variable, HBF production. One AS parent was homozygous (II-3) and the other heterozygous (II-2) for a −158 C → T substitution (*Xmn*I-positive site), and both had increased HBF production. Two normal (AA) family members (I-1 and I-3) who were heterozygous for a −158 *Xmn*I positive site and whose 3′ β haplotypes were + + + rather than + + − did not have increased HBF programs, consistent with previous observations of normal HbF production in AA Saudis.[3]

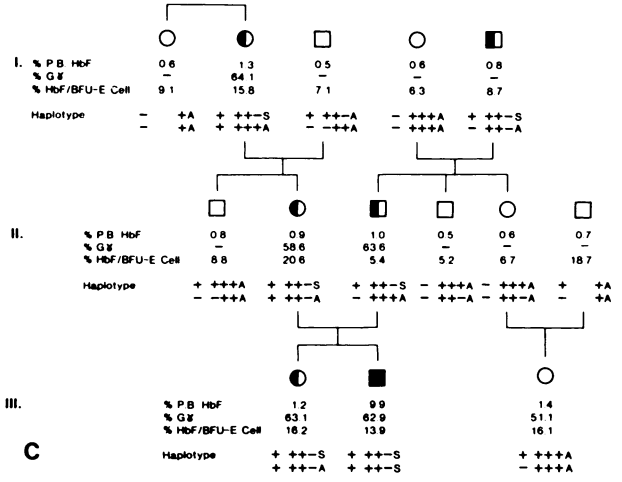
In family 2, the SCA patient III-1 was heterozygous for a + + + − S β region haplotype and for another sickle chromosome for which the β region haplotype was − + − + S, a haplotype that is not associated with elevated hemoglobin F.[3,8,9] The marked elevations of hemoglobin F, $^{G}\gamma/^{A}\gamma$ ratio, and HBF production in vitro observed in the SCA patient were all consistent with levels often observed in Saudi Arabian sickle cell disease. These data suggest that only one chromosome with the + + + − S β region haplotype is necessary to induce elevated HBF and high $^{G}\gamma$ production in Saudi SCA. The paternal grandfather, I-4, who was heterozygous for the + + + − S β region haplotype also demonstrated increased HBF production in vitro. However, his son (II-3), who inherited the same sickle chromosome, did not. The patient's mother II-2 was heterozygous for − + − + S and + + + + A β region haplotypes. Neither of these haplotypes would have been expected to be associated with elevated HBF production. Nevertheless, such an increase was clearly evident. The increased HbF production in culture was not itself due to the − + − + S haplotype because the patient's maternal grandmother (I-1) was heterozygous for this chromosome and did not have an increased HBF production. Similarly, the accompanying + + + + A haplotype was not itself responsible for the high HBF production in II-2 because its presence in I-2 was not associated with increased HBF. We must conclude that in II-2 the interaction of − + − + S and/or the + + + + A β regions with a factor(s) not detected in this analysis may have resulted in the high HBF production. This single family study strongly suggests that the + + + − S β region haplotype is not solely responsible for the increased HBF production observed in Saudis.

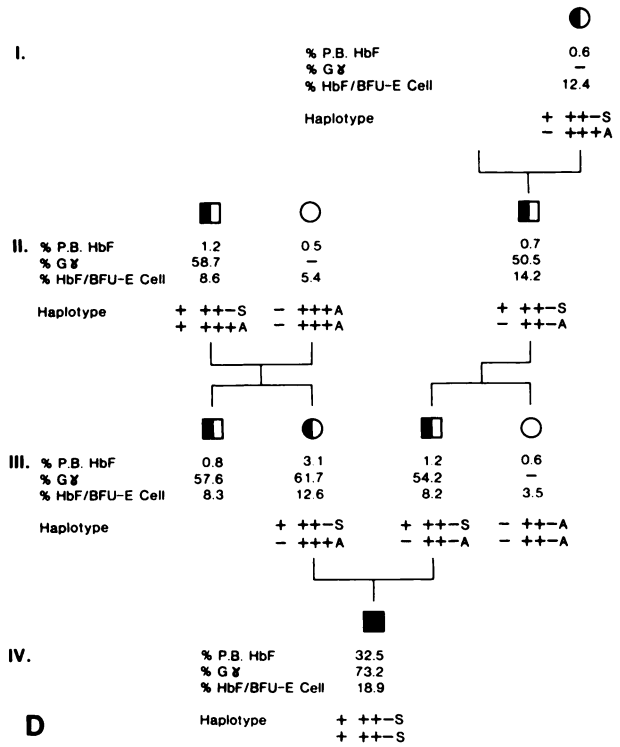
The three other family studies confirmed this impression. In family 3, the SCA patient (III-2) was homozygous for the classic Saudi + + + − S β region, but neither his circulating HbF nor percentage of HbF in BFU-E–derived cells was exceptionally increased. The remaining data in this family suggests that the two + + + − S containing chromosomes had different capacities for HbF production. The progenitor-derived erythroblasts with the maternal (II-2) + + + − S–containing chromosome had the expected increased HBF phenotype, whereas the paternal (II-3) phenotype was normal. This finding was recapitulated in the respective grandparents I-2 and I-5. That the + + + + A β region could, on rare occasions, also be associated with increased HBF production was suggested by II-6 and his daughter III-3. These two are the only exceptions that we have detected to the observation that high HBF production by BFU-E–derived erythroblasts in Saudis is only associated with β^{S}.

The results obtained from study of family 4 further emphasize the lack of tight linkage of the classic Saudi + + + − S β region to elevation of HBF production and circulating $^{G}\gamma/^{A}\gamma$ ratios. The proband (IV-1) was homozygous for the + + + − S β region haplotype and had very high circulating HBF and $^{G}\gamma$ globin. His HBF production in vitro was substantially increased as well. Eight family members were studied, six of whom had sickle trait. All had at least one copy of the classic + + + − S β region haplotype. All of these had substantially higher hemoglobin F production in vitro than did the two family members with various β^{A}-containing haplotypes, but there was variability of both HBF production and percentage of $^{G}\gamma$ in the sickle trait individuals. Again, this suggests that precise control of the HBF phenotype is not tightly linked to the particular haplotype involved. This impression was confirmed in family 5, in which the proband (III-1) had the expected elevated hemoglobin F, $^{G}\gamma/^{A}\gamma$ ratio and HBF production but was heterozygous for the classic + + + − S β region haplotype. His other β^{S}-containing chromosome − + − + S was, as expected, not associated with an elevated hemoglobin F production when found in the heterozygous state in other members of his family. The classic + + + − S β region haplotype present in his paternal lineage, while it produced a substantial elevation of $^{G}\gamma$, was not associated with particularly high HBF production in vitro.









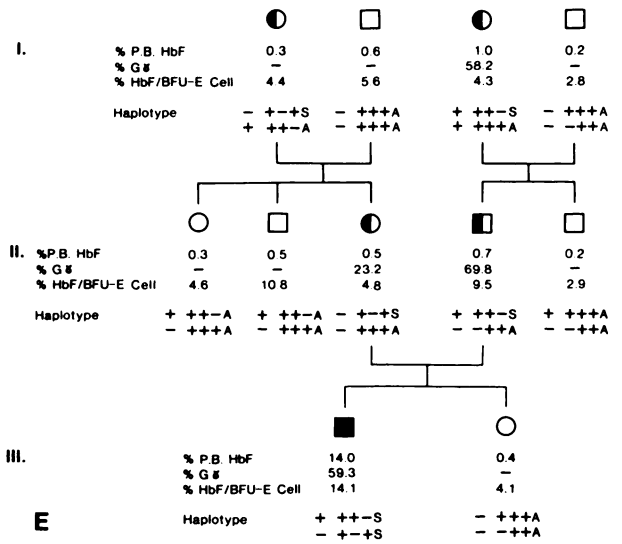


Fig 1. Analysis of percentage peripheral blood (PB) hemoglobin F, percentage of circulating $^{A}\gamma$ and percentage of hemoglobin F/BFU-E–derived cell in members of five families (families 1 through 5) with sickle cell anemia. Polymorphic restriction sites studied in the β globin gene cluster include, sequentially, the −158 *Xmn*I site 5′ to $^{A}\gamma$, the *Ava*II site in the IVS-2 of the β globin gene, and the *Hpa*I and *Bam*HI site 3′ to the β globin gene (described in the Materials and Methods section). Symbols: Male (□), female (○), AA (○, □), AS (◐, ◧), SS (●, ■). (A) Family 1; (B) Family 2; (C) Family 3; (D) Family 4; (E) Family 5.

Relationship of hemoglobin F production to zygosity for the Hb S gene. All members of these five families were grouped based on zygosity for the Hb S gene and the presence of the −158 *Xmn*I-positive site. The average percentage of HbF/BFU-E–derived cell for each individual is shown in Fig 2. There was no significant difference in hemoglobin F production between AA (−/−) and AA (+/−), between AS (+/−) and AS (+/+), or between SS (+/−) and SS (+/+). If more patients had been studied in several of these groups, significant differences between groups might have been demonstrated, but the differences would be small. As noted previously,[3] significant differences were noted between AA (+/−) and SS (+/−) ($P < .01$), AS (+/−) and SS (+/−) ($P < .025$), and between AS (+/+) and SS (+/+) ($P < .05$). As a group, AA individuals had significantly lower hemoglobin F production in culture than that of AS individuals ($P < .025$), and AS individuals had significantly lower hemoglobin F production than that of SS ($P < .001$). Thus, the percentage HbF produced in culture appeared to be dependent on the degree of zygosity for the sickle gene. The −158 C → T substitution recognized by *Xmn*I cannot be solely responsible for increased fetal hemoglobin production, since AS (+/+) individuals produced significantly less fetal hemoglobin in culture and in blood than did SS (+/+). The SD in each group is large, suggesting multifactorial control of expression of γ globin genes.

Relationship of $^{G}\gamma$ production to zygosity for the Hb S gene. Data on $^{G}\gamma/^{A}\gamma$ ratios in these patients are incomplete because insufficient quantities of blood were available for isolation of hemoglobin F in all patients, particularly in those patients with <1% circulating fetal hemoglobin. $^{G}\gamma/^{A}\gamma$ ratios are shown in Fig 3, together with the sickle phenotype and presence or absence of digestion by *Xmn*I at the −158 polymorphic site.

Saudis with AS or SS and a −158 C → T substitution, even when bearing this site on one chromosome, had an elevated level of $^{G}\gamma$ (≥50%), suggesting that an increase in $^{G}\gamma$ globin production can occur in association with this site without a measurable increase in total fetal hemoglobin production. No significant differences in $^{G}\gamma$ production were noted in patients with AS (+/−) compared to AS (+/+), or in the two patients with SS (+/−) as compared with those with SS (+/+). However, individuals with AS (+/+) had significantly lower $^{G}\gamma/^{A}\gamma$ ratios than did those with SS (+/+) ($P < .025$). $^{G}\gamma/^{A}\gamma$ ratios were characterized by wide SD within a group, also suggesting that multiple factors control $^{G}\gamma$ globin gene expression in these patients.

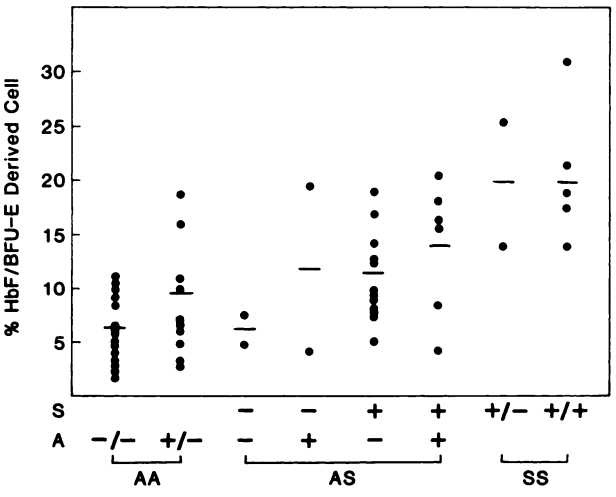


Fig 2. Assessment of percentage of HBF/BFU-E–derived erythroblast in AA, AS, and SS family members whose β globin gene clusters contain (+) or do not contain (−) the −158 C → T substitution 5′ to $^{G}\gamma$. Bar indicates mean for each group.

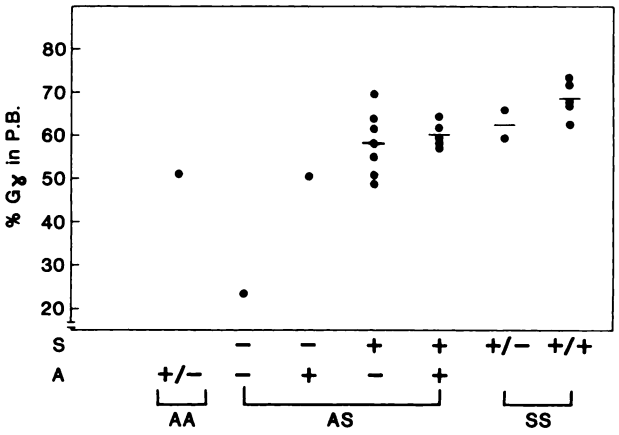


Fig 3. Assessment of the percentage of $^{G}\gamma$ in the peripheral blood in AA, AS, and SS family members whose β globin gene clusters contain (+) or do not contain (−) the −158 C → T substitution 5′ to $^{G}\gamma$. Bar indicates mean for each group.

DISCUSSION

Sickle cell anemia is characterized by marked clinical diversity, which may partially reflect the different genetic backgrounds on which β^{S} arose.[10,11] Increased hemoglobin F and/or $^{G}\gamma$ production noted in some sickle cell and thalassemia patients has been associated with inheritance of a specific 5′ haplotype of the β globin gene cluster (+ − + +) and with the presence of a C → T substitution at −158 5′ to the $^{G}\gamma$ globin gene.[1,8,9,12,13]

We recently completed an analysis of Saudis from the Eastern Province with SS, AS, and AA genotypes, most of whom were at least heterozygous and often homozygous for the C → T substitution at −158 and β^{S3}. That analysis revealed an association of the C → T substitution with elevated HbF production and also showed that the β^{S} gene in these Saudis is usually linked to a distinctive 3′ haplotype (+ + −). The present study of five Eastern Province Saudi families, in which there was at least one member with sickle cell anemia, was performed to determine whether the specific γβ globin gene cluster frequently found in these individuals, could itself explain the high circulating hemoglobin F, increased $^{G}\gamma/^{A}\gamma$ ratio, and elevated hemoglobin F production by progenitor-derived erythroblasts that characterizes these patients. Unlike Blacks and Mediterraneans heterozygous for deletion or nondeletion HPFH genes,[14-16] Saudis with one copy of the "common" Eastern oasis haplotype, + + + −S do not have elevated total circulating HbF, although they do

tend to have variable increases in the $^{G}\gamma/^{A}\gamma$ ratio.[1,3] To determine whether the + ++ −S $\gamma\beta$ globin gene cluster is tightly linked to increase HbF production, we measured HbF production in the progenitor-derived erythroblasts of 55 family members of probands with sickle cell anemia and determined whether increased HbF production in these cells was associated with the presence of at least one copy of the + ++ −S haplotype. We chose this approach because we had determined in previous studies that individual hemoglobin F production in culture is reproducible over relatively long periods of time and because the hemoglobin F production by BFU-E–derived erythroblasts in Saudis correlates with the total circulating HbF.[1,2]

Five families provided an opportunity to study 11 different β^{S}-bearing chromosomes, nine of which had the + ++ −S haplotype. We confirmed our previous finding[3] that zygosity for the + ++ −S $\gamma\beta$ haplotype correlates with the extent of HbF production in Saudis, consistent with the observation of other researchers that zygosity for HbS correlates with circulating HbF levels.[17-19] However, the association does not suggest tight linkage. Other mechanisms appear to be involved. For example, the −158 C → T substitution 5′ to the $^{G}\gamma$ gene of the + ++ −S chromosome may simply increase the capacity of the $^{G}\gamma$ region to respond to certain transacting regulatory proteins. The level of total HbF produced in erythroblasts might depend on the intracellular level of such proteins as well as on specific DNA sequences, both immediately 5′ or at considerable distance from the γ globin genes.[16,20-22] Erythropoietic stress, such as the hemolytic anemia of sickle cell disease, might affect *trans*-acting regulatory protein levels and thereby lead to increased expression of the $^{G}\gamma$ genes and total hemoglobin F.

Clearly, ultimate understanding of regulatory mechanisms controlling high hemoglobin F production in Saudis and in other individuals with SCA such as Asian Indians,[23] awaits identification of the proteins that regulate globin gene expression and the development of in vitro expression systems that permit assessment of the function of such regulators in the presence of natural and artificial globin gene constructs.

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