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A 3-Mb Region for the Familial Hemiplegic Migraine Locus on 19p13.1–p13.2: Exclusion of PRKCSH as a Candidate Gene

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Abstract
Familial hemiplegic migraine (FHM) is an autosomal dominant subtype of migraine with attacks, associated with transient episodes of hemiparesis. One of the genes for FHM has been assigned to chromosome 19p13. Detailed analysis of critical recombinants from two different chromosome 19-linked FHM families, using new markers indicated a 6-cM candidate region on 19p13.1–p13.2 flanked by loci D19S394 and D19S226. Another paroxysmal neurological disorder, episodic ataxia type 2 (EA-2), has also been linked to the same chromosomal region. Most of the interval was completely covered by YAC and cosmid contigs; the physical map yielded approximately 3 Mb encompassing several genes including the protein kinase substrate 80K-H (PRKCSH) gene. Since PRKCSH is involved in neuronal signal transduction, it was considered to be an FHM candidate gene. The genomic structure of this gene was established and mutation analysis for all exon and flanking intron sequences was performed in FHM- and EA-2-affected individuals. Five polymorphisms were identified, including a trinucleotide repeat length variation in the coding sequence. However, no potential disease causing mutation was found and therefore the PRKCSH gene can be excluded for both FHM and EA-2.

Introduction

Migraine is a chronic, paroxysmal disease, affecting up to 16% of the general population [1]. Migraine attacks usually last one day, and present with severe, incapacitating, unilateral, pulsating headache, associated with nausea, vomiting, photophobia and phonophobia (migraine without aura). In about 15% of patients attacks are preceded by transient focal neurological aura symptoms, which are usually visual, but sometimes consist of hemiparesis, hemisensory symptoms, or aphasia (migraine with aura).

Migraine is frequently familial, suggesting that genetic components are involved [2]. Family and twin studies have yielded conflicting results with respect to the mode of inheritance of migraine [3, 4]. Migraine can be considered as a genetic disorder with variable expression of clinical symptoms, a complex mode of inheritance, and influenced by environmental factors. The migraine spectrum comprises the common types of migraine, (with and without aura) as well as rare autosomal dominant variants of migraine such as familial hemiplegic migraine (FHM).

A list of all members of the Dutch Migraine Genetics Research Group is given elsewhere [4].

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Table 1. Sizes, splice junctions and primers of PRKCSH exons

Exon No.	Nucleotide position	Exon size, bp	3′ splice site	5′ splice site	Intron size, kbp	Exon	Primer	sequence	Product (bp)
1	1–61	>60	–	CGT/gtagg	0.4	1–2	for	gag ggg tgc ggt gga tac tga	640
							rev	atc cct aag gcc caa tgt tg	
2	62–215	154	tgcag/CTG	CCA/gtgag	0.3				
3	216–332	117	cgcag/ATC	CAG/gtgag	1.1	2–3	for	gcg cat ctc ccc gct gta	540
							rev	cag cag agg tag tat ctt gg	
4	333–428	96	cacag/GCA	GTG/gtaag	0.093	4–5	for	ctc tta tct gtg gat gga tg	370
							rev	agc cag gca aag tct tgg ggt	
5	429–486	58	cacag/ACT	CAA/gtacg	~3				
6	487–604	118	cgcag/AGA	CAG/gtaag	1.1	6–7	for	gga tcc taa gtg ccc cag tg	1300
							rev	tag tcc ttg gcc gat ttc tg	
7	605–734	130	ccgag/AAA	AAG/gtatg	~3				
8	735–819	85	tacag/AGC	GAC/gtcag	0.25	8–9	for	cat ata gta ggc gct tgg tg	1300
							rev	ggg att ggg tgc tca aca gc	
9	820–898	79	agcag/GGT	CAG/gtacc	0.7				
10	899–985	87	ctcag/GCC	GAG/gtagt	0.3	10–11	for	acg tgg tgg cct aga tc	800
							rev	cgt agg gcg gca ttt tgt ctt	
11	986–1141	156	ccgag/GCA	GAG/gtgca	0.095				
12	1142–1238	97	cccag/GAG	ATG/gtcga	0.3	12–13	for	tgt ttg ggg gag aag tgg	800
							rev	gtc taa gct cag gat ctt cc	
13	1239–1308	70	cccag/CTG	CAG/gtagc	0.25				
14	1309–1398	90	tccag/GAA	CGA/gtgcg	0.4	14	for	agg aca gcc tgg gca cca tt	300
							rev	caa cca tca gga aac tgc cg	
15	1399–1473	75	cacag/ATA	TGG/gtgag	0.087				
16	1474–1573	100	cccag/CAC	ACC/gtgag	0.09	15–17	for	cgt tcc cca acc cat atg tc	660
							rev	ttg ata gag tgg cca tgt gg	
17	1574–1734	161	cccag/GTG	CAG/gtggg	1.1				
18	1735–2019	>280	cccag/AAC	–		18	for	cca ggc ttt cta atc tgg tc	440
							rev	atc tgc aga aag ctg cgt cc	

Exon and intron sequences are shown in uppercase and lowercase letters, respectively; cDNA positions as published by Sakai et al. [13]; intron sizes were determined by PCR or Southern blotting; primers for primary PCR amplification are shown. Sequences are available via Genbank access number U50316 to U50327.

FHM (MIM141500) is an autosomal dominant familial disorder characterized by migraine attacks associated with a transient hemiparesis in addition to other aura symptoms. A gene for FHM has been mapped to chromosome 19p [5, 6]. Recently, we showed the involvement of the chromosome 19p FHM locus in *normal* migraine with and without aura by sib-pair analysis [7], supporting the hypothesis of a continuous migraine spectrum encompassing FHM as well as the common types of migraine. Consequently, a genetic study in rare monogenic types of migraine such as FHM will provide clues to hereditary factors in the common types of migraine with a more complex inheritance pattern.

Since genetic heterogeneity was shown in about half of the FHM families [6, 8], at least one additional FHM locus must exist. A clinical comparison of FHM families linked and unlinked to chromosome 19p did not show significant differences for age of onset or frequency and duration of attacks [Terwindt, pers. commun.]. However, in 3 out of 8 chromosome 19-linked families, FHM was associated with chronic cerebellar ataxia, whereas none of the unlinked FHM families displayed cerebellar ataxia [4–6, 8]. A gene for an episodic type of cerebellar ataxia, episodic ataxia type-2 (MIM108500), has recently been mapped to the same chromosomal area on chromosome 19p13 [9–12]. In this paper, we describe the further analy-

sis of previously reported recombinants [6] and we confined the FHM candidate region on chromosome 19p13–p13.2 to 6 cM, representing approximately 3 Mb of DNA. The protein kinase C substrate 80K-H (PRKCSH) is located within this interval [13]. Protein kinase C plays an important role in the signal transduction of for example hormones, neurotransmitters and growth factors. Based on its putative function in the neuronal signal transduction [13–15], PRKCSH was considered a candidate gene for FHM. The PRKCSH gene was therefore examined, the intron/exon structure was clarified and mutation analysis was performed by single-stranded conformation polymorphism (SSCP) analysis and direct sequencing.

Materials and Methods

Subjects

Family members of 3 previously described chromosome 19-linked FHM pedigrees [6] were used to genetically confine the FHM candidate region. Thirty-four individuals were subjected to mutation analysis in the PRKCSH gene. This group consisted of (1) 9 individuals from 7 unrelated chromosome 19-linked FHM families: family A, B, C, 1 affected person from an American family (kindly provided by Dr. S. Peroutka, Menlo Park, Calif., USA) and 3 Italian FHM patients from 3 unrelated families (kindly provided by Dr. M. Ferrari, Milan, Italy); (2) 3 unrelated small FHM families: 2 Dutch families and 1 American family (kindly provided by Dr. K.M.A. Welch, Detroit, Mich., USA); (3) 10 unrelated sporadic patients with hemiplegic migraine from the Netherlands; (4) 2 unrelated individuals with migraine with and without aura, and (5) a set of 10 individuals with 4 different chromosome-19-linked episodic ataxia and 6 healthy controls (kindly provided by Dr. M. Litt, USA).

Fifty randomly collected individuals from the Dutch population [16] were used as controls to determine the allele frequencies of polymorphic sites.

Genetic Analysis

DNA from peripheral blood cells was isolated by standard methods [17]. Microsatellite markers D19S216, D19S413, D19S394, EPOR, D19S221, D19S840, D19S226 and D19S179 were tested by PCR as described previously [6]. Oligonucleotide primer sequences were obtained via the Human Genome Data Base.

Generation of cDNA Probes

RNA from peripheral blood cells was isolated according to the RNAzol™ procedure (Campro Scientific B.V.); cDNA synthesis was performed using MMLV revese transcriptase (Gibco BRL) according to the manufacturer.

Three sets of primers were chosen to produce 3 overlapping fragments encompassing the whole coding region and flanking sequences of the gene: PRKCSH-A (forward 5′-GAGGGGTGCGGTGGATACTGA-3′ and reverse: 5′-AGCAGTCACAAACACCATCG-3′ producing a 413-bp fragment covering the cDNA from position 22 to 434, PRKCSH-B (forward: 5′-GCTGCCTGTCCTAATGGC-3′ and reverse: 5′-ACGAGCAGACGCAGGC-3′) generating an 874-bp product containing nucleotide 338–1211 and PRKCSH-C (forward: 5′-CCAAGGAGGAGCAGCC-3′ and reverse 5′-TGGGGGTGGTGGGGCGAGTCA-3′), a 938-bp PCR product from cDNA position 1035 to 1972. These three PCR products were subcloned into the TA vector (Invitrogen) and subjected to dideoxy sequence analysis (T7 Sequencing kit, Pharmacia Biotech). Probes were labeled using the Megaprime DNA Labeling system (Amersham).

Table 2. Primer sequences for secondary PCR amplification[1]

Exon	Primer sequence (5′→3′)		Product, bp
2	for	gcg cat ctc ccc gct gta	150
	rev	atc cct aag gcc caa tgt tg	
6	for	gga tcc taa gtg ccc cac tg	240
	rev	agt cac ttg ccc cag aac ag	
7	for	agt caa tga gga gga ggc	210
	rev	tag tcc ttg gcc gat ttc tg	
8	for	cat ata gta ggc gct tgg tg	170
	rev	aaa ggt ggg cag ctc caa cta	
9	for	cct cca ggt tgc ttc tgc	140
	rev	gca atg ctc cct aga agt c	
10	for	acg tgg tgg cct aga tc	180
	rev	agg cag ctc ctt tgt gag g	
11	for	aca cag gtg ttg tgg ctg cg	440
	rev	cgt agg gcg gca ttt tgt ctt	
12	for	tgt ttg ggg gag aag tgg	200
	rev	gtc taa gct cag gat ctt cc	
13	for	aca tcc atg aac ccc gtt cc	170
	rev	gtg gtt gcc tca gtg att c	

[1] Primers for second amplification of exon 2–3, 4–5, 14, 15–17 and 18 are shown in table 1.

Genomic Analysis

Cosmid LLNL No. 18069 was known to be positive for PRKCSH and an *Eco*RI restriction map was already available through the Genome Center at Lawrence Livermore National Laboratory (LLNL). The cosmid was digested with *Bam*HI, *Hind*III and double digested with *Bam*HI/*Eco*RI and *Hind*III/*Eco*RI. The resulting fragments were subcloned into pBluescript II KS(–) vector (Stratagene); PRKCSH cDNA probes were used for hybridization screening. Positive clones were sequenced (T7 Sequencing kit, Pharmacia Biotech) using vector primers. cDNA-specific oligonucleotides were used for sequencing from exon into the adjacent intron. Subsequently, intron-specific primers were used for sequencing the reverse strand. On average, 100 bp of exon flanking intron sequence was determined.

Mutation Analysis

PCR of Exon-Containing Fragments. A primary PCR was performed in a reaction volume of 30 µl. Ten sets of primers were chosen to produce fragments sizing from 370 to 1,300 bp encompassing all exon and flanking intron sequences of the coding region of the PRKCSH gene (table 1).

Table 3. Digestion of secondary PCR products for SSCP analysis

PCR product	Size, bp	Enzyme	Fragments, bp	Buffer
exon 3	540	*Rsa*I	220, 320	2 × One-Phor-All[1]
exons 4-5	370	*Mbo*I	240, 130	1 × One-Phor-All[1]
exon 11	440	*Ava*II	20, 200, 220	1 × One-Phor-All[1]
exons 15-17	660	*Bbv*I	200, 210, 250	1 × NEbuffer[2]
exon 18	440	*Hin*fI	190, 250	2 × One-Phor-All[1]

[1] From Pharmacia.
[2] From Biolabs.

Primary PCR products were labeled by a second (semi-nested) PCR in a 15-µl reaction volume using 1 µl primary PCR product and 0.7 µCi [α-^{32}P]-dCTP (3,000 Ci/mmol; Amersham). The sets of PCR primers amplified 14 fragments ranging from 150 to 660 bp (table 1, 2).

SSCP Analysis. Subsequent to the secondary PCR, 5 PCR products encompassing exons 3, 4–5, 11, 15–17, 18 and their flanking intron sequences were digested to yield shorter fragments suitable for SSCP analysis; the remaining amplified fragments already had an appropriate size. The digestion of the large PCR products was performed by adding 5 µl mixture with a specific buffer and 3 U restriction enzyme, directly to the secondary PCR product (15 µl) for 2 h at 37 °C (table 3).

Secondary PCR product was subjected to SSCP analysis [18] on either an 8% polyacrylamide gel with 10% glycerol and 1 × TBE, or a Mutation Detection Enhancement gel without glycerol buffer (AT Biochem). Primary PCR products were cloned in the TA vector (Invitrogen) and subjected to dideoxy sequence analysis (T7 Sequencing kit, Pharmacia Biotech) using T7 and SP6 primers.

Results

The FHM Candidate Region

A re-evaluation of the critical recombinants published previously [6] by using new markers, demonstrated that individual A-I-1 positioned the FHM gene telomeric of D19S226 by a meiotic crossover between D19S221 and D19S226. The marker between these loci, D19S840, was not informative. Another individual, C-II-6, reveals a recombination between D19S394 and EPOR, favoring a position centromeric of D19S394. Consequently, the FHM locus can be positioned between D19S394 and D19S226, a 6-cM interval (fig. 1). In collaboration with the Chromosome 19 Human Genome Center, Lawrence Livermore National Laboratory (LLNL) California, a contig was built between D19S394 and D19S226. The whole region was almost covered by YACs, PACs and cosmids. The physical map yielded approximately 3 Mb as a target region for the FHM gene. The physical map of chromosome 19 is available via Internet (http://www-bio.llnl.gov/bbrp/genome/genome.html). Several genes are known to be located within this FHM interval (fig. 1): the low-density lipoprotein receptor (LDLR) gene, the gene for erythropoietin receptor (EPOR), the protein kinase C substrate heavy-chain (PRKCSH) gene, a locus containing zinc finger motifs (ZNF58), the mannosidase alpha B locus (MANB), the jun B proto-oncogene (JUNB), the human homolog of the *Saccharomyces cerevisiae* gene RAD23A, a gene involved in lymphoblastic leukemia (LYL1), and MHC class II regulatory factor RFX1.

Table 4. Genes located in FHM region on 19p13

Locus	MIM No.	Associated disorder/function
LDLR	143890	hypercholesterolemia
EPOR	133100	benign erythrocytosis
MANB	248500	lysosomal alpha-*D*-mannosidase deficiency
JUNB	165161	proto-oncogene
RAD23A	600061	xeroderma pigmentosa
LYL1	151440	acute lymphoblastic leukemia
RFX1	600006	bare lymphocyte syndrome
ZNF58	–	unknown
PRKCSH	177060	unknown

PRKCSH as Candidate Gene

Although none of these genes can directly be excluded as candidate gene for FHM, the postulated role of PRKCSH in neuronal signal transduction made it a strong candidate gene for FHM. To test this hypothesis, we established the genomic structure of the gene and performed a mutation analysis. The cDNA of PRKCSH has been isolated and described by Sakai et al. [13]. The sequence is available through Genbank at accession num-

ber J03075. The nucleotide sequence contains an open reading frame of 1,581 base pairs, encoding an acidic protein of 527 amino acid residues with several phosphorylation sites and an extremely Glu-rich region. Similarity studies in Genbank yielded several homologous human ESTs, originating from different tissues like brain, breast, spleen and placenta suggesting a widely expressed gene. Genbank accession numbers of the ESTs homologous to PRKCSH are R42605, M77871, M78134, R48768, T50679 and T51209, respectively.

Exon-Intron Structure of the PRKCSH Gene

The PRKCSH gene consists of 18 exons that range in size from 58 bp (exon 5) to ≥200 bp (exon 18). The sizes of introns were estimated by PCR and Southern blotting and range from 87 bp (intron 15) to almost 3 kb (introns 5 and 7). Exon 2 includes the start codon (position 137) and part of the untranslated 5′ region. The stop codon is located in exon 17. Exon 18 contains the 3′-terminal noncoding region only. The putative polyadenylation site AATAAA is identified at position 2036, approximately 300 bp downstream (cDNA) of the termination codon; the exact position of the poly-A tract is not known. The complete PRKCSH gene spans nearly 18 kb at the genomic level (fig. 2).

Mutation Analysis

All exons and their flanking intron sequences, containing the complete coding region of PRKCSH and part of flanking untranslated sequences were screened for the

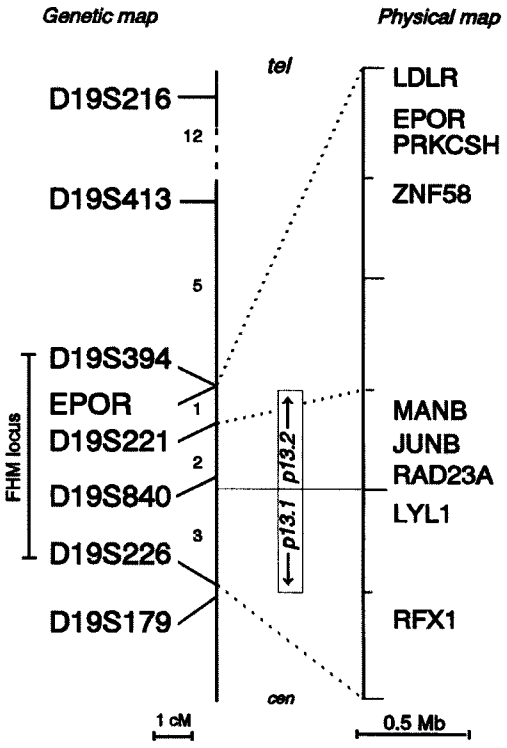

Fig. 1. Genetic and physical map of the FHM candidate region on chromosome 19p13. Abbreviations of gene names, as shown in physical map, are explained in Results – The FHM Candidate Region and table 4.

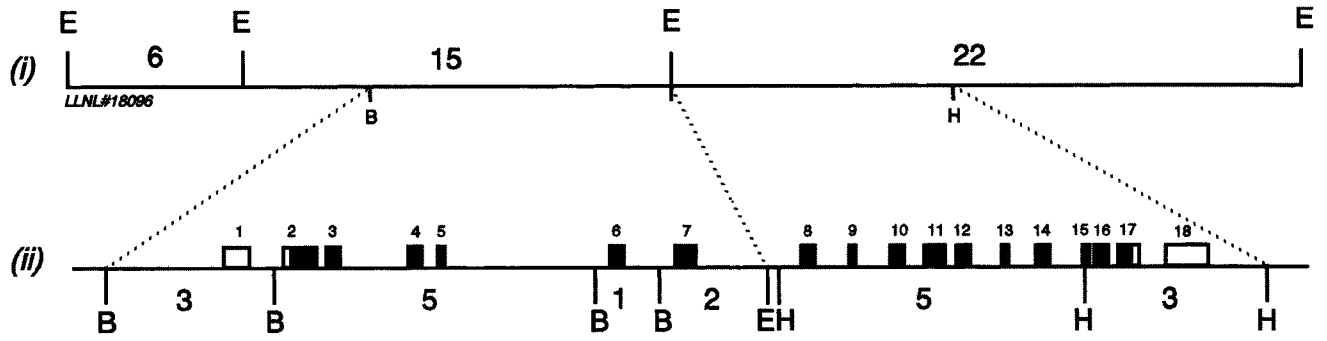

Fig. 2. Genomic organization of PRKCSH gene; (i) *Eco*RI restriction map of cosmid LLNL No. 18096 obtained from Lawrence Livermore National Laboratory and (ii) the exon distribution of PRKCSH relative to *Eco*RI (E), *Bam*HI (B) and *Hin*dIII (H) restriction sites; distance is given in kilobase pairs (kb).

presence of mutations by SSCP analysis in 34 individuals described in section Materials and Methods. Five polymorphisms were identified by sequencing and their presence was observed in 50 unrelated individuals. No potential disease causing mutation could be identified in either FHM or EA-2 patients. One polymorphism is located in exon 11 and alters the sequence of the functional gene product; the remaining four polymorphic sites are located in introns or in the 3′ untranslated region.

Exon 11 harbors a polymorphic GAG trinucleotide repeat, encoding a glutamic acid stretch. The number of GAG repeats varied from 8 to 10 in the individuals we tested. No specific allele was associated with either FHM or episodic ataxia. A subsequent screening of 50 random individuals from the Dutch population also showed the presence of this polymorphism showing four different alleles with a frequency of 0.04 (n = 8), 0.28 (n = 9), 0.67 (n = 10) and 0.01 (n = 11).

In the second intron another polymorphism was detected. Sequence analysis showed a 3-bp deletion 10 bp downstream of the exon 2 boundary, changing CCTCCT into CCT. The observed frequency of the shortened allele in the control subjects was 0.03.

A third variation was identified 51 bp downstream of exon 13 showing an A to G transition with an allele frequency of 0.04 in random controls. The fourth polymorphic site was positioned in intron 14 showing a C to T substitution 45 bp downstream of exon 14 with an allele frequency of 0.06 in the control subjects. The last variation observed was a T to C transition at cDNA position 1905 in the 3′ untranslated region of the gene, located in exon 18. The observed frequency of the rare allele in the control subjects was 0.05.

Discussion

The FHM candidate region on chromosome 19p13 has been narrowed down by studying recombinants from two different FHM families. Our results suggest a most likely position of an FHM gene between loci D19S394 and D19S226, a 6-cM interval (fig. 1). Joutel et al. [5] reported an obligate crossover in an FHM carrier (individual II-12, family 1), suggesting an FHM locus distal from D19S221. Without allowing for a double recombination event, these data imply that the chromosome 19 FHM gene is located within a 1-cM region flanked by D19S394 and D19S221. This crucial recombinant has subsequently not been discussed [8]. Accordingly, we focussed primarily on the region between D19S394 and D19S226 with a particular interest for the 1-cM region flanked by D19S394 and D19S221.

A locus for CADASIL, the acronym for cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (MIM125310), is located centromeric of D19S226 [19, 20]. Although CADASIL presents with attacks of severe headaches, the genetic data suggest that mutations in two different genes cause FHM and CADASIL, respectively. The LLNL physical map of chromosome 19 shows that the interval between D19S394 and D19S226 spans about 3 Mb, mostly covered by YAC and cosmid contigs. So far, 9 genes have been localized in this region and several clinical phenotypes have been described caused by mutations in those genes (fig. 1, table 3). However, none of the mutation-linked clinical features, as described so far, suggest an involvement in the etiology of hemiplegic migraine.

Interestingly, two genes are located in the 1-cM region between D19S394 and D19S221 in which no mutations have been described so far: ZNF58 and PRKCSH. The ZNF58 locus probably contains a zinc finger gene or gene cluster, though the structure has not been described yet. Proteins containing zinc finger motifs are potentially capable of binding nucleic acids and may act as regulatory factors in gene expression. The PRKCSH gene encodes an 80 kD substrate for Ca^{2+}/phospholipid-dependent protein kinase C, a large gene family with multiple enzymological characteristics [13]. Different members of the kinase family have distinct functions in the processing and response to external signals [14]. Protein kinase C has an important role in signal transduction of, for example, hormones, neurotransmitters and growth factors. Alterations in the PRKCSH protein, a substrate for protein kinase C, may therefore result indirectly in a disturbance of signal transduction which could be implied in attacks of (hemiplegic) migraine. We therefore considered PRKCSH a good candidate gene for hemiplegic migraine; the exon/intron structure of the gene was elucidated and mutation analysis was performed.

The genomic structure of PRKCSH revealed 18 exons within an 18-kb interval (table 1, fig. 2). The exon sequence was consistent with the cDNA sequence published by Sakai et al. [13]. Exon-specific analysis of 9 affected individuals from 7 unrelated chromosome-19-linked FHM families, 10 sporadic FHM patients and 2 individuals suffering from migraine with and without aura revealed no potential FHM-causing mutations in the coding sequence of the PRKCSH gene. However, 5 polymorphic sites were observed of which 1 alters the protein sequence: a trinucleotide repeat at position 1073 of the cDNA

sequence encoding a stretch of glutamic acids. The function of this acidic domain is unknown. The observed alleles of $(GAG)_n$ varied from n = 8 to n = 11 and did not reach values of $n \geq 40$ as the highly unstable expanded trinucleotide repeats for example in individuals affected for spinocerebellar ataxia type 1 [21] or Huntington disease [22].

Recently, a gene for episodic ataxia (EA-2; MIM 108500) was assigned to chromosome 19p13 [9–12]. The most likely location of the EA-2 gene is between D19S226 and D19S413, overlapping the entire region of the FHM gene reported here (fig. 1). EA-2 is a rare neurological autosomal dominant disorder characterized by attacks of generalized ataxia, generally associated with an interictal nystagmus. Interestingly, in about 40% of the chromosome-19p-linked FHM families, hemiplegic migraine is associated with progressive cerebellar ataxia and nystagmus [6, 8]. Notwithstanding the clinical differences between EA-2 and FHM, the episodic character of both disorders, the associated migraine in EA-2 patients [10], the presence of cerebellar ataxia and nystagmus in FHM as well as in EA-2, the reported relationship betwen basilar migraine and paroxysmal ataxia [23], and the genetic localizations of both genes to the same region on chromosome 19p13.1–p13.2 suggest that FHM and EA-2 could be allelic. Accordingly, we also screened 4 independent EA-2 affected individuals for mutations in the PRKCSH gene. Besides the presence of polymorphisms described in this paper, no evidence for a causative alteration in this gene was found. Consequently, the PRKCSH gene can also be excluded for EA-2.

Another type of episodic ataxia, EA-1, which is characterized by brief episodes of ataxia with interictal myokymia (twitching of small muscles), is caused by point mutations in a potassium channel gene (KCNA1) located on chromosome 12p [24]. In addition to EA-1, other inherited ion-channel mutations have recently been described that produce episodic signs: mutations in a sodium channel induce hyperkalemic periodic paralysis [25] and hypokalemic periodic paralysis is caused by mutations in a calcium channel [26]. FHM and EA-2 are clinically characterized by *episodic* signs, suggesting that both disorders could also be regarded as 'channelopathies' [27]. Interestingly, an α1A subunit of a neuronal-voltage-dependent calcium channel (CACNL1A4) was recently mapped to chromosome 19p13.1–p13.2 [28]. Accordingly, it will be worthwhile investigating the possible involvement of this gene in both hemiplegic migraine and episodic ataxia.

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