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Replication of linkage of familial hypobetalipoproteinemia to chromosome 3p in six kindreds

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Abstract **Familial hypobetalipoproteinemia (FHBL) is a genetically heterogeneous condition characterized by very low apolipoprotein B (apoB) concentrations in plasma and/or low levels of LDL-cholesterol (LDL-C) with a propensity to developing fatty liver. In a minority of cases, truncation-specifying mutations of the apoB gene (*APOB*) are etiologic, but the genetic basis of most cases is unknown. We previously reported linkage of FHBL to a 10 cM region on 3p21.1-22 in one kindred. The objectives of the current study were to identify other FHBL families with linkage to 3p and to narrow the FHBL susceptibility region on 3p. Six additional FHBL kindreds unlinked to the *APOB* region on chromosome 2 were genotyped with polymorphic markers spanning a region of approximately 13 cM on chromosome 3. Quantitative linkage analyses indicated that the FHBL in these families was linked to 3p21.1-22. Haplotype analysis identified several meiotic crossover events, allowing us to narrow the critical region from 10 cM to 2.0 cM, between markers D3S2407 and D3S1767.**—Neuman, R. J., B. Yuan, D. S. Gerhard, K-Y. Liu, P. Yue, S. Duan, M. Averna, and G. Schonfeld. **Replication of linkage of familial hypobetalipoproteinemia to chromosome 3p in six kindreds.** ***J. Lipid Res.*** **2002.** 43: **407–415.**

Supplementary key words linkage analysis • genetic • Markov chain Monte Carlo • variance components • oligogenic

Familial hypobetalipoproteinemia (FHBL) is a disorder of lipoprotein metabolism characterized by extremely low levels of LDL-cholesterol (LDL-C) and/or apolipoprotein B (apoB), segregating as an autosomal dominant trait (1–3). Mutations of the apoB gene (*APOB*) on chromosome 2p23-24 are etiologic for FHBL. These mutations result in a variety of truncated forms of apoB protein that are designated according to a centile nomenclature (4). The normal forms in plasma are apoB-100 consisting of 4,536 amino acids, and apoB-48, a product of apoB mRNA editing that is 2,152 amino acids or 48% of the full-length protein. In humans, apoB-100 is secreted by the liver in VLDL particles and apoB-48 is secreted by the small intestine in chylomicrons (2). Approximately 35 different truncations ranging from apoB-2 to apoB-89 have been described. Those longer than apoB-26 are usually detectable in plasma, whereas shorter forms, although secreted by liver and intestine, are not (5). Truncation-producing mutations of *APOB* are found only in a small minority of kindreds with FHBL (6–8), and the genetic etiologies are not well understood in the overwhelming majority of cases (7). However, it is clear that FHBL is genetically heterogeneous (9–12). Fazio et al. (13) and Pulai et al. (10) reported on 2 FHBL families in which linkage to the apoB gene was ruled out. We reported linkage of FHBL to a 10 cM region of 3p21.1-22 in one of those families, the F-family (11), suggesting that a FHBL susceptibility gene may reside at that location.

As part of an ongoing investigation seeking to discover the genetic etiologies of FHBL, we have been ascertaining and studying families with low plasma concentration of LDL-C and apoB. We analyzed 21 hitherto unreported kindreds with FHBL in which no unusual truncated forms of apoB were present in plasma. Initially, we had sought linkage to chromosome 2p (the well-known locus at or near *APOB*) by genotyping in the appropriate regions and performing linkage analysis (Pin et al., unpublished observations). Quantitative trait loci (QTL) analysis indicated that 7 of the 21 pedigrees were not linked to the apoB region. These families were then genotyped with markers mapped to 3p21.1-22. Quantitative linkage analysis indicated that six of these pedigrees were linked to this region, confirming and extending our original observation (11). To further narrow the susceptibility locus, we performed haplotype analysis in one of the pedigrees with crossovers in the critical region. These analyses implicated a 2 cM region on 3p that may harbor a genetic susceptibility locus for FHBL.

Abbreviations: apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; BMI, body mass index; FHBL, familial hypobetalipoproteinemia; QTL, quantitative trait loci.

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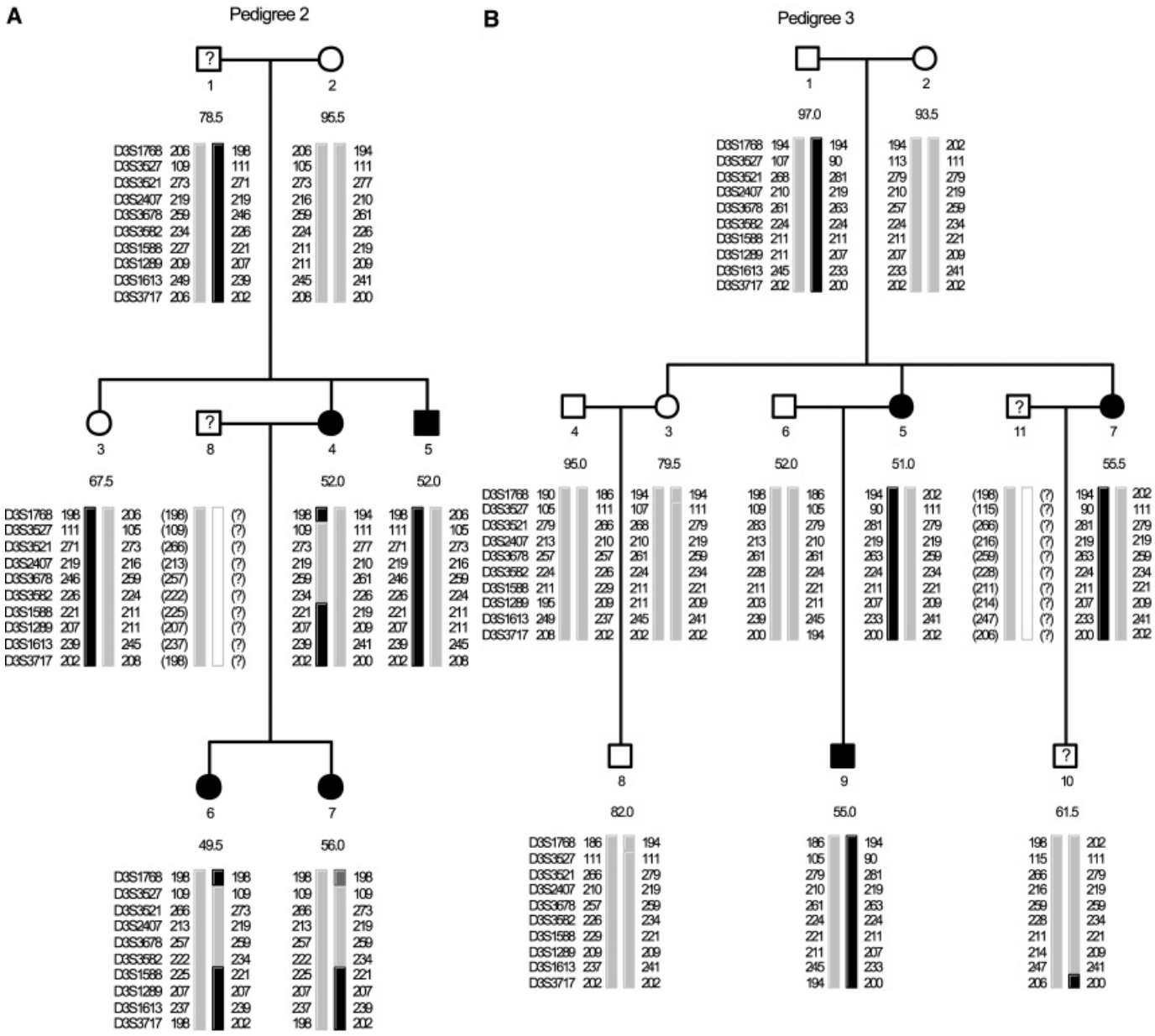

Fig. 1. Familial hypobetalipoproteinemia (FHBL) kindreds, showing haplotypes consisting of 10 markers on chromosome 3p in pedigrees 2, 3, 7, 9, and 50. Pedigree 4 haplotypes include two additional markers. ApoB levels are given above the haplotype symbols. Solid symbols denote subjects with apoB levels in the lower 5% for age and sex. Question marks denote borderline or unknown apoB levels. The black bars represent purported haplotypes segregating with affected FHBL subjects.

MATERIALS AND METHODS

Pedigrees

Ascertainment of potential probands with FHBL occurred by screening volunteer populations in the metropolitan areas of St. Louis, MO, for individuals with total plasma cholesterol levels below 150 mg/dl. Probands provided information on their own health status and on the structure of their kindred. Members of 21 pedigrees were invited to participate based on the presence of two or more affected individuals in two or more generations, the absence of truncated forms of apoB in plasma on immunoblotting (14), the absence of diabetes mellitus, thyroid, liver, and kidney diseases, and a willingness to participate. None of the participants was taking medications known to affect lipid metabolism. The Human Studies Committee of the Washington University Medical Center approved all protocols and informed consent procedures.

Initially, the 21 families were genotyped for seven markers in the apoB region on chromosome 2. Linkage analysis indicated that seven of these pedigrees were not linked to the apoB gene (Pin et al., unpublished observations). These families were then genotyped using ten chromosome 3 polymorphic tetra-, tri-, or di-nucleotide markers in a 13 cM region around marker D3S3678, the area of maximum linkage previously identified in the F-kindred (11). Marker order was obtained from the Marshfield Medical Research Foundation's Web site (http://www.marshfieldclinic.org/research/genetics/) (15). Preliminary analysis indicated that one family was not linked to chromosome 3 and was dropped from further consideration.

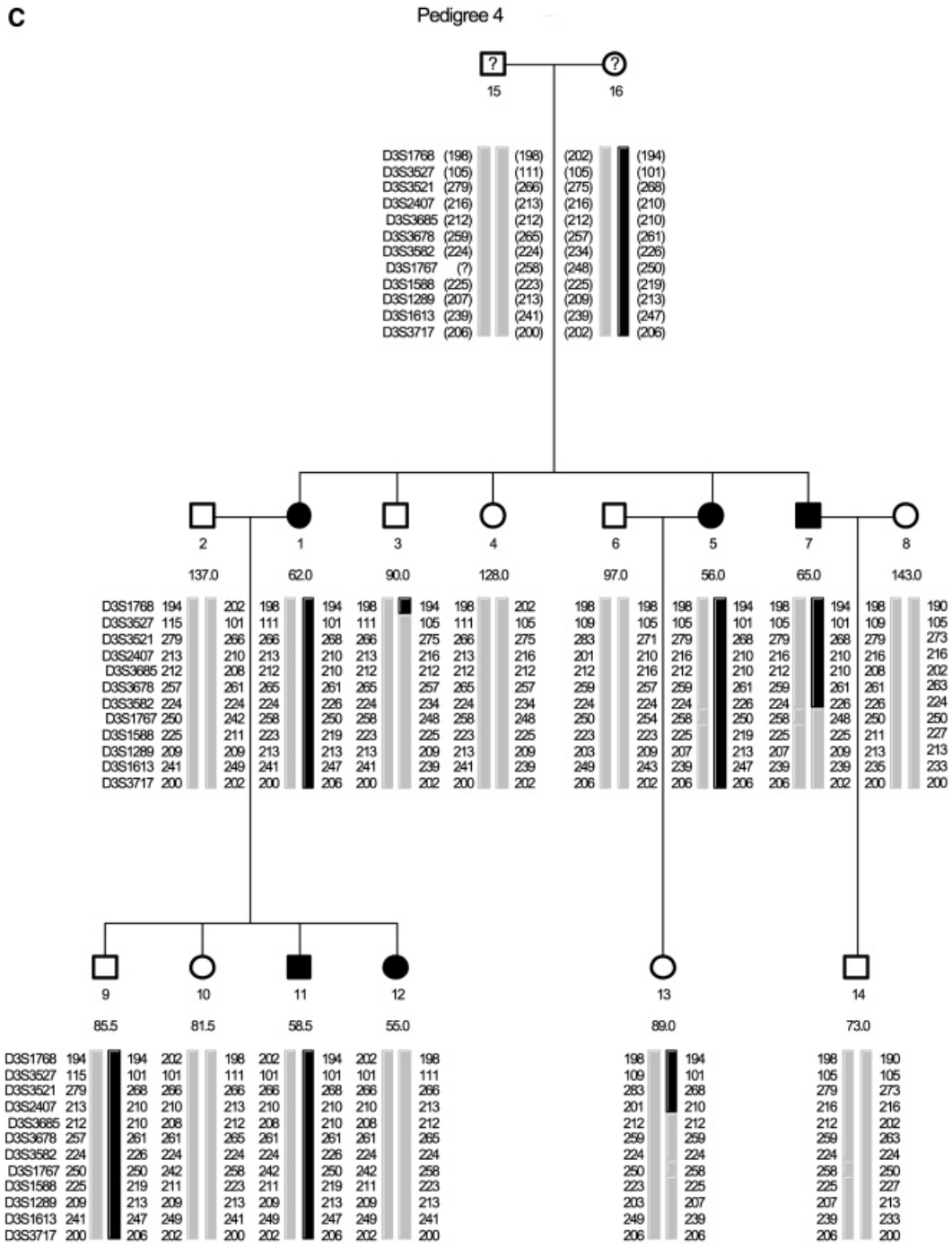

Fig. 1. (*Contined*)

Laboratory procedures

Lipid and apolipoprotein analyses. Bloods were drawn after 12 h of fasting into EDTA tubes (1 mg/ml). Plasmas were analyzed for total TGs and cholesterol using enzymatic methods (Wako Cholesterol and TG kits) in the Core Laboratory for Clinical Studies, which is CDC-standardized and monitored. VLDL, LDL, and HDL were isolated by ultracentrifugation and dextran sulfate precipitation (16), and their TG and cholesterol contents assessed by enzymatic methods as above. The coefficients of variation of the lipid assays are <3%. Total plasma apolipoprotein (apo)A-I and apoB concentrations were determined by immunonephelometry [coefficients of variation (CVS) of 7% and 8%] (17). Aliquots of whole blood were frozen for DNA analysis.

Genotyping. Genomic DNA was extracted from the peripheral blood leukocytes using the Gentra Puregene™ DNA extraction kit. PCR amplifications were carried out with 30 ng of genomic DNA in a 15 ul reaction volume containing 3 pmol of each primer, 0.9 U *Taq* DNA polymerase (PE Applied Biosystem), 200 uM dNTPs, 1.5 mM $MgC1_2$, and PCR buffer (10 mM Tris-HC1, pH 8.3; 50 mM KC1; 1% Triton-X-100). The thermal cycling conditions were as follows: initial denaturation for 5 min at 95°C, followed by 30 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 30 s, and a terminal extension for 30 min at 72°C in a thermocycler

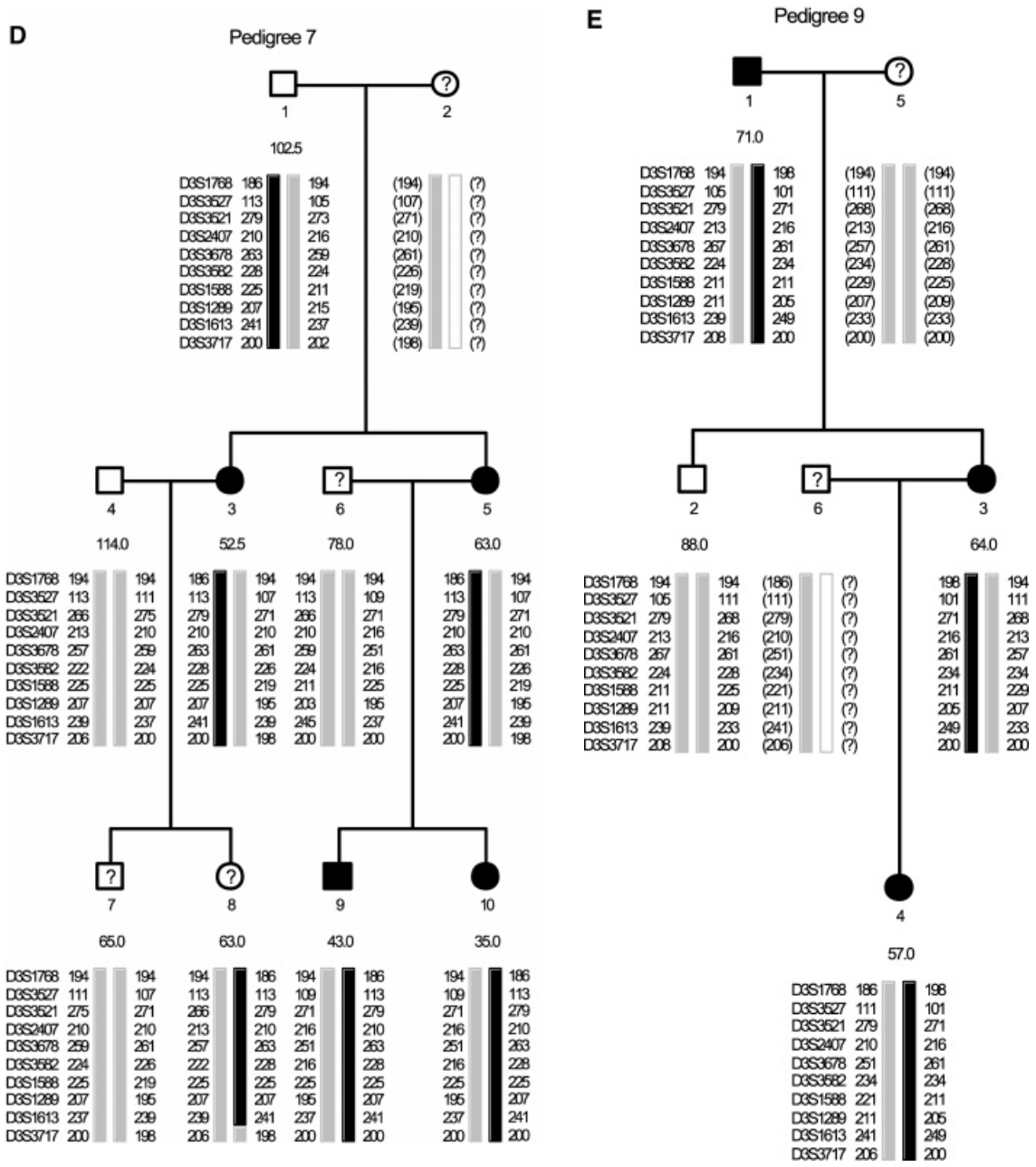

Fig. 1. (*Continued*)

(PTC-200, MJ Research). The PCR products were analyzed using ABI 377 automated DNA sequencer (PE Applied Biosystem) with the ABI PRISM GeneScan Analysis Software (V2.1.2 and 3.1 PE Applied Biosystem) and ABI PRISM Genotyper version 2.0 (PE Applied Biosystem). Allele sizes were determined using GENESCAN™ -500 Rox as a size standard (PE Applied Biosystem). Primer sequence information was obtained from the Genome Database (http://www.gdb.org/), GenBank (http://www.ncbi.nlm.nih.gov/Genbank/), and the Genethon database (http://www.genethon.fr/genethon_en.html). Genotypes were checked for typing errors with the GenoCheck program (18, 19).

Linkage analyses

Linkage analyses were performed using the computer programs SOLAR release 1.6.6 (20) and Loki 2.3 (21). Neither specifies a putative genetic model for the trait (such as dominant, additive, or recessive) with an accompanying penetrance function. However, the mathematical underpinnings of these two software packages are quite different. SOLAR conducts two point and multipoint linkage analysis within the variance component framework. Briefly, the goal of variance component analyses is to partition the observed variation of the trait into a sum of variances, each of which is genetic or environmental in nature. We proceeded by first comparing a polygenic model to a sporadic model (no genetic components). Logarithm of odds (LOD) scores were then computed to test the null hypothesis that the additive genetic variance due to a potential QTL equals 0, by comparing the maximum likelihoods between a model that assumes the presence of a trait-related QTL and an unlinked polygenic background, to a model without the susceptibility QTL. SOLAR assumes a normal distribution of the trait phenotype.

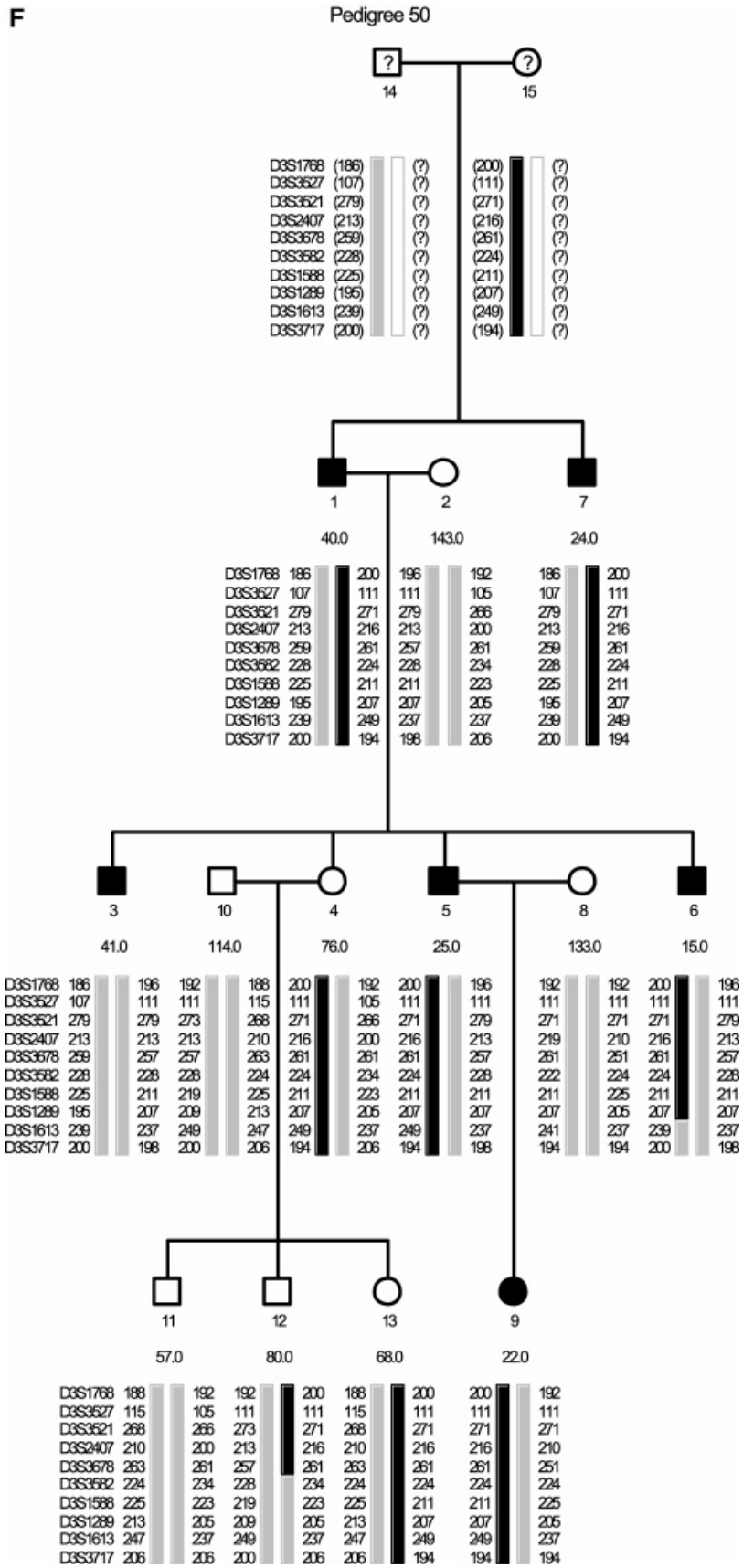

Fig. 1. (*Continued*)

Accordingly, all analyses were performed on the square root of the apoB level.

Loki performs multipoint linkage analysis on quantitative traits using oligogenic models (models with multiple putative QTLs) without assigning a fixed number of potential QTLs. The number of QTLs and their chromosomal locations are estimated using a sampling method called reversible jump Markov chain Monte Carlo (MCMC) (21). To determine linkage, a statistic known as the Bayes Factor (22) or L-Score is computed as follows: For each sampling iteration (300,000 iterations were used for each analyses), the prior probability of finding a QTL linked to a 1 cM bin is 1/t where t is the total map length of the genome (approximately 33 Morgans). If, for a particular iteration, there are n QTLs in the model, the prior probability, p, of at least one QTL located in the bin is $1 - (1 - 1/t)^n$. The posterior probability, q, is 1 or 0 depending on whether at least one QTL is located in the 1 cM bin. The Bayes Factor (L-score) for each bin is estimated by averaging q/p over all iterations. Regions that have a high probability of containing a QTL will have considerably elevated L-scores compared with surrounding regions. The Bayes Factors cannot be quantified in terms of a LOD score, but guidelines for estimating the importance of the Bayes factor are given by Kass and Raftery (22). Scores in the range of 3 to 20 are considered positive signals, 20 to 150 are considered strong, and over 150 very strong. Loki has successfully identified two known genes in an Alzheimer data set (23) and the trait genes in simulated complex traits (21, 24). Loki was initially executed 20–30 times, with different starting seeds, on all seven pedigrees to be sure about the consistency of the results. We then ran Loki multiple times with six pedigrees, leaving out one pedigree each time, to see if results would be influenced by the omission of any one pedigree. One pedigree significantly lowered the L-score. This pedigree was quite small with only four genotyped family members. Consequently, this pedigree was omitted from any further analyses. Sex and age were included as covariates in all analyses.

To identify crossover events, haplotypes were constructed using the computer program Simwalk (v. 2.6) based on MCMC and simulated annealing methods to compute the most likely haplotypes (25).

RESULTS

The six families included in our quantitative analysis study consisted of 57 genotyped individuals (**Fig. 1**, **Table 1**). For purposes of haplotype analysis (see below), pedi-

TABLE 1. Clinical characteristics of the FHBL families

Family	Status	Values	Age	BMI	Total Cholesterol	TG	LDL	ApoB	HDL	ApoA-I
			years	*kg/m²*			*mg/dl*			
2	Affected	Mean	19.4	20.6	130.4	66.3	67.0	52.4	52.3	128.8
	n = 4	Minimum	6.0	16.5	118.5	59.0	51.5	49.5	57.5	116.0
		Maximum	34.5	24.6	139.0	71.0	75.5	56.0	58.0	145.5
	Normal	Mean	50.0	30.1	168.8	123.5	100.5	81.5	45.8	134.3
	n = 2	Minimum	37.5	24.2	141.5	121.0	81.0	67.5	36.0	111.0
		Maximum	62.5	36.0	196.0	126.0	120.0	95.5	55.5	157.5
3	Affected	Mean	28.2	21.2	139.9	55.2	69.4	53.8	61.22	140.8
	n = 3	Minimum	9.0	20.9	137.3	44.7	66.3	51.0	60.5	133.0
		Maximum	38.0	21.5	143.5	69.0	73.0	55.5	62.7	149.5
	Normal	Mean	47.8	21.0	197.1	72.3	120.8	89.8	62.6	151.3
	n = 6	Minimum	10.0	14.6	176.0	50.0	110.5	79.5	46.5	128.0
		Maximum	72.0	25.8	231.0	117.0	142.0	97.0	80.5	182.5
4	Affected	Mean	46.7	23.2	133.7	76.4	75.5	59.3	44.0	121.1
	n = 5	Minimum	33.0	18.8	122.0	48.0	64.0	55.0	38.0	106.0
		Maximum	63.0	27.0	167.0	92.0	96.7	65.0	52.0	146.7
	Normal	Mean	44.2	25.0	186.8	176.8	114.4	102.7	37.5	110.2
	n = 9	Minimum	14.0	19.8	134.0	67.0	80.0	73.0	23.5	1.0
		Maximum	66.0	32.3	255.0	341.0	175.0	143.0	49.0	149.0
7	Affected	Mean	32.3	22.0	114.0	47.6	58.4	48.4	48.3	126.6
	n = 4	Minimum	18.0	20.0	98.5	31.0	47.5	35.0	44.5	117.0
		Maximum	47.0	25.1	130.0	61.0	73.5	63.0	52.5	132.0
	Normal	Mean	62.3	29.4	200.8	85.5	141.8	111.6	45.3	133.3
	n = 2	Minimum	48.5	26.6	192.5	69.0	138.5	110.0	42.5	118.0
		Maximum	76.0	32.2	209.0	102.0	145.0	113.5	48.0	148.5
9	Affected	Mean	53.3	24.2	136.7	77.0	73.0	64.0	48.0	145.0
	n = 3	Minimum	23.0	22.5	132.0	45.0	67.0	57.0	37.0	129.0
		Maximum	90.0	26.1	140.0	105.0	80.0	71.0	56.0	154.0
	Normal	Mean	50.0	29.7	157.0	126.0	100.0	88.0	32.0	130.0
	n = 1	Minimum	50.0	29.7	157.0	126.0	100.0	88.0	32.0	130.0
		Maximum	50.0	29.7	157.0	126.0	100.0	88.0	32.0	130.0
50	Affected	Mean	43.7	24.7	102.0	50.7	32.8	27.8	59.2	150.3
	n = 6	Minimum	11.0	18.2	66.0	10.0	20.0	15.0	26.0	100.0
		Maximum	82.0	30.7	139.0	89.0	45.0	41.0	82.0	215.0
	Normal	Mean	31.3	24.7	191.9	98.1	119.4	95.7	53.0	148.6
	n = 7	Minimum	9.0	18.1	124.0	27.0	70.0	57.0	44.0	121.0
		Maximum	63.0	29.8	272.0	161.0	185.0	143.0	62.0	181.0

TABLE 2. Two-point solar results

Marker	Location	LOD Scores	QTL[a] h^2	Residual[b] h^2
D3S1768	61.52	0.40	0.30	0.63
D3S3527	63.12	0.12	0.20	0.73
D3S3521	63.12	0.22	0.22	0.71
D3S2407	67.94	0.12	0.15	0.78
D3S3678	68.47	2.93	0.86	0.00
D3S3582	69.19	0.74	0.36	0.53
D3S1588	70.61	0.53	0.32	0.61
D3S1289	71.41	0.66	0.37	0.53
D3S1613	72.21	0.15	0.29	0.57
D3S3717	74.35	0.47	0.35	0.53

[a] Variance due to the QTL.
[b] Variance due to residual polygenes.

gree members with apoB levels ⩽5th percentile for age and sex (17) were considered affected. The FHBL affectation status of five genotyped individuals was declared unknown either because of borderline apoB levels or disparate apoB, and/or LDL-C concentrations on repeat determinations, probably due to biologic variation. (In general, the precision of apoB and LDL-C measurements is high. See the Materials and Methods section.)

Linkage analysis

Two-point and multipoint point analyses were conducted using 10 markers spanning 13 cM on chromosome 3 in the region of interest (**Table 2**). For both analyses the phenotype was defined to be the square root of the apoB level to reduce kurtosis. Because Loki is a MCMC method, analyses were repeated multiple times, each with different starting values. Positive L-scores were seen at approximately 69 cM, between D3S3678 and D3S3582, over all replicate runs. The average L-score for 20 runs was 30.4 (SD = 3.1), that is, the ratio between observed and expected (under the null hypothesis of no linkage) number of QTLs in a particular bin was, on average, over 30, considered to be a strong signal (22). **Figure 2** displays a representative histogram of map position versus L-score (Bayes Factor).

Results from the 2-point analysis using SOLAR were consistent with the Loki results. The LOD score obtained when comparing a polygenic model with a sporadic model (no genetic component) was highly significant ($P \leqslant 0.0004$), indicating the presence of genetic factors. Two-point linkage analysis gave the strongest evidence for linkage to a chromosome 3 QTL at marker D3S3678 located at 68.5 cM on the Marshfield map where the LOD score was 2.9 (Table 2). Multipoint linkage analysis with SOLAR yielded a maximum multipoint LOD score of 1.6 between markers D3S3678 and D3S3582 (**Fig. 3**). The QTL and residual heritabilities at this location were 0.6 and 0.3, suggesting that additional genetic factors may contribute to apoB variation.

Haplotype analysis

Haplotypes spanning approximately 13.4 cM on chromosome 3 were constructed using 10 polymorphic DNA markers (Fig. 4). Crossovers were found in family 4 (individuals 3, 7, and 13) that helped to define the borders of the critical region (**Fig. 4**). The telomeric border of the critical region was defined by a crossover in the mother of unaffected subject 13 of family 4 between D3S2407 and D3S3678. The centromeric border was defined by a recombination event in the parent of affected individual 7 between D3S3582 and D3S1588 within a span of 2.67 cM. Additional markers were subsequently genotyped in this region for family 4, resulting in narrowing the region to 2 cM between D3S2407 and D3S1767, a physical distance of approximately 6 million base pairs (Fig. 4). Unaffected subject 9, 40.5 years of age, had an apoB level of 85.5, placing him in the lower 25th percentile for his age and sex, also carried the affected haplotype, indicating unknown environmental or additional genetic factors influencing systemic apoB level. Thus, we have considerably narrowed the size of the critical region from our previous analysis (11).

DISCUSSION

We had previously reported that chromosome 3 contained a susceptibility gene for FHBL based on linkage analysis in a large multigenerational family. Twenty-one

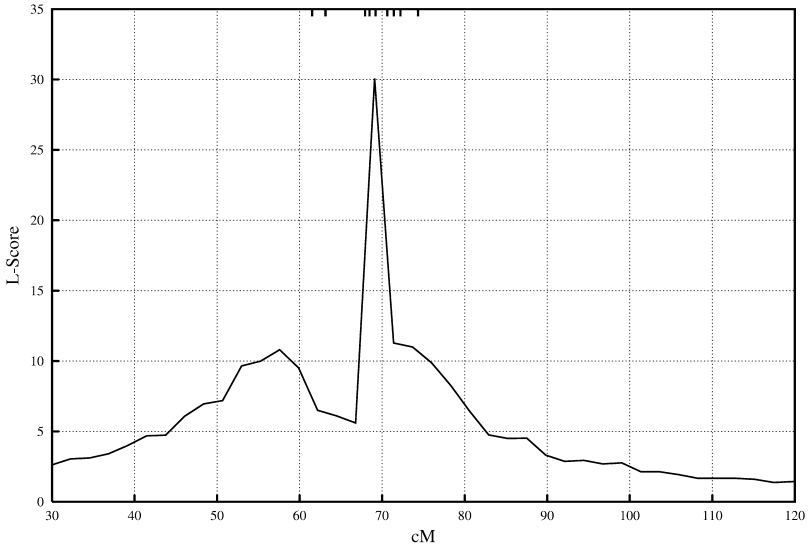

Fig. 2. Histogram of map position (cM) versus L-score. The tick marks at the top of the graph indicate the location of the markers used in the analysis. See Table 2 for marker names and locations.

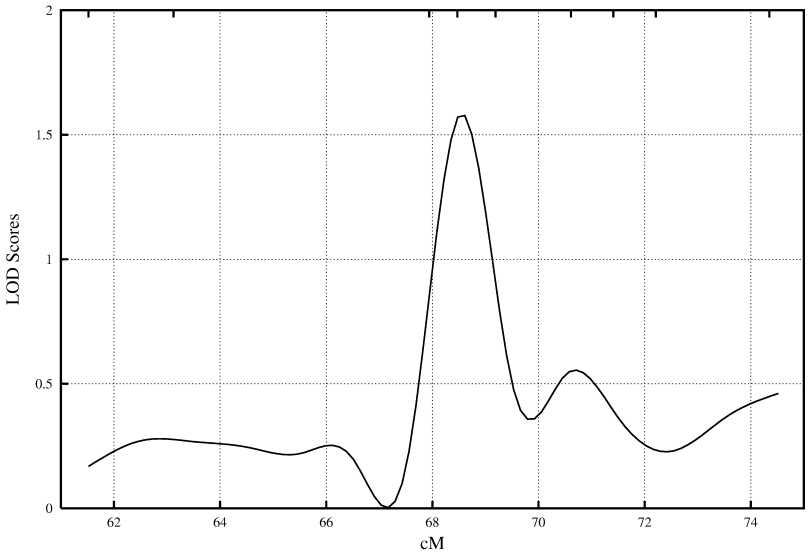

Fig. 3. SOLAR multipoint logarithm of odds (LOD) scores. The tick marks at the top of the graph indicate the location of the markers used in the analysis. See Table 2 for marker names and locations.

new kindreds with FHBL were identified as suitable for further study, based on our selection criteria as described. Seven of these gave no evidence of linkage to the *APOB* region on chromosome 2. We hypothesized that some of these families would be linked to chromosome 3. Significant evidence for QTL activity was detected on chromosome 3 for six of these families using two different QTL approaches. This confirms our previous finding of a susceptibility locus for FHBL at 3p21.1-22 (11).

Further analyses identified crossovers in family 4, permitting the narrowing of the critical region. Individual 13 in family 4 defined the telomeric border of the region near D3S2407. This was consistent with our previous report (11) in which individual 314 of the F-family had a crossover between D3S3521 and D3S2407, confirming that the telomeric border of the critical region was in the vicinity of D3S2407. Individual 7 of family 4 defined the centromeric border near D3S3582. Thus, we were able to narrow the region from 10 cM to 2 cM.

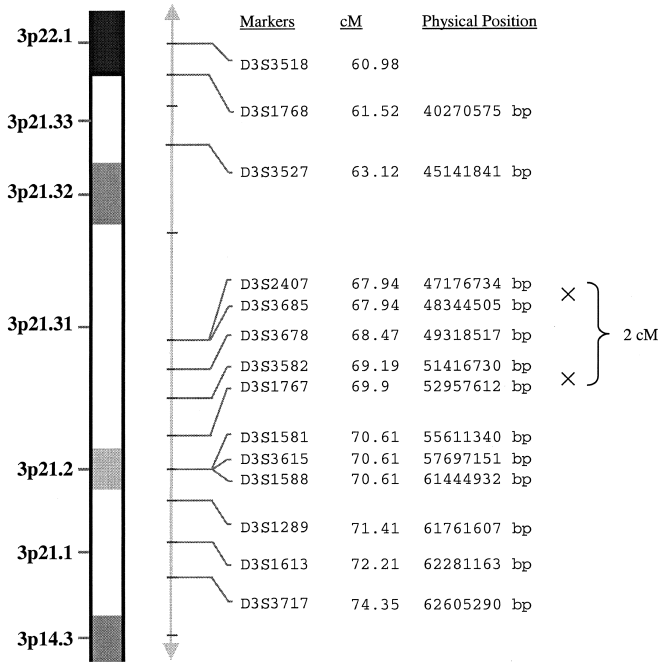

Fig. 4. Map of FHBL region taken from Marshfield integrated map of Santa Cruz physical map (*based on December 12, 2000, freeze). The recombination events occurred between polymorphic markers D3S2407 and D3S3685, and D3S3582 and D3S1767. The critical region is between D3S2407 and D3S1767.

A recent paper by Ko et al. (26) further supports the possibility of a susceptibility locus for FHBL with human chromosome 3. Using F2 and N2 (backcrossed) offspring of two mice strains that are high and low human apoB producers, they identified two quantitative trait loci as the major regulators of the protein levels. One locus was localized to mouse chromosome 6 and the other to chromosome 4. The mouse chromosome 6 region is syntenic with human chromosome 3p.

The importance of finding the genetic causes of low cholesterol is many fold. First, low cholesterol levels confer longevity and protection against coronary heart disease (27–29). Second, several reports have shown that subjects with FHBL may have increased susceptibility for developing fatty livers (30–36), including subjects from the F-family where no apoB defects are apparent and linkage to 3p21.1-22 has been reported (30). The predisposition to fatty liver appears to be due to the limited capacities of the liver in these individuals to export TGs in VLDL particles, as apoB-100 production rates are reduced to about 30% of normal rates (14, 37, 38). Similarly, there are elevated levels of TGs in livers of engineered mice bearing two different truncation-producing mutations of *APOB* (39, 40). In these mice, the limitation on hepatic TG export in VLDL particles also seems to be responsible for the fatty livers. The fatty liver of FHBL differs from the fatty livers seen in obesity and diabetes mellitus, which appear to be due to overproduction of TGs in liver (41). It is our hope that identifying the mutations causing FHBL will provide new insights into the assembly and secretion of VLDL particles, i.e., the hepatic TG-exporting system, and the complexities of fatty liver. It may also provide new opportunities in the prevention and treatment of coronary heart disease.

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