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# A PCR Amplification Method Reveals Instability of the Dodecamer Repeat in Progressive Myoclonus Epilepsy (EPM1) and No Correlation between the Size of the Repeat and Age at Onset

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## Summary

**Progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1) is a rare, autosomal recessive disorder characterized by onset at age 6–16 years, generalized seizures, incapacitating myoclonus, and variable progression to cerebellar ataxia. The gene that causes EPM1, *cystatin B*, encodes a cysteine proteinase inhibitor. Only a minority of EPM1 patients carry a point mutation within the transcription unit. The majority of EPM1 alleles contain large expansions of a dodecamer repeat, CCC CGC CCC GCG, located upstream of the 5′ transcription start site of the *cystatin B* gene; normal alleles contain two or three copies of this repeat. All EPM1 alleles with an expansion were resistant to standard PCR amplification. To precisely determine the size of the repeat in affected individuals, we developed a detection protocol involving PCR amplification and subsequent hybridization with an oligonucleotide containing the repeat. The largest detected expansion was ~75 copies; the smallest was ~30 copies. We identified affected siblings with repeat expansions, of different sizes, on the same haplotype, which confirms the repeat's instability during transmissions. Expansions were observed directly; contractions were deduced by comparison of allele sizes within a family. In a sample of 28 patients, we found no correlation between age at onset of EPM1 and the size of the expanded dodecamer. This suggests that once the dodecamer repeat expands beyond a critical threshold, *cystatin B* expression is reduced in certain cells, with pathological consequences.**

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## Introduction

The *cystatin B* gene is localized in 21q22.3 (Pennacchio et al. 1996), and it encodes a cysteine protease inhibitor (Turk and Bode 1991). Mutations in this gene cause progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1; MIM 254800 [http://www.ncbi.nlm.nih.gov/omim]) (Pennacchio et al. 1996; Lafrenière et al. 1997; Lalioti et al. 1997*a*). EPM1 is a rare, autosomal recessive disease with onset at age 6–16 years. Patients have generalized, clonic or tonic-clonic seizures and marked progressive and incapacitating myoclonus. They may develop ataxia and mild mental deterioration. Only a small minority (~14%) of the EPM1 alleles harbor mutations within the transcriptional unit of the gene (Pennacchio et al. 1996; Lafrenière et al. 1997; Lalioti et al. 1997*a;* Virtaneva et al. 1997). In the majority of patients, the disease is associated with expansion of a dodecamer sequence (CCC CGC CCC GCG), estimated, by Southern blot analysis, at >60 copies (Lalioti et al. 1997*b*, 1997*c*). Normal alleles contain two or three tandem copies of this sequence. Additional, rare, normal alleles containing 12–17 repeats were identified in two CEPH families. However, these repeats are unstably transmitted to offspring (Lalioti et al. 1997*c*). The repeat is located in the 5′ flanking region of *cystatin B* and therefore, unlike the trinucleotide repeats in other repeat-expansion disorders, is not contained in the primary transcript (Richards and Sutherland 1992; Ashley and Warren 1995; Paulson and Fischbeck 1996). The expansion is associated with a marked reduction of *cystatin B* mRNA in patients' blood but not in their cell lines.

Because of the nature of the repeat, which consists only of C and G nucleotides, PCR amplification of expanded EPM1 alleles of patients and heterozygotes was not possible. To date, the expanded alleles have been visualized only by Southern blot analysis. Because of the limited resolution of the Southern blot, it was not pos-

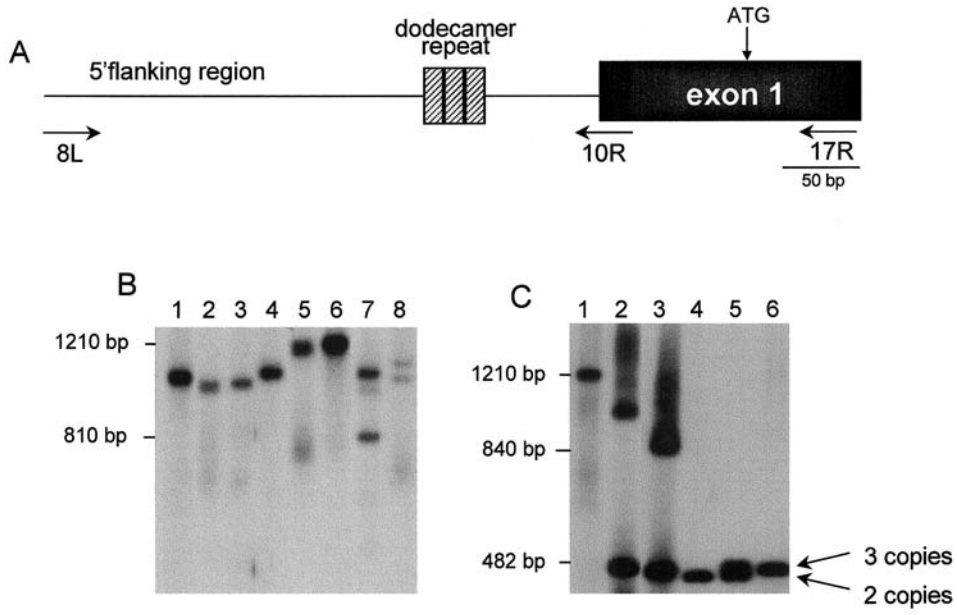


**Figure 1** *A,* Schematic representation of exon 1 of *cystatin B* and the 5′ flanking region containing the dodecamer repeats. The primers are shown as arrows below the gene. *B,* PCR amplification using primers 8L–17R from homozygous (lanes 1–6) and compound heterozygous patients with two expanded alleles (lanes 7–8). *C,* PCR amplification from a homozygous patient (lane 1), a compound heterozygote with a point mutation and an expansion (lane 2), a compound heterozygote with a two-nucleotide deletion and an expansion (lane 3), and normal individuals with two and/or three copies of the repeat (lanes 4–6). The size of the normal fragment with three repeats is 482 bp. Estimated sizes of the largest and smallest expanded alleles are indicated on the left of panels B and C.

sible to assess the instability of the large repeats, except in an indirect manner, and then only if the differences were substantially large (Lafrenière et al. 1997; Lalioti et al. 1997*c;* Virtaneva et al. 1997). Moreover, the sizes of the DNA fragments could not be determined, with precision, to evaluate their correlation with the phenotype. To determine the exact sizes of alleles within a single family, PCR amplification, with primers immediately flanking the repeat, is the method of choice. We have tried many PCR and electrophoresis conditions and detection techniques, with the aim of consistent amplification of the repeat expansions. Here we report our most successful procedure, which consists of a PCR amplification, under special conditions, and subsequent hybridization of the products by use of an oligonucleotide probe containing the repeat.

We have observed instability of the repeat, including both expansions and contractions, between siblings who share the same perigenic haplotype. Precise sizing of the repeat in different individuals showed no correlation between the size of the expansion and the age at onset of the disease.

## Material and Methods

### *Patients*

Descriptions of both the patient group and the diagnostic criteria have been reported elsewhere (Malafosse et al. 1992; Lalioti et al. 1997*a*; R. Gouider, S. Ibrahim, M. Fredj, R. Ouazzani, A. Gargouri, A. Malafosse, M. Baldy-Moulinier et al., unpublished data). The study was approved by the appropriate institutional review committees, and informed consent was obtained from the patients and from other members of their families.

Age at onset was determined by examination of all clinical records and detailed family history. Patients for whom these data were not available were excluded from the study.

### *PCR Amplification and Hybridization*

PCR amplification of the large and normal alleles was performed in a 15-$\mu$l volume containing 1 × cloned-*Pfu* polymerase buffer, 100 nM of each nucleotide (except that 7-deaza-2′-dGTP was substituted for all dGTP), 0.5 $\mu$M of each primer, 10% dimethyl sulfoxide (DMSO), and 1 U cloned-*Pfu* polymerase (Stratagene). Primers 8L (5′-CTG CAG GAT TGC CCC TAC TCC GAC TG-3′), 17R (5′-CTG GTC GGC GAT GTG CTG GGT CTC-3′), and 10R (5′-GGG GTC ACG TGA CGC GCG GGC GGA ACC AAG-3′) were used. Reverse primers 10R and 17R, in combination with forward primer 8L, produced comparable results. After the initial denaturation at 94°C for 5 min, a five-cycle "touchdown" reaction (94°C for 20 s, 65°C→60°C for 20 s, and 72°C for 2 min) was followed by another 25 cycles, with annealing at 60°C

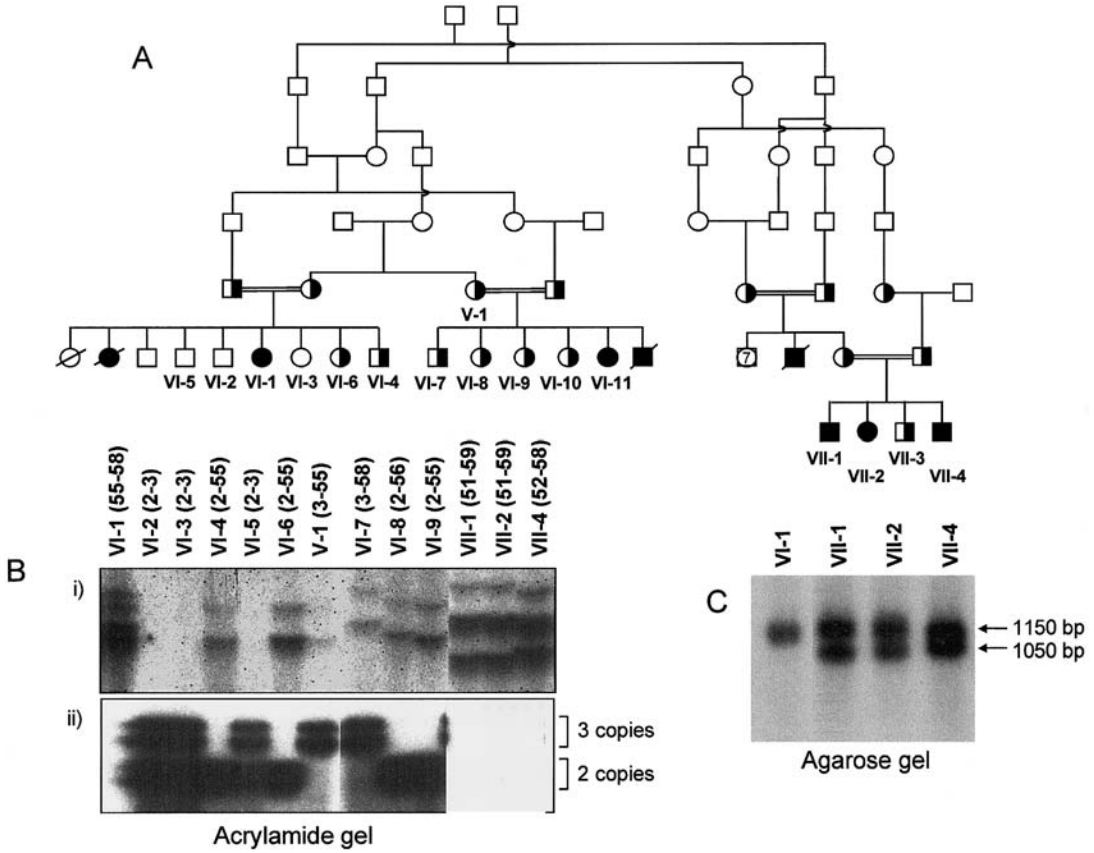


**Figure 2** *A,* Pedigree of family GVA08, from Valais, Switzerland, showing repeat instability. Affected and heterozygous individuals in the last three generations are indicated. All EPM1 alleles share the same haplotype around *cystatin B. B,* PCR amplification, with primers 8L–17R, and incorporation of $\alpha$-[$^{32}$P]dCTP, run on a denaturing acrylamide gel. Panel *i,* which shows the expanded alleles, was exposed for several days. Panel *ii,* which shows normal alleles, represents a shorter exposure of a lower region of the same gel. Because of the denaturation, each allele shows two bands. The large allele of individual V-1 (mother) has further expanded, by one repeat unit, on transmission to her carrier daughter, VI-8. Individual VII-4 (GVA08e, table 1) has expanded allele sizes that are different from those of his affected siblings, VII-1 and VII-2 (GVA08c and GVA08d, respectively, table 1). For each individual, the number of repeats of each allele is shown in parentheses. *C,* Detection of expanded EPM1 alleles in selected affected individuals, by PCR amplification and hybridization (see Material and Methods).

and a final extension at 72°C for 10 min. Products were electrophoresed overnight, in a 2% agarose gel, and were then transferred on a nylon hybridization filter (Zeta-probe, Biorad). The oligonucleotide CSTB.R2, used in hybridization, contains two copies of the dodecamer repeat 5′-(CCC CGC CCC GCG)$_2$-3′. It was labeled by use of terminal transferase (Boehringer) and $\alpha$-[$^{32}$P]dATP. Hybridization was performed in 50% formamide, 4 × SSC, 1% SDS, 10 × Denhardt's reagent, 5% dextran sulfate, and 100 $\mu$g/ml herring sperm DNA, at 50°C, overnight. Posthybridization washes were performed twice with 2 × SSC at 50°C for 30 min and then twice with 0.1 × SSC/0.1% SDS at 70°C for 30 min. The filters were exposed overnight with intensifying screens, at −80°C.

For determination of the heterozygotes' expanded allele size, radioactive PCR amplification was performed as described above, with the addition of $\alpha$-[$^{32}$P]dCTP. Loading buffer, with a final concentration of 55% formamide, was added, and the products were denatured at 95°C for 10 min, were loaded on a denaturing 5% acrylamide 8M urea gel, 0.8 × Tris-taurine-EDTA (20 ×:1.78 M Tris base, 0.58 M taurine, 10 mM EDTA), and were electrophoresed for 6 h at 80 W. The gel was dried and autoradiographed for several days, at −80°C.

## Results and Discussion

### *PCR Amplification and Hybridization*

Previously published PCR protocols for amplification of CG-rich sequences, such as the fragile X alleles (Brown et al. 1993; Chong et al. 1994; Levinson et al. 1994), failed to reproducibly amplify the EPM1 alleles. We have tested a number of PCR conditions, varying the DMSO concentrations, primer combinations, 7-

**Table 1**

**Estimates of the Number of Dodecamer Repeats in Patients with EPM1**

| Case No. | Patient[a] | Age at Onset (years) | Large Expansion | Small Expansion | Mean Expansion |
|---|---|---|---|---|---|
| 1 | GVA16 | 15 | 75 | 75 | 75 |
| 2 | GVA14 | 13 | 75 | 54 | 65 |
| 3 | GVA13 | 10 | 75 | 75 | 75 |
| 4 | GVA15a | 12 | 64 | 64 | 64 |
| 5 | GVA15b | 9 | 64 | 64 | 64 |
| 6 | GVA15c | 11 | 64 | 64 | 64 |
| 7 | GVA11a | 12 | 64 | 62 | 63 |
| 8 | GVA11b | 12 | 64 | 64 | 64 |
| 9 | GVA11c | 14 | 64 | 64 | 64 |
| 10 | GVA24 | 9 | 63 | 63 | 63 |
| 11 | GVA25a | 12 | 60 | 58 | 59 |
| 12 | GVA25b | 12 | 58 | 58 | 58 |
| 13 | GVA25c | 10 | 58 | 58 | 58 |
| 14 | GVA08a (VI-1) | 13 | 58 | 55 | 57 |
| 15 | GVA08b | 14 | 58 | 55 | 57 |
| 16 | GVA08c (VII-1) | 11 | 59 | 51 | 55 |
| 17 | GVA08d (VII-2) | 14 | 59 | 51 | 55 |
| 18 | GVA08e (VII-4) | 11 | 58 | 52 | 56 |
| 19 | GVA18 | 13 | 55 | 49 | 52 |
| 20 | GVA19 | 9 | 52 | 30 | 41 |
| 21 | GVA20a | 10 | 50 | 50 | 50 |
| 22 | GVA12 | 8 | 50 | 50 | 50 |
| 23 | GVA17 | 12 | 50 | 50 | 50 |
| 24 | GVA21b | 8 | 50 | 50 | 50 |
| 25 | GVA22 | 15 | 49 | 49 | 49 |
| 26 | GVA23 | 14 | 47 | 47 | 47 |
| 27 | GVA26 | 9 | 47 | 47 | 47 |
| 28 | GVA27 | 8 | 46 | 46 | 46 |

[a] Numbers in parentheses represent members of the pedigree GVA08, shown in figure 2*A*.

deaza-2′-dGTP concentration, polymerase, and PCR program. After PCR amplification, the products were electrophoresed overnight, transferred, and hybridized with an oligonucleotide primer containing two dodecamers. The hybridization with an oligonucleotide was preferred to direct labeling of the products by incorporation, because it takes advantage of the number of repeats in the expansion to amplify the signal strength, which would otherwise be weak because of poor amplification of the large alleles.

The use of *Pfu* polymerase, 7-deaza-2′-dGTP in place of dGTP, and 10% DMSO resulted in the most consistent amplification of the large alleles (fig. 1). The choice of PCR program, either two-step PCR (94°C and 68°C) or three-step touchdown PCR (94°C, 65°C→60°C, and 72°C), did not affect the outcome of the reaction. However, the choice of nucleotide primers was important, and reproducible results were obtained with the combinations 8L–17R and 8L–10R (fig. 1).

The amplifications from homozygous patients or from compound heterozygous patients with two different large alleles resulted in distinct amplified products (fig. 1*B*). However, in the heterozygotes with one normal and one expanded allele, additional bands, possibly due to heteroduplexes, were detected. Calculation of the exact size of the large allele is difficult in such cases. Thus, for these cases, PCR products were labeled, by incorporation of $\alpha$-[$^{32}$P]dCTP, and were electrophoresed in denaturing acrylamide gels. This method resulted in a better estimation of the large allele size, but signal strength was decreased (fig. 2*B*).

This method allowed us to calculate the sizes of the expanded alleles with greater precision than that afforded by Southern blot analysis. The estimated size of the largest allele, which was found in homozygosity, was 75 copies of the dodecamer (table 1). The smallest EPM1 allele contained 30 dodecamers and was detected in a compound heterozygous patient who had a second allele that contained 52 repeats.

### *Instability of the Expanded Alleles*

The instability of alleles with 12–17 repeats (Lalioti et al. 1997*c*) suggests that a similar situation may exist for the expanded alleles. Furthermore, Southern blot analyses of Finnish EPM1 alleles with the same haplotype revealed expanded alleles with different sizes, again suggesting instability (Lafrenière et al. 1997; Virtaneva

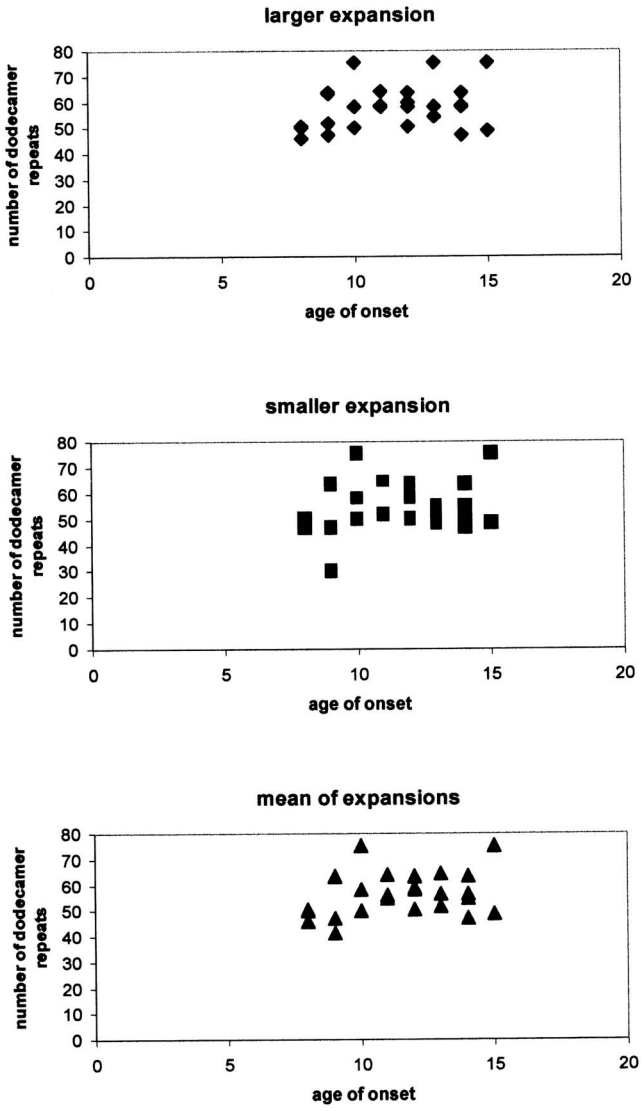


**Figure 3** Plots of the age at onset of EPM1 (*x* axes) with the number of dodecamer repeats (*y* axes) for the large, small, and mean allele sizes.

et al. 1997). Using the PCR method described here, we detected alleles with different numbers of repeats among affected siblings, between parent and child, and in more distant relatives, which directly confirms the instability of the disease-related expansion (fig. 2). All affected individuals in the consanguineous family GVA08, shown in figure 2, share the same haplotype around *cystatin B* (data not shown). However, there are six different sizes of expanded alleles (containing 51, 52, 55, 56, 58, and 59 repeats), all of which must have originated from a single, ancestral expanded allele (fig. 2; table 1, family GVA08). Because the most commonly observed allele in the pedigree contained 55 repeats, we hypothesized that both expansions and contractions have occurred in this family. An alternative hypothesis might be that the ancestral allele contained $\leqslant 51$ repeats and that all the individuals in the pedigree have expanded alleles.

Another expansion has occurred in heterozygous individual VI-8. The expanded allele was transmitted from her mother, since her normal allele, which contains two repeats, was paternal. We have previously observed only expansions of intermediate-sized alleles (12–13 copies of the dodecamer) during paternal transmissions (Lalioti et al. 1997*c*). The expansion of the dodecamer repeat, as seen in individual VI-8, is the first to be associated with maternal transmission. The PCR-amplification results showed no evidence of substantial somatic instability of the repeat in DNA from blood or from cell lines.

### *Lack of Correlation between the Expanded-Allele Size and Age at Onset of EPM1*

The age at onset of the trinucleotide-repeat–expansion disorders often correlates with the size of the expanded alleles (Ashley and Warren 1995; Filla et al. 1996; Paulson and Fischbeck 1996; Lamont et al. 1997; Monros et al. 1997; Montermini et al. 1997). We have investigated whether there is such a correlation between the size of the dodecamer-repeat expansion in EPM1 and the age at onset of disease. Since EPM1 is an autosomal recessive disorder and since some of our patients were compound heterozygotes with two different expansions, we have examined potential associations between the large, small, and mean allele sizes and the age at onset. Clinical data from 28 patients were collected, and the allele sizes were determined. As shown in figure 3, there is no apparent correlation, in this patient group, between the size of the repeat and the age at onset.

In Friedreich ataxia (FA), another autosomal recessive disorder that is due to a trinucleotide-repeat expansion, the age at onset is correlated with the size of the expansion. In compound-heterozygous FA patients with two different expansions, the size of the smaller one was more strongly correlated with the age at onset and the severity of the disease (Filla et al. 1996; Lamont et al. 1997; Monros et al. 1997; Montermini et al. 1997). The fact that this is not the case for EPM1 may suggest that once the dodecamer repeat expands beyond a critical threshold, *cystatin B* gene expression is reduced in certain cells, with pathological consequences. Therefore, the age at onset of this disorder no longer depends on the size of the alleles after this threshold is passed by both alleles of a patient. This threshold is likely to be within 13–30 copies of the dodecamer. The different ages at onset among EPM1 siblings (table 1) could be due to modifier genes and/or environmental factors. It is still possible that there is correlation between EPM1 allele size and another phenotypic determinant of the disorder.

Detailed, standardized evaluation of phenotype is needed to uncover such a correlation.

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