# An $^{A}\gamma$ Type of Nondeletional Hereditary Persistence of Fetal Hemoglobin With a T → C Mutation at Position −175 to the Cap Site of the $^{A}\gamma$ Globin Gene

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**The nondeletional types of hereditary persistence of fetal hemoglobin (ndHPFH) concern the continued synthesis of hemoglobin (Hb) F with either $^{G}\gamma$ or $^{A}\gamma$ chains in amounts varying from 5% to 30%. Several mutations have been identified in either the $^{A}\gamma$ or $^{G}\gamma$ promoter which are considered causative to the continued production of one of the two γ chains because the substitutions occur in sequence motifs essential for the expression characteristics of the γ-globin gene in the 3′ position. We report the discovery of a T → C mutation at position −175 in the $^{A}\gamma$ promoter which was associated with a greatly increased level of Hb F (with mainly $^{A}\gamma$) and a decreased level of Hb A in the one (Black) heterozygote who had a $\beta^{C}$ gene in *trans*. The same mutation has been observed in the $^{G}\gamma$ promoter of a Black heterozygote who had high levels of Hb F with $^{G}\gamma$ chains only. A detailed comparison between these two individuals indicated significant differences in the levels of Hb F and Hb A which may result from an additional mutation at position −158 in the $^{G}\gamma$ promoter.**


NONDELETIONAL types of hereditary persistence of fetal hemoglobin (ndHPFH) are relatively rare, but have been observed among Blacks, Italians, Greeks, English, and Chinese.[1-18] Two different forms are known, characterized by the type of chain (either $^{G}\gamma$ or $^{A}\gamma$) present in the hemoglobin (Hb) F found in quantities of 5% to 30% in heterozygotes. Recent analyses of the promoter regions 5′ to their $^{G}\gamma$ or $^{A}\gamma$ globin genes have indicated that specific nucleotide (nt) substitutions are present in sequences suspected of binding regulatory proteins (reviewed in refs 19-22). Thus, mutations within these regions may interfere with promoter activities.

One specific mutation concerns the T → C replacement at nt position −175 relative to the cap site of the $^{G}\gamma$ gene; this mutation was observed in a Black family from Philadelphia and in an Italian family.[17,18] This mutation occurs in an ATGCAAAT octamer which is known to bind a regulatory protein in other genetic systems.[23-25] We report the same mutation, but in the promoter of the $^{A}\gamma$ gene of a Black male with elevated Hb F.

## MATERIALS AND METHODS

Blood samples from the propositus, his daughter, mother, brother, and sister were collected in vacutainers with EDTA as anticoagulant and transported to the laboratory in Augusta, GA. At least ten collections have been made since 1970 when the abnormality in the propositus was detected. Informed consent was obtained.

Hematologic data were collected with automated cell counters. Hbs in RBC lysates were studied with different electrophoretic methods.[26] The Hbs were quantitated by diethylaminoethyl (DEAE) cellulose chromatography[27] and by cation exchange high-performance liquid chromatography (HPLC),[28] which allows the separation of Hb $A_2$ and Hb C. Hb F was also quantitated with an alkali denaturation procedure.[29] Hb F was isolated by preparative DEAE-cellulose chromatography,[27] and the relative quantities of $^{G}\gamma$ and $^{A}\gamma$ chains were determined by a chemical procedure which determines the glycine and alanine contents in the isolated γCB-3 peptide,[30] by a reversed-phase HPLC method using phosphate buffers at low pH[31] and by reversed-phase HPLC on the large-pore $C_4$ column using acetonitrile-$H_2O$-trifluoroacetic acid (TFA) developers.[32,33] In vitro chain synthesis followed a procedure described previously.[26]

DNA was isolated from circulating WBCs by the method of Poncz et al.[34] Amplification of the promoter region of the $^{A}\gamma$ globin gene used a 5′ primer selected for region −622 through −597 5′ to the cap site of the $^{A}\gamma$ globin gene because this region shows a maximum number of differences between the promoters of the $^{A}\gamma$ and $^{G}\gamma$ globin genes.[35] The 5′ primer:

5′ TTTAAGCTTTGAAACTGTGGTCTTTATGAAAATTG 3′

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−622 −597

of 26 nts was supplemented with nine additional nts (marked with asterisks) at the 5′ end which are not homologous to the promoter sequence but are attached because they represent a *Hind* III site which will be used in future experiments involving expression systems. The $^{G}\gamma$ primer was chosen for the region −623 through −598 5′ to the $^{G}\gamma$ cap site:

5′ TTTAAGCTTGCACTGAAACTGTTGCTTTATAGGAT 3′

************

−623 −598

The reverse complementary primer:

5′ TTTAAGCTTGGCGTCTGGACTAGGAGCTTATTG 3′

************

+56 +30

was located at nt positions +56 through +30 relative to the cap site; the sequence of this region of the $^{A}\gamma$ globin gene is the same as that of the $^{G}\gamma$ globin gene. With these primer sets, the $^{A}\gamma$ promoter region can be selectively amplified. Amplification was done as described previously[36,37] except that the reaction buffer contained 20 mmol/L morpholinopropane sulfonic acid (MOPS), pH 7.5, 2.5 mmol/L

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$MgCl_2$, 40 mmol/L NaCl, 240 μmol/L of each dNTP, 1 μmol/L of each primer, and 0.5 μg DNA. Sequence analysis was done with the dideoxy procedure of Sanger et al[38] using appropriate primers. Both the $^{G}\gamma$ and $^{A}\gamma$ promoter regions were amplified and sequenced. These analyses were conducted from −450 5′ to the cap site to the reverse primer site. A problem arose with the choice of one primer [CAGCCTTGCCTTGACCAAT] which spanned the region −129 to −111 5′ to the cap site. A computer analysis showed that this primer had a 63% homology to another segment of the $^{A}\gamma$ gene which was 27 nts away from the original primer site. This primer is completely homologous

5′ CAGCCTTGCCTTGACCAAT CAGCCTTGCCTTGACCAAT 3′

TTGGCCAGCCTTGCCTTGACCAATAGGCTTGACAAGGCAAACTTGACCAATA ⟶ Cap

−130 −120 −110 −100 −90

with the $^{A}\gamma$ gene in the last 10 nts on the 3′ end of the second site (see scheme). This would readily account for its ability to serve as a primer at two locations, and results in data which can be interpreted as having 27 base pairs (bp) deletion (between nt positions −84 and −110 5′ to the cap site of $^{A}\gamma$). We obtained the same data (ie, the "27 nt deletion") when this primer was used on amplified DNAs from the propositus and a normal control. Selection of another sequencing primer (between nt −167 and −150) away from the CAAT duplication showed that no deletion was present.

One mutation observed by sequencing, ie, the T → C mutation at −175 of the $^{A}\gamma$ promoter was confirmed by dot-blot analysis of amplified DNA using probes with sequences as listed Fig 1; methodology has been given elsewhere.[36]

Haplotyping involved the following restriction sites: *Hinc* II 5′ to ε; *Xmn*I 5′ to $^{G}\gamma$; *Hind* III at $^{G}\gamma$ and $^{A}\gamma$; *Hinc*II at ψβ and 3′ to it; *Ava*II at β, and *Bam*HI 3′ to β. Methodology was as described before.[39-41] The number of α-globin genes was determined by gene mapping using the enzymes *Eco*RI, *Bam*HI, and *BGL*II.[42,43]

## RESULTS

The patient, R.E., is a 25-year-old Black man who in 1970 at age 7 years, was found to have three Hb types by starch gel electrophoresis: Hb C, Hb F, and Hb A. His mother, child, and two siblings are Hb C heterozygotes; his father is dead and paternal relatives cannot be located. The patient was studied on numerous occasions; some of the data are summarized in Table 1. Hematologic data were normal except for a mild microcytosis which was likely caused by Hb C. In vitro chain synthesis in reticulocytes showed a nearly balanced ratio of 0.94 between α and the sum of the $\beta^{A}$, $\beta^{C}$, and γ chains. The γ/non-α ratio was 0.32, indicating a continuous synthesis of Hb F. This Hb F was distributed equally among the RBCs, using the Kleikhauer-Betke elution procedure.[26] Gene mapping analyses indicated the presence of four α-globin genes (αα/αα).

The quantities of the Hb components were determined over the years with different chromatographic procedures which gave comparable data (Table 1). The average Hb $A_2$ + Hb C level was ~46% (Hb $A_2$ was a low 1.6%), that of Hb A was ~16%, and that of Hb F was a high ~38%. Data obtained for Hb F with an alkali denaturation procedure averaged ~31% which, as expected, is somewhat lower than the values obtained by chromatography. The $^{A}\gamma$ value in isolated Hb F was a high 80% which identified this HPFH as an $^{A}\gamma$ type. The $^{G}\gamma$ value was consistently estimated at ~20% which is higher than the values observed in subjects with other types of $^{A}\gamma$-ndHPFH.[7-10,14] These values correspond to peripheral blood values of ~8% for Hb F of the $\alpha_2^G\gamma_2$ type and ~30% for Hb F of the $\alpha_2^A\gamma_2$ type.

Sequence analysis of amplified DNA of the $^{A}\gamma$ promoter showed two nts (T and C) at position −175 to the cap site (Fig 1). This T→C mutation was readily confirmed through dot-blot analysis of another sample of amplified DNA. Other substitutions were observed at position −369 (C→G), at position −16 (C→G), and at position +24 (A→C). The mutation at −369 appears most common as it was found in five additional chromosomes (from adults without ndHPFH), whereas that at position +24 was present in two other nonrelated chromosomes. The C→G mutation at position −16 needs to be evaluated further; however, it was also present in the DNA of the mother who is a Hb C heterozygote.

Haplotype analyses identified the type [− + + − + + + +] for the chromosomes with the −175

**Table 1. Hematologic and Hb Composition Data for Subject R.E.**

| Factor | 1975 | 1982 | 1988 |
|---|---|---|---|
| Age (yr) | 12 | 19 | 25 |
| Hb (g/dL) | 13.5 | 15.3 | 15.0 |
| PCV (L/L) | 0.37 | 0.465 | 0.405 |
| RBC ($10^{12}$/L) | 5.39 | 5.84 | 5.77 |
| MCV (fL) | 69 | 79 | 70 |
| MCH (pg) | 25.0 | 26.2 | 26.0 |
| MCHC (g/dL) | 36.5 | 32.9 | 37.0 |
| Hb $A_2$ (%) | (+ Hb C) | (+ Hb C) | 1.6† |
| Hb C (%) | 42.8* | 47.9† | 45.4† |
| Hb A (%) | 16.3* | 15.8† | 15.7† |
| Hb F (%) | 40.9* | 36.3† | 37.3† |
| $F_{AD}$ (%)‡ | 31.0 | 30.8 | 29.9 |
| $^{G}\gamma$ (%) | (0.32)§ | 22.6‖ | 19.0¶ |
| $^{A}\gamma$ (%) | (2.85) | 77.4 | 81.0 |

*By DEAE-cellulose chromatography.[27]

†By cation-exchange HPLC[28] allowing separation of Hb $A_2$ and Hb C.

‡By an alkali denaturation procedure.[29]

§By a chemical procedure[30] which determines the number of glycine (0 to 1) and alanine (3 to 2) residues in peptide γCB − 3 of isolated Hb F.

‖By reversed-phase HPLC using phosphate developers.[31]

¶By reversed-phase HPLC with acetonitrile-$H_2O$-TFA developers.[32,33]

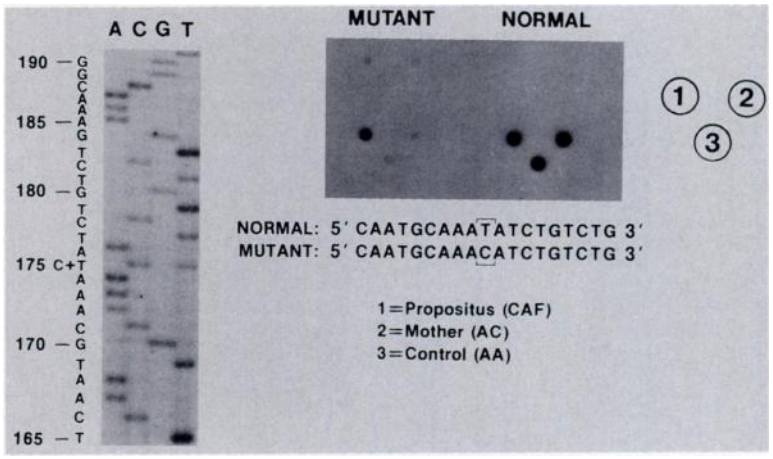



**Fig 1. Left: Sequencing gel of amplified DNA from subject R.E. showing two nts (C and T) at position −175 relative to the Cap site of the $^{A}\gamma$ globin gene. (Right) Hybridization of amplified DNA from R.E. (slot 1), his mother (slot 2), and normal control (slot 3) with normal and mutant probes.**

T→C mutation and the type [− − + − − − + +] for the chromosome with the $\beta^{C}$ mutation.

Sequencing of the amplified $^{G}\gamma$ promoter showed the T→C mutation at −158 (*Xmn*I positive) as the only unusual substitution. The data were confirmed by dot-blot analysis as shown in Fig 2. Figure 2 also shows the specificity of the amplification primers because the amplified $^{A}\gamma$ promoter did not hybridize with the *Xmn*I-positive probe. Finally, Fig 2 shows that the *Xmn*I site is present on the same chromosome as the −175 T→C $^{A}\gamma$ mutation as the mother was *Xmn*I negative.

## DISCUSSION

The number of subjects with an ndHPFH (either $^{G}\gamma$ or $^{A}\gamma$) with substitutions in the promoter regions of the $^{G}\gamma$ or $^{A}\gamma$ globin genes reported in the literature is steadily increasing.[1-18] The positions where substitutions have been observed, namely at −202 ($^{G}\gamma$ and $^{A}\gamma$), −198 ($^{A}\gamma$), −196 ($^{A}\gamma$), −175 ($^{G}\gamma$ and $^{A}\gamma$), −161 ($^{G}\gamma$), −158 ($^{G}\gamma$), and −117 ($^{A}\gamma$) relative to the cap site, are within sequence motifs involved in the binding of regulatory factors.[19-21] Mutations within these sequences may result in an alteration of the binding affinity for such proteins, which will indirectly influence the activity of the $\gamma$ gene in the 3′ position. Variations in the quantities of Hb F in nonanemic ndHPFH heterozygotes (10% to 15% at −117 relative to $^{A}\gamma$; 20% to 30% at −175 relative to $^{G}\gamma$ or $^{A}\gamma$; 15% to 20% at −196 relative to $^{A}\gamma$; 5% to 8% at −198 relative to $^{A}\gamma$; 2% to 3% at −202 relative to $^{A}\gamma$, and 15% to 25% at −202 relative to $^{G}\gamma$) suggest that the location of the substitution is an important determining factor. However, the importance of the type of mutation is evident when the Hb F values in subjects with a C→T mutation at position −202 ($^{A}\gamma$) (2% to 3% Hb F) are compared with those for subjects with a C→G mutation at the same position but at $^{G}\gamma$ (15% to 25% Hb F). Such comparisons make it of interest to evaluate carefully the data obtained for the two ndHPFH heterozygotes, one with a T→C mutation at −175 ($^{G}\gamma$) and the other with the same mutation but at −175 ($^{A}\gamma$).

Both subjects, ie, our male propositus with the −175 T→C ($^{A}\gamma$) mutation and the adult woman with the same mutation at $^{G}\gamma$,[17] have a normal hematology, low levels of Hb $A_2$, a balanced in vitro chain synthesis which excludes $\beta$-thalassemia. The woman with $^{G}\gamma$-ndHPFH has a $\beta^{S}$ gene in *trans* (our patient has the $\beta^{C}$ gene), and has nearly equal quantities of Hb A and Hb F (27.5% Hb A; 29.5% Hb F; 43% Hb S + Hb $A_2$), whereas in our patient the Hb F level is more than twice that of Hb A (16% Hb A; 38% Hb F; 45% Hb C + Hb $A_2$). Another difference concerns the relative synthesis of the $\gamma$ chain regulated by the $\gamma$-globin gene with a normal promoter; the woman with Hb S-$^{G}\gamma$-ndHPFH had 100% $^{G}\gamma$ and no $^{A}\gamma$,[17] whereas the man with Hb C-$^{A}\gamma$-ndHPFH had 80% $^{A}\gamma$ and 20% $^{G}\gamma$ (Table 1).

The $^{G}\gamma$ and $^{A}\gamma$ promoters have the ATGCAAAT octamer

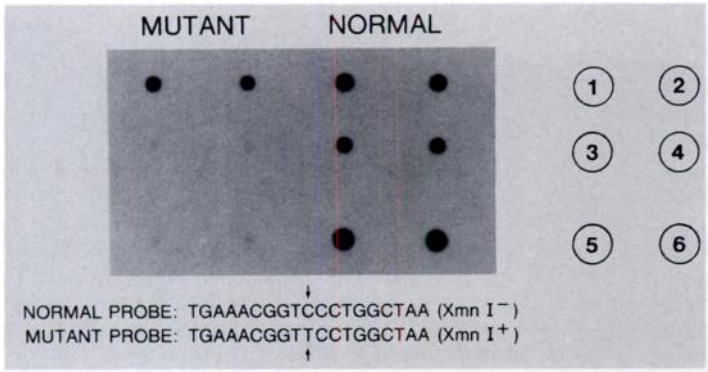
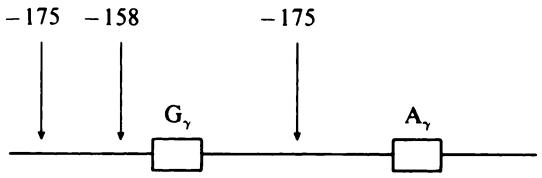



**Fig 2. Hybridization of amplified $^{G}\gamma$ DNA from subject R.E. (slots 1 and 2), his mother (slots 3 and 4), along with amplified $^{A}\gamma$ DNA from R.E. (slot 5) and his mother (slot 6), using normal (*Xmn*I$^{-}$) and mutant (*Xmn*I$^{+}$) probes.**

motif (position −182 through −175) which is present in promoters and enhancers of several other genes and has been identified as being potentially important for the expression of a fetal globin gene.[17] Because the same mutation is present in both conditions but in different promoters, a more or less similar in vivo expression can be expected. However, an additional difference is present at position −158 relative to the cap site of the $^{G}\gamma$ gene; the $^{G}\gamma$ promoter of the female with Hb S-$^{G}\gamma$-ndHPFH had a C at −158 (or is *Xmn*I negative), whereas the $^{G}\gamma$ promoter of the male with Hb C-$^{A}\gamma$-ndHPFH had a T at −158 (*Xmn*I positive) (scheme):

The T→C mutation is known to increase the $^{G}\gamma$ level in normal persons[44-46] and in patients with sickle cell anemia[5,47-50] and β-thalassemia.[40,41] Moreover, an increased production of Hb F (with mainly $^{G}\gamma$ chains) occurs when an anemia is present in such patients.[50,51] Thus, the nt difference at position −158 ($^{G}\gamma$) between the two subjects with ndHPFH may be a possible cause for the higher Hb F level in the subject with Hb C-$^{A}\gamma$-ndHPFH and for the relatively large production of Hb F with $^{G}\gamma$ chains. The mechanism responsible remains obscure, but in vitro expression analysis may be helpful in evaluating this possibility further.




| | | | |
|---|---|---|---|
| Normal | T | C/T | T |
| S-$G_\gamma$-$\beta^+$-HPFH | C | C | T |
| C-$A_\gamma$-$\beta^+$-HPFH | T | T | C |

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