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Episodic ataxia type 2 (EA2) and spinocerebellar ataxia type 6 (SCA6) due to CAG repeat expansion in the *CACNA1A* gene on chromosome 19p

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Point mutations of the *CACNA1A* gene coding for the α_{1A} voltage-dependent calcium channel subunit are responsible for familial hemiplegic migraine (FHM) and episodic ataxia type 2 (EA2). In addition, expansions of the CAG repeat motif at the 3′ end of the gene, smaller than those responsible for dynamic mutation disorders, were found in patients with a progressive spinocerebellar ataxia, named SCA6. In the present work, the analysis of two new families with small CAG expansions of the *CACNA1A* gene is presented. In one family, with a clinical diagnosis of EA2, a CAG_{23} repeat allele segregated in patients showing different interictal symptoms, ranging from nystagmus only to severe progressive cerebellar ataxia. No additional mutations in coding and intron–exon junction sequences in disequilibrium with the CAG expansion were found. In the second family, initially classified as autosomal dominant cerebellar ataxia of unknown type, an inter-generational allele size change showed that a CAG_{20} allele was associated with an EA2 phenotype and a CAG_{25} allele with progressive cerebellar ataxia. These results show that EA2 and SCA6 are the same disorder with a high phenotypic variability, at least partly related to the number of repeats, and suggest that the small expansions may not be as stable as previously reported. A refinement of the coding and intron–exon junction sequences of the *CACNA1A* gene is also provided.

INTRODUCTION

Episodic ataxia type 2 (EA2), also known as acetazolamide-responsive/hereditary paroxysmal cerebellar ataxia (APCA/HPCA), is an autosomal dominant disorder characterized by attacks of vertigo, visual disturbance, dysarthria and ataxia lasting from minutes to days, which respond to acetazolamide treatment. Interictally, nystagmus and other cerebellar signs of variable severity are present (1–3). EA2 is genetically and clinically distinct from episodic ataxia type 1 (EA1), the latter being due to mutations in the *KCNA1* gene on chromosome 12p13 (4,5) and characterized by shorter attacks and interictal myokymia without cerebellar signs.

The gene responsible for EA2, *CACNA1A* (6), previously named *CACNL1A4*, maps on chromosome l9p13 and codes for an α_{1A} voltage-dependent calcium channel subunit (7). Mutations of this gene were also found to be associated with familial hemiplegic migraine (FHM). At present, four missense mutations causing FHM and two mutations, disrupting the reading frame, responsible for EA2 are known (7).

Recently, Zhuchenko *et al.* (8) reported eight families affected with an autosomal dominant cerebellar ataxia (ADCA), named spinocerebellar ataxia 6 (SCA6) by the authors, in which the disease segregated with a small stable expansion, ranging from 21 to 27 units, of the CAG repeat contained at the 3′ end of the *CACNA1A* gene. Since none of the 475 tested normal subjects had an allele size exceeding 16 repeats, the authors postulated that the expansion, although not reaching the size observed in dynamic mutation disorders (e.g. SCA1, SCA2, etc.), is responsible for the disease. Analysis of different cDNA isoforms revealed that the CAG repeat is translated into a polyglutamine stretch in three isoforms in which an alternative splice site five nucleotides upstream extends the open reading frame (ORF) beyond the stop codon reported by Ophoff *et al.* (7). SCA6 and EA2 are considered by Zhuchenko *et al.* (8) to be not only genetically but also phenotypically different disorders. The former, due to small CAG expansions, is characterized by a permanent and progressive cerebellar functional deficit and atrophy preceded by momentary imbalance and ‘wooziness’, while the latter, due to truncated protein, presents only mild and intermittent cerebellar dysfunction.

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Table 1. Main clinical features in eight EA2 Italian families

Family	No. of patients examined	Age at examination (years)	Onset of attacks (years)	Response to acetazolamide	Interictal nystagmus ± mild cereb. signs	Severe progressive ataxia	Cerebellar atrophy at MRI
1	3	34–74	25–45	1/1	2/3	1/3	1/1
2	2	33–63	9–50	–/–	2/2	0/2	1/1
3	2	18–55	12–30	1/1	2/2	0/2	1/1
4	2	61–83	54–61	1/1	1/2	1/2	1/1
5	3	16–60	7–20	–/–	2/3	0/3	1/1
6	2	31–61	30	–/–	1/2	0/2	0/1
7	3	36–60	25–35	1/1	2/3	0/3	0/1
8	13	23–74	20–52	7/7	9/13	4/13	11/11

The present work shows that small CAG expansions of the *CACNA1A* gene can (i) cause both an intermittent and a progressive permanent cerebellar deficit in the absence of other mutations, leading us to question the distinction between SCA6 and EA2; and (ii) can exhibit inter-generational changes of allele size suggesting that they might be prone to a low level of instability. A refinement of the coding and intron–exon junction sequences of the *CACNA1A* gene is also provided.

RESULTS

CAG expansion and the EA2 phenotype

Within a long-term project on ADCAs, eight families were diagnosed as affected with EA2. Criteria for the clinical diagnosis are described in Materials and Methods. The main clinical features observed in these families are reported in Table 1.

As a part of a project aimed at identifying polymorphic trinucleotide repeats possibly involved in EA2 in the 1.5 Mb region where the gene was mapped by linkage (9), a CAG repeat was detected before the identification of the *CACNA1A* gene by Ophoff *et al.* (7), later found to correspond to that reported at the 3′ end of the gene. All the eight EA2 families were typed for this repeat as well as unrelated normal subjects. The CAG allele size in 86 normal chromosomes from random and affected subjects ranged between seven and 14 repeats, in agreement with other reports (7,8,10,11). The size of the repeat motif segregating with the disease in seven out of eight EA2 pedigrees ranged from nine to 13 repeats and in family 8 was 23 repeats, as also confirmed by sequencing. This CAG_{23} allele was found in all 13 patients of the same family and in five asymptomatic 50% at-risk subjects, aged 23–37 years. No instability of the repeat length was detected in this family. None of the other 52 healthy relatives had the expanded allele.

The detailed clinical phenotype of family 8, with the CAG_{23} allele, has been reported previously (9). The main features were repeated attacks of vertigo and unsteadiness responsive to acetazolamide treatment, interictal cerebellar signs of variable severity ranging from nystagmus only to severe progressive ataxia, and vermian plus or minus hemisphere cerebellar atrophy at magnetic resonance imaging (MRI) scan. Age at onset of attacks and of permanent ataxia were highly variable (range 20–52 and 35–70 years, respectively), showing that factors other than repeat number were modulating the natural history of the disease. Progression to severe ataxia was observed in four patients with a disease duration ranging from 8 to 25 years. Other relatives with a similar disease duration showed only mild permanent cerebellar signs. The distinctive features in this family were a strong vestibular component of attacks without clear cerebellar signs, an age at onset in adulthood and a high frequency of cerebellar atrophy.

This phenotype, however, was not qualitatively different from that observed in the other EA2 families with CAG alleles in the normal range, in terms of progression to severe ataxia and cerebellar atrophy: both family 1 and 4 had severely ataxic patients, and cerebellar atrophy in one patient was found in five families.

In order to test whether CAG expansions could also be found among families diagnosed as affected with ADCA or with idiopathic late onset cerebellar ataxia (ILOCA), nine ADCA families and four ILOCA patients without SCA1, SCA2, SCA3 or DRPLA expansions were screened. Only one family, DA (Fig. 1), was found to have a CAG_{25} allele segregating with ADCA, in a mother, II-3, and in two affected children, III-2 and -3. The onset of the disease in these patients was at 39, 33 and 24 years of age, respectively. Extending the examination to new DA family members, reported as normal, was planned as a means of testing if non-ataxic relatives could have an EA2 phenotype with short vertigo/dizziness episodes and interictal nystagmus. Preliminary results showed that three members (54, 58 and 64 years old) had a history of vertigo/dizziness episodes of variable duration (4–23 years) and two of them were showing interictal nystagmus on the lateral gaze. Some of their children (not reported in the pedigree) also showed similar signs, and therefore all the criteria for a diagnosis of EA2, used for the previous families, were met (see Materials and Methods). DNA typing of these three subjects revealed that they were carrying a CAG_{20} allele. Overall, genotypes in this family (Fig. 1) imply that the expanded CAG allele was inherited from the father I-1, and strongly suggest his carrying a CAG_{20} allele passed on to three children. Subject I-1, deceased when 72 years old, was in fact reported as having had vertigo episodes. An increase from 20 to 25 repeats in the paternal expanded allele must have given origin to the CAG_{25} allele carried by II-3 and her two children. In order to confirm such a hypothesis and to exclude possible, although unlikely, errors in the reconstruction of the paternal genotype, e.g. due to non-paternity, markers flanking the CAG repeat were typed and the haplotype segregating with the expanded allele reconstructed. Markers *D19S221*, *D19S1150* telomeric and *D19S840*, *RFX1* centromeric to the CAG repeat, were used. The results, reported in Figure 1, clearly show that the two expanded alleles share an identical haplotypic context, not found in 109 independent normal chromosomes.

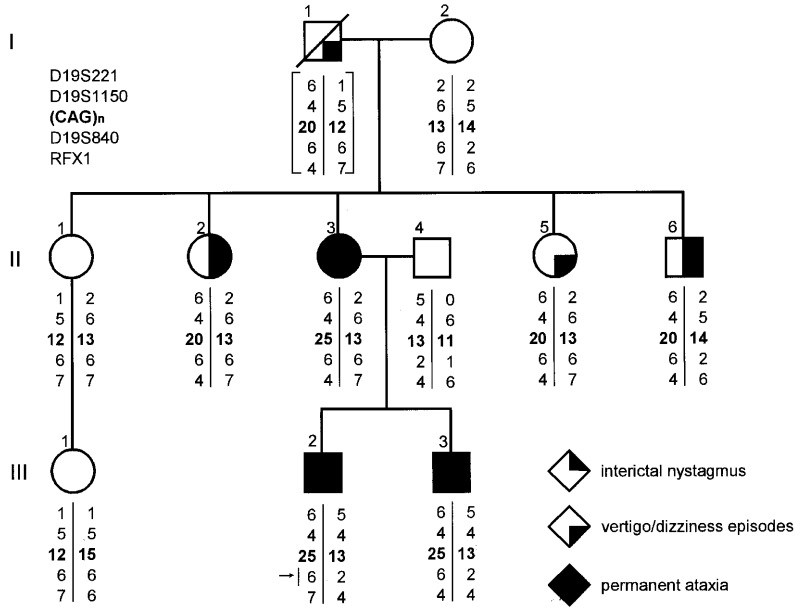

Figure 1. Pedigree of family DA showing CAG repeat alleles and their haplotypic context. The haplotype in brackets is inferred. The arrow indicates a recombination either between the CAG_n repeat and *D19S840*, or between the latter marker and *RFX1*.

Table 2. Sequence changes found in 47 exons and intron-exon junctions as reported by Ophoff *et al.* (7)

Exon	Position[a]	Mutation	Comments
10	–2 at 5′ end	del A	changes the reported 5′ splice site, and moves it 3 nt forward
18	–3 at 5′ end	del C	
18	+7 at 3′ end	ins G	
19	2961	ins G	changes corresponding aa from D to G
19	2963	del C	changes corresponding aa from D to G
21	3894–6	del AAG	eliminates a codon for glutamic acid
21	+16 at 3′ end	del G	
26	+11 at 3′ end	G→A	
36	–12 at 5′ end	ins CG	
36	–12 at 5′ end	C→T	patient is heterozygote C/T while the normal is homozygote C/C
40	–8 at 5′ end	ins G	
43	–11 at 5′ end	ins C	
43	–8–9 at 5′ end	CG→GC	
44	+8 at 3′ end	ins C	
44	+9 at 3′ end	ins C	
44	+19 at 3′ end	G→C	

[a]Negative and positive figures refer to distance from acceptor and donor splice sites, respectively; position of changes in the coding sequence refers to cDNA sequence (accession no. X99897).

Screening for mutations in *CACNA1A* gene bearing the CAG_{23} allele and refinement of exon and intron–exon junction sequences

In order to test whether the CAG repeat expansion was the only mutation causing the EA2 phenotype, screening for genomic rearrangements and point mutations was performed in one patient from family 8.

Hybridization of *Eco*RI-, *Bam*HI- and *Hin*dIII-digested genomic DNA with *CACNA1A* cDNA provided no evidence for visible rearrangements in the gene. A sequence analysis of the coding exons and the intron–exon junctions of the *CACNA1A* gene provided the following results.

The 47 coding exons and intron–exon junctions reported by Ophoff *et al.* (7) initially were sequenced and changes from the sequences reported in GeneBank (accession Nos X99897, Z80114-5 and U79663-8) were considered potential mutations when found either in exons or in introns up to 20 nucleotides from the splice site. These changes, listed in Table 2, were homozygous in the patient, except for the C→T transition in exon 36. All

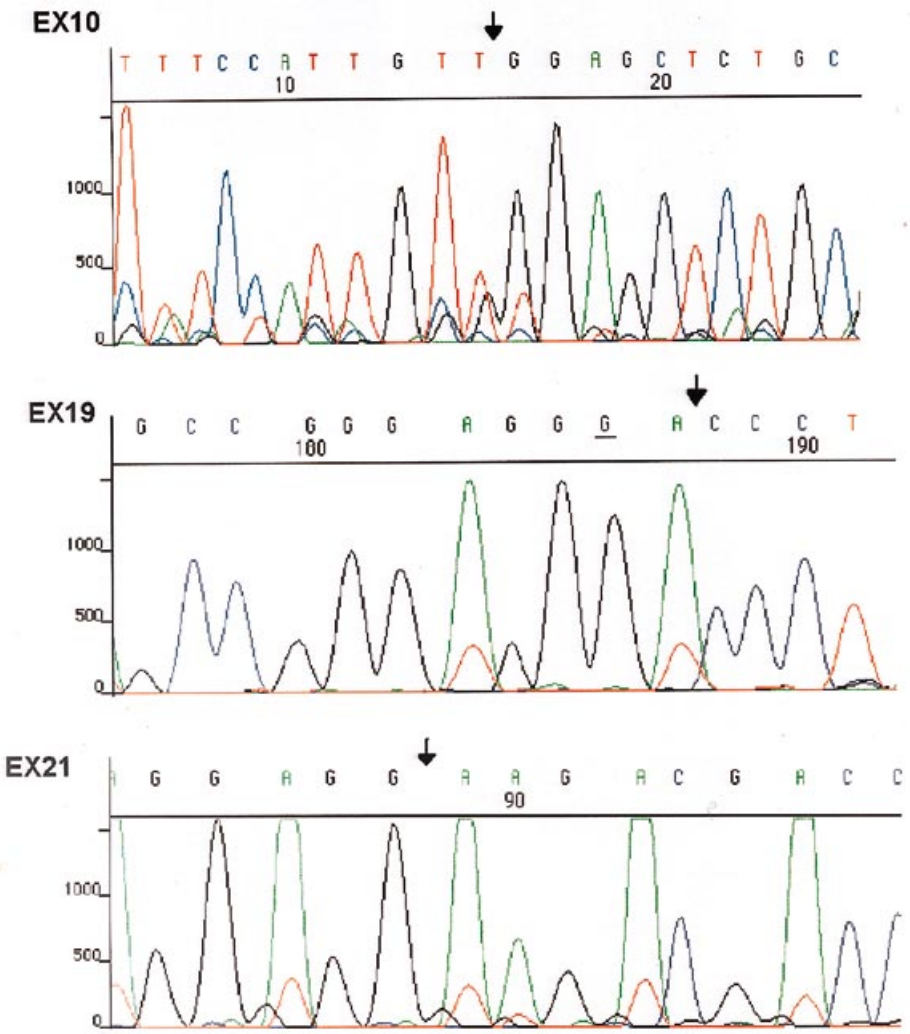

Figure 2. Sequences of exons 10, 19 and 21 of an EA2 patient (family 8) showing nucleotide changes that modify the amino acid sequence as compared with previous data (7). Arrows indicate the site of nucleotide deletions; inserted nucleotide is underlined (see Table 2 for details).

homozygous changes turned out to be homozygous also in a normal control, whose DNA was sequenced. Exons 10, 19 and 21 showed changes modifying the amino acid sequence (Table 1 and Fig. 2) and were tested further by sequencing the DNA samples of two additional normal subjects and one additional patient from family DA. All of them were homozygotes for the changes reported in Figure 2. It should also be noted that the new amino acid sequence coded by the modified exons 19 and 21 is identical to that of the homologous mouse protein at the same positions (accession No. U76716). Moreover, 15 unrelated subjects of different European origin and two patients of family 8 were tested by single strand conformation polymorphism (SSCP) analysis for the exons 10, 18, 19, 21, 40, 43 and 44. All of them showed a pattern identical to that of the patients. All the above evidence indicates that changes in these exons should be considered amendments of the previously reported sequences (7). The C→T transition of exon 36 caused a loss of an *Msp*I restriction site. Digestion with this enzyme of the DNA of 24 normal subjects provided evidence of three distinct genotypes: C/C, C/T and T/T. The site is therefore polymorphic, with an estimated T allele frequency of 0.125 ± 0.047.

Other homozygous changes in the patient DNA were not tested in a normal control since: (i) substitutions T→C at positions 3263 and 4898 of the cDNA (accession No. X99897) of exons 19 and 29, respectively, do not change the coded amino acids; (ii) substitutions G→T, C→T and C→T at positions 5054, 5089 and 5128, respectively, of the cDNA (accession No. X99897) of exon 30, are consistent with the cDNA sequence reported by Zhuchenko *et al.* (8). In addition, the patient is heterozygote A/C at position 623 of the cDNA, with no change in the corresponding amino acid. On the whole, none of the above changes could be considered a mutation affecting the protein and, therefore, responsible for the disease.

A comparison of the six isoforms reported by Zhuchenko *et al.* (8) showed the presence of an alternative splice site occurring five nucleotides upstream from the reported exon 47, leading to a longer ORF. Additional sequencing of the extended ORF of exon 47 did not reveal any change, as compared with sequences reported in GenBank.

DISCUSSION

In the present work, we report on small CAG expansions of the *CACNA1A* gene in families presenting with an EA2 phenotype, i.e. episodes of vertigo/dizziness and variable interictal cerebellar signs, from nystagmus only to severe progressive cerebellar ataxia. The first family was diagnosed originally as affected by EA2 and reported as such in the literature (9). The main clinical features of these patients were not qualitatively different from those found in other EA2 families with CAG alleles in the normal range, in terms of age at onset, progression to severe ataxia and cerebellar atrophy. This appears to be in good agreement with literature data showing that the EA2 phenotype is highly variable within families and that intermittent ataxia may evolve to severe permanent ataxia in some patients but not in others (12–14). No additional mutation responsible for the EA2 phenotype in the family could be found in the complete coding sequence of the gene and in the intron–exon junctions. This excludes the presence of mutations altering the predicted protein structure, while mutations affecting the still unknown regulatory regions of the gene cannot be ruled out at present. The variability of the phenotype could not be accounted for by the number of CAG repeats, which was stable in all the patients examined.

The second family, originally diagnosed as affected with ADCA of an unknown genetic type, was ascertained through three family members presenting with severe progressive ataxia. These patients had a CAG_{25} allele. When new family members, reported as normal, were examined, some of them met the criteria for the diagnosis of EA2 used for selecting other families with this disease. All of them carried a CAG_{20} allele. In this family, therefore, the two expanded alleles, resulting from an inter-generational allele size change, were associated with different clinical phenotypes.

Overall, these results have several implications. First of all, the genotype–phenotype correlation proposed by Zhuchenko *et al.* (8), i.e. stop mutations leading to an EA2 and expansion of the CAG motif to a SCA6 phenotype, is not confirmed by the present evidence. The CAG_{23} allele segregating in family 8 with an EA2 phenotype is well within the range of expansions reported for SCA6 patients by Zhuchenko *et al.* (8), and family DA shows a continuum between the two phenotypes as a function of the size of the CAG allele. EA2 and SCA6 should, therefore, be considered the same disorder with a high phenotypic variability, only in part related to the number of repeats. Other factors involved in the progression of the disease to severe permanent ataxia should be hypothesized. In the absence of any knowledge about underlying pathogenic mechanisms, only highly speculative hypotheses can be proposed. For example, a variable degree of permanent neuronal damage could be produced during attacks, as a function of their frequency, length and severity. Since attacks are triggered by a variety of stimuli, such as quick head movements and physical and emotional stress, a gene–environment interaction can contribute to the development of an SCA6 phenotype.

Secondly, if a family affected with EA2 is ascertained through severely ataxic subjects, the presence of short vertigo episodes and of mild cerebellar signs in non-ataxic subjects may be overlooked, as occurred in the second family. From this point of view, it would be interesting to know whether the non-ataxic relatives of SCA6 patients reported by Zhuchenko *et al.* (8) were checked accurately in order to exclude any possible sign reminiscent of EA2. It should be noted that among these patients, four sporadic cases are reported. If all of them carry new mutations, this would imply a very high mutation rate. On the other hand, if the expanded allele is inherited from one parent, the latter should show some clinical signs.

The size of the expanded alleles in the *CACNA1A* gene appears to be inter-generationally stable, both in family 8 and in the reported SCA6 families (8,10), as expected on the basis of the small number of repeats compared with that observed in disorders such as SCA1, 2 or 3, due to highly unstable large CAG expansions (15). The presence, in one family, of an inter-generational change in the expanded allele size could be due to a rare mutational event, such as those that occur in microsatellites with an estimated frequency between 10^{-3} and 10^{-5} (16,17). However, the number of meioses observed so far in families with expanded CAG repeat alleles is relatively small, ~50 (8,10,11), and among them another increase in the allele size is reported (11). An instability of the expanded CAG repeat alleles, although at a lower level than that reported for 36 repeats or more, should be considered. CAG typing in an extensive number of relatives of EA2/SCA6 patients would be very useful for providing information on this issue.

It is not clear how small CAG expansions produce their phenotypic effects. Zhuchenko *et al.* (8) favor a hypothesis that links the detrimental effects to the altered protein structure, i.e. to the expanded polyglutamine stretch in protein isoforms where the CAG repeat is translated. However, their pathogenic effect is not easily explainable, given that the number of polyglutamines in the CACNA1A protein is well within the normal range of polyglutamine tracts observed in other loci, and the ratio of isoforms with and without polyglutamines in the neuronal cells involved in the disease is not known.

An alternative hypothesis is that the expanded CAG stretch might affect mRNA stability, splicing efficiency or transcriptional/translational processes (7), leading to an insufficient production of the protein. Such a hypothesis could explain the EA2 phenotype in patients both with a mutation disrupting the reading frame and with CAG repeat expansions.

MATERIALS AND METHODS

Clinical diagnosis and CAG repeat genotypes

The criteria used for the diagnosis of EA2 patients were repeated attacks of vertigo and/or ataxia with interictal cerebellar signs (from nystagmus only to severe ataxia) transmitted as an autosomal dominant trait in at least two generations. Ataxia was considered severe when patients showed marked difficulty or inability to stand or walk without support. Response to acetazolamide treatment could be assessed in five families only, since in the others either the treatment had uncomfortable side effects, or the patients' compliance with drug treatment was very poor.

In the nine families affected with ADCA and in four subjects affected with ILOCA without SCA1, SCA2, SCA3 or DRPLA CAG expansions, the clinical phenotype included progressive cerebellar ataxia, cerebellar atrophy, dysarthria, dysmetria, dysdiadocokinesia, and nystagmus with or without other functional deficits.

Blood samples from EA2, ADCA and ILOCA family members were obtained after informed consent, as well as from 36 normal random subjects. DNA was extracted according to standard procedures and typed for the size of CAG repeat of the *CACNA1A* gene by PCR according to Ophoff *et al.* (7). Allele size was

assessed by comparison with sequenced genomic fragments containing seven, 11 and 23 repeats.

PCR analysis of DNA markers

PCR analyses of the markers *D19S221*, *D19S1150*, *D19S840* and *RFX1*, used to reconstruct the haplotype associated with the CAG expansions of the DA family, were performed according to methods reported in refs 7 and 18–20. The relative position of these markers and of the CAG repeat, as reported in the 19 chromosome map available at Lawrence Livermore National Laboratory (LLNL, unpublished data), is: tel–*D19S221*–*D19S1150*–$(CAG)_n$–*D19S840*–*RFX1*–cen.

Haplotypes on normal chromosomes were derived from 34 random subjects married into families already typed for chromosome 19p markers and from 41 independent normal chromosomes segregating in EA2 or CADASIL (18) families.

Screening for rearrangements

Genomic DNA samples (7 µg) from one affected and one unaffected individual from family 8 were digested with *Eco*RI, *Bam*HI and *Hin*dIII, separately, run in 0.8% agarose gel and blotted onto a nylon membrane (Hybond N+). The membrane was then hybridized with ^{32}P-oligolabeled cDNA fragment probes, obtained by PCR from total human cerebellum cDNA (Clontech) (7). After a high stringency wash with 0.1× SSC and 0.1% SDS for 20 min at 65°C, the blot was exposed to X-ray film at –70°C for 1–2 days. Genomic DNA from the same affected subject was used as substrate to generate PCR products for sequencing analysis. Amplifications of the 47 *CACNA1A* exons reported by Ophoff *et al.* (7) were performed according to procedures described, except for exons 37 and 42 where the following primers were used: exon 37 reverse 5′-TCATTCCATGGATGCTAGC-3′ and exon 42 reverse 5′-AACCACCCGGGCCCTG-3′. In addition, the coding region of exon 47 was amplified completely with primers: EX47F2 5′-TCCCCTGTGATCCGTAAG-3′ ; EX47F3 5′-TCCCAATCCCGTGTCTCCTTTG-3′; EX47F4 5′-GCACCATGGCTACTACC-3′; EX47F5 5′-TGCGCCTCGCCTTCTCG-3′; EX47R2 5′-ATTCCCTCGCCCGGGGCTTAG-3′; and EX47R3 5′-ATCGGCCTCGTCGTAGTC-3′. Sequencing of PCR products was performed with an ABI 310 automated sequencing apparatus with a Cycle Sequencing Perkin Elmer kit according to the manufacturer. All the PCR products were sequenced completely with both forward and reverse primers. Results discordant with the *CACNA1A* exon and splice site sequences (accession Nos X99897 and Z80114-5) were checked by sequencing the same exon in DNA of normal subjects drawn either from the CEPH collection (#134010 and #144107) or from a panel of random samples of the Italian population (BK17).

SSCP analysis was performed according to Ophoff *et al.* (7) on subjects belonging to 13 EA2 families without CAG repeat expansions, two normal random subjects from the Dutch population and two patients of family 8.

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