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Haplotype Mapping of a Major Quantitative-Trait Locus for Fetal Hemoglobin Production, on Chromosome 6q23

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Summary

Fetal hemoglobin (Hb F) and fetal cell (FC) levels in adults show considerable variation and are influenced by several genetic variants; the major determinants appear to be unlinked to the β-globin gene cluster. Recently, a *trans*-acting locus controlling Hb F and FC production has been mapped to chromosome 6q23 in an Asian Indian kindred that includes individuals with heterocellular hereditary persistence of Hb F (HPFH) associated with β thalassemia. We have extended the kindred by 57 members, bringing the total studied to 210, and have saturated the region with 26 additional markers. Linkage analysis showed tight linkage of the quantitative-trait locus (QTL) to the anonymous markers D6S976 (LOD score 11.3; recombination fraction .00) and D6S270 (LOD score 7.4; recombination fraction .00). Key recombination events now place this QTL within a 1–2-cM interval spanning ~1.5 Mb between D6S270 and D6S1626. Furthermore, haplotype analysis has led to a reevaluation of the genealogy and to the identification of additional relationships in the kindred.

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 unevenly distributed and restricted to a subset of erythrocytes named "F cells" (FC) (Boyer et al. 1975). Surveys of healthy blood donors in several population groups show that the levels of Hb F and FC vary considerably (as much as 20-fold), with 10%–15% of the population having ⩾4.5% FC, corresponding to ⩾0.6% Hb F (Zago et al. 1979; Miyoshi et al. 1988; Sampietro et al. 1992). The high FC and Hb F (1%–4% of total hemoglobin) levels are transmitted in the condition referred to as heterocellular "hereditary persistence of fetal hemoglobin" (HPFH) (Stamatoyannopoulos and Nienhuis 1994). Unlike the rare forms of HPFH, which are caused either by extensive deletions within the β-globin gene cluster or by point mutations in the γ-globin gene promoters, no mutations are identifiable within the β-globin complex in heterocellular HPFH. Furthermore, in many families, the high-FC phenotype segregates independently of the β complex, implicating the presence of *trans*-acting factors (Gianni et al. 1983; Giampaolo et al. 1984; Jeffreys et al. 1986; Martinez et al. 1989; Thein and Weatherall 1989). Recently, two such loci involved in the control of FC production have been mapped by linkage analysis. One locus has been mapped on chromosome 6q23 in an extensive inbred Asian Indian kindred with β thalassemia (Craig et al. 1996). The other locus (the FC-production, or FCP, locus) which is associated with variation in FC levels in sickle-cell disease, has been mapped to the Xp22.2-p22.3 region (Dover et al. 1992).

Apart from the two *trans*-acting loci, other factors known to influence Hb F/FC levels in adults include age (levels fall as one becomes older) (Rutland et al. 1983), gender (women have higher levels than men) (Miyoshi et al. 1988), a common sequence variation (T-C) at position −158 upstream of the $^{G}\gamma$-globin gene, which is detectable by the restriction enzyme *Xmn*I (the *Xmn*I-$^{G}\gamma$ site) (Gilman and Huisman 1985; Sampietro et al. 1992), and the inheritance of β thalassemia (Weatherall and Clegg 1981). Thus, several quantitative-trait loci (QTLs) are involved in the control of Hb F/FC levels after birth.

Previously, the 6q gene was positioned within an interval of 11 cM between the anonymous markers D6S408 and D6S292, with tight linkage to D6S976 (LOD score 6.3 at recombination fraction [θ] .00) (Craig et al. 1996). However, linkage of the QTL to 6q was evident in only one half of the kindred (pedigree A). A

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major QTL for FC could also be detected by segregation analysis in the other half of the kindred (pedigree B), but there was no evidence for linkage of that gene to any of the markers in the 6q interval, and genetic heterogeneity was concluded.

We have studied an extra 57 family members (bringing the total analyzed to 210) and have genotyped the kindred for an additional 26 microsatellite markers in the 6q22.3-q23.2 region (Dib et al. 1996). Segregation analysis confirmed that the FC trait in both halves of the kindred (pedigrees A and B) is significantly influenced by three genetic factors: β thalassemia, the *Xmn*I-$^{G}\gamma$ site, and the 6q gene. Multipoint linkage analysis, together with haplotype analysis, positions the QTL within an interval of 1–2 cM between D6S270 and D6S1626. Haplotype analysis confirmed that the kindred, including pedigrees A and B, is descended from a common ancestor dating back seven generations.

Families and Methods

Family Data

The kindred is of Asian Indian origin and consists of 210 individuals, but 3 members (the proband and 2 β thalassemia homozygotes) with extreme FC values (100%) were excluded from analysis; thus, a total of 207 individuals were studied. Heterocellular HPFH, defined by an immunological FC assay, has been observed to cosegregate with β thalassemia and the *Xmn* I-$^{G}\gamma$ site in the family (Thein et al. 1994). The kindred was composed of two units, pedigrees A and B, initially thought to be unrelated; two siblings in pedigree B are married to individuals belonging to different generations in pedigree A. Subsequently, instigated by haplotype analysis, further inquiries revealed that family members of pedigrees A and B can be traced to a common ancestor, dating back seven generations. Figure 1 shows the interrelationship between the two pedigrees. For details on the pedigrees, see the work of Thein et al. (1994).

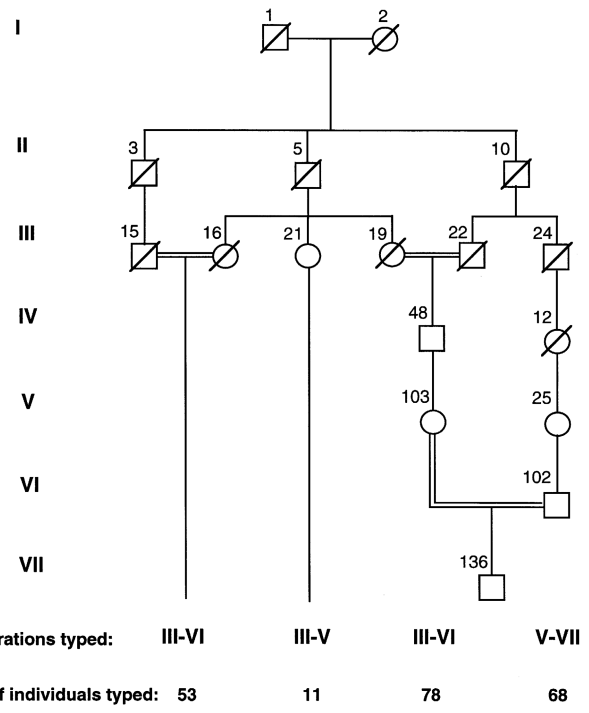

Figure 1 Family branches in the seven-generation Asian Indian kindred (Thein et al. 1994). Individuals 15 and 16 and individuals 19 and 22 are first cousins belonging to the third generation of the kindred. Descendants of the other founders (individuals 15, 16, 19, 21, and 22) form the subpedigree designated "A," whereas descendants of individual 24 form the subpedigree designated "B" (Thein et al. 1994). Individual 102 is a great-grandson of the founder (individual 24) of pedigree B, and his marriage to individual 103 in pedigree A represents the earliest joining of the two pedigrees. In the initial analysis, pedigree B was thought to be essentially unrelated to pedigree A, since two unrelated married-in siblings in pedigree B were married to two other individuals belonging to different generations descended from individuals 19 and 22. Although a total of 210 individuals were typed, only 207 were analyzed.

Phenotyping

FC assays were performed on peripheral blood by microscopy and/or fluorescence-activated cell sorting using a monoclonal mouse anti–γ-globin chain antibody (Thorpe et al. 1994). The T-C polymorphism at position -158 of the $^{G}\gamma$-globin gene was determined by two approaches: hybridization of *Xmn*I-digested DNA with a γ probe (*Hin*dIII 3.3-kb $^{A}\gamma$) (Gilman and Huisman 1985) and/or *Xmn*I restriction analysis of the $^{G}\gamma$-globin promoter region amplified by PCR (Craig et al. 1993). Hematologic data were obtained, by standard methods, on blood samples freshly collected in EDTA. Levels of Hb A2 were determined by elution following cellulose acetate electrophoresis, and Hb F levels were determined by alkaline denaturation (Sharpe and Wood 1989). The diagnosis of β thalassemia was made from a combination of the results of the full blood counts, Hb A2 and Hb F levels, and ferritin values; and the mutations were characterized by DNA analysis (Thein et al. 1994).

Genotyping

The kindred was genotyped for 26 additional microsatellite markers that mapped within the interval spanned by D6S408 and D6S292. Of these 26 markers, 11 were uninformative in the family. A total of 15 markers in this interval were analyzed; the order of these markers (cen-D6S408-D6S407-D6S1620-D6S1572-D6S435-D6S262-D6S457-D6S413-D6S975-D6S1722-D6S1038-D6S976-D6S270-D6S1626-tel) was determined on the basis of a combination of published sources

(Dib et al. 1996), authors' unpublished results, and analysis of the family presented here.

Genotyping was performed as described elsewhere (Craig et al. 1996). Microsatellites were amplified by PCR and were analyzed on 7 M urea/6% polyacrylamide gels. The PCR conditions were 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 45 s and a final extension of 72°C for 2 min. The separated PCR products were transferred onto positively charged nylon membranes (Hybond N^+; Amersham) and hybridized with radio-labeled $(CA)_n$ primers. Hybridizations were performed at 42°C for 3 h in 7% polyethylene glycol (PEG 6000 or 8000) and 10% SDS. Membranes were washed once in 2 × SSC and 0.1% SDS, at room temperature for 10 min. Genotypes were scored relative to those of an individual (134702) from the CEPH panel.

Statistical Analysis

Combined segregation/linkage analysis.—Although the sample can be represented as a single kindred of seven generations, we choose to divide the family into six groups, as previously described, because the computer programs used in the analysis do not currently accept extended families with a high degree of consanguinity. Segregation parameters and θ values were estimated simultaneously by maximum likelihood under both a mixed model and a major-gene model (Morton and Maclean 1974), by the Pedigree Analysis Package (PAP), version 4.0 (Hasstedt 1994). In our analysis, the mixed model included nine parameters: (1) the frequency of the allele responsible for high ln(FC) values, *q;* (2–4) three genotype-specific means—μ_{AA}, μ_{Aa}, and μ_{aa}—with μ_{aa} representing the high-trait homozygote; (5) the within-genotype variance, expressed as SD; (6) the fraction of the within-genotype variance attributable to polygenes, or "polygenic heritability"; (7 and 8) regression coefficients for the covariates (β thalassemia and *Xmn* I-$^G\gamma$); and (9) the θ between the marker and the QTL. The major-gene model assumes that all the genetic variability in the trait can be accounted for by a single QTL; thus, the polygenic heritability parameter is fixed at zero, and the model only assumes eight parameters. All models assume Mendelian transmission with transmission probabilities τ_1, τ_2, and τ_3 fixed at 1.00, .50, and .00, respectively. The segregation/linkage analysis was conducted with the marker D6S976. Maximum-likelihood estimates of the allele frequencies for D6S976 were calculated from the data and were fixed in the subsequent analysis. ln(FC) values were preadjusted for the effects of age and gender, by the generalized linear model (GLM) procedure of SAS (1997). A single-point LOD score was calculated as the difference in the log10 likelihood between the best-fitting model including all the parameters above and the same model in the unlinked state ($\theta = .5$).

Multipoint linkage analysis.—The 15 markers used in the analysis were ordered, and genetic distances were determined, on the basis of published and unpublished genetic- and physical-map resources and on the basis of the data as used by CRIMAP (Green et al. 1990). Recombination events between the ordered markers were identified by use of the CRIMAP and SIMWALK (Sobel and Lange 1996) programs. No multiple recombination events were present within the map of ordered markers. The LINKMAP program of VITESSE (O'Connell and Weeks 1995) was used for the nine-point linkage analysis. Because VITESSE does not currently allow for either a polygenic-heritability parameter or covariate effects, ln(FC) values were preadjusted for the effects of the β thalassemia and *Xmn*I-$^G\gamma$ genotypes and for sex and age, by use of the GLM procedure of SAS (1997), and multipoint analysis was conducted under the major-gene genetic model. The multipoint LOD score is calculated as a ratio of maximum likelihoods, analogous to the single-point case: $Z(x) = \log[L(x)/L(\infty)]$, where x is the position of the trait at a specified point in the ordered marker map and $x = \infty$ denotes the infinite position where the test locus is unlinked to all the markers (Lathrop et al. 1985). The multipoint location score is a multiple of the multipoint LOD score: $S(x) = 2\ln(10)Z(x)$, where $2\ln(10) \approx 4.6$.

QTL genotype probabilities and haplotype analysis.—The genetic-model parameters were used to estimate probabilities of each individual's QTL genotypes, conditional on the individual's and his or her relatives' ln(FC) values and genotypes at marker D6S976. Individuals were assigned the most probable ln(FC) genotype—AA, Aa, or aa. Haplotype assignment was conducted for 15 markers after the manual editing of results from the SIMWALK program. Haplotypes were constructed blind to the individuals' ln(FC) values. The kindred was represented as a single family unit in the haplotype analysis with the consanguineous relationships.

Table 1

Genetic-Model Parameters and Segregation/Linkage Analysis of D6S976 and ln(FC)

Segregation Model	P	μ_{AA}	μ_{Aa}	μ_{aa}	SD	β_T	β_X	h^2	θ	−2 ln(L)	LOD Score
Mixed	.74	−3.88	−2.82	−1.94	.46	1.00	.29	.65	.054	920.92	12.41
Major gene	.71	−3.82	−2.83	−2.00	.43	1.02	.35	.00	.027	933.73	11.64

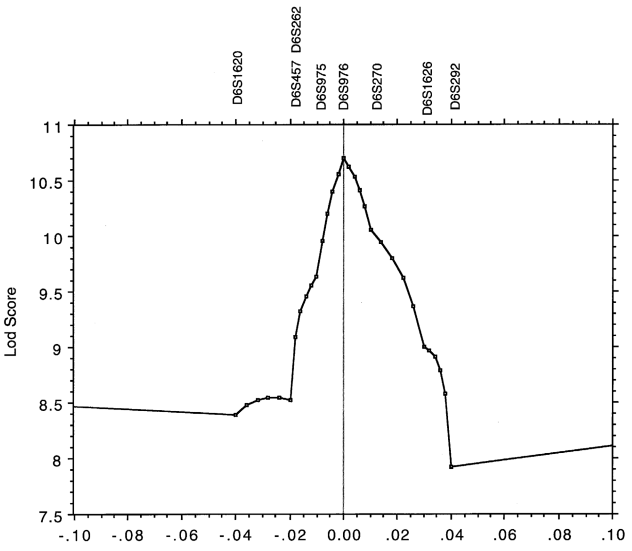

Figure 2 Plot of multipoint LOD scores over a 2-cM interval. Locations of eight markers fixed in the analysis are given at the top. Recombination distances, along the bottom, are relative to marker D6S976.

The ln(FC) locus was ordered within a region of the haplotype, on the basis of data for recombinant offspring from parents with informative ln(FC) genotypes.

Results

Genealogy

In figure 1, individuals 22 and 19 are first cousins and are members of what has been defined as pedigree A (Craig et al. 1996). Individuals 22 and 19 are in the third generation of pedigree A. Descendants of individual 24 (brother of individual 22) in the third generation are members of what has been defined as pedigree B, whereas the rest of the kindred are from pedigree A. Individual 102 is a son of the founders of pedigree B, and his marriage to individual 103 represents the earliest link between the two pedigrees.

Linkage Analysis

Table 1 shows the results of the combined segregation/linkage analysis, both under the mixed model and under the major-gene model. The central position of the heterozygote mean relative to the homozygote mean indicates a codominant effect. The low-trait homozygote mean was ~4.2 SD from the high-trait homozygote mean and 2.3 SD from the heterozygote mean, indicating considerable overlap between the genotype-specific distributions (table 1). Single-point LOD scores were 12.41 and 11.64 under the mixed and major-gene models, respectively. The major-gene–model parameters were used in the nine-point linkage analysis of the adjusted ln(FC) values, with a fixed map of eight markers (fig. 2). There were no crossover events observed between markers D6S457 and D6S262 in the six pedigrees. Marker D6S976 had the highest multipoint LOD score, 10.71. A confidence interval of 1 LOD unit below the peak of the multipoint plot spans ~3 cM and includes markers D6S975, D6S976, and D6S270 and ~1 cM centromeric, toward D6S1626.

Haplotype Analysis

Haplotypes were constructed from 15 markers spanning ~16 cM and subsequently were screened for recombinant chromosomes. Allele 8 at marker D6S976 was the first to be identified as being associated with a high trait value (table 2). Haplotype analysis identified a chromosome segment that had the associated allele at D6S976 and that showed the clearest segregation with the high trait value. Individuals were assigned their most probable QTL genotypes. Seventy-one percent of QTL genotypes were assigned with a probability >.90. Recombinants for the associated chromosome reduced the candidate region to ~5 cM between the telomeric side of D6S262 and the centromeric side of D6S1626. Recombinant individuals 50 and 133 in figure 3 define the telomeric boundary. Individual 49 is a married-in founder in the kindred and, at 13 of the 15 markers, carries a haplotype with the same alleles as are seen in the high trait–associated haplotype. The conserved region is con-

Table 2

Probabilities of D6S976 Haplotype and of QTL Genotype, for the Three-Generation Family Depicted in Figure 3

Individual	D6S976 Haplotype	*P*(AA)	*P*(Aa)	*P*(aa)
19	8 | 4	NA	NA	NA
22	8 | 6	NA	NA	NA
40	6 | 4	.9989	.0000	.0011
42	8 | 8	.0000	.0021	.9979
43	8 | 8	.0000	.0002	.9998
44	4 | 6	.9973	.0000	.0026
45	8 | 4	.2050	.7155	.0795
47	6 | 4	.9985	.0015	.0000
48	8 | 4	.0000	.9735	.0264
49	8 | 4	.9996	.0000	.0004
50	8 | 8	.0000	.0116	.9884
53	6 | 8	.0000	1.0000	.0000
54	6 | 8	NA	NA	NA
57	6 | 4	.9283	.1194	.0000
88	4 | 4	.0236	.9678	.0086
89	8 | 4	.0000	.9976	.0024
102	4 | 4	.0030	.9921	.0049
103	8 | 8	.0000	1.0000	.0000
104	4 | 8	.9218	.0436	.0346
105	4 | 4	.9647	.0135	.0217
133	4 | 4	.9618	.0199	.0183
136	4 | 8	.0046	.9448	.0505
175	4 | 8	1.0000	.0000	.0000

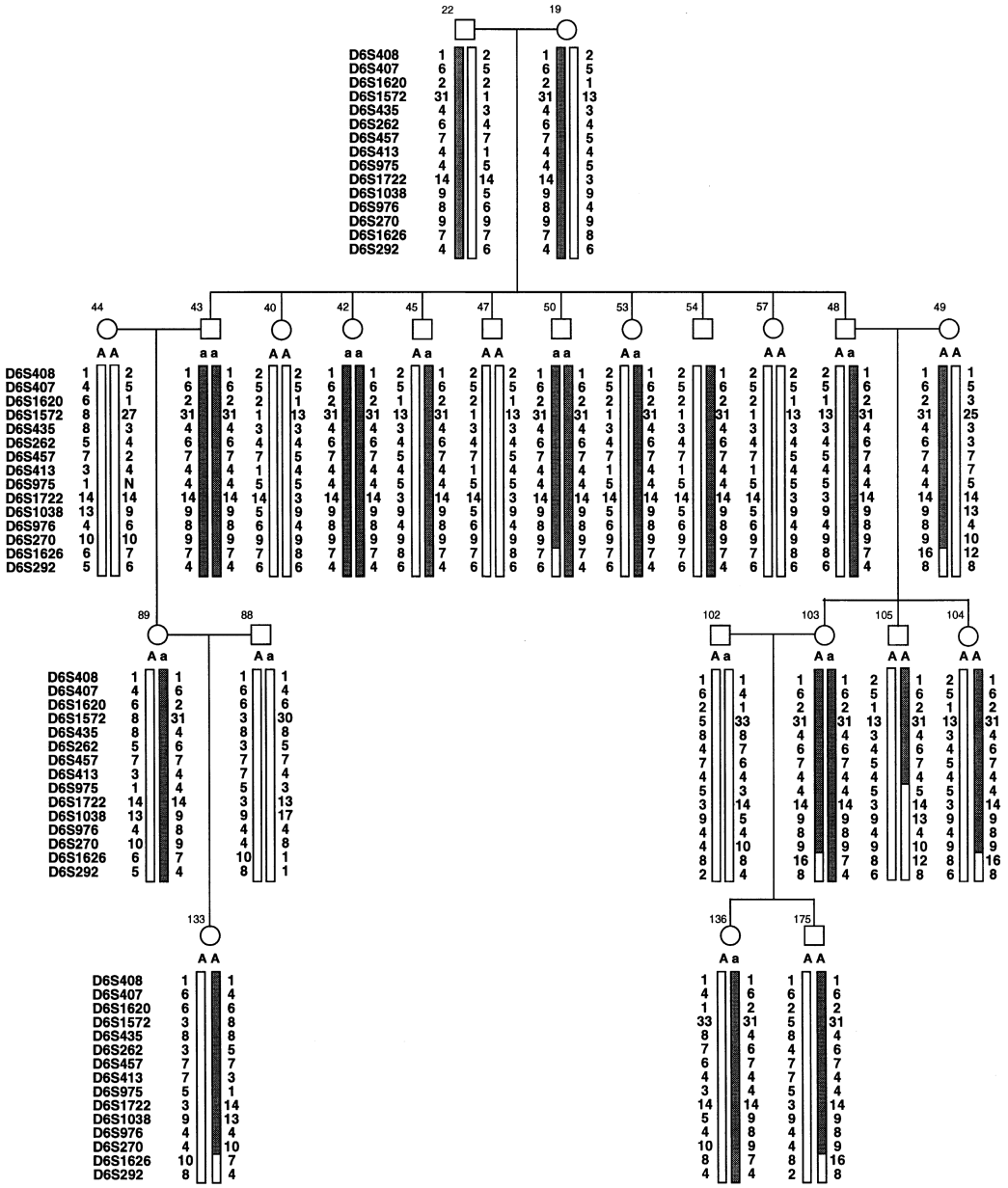

Figure 3 Twenty-three members of the complete pedigree, showing segregation of haplotypes and informative recombination events. ln(FC) genotype probabilities are given directly below the individuals' identification numbers, with an "a" representing the trait-increasing allele. Gray-shaded boxes represent the haplotype associated with the high trait value. The recombination in individuals 50 and 133 defines the telomeric boundary of the candidate region, whereas the centromeric boundary is defined by the recombination in individual 49, narrowing the region of the QTL to between markers D6S270 and D6S1626. The recombinant haplotype in individual 49 is not associated with a high trait value, as is confirmed in individuals 103, 104, and 175, who have inherited this recombinant haplotype from individual 49. Individual 54 is a homozygote for β thalassemia, with 100% FC and, consequently, is not included in the analysis. Although individual 102's genotype is Aa, the a allele is derived from a different lineage, which is unlinked to 6q (see text).

tinuous and extends from the centromeric end of the haplotype to marker D6S270. Considering the high observed heterozygosity within the kindred (table 3), the relatively high degree of inbreeding, and the founding nature of the population, it is most likely that individual 49 is a carrier of a haplotype that is of an ancestry common to the high trait–causing haplotype. Individual 49 has an AA genotype at the ln(FC) locus, narrowing the region of the high trait–causing locus to 1–2 cM between markers D6S270 and D6S1626. Only 1 cM

Table 3

Genetic Map and Observed Heterozygosity of 15 Markers on Chromosome 6q

Locus	Cumulative Genetic Distance (cM)	Observed Heterozygosity
D6S408	.00	.45
D6S407	5.30	.74
D6S1620	6.40	.67
D6S1572	8.40	.72
D6S435	8.40	.87
D6S262	8.40	.70
D6S457	8.40	.70
D6S413	8.40	.60
D6S975	9.50	.74
D6S1722	9.50	.52
D6S1038	9.50	.50
D6S976	10.50	.74
D6S270	11.50	.72
D6S1626	13.50	.70
D6S292	14.00	.75

telomeric of D6S270 lies within the 1-LOD-unit multipoint confidence interval. Allele 8 at marker D6S976 is present only on the high-trait chromosome, except in the case of individuals 49, 103, 104, and 175 (individuals 103, 104, and 175 are descendants of individual 49; fig. 3). This is reflected in the high LOD score at D6S976, whereas allele 9 at marker D6S270 is a common allele in the kindred. Although individual 102 is a heterozygote at the QTL genotype, he has not inherited a copy of the high trait–causing haplotype. This individual is a member of pedigree B and has inherited his QTL genotype through a lineage that is unlinked to chromosome 6q. The QTL genotypes in pedigree B indicate that the a allele in individual 102 was maternally inherited. Individual 175 has inherited this grandmaternal allele but has only a 2% FC level, providing evidence that other unlinked genes may be involved in the determination of FC levels.

Discussion

The results presented here confirm the initial localization of an Hb F/FC QTL on chromosome 6q23. Heterocellular HPFH is a multifactorial quantitative trait, and we have mapped the QTL to a small region, using techniques that take advantage of the extended, consanguineous kindred. Multipoint linkage analysis and haplotype analysis have reduced the candidate region from 11 cM to an interval of 1–2 cM between markers D6S270 and D6S1626. The two methods show concordance in results even though the multipoint analysis was conducted on a divided pedigree and the haplotype analysis considered all the family relationships. The 1-LOD-unit confidence interval of the multipoint-linkage curve spans ~3 cM and has a 1-cM region in common with the haplotype location.

Although there was considerable overlap in the ln(FC) genotype-specific distributions, the extended-family information allowed for assignment of QTL genotypes with a high degree of confidence. With the inheritance pattern of 15 tightly linked, phase-known markers defined over six generations, we were able to unambiguously map the QTL by assigning phenotype to haplotype. Any marker between D6S270 and D6S1626 that is found to be informative in individual 49 will test the candidacy of part of the region. We are currently isolating new polymorphic sequence variants—biallelic or short-sequence tandem repeats—in the candidate region and are typing the key individuals for the newly generated markers.

Preliminary physical mapping has identified three overlapping YAC clones spanning this interval, estimated to be ~1.5 Mb, which has been confirmed by FISH analysis of bacterial artificial chromosome clones containing the flanking markers on interphase spreads. At present, the physical map of this 6q region is relatively sparse; the region spanned by D6S270 and D6S1626 contains seven unique loci identified as expressed sequence tags (ESTs) (from dbEST at the National Center for Biotechnology Information, Bethesda). cDNA clones for two of these loci have been isolated; one encodes a novel protein kinase designated "MAP-KKK 5" (mitogen-activated protein kinase kinase kinase 5), and the other (human c-myb proto-oncogene) encodes a 72-kD protein, c-myb protein, highly homologous to the corresponding chicken and mouse proteins. These ESTs are currently being investigated as candidates, by DNA sequence analysis, in a panel of selected members of the pedigree.

Our results provide significant evidence for the presence, on chromosome 6q23, of a QTL modulating Hb F and FC production. It is not clear whether the effect (*a*) lies in a *trans*-acting factor that directly interacts with the γ-globin genes or (*b*) is indirect (e.g., protein-protein) but still specific for the γ-globin genes (Crossley and Orkin 1993). Alternatively, the 6q gene could also code for a factor involved in the erythroid-maturation pathway leading to a premature commitment of progenitor cells and increased FC (and Hb F) levels. Because high levels of Hb F are clearly beneficial to patients with sickle-cell disease or and β thalassemia (Blau and Stamatoyannopoulos 1994), identification of this gene could provide insights into *trans* components involved in hemoglobin switching and could lead to development of novel approaches for the pharmacological reactivation of Hb F.

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