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Research Article

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A Gene Conversion Located 5′ to the $^{A}\gamma$ Gene in Linkage Disequilibrium with the Bantu Haplotype in Sickle Cell Anemia

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Abstract

Cloning and sequencing of the γ-globin gene of a sickle cell anemia patient homozygous for the Bantu haplotype has revealed a gene conversion that involves the replacement of an $^{A}\gamma$ sequence by a $^{G}\gamma$ sequence in the promoter area of the $^{A}\gamma$ gene. This event is similar to another gene conversion believed to be responsible for the very high homology between γ-globin genes, suggesting that the promoter area of these genes is prone to this type of genetic rearrangement. Further analysis demonstrated that the chromosome bearing this gene conversion has a very high frequency among Bantu chromosomes and a very low or nil frequency in other haplotypes linked to the β^{s} gene. No correlation was found between the $^{G}\gamma/^{A}\gamma$ ratio and the presence of the gene conversion among Bantu haplotype patients, thus excluding a portion of the γ gene sequence in the determination of this ratio.

Introduction

The β^{s} gene is linked to three different and geographically specific haplotypes in Africa (1–3). These haplotypes have been demonstrated to differ sharply in $^{G}\gamma$ expression (4, 5). Moreover, they exhibit different tendencies for hemoglobin F (HbF)[1] response in the presence of anemia (4, 5). Nevertheless, it has become apparent that within these haplotypes a significant amount of variation exists in $^{G}\gamma$ expression, and particularly the HbF expression (4, 5). It is reasonable, then, to think that within each haplotype an amount of genetic variability must exist to account for these differences. In this context we have begun the systematic analysis of the DNA sequences, particularly around the $^{G}\gamma$ genes in different β^{s}-linked haplotypes, to try to define the molecular basis of HbF expression during erythropoietic stress.

Of particular interest is the Bantu haplotype, which exhibits unusual variability in the percentage of both $^{G}\gamma$ and peripheral HbF (5). We report here the unexpected finding of a gene conversion event that brings $^{G}\gamma$ sequences to the 5′ region of $^{A}\gamma$, a situation that seems to be commonly found in the Bantu haplotype linked to the β^{s} gene. Although this finding cannot account for the differences in $^{G}\gamma$ expression found within the bearers of this haplotype (5), the finding is useful in excluding a portion of the sequence 5′ to the γ genes in the determination of the $^{G}\gamma/^{A}\gamma$ ratio.

Methods

β-Globin gene cluster haplotypes and % $^{G}\gamma$ determination were performed as described previously (1, 4).

Cloning and sequencing. Genomic DNA (50 μg) was digested by Bgl II and fractionated according to size on a 5–20% NaCl gradient. The 12–14-kb fraction containing both γ-globin loci was ligated into the Bam HI site of the phage vector EMBL3. Approximately 500,000 clones from this library were then screened using a radiolabeled probe encoding the $^{G}\gamma$ second intervening sequence; three positive clones were found. Subclones containing the $^{A}\gamma$ and $^{G}\gamma$ promoter regions were derived by digesting the Bgl II inserts with Sal I and Bam HI and subcloning the fragments into plasmid pGEM3. To sequence the promoter area of both the γ-globin gene and the second intervening sequence of $^{A}\gamma$, various subclones were constructed from one of these positive clones. Digestion of the 13-kb insert by Bam HI and Sal I yielded three fragments: Sal I-Bam HI from site −1,700 ($^{G}\gamma$) to +471 ($^{G}\gamma$); Bam HI-Bam HI from site +471 ($^{G}\gamma$) to +471 ($^{A}\gamma$); and Bam HI-Sal I from site +471 to +6,287 ($^{A}\gamma$) (see Table I). The numbering is relative to the cap site of $^{G}\gamma$ or $^{A}\gamma$. These three fragments were gel purified, ligated with the plasmid pGEM-3 previously cut by Bam HI or Sal I/Bam HI, and introduced into HB101 competent cells by standard procedures. Smaller subclones were derived by partial deletion of the Bam HI/Sal I inserts by restriction digestion with two restriction enzymes (see Table I), followed by gel purification (using low melting point agarose) of the restriction fragment containing the plasmid plus a portion of the insert. The purified fragments were blunt ended, circularized using T4 DNA ligase, and used to transform HB101 competent cells. These subclones were then sequenced by the Sanger dideoxy method (6) using the SP6 and T7 promoter primers (Promega Biotec; Madison, WI) and primers AS-10, Na-3, and Na-4 (see Table II). Gel compression artifacts at GC-rich sequences were resolved by running sequencing gels containing 7 M urea and 30% formamide at 60–65°C.

PCR amplification. Fragments −597 to +16 5′ of $^{G}\gamma$ and −595 to +16 of $^{A}\gamma$ gene were amplified by the polymerase chain reaction (PCR) technique (7) using a DNA thermal cycler and a DNA amp kit according to the manufacturer's instructions (Perkin-Elmer Cetus Corp., Norwalk, CT). After 40 cycles of amplification, ~ 5 μg of amplified product was obtained.

The 5′ $^{G}\gamma$ region was amplified using primers AS-8 and AS-10, whereas primers AS-9 and AS-10 were used to amplify the 5′ $^{A}\gamma$ region. The sequence and position of all the primers are given in Table II.

Single base mismatch detection. Approximately 50 ng of PCR-amplified DNA was denaturated, neutralized, and applied to nitrocellulose with a slot blot apparatus (Schleicher & Schuell, Inc., Keene, NH) following the recommendation of the manufacturer. 100 ng of oligonucleotide Na-3 and Na-4 (see Table II) were 5′ end-labeled using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and 50 μCi of γ-32P ATP (>3,000 Ci/mmol). Unincorporated nucleotides were removed by chromatography through Sephadex G50. The nitro-

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1. *Abbreviations used in this paper:* HbF, hemoglobin F; PCR, polymerase chain reaction.

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Table I. Plasmid Constructed To Determine the Sequence of Part of the γ Genes

Initial plasmid	Final plasmid	Enzyme used
−1,700 ($^{G}\gamma$) to +471 ($^{G}\gamma$)	−1,100 ($^{G}\gamma$) to −202 ($^{G}\gamma$)	Apa I/Bam HI
−1,700 ($^{G}\gamma$) to +471 ($^{G}\gamma$)	−386 ($^{G}\gamma$) to +471 ($^{G}\gamma$)	Sal I/Stu I
−386 ($^{G}\gamma$) to +471 ($^{G}\gamma$)	−202 ($^{G}\gamma$) to +471 ($^{G}\gamma$)	Apa I/Hinc I
+471 ($^{G}\gamma$) to +471 ($^{A}\gamma$)	+471 ($^{G}\gamma$) to −386 ($^{A}\gamma$)	Stu I/Sal I
+471 ($^{G}\gamma$) to +471 ($^{A}\gamma$)	+471 ($^{G}\gamma$) to −202 ($^{A}\gamma$)	Apa I/Sma I
+471 ($^{G}\gamma$) to +471 ($^{A}\gamma$)	−202 ($^{A}\gamma$) to +471 ($^{A}\gamma$)	Apa I/Hinc II
+471 ($^{A}\gamma$) to +6,287 ($^{A}\gamma$)	+1,243 ($^{A}\gamma$) to +6,297 ($^{A}\gamma$)	Hpa I/Sma I
+471 ($^{A}\gamma$) to +6,287 ($^{A}\gamma$)	+471 ($^{A}\gamma$) to +1,243 ($^{A}\gamma$)	Hinc II

The numbering is relative to the cap site of either $^{G}\gamma$ or $^{A}\gamma$. The extremities used for sequencing are italicized.

cellulose filters were then hybridized and washed as described by Orkin et al. (8) and autoradiographed for 1 h.

Results

β-Globin gene cluster haplotype. The sickle cell patient studied, selected because of his low level of $^{G}\gamma$ expression (<38%), was homozygous for the β^{s} gene. We digested DNA with restriction enzyme known to be polymorphic in the β gene cluster. The patient was found to correspond to the Bantu haplotype (data not shown). Haplotype analysis was also performed on another 60 β^{s} chromosomes derived from DNA from SS patients in Senegal, Central African Republic, and Benin.

DNA sequence. The 5′ region of $^{G}\gamma$ and $^{A}\gamma$ was sequenced from −386 to +30. In addition, we sequenced the $^{A}\gamma$ gene from +900 to +1,500. Both strands were sequenced in most cases.

The patient DNA exhibited several differences in the 5′ region of the $^{A}\gamma$ gene compared with published sequence information from a normal individual: the C → G mutation in site −369, which has already been described (9) and seems to be a common polymorphism and two transversions (C → T in site −271; A → G in site +25) (Fig. 1). These two transversions create a 100% sequence homology between the 5′ regions of $^{A}\gamma$ and $^{G}\gamma$, making the 5′ $^{A}\gamma$ region identical to the 5′ $^{G}\gamma$ region. To fully interpret this result we needed to determine which of the two previously reported types of $^{A}\gamma$ gene (chromosome A or B) corresponded to our case (10) (see Discussion). The second intron contains the larger number of differences between these two types of chromosomes. According to the sequence in intron 2, the chromosome studied here was of the A type. Moreover, the TG-repeat region around +1,062, which is highly polymorphic, was $(TG)_{10}(CG)_3(TG)_9$.

The most probable explanation for these observations is that a gene conversion has occurred, replacing the normal 5′ $^{A}\gamma$ sequences with the corresponding $^{G}\gamma$ sequence. The left border of the conversion is between bases −307 and −271, the right border between bases +25 and +1,107.

No mutations were observed in the 5′ $^{G}\gamma$ region as compared with the chromosome A sequence.

PCR reaction. To check the specificity of the PCR primers AS-8 and AS-9, both $^{G}\gamma$ and $^{A}\gamma$ 5′ regions were amplified and subsequently digested by Hph I and Stu I. As expected, Stu I digestion produced a 230- and a 427-bp fragment from both genes. The Hph I enzyme produced a 275- and a 400-bp fragment for the $^{G}\gamma$ 5′ region and did not cut in the $^{A}\gamma$ 5′ region (thus leading to the observation of a 657-bp fragment) for the $^{A}\gamma$ 5′ region (Fig. 2), demonstrating that the PCR amplification of the $^{G}\gamma$ and $^{A}\gamma$ 5′ regions, using the synthetic oligonucleotide primers AS-8/AS-10 and AS-9/AS-10, is specific for $^{G}\gamma$ and $^{A}\gamma$, respectively.

Oligonucleotide hybridization. To establish the frequency of this conversion among Bantu haplotypes and its presence or absence among the two other haplotypes linked to the β^{s} gene, we tested 60 chromosomes (23 Bantu, 27 Benin, and 11 Senegal) with two oligonucleotides capable of differentiating the 5′ $^{A}\gamma$ sequence from the 5′$^{G}\gamma$ in the PCR amplified $^{A}\gamma$ 5′ region (see Methods). We found that > 80% of the chromosomes bearing the Bantu haplotype have the gene conversion in front of $^{A}\gamma$ and that none of the Senegal and Benin chromosomes have it. These results are illustrated in Fig. 3 and are summarized in Table III. In addition, we determined that the gene conversion reported here is present in normal black Americans: 3 of a total of 28 chromosomes tested were positive. Because of the absence of complete family information, precise haplotype assignments were not possible among the positive normal individuals. The normal black individuals positive for the gene conversion were heterozygous for two 5′ subhaplotypes which could be Benin, −+−+, or +−−+ (Hind III sites of the γ gene and Hinc II site 5′ and in the ψβ gene).

Finally, no correlation was found between the presence of the gene conversion and the level of $^{G}\gamma$ expression of the 11 SS patients homozygous for the Bantu haplotype studied.

Discussion

The $^{G}\gamma$- and $^{A}\gamma$-globin genes are duplicated genes lying in tandem on chromosome 11 (10). Two different chromosomes 11 (called chromosome A and B) have been described. The $^{G}\gamma$ and $^{A}\gamma$ genes from chromosome A are 85% homologous over a 5-kb region (1.5 kb upstream of the gene, 1.6 kb constituting

Table II. Primers and Probes

Name	Sequence	Position	Specificity	Function
AS-8	5′ AACTGTTGCTTTATAGGATTTT	5′ $^{G}\gamma$ −616 to −598	$^{G}\gamma$	PCR primer
AS-9	5′ CTGTGGTCTTTAGAAAATTGT	5′ $^{A}\gamma$ −614 to −596	$^{A}\gamma$	PCR primer
AS-10	5′ AGGAGCTTATTGATAACTCAGAC	$^{G}\gamma$ and $^{A}\gamma$* +17 to +41	$^{G}\gamma$ and $^{A}\gamma$	PCR primer
Na-3	5′ GTCATTCCAATTTTTCTCT	$^{G}\gamma$ −262 to −281	$^{G}\gamma$	Detection of the C-T mutation position −271
Na-4	5′ AGAGAAAAACTGGAATGAC	$^{A}\gamma$ −262 to −281	$^{A}\gamma$	Id

* Complementarity between the $^{A}\gamma$ gene site +17 to +41 is not total. There is a mismatch in site +25 that slightly affected the yield but not the specificity of the PCR reaction.

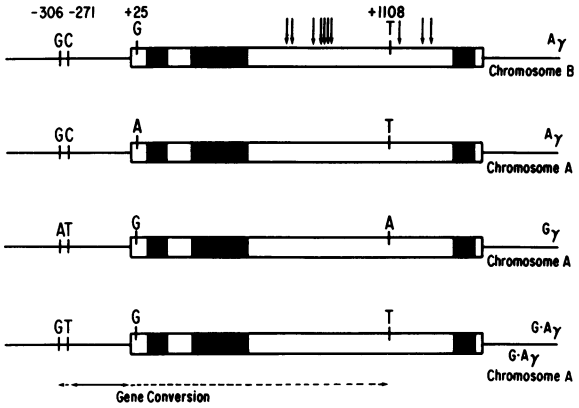

Figure 1. Comparison of different γ-globin genes. The black boxes represent exons. The vertical arrows point to the sequence differences between chromosomes A and B (reference 11, and Slightom, S. L., personal communication). The $^{A}\gamma$ gene of the Bantu chromosome shown (labeled $^{GA}\gamma$) is $^{G}\gamma$-like at sites −271 and +25. The horizontal arrows show the limit of the gene conversion and the dotted portion the uncertainty introduced by high homology.

the gene, and 1.9 kb downstream of the gene). In addition, they are 98% homologous in a region starting at site 500 and extending to a TG-rich region near the end of the IVS-2 located around site +1,060. It has been suggested that this 98% homology stems from a gene conversion between the two γ genes that occurred about 1 million years ago, long after the primary duplication event (which established the γ-globin genes) (11). The $^{A}\gamma$ and $^{G}\gamma$ genes from chromosome B are also 85% homologous but do not have the converted area.

We describe here an $^{A}\gamma$ gene which, like a $^{G}\gamma$ gene from chromosome A, has a T at site −271 and a G at site +25. Normal $^{A}\gamma$ genes from chromosome B also have a G in site +25 but exhibit a G in site −271. The $^{A}\gamma$ gene described here could be either an $^{A}\gamma$ gene chromosome B type with a point mutation (or a small gene conversion) in site −271 or an $^{A}\gamma$ gene chromosome A type converted by a $^{G}\gamma$ gene chromosome A type. To resolve this point we sequenced part of the IVS-2 of the mutated $^{A}\gamma$ gene because this is a region rich in differences between chromosomes A and B. We found that the $^{A}\gamma$ gene being studied was of the chromosome A type, demonstrating consequently that a gene conversion had taken place in front of the $^{A}\gamma$ gene: a $^{G}\gamma$-like sequence starting between basepairs

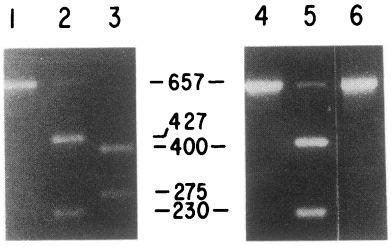

Figure 2. Specific amplification of $^{A}\gamma$ or $^{G}\gamma$ promoter. 10% of the PCR reaction (30 cycles for $^{G}\gamma$, 40 cycles for $^{A}\gamma$) were loaded on a 1.5% ethydium bromide-containing agarose gel after ethanol precipitation and restriction enzyme digestion. Lanes *1–3*, PCR reaction with primers AS-10 and AS-8 ($^{G}\gamma$ specific). Lanes *4–6*, PCR reaction with primer AS-10 and AS-9 ($^{A}\gamma$ specific). Lanes *1* and *4*, PCR-amplified DNA. Lanes *2* and *5*, PCR-amplified DNA digested by Stu I. Lanes *3* and *6*, PCR-amplified DNA digested by Hph I. The $^{G}\gamma$ 5′ region has an Hph I site not present in front of $^{A}\gamma$. The absence of this site in PCR-amplified DNA with primers AS-9 and AS-10 (lane *6*) shows that these two primers amplify specifically the $^{A}\gamma$ 5′ region.

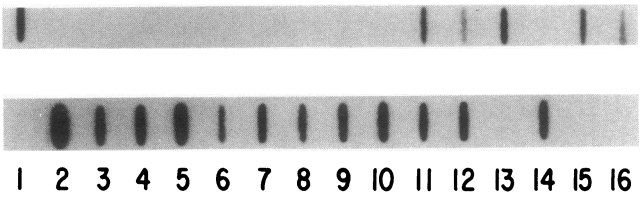

Figure 3. Detection of the gene conversion among patients of various haplotypes. Autoradiogram showing the result of a slot blot analysis of amplified DNA hybridized with end-labeled oligonucleotides specific for the $^{A}\gamma$ and $^{G}\gamma$ 5′ areas. The two nitrocellulose filters shown contained PCR-amplified DNA from some of the patients reported here: eight patients with Benin haplotype (lanes *3–10*) and six patients with Bantu haplotype (lanes *11–16*). Lane *1* is $^{G}\gamma$ control and lane *2* is $^{A}\gamma$ control from PCR-amplified cloned γ-globin genes. The upper strip was hybridized with oligonucleotide Na-4 ($^{G}\gamma$ specific) and the lower strip was hybridized with oligonucleotide Na-3. Using appropriate conditions the oligonucleotide Na-3 hybridized only to $^{G}\gamma$ type promoter (T in the −271 site) and the oligonucleotide Na-4 with $^{A}\gamma$-type promoter (C at −271), allowing a rapid screening of patients for the gene conversion (assuming that T in site −271 of $^{A}\gamma$ gene is always associated with the gene conversion). The pattern observed allows us to conclude that patients 3–10 and 14 do not exhibit the gene conversion, that 11 and 12 are heterozygous for the conversion, and that 13, 15, and 16 are homozygous for the gene conversion. Lanes *1* and *2* are controls to show the specificity of the oligonucleotides.

−306 and −271 (from the cap site of the $^{A}\gamma$ gene) and extending to between basepairs +25 and +1,107 had replaced the corresponding $^{A}\gamma$ sequence. The uncertain limits of this gene conversion stem from the extraordinary homology in the sequence of the two γ genes and the resulting absence of sequence differences between the sites quoted.

The TG repeat around +1,062 in the second intervening sequence is of particular interest because (*a*) it has been shown that this region is highly polymorphic, probably due to very short gene conversions (12), and (*b*) it is thought that the gene conversion that occurred 1 million years ago in chromosome A has its 3′ boundary in this repeat (11). The novel TG repeat we described in the chromosome studied has the sequence $(TG)_{10}(CG)_3(TG)_9$, a sequence different from all others previously described (11). Hence, we cannot ascertain with precision the actual 3′ end of the gene conversion.

If the TG repeats are involved mechanistically in this mutational event, the gene conversion described here involving the $^{A}\gamma$ gene in a Bantu chromosome could closely resemble the old one on chromosome A. In any case, this finding demonstrates that this region is prone to this type of DNA rearrangement, and documents an ongoing genetic process that tends to conserve the similarities between the two genes.

To establish whether this gene conversion was a private event of the chromosome studied or a more general phenomenon among Bantu haplotypes, we studied the corresponding region of several chromosomes by PCR amplification and hybridization with specific oligonucleotide probes. The results in Table III demonstrate that this conversion is highly associated with the Bantu haplotype bearing the β^{s} gene and appears at low or nil frequency in other haplotypes. The gene conversion is nevertheless also present in normal individuals with different haplotypes. Until data are collected on a considerable number of β^{A} chromosome haplotypes (clearly identified by pedigree studies) in Africa, any interpretation as to the history

Table III. Incidence of Gene Conversion 5' to the $^{A}\gamma$ Gene in Different Haplotypes Linked to the β^{s} Gene

Haplotype	*n*	% $^{G}\gamma$ (mean±SD)	$^{GA}\gamma/^{GA}\gamma$	$^{GA}\gamma/^{A}\gamma$	$^{A}\gamma/^{A}\gamma$	Conversion frequency
Bantu/Bantu						
Low $^{G}\gamma$ (<38%)	5	27.5±3.6	3	1	1	
High $^{G}\gamma$ (>38%)	6	46.8±1.8	5	1	0	
Low $^{G}\gamma$ + high $^{G}\gamma$	11	38.8±10.3	8	2	1	0.82
Benin/Benin	13	42.7±5.9	0	0	13	0
Senegal/Senegal	5	71.5±1.3	0	0	5	0
Senegal/Bantu	1	59.4	0	1	0	

$^{GA}\gamma$, Chromosome bearing the gene conversion at the $^{A}\gamma$ promoter region; $^{A}\gamma$, chromosome with no gene conversion. All the patients studied were homozygote for the β^{s} gene.

of the gene conversion has to be tentative. The limited data available allow us only to say that the gene conversion is present in β^{A} chromosome on at least one non-Bantu haplotype. This could mean that the gene conversion occurred before the divergence between Bantu haplotype and the non-Bantu haplotype, or that a third gene conversion of the same area, independent from the Bantu and ancestral chromosome A conversion, occurred in the β^{A} chromosome. The fact that only some of the Bantu have the gene conversion supports the second hypothesis, since evidence has been accumulated (1, 4, 5) showing that the β^{S} mutation linked to the Bantu haplotype probably occurred only once. This would mean that the Bantu-linked gene conversion is a very recent event and could have occurred in the last few thousand years, that is, after the appearance of the β^{S} mutation. None of these issues can be entirely ascertained until further and extensive analyses on African β^{A} chromosomes are completed.

The fact that the presence of this conversion does not alter the basic adult $^{G}\gamma/^{A}\gamma$ ratio (< 50% $^{G}\gamma$) in the bearers of the Bantu haplotype strongly suggests that the sequences between the −307 and +1,062 sites of both γ genes are not involved in determining this ratio. Moreover, as a majority of Bantu haplotype bearers carry the same gene conversion, the variability of $^{G}\gamma$ expression (< 38% or > 38%, Table II) within this group (5) is unrelated to this genetic event. Further work will have to establish the impact of this gene conversion on the phenotypic expression.

This conclusion does not contradict the observations that various point mutations 5' to each γ gene can modify the synthesis of the corresponding γ gene and produce more HbF (9, 12–15), but suggests that the regulatory sequences (in the absence of such mutations) that normally determine the ratio of expression between these two genes will have to be found elsewhere.

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