Short GCG expansions in the *PABP2* gene cause oculopharyngeal muscular dystrophy

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Autosomal dominant oculopharyngeal muscular dystrophy (OPMD) is an adult-onset disease with a world-wide distribution[1]. It usually presents in the sixth decade with progressive swallowing difficulties (dysphagia), eyelid drooping (ptosis) and proximal limb weakness. Unique nuclear filament inclusions in skeletal muscle fibres are its pathological hallmark[2]. We isolated the poly(A) binding protein 2 gene (*PABP2*) from a 217-kb candidate interval on chromosome 14q11 (B.B. *et al.*, manuscript submitted). A $(GCG)_6$ repeat encoding a polyalanine tract located at the N terminus of the protein was expanded to $(GCG)_{8-13}$ in the 144 OPMD families screened. More severe phenotypes were observed in compound heterozygotes for the $(GCG)_9$ mutation and a $(GCG)_7$ allele that is found in 2% of the population, whereas homozygosity for the $(GCG)_7$ allele leads to autosomal recessive OPMD. Thus the $(GCG)_7$ allele is an example of a polymorphism which can act either as a modifier of a dominant phenotype or as a recessive mutation. Pathological expansions of the polyalanine tract may cause mutated PABP2 oligomers to accumulate as filament inclusions in nuclei.

In order to identify the gene mutated in OPMD, we constructed a 350-kb cosmid contig between flanking markers *D14S990* and *D14S1457* (Fig. 1*a* and B.B. *et al.*, manuscript submitted). Twenty-five cDNAs were isolated by cDNA selection from the candidate interval[3]. Three of these hybridized to a common 20-kb *Eco*RI restriction fragment and showed high sequence homology to the bovine poly(A) binding protein 2 gene (Fig. 1*a*). An EST coding for human *PABP2* was previously localized to both chromosome 14q11 and Xq12-q13 by fluorescent *in situ* hybridization[4]. The X-linked *PABP2* pseudogene was identified through genomic sequencing; at this point, more than 40 human EST sequences corresponding to *PABP2* have been deposited to GenBank. *PABP2* was a good candidate for OPMD because it mapped to the genetically defined 0.26-cM candidate interval in 14q11(Fig. 1*a*, and B.B. *et al.*, manuscript submitted), its mRNA is highly expressed in skeletal muscle (data not shown), and the PABP2 protein is exclusively localized to the nucleus[5], where it acts as a factor in mRNA polyadenylation[5-8].

We subcloned an 8-kb *Hin*dIII genomic fragment containing the *PABP2* gene, and sequenced 6,002 bp (Fig. 1*b*). The coding sequence was determined based on the previously published bovine sequence and the sequence of 31 human cDNAs and ESTs; it compromises seven exons and is transcribed in the cen→qter orientation (Fig. 1*b*). Multiple splice variants are represented by ESTs and suggested by northern blots (ref. 4, and data not shown). In particular, introns 1 and 6 are present in more than 60% of clones (Fig. 1*b*). The coding sequences are highly conserved between the human, bovine and mouse genes. 93% of the *PABP2* sequence was readily amenable to RT-PCR- or genomic-SSCP screening, but no mutations were revealed by either technique. However, a 400-bp region of exon 1, which contains the start codon, could not be readily amplified. This region is 80% GC-rich. It includes a $(GCG)_6$ repeat which codes for the first six alanine residues of a homopolymeric stretch of ten (Fig. 2*g*). Special conditions were designed to PCR-amplify a 242-bp genomic fragment including this GCG-repeat. The $(GCG)_6$ allele was found in 98% of French Canadian control chromosomes, whereas 2% of them carried a $(GCG)_7$ polymorphism (n=86; ref. 9).

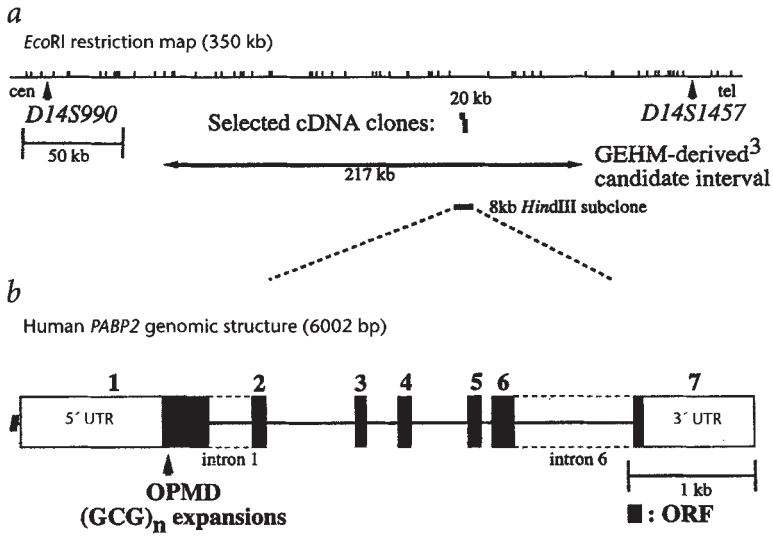

Fig. 1 Positional cloning of the *PABP2* gene. ***a***, Positions of the *PABP2* selected cDNA clones in relation to the *Eco*RI restriction map and the Genealogy-based Estimate of Historical Meiosis (GEHM)-derived candidate interval (B.B. *et al.*, manuscript submitted). ***b***, Genomic structure of the *PABP2* gene, and position of the OPMD $(GCG)_n$ expansions. Exons are numbered. Introns 1 and 6 are variably present in 60% of cDNA clones. ORF, open reading frame; cen, centromere; tel, telomere.

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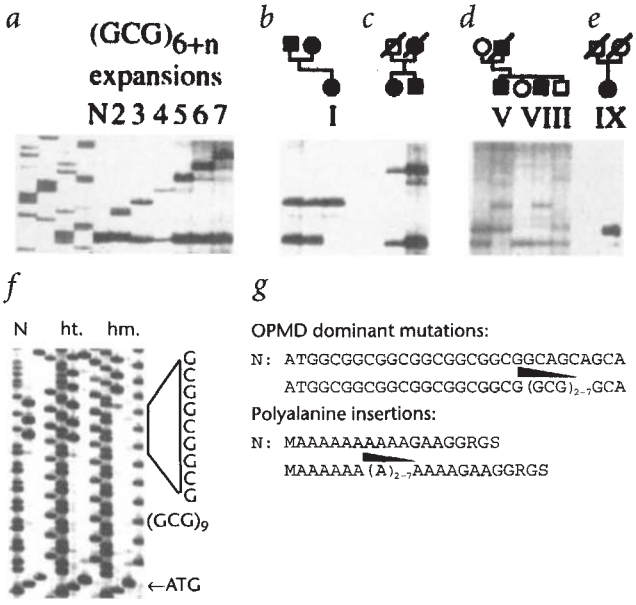

Fig. 2 OPMD $(GCG)_n$ expansion sizes and sequence of mutations. ***a***, $(GCG)_6$ normal allele (N) and the six different $(GCG)_n$ expansions observed in 144 families. ***b***, Genotype of a homozygous $(GCG)_9$ case and her parents. ***c***, The doubling of the French-Canadian $(GCG)_9$ expansion is demonstrated in Family F151. ***d***, Independent segregation of the $(GCG)_7$ allele. Case V has a more severe OPMD phenotype. ***e***, Case IX, who has a recessive form of OPMD, is shown to have inherited two copies of the $(GCG)_7$ polymorphism. ***f***, Partial sequence of exon 1 in a normal $(GCG)_6$ control (N), a heterozygote (ht.) and a homozygote (hm.) for the $(GCG)_9$-repeat mutation. ***g***, Nucleotide sequence of the mutated region of *PABP2*, amino acid sequences of the N-terminal polyalanine stretch and position of the OPMD alanine insertions.

Screening OPMD cases in 144 families revealed a PCR product which exceded that found in controls in all affected individuals by 6 to 21 bp (Fig. 2*a*). Sequencing the GC-rich region of *PABP2* in at least two carriers revealed expansions of the GCG-repeat (Fig. 2*g*). The $(GCG)_9$ expansion found in 70 French Canadian families is the most frequent mutation we observed (Table 1). The $(GCG)_9$ expansion is quite stable. A single increase from a $(GCG)_9$ to a $(GCG)_{12}$ mutation was observed in family F151 in an estimated 598 French Canadian meioses (Fig. 2*c*), contrasting with the unstable nature of previously described disease-causing triplet-repeats[10].

PABP2 genotyping of all participants in our French-Canadian OPMD study provided molecular insights into the clinical variability observed in this condition. The genotypic information was added to an anonymous version of our clinical database of 176 $(GCG)_9$ mutation carriers[9]. Severity of the OPMD phenotype can be assessed by the swallowing time (*ST*), in this case, the length of time required to drink 80 ml of ice-cold water[9,11]. The late onset and progressive nature of the muscular dystrophy is clearly illustrated in heterozygous carriers of the $(GCG)_9$ mutation (bold curve in Fig. 3) when compared the average *ST* of control $(GCG)_6$ homozygous participants (n=76, thinner line in Fig. 3). Thus, two groups of genotypically distinct OPMD cases have more severe swallowing difficulties. Individuals I, II, and III have an early-onset disease and are homozygous for the $(GCG)_9$ expansion ($P<10^{-5}$; Fig. 2*b*,*f*). Cases IV, V, VI and VII have more severe phenotypes and are compound heterozygotes for the $(GCG)_9$ mutation and the $(GCG)_7$ polymorphism ($P<10^{-5}$). The independent segregation of the two alleles is shown in Fig. 2*d*. Case V, who inherited the French Canadian $(GCG)_9$ mutation and the $(GCG)_7$ polymorphism, has more symptoms than his brother VIII who carries the $(GCG)_9$ mutation and a normal $(GCG)_6$ allele (Figs 2*d*,3). The $(GCG)_7$ polymorphism thus appears to be a modifier of severity of dominant OPMD. Furthermore, the $(GCG)_7$ allele can act as a recessive mutation. This was documented in the patient IX, who inherited two copies of the $(GCG)_7$ polymorphism and has a late-onset biopsy-proven autosomal recessive form of OPMD (Fig. 2*e*).

This is the first description of short trinucleotide-repeat expansions causing a human disease. The addition of only two GCG repeats is sufficient to cause dominant OPMD. OPMD expansions do not share the cardinal features of 'dynamic' mutations (ref. 12). The GCG expansions are not only short; they are also meiotically quite stable. Furthermore, there is a clear cut-off between the normal and abnormal alleles, with a single GCG expansion being a recessive mutation. The *PABP2* $(GCG)_7$ allele is the first example of a relatively frequent allele which can act as either a modifier of a dominant phenotype or as a recessive mutation. This dosage effect is reminiscent of the one observed in a homozygote for two dominant synpolydactyly mutations[13]. In this case, the patient had more severe deformities because she inherited two duplications causing an expansion in the polyalanine tract of the HOXD13 protein[13,14]. A duplication causing a similar polyalanine expansion in the gene encoding the α1 core-binding factor has also been found to cause dominant cleidocranial dysplasia[15]. The mutations in these two rare diseases are not triplet-repeats. They are duplications of 'cryptic repeats' composed of mixed synonymous codons, and are thought to result from unequal crossing over[16,17]. That the French Canadian F151 family shares the same centromeric and telomoric haplotypes with two other families, while bearing an increase in the size of the GCG expansion, suggests that slippage during replication may be the underlying mutational mechanism in OPMD (Fig. 2*c*; refs 12,18).

Different observations suggest that a gain of function of PABP2 may cause the accumulation of nuclear filaments observed in OPMD[2]. PABP2 is found mostly in dimeric and oligomeric form[4]. It is possible that the polyalanine tract plays a role in polymerization. Polyalanine stretches have been found in many other nuclear proteins such as the HOX proteins, but their functions is still unknown[19,20]. Alanine is a highly hydrophobic amino acid present in the protein cores. In the fibres of dragline spider silk, polyalanine stretches are thought to form β-sheet structures important for the fibres' strength[21]. Polyalanine oligomers have also been shown to be extremely resistant to chemical denaturation and enzymatic degradation[22]. PABP2 oligomers comprised of a sufficient number of mutated molecules might accumulate in the nuclei by forming undegradable polyalanine-rich macromolecules. The rate of accumulation would then depend on the ratio of mutated to non-mutated protein. The more severe phenotypes observed in homozygotes for the $(GCG)_9$ mutations and compound heterozygotes carrying both $(GCG)_9$ and $(GCG)_7$ alleles may result from the fact that their PABP2 oligomers are composed only of mutated proteins. Faster filament accumulation could

Table 1 • Number of families sharing the different mutations

Mutations	Polyalanine[a]	Families	Countries[b]
$(GCG)_8$	12	4	1
$(GCG)_9$	13	99	6
$(GCG)_{10}$	14	19	8
$(GCG)_{11}$	15	16	6
$(GCG)_{12}$	16		3
$(GCG)_{13}$	17		1
Total		144	

[a] 10 alanine residues in normal PABP2. [b] from 15 different countries

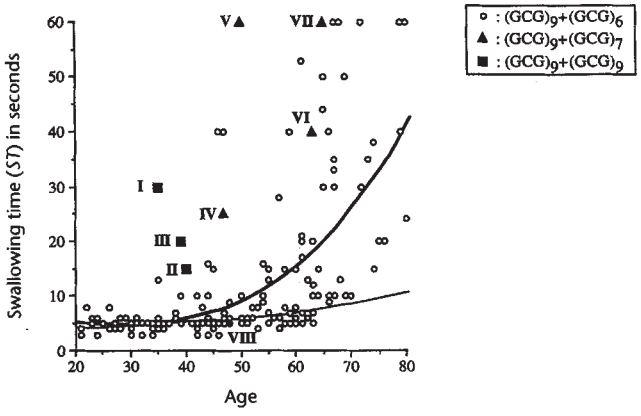

Fig. 3 Age distribution of swallowing time (ST) for French-Canadian OPMD carriers of the $(GCG)_9$ mutation. The bold curve represents the average OPMD *ST* for carriers of only one copy of the $(GCG)_9$ mutation (n=169), while the thinner line corresponds to the average *ST* for $(GCG)_6$ homozygous normal controls (n=76). The black dot corresponds to the *ST* value for individual VIII. Roman numerals refer to individual cases shown in Fig. 2*b,d*.

cause accelerated cell death. The recent description of nuclear filament inclusions in three CAG repeat diseases—Huntington's disease and spinocerebellar ataxia type 1 and type 3—raises the possibility that 'nuclear toxicity' caused by the accumulation of mutated homopolymeric domains is involved in the molecular pathophysiology of triplet-repeat diseases[19,23–26]. Future immunocytochemical and expression studies will test this hypothesis and may provide insight into why certain muscle groups are more affected in OPMD patients even though all tissues express *PABP2*.

Methods

Contig and cDNA selection. The cosmid contig was constructed by standard cosmid walking techniques using a gridded chromosome 14-specific cosmid library[27]. The cDNA clones were isolated by cDNA selection as previously described[3].

Cloning of *PABP2*. Three cDNA clones corresponding to *PABP2* were sequenced (Sequenase, USB). Clones were verified to map to cosmids by Southern hybridization. The 8-kb *Hin*dIII restriction fragment was subcloned from cosmid 166G8 into pBluescriptII (SK) (Stratagene). The clone was sequenced using primers derived from the bovine *PABP2* gene and human EST sequences. Sequencing of the *PABP2* introns was carried out by primer walking.

***PABP2* mutation screening and sequencing.** All cases were diagnosed as having OPMD on clinical grounds[9]. RT-PCR- and genomic SSCP analyses were carried out according to standard protocols[28]. The primers used to amplify the *PABP2* mutated region were: 5'–CGCAGTGCCCCGCCTTAGA–3′ and 5′–ACAAGATGGCGCCGCCGCCCGGC–3′. PCR reactions were performed in a total volume of 15 µl containing 40 ng of genomic DNA, 1.5µg of BSA, 1 µM of each primer, 250 µM dCTP and dTTP, 25 µM dATP, 125 µM dGTP and 125 µM 7-deaza-dGTP (Pharmacia), 7.5% DMSO, 3.75 µCi[^{35}S]dATP, 1.5 units of *Taq* DNA polymerase and 1.5 mM $MgCl_2$ (Perkin Elmer). For non-radioactive PCR reactions the [^{35}S]dATP was replaced by 225 µM dATP. The amplification procedure consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 70 °C for 30 s, elongation at 74 °C for 30 s and a final elongation at 74 °C for 7 min. Samples were loaded on 5% polyacrylamide denaturing gels. Following electrophoresis, gels were dried and autoradiographs were obtained. Sizes of the inserts were determined by comparing to a standard M13 sequence (Sequenase, USB). Fragments used for sequencing were gel-purified. Sequencing of the mutated fragment with the Amplicycle kit (Perkin Elmer) used the 5′–CGCAGTGCCCCGCCTTAGAGGTG–3′ primer at an elongation temperature of 68 °C. The OPMD cases tested originate from the following countries: Armenia, Australia, Belgium, Canada, France, Germany, Israel, Italy, Japan, Netherlands, Spain, Switzerland, United Kingdom, United States and Uruguay.

Stability of (GCG)-repeat expansions. The carrier frequency of the French-Canadian OPMD mutation is estimated to be 1 in 1,000 in the Province of Québec, approximately 1 in 200,000 in France and 1 in 700 in Bukhara Jews living in Israel[9,29,30]. The meiotic stability of the $(GCG)_9$ repeat was estimated based on our large French Canadian OPMD cohort. We previously established that a single ancestral OPMD carrier chromosome was introduced to the French-Canadian population by three sisters in 1648 (B.B. *et al.*, manuscript submitted). Seventy of the seventy one French Canadian OPMD families tested to date segregate a $(GCG)_9$ expansion. However, in family F151, the affected brother and sister, despite sharing the French Canadian ancestral haplotype, carry a $(GCG)_{12}$ expansion including twice the number of abnormal GCG repeats as the ancestral $(GCG)_9$ mutation. In our founder-effect study, we estimated that 450 (304-594) historical meioses shaped the 123 OPMD cases belonging to 42 of the 71 enrolled families. Screening the full set of participants in our study allowed us to identify another 148 $(GCG)_9$ carrier chromosomes. Therefore, we estimate that a single mutation of the $(GCG)_9$ expansion has occurred in 598 (452–742) meioses.

Genotype-phenotype correlations. One-hundred and seventy-six carriers of at least one copy of the $(GCG)_9$ mutation were examined during the early stage of the linkage study. All were asked to swallow 80 ml of ice-cold water as rapidly as possible. Testing was stopped after 60 s. The swallowing time (*ST*) was validated as a sensitive test to identify OPMD cases[9,11]. Analyses of variance were computed by two-way ANOVA (SYSTAT package). The mean *ST* value of the $(GCG)_9$ homozygotes was compared to the mean value of all $(GCG)_9$ heterozygotes aged 35–40 yr ($P<10^{-5}$). For the $(GCG)_9$ and $(GCG)_7$ compound heterozygotes their mean *ST* value was compared to the mean value of all $(GCG)_9$ heterozygotes aged 45–65 yr ($P<10^{-5}$).

GenBank accession numbers. X-linked *PABP2* pseudogene, Y08772; genomic sequence of human *PABP2*, AF026029; sequence of bovine *PABP2* , X89969; sequence of mouse *Pabp2*, U93050.

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