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A fine physical map of the *CACNA1A* gene region on 19p13.1–p13.2 chromosome

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Abstract

The P/Q-type Ca^{2+} channel α_{1A} subunit gene (*CACNA1A*) was cloned on the short arm of chromosome 19 between the markers D19S221 and D19S179 and found to be responsible for Episodic Ataxia type 2, Familial Hemiplegic Migraine and Spinocerebellar Ataxia type 6. This region was physically mapped by 11 cosmid contigs spanning about 1.4 Mb, corresponding to less than 70% of the whole region. The cosmid contig used to characterize the *CACNA1A* gene accounted only for the coding region of the gene lacking, therefore, the promoter and possible regulation regions. The present study improves the physical map around and within the *CACNA1A* by giving a complete cosmid or BAC contig coverage of the D19S221–D19S179 interval. A number of new STSs, whether polymorphic or not, were characterized and physically mapped within this region. Four ESTs were also assigned to cosmids belonging to specific contigs.

Keywords: EEF1D; HSPF1; Polymorphic STR; PRKCA

1. Introduction

The gene responsible for Episodic Ataxia type 2 (EA2) was mapped within a region of the short arm of chromosome 19 between D19S221 and D19S226 markers (Calandriello et al., 1996). In this region, the P/Q-type Ca^{2+} channel α_{1A} subunit gene (*CACNA1A*) was recently cloned and found to be responsible for EA2 and for Familial Hemiplegic Migraine (FHM) (Ophoff et al., 1996). Although, the coding region of the *CACNA1A* gene has been completely sequenced, the characterization of the gene does not comprise the promoter, the 5′ and 3′ UTR regions and possible transcriptional and translational regulating segments located in introns and flanking regions.

The portion of the 'high-resolution metric map of chromosome 19' spanning from D19S221 to D19S179 consisted of 11 ordered cosmid contigs (Ashworth et al., 1995). The sum of intercosmid distances and cosmid sizes suggested the span between the proximal marker D19S179 and the distal marker D19S221 to be about 1.8 Mb. This physical distance corresponds to a recombination distance of about 6 cM on the genetic map (Dib et al., 1996). As determined by *Eco*RI mapping, these 11 contigs ranged from 55 to 341 kb in size and covered a total of 1396 kb, corresponding to less than 70% of the actual whole region. The present study improves the physical map around and within the *CACNA1A* gene by giving a complete cosmid/BAC contig coverage. This was done by extending and merging the cosmid contigs coverage of the *CACNA1A* gene and its controlling region and in a change of the 5′–3′ direction of the gene

Abbreviations: BAC, bacterial artificial chromosome; CACNA1A, Ca^{2+} channel α_{1A} subunit gene; EA2, episodic ataxia type 2; EEF1-D, eukaryotic translation elongation factor 1 delta; EST, expressed sequence tag; FHM, familial hemiplegic migraine; FISH, fluorescence in-situ hybridization; HSPF1, heat shock 40 kD protein 1; PAC, P1 artificial chromosome; PCR, polymerase chain reaction; PRKACA, protein kinase, cAMP-dependent catalytic alpha; STR, short tandem repeat; STS, sequence-tagged site.

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previously reported (Ophoff et al., 1996), with respect to the centromere. Furthermore, a number of new STSs were characterized and physically mapped within this region. Some were found to be polymorphic, whereas other were not. Four ESTs previously located by radiation hybrid in the region were assigned to cosmids belonging to specific contigs.

2. Materials and methods

2.1. Cosmids

All cosmids were derived from the human chromosome 19 flow-sorted library prepared at Lawrence Livermore National Laboratories (LLNL) (de Jong et al., 1989). The cosmids used in this study were identified using the LLNL nomenclature. The cosmid DNA was isolated and purified following standard procedures (Sambrook et al., 1989).

The cosmids representing the 11 contigs between D19S221 and D19S179 are: 31419, 30649, 30645, 10128, 31000, 15929, 15566, 31240, 30272, 33071, 20236, 28333, 16922, 23946, 30762, 29174, 29355, 23127, 17208, 30633, 32236, 27105, 27415, 26540, 30723, 22159, 25980, 23890, 33890, 30041, 31192, 30720, 31614, 31606.

2.2. Southern blotting and filter hybridization

*Eco*RI, *Bam*HI, *Hin*dIII, *Bgl*I single and double digestions and southern blotting were performed following standard procedures according to the manufacturers. Hybridizations of Southern blotting and colony plasmid, cosmid, BAC and PAC filters with probes and oligonucleotides were performed, with ^{32}P-oligolabeled DNA fragments or ^{32}P end-labeled oligonucleotides, according to standard procedures (Sambrook et al., 1989).

2.3. FISH

FISH was performed as described previously (Brandriff et al., 1994).

2.4. Cosmid fragments subcloning

Cosmid DNA were double-digested with *Eco*RI and *Taq*I restriction enzymes, and the resulting fragments were subcloned with the SureClone™ Ligation Kit (Pharmacia-Biotech) according to the manufacturer.

2.5. PCR and sequencing analyses

Alu PCR, Alu-Vector PCR and splice-PCR were performed as described by Nelson et al. (1991) and Fuentes et al. (1997). Standard PCR analyses were performed in 25 μl reactions containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton® X-100, 1.5 mM $MgCl_2$, 200 μm dNTPs, 20 pmol of each primer, 1 U of Taq polymerase, and 200–400 ng of genomic or 2 ng of cosmid or plasmid DNA template, using a program of initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at a temperature depending on the primer pair for 30 s, extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. Di- and tri-nucleotide repeat STSs were analyzed by PCR under the same conditions described above but in a final volume of 12.5 μl, 10 pmol of each primer [one of which was previously ^{32}P end-labeled (Sambrook et al., 1989)], 0.5 U of Taq polymerase and 40 ng of genomic DNA template. The reaction products were separated by electrophoresis on 1–2% agarose gel, stained with ethidium bromide and visualized by a BioRad Gel Doc 2000 or, in the case of radiolabeled products, on a 6% polyacrylamide denaturing gel and visualized by autoradiography. Table 1 shows the PCR annealing temperatures and conditions if they differ from the described standard PCR. The PCR analyses for the 18 ESTs markers were performed with the primers and under the same conditions reported in GenBank. PCR amplification of the exon 37B was performed by the standard PCR method at 54°C with the following primers: ex37Bforw 5′-GTTCTCTGTTCCCATTCCCG-3′ and ex37Brev 5′-AACCCAGTGCCTGGACGTC-3′; the PCR product was 208 nt long.

Sequencing of cosmid and plasmid inserts and of PCR products was performed with an ABI 310 automated sequencing apparatus with a Cycle Sequencing Perkin Helmer kit according to the manufacturer.

3. Results

3.1. Extension and merging of cosmid contigs

At first, several gaps between the 11 ordered cosmid contigs were present in the region, including a wide gap next to the *CACNA1A* gene. To complete the physical coverage of this region, probes generated by Alu–Alu and Alu–Vector PCR of cosmids at the ends of each contig were hybridized to colony filters of chromosome 19 cosmid libraries.

In three cases (between contigs: 1596 and 792; 792 and 2321; 1009 and 847), a single walk step was sufficient to join two previously assembled contigs (for details of restriction maps, see http://www-bio.llnl.gov/rmap/). However, during cosmid walking between other contigs, apparent gaps in the cosmid library were found. Such gaps could represent either sequences that are unstable in the vector/host system used, or a biased distribution of the restriction sites used for library construction or, simply, the statistics of DNA sampling in library genera-

Table 1
New STSs characterized in the D19S221–D19S179 chromosome region[a]

STS (dbSTS_Id)	Polymorphism		PCR	Primers
	Allele	Freq. ± e.s.		
AAG18 (71914)	1	0.02 ± 0.02	$A = 57°C$	F: 5′-gctatcgctcccagcgtg-3′
	2	0.34 ± 0.07	Note: plus 10% DMSO	R: 5′-tcgaagctgcagctgttcc-3′
	3	0.02 ± 0.02		
	4	0.04 ± 0.03		
	5	0.57 ± 0.07		
GT8 (71911)	1	0.04 ± 0.04	$A = 55°C$	F: 5′-cagtctgtcccaggaagc-3′
	2	0.58 ± 0.10		R: 5′-accacatagactcacagaatc-3′
	3	0.25 ± 0.09		
	4	0.08 ± 0.06		
	5	0.04 ± 0.04		
GT3 (76855)		Not pol.	$A = 53°C$	F: 5′-aattataggcatgtgccacc-3′
				R: 5′-gcattttggaaggctgagtc-3′
CCG7 (71913)		Not pol.	$A = 57°C$	F: 5′-tgcaggtcgactctagagg-3′
			Note: plus 10% DMSO	R: 5′-tcgccggcaagcagcaag-3′
GT18 (71912)		Not pol.	$A = 55°C$	F: 5′-tggtaaggcagtagtagac-3′
				R: 5′-tgctagatgctggagatac-3′
SPL1.4 (71909)		Not pol.	$A = 62°C$	F: 5′-cacagctgctcagagggagc-3′
				R: 5′-gccgtcccatggggtggac-3′
SPL0.3 (71910)		Not pol.	$A = 53°C$	F: 5′-aggtcaaagtctggagttg-3′
				R: 5′-ggacattcctatcttcttcc-3′

[a] A, annealing temperature. Only differences from the standard PCR method described in Section 2.5 are reported.

tion. In an effort to obtain clonal coverage across these gaps, total genomic PAC and BAC libraries (Shizuya et al., 1992; Ioannou et al., 1994) were screened with the same probes generated by cosmid walking. Since cosmids are more reliable and easy to handle, once BAC or PAC clones were found extending the contigs, they were used to generate new probes by Alu–Alu and Alu–Vector PCR, the latter being hybridized back to chromosome 19 cosmid libraries.

A set of cosmids representative of the region (see Section 2.1) has been analyzed by PCR for the presence of D19S840 and D19S914 STSs genetically mapped in the region (Dib et al., 1996). D19S914 was located to cosmid 29972, whereas D19S840 was not present in this set of cosmids. It was thus hybridized to an LLNL chromosome 19 specific library, and was very useful in locating a new contig, mapped by FISH in the wide gap between D19S1150 (an internal marker to *CACNA1A*) and *RFX1* markers. This new contig was merged to the flanking *CACNA1A* contig using the same procedure described above (Fig. 1).

3.2. Orientation and refinement of CACNA1A gene genomic structure

Due to the connection of the contig, where the gene itself was characterized, with the adjacent contigs, a new *CACNA1A* orientation was obtained with the 5′ end proximal with respect to the 3′ end (Fig. 1).

A comparison between the *CACNA1A* cDNA and exon sequences (Accession Nos. X99897, Z80114–Z80155) reported by Ophoff et al. (1996) and the six isoforms (Accession Nos. U79663–U79668) reported by Zhuchenko et al. (1997) revealed that two of the latter differ from X99897 cDNA sequence by 97 nucleotides. The position of these nucleotides and the length of the stretch suggested the presence, in these two isoforms, of an alternative exon 37, instead of the canonical exon 37, referred to here as 37B and A, respectively.

In order to localize and characterize the alternative exon 37B, we hybridized a colony filter of all the cosmids belonging to the contig containing the *CACNA1A* gene (Ophoff et al., 1996), with primers specific for exons 36, 37A, 37B and 38. The cosmid 15496 was found to be positive for all exons 36–38. We obtained a restriction map of the cosmid by hybridization of a panel of *Eco*RI, *Bam*HI, *Hin*dIII, *Bgl*I single and double digestions of cosmid 15496 with these primers and inferred the following relative order of the exons: 36–37A–37B–38 (Fig. 2). The sequence of exon 37B and its intron/exon boundaries was then characterized (Accession No. AF144098), and a primer pair was designed from it (see Section 2.5) for exon 37B mutation analysis, including 49 bp upstream and 23 bp downstream of the exon boundaries.

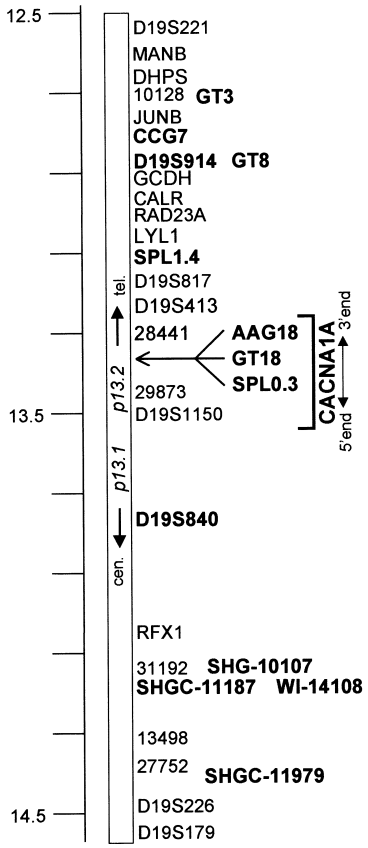

Fig. 1. Schematic physical map of the D19S221–D19S179 interval of the short arm of chromsome 19. The markers whose physical localization are described in this paper are in bold (distances in Mb from the telomere). Modified from: Chromosome 19-p arm metric physical map, http://www-bio.llnl.gov/genome/html/chrom_map.html.

3.3. Characterization of new polymorphic and non-polymorphic STSs

A set of cosmids, representative of the region under study, was hybridized with oligonucleotides made up of di- and tri-nucleotides: $(GT)_{15}$, $(ACG)_{10}$, $(AGG)_{10}$, $(ACC)_{10}$, $(AGC)_{10}$, $(ACT)_{10}$, $(ATC)_{10}$, $(AAC)_{10}$, $(AAG)_{10}$, $(AAT)_{10}$ and $(CCG)_{10}$. The positive cosmids were double-digested with *Eco*RI and *Taq*I restriction enzymes, subcloned in pUC18 and screened again with the positive oligonucleotide. The selected subclones were thus sequenced and the repeat characterized. In addition, PCR products were generated with a pool of primers designed on splicing donor and acceptor site consensus sequences (Fuentes et al., 1997) from the set of cosmids, subcloned in pUC18 and then sequenced. A total of seven new STSs have been detected (Table 1). Two of these were found to be polymorphic for an internal STR, three were found to contain non-polymorphic STRs, and the last two contained splicing consensus sequences (SPL). They were: AAG18 (dbSTS_Id 71914), an imperfect GAAA/GAA repeat showing five common alleles, mapping on overlapping cosmids 26540 and 27415 (Accession No. AC005513) and located within the first intron of *CACNA1A* gene; GT8 (dbSTS_Id 71911), an imperfect TG repeat $[(TG)_{15}TC(TG)_2 TC(TG)_2TA(TG)_8]$ showing five common alleles, mapping on the overlapping regions of cosmids 31240 and 30272 (Accession No. AD000092); GT3 (dbSTS_Id 76855) mapping on cosmid 10128 and containing a non-polymorphic GT repeat; CCG7 (dbSTS_Id 71913) mapping on cosmid 15566 and containing a $(CGG)_2AGG(CGG)_5$ segment; GT18 (dbSTS_Id 71912) mapping on 26540 and containing a non-polymorphic $(GT)_{10}T(GT)_3$; SPL1.4 (dbSTS_Id 71909) mapping on overlapping cosmids 16922 and 23946; and SPL0.3 (dbSTS_Id 71910) mapping on overlapping cosmids 26540 and 27415.

3.4. Localization of ESTs

The set of cosmid representative of the region under study was further analyzed by PCR for the presence of the following EST markers, mapped by a radiation hybrid in the region (Gyapay et al., 1996; Stewart et al., 1997): Bda20d07, SGC31653, SGC35402, SHGC-9937, SHGC-10107, SHGC-11979, SHGC-11187, SHGC-30311, stSG4364, stSG4782, WI-7557,

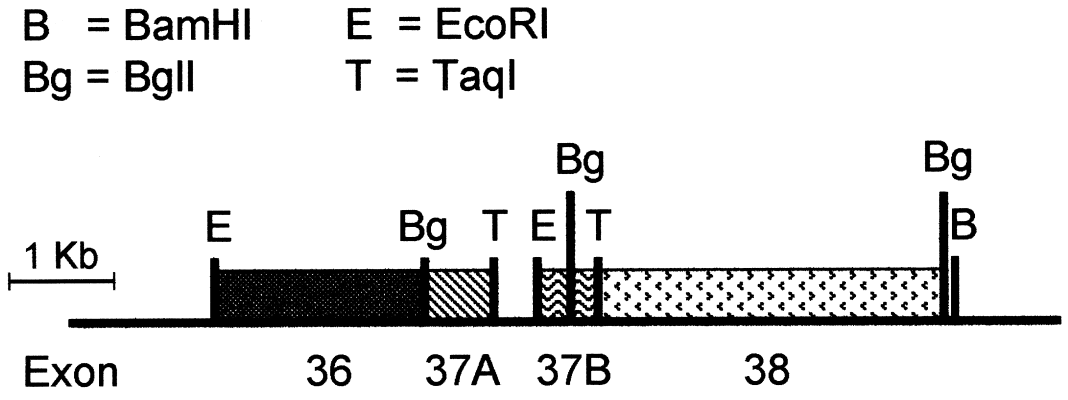

Fig. 2. Restriction map of the 15496 cosmid portion containing exons 36–38 of *CACNA1A* gene.

Table 2
ESTs positive for the D19S221–D19S179 chromosome region

Marker	Positive cosmid(s)	Protein similarity
SHGC-11979	21020, 18656	100% *Homo sapiens* heat shock 40 kD protein 1, HSPF1
SHGC-11187	7503, 28320	99% *Homo sapiens* protein kinase, cAMP-dependent, catalytic alpha, PRKACA
SHGC-10107	31192	96% *Homo sapiens* eukaryotic translation elongation factor 1 delta, EEF1D
WI-14108	28320	51% *Saccharomyces cerevisiae* anti-silencing protein-1

WI-14108, WI-14932, WI-15958, WI-18593, U10324. Four of them, SHGC-11979, SHGC-11187, SHGC-10107 and WI-14108, gave a positive signal. Table 2 shows the positive ESTs, the corresponding positive cosmid(s), and best protein similarity.

4. Discussion

With the enlargement of the *CACNA1A* containing the cosmid contig, it is possible to analyze the 5′ and 3′ flanking regions of the gene coding segment and thus characterize the promoter and possible regulation regions not yet identified. Since a number of FHM/EA2 families, whose lod score analysis gave a significant linkage to *CACNA1A* closely linked markers, were screened and no mutations were found in the coding region (Ophoff et al., 1996; M. Frontali unpublished data), it is likely that a considerable amount of the FHM and EA2 mutations reside in the *CACNA1A* controlling regions. The extension of the mutation screening to these regions is thus necessary for a complete comprehension of the function of the gene itself. Moreover, the characterization of the new exon 37B with its intron/exon splicing boundaries will extend *CACNA1A* mutation screening to this additional exon.

The finding of a polymorphic trinucleotide repeat (AAG18, dbSTS_Id 71914) in the first *CACNA1A* intron could be very useful to those who are interested in studying *CACNA1A*-associated diseases. In fact, this locus is similar to the GAA polymorphic repeat, whose expansion is responsible for Friedreich's ataxia (Campuzano et al., 1996). Therefore, an analysis of a possible expansion should be taken into account when planning a screening of mutations for EA2/FHM and other 19p13 linked diseases.

Furthermore, in this chromosome 19 region, four potential genes — heat shock 40 kD protein 1 (HSPF1), protein kinase, cAMP-dependent, catalytic alpha (*PRKACA*), eukaryotic translation elongation factor 1 delta (*EEF1D*), and anti-silencing protein-1 — have been physically localized. The physical localization on cloned and ordered genomic DNA fragment makes the genomic organization analysis of these genes possible.

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