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Detection of a Major Gene for Heterocellular Hereditary Persistence of Fetal Hemoglobin after Accounting for Genetic Modifiers

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Summary

"Heterocellular hereditary persistence of fetal hemoglobin" (HPFH) is the term used to describe the genetically determined persistence of fetal hemoglobin (Hb F) production into adult life, in the absence of any related hematological disorder. Whereas some forms are caused by mutations in the β-globin gene cluster on chromosome 11, others segregate independently. While the latter are of particular interest with respect to the regulation of globin gene switching, it has not been possible to determine their chromosomal location, mainly because their mode of inheritance is not clear, but also because several other factors are known to modify Hb F production. We have examined a large Asian Indian pedigree which includes individuals with heterocellular HPFH associated with β-thalassemia and/or α-thalassemia. Segregation analysis was conducted on the HPFH trait FC, defined to be the percentage of Hb F–containing cells (F-cells), using the class D regressive model. Our results provide evidence for the presence of a major gene, dominant or codominant, which controls the FC values with residual familial correlations. The major gene was detected when the effects of genetic modifiers, notably β-thalassemia and the *Xmn*I-$^{G}\gamma$ polymorphism, are accounted for in the analysis. Linkage with the β-globin gene cluster is excluded. The transmission of the FC values in this pedigree is informative enough to allow detection of linkage with an appropriate marker(s). The analytical approach outlined in this study, using simple regression to allow for genetic modifiers and thus allowing the mode of inheritance of a trait to be dissected out, may be useful as a model for segregation and linkage analyses of other complex phenotypes.

Introduction

In normal adults the synthesis of fetal hemoglobin (Hb F) is reduced to very low levels, usually accounting for <0.6% of the total hemoglobin. The Hb F is restricted to a subpopulation of erythrocytes termed "F-cells" (Boyer et al. 1975), and, generally, there is a good correlation between the amount of Hb F and the proportion of F-cells. There is a considerable variation in the amount of the Hb F in normal adults, with levels varying by more than 10-fold. Among the factors known to influence Hb F levels are age (Rutland et al. 1983), sex (Miyoshi et al. 1988), a common sequence variation (T-C) at position −158 upstream of the $^{G}\gamma$-globin gene and detectable by the restriction enzyme *Xmn*I (Gilman and Huisman 1985; Sampietro et al. 1992), and the coinheritance of β-thalassemia and α-thalassemia (Weatherall and Clegg 1981). Thus Hb F production is influenced by several genetic modifiers, both linked and unlinked to the β-globin gene complex.

Synthesis of much higher levels of Hb F can persist into adult life as a heterogeneous condition referred to as "hereditary persistence of fetal hemoglobin" (HPFH) (Stamatoyannopoulos and Nienhuis 1987). In one group, the HPFH determinants demonstrate a clear Mendelian inheritance as alleles of the β-globin gene

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complex on the short arm of chromosome 11; the levels of Hb F in heterozygotes are in the range of 5%–25% of total hemoglobin, and these are caused by large deletions within the β-globin gene cluster or by mutations in the promoters of the γ-globin genes. There is another form which is characterized by smaller (<5%) increases in the levels of Hb F and in the proportion of F-cells in otherwise normal individuals but which, on interaction with homozygous β-thalassemia or sickle cell disease, can nevertheless increase Hb F output to levels which are clinically beneficial. Because the Hb F is unevenly distributed among the F-cells, this form is often referred to as "heterocellular HPFH" (or "Swiss HPFH," from the original report [Marti and Butler 1961]). It has only been defined phenotypically by the levels of Hb F, but it is likely that it is genetically heterogeneous.

In some families, the heterocellular HPFH determinant behaves as an allele of the β-globin gene complex (Stamatoyannopoulos et al. 1975; Altay et al. 1977; Old et al. 1982), while in others it segregates independently of the β-globin gene cluster (Gianni et al. 1983; Giampaolo et al. 1984; Jeffreys et al. 1986; Martinez et al. 1989; Thein and Weatherall 1989), suggesting that the locus controlling the F-cell production is either *trans*-acting or, if on chromosome 11, located at a considerable distance from the β-globin gene complex. While X-linked inheritance has been suggested in some cases (Miyoshi et al. 1988; Dover et al. 1992), a father-to-son transmission has been demonstrated in several families (Gianni et al. 1983; Thein and Weatherall 1989).

The underlying genetic heterogeneity makes it difficult to interpret studies of segregation and linkage analyses in heterocellular HPFH, particularly those involving several different small families. Analysis of data from one large pedigree, assuming that the disorder is genetically homogeneous, should, in contrast, be useful for determining the mode of inheritance. We have examined an extended Asian Indian pedigree in which heterocellular HPFH segregates in six generations. The pedigree includes individuals with the *Xmn*I-$^{G}\gamma$ polymorphic site, β-thalassemia, and/or α-thalassemia. DNA analysis of several family members with HPFH demonstrated that the β-globin gene cluster is intact, and sequence analysis of the γ-globin gene promoters showed no abnormality.

In order to determine the mode of inheritance, segregation analysis was carried out on the HPFH trait FC, defined to be the percentage of F-cells, using the regressive models (Bonney 1984) and accounting for other factors which may modify the FC value, including age, sex, β-thalassemia, α-thalassemia, and the *Xmn*I-$^{G}\gamma$ site.

Lod scores for linkage between the gene(s) controlling the F-cell production led to exclusion of the β-globin gene complex as having a major contribution to the HPFH in this pedigree. Generating a hypothetical diallelic marker tightly linked to the trait indicated that transmission of the FC values is informative enough in this pedigree to allow detection of linkage with an appropriate marker(s).

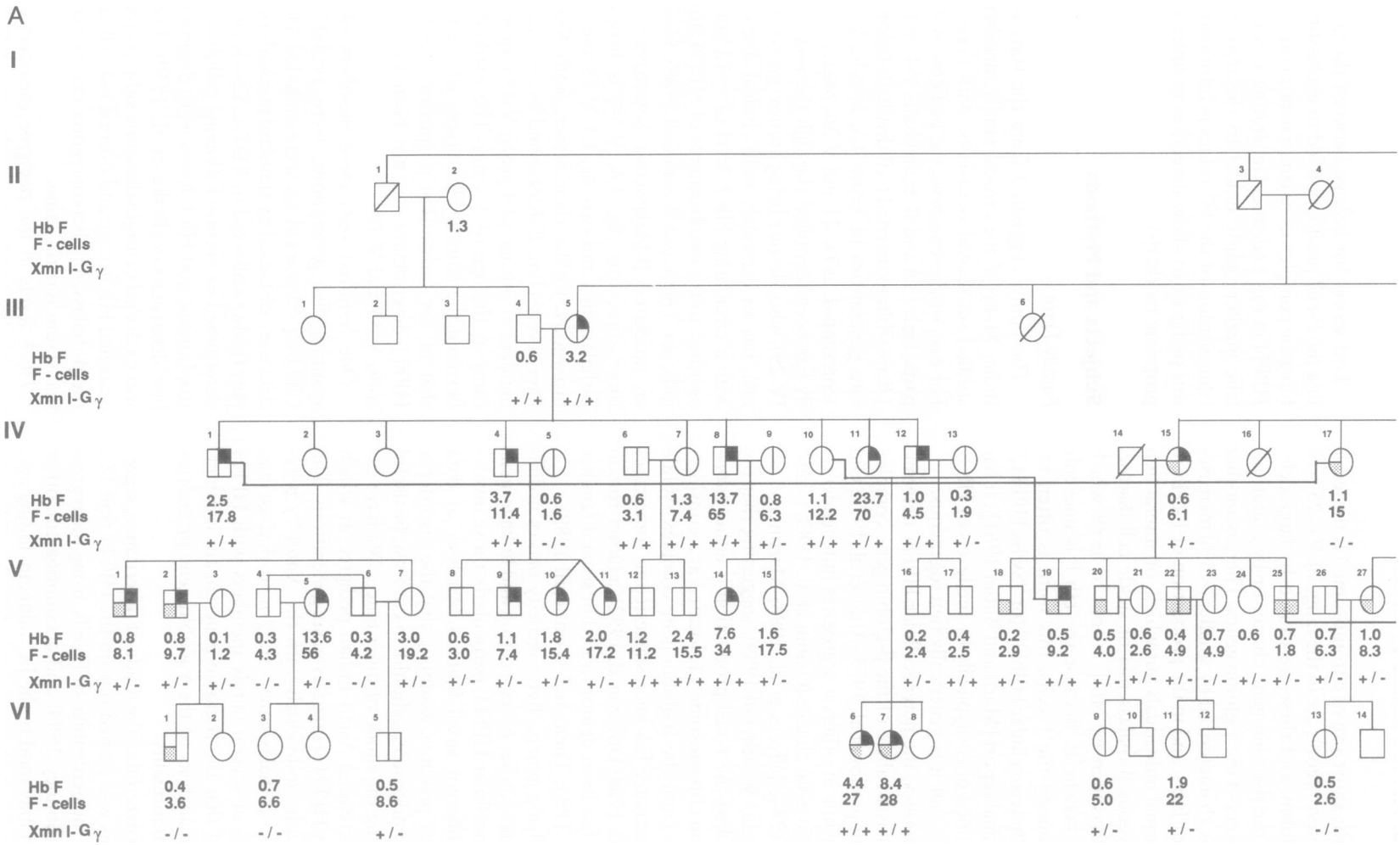
Subjects and Methods

Family Data

The family originated from the state of Gujarat in India. Many of the critical family members have been studied on several occasions, with consistent results. For ease of presentation, the pedigree is shown in two parts, figure 1*A* and *B*. Individuals V-1 and V-2 in figure 1*B* are siblings married to individuals belonging to separate generations in figure 1*A*, in which they are also represented, as IV-21 and V-26, respectively. The family has been identified through the propositus (fig. 1*A*, IV-29), who, despite being homozygous for β°-thalassemia, has an extremely mild clinical disease associated with a circulating Hb F level of ~12 gm/dl which is ascribed to the coinheritance of HPFH (for clinical details, see Thein and Weatherall 1989). He is married to an unrelated β-thalassemia heterozygote; they have three sons; one (fig. 1*A*, V-54) is heterozygous for β-thalassemia and two (fig. 1*A*, V-55 and V-56) are homozygous. Unlike their father, both V-55 and V-56 have severe clinical disease and became transfusion dependent at the age of 4 years; V-55 required a splenectomy at the age of 5 years. The marked discrepancy between the clinical phenotypes of the two sons and that of the father (the propositus) suggests that the HPFH determinant has not been transmitted to the sons, V-55 and V-56.

One hundred sixty-three members of the family, spanning five generations, were available for study. Children <2 years of age were excluded. Hematological data were obtained by standard methods on blood samples freshly collected in EDTA. Levels of Hb A_2 were determined by elution following cellulose acetate electrophoresis, and Hb F levels were determined by alkaline denaturation (Betke et al. 1959). Iron deficiency was excluded in nonthalassemic subjects on the basis of a normal Hb for age and normal red-cell indices and in the β-thalassemic heterozygotes on the basis of a normal serum ferritin value.

Hb F levels in the pedigree members.—Hb F was measured in 163 family members (fig. 1*A* and *B*). Analy-


A
I
II
III
IV
V
VI
Hb F
F - cells
Xmn I-Gγ
1.3
0.6
3.2
+/+
+/+
2.5
17.8
+/+
3.7
11.4
+/+
0.6
1.6
-/-
0.6
3.1
+/+
1.3
7.4
+/+
13.7
65
+/+
0.8
6.3
+/-
1.1
12.2
+/+
23.7
70
+/+
1.0
4.5
+/+
0.3
1.9
+/-
0.6
6.1
+/-
1.1
15
-/-
0.8
8.1
+/-
0.8
9.7
+/-
0.1
1.2
+/-
0.3
4.3
-/-
13.6
56
+/-
0.3
4.2
-/-
3.0
19.2
+/-
0.6
3.0
+/-
1.1
7.4
+/-
1.8
15.4
+/-
2.0
17.2
+/-
1.2
11.2
+/+
2.4
15.5
+/+
7.6
34
+/+
1.6
17.5
+/-
0.2
2.4
+/-
0.4
2.5
+/+
0.2
2.9
+/-
0.5
9.2
+/+
0.5
4.0
+/-
0.6
2.6
+/-
0.4
4.9
+/-
0.7
4.9
-/-
0.6
+/-
0.7
1.8
+/-
0.7
6.3
+/-
1.0
8.3
+/-
0.4
3.6
-/-
0.7
6.6
-/-
0.5
8.6
+/-
4.4
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+/+
0.6
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1.9
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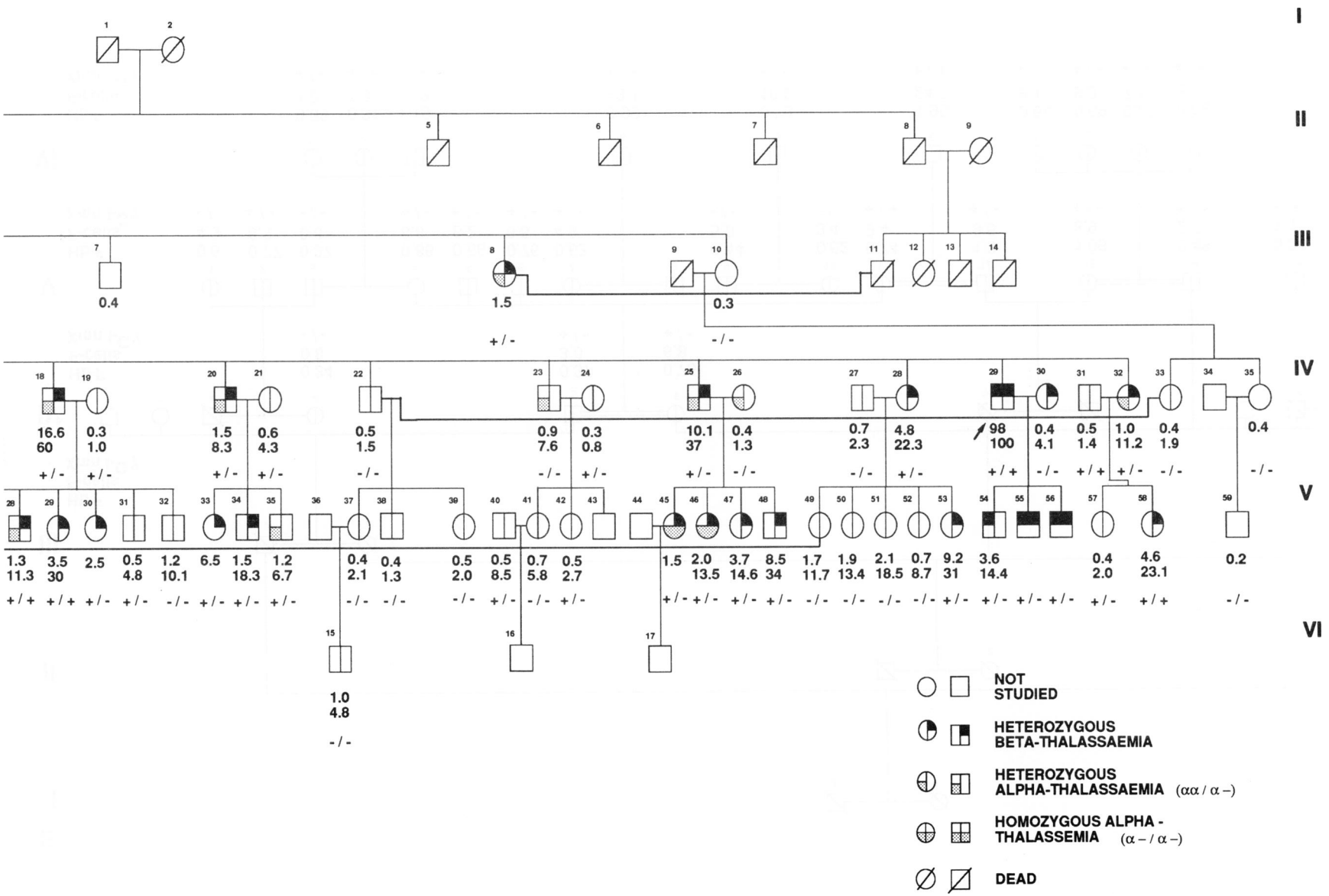
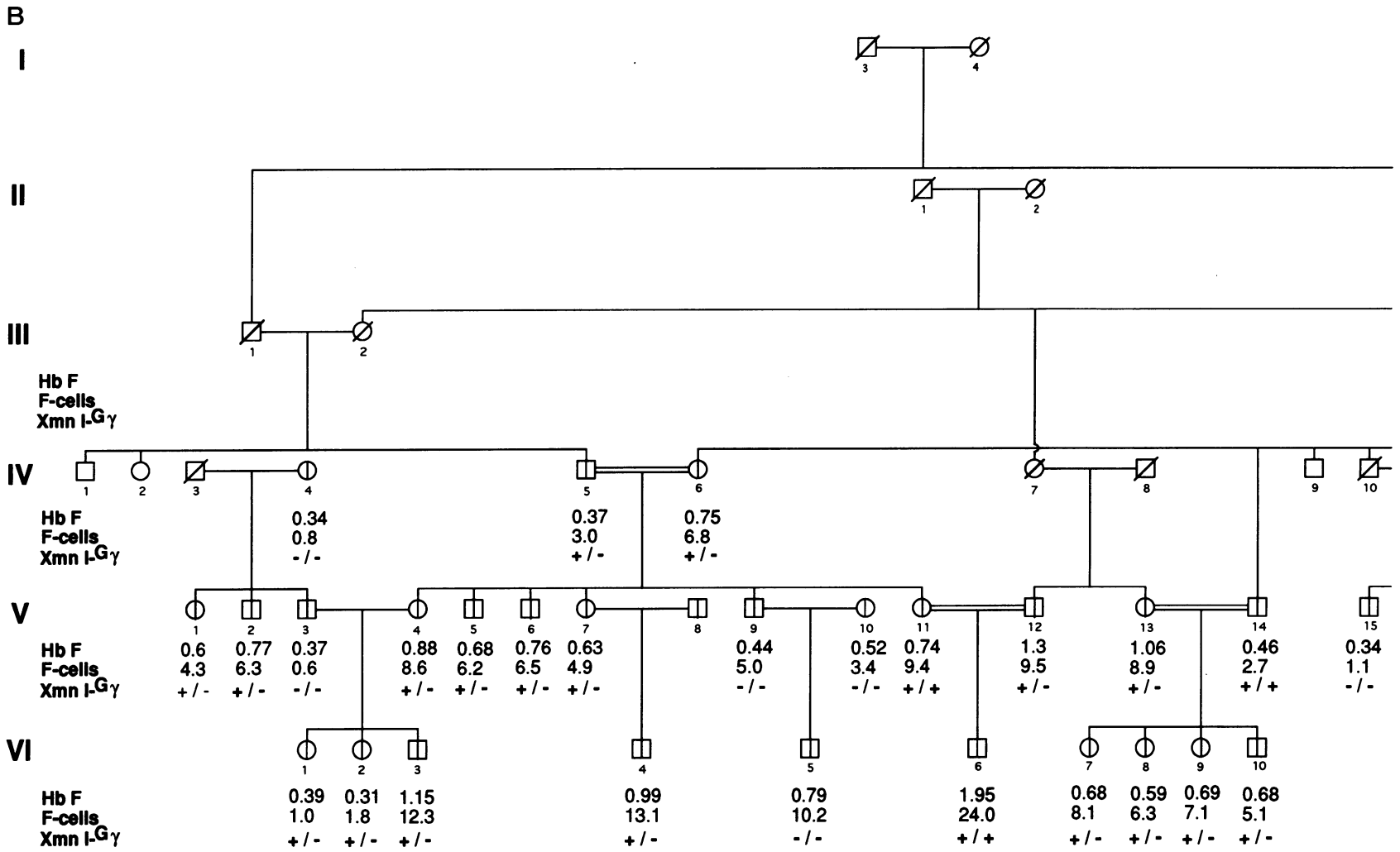
2.6
-/-


I
II
III
IV
V
VI
0.4
1.5
+ / -
0.3
- / -
16.6 60 + / -
0.3 1.0 + / -
1.5 8.3 + / -
0.6 4.3 + / -
0.5 1.5 - / -
0.9 7.6 - / -
0.3 0.8 + / -
10.1 37 + / -
0.4 1.3 - / -
0.7 2.3 - / -
4.8 22.3 + / -
98 100 + / +
0.4 4.1 - / -
0.5 1.4 + / +
1.0 11.2 + / -
0.4 1.9 - / -
0.4 - / -
1.3 11.3 + / +
3.5 30 + / +
2.5 + / -
0.5 4.8 + / -
1.2 10.1 - / -
6.5 + / -
1.5 18.3 + / -
1.2 6.7 + / -
0.4 2.1 - / -
0.4 1.3 - / -
0.5 2.0 - / -
0.5 8.5 + / -
0.7 5.8 - / -
0.5 2.7 + / -
1.5 + / -
2.0 13.5 + / -
3.7 14.6 + / -
8.5 34 + / -
1.7 11.7 - / -
1.9 13.4 - / -
2.1 18.5 - / -
0.7 8.7 - / -
9.2 31 + / -
3.6 14.4 + / -
+ / -
+ / -
0.4 2.0 + / -
4.6 23.1 + / +
0.2 - / -
1.0 4.8 - / -
NOT STUDIED
HETEROZYGOUS BETA-THALASSAEMIA
HETEROZYGOUS ALPHA-THALASSAEMIA (αα / α –)
HOMOZYGOUS ALPHA - THALASSEMIA (α – / α –)
DEAD


B
I
II
III
IV
V
VI
Hb F
F-cells
Xmn I-Gγ
0.34
0.8
- / -
0.37
3.0
+ / -
0.75
6.8
+ / -
0.6
4.3
+ / -
0.77
6.3
+ / -
0.37
0.6
- / -
0.88
8.6
+ / -
0.68
6.2
+ / -
0.76
6.5
+ / -
0.63
4.9
+ / -
0.44
5.0
- / -
0.52
3.4
- / -
0.74
9.4
+ / +
1.3
9.5
+ / -
1.06
8.9
+ / -
0.46
2.7
+ / +
0.34
1.1
- / -
0.39
1.0
+ / -
0.31
1.8
+ / -
1.15
12.3
+ / -
0.99
13.1
+ / -
0.79
10.2
- / -
1.95
24.0
+ / +
0.68
8.1
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0.59
6.3
+ / -
0.69
7.1
+ / -
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5.1
+ / -

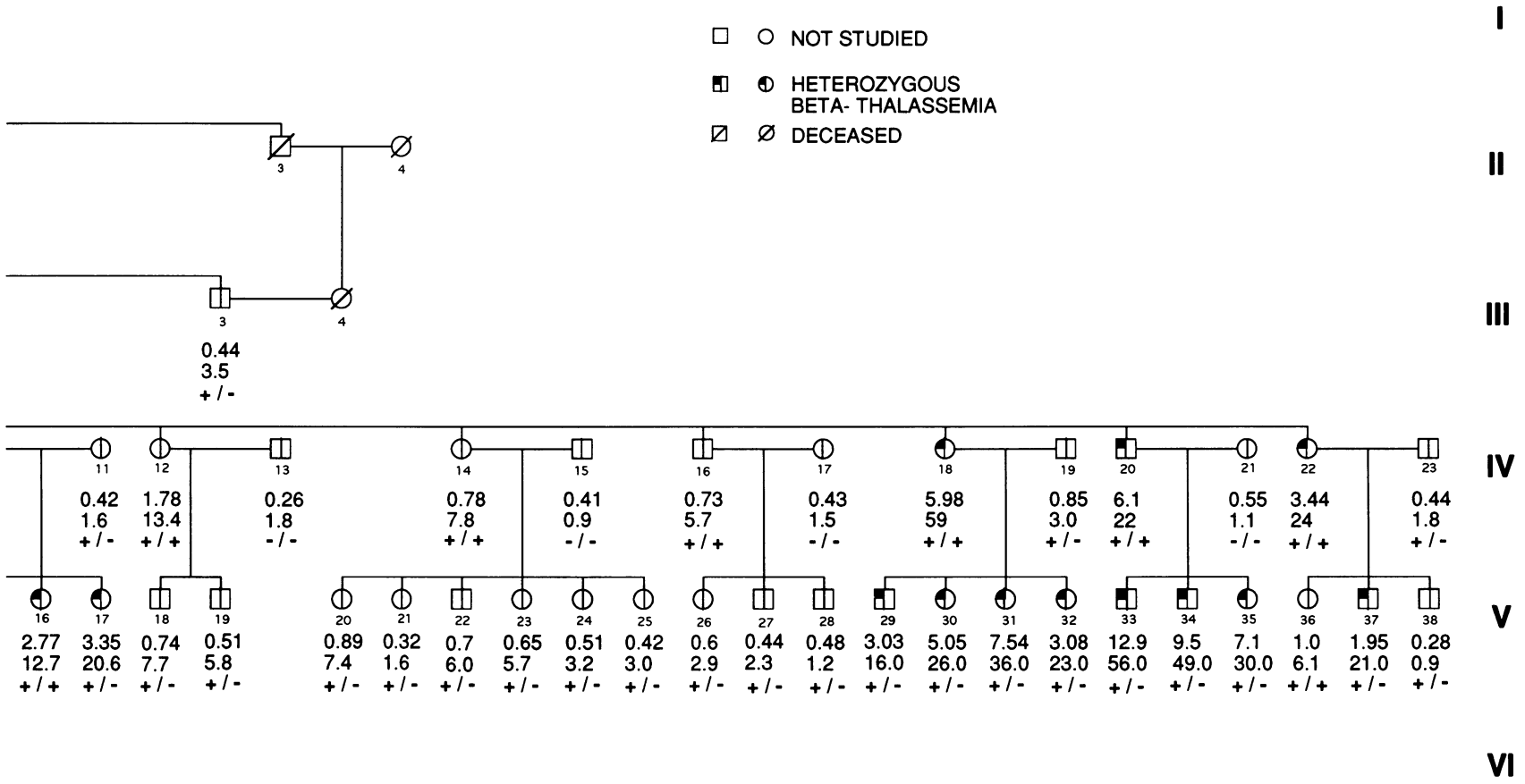

Figure 1 Extended pedigree of the Asian Indian family, with the Hb F levels, FC ("F-cells") values, and *Xmn*I-$^{G}\gamma$ polymorphism shown. β-thalassemia status and α-thalassemia status are as indicated in the definition of symbols.

sis of the Hb F levels in the pedigree members showed that several individuals, with or without β-thalassemia trait, had levels much higher than normal, while the Hb F levels of the other family members were within normal limits. The increased Hb F levels had a range of 0.8%–3.4% in otherwise normal members and of 2.5%–24% in β-thalassemia heterozygotes.

Distributions of Hb F levels have been reported in several studies; in a survey of 750 normal blood donors, Zago et al. (1979) showed that only 2.8% had Hb F levels >0.8% and that family studies of these individuals indicated that the high levels of Hb F were genetically controlled. The levels of increased Hb F observed in members of this pedigree are consistent with the presence of heterocellular HPFH.

FC values in the pedigree members.—F-cell assays were performed on peripheral blood by using a monoclonal mouse anti-γ chain antibody (gift from Professor Peter Beverley, London) by microscopy (Wood et al. 1975) ($\geq 2 \times 10^3$ red blood cells [RBCs] counted per blood smear) and/or fluorescence-activated cell sorting (FACS) (Thorpe 1981) (10^4 RBCs counted per assay). All counts were performed in duplicate by two observers, and the results were averaged; a heterocellular distribution of Hb F was demonstrated. FC values obtained by microscopy and FACS were generally in good agreement. FC values estimated by microscopic scoring were used in the analysis, as some of the initial F-cells have not been assessed by FACS. FC values of the pedigree members are shown in figure 1*A* and *B*.

Although there is good correlation between the Hb F levels and the FC values, the amount of Hb F as determined by the alkaline denaturation procedure is not consistently reproducible, particularly in the very low range. Thus the FC value was the phenotype analyzed in this pedigree.

β-Globin haplotype and α-globin genotype analysis.—DNA was extracted from the peripheral blood leukocytes and was analyzed for seven RFLPs in the β-globin gene cluster, and the β-globin haplotypes were determined for all the subjects (Antonarakis et al. 1982). The T-C polymorphism at position −158 of the $^{G}\gamma$-globin gene was determined by two approaches: hybridization of *Xmn*I-restricted DNA with a γ probe (*Hin*dIII 3.3-$^{A}\gamma$) and/or *Xmn*I-restriction analysis of the $^{G}\gamma$-globin gene 5′ region amplified by the PCR (Saiki et al. 1988). The number of α-globin genes located on the short arm of chromosome 16 was determined by hybridizing *Bam*HI-digested DNA and *Bgl*II-digested DNA with α- and ζ-globin gene probes, respectively. All DNA probes were radiolabeled with ^{32}P-dCTP by random hexamer priming.

Characterization of β-thalassemia mutations.—The β-thalassemia mutations in the propositus (IV-29) and his wife (IV-30) were characterized by direct sequence analysis of the β-thalassemia genes, which were amplified by the PCR (Thein and Hinton 1991). The mutations were independently confirmed by the technique of allele-specific oligoprobe hybridization (Thein and Wallace 1986).

Sequence analysis of γ-globin promoter regions.—The PCR was used to specifically amplify the region immediately upstream of both the $^{G}\gamma$- and $^{A}\gamma$-globin genes from positions −635 to +50 relative to the cap site (Craig et al. 1993). In the PCR, the upstream primer (either 5′-AGI or 5′-GGI) was 5′ biotinylated to facilitate the preparation of single-stranded (ss) template DNA by using magnetic beads (Thein and Hinton 1991). The ssDNA was directly sequenced using the dideoxy chain-termination method.

Confirmation of family relationships.—The genetic relationships of the kindred were investigated by a combination of approaches—DNA fingerprinting analysis using the hypervariable minisatellite probes (Jeffreys et al. 1985, 1986) and analysis of human leukocyte antigens, serum proteins, and blood groups. Nonpaternity was excluded in all cases.

Statistical Analysis

All analyses were carried out on the FC trait as assessed by microscopic scoring. We first checked that this trait was highly correlated (correlation coefficient = .92) with the level of Hb F. Since the FC values were highly skewed (coefficient of skewness = 2.26), a log transformation of the data was performed. Prior to segregation and linkage analyses, we assessed which covariates were significantly associated with the FC trait. These included age, sex, presence/absence of α-thalassemia, presence/absence of β-thalassemia, and the T-C polymorphism determined by the presence/absence (+/−) of the *Xmn*I-$^{G}\gamma$ restriction site. Covariate-trait associations were first detected by one-way analyses of variance. Simultaneous effects of covariates on the trait were estimated by stepwise multiple regression, considering the observations on family members as independent and using the BMDP package.

Segregation and Linkage Analyses

To detect a major gene effect among all factors causing the familial correlations of FC values, segregation analysis was conducted on the ln(FC) values, using the

regressive models introduced by Bonney (1984). Major gene effects are modeled against a background of familial covariability due to the joint effects of factors of unspecified origin, such as polygenic, cultural, and common environmental transmission.

Let us assume that the major gene is represented by an autosomal locus with two alleles (A and a). This major gene effect is defined in terms of *q*, the allele A frequency responsible for high values of the trait, and the three genotype-specific means of the phenotype, μ_{AA}, μ_{Aa}, and μ_{aa}. For individuals with no parents in the pedigree, the three genotype frequencies are functions of *q*, if Hardy-Weinberg equilibrium is assumed. For individuals with ancestors in the pedigree, the three genotypic probabilities for a given parental mating type are expressed in terms of the three transmission probabilities of Elston and Stewart (1971). These transmission parameters, denoted τ_{AAA}, τ_{AaA}, and τ_{aaA}, are the conditional probabilities of transmitting to offspring allele A for parental genotypes AA, Aa, and aa, respectively; they are equal to 1, .5, and 0 under the Mendelian hypothesis, whereas they can take any value between 0 and 1 under the general transmission model. Conditioned on major genotypes, the phenotypes of all pedigree members are assumed to follow a multivariate normal distribution. This joint multivariate normal density is decomposed in a product of univariate densities by regressing the residual phenotype (i.e., adjusted for major genotype) of each individual on the residual phenotypes of preceding relatives, with the regression coefficients expressed in terms of the observed phenotypic correlations. Different patterns of dependence between a person and preceding relatives have been described. We consider here the general class D regressive model, which includes several patterns of correlations as particular cases (Demenais and Bonney 1989). Under the class D model, the residual covariation among family members is expressed in terms of the variance conditional on major genotypes (σ^2) and the four phenotypic correlations: the father-mother correlation (ρ_{FM}), the father-offspring correlation (ρ_{FO}), the mother-offspring correlation (ρ_{MO}), and the sib-sib correlation (ρ_{SS}). The regressive models can accommodate for measured covariates simultaneously with the other factors in the regression, and genotype-specific effects of these covariates can be incorporated. The regressive approach has also been extended to linked marker loci (Bonney et al. 1988).

The likelihood of the pedigree was computed using the computer program REGRESS, a Pascal program which incorporates the regressive approach in the ILINK program of the LINKAGE package (Lathrop et al. 1984). To lessen the computer time in calculating the likelihood function under the class D model, we used the approximation proposed by Demenais et al. (1990). Correction for ascertainment was done by removing the proband leading to selection of this pedigree. Since this large pedigree included several loops, it had to be broken into six independent subpedigrees. Parameter estimation and tests of hypotheses were carried out using maximum-likelihood methods. Evidence for the presence of a major effect was provided by testing the null hypothesis of no major effect against a model including a major effect and residual correlations. If this test was significant, two additional criteria had to be fulfilled before inference of segregation of a major gene (Demenais et al. 1986): (1) the hypothesis of Mendelian transmission ($\tau_{AAA} = 1$, $\tau_{AaA} = .5$, and $\tau_{aaA} = 0$) should fit the data, and (2) the absence of transmission of the major effect from parents to offspring ($\tau_{AAA} = \tau_{AaA} = \tau_{aaA}$) should be rejected against the general transmission probability (where the three τs can take any value between 0 and 1).

Linkage Analysis

Linkage analysis was conducted between the adjusted ln(FC) values and the β-globin haplotypes. The best-fitting mode of inheritance for the trait and corresponding parameter estimates obtained from segregation analysis were used. Allelic frequencies of the different β-globin haplotypes were estimated from the data. Lod scores at different values of the recombination fraction, θ, were computed with the REGRESS program.

Results

Family Data

The FC values and Hb F levels, β-thalassemia status, and α-genotype of the family members are shown in figure 1*A* and *B*. The diagnosis of heterozygous β-thalassemia was established on the basis of hypochromic microcytic RBC indices and an elevated level of Hb A_2.

Gene mapping showed the β-globin gene complex to be intact with no deletion or rearrangement. RFLP analysis of the β-globin gene complex by using seven polymorphic restriction-enzyme sites (*Hin*dII-ε, *Hin*dIII-^{G}γ, *Hin*dIII-^{A}γ, *Hin*dII-ψβ, *Hin*dII-3′ψβ, *Ava*II-β, and *Bam*HI-β) showed the β-thalassemia allele in the propositus (fig. 1*A*, IV-29) to be associated with the −+−++++ β-globin haplotype and showed that in his wife (IV-30) to be associated with the −−−−−+

Table 1

Details of *Xmn*I-$^{G}\gamma$ Status, β-Globin Genotype and α-Globin Genotype in the 150 Pedigree Members Analyzed

α-Globin Genotype	*Xmn*I-$^{G}\gamma$ (−/−)		*Xmn*I-$^{G}\gamma$ (+/−)		*Xmn*I-$^{G}\gamma$ (+/+)		Total
	β^{Th}/β^{N}	β^{N}/β^{N}	β^{Th}/β^{N}	β^{N}/β^{N}	β^{Th}/β^{N}	β^{N}/β^{N}	
αα/αα	1	28	18	46	11	10	114
αα/α−	0	5	8	11	5	4	33
α−/α−	0	0	1	2	0	0	3
Total	1	33	27	59	16	14	150

β-globin haplotype and the normal β alleles to be associated with 11 haplotypes (results not shown). Direct sequence analysis and oligonucleotide probe hybridization showed the propositus to be homozygous for the G→T substitution at position 1, IVS-1 of the β-globin gene, while his wife is heterozygous for the frameshift codon 8/9 (+G) mutation. Both these mutations result in β°-thalassemia. Of their children, V-54 is heterozygous for the β° IVS-1 position 1 G-T mutation, while V-55 and V-56 are compound heterozygotes for the β° IVS-1 position 1 G-T and the β° frameshift codon 8/9 mutations. Sequence analysis of both the $^{G}\gamma$ and $^{A}\gamma$ promoter regions did not show any difference from the published normal sequences.

Of the 163 pedigree members, FC values were determined in 153. Since the proband and the two β-thalassemia homozygotes (V-55 and V-56) had extreme (100%) FC values, they were excluded from the analysis. The family sample analyzed with measured FC values thus included a total of 150 individuals. Of the 150 members, 44 were heterozygous for β-thalassemia, 33 had one α-globin gene deleted (αα/α−), 3 had two α-globin genes deleted (α−/α−), 34 were *Xmn*I-$^{G}\gamma$ (−/−), 86 were *Xmn*I-$^{G}\gamma$ (+/−), and 30 were *Xmn*I-$^{G}\gamma$ (+/+). The distribution of these values is shown in table 1 and figure 1*A* and *B*.

Influence of Covariates on FC Values

A log transformation of the FC values led to nonsignificant skewness (.02) and kurtosis (−.67). Table 2 presents the estimated means and SDs of the FC and ln(FC) values, classified by covariates. One-way analyses of variance showed a borderline significant association of ln(FC) with age ($P = .02$) and highly significant associations with the presence of β-thalassemia ($P < .0001$) and the presence of the *Xmn*I-$^{G}\gamma$ polymorphism ($P < .0001$). There was no significant difference of the ln(FC) values according to sex and presence of α-thalassemia. Stepwise multiple regression confirmed that the factors significantly associated with the ln(FC) values were age, β-thalassemia, and the *Xmn*I-$^{G}\gamma$ poly-

Table 2

Distribution of FC and ln(FC) Values by Age, Sex, Presence of α-Thalassemia, Presence of β-Thalassemia, and Presence/Absence (+/−) of a *Xmn*I-$^{G}\gamma$ Site

Covariate	*n*	FC Mean	FC SD	ln(FC) Mean	ln(FC) SD
Age (years):					
2–15	50	14.82	12.98	2.23	1.09
16–35	57	9.86	12.18	1.82	.96
>35	43	10.29	15.70	1.59	1.18
P value		ns		.02	
Sex:					
Male	71	10.89	13.79	1.83	1.06
Female	79	12.31	13.54	1.95	1.13
P value		ns		ns	
α-Thalassemia mutation:[a]					
None (αα/αα)	114	11.54	14.16	1.83	1.14
At least one (αα/α− and α−/α−)	36	11.95	11.97	2.08	.94
P value		ns		ns	
β-Thalassemia mutation:[a]					
None	106	5.80	4.80	1.42	.86
At least one	44	25.69	17.36	3.02	.72
P value		≪.0001		≪.0001	
*Xmn*I-$^{G}\gamma$ site:					
−/−	34	4.93	4.52	1.20	.92
+/−	86	11.70	13.08	1.94	1.05
+/+	30	19.05	17.90	2.54	.97
P value		≪.0001		≪.0001	

NOTE.—ns = not significant.

[a] Presence of α-thalassemia and presence of β-thalassemia were each grouped into one class since there were only three individuals with (α−/α−) and none with homozygous β-thalassemia.

Table 3

Segregation Analysis of ln(FC) Values Adjusted for Age and Sex

Model	q	Parameter											
		μ_{aa}	μ_{Aa}	μ_{AA}	σ^2	τ_{AAA}	τ_{AaA}	τ_{aaA}	ρ_{FM}	ρ_{FO}	ρ_{MO}	ρ_{SS}	$-2 \ln L^a$
1. No major effect, no familial correlation	(0)	.02	(=μ_{aa})	(=θ_{aa})	1.12	...	...	...	(0)	(0)	(0)	(0)	170.11
2. No major effect, familial correlation:													
a. All familial correlations	(0)	−.34	(=μ_{aa})	(=μ_{aa})	1.08	...	...	...	−.09	.49	.35	.44	110.74
b. Familial correlation with $\rho_{FM} = 0$	(0)	−.36	(=μ_{aa})	(=μ_{aa})	1.08	...	...	...	(0)	.49	.39	.44	111.98
3. Mendelian effect, no familial correlation:													
a. Codominant	.20	−.98	.48	1.62	.38	(1)	(.5)	(0)	(0)	(0)	(0)	(0)	119.74
b. Dominant	.20	−.86	.69	.69	.53	(1)	(.5)	(0)	(0)	(0)	(0)	(0)	138.06
c. Recessive	.68	−.95	−.95	.62	.54	(1)	(.5)	(0)	(0)	(0)	(0)	(0)	135.86
4. Codominant Mendelian effect, familial correlation	.12	−.69	.63	1.84	.58	(1)	(.5)	(0)	(0)	.49	.39	.44	106.19

NOTE.—Parameters are fixed at the values shown in parentheses, for the corresponding model.
[a] Maximum likelihood of the pedigree, computed under each model.

morphism. Higher-order terms for age and interactions among the covariates in the regression were not significant. Since the ln(FC) values were slightly higher in females (1.95 ± 1.13) than in males (1.83 ± 1.06), although nonsignificantly different, ln(FC) was also regressed for sex.

Segregation Analysis

Since some of the covariates associated with the FC values are genetically transmitted, these data can serve as a model to study how taking into account genetic covariates may influence the outcomes of segregation analysis. Thus, segregation analysis was first performed on the ln(FC) values adjusted for age and sex. The analysis was then conducted on these adjusted ln(FC) values, simultaneously taking into account the effects of β-thalassemia and the *Xmn*I-$^G\gamma$ polymorphism. The presence/absence of β-thalassemia mutation was coded as a binary variable and the *Xmn*I-$^G\gamma$ polymorphism as a trichotomous variable corresponding to the number of *Xmn*I-$^G\gamma$ restriction sites.

Segregation analysis of ln(FC) adjusted for age and sex is presented in table 3. There is strong evidence for familial correlations (model 1 vs. model 2a, $\chi^2_4 = 59.37$, $P < .0001$). Tests of familial correlations against the general class D pattern show that the spouse correlation, ρ_{FM}, does not differ from zero (model 2b vs. model 2a, $\chi^2_1 = 1.24$, $.20 < P < .30$). Thus, ρ_{FO} and ρ_{MO} are subsequently estimated together with the sib-sib correlation. Under a model including a Mendelian major gene alone, both recessive and dominant inheritance are rejected against a codominant model (model 3c vs. model 3a, $\chi^2_1 = 16.12$; model 3b vs. model 3a, $\chi^2_1 = 18.32$; $P < .0001$). With respect to a model including a major gene effect and residual familial correlations, presence of a codominant major gene effect is not significant (model 4 vs. model 2b, $\chi^2_3 = 5.79$, $.10 < P < .20$). Thus, the familial transmission of ln(FC) adjusted for age and sex can be accounted for by high familial correlations ($\rho_{FO} = .49$, $\rho_{MO} = .39$, and $\rho_{SS} = .44$) without a major gene factor being distinguishable.

Table 4 shows the results of segregation analysis of age- and sex-adjusted ln(FC) values when account is taken of the effects of the genetic covariates, β-thalassemia and the *Xmn*I-$^G\gamma$ polymorphism. Again, familial correlations for this trait are highly significant (model 1 vs. model 2, $\chi^2_4 = 33.87$, $P < .0001$). We checked that the spouse correlation did not differ from zero, and for the same reasons as above, the three correlations, ρ_{FO}, ρ_{MO}, and ρ_{SS}, were independently estimated. As opposed to the preceding situation, presence of a significant major gene effect can be detected (model 4a vs. model 2, $\chi^2_3 = 19.72$, $P < .0005$). Residual correlations are also needed (model 4a vs. model 3a, $\chi^2_3 = 11.19$, $P < .02$). As before, recessive inheritance was rejected (results not shown). However, whereas dominant in-

Table 4

Segregation Analysis of Age- and Sex-adjusted ln(FC) Values When Accounting for the Genetic Covariates β-Thalassemia and the *Xmn*I-$^{G}\gamma$ Polymorphism

Model	q	Parameter												
		μ_{aa}	μ_{Aa}	μ_{AA}	σ^2	τ_{AAA}	τ_{AaA}	τ_{aaA}	ρ_{FO}	ρ_{MO}	ρ_{SS}	b_T[a]	b_X[b]	$-2 \ln L$[c]
1. No major effect, no familial correlation	(0)	−2.40	(=μ_{aa})	(=μ_{aa})	.58	...	...	...	(0)	(0)	(0)	1.39	.31	69.20
2. No major effect, familial correlation	(0)	−2.29	(=μ_{aa})	(=μ_{aa})	.59	...	...	...	.46	.31	.33	1.19	.30	35.33
3. Mendelian effect, no familial correlation:														
a. Codominant	.31	−2.83	−1.78	−1.02	.18	(1)	(.5)	(0)	(0)	(0)	(0)	1.22	.21	26.80
b. Dominant	.27	−3.05	−1.84	−1.84	.22	(1)	(.5)	(0)	(0)	(0)	(0)	1.56	.20	36.25
4. Mendelian effect and familial correlation:														
a. Codominant	.27	−2.96	−1.75	−1.55	.21	(1)	(.5)	(0)	.49	.18	.27	1.34	.27	15.61
b. Dominant	.27	−2.97	−1.76	−1.76	.21	(1)	(.5)	(0)	.49	.19	.27	1.34	.29	16.05
5. No transmission of major effect:														
a. Codominant	→.0	−2.39	−1.79	−1.76	.47	.69	.69	.69	.49	.29	.34	1.17	.28	29.40
b. Dominant	→.0	−2.39	−1.74	−1.74	.45	.65	.65	.65	.51	.29	.35	1.17	.27	29.62
6. General transmission of major effect:														
a. Codominant	.25	−2.91	−1.69	−1.32	.19	1.0	.42	.04	.46	.16	.28	1.33	.23	14.19
b. Dominant	.24	−2.99	−1.75	−1.75	.21	1.0	.42	.08	.51	.18	.29	1.35	.29	14.36

NOTE.—Parameters are fixed at the values shown in parentheses, for the corresponding model.

[a] Regression coefficient of the age and sex-adjusted ln(FC) on β-thalassemia.

[b] Regression coefficient of the age- and sex-adjusted ln(FC) on *Xmn*I-$^{G}\gamma$ polymorphism.

[c] Maximum likelihood of the pedigree, computed under each model.

heritance was rejected against a codominant model with no residual correlations (model 3b vs. model 3a, $\chi^2_1 = 9.45$, $P < .001$), a dominant major gene effect fits the data when residual correlations are accounted for (model 4b vs. model 4a, $\chi^2_1 = 0.44$, $P = .50$). Mendelian transmission of this major gene effect, codominant or dominant, fits the data (model 4a vs. model 6a, $\chi^2_3 = 1.42$; model 4b vs. model 6b, $\chi^2_3 = 1.69$; $P > .50$). The nontransmission of this effect (equal tau model) is rejected in either case (model 5a vs. model 6a, $\chi^2_3 = 15.21$; model 5b vs. model 6b, $\chi^2_3 = 15.26$; $P < .001$). Therefore, while accounting for the effects of the genetic covariates, β-thalassemia and the *Xmn*I-$^{G}\gamma$ polymorphism, the familial transmission of the age- and sex-adjusted ln(FC) values can be explained by the segregation of a major gene, codominant or dominant, with residual familial correlations. This major gene accounts for 64% of the variance of the trait, after adjustment for the effects of age, sex, β-thalassemia, and the *Xmn*I-$^{G}\gamma$ polymorphism, as can be deduced from the reduction of the residual variance from .59 (model 2) to .21 (model 4a) in table 4. The residual father-offspring correlation ($\rho_{FO} = .49$) is more than twice as high as the mother-offspring correlation ($\rho_{MO} = .18$), and the sib-sib correlation ($\rho_{SS} = .27$) is between the parent-offspring correlations. This pattern of residual correlations does not differ from a class A pattern, where the children are correlated only because of common parentage, with $\rho_{SS} = \rho^2_{FO} + \rho^2_{MO}$ (Bonney 1984). Moreover, a classical polygenic pattern, where the three residual correlations are constrained to be equal ($\rho_{FO} = \rho_{MO} = \rho_{SS}$), is rejected.

We investigated whether the effect of the major gene was similar according to the presence or absence of β-thalassemia and the *Xmn*I-$^{G}\gamma$ polymorphism, by allowing the regression of ln(FC) on these two covariates to be genotype dependent (table 5). A genotype-specific effect of either β-thalassemia or the *Xmn*I-$^{G}\gamma$ polymorphism cannot be detected (model 1 vs. model 2, $\chi^2_2 = 4.95$; model 1 vs. model 3, $\chi^2_2 = 4.18$; $.10 < P < .20$). However, as seen from table 5, the estimates of the regression coefficients of ln(FC) on β-thalassemia and *Xmn*I-$^{G}\gamma$ differ according to the genotype at the major gene, especially when these two covariates are made

Table 5

Tests for Interactions between a Codominant Major Gene for the Age- and Sex-adjusted ln(FC) Values and the Genetic Modifiers β-Thalassemia and the *Xmn*I-$^{G}\gamma$ Polymorphism

Model	q	Parameter																
		μ_{aa}	μ_{Aa}	μ_{AA}	σ^2	τ_{AAA}	τ_{AaA}	τ_{aaA}	ρ_{FO}	ρ_{MO}	ρ_{SS}	b_{Taa}[a]	b_{TAa}[a]	b_{TAA}[a]	b_{Xaa}[b]	b_{XAa}[b]	b_{XAA}[b]	$-2 \ln L$[c]
1. No interaction[d]	.27	−2.96	−1.75	−1.55	.21	(1.0)	(0.5)	(0)	.49	.18	.27	1.34	1.34	1.34	.27	.27	.27	15.61
2. Gene × β-thalassemia	.28	−3.14	−1.41	−1.45	.16	(1.0)	(0.5)	(0)	.31	.15	.10	1.55	1.05	1.68	.19	.19	.19	10.66
3. Gene × *Xmn*I-$^{G}\gamma$	.29	−3.07	−1.48	−2.01	.18	(1.0)	(0.5)	(0)	.46	.21	.29	1.35	1.35	1.35	.29	.10	.51	11.43
4. Gene × β-thalassemia, Gene × *Xmn*I-$^{G}\gamma$	.28	−3.24	−1.47	−1.22	.15	(1.0)	(0.5)	(0)	.28	.12	.00	1.52	1.05	1.84	.28	.23	−.01	8.18
5. No transmission of major effect	.27	−3.53	−1.57	−2.22	.29	.50	.50	.50	.60	.32	.36	1.84	.46	1.73	.36	.49	.21	17.04
6. General transmission of major effect	.28	−3.22	−1.49	−1.23	.14	1.0	.39	.17	.34	.14	.16	1.50	1.08	1.74	.27	.23	.03	3.47

NOTE.—Parameters are fixed at the values shown in parentheses, for the corresponding model.
[a] Genotype-specific regression coefficient of the age- and sex-adjusted ln(FC) on β-thalassemia.
[b] Genotype-specific regression coefficient of the age- and sex-adjusted ln(FC) on *Xmn*I-$^{G}\gamma$ polymorphism.
[c] Maximum likelihood of the pedigree computed under each model.
[d] This model corresponds to model 4a of table 4.

genotype dependent (model 4). Interestingly, accounting for these genotype-dependent effects of covariates leads to a decrease in the residual correlations, especially the sib-sib correlation, which converges to zero (model 4). With these genotype-specific effects, we confirmed that the Mendelian transmission of the major gene fitted the data (model 4 vs. model 6, $\chi^2_3 = 4.71$, $.10 < P < .20$), and the no-transmission model was rejected (model 5 vs. model 6, $\chi^2_3 = 13.57$, $P < .01$). We also checked that the pattern of residual correlations, when we accounted for interactions between the major gene and either one or both of the covariates, was consistent with a class A pattern, whereas a polygenic pattern was rejected.

Linkage Analysis

Lod scores for linkage between adjusted ln(FC) values and the β-globin gene complex are presented in table 6. We used the best-fitting model, codominant and dominant, with residual correlations, when accounting for the effects of β-thalassemia and the *Xmn*I-$^{G}\gamma$ polymorphism. The estimates of the parameters are from models 4a and 4b in table 4. As seen in table 6, linkage with the β-globin gene cluster can be excluded (lod scores < -2) at $\theta \leq .10$. This shows that the gene controlling the F-cell production in this pedigree is located outside the β-globin gene complex.

Discussion

The propositus, despite being homozygous for β°-thalassemia, has an unusually mild clinical disorder which is attributed to an inherent ability to produce Hb F. Although this may partly reflect the presence of the *Xmn*I-$^{G}\gamma$ site, many of the normal and β-thalassemic heterozygous family members, with or without the *Xmn*I-$^{G}\gamma$ site, also have unusually high Hb F levels and FC values, suggesting the presence of an HPFH determinant.

The situation is similar to that described as heterocellular HPFH, which is defined phenotypically by the heterocellular distribution of a slight (≤4%) but persistent increase in levels of Hb F in otherwise normal individuals. However, as in previous studies, the variable increase in Hb F becomes even more marked when coinherited with β-thalassemia, the Hb F levels being 2.5%–24% of the total hemoglobin. Although previous studies, without formal segregation analyses, have suggested a dominant or codominant gene causing heterocellular HPFH, the wide variation in the increase of Hb F levels and FC values could be explained by a more complex genetic mechanism.

The mode of inheritance of heterocellular HPFH in the extended Asian pedigree presented here was investigated by using the regressive models. Our results show that the familial transmission of FC values can be explained by the segregation of a major gene, codominant or dominant, with residual familial correlations, when the effects of genetic modifiers—β-thalassemia and the *Xmn*I-$^{G}\gamma$ polymorphism—influencing the trait are accounted for. This major gene is located outside the β-globin gene cluster, at a position yet to be determined.

Table 6

Lod Scores for Linkage between the FC Values and the β-Globin Gene Complex, Using the Best-fitting Models, Codominant or Dominant Major Gene (MG) with Residual Familial Correlations, as Provided by Segregation Analysis

Model	Lod Score at θ =						
	.0	.01	.05	.10	.20	.30	.40
Codominant MG with residual correlations[a]	−8.51	−6.31	−3.85	−2.47	−1.13	−.49	−.16
Dominant MG with residual correlations	−8.81	−6.17	−3.80	−2.45	−1.12	−.48	−.15

[a] The parameter values are those shown in table 4 (models 4a and 4b).

Comparison of the outcomes of segregation analyses clearly demonstrates that regressing a quantitative trait on genetic cofactors modifying its expression leads to clarification of the genetic determinism of that trait. Whereas no major gene factor could be detected underlying the familial transmission of the ln(FC) values simply adjusted for age and sex, the effect of a major gene could be distinguished on a background of familial covariation when the genetic modifiers, β-thalassemia and the *Xmn*I-$^{G}\gamma$ polymorphism, were taken into account. Accounting for the genetic covariates leads to a more accurate assignment of major genotypes among family members and to a reduction of the residual variance. The proportion of the total trait variance due to the regression on these covariates is 50%, of which 82% is due to β-thalassemia and 8% to the *Xmn*I-$^{G}\gamma$ polymorphism. The major gene was found to account for 64% of the residual variance of the adjusted trait; this gene would thus be responsible for ~32% of the total phenotypic variance. However, this may be slightly overestimated, since the pedigree had to be broken into subpedigrees in which the founders are third-generation siblings with parents of unknown status. The excess of gene carriers among these founders may have inflated the gene frequency. As can be observed from the estimates of the residual familial correlations, the major gene accounts for 42% of the mother-offspring correlation and for 18% of the sib-sib correlation of the ln(FC) values adjusted for all covariates, whereas the high father-offspring correlation is not changed by inclusion of the major gene into the model. Introducing interactive effects between the major gene and the genetic covariates leads to a decrease of all residual familial correlations, with the sib-sib correlation reaching zero. However, these interactive effects were not significant, which can be partly due to a lack of power in this relatively small sample. Interestingly, we found that the pattern of residual correlations, with or without gene-covariate interactions, fitted a simple class A pattern, as originally described by Bonney (1984). Such a pattern, where the sibs are correlated since they have parental phenotypes in common, may be due to shared genes and/or environment. However, these residual correlations were not consistent with a classical polygenic pattern caused by the small and additive effects of many genes. Overall, our results clearly show that regressing on genetic factors influencing a trait, without needing complex oligogenic models, is a powerful method to elucidate the genetic determinants of that trait. Relaxing the classical assumption of additivity of the different effects may also bring more insight into the underlying mechanisms.

Linkage analysis with the β-globin gene complex excluded the major gene being located in the vicinity of this complex. Exclusion of the β-globin gene cluster confirms the DNA analysis, which failed to show a mutation/deletion in the β-globin gene complex, a common cause of HPFH. Linkage analysis was repeated by setting the residual familial correlations to zero as classically done in linkage analysis. More negative lod scores were obtained when residual correlations were accounted for than when they were ignored. This is in agreement with our recent results of a simulation study showing that taking into account residual familial correlations improves the detection of linkage of gene(s) involved in multifactorial traits, provided the major gene effect is known or correctly estimated (Demenais and Lathrop, in press). We then went on to check whether transmission of the FC values in this pedigree was informative enough to detect linkage. We constructed a hypothetical diallelic codominant marker by assigning to each individual a marker genotype based on his or her adjusted ln(FC) value, using the genotypic mean estimates obtained from segregation analysis under the best-fitting codominant model with residual familial correlations. For instance, a marker

genotype 11 was assigned to all individuals with regressed trait values close to the mean μ_{aa}, and similarly for the genotypes Aa and AA. The so-called constructed marker was compatible with the family structure, except in some cases where a person's marker genotype was assigned according to those of the parents. A maximum lod score of 10.25 was obtained at $\theta = .01$. The maximum lod score was increased further to 13.81 when genotype-dependent effects of the covariates were specified. This clearly demonstrates that the transmission of the regressed FC trait in this pedigree is informative enough to allow detection of linkage with an appropriate marker(s). Subsequent segregation analysis accounting for a newly mapped gene as a genetic covariate will make it possible to uncover any further locus/loci which could contribute to the high Hb F phenotype.

The present study has clearly indicated that regressing on genetic cofactors influencing a multifactorial trait can lead gradually to identification of the different genes involved. The development of highly polymorphic markers saturating the human genome at a high resolution (NIH/CEPH Collaborative Mapping Group 1992; Weissenbach et al. 1992) should ultimately make it possible to identify the *trans*-acting gene(s) controlling the expression of γ-globin genes.

The *cis*-acting DNA sequences which are important in the regulation of globin gene expression now seem fairly well defined (Grosveld et al. 1993). The question arises as to how a gene or genes which are quite separate from the β-globin gene complex regulate Hb F production. Two potential mechanisms have been suggested: (1) that the *trans*-acting locus (or loci) produces proteins which interact with the effector sites, the hypersensitive sites in the β locus control region, or the promoter regions of the γ-globin genes and (2) that the *trans*-acting gene(s) acts indirectly by influencing the maturation of the F-cells (Orkin 1990). Identification of the *trans*-acting gene(s) should provide another angle and insight into the understanding of regulation of expression of the globin genes.

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