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ARTICLE

Foetal haemoglobin in normal healthy adults: relationship with polymorphic sequences *cis* to the β globin gene

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We evaluated the effects of polymorphic markers within the β globin gene cluster on HbF expression in two groups. These groups were randomly selected from a survey of HbF distribution in a large population study of unrelated healthy Algerian adults (*n*=827). The first group contained individuals with normal HbF levels (0.1–0.5%) and the second group contained individuals with raised HbF levels (0.8–2.3%). Of the various polymorphic markers analysed, only the -309 Gγ A→G, the -158 Gγ C→T, the Gγ IVS2 TC $(TG)_9$ AG $(TG)_2$ $(CG)_2$ and the -540 β $(AT)_9$ T_5 sequence configurations were significantly associated with increased HbF levels. More than 84% of the subjects with elevated HbF levels carried one or several of these four marker configurations, suggesting that the β globin gene cluster exerts a significant effect on HbF expression in healthy individuals.
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Keywords: HPFH; β globin gene cluster; polymorphisms

Introduction

The developmental switch from foetal (α_2 γ_2) to adult (α_2 β_2) haemoglobin (Hb) occurs just before birth. In normal subjects: (i) foetal Hb (HbF) constitutes less than 1% of the total Hb by the end of the first year of life; (ii) the synthesis of HbF is restricted to a subpopulation of red cells, known as F-cells (FC),[1,2] (iii) HbF levels are directly correlated to the number of FC.[3,4] Hereditary Persistence of Foetal Haemoglobin (HPFH) is characterised by persistently high levels of HbF after birth. Large deletions of varying sizes within the β globin cluster and single base substitutions in the promoter region of one of the two γ globin genes result in HPFH, with HbF in the range of 5–30% in heterozygous individuals.[5] These rare forms of HPFH show a clear Mendelian pattern of inheritance and HbF is uniformly distributed among all red blood cells (pancellular HPFH). In the more common form of HPFH, known as Swiss-type HPFH[6] or heterocellular HPFH, HbF levels only increase slightly (1–4%). This form is genetically heterogeneous and seem to result from complex interactions between a variety of genetic determinants, some of which are linked to the β globin gene cluster. Studies have mapped some of the determinants to chromosomes Xp22.2[7] and 6q23,[8,9] but the precise loci involved have not yet been identified.

The degree to which the β globin gene cluster contributes to HbF expression is still under debate.[10,11] Some studies have suggested that this cluster is involved in the variability of HbF expression and FC number in individuals with one (heterozygote) or two copies of the β thalassaemic (β thal) or the

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sickle cell gene, whereas others failed to confirm these findings.[12,13] Studies of sequence variations in the Locus Control Region hypersensitive site-2 (β LCR 5′HS2),[14–16] the second intron of the A γ gene (Aγ IVS2),[17] the promoter region of the β globin gene[18,19] and the -158 5′ of the Gγ gene[20,21] have found a genetic association with HbF levels, FC number or both. These association studies have so far been performed exclusively on populations subjected to different degrees of anaemic stress or on carriers of various β globin gene defects.

The aim of this study was to determine the effect of these putative genetic markers on HbF expression in healthy adults free from any globin gene defects. We studied seven polymorphic regions of the β globin gene cluster, including four repeat sequences, and evaluated their association with HbF expression in healthy Algerian adults. Our data suggest strongly that there is a significant association between markers of the β globin gene cluster and HbF levels in healthy adults.

Materials and methods

Haematology and haemoglobin analysis

Haematological parameters were measured by use of automated counters (STKB Coultronic, Margency, France). HbA_2 and HbF levels were estimated by use of a highly sensitive HPLC method.[22]

Population survey for HbF distribution

Informed consent was obtained from 859 unrelated Algerian adults. Their age ranged from 18 to 60 years and around 90% of them were younger than 40 years. Thirty-two of the subjects were carriers of S, C or the β thalassemia trait and were excluded from the study. The HbF level was measured in the remaining 827 healthy adults (417 males and 410-non pregnant females).

DNA analysis

Genomic DNA was extracted from the peripheral blood leukocytes of 97 randomly selected subjects: 51 with raised HbF levels and 46 with normal HbF levels. We analysed the entire β globin gene segment and the foetal globin gene promoter region for mutations by use of denaturing gradient gel electrophoresis (DGGE).[23,24] Deletions in the α globin gene locus were sought by a PCR-based procedure.[25]

The following polymorphisms were investigated by use of previously described PCR-based procedures and, when necessary, by sequencing the cloned PCR products. The $(AT)_x\ N_{12}\ (AT)_y$ repeat configurations within the 5′HS2 of β LCR,[14] the $(TG)_n\ (CG)_m$ configurations in the IVS2 Gγ and Aγ,[17] the $(AT)_x\ T_y$ repeat configuration in the 5′ β globin gene, were characterised by size separation and, when appropriate, by direct nucleotide sequencing.[26] The sequence variations, C→G at -369; A→G at -309 and C→T at -158 in the Gγ promoter region were explored by PCR-RFLP using *Bfa*I, *Mbo*II and *Xmn*I enzymes, respectively.

Statistical analysis

Due to the large size of the study population (males=417; females=410), the Z test[27] was used to compare the mean HbF levels of the two genders. The 2×2 χ test was performed to detect differences in the distribution of repeat sequence configurations and single nucleotide polymorphisms between the phenotype groups. Statistical significance was set at $P < 0.05$.

Given that some of our genotypes have an unknown gametic phase and include a large number of alleles, Arlequin, a program for the analysis of population genetic data, was used (http://lgb.unige.ch/arlequin) to perform a likelihood method for the analysis of linkage disequilibrium between the pairs of genetic marker configurations in each subgroup and also to establish the maximum likelihood haplotype frequency using an Expectation-Maximization (E.M) algorithm.[28] The relationship between absolute HbF levels and genetic markers was investigated by use of the Students' *t*-test.

Results

HbF levels in the population

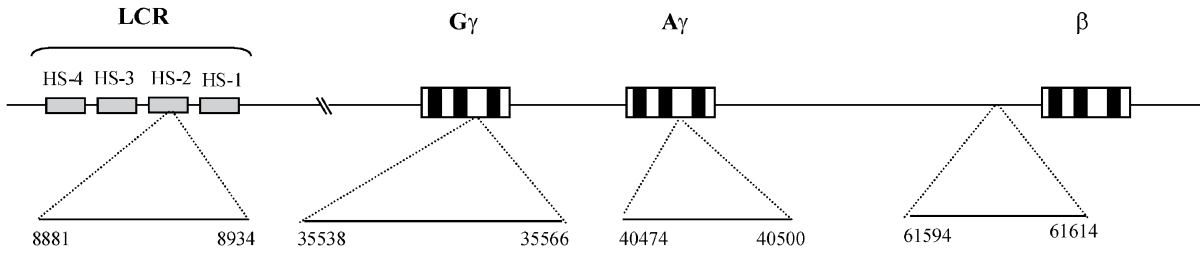
We studied 827 healthy adults (417 males and 410 females) with normal haematologic and Hb profiles. HbF levels ranged between 0.1 and 2.3%. In 520 subjects (62.9%), HbF levels were between 0.2 and 0.3%. In 86 subjects (10.4%) HbF levels were equal or above 0.8%. In 47 of these subjects (54.7%), HbF levels were $\geqslant 1\%$. The average HbF level in the population was $0.39 \pm 0.29\%$ (mean $\pm$ SD). A significant difference ($P < 0.01$; Z test) was noted between males (HbF, $0.35 \pm 0.26\%$) and females (HbF, $0.435 \pm 0.312\%$). A higher proportion of females had increased HbF levels.

Phenotype analysis

We randomly selected 97 subjects for genetic studies: 51 with raised HbF levels (0.8–2.3%; High F group) and 46 with normal range of HbF levels (0.1–0.5%; NRF group). The haematologic indices of these 97 subjects were in the normal range. The means $\pm$ SD of the High F and NRF subjects were respectively $2.58 \pm 0.26\%$ and $2.54 \pm 0.25\%$ for HbA2; 14 g/dl $\pm$ 1.12 and 13.42 g/dl $\pm$ 1.43 for total Hb; $41.56\% \pm 3.34$ and $39.27 \pm 4.30\%$ for hematocrit; 85.8 fl $\pm$ 5.06 and 86.15 fl $\pm$ 5.49 for MCV, and 29g/dl $\pm$ 1.88 and 29.98g/dl $\pm$ 2.55 for MCH. These haematologic indices suggest that the β thalassaemic allele is absent in these subjects. This was further confirmed by DNA analysis using a variety of techniques, including nucleotide sequencing when appropriate.

Marker analysis

The DNA regions analysed and the sequence configurations encountered in this study are listed in Figure 1. The $(AT)_x\ N_{12}$



	$(AT)_x N_{12} (AT)_y$			$(TG)_n (CG)_m$			$(TG)_n (CG)_m$		$(AT)_x T_y$	
A	$(AT)_{10}$	N_{12}	$(AT)_{11}$	K	$(TG)_{11}$	$(CG)_3$		T $(TG)_{13}$	V	$(AT)_7$ T_7
B	$(AT)_9$	N_{12}	$(AT)_{12}$	L	$(TG)_{19}$	$(CG)_2$ CACG		U Other	W	$(AT)_9$ T_5
C	$(AT)_8$	N_{12}	$(AT)_{13}$	M	$TC(TG)_9$	$AG(TG)_2$ $(CG)_2$			X	$(AT)_8$ T_4
D	$(AT)_9$	N_{12}	$(AT)_{10}$	N	$(TG)_{19}$	$(CG)_3$ CACG			Y	$(AT)_8$ T_5
E	$(AT)_8$	N_{12}	$(AT)_{11}$	O	$(TG)_{18}$	$(CG)_2$ CACG			Z	$(AT)_6$ T_9
F	$(AT)_9$	N_{12}	$(AT)_{11}$	P	$(TG)_{13}$	$(CG)_2$ CACG				
G	$(AT)_8$	N_{12}	$(AT)_{10}$	Q	$TG)_{21}$	$(CG)_2$ CACG				
H	$(AT)_8$	N_{12}	$(AT)_7$	R	$TC(TG)_{15}$	$(CG)_2$ CACG				
I	$(AT)_{10}$	N_{12}	$(AT)_{12}$	S	$TC(TG)_{16}$	$(CG)_2$ CACG				
J	$(AT)_8$	N_{12}	$(AT)_{12}$							

Figure 1 βLCR 5′ HS2, Gγ, Aγ IVS2 and 5′ of β globin gene sequence configurations in NRF and High F subjects. The configurations of LCR 5′HS2 were named A to J, those of Gγ IVS2 were named K to S, those of Aγ IVS2 were named T and U and those 5′ of β globin were named V to Z. A, K, T and V are the reference sequence configurations from the HUMHBB reference sequence. The nucleotides are numbered according to the HUMHBB sequence.

$(AT)_y$ configurations of β LCR HS2 were named A to J, the $(TG)_n$ $(CG)_m$ configurations of Gγ IVS2 were named K to S, the $(TG)_n$ $(CG)_m$ configurations of Aγ IVS2 were named T and U and the $(AT)_x$ T_y configurations 5′ of β were named V to Z. The allele and genotype frequencies for each site are given in Tables 1a and b, respectively. The frequency of the repeat sequence configurations and simple nucleotide variations whose distributions differed significantly between the High F and NRF groups are given in Figure 2.

Gγ and Aγ promoter regions

None of the individuals in the High F group harboured any sequence changes in the Gγ and Aγ promoter regions (up to −264 bp), except for the common C→T polymorphism at −158 of Gγ and the 4 bp deletion upstream of Aγ (−222 to −225). The G at −369 5′ of Gγ was not polymorphic in either of the groups, whereas the A→G substitution at −309 was only found in the High F group (*n*=9). The frequency of the −158 Gγ C→T polymorphism (detected as *Xmn*I- →*Xmn*I+ polymorphism) was statistically different between the two groups (*P*= 0.02): *Xmn*I+ was present in 51% of High F subjects *vs* 28% in the NRF group (Figure 2).

Configurations of the $(AT)_x$ N_{12} $(AT)_y$ motif in β LCR HS2

Ten different sequence configurations of the $(AT)_x$ N_{12} $(AT)_y$ motif were found among the 194 chromosomes studied. The most common configuration in both groups was the A configuration (Table 1a), which is the reference sequence (GenBank coordinates: 8881-8934). Although the configuration D was much more common in the High F group (52%) than in the NRF group (32.6%) (Table 1b), the difference was not statistically significant (*P*=0.06).

Gγ IVS2 $(TG)_n$ $(CG)_m$ configurations

In the Gγ IVS2, two novel sequence configurations for $(TG)_n$ $(CG)_m$ motifs (configurations Q and R) were found in three High F subjects. The prevalence of configurations M and O differed significantly between the two groups: 25.5% of the High F subjects carried configuration M *vs* 2.27% in the NRF group (*P*=0.002) and 40.9% of the NRF subjects carried configuration O compared to 5.76% in the High F group (*P*=0.0001) (Figure 2).

5′ β globin gene promoter $(AT)_x$ $(T)_y$ motif

Configuration V (reference sequence) was the most frequent configuration in both two groups (Table 1a), but the configuration W was significantly more common in the High F group than in the NRF group (*P*=0.012) (Figure 2).

Marker combinations

Over 84% of the High F subjects (43/51) harboured at least one of four sequence configurations, namely G at −309 of Gγ, *Xmn*I+, M and W configurations, compared to only 43% (20/46) of the NRF group (Table 2). We found that 22

Table 1a Allele frequency of sequence configurations in the High F and NRF groups

Marker		*Allele frequency* High F group	NRF group
Configurations			
β LCR 5′HS2			
	A	0.286	0.413
	B	0.03	0.054
	C	0.019	0
	D	0.265	0.164
	E	0.079	0.12
	F	0.189	0.164
	G	0.001	0.021
	H	0.002	0.01
	I	0.079	0.032
	J	0.03	0.021
Gγ IVS2	K	0.345	0.326
	L	0.305	0.364
	M	0.138	0.011
	N	0.118	0.07
	O	0.03	0.205
	P	0.04	0
	Q	0.02	0
	R	0.001	0
	S	0	0.023
Aγ IVS2	T	0.275	0.152
	U	0.725	0.847
β globin promoter	V	0.53	0.86
	W	0.236	0.087
	X	0.068	0
	Y	0.098	0.044
	Z	0.068	0.011
A at −309 of Gγ		0.911	1
G at −309 of Gγ		0.089	0
*Xmn*I+		0.275	0.153
*Xmn*I−		0.725	0.847

Table 1b Genotype frequency at each polymorphic site studied in the High F and NRF groups

Marker		*Genotype frequency* High F group	NRF group
β LCR 5′HS2	A/D	0.176	0.152
	D/J	0.157	0.022
	A/A	0.117	0.152
	D/F	0.1	0.065
	F/F	0.08	0.43
	F/E	0.06	0.022
	A/E	0.04	0.13
	A/F	0.04	0.13
	A/B	0.04	0.065
	F/I	0.04	0
	H/B	0.02	0
	B/E	0.02	0.022
	D/G	0.02	0
	E/E	0.02	0
	A/C	0.02	0
	D/D	0.02	0.043
	D/I	0.02	0
	E/E	0.02	0
	J/A	0	0.022
	D/B	0	0.022
	H/E	0	0.022
	J/F	0	0.022
	G/E	0	0.022
	D/E	0	0.022
	G/A	0	0.022
Gγ IVS2	L/K	0.294	0.29
	O/L	0.039	0.25
	K/K	0.098	0.068
	L/L	0.078	0.023
	P/K	0.059	0
	N/Q	0.039	0
	L/N	0.02	0.09
	O/K	0.02	0.114
	L/Q	0.02	0
	M/K	0.115	0
	M/L	0.059	0
	N/M	0.039	0
	R/M	0.02	0
	N/N	0.039	0
	M/M	0.02	0
	L/P	0.02	0
	K/N	0.02	0.023
	S/K	0	0.046
	O/N	0	0.046
	R/L	0	0.046
	S/L	0	0.046
	O/M	0	0.046
Aγ IVS2	T/T	0.04	0.02
	T/U	0.470	0.26
	U/U	0.49	0.72
β globin promoter	V/V	0.273	0.74
	V/W	0.234	0.152
	Y/W	0.117	0.043
	V/Z	0.117	0.022
	V/Y	0.059	0.043
	Y/Z	0.022	0
	W/W	0.04	0
	V/X	0.098	0
	X/W	0.04	0
*Xmn*I	−/−	0.49	0.72
	+/−	0.47	0.26
	+/+	0.04	0.02
−309 of Gγ	A/G	0.176	0
	A/A	0.824	1

High F and 17 NRF subjects carried one of these four sequence configurations. Significantly, more of the subjects in the High F group (37.5 *vs* 6.5%) contained two configurations. None of the NRF group subjects had three configurations.

Arlequin tests

Table 3 summarises the results of the linkage disequilibrium test between pairs of genetic markers in High F subjects. This test showed linkage disequilibrium (i) between configurations D (β LCR-HS2) and M (Gγ IVS2) and between the *Xmn*I+ polymorphism and configurations D, M and W (−540 of β globin gene). However, configuration W was not associated with either D or M. The Arlequin data confirmed that the *Xmn*I+ site is in strict linkage disequilibrium with configuration T in Aγ IVS2 as demonstrated earlier,[18] which validates our statistical testing procedure. The maximum likelihood haplotype frequency test failed to reveal any specific associations between the β globin gene cluster haplotypes (constituted by all the studied markers) and the HbF phenotype groups.

Genotype/phenotype association

Since more than 84% of the High F subjects had at least one of the four sequence configurations compared to 43% of the NRF subjects, we further investigated their combined association with HbF expression for all the studied individuals (Table 4). Our data revealed that subjects carrying one, two or three configurations had significantly higher mean absolute HbF values (g/dl) than those not carrying any of these ($P<0.001$). Furthermore, the effect on HbF level was more pronounced when two markers were present than when only one was present ($P<0.005$).

Discussion

The amount of HbF in normal adults varied by more than 20-fold. The distribution curve was skewed towards right with around 10% of the subjects having HbF levels above 0.8%. Family studies have shown that a strong genetic component determines the HbF level in subjects at the extremes of this spectrum.[3,4] This is the reason why we chose subjects at the extreme ends of the spectrum for the further genetic analysis (NRF and High F groups).

None of these subjects harboured any β globin gene defects or any anomalies in the foetal globin gene regions. We studied seven polymorphic markers spread through the 40 Kb segment of β globin gene cluster and assessed their association with HbF expression. Four polymorphisms (-309 $G\gamma$ A→G, -158 $G\gamma$ C→T (*Xmn*I+), TC $(TG)_9$ AG $(TG)_2$ $(CG)_2$ in $G\gamma$ IVS2 and $(AT)_9$ T_5 5′ of the β globin gene) showed a statistically significant independent association with the HbF expression. The -309 $G\gamma$ A→G polymorphism was found exclusively in High F subjects. These specific

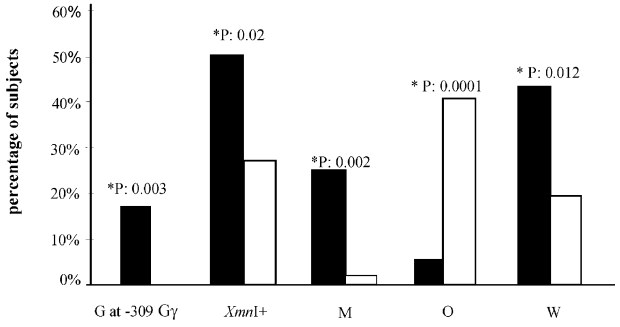


Figure 2 Distribution of the polymorphic markers among High F and NRF groups. Filled bars: high F group; empty bars: normal range of the HbF group (NRF), *: statistically significant, *P*: *P* value.

Table 3 Arlequin test for linkage disequilibrum between paired markers in High F subjects

Allelic association	*Frequency*
*Xmn*I+/D	0.17
*Xmn*I+/M	0.021
*Xmn*I+/W	0.125
M/A	0.009
M/D	0.052
M/V	0.063
W/M	0
W/D	0

The Arlequin test failed to reveal any specific pattern of marker association with HbF expression in High F subjects, suggesting that the contribution of sequence configurations is independent.

Table 2 Marker combinations in the High F and NRF subjects

Presence of	*High F subjects (%)*	*NRF subjects (%)*	P *value*
No marker	8 (15.7%)	26 (56.5%)	0.0001
Single marker			
G at -309 of $G\gamma$ alone	4 (7.7%)	0	
*Xmn*I+ alone	8 (15.7%)	11 (24%)	
M configuration alone	4 (7.7%)	1 (2%)	
W configuration alone	6 (11.7%)	5 (11%)	
Total	22 (43.14%)	17 (36.95%)	0.54
Two markers			
G at -309 $G\gamma$ and *Xmn*I+	1 (2%)	0	
G at -309 $G\gamma$ and M configuration	2 (4%)	0	
G at -309 $G\gamma$ and W configuration	1 (2%)	0	
*Xmn*I+ and M configuration	2 (4%)	0	
*Xmn*I+ and W configuration	13 (25.5%)	3 (6.5%)	
Total	19 (37.5%)	3 (6.5%)	0.0005
Three markers			
G at $-\gtrless\leqslant \Gamma\gamma\cdot,\cdot$ *Xmn*$\lambda\pm$ $\alpha\nu\delta$ ϑ $\psi\omega\nu\phi\iota\gamma\upsilon\rho\alpha\tau\iota\omega\nu$	1 (2%)	0	
*Xmn*I+, M and W configuration	1 (2%)	0	
Total	2 (3.92%)	0 (0%)	
	n: 51	n: 46	

The distribution of four sequence configurations that showed a significant positive association with HbF expression were analysed in High F and NRF subjects. Members of the NRF group were more likely to contain none of the four configurations than were members of the High F group. Conversely, members of High F group were more likely to harbour two or three configurations.

Table 4 Marker genotype-HbF phenotype association

	No marker	*1 marker*	*2 markers*	*3 markers*	P *value (Student's* t*-test)*
HbF (g/dl)	0.058 ± 0.052	0.110 ± 0.072	0.168 ± 0.083	0.14 ± 0.014	$P<0.001$* $P<0.005$**
n	34	39	22	2	

Comparison of the absolute levels of HbF expression in individuals with none of the four markers and in individuals with one or two markers revealed a statistically significant association (*). Similarly, the absolute level of HbF expression was significantly higher in subjects with two markers than in those with one marker (**), suggesting that these markers contribute to HbF expression in an independent manner.

sequence configurations were found in more than 84% of the high F subjects and only in 43% of the subjects with HbF in the normal range.

It is interesting that the −309 Gγ A→G variation was only found in the High F group because we (unpublished observations) and others[16,29] have shown that this variation is specific to the Benin β^S haplotype, which is usually associated with low HbF expression. In our study, none of the subjects with −309 Gγ A→G variation had this haplotype and no other population data is available so we can not determine whether, in a context different from the Benin β^S haplotype, this polymorphism plays a role in HbF expression. Similarly, the Gγ IVS2 TC $(TG)_9$ AG $(TG)_2$ $(CG)_2$ configuration was previously found on two chromosomes associated with the Benin β^S haplotype.[29] Our findings show that these two polymorphic markers are also present in normal chromosomes and are associated with raised HbF levels. Conversely, the *Xmn*I+ is a common polymorphism that has been extensively explored in different populations and has been shown to influence the number of FC and HbF levels in normal[30,31] and anaemic subjects.[20,21]

The $(AT)_9$ T_5 configuration (configuration W), which is located within a putative transcriptional silencer[32] of the β globin gene, was originally described in a silent β thalassaemia case[33] and has been shown to be associated with reduced sickle gene expression.[34] However, another study involving a few non-anaemic subjects, did not confirm this effect on β globin gene expression.[12] Our data, based on a much larger number of subjects, reveal that this configuration is associated with raised HbF levels. Genes that have minor effects cannot be studied on an individual basis, but their effects may be revealed in a statistical manner through a population study. This might explain why some studies with small number of individuals failed to demonstrate this association.

Of the 22 High F subjects with the $(AT)_9$ T_5 sequence configuration, 15 had *Xmn*I+. The co-presence of these sequences both in *cis* and in *trans* is associated with elevated HbF levels in homozygous and heterozygous β thalassaemia.[18,19] Our data suggest that even in the absence of any β globin defects, *Xmn*I+ and $(AT)_9$ T_5, are strongly associated with elevated levels of HbF.

As more than 84% of individuals (43/51) carry at least one of the four marker configurations and as those possessing one of these marker configurations have higher mean HbF levels than those lacking them, these markers may contribute to HbF expression in an independent manner. We cannot totally exclude the possibility that these markers have a direct effect, as they are located in evolutionarily conserved regulatory regions of the globin genes. Alternatively they may act indirectly, as a marker in linkage disequilibrium with an unidentified sequence in the region surrounding this cluster.

This is the first elaborate analysis to have explored the contribution of the β globin gene cluster in the expression of HbF in healthy adults, free of any globin gene defects. We have demonstrated that this cluster makes a significant genetic contribution to HbF.

Acknowledgments

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