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Localization of Familial Benign Hypercalcemia, Oklahoma Variant (FBH_{Ok}), to Chromosome 19q13

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Summary

Calcium homeostasis by the kidneys and parathyroids is mediated by the calcium-sensing receptor (CaSR), which is located on 3q21-q24 and belongs to family C of the superfamily of G-protein coupled receptors that includes those for metabotropic glutamate, certain pheromones, and γ-amino butyric acid (GABA-B). Inactivating CaSR mutations result in familial benign hypercalcemia (FBH), or familial hypocalciuric hypercalcemia (FHH), whereas activating mutations result in hypocalcemic hypercalciuria. However, not all FBH patients have CaSR mutations, which, together with the mapping of another FBH locus to 19p13.3, suggests that additional CaSRs or second messengers may be involved. These may be identified by positional cloning, and we therefore performed a genomewide search, using chromosome-specific sets of microsatellite polymorphisms, in an Oklahoma family with an FBH variant (FBH_{Ok}), for which linkage to 3q and 19p had been excluded. Linkage was established between FBH_{Ok} and eight chromosome 19q13 loci, with the highest LOD score, 6.67 (recombination fraction .00), obtained with D19S606. Recombinants further mapped FBH_{Ok} to a <12-cM interval flanked by D19S908 and D19S866. The calmodulin III gene is located within this interval, and DNA sequence analysis of the coding region, the 5′ UTR, and part of the promoter region in an individual affected with FBH_{Ok} did not detect any abnormalities, thereby indicating that this gene is unlikely to be implicated in the etiology of FBH_{Ok}. This mapping of FBH_{Ok} to chromosome 19q13 will facilitate the identification of another CaSR or a mediator of calcium homeostasis.

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Introduction

Familial benign hypercalcemia[4] (FBH; MIM 145980) is a heritable disorder of mineral metabolism that is transmitted as an autosomal dominant trait with a high degree of penetrance (Chou et al. 1992; Brown 1997; Hebert et al. 1997). FBH is biochemically characterized by a lifelong elevation of serum calcium concentrations and is associated with inappropriately low urinary calcium excretion and, usually, a normal circulating parathyroid hormone (PTH) concentration (Brown 1997; McKusick 1998). Hypermagnesemia is also typically present. The disorder is considered to be benign, since patients with FBH are usually asymptomatic. However, an increased prevalence of chondrocalcinosis and possibly of pancreatitis (Pearce et al. 1996*a*) has been observed in adults, and some children may suffer from neonatal severe primary hyperparathyroidism (NSHPT), a life-threatening disorder associated with severe hypercalcemia, hypotonia, bone demineralization, fractures, and respiratory distress (Pollak et al. 1993; Pearce et al. 1995; Bai et al. 1997). The genetic abnormalities causing FBH and NSHPT in some patients involve loss-of-function mutations of the calcium-sensing receptor (CaSR) located on chromosome 3q21-q24; these FBH patients are heterozygous (wild type/mutant) for the CaSR mutations, and the NSHPT patients either may be homozygous for the CaSR mutations or have de novo heterozygous CaSR

[4] Familial benign hypercalcemia (FBH) has also been referred to as "familial hypocalciuric hypercalcemia" (FHH), "familial benign hypocalciuric hypercalcemia" (FBHH), "familial hypercalcemia," and "hypocalciuric hypercalcemia, familial" (HHC). In addition, three types of FBH are recognized: FBH1 (MIM 145980), FBH2 (MIM 145981), and FBH3 (MIM 600740) (or HHC1, HHC2, and HHC3, respectively; McKusick 1998). FBH3 is also referred to as "hypercalcemia, familial benign, Oklahoma type," "FBH, Oklahoma variant," or "FBH_{Ok}." The chromosomal locations of FBH1, FBH2, and FBH3 are 3q21-q24 (Chou et al. 1992), 19p13.3 (Heath et al. 1993), and 19q13 (this report), respectively. Neonatal severe primary hyperparathyroidism (NSHPT), which is also referred to as "neonatal severe hyperparathyroidism" (NSH), may arise in some families with FBH1 and represents a homozygous manifestation of the benign heterozygous disorder.

mutations. The great diversity of the reported CaSR mutations in FBH and NSHPT—which include nonsense, missense, deletion, and insertion mutations—together with their scattered locations throughout the 3,234-bp coding region (Brown 1997), have made it difficult to establish the use of CaSR-mutation analysis as a method for the diagnosis of these disorders (Chou et al. 1992; Pollak et al. 1993; Pearce et al. 1995, 1996*b;* Bai et al. 1997).

The human CaSR is a 1,078–amino acid cell-surface protein that is expressed in the parathyroids and kidneys, where it allows regulation of PTH secretion and renal tubular calcium reabsorption appropriate to the prevailing extracellular calcium ($[Ca^{2+}]_o$) concentration (Brown 1997; McKusick 1998). The CaSR is a member of family C of the superfamily of G-protein coupled receptors (GPCRs) that includes the metabotropic glutamate receptors, some pheromone receptors, and the γ-amino butyric acid (GABA-B) receptors (Birnbaumer 1995; Brown 1997; Hebert et al. 1997). Ligand binding by the CaSR results in G-protein–dependent stimulation of phospholipase C (PLC) activity, causing accumulation of inositol 1,4,5-triphosphate (IP_3) and the rapid release of calcium ions from intracellular stores ($[Ca^{2+}]_i$), followed by an influx of $[Ca^{2+}]_o$ (Pearce et al. 1996*b;* Bai et al. 1997; Brown 1997; Hebert et al. 1997). These intracellular events are thought to mediate a decrease in the rate of PTH secretion from the parathyroid cell and a reduction in renal tubular calcium reabsorption. Thus, the CaSR plays a key role in the regulation of extracellular calcium homeostasis, with loss-of-function mutations resulting in the hypocalciuric hypercalcemia of FBH and gain-of-function mutations resulting in the opposite phenotype, hypercalciuria with hypocalcemia (Pollak et al. 1993, 1994; Pearce et al. 1995, 1996*c;* Brown 1997; Hebert et al. 1997). However, such mutations involving the 3,234-bp coding region of the CaSR gene have been found only in one-half to two-thirds of FBH patients. Thus, it appears likely that other mutations involving the as-yet-uncharacterized regions of the CaSR gene, which include the untranslated, perigenic, and distant regulatory regions, or mutations involving other receptors and mediators of calcium regulation may occur in FBH patients.

The identification of two other FBH loci not linked to chromosome 3q21-24 indicates genetic heterogeneity and supports the possibility that there may be additional CaSRs or second messengers involved in calcium homeostasis. Of these two FBH loci, one has been mapped to chromosome 19p13.3 (Heath et al. 1993), and the other—identified in an extensive Oklahoma kindred with an FBH type called "FBH, Oklahoma variant" (FBH_{Ok}; McMurtry et al. 1992)—has been excluded from chromosomes 3q and 19p13.3, by linkage analysis (Trump et al. 1995). The FBH_{Ok} kindred is notable for additional clinical features that arise in some adults as they age; these features include elevations in serum PTH concentrations, hypophosphatemia, and osteomalacia (McMurtry et al. 1992). In order to identify the location of the FBH_{Ok} locus, we undertook a genomewide search using the available 254 microsatellite polymorphisms in chromosome-specific sets, with an average intermarker distance of 13 cM (Reed et al. 1994).

Patients and Methods

Patients

Thirty-eight members, from five generations, of the previously reported (McMurtry et al. 1992; Trump et al. 1995) Oklahoma kindred with autosomal dominant FBH and developmental increases in serum PTH levels were investigated. For these family members, the biochemical phenotype of FBH was established (McMurtry et al. 1992; Trump et al. 1995) by documenting hypercalcemia associated with a low ratio (<.010) of calcium clearance to creatinine clearance. Supranormal concentrations of serum PTH had been observed in some affected individuals >30 years of age (II-2, II-4, II-6, II-9, and III-6). Three adults >40 years of age (II-4, II-9, and III-3) also showed evidence of osteomalacia. An individual taking anticonvulsants that may alter Ca^{2+} homeostasis, an individual (IV-4) described previously (McMurtry et al. 1992) as suffering from hypocalcemia due to hypoparathyroidism, and four individuals who were normocalcemic children of unaffected parents were excluded from the linkage study; the haplotypes of individual IV-4 were determined but were not included for analysis at the FBH_{Ok} locus. Thus, the 32 family members, from four generations, included for genetic analysis of the FBH_{Ok} locus consisted of 17 affected members, 13 unaffected members, and 2 unaffected spouses (fig. 1).

Genotyping and Linkage Analysis

Leukocyte DNA was prepared from venous blood samples, by standard methods (Thakker et al. 1990), from 33 members (17 affected and 16 unaffected) of the four-generation FBH_{Ok} family (fig. 1). Genomewide linkage analysis was performed by use of chromosome-specific sets of fluorescently labeled primers (Oswel DNA Service; Reed et al. 1994) that identified 254 polymorphic microsatellites, 80% of which were derived from the Généthon genetic linkage map and were arranged into 39 sets covering all 22 autosomes and the X chromosome, with an average intermarker distance of 13 cM (Gyapay et al. 1994; Reed et al. 1994; Ashworth et al. 1995). Additional markers from chromosome 19 were also obtained from the Généthon linkage map (Gyapay et al. 1994). PCRs were performed in a total volume of

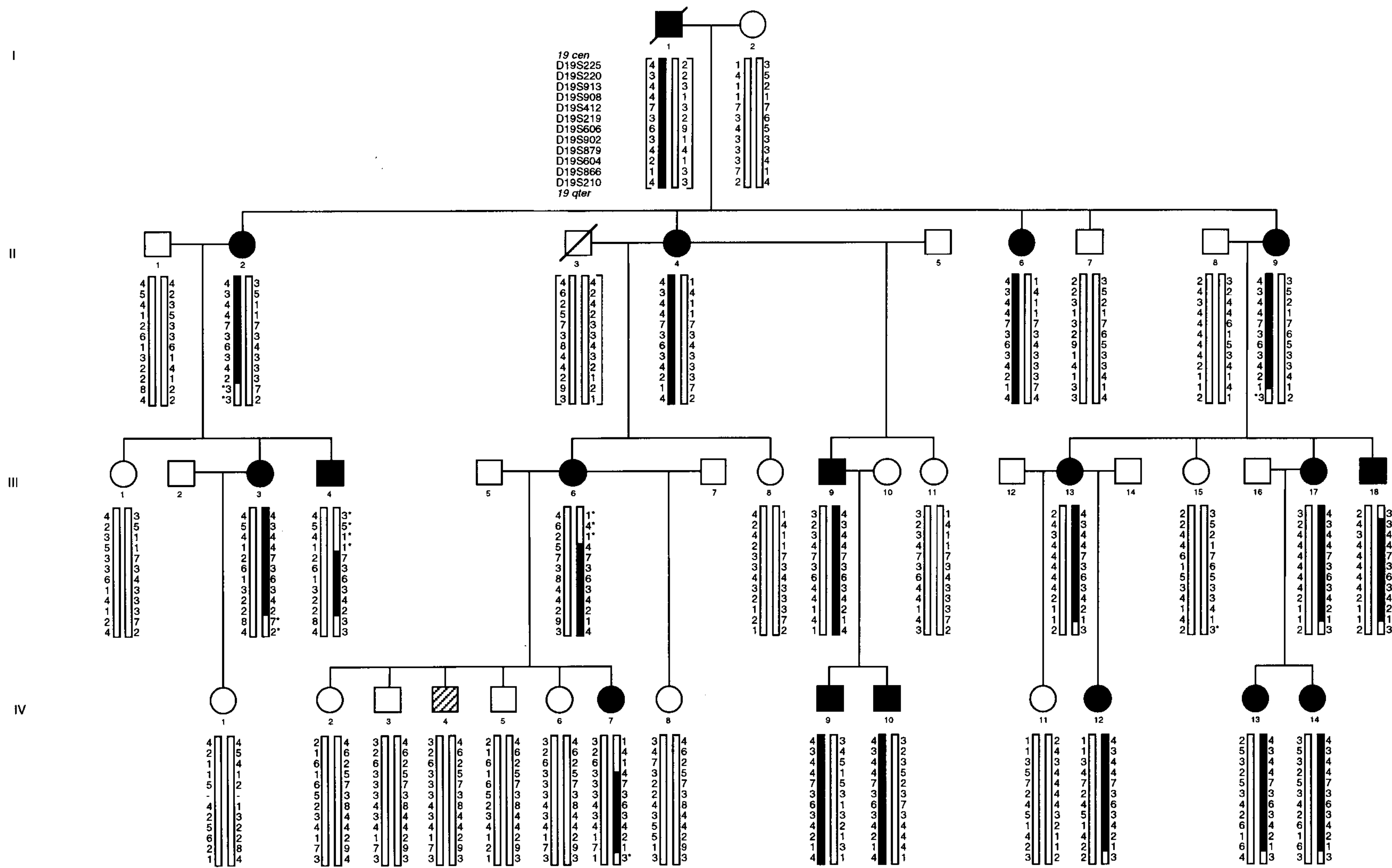

Figure 1 Pedigree of family segregating for FBH_{Ok}, and chromosome 19q13 loci. The pedigree has been truncated, and the numerals identifying individuals have been altered from the original descriptions (McMurtry et al. 1992; Trump et al. 1995), to indicate those family members who yielded information for the localization of FBH_{Ok}. Squares indicate males, and circles indicate females. Unblackened symbols indicate unaffected individuals, and blackened symbols indicate affected individuals. The hatched square indicates an individual (IV-4) with idiopathic hypoparathyroidism who was exluded from the linkage analysis. The paternal haplotypes are on the left, and the maternal haplotypes are on the right. Deduced haplotypes are within brackets. FBH_{Ok} is segregating with the haplotype [4, 3, 4, 4, 7, 3, 6, 3, 4, 2, 1, 4], defined by the loci listed to the left of the haplotypes of individual I-1 (e.g., in individuals II-4, II-6, III-9, IV-9, and IV-10). Blackened bars indicate affected haplotypes, and unblackened bars indicate unaffected haplotypes. Recombinants (indicated by an asterisk [*]) between FBH_{Ok} and the loci listed were observed in individuals II-2, II-9, III-3, III-4, III-6, III-18, and IV-7.

Table 1

Two-Point Linkage Analysis between FBH_{Ok} and 17 Loci from Chromosome 19

Locus	Peak LOD Score (θ)	LOD Score at θ =[a]							Distance[b] (cM)
		.00	.001	.01	.05	.10	.15	.20	
D19S221	.28 (.657)	−31.41	−17.13	−12.77	−7.31	−4.79	−3.36	−2.39	35.5
D19S49	.61 (.309)	−20.03	−10.22	−5.57	−2.12	−.74	−.05	.34	?
D19S225	2.74 (.122)	−11.52	−2.31	.61	2.32	2.71	2.71	2.53	55.9
D19S220	4.45 (.075)	−9.47	1.52	3.42	4.38	4.41	4.16	3.77	61.4
D19S913	3.27 (.093)	−3.69	.15	2.08	3.12	3.27	3.13	2.87	67.1
D19S908	4.38 (.046)	−1.80	3.14	4.05	4.38	4.19	3.86	3.44	69.2
D19S412	2.53 (.000)	2.53	2.52	2.48	2.32	2.09	1.86	1.61	69.9
D19S219	4.11 (.000)	4.11	4.10	4.05	3.78	3.44	3.07	2.69	69.9
D19S606	6.67 (.000)	6.67	6.65	6.56	6.12	5.56	4.96	4.34	76.2
D19S902	4.31 (.000)	4.31	4.30	4.24	3.95	3.58	3.19	2.78	76.2
D19S879	4.56 (.000)	4.56	4.55	4.49	4.22	3.86	3.48	3.08	78.9
D19S604	5.37 (.000)	5.37	5.36	5.28	4.93	4.47	3.98	3.48	79.0
D19S866	2.20 (.120)	−10.16	−1.17	.78	1.92	2.18	2.17	2.03	81.1
D19S907	2.60 (.101)	−3.82	.23	1.51	2.43	2.60	2.51	2.31	81.7
D19S572	2.49 (.157)	−17.97	−3.84	−.59	1.65	2.32	2.49	2.42	93.4
D19S180	1.65 (.154)	−5.13	−1.10	.08	1.16	1.55	1.65	1.60	?
D19S210	1.08 (.259)	−20.89	−8.79	−4.47	−1.14	.13	.71	.98	104.9

[a] LOD scores were calculated under an autosomal dominant mode of inheritance, a penetrance of 90%, and a phenocopy rate of 0.

[b] Distances from 19pter and the order of loci are from maps reported by Gyapay et al. (1994), Ashworth et al. (1995), and the Whitehead Institute for Biomedical Research/MIT Center for Genome Research. A question mark (?) indicates that the distance is unknown.

10 μl containing 50 ng genomic DNA, 20 ng each primer, 0.5 U *Taq* polymerase, and the recommended PCR buffer, which included 1–3 mM $MgCl_2$, for 24–35 cycles under conditions described previously (Pearce et al. 1995, 1996*a;* Lloyd et al. 1996; The European Consortium on MEN1 1997). Reactions for individuals were either multiplexed or multipooled, as appropriate for the PCR conditions (Reed et al. 1994), allele sizes, and fluorescent label. Alleles were detected on a 6% denaturing polyacrylamide gel (12-cm well-to-read plates), by use of a semiautomated DNA fragment analyzer (ABI 377) and GENESCAN software (The European Consortium on MEN1 1997). Alleles were scored directly by use of the GENOTYPER program. Two-point LOD scores were calculated by use of the MLINK and ILINK programs (Thakker et al. 1990), with the frequency and penetrance of FBH_{Ok} set at 10^{-4} and 90%, respectively (Trump et al. 1995); variation of these values had no significant effect on the results of the linkage analysis.

DNA Sequence Analysis of Calmodulin (CALM) III

CALM III–specific primers (details available on request) were designed from the published sequence (Koller et al. 1990) and were used for PCR amplification of the six exons and their splice sites, all of the 123-bp 5′ UTR and 238 bp of the upstream promoter region that contains minor transcriptional sites, and 68 bp of the 1,621-bp 3′ UTR. The size of each CALM III exon is as follows: exon 1, 126 bp; exon 2, 31 bp; exon 3, 144 bp; exon 4, 107 bp; exon 5, 136 bp; and exon 6, 1,650 bp (Koller et al. 1990). The first 123 bp of exon 1 consist of the 5′ UTR, and the initiator methionine is encoded by 124–126 bp. The remaining 147 amino acids of the 148–amino acid CALM III are encoded by exons 2–5 and the 5′ 29 bp of exon 6. The proximity of exons 5 and 6 made it feasible to amplify their coding regions together with intron 5, with one set of primer pairs. Thus, the sizes of the PCR products that contained the promoter region, 5′ UTR, and exon 1; exon 2; exon 3; exon 4; and exon 5, exon 6, and the 68 bp of the 3′ UTR were 419, 300, 285, 313, and 465 bp, respectively. DNA from an FBH_{Ok}-affected member (III-3, fig. 1) and from an unaffected unrelated individual was used, and the DNA sequences of the PCR products were determined as described previously (Pearce et al. 1995, 1996*a,* 1996*c;* Lloyd et al. 1996), by use of a semiautomated system (ABI 373).

Results

We undertook a genomewide linkage search for the FBH_{Ok} locus, using microsatellite-repeat markers spaced an average of 13 cM apart (Reed et al. 1994). Following an analysis with 76 polymorphic loci, which consisted of 12, 14, 16, 11, 6, 4, 6, and 7 loci (details available on request) from chromosomes 5, 7, 8, 9, 11, 15, 19, and 21, respectively, linkage was established between FBH_{Ok} and the chromosome 19q locus D19S220, with a peak LOD score of 4.45 at recombination fraction (θ) .075 (table 1). In addition, the recombinants observed between FBH_{Ok} and the chromosome 19q loci suggested that FBH_{Ok} was located between D19S220 and D19S210

(fig. 1), an interval estimated to be 43.5 cM (table 1). Therefore, eleven additional polymorphic loci (D19S913, D19S908, D19S412, D19S219, D19S606, D19S902, D19S879, D19S604, D19S866, D19S907, and D19S572) from this interval were used (Gyapay et al. 1994; Ashworth et al. 1995) for further studies. Linkage between FBH_{Ok} and eight polymorphic loci—D19S220, D19S913, D19S908, D19S219, D19S606, D19S902, D19S879, and D19S604—was established (table 1), with peak LOD scores of 3.27–6.67 (θ = .000–.093). Thus, FBH_{Ok} was mapped to chromosome 19q13. An analysis of recombinants helped to further localize FBH_{Ok}. The pedigree in figure 1 shows 43 members (41 surviving and 2 deceased), from four generations, with data from 12 chromosome 19q13 microsatellite polymorphic loci. The haplotype of individual I-1, a deceased affected father, was ascertained by an examination of his four affected children (II-2, II-4, II-6, and II-9) and one unaffected son (II-7). His affected daughter, II-2, inherited a recombinant haplotype that locates FBH_{Ok} centromeric to D19S866. Her affected son, III-4, inherited a recombinant haplotype that locates FBH_{Ok} telomeric to D19S908. The combined observations of the recombinants observed in II-2 and III-4 locate FBH_{Ok} within the <12-cM interval (table 1) flanked centromerically by D19S908 and telomerically by D19S866.

A survey of human–chromosome 19 maps (Lawrence Livermore National Laboratory) and of human-gene databases (Genome Database and Genbank) revealed that the gene encoding CALM III was located within this <12-cM interval (Berchtold et al. 1993; Ashworth et al. 1995). CALM is a highly conserved, ubiquitously expressed, intracellular Ca^{2+}-binding protein that is known to have a function in signal transduction, through its role as the delta subunit of phosphorylase kinase (Bachs et al. 1994). There are at least three expressed copies of CALM in the human genome, with several additional CALM-like genes and pseudogenes (Berchtold et al. 1993; Rhyner et al. 1994). The three human CALM genes, referred to as "CALM I," "CALM II," and "CALM III," each of which contains four calcium-binding domains, have different sequences in the promoter regions, suggesting differential regulation at the transcriptional level (Berchtold et al. 1993; Bachs et al. 1994; Rhyner et al. 1994). The chromosomal location and functional properties of the CALM III gene, which consists of six exons that span 10 kb of genomic DNA (Koller et al. 1990), suggested that it may represent a candidate gene for FBH_{Ok}. However, DNA sequence analysis of the 1,782 bp that encompass the coding region in the six exons, their consensus splice sites, all of the 5′ UTR, the 238 bp of the upstream promoter region, and <5% of the 3′ UTR of CALM III from an individual affected with FBH_{Ok} and from an unrelated normal individual did not detect any abnormalities, thereby indicating that the CALM III gene is unlikely to be the gene responsible for FBH_{Ok}.

Discussion

Our mapping of FBH_{Ok} to chromosome 19q13 and to the <12-cM interval flanked by D19S908 and D19S866 represents an important advance in the identification of this gene, which regulates calcium homeostasis. This gene may possibly encode another CaSR or a downstream mediator in the CaSR pathway. The CaSR pathway, which begins with ligand binding by the CaSR, involves G-protein–dependent stimulation of PLC activity that causes an accumulation of IP_3 and a rapid release of $[Ca^{2+}]_i$ from intracellular stores, followed by an influx of $[Ca^{2+}]_o$ (Pearce et al. 1996*b;* Bai et al. 1997; Brown 1997; Hebert et al. 1997). The intermediary between elevation of $[Ca^{2+}]_i$ and the physiological response of a decrease in the rate of PTH secretion from the parathyroid cell and a reduction in renal tubular calcium reabsorption is likely to involve a type of calcium-binding protein that may be either a calcium-dependent enzyme or a calcium-dependent modulating protein such as CALM (Bachs et al. 1994; Crivici and Ikura 1995; Gnegy 1995). Our database searches of human–chromosome 19q13 maps (Lawrence Livermore National Laboratory, Genome Database, and Genbank) and of the syntenic regions on mouse chromosome 7, for CaSRs and putative mediators, revealed two potential candidate genes for FBH_{Ok}. These were a mouse CaSR-related sequence, referred to as "Casr-rs1" (Hinson et al. 1997), and the human CALM III gene (Berchtold et al. 1993).

Casr-rs1 is one of five CaSR-related sequences (Casr-rs1 to Casr-rs5) that have been identified in mice (Hinson et al. 1997). Casr-rs1 has been located on mouse chromosome 7 and, hence, is in the region syntenic to human chromosome 19q13. However, our BLAST analysis of Casr-rs1, which has 40% amino acid identity to the human CaSR, revealed 81% identity to the mouse pheromone receptor VR6 (Matsunami and Buck 1997). Furthermore, recent analysis of the compact genome of the fish *Fugu rubripes* has revealed a large family of receptors that are closely related to CaSR (Naito et al. 1998). These appear to be the fish homologues of the vomeronasal receptors, which are GPCRs activated by some volatile odorant pheromones, reported in mice and rats (Herrada and Dulac 1997; Matsunami and Buck 1997). However, to date, human homologues of pheromone receptors and Casr-rs1 have not been isolated. Thus, the relationships between these pheromone receptors, Casr-rs1, and the FBH_{Ok} gene cannot be defined and must await the characterization of the human homologues of these fish and mouse receptors.

The second candidate gene identified for FBH_{Ok} encoded for a CALM, which is a ubiquitous, intracellular calcium-binding regulatory protein that is involved in many signaling pathways (Crivici and Ikura 1995; Gnegy 1995). Thus, CALM may activate the function of various kinases (CALM kinases I and II and myosin light-chain kinase), phosphatases (calcineurin), ion channels (plasma membrane Ca^{2+} pump), phosphodiesterase, adenylate cyclase, and nitric oxide synthetase. In addition, CALM may regulate cell-surface expression of the integral membrane protein, L-selectin, through a proteolytic mechanism (Kahn et al. 1998). Three human CALM genes, CALM I, CALM II, and CALM III, have been identified, and CALM III has been mapped to chromosome 19q13 (Berchtold et al. 1993; Rhyner et al. 1994). The chromosomal location and the likely functional properties of CALM III, on the basis of those of the other CALMs, indicated it as a strong candidate gene for FBH_{Ok}. However, the absence of CALM III DNA-sequence abnormalities in an individual with FBH_{Ok} indicated that the CALM III gene was unlikely to be implicated in the etiology of this disorder and that other candidate genes need to be identified.

Our localization of FBH_{Ok} to a well-defined region of chromosome 19q13 represents an important step for the identification of the basis of this disorder of calcium homeostasis. The <12-cM interval containing the FBH_{Ok} gene appears to be, on a physical map, as small as 4 Mb in size, and >30 genes and expressed sequence-tagged sites have been mapped to this region (Ashworth et al. 1995; Durocher et al. 1995; Lawrence Livermore National Laboratory). In addition, the region has been prepared for long-range sequencing (Lawrence Livermore National Laboratory), and, thus, our mapping of FBH_{Ok} may help in the identification of disease associations and functional properties of genes isolated from this region.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genbank, http://www.ncbi.nlm.nih.gov/Web/Genbank/ (for map of human chromosome 19)

Généthon, http://www.genethon.fr/ (for map of microsatellite markers used in the linkage analysis)

Genome Database, http://gdbwww.gdb.org/ (for map of human chromosome 19)

Lawrence Livemore National Laboratory, Human Genome Centre, http://www-bio.llnl.gov/genome/genome.html (for map of human chromosome 19)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for FBH [MIM 145980], FBH1 [MIM 145980], FBH2 [MIM 145981], and FBH3 [MIM 600740])

Whitehead Institute for Biomedical Research/MIT Center for Genome Research, http://www-genome.wi.mit.edu/cgi-bin/contig/sts_info (for map of locations and distances between loci used in the linkage analysis)

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