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CAG repeat instability, cryptic sequence variation and pathogeneticity: evidence from different loci

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Different aspects of expanded polyglutamine tracts and of their pathogenetic role are taken into consideration here. (i) The $(CAG)_n$ length of wild-type alleles of the Huntington disease gene was analysed in instability-prone tumour tissue from colon cancer patients to test whether the process leading to the elongation of alleles towards the expansion range involves single-unit stepwise mutations or larger jumps. The analysis showed that length changes of a single unit had a relatively low frequency. (ii) The observation of an expanded spinocerebellar ataxia (SCA)1 allele with an unusual pattern of multiple CAT interruptions showed that cryptic sequence variations are critical not only for sequence length stability but also for the expression of the disease phenotype. (iii) Small expansions of the $(CAG)_n$ sequence at the *CACNA1A* gene have been reported as causing SCA6. The analysis of families with SCA6 and episodic ataxia type 2 showed that these phenotypes are, in fact, expressions of the same disorder caused either by point mutations or by small $(CAG)_n$ expansions. A gain of function has been hypothesized for all proteins containing an expanded polyglutamine stretch, including the α_{1A} subunit of the voltage-gated calcium channel type P/Q coded by the *CACNA1A* gene. Because point mutations at the same gene with similar phenotypic consequences are highly unlikely to have this effect, an alternative common pathogenetic mechanism for all these mutations, including small expansions, can be hypothesized.

Keywords: *CACNA1A* gene; episodic ataxia type 2; Huntington's disease; spinocerebellar ataxia type 1 (SCA1); spinocerebellar ataxia type 6 (SCA6)

1. INTRODUCTION

It is well known that several autosomal dominant disorders (Huntington's disease (HD), spinocerebellar ataxia (SCA)1, SCA2, SCA3, SCA6, SCA7, dentatorubral–pallidoluysian atrophy (DRPLA) and spinal and bulbar muscular atrophy (SBMA)) are associated with unstable expansions of CAG repeat sequences in the coding region of the corresponding genes. Three aspects of these mutations are explored here: (i) the process leading wild-type alleles to expand above the threshold of instability; (ii) the phenotypic effect of expanded alleles with an unusual type of interruption of the polyglutamine repeat; and (iii) the possibility that one of the mutations, namely SCA6, acts through a pathogenetic mechanism different from that hypothesized for the other disorders owing to $(CAG)_n$ expansions.

2. LENGTH CHANGE AND VARIABILITY OF NORMAL HD ALLELES

In all expanded-polyglutamine disorders new mutant alleles arise from wild-type alleles that show a length polymorphism of variable structure and degree (Jodice *et al.* 1997*a*). Expanded alleles are prone to further length variation (instability) during both meiotic and mitotic cell divisions (see, for example, Chong *et al.* 1995). At present, little is known about the mechanism leading to the elongation of alleles from the normal range into the unstable range. The presence of variant trinucleotides, interrupting the $(CAG)_n$ sequence, has been shown to have a stabilizing effect. For example, in most SCA1 and SCA2 normal alleles, the $(CAG)_n$ sequence is interrupted by CAT or CAA trinucleotides, respectively. In these genes, pure $(CAG)_n$ stretches longer than 21 units have never been observed (Chung *et al.* 1993; Imbert *et al.* 1996), whereas the unstable expanded alleles have stretches of 35 or more pure CAG units. It is the loss of the variant trinucleotide that presumably triggers the expansion process in SCA1 and SCA2. Pearson *et al.* (1998) have proposed a model in which CAG interruptions exert their stabilizing effect by inhibiting DNA strand slippage.

Other genes, such as HD or DRPLA or SCA3, have normal alleles with no interruptions, except for the presence of a variant trinucleotide at the 3′ or 5′ end of the repeat tract in both the normal and expanded alleles (McNeil *et al.* 1997). In SCA3 and DRPLA the $(CAG)_n$ length distributions of normal and expanded alleles are widely separated and events producing new mutant

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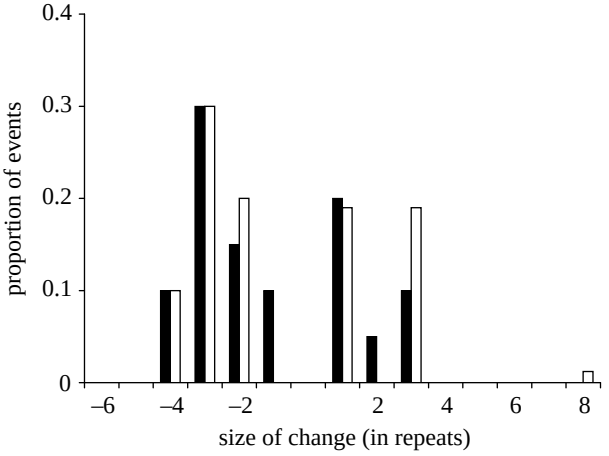

Figure 1. Histogram of HD $(CAG)_n$ size changes in colorectal tumour DNAs compared with non-tumour DNA. New sizes were scored as additional bands in the tumour DNA, compared with the patient genotype. Two methods were used to estimate the length change leading to the variant allele from either of the patient normal alleles: (i) each variant allele was assumed to derive from the normal allele more similar to it (dark bars); (ii) size change was estimated by the EM algorithm (Di Rienzo *et al*. 1998) (light bars).

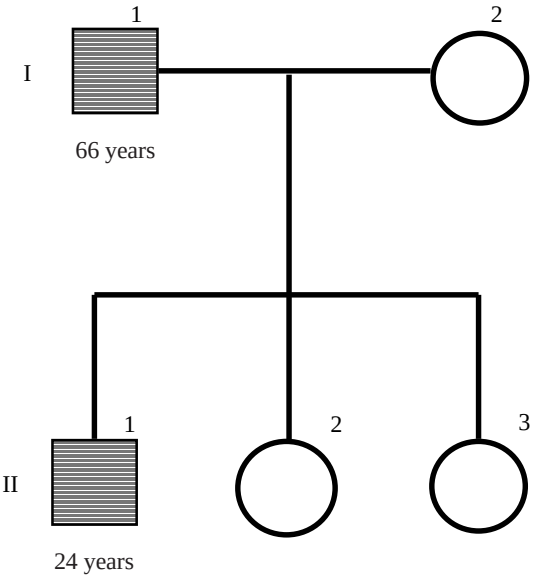

Figure 2. Family with an expanded SCA1 allele (45 repeats) containing unusual CAT interruptions. Grey squares indicate heterozygotes for an allele $(CAG)_{12}$-CAT-CAG-CAT-$(CAG)_{12}$-CAT-CAG-CAT-$(CAG)_{15}$, showing no signs of disease. The ages of these subjects are also reported.

alleles must be assumed to involve jumps of several units. For HD, which shows continuity between the range of normal and expanded alleles, Rubinsztein *et al*. (1994) have proposed a model that favours single unit changes leading progressively towards the instability threshold. To investigate this issue further, the HD CAG repeat was analysed in a model system consisting of normal and tumour tissues from sporadic patients affected by colon cancer (Di Rienzo *et al*. 1998). It is known that microsatellites in colon cancer cells are particularly prone to instability, owing partly to defects in the mismatch repair system (Liu *et al*. 1995). Overall, ten tumour DNAs with variant $(CAG)_n$ sizes on a total of 115 pairs of matched normal-tumour DNAs were observed. The results, reported in figure 1, show that increments of a single unit account for only 20% of all changes and that the overall frequency of single unit variations is *ca*. 30%, under the conservative assumption that every variant allele was derived from the normal allele more similar to it. The frequencies were still lower when the distribution of estimated changes was obtained through the EM algorithm (Di Rienzo *et al*. 1998), which optimizes the relative probability of the variant size's being generated by one or the other of the parental alleles. In addition, a slight excess of shortenings was observed, mainly contributed by changes of −3. The present and the previous data on HD normal polymorphism in different world populations (Jodice *et al*. 1997*a*) fit the theory by Di Rienzo *et al*. (1998), which predicts that the square of length change observed in this model system is linearly related to the variance of the $(CAG)_n$ size in the population. The experimental verification (Di Rienzo *et al*. 1998) of this expectation strengthens the idea that the events scored in this model are similar to those occurring in meioses *in vivo*. It should be noted that large meiotic series were analysed by Brinkmann *et al*. (1998), showing that most changes in microsatellites involve a single unit. However, this report did not include the HD gene, leaving open the possibility of a locus-specific mutational pattern of changes larger than one unit. In this context it should also be remembered that the frequency of expanded HD alleles in the population can be maintained not only through the length mutation of wild-type alleles but also by means of the increased fitness in carriers of alleles in the medium–low expansion range, as proposed by Frontali *et al*. (1996).

Table 1. *Published data on age at onset in SCA1 patients*

source	no. of patients	range of CAG units	range of age at onset	no. with onset at 60–65 years
Sasaki *et al*. (1996)	35	42–63	15–63	2
Dubourg *et al*. (1995)	42	42–67	21–52	0
Kameya *et al*. (1995)	20	42–58	19–55	0
Genis *et al*. (1995)	22	41–59	26–52	0
Ranum *et al*. (1994)	113	42–81	3–65	2
Jodice *et al*. (1994)	55	47–66	15–51	0
Orr *et al*. (1993)	27	43–81	3–65	1

3. PHENOTYPIC EFFECT OF INTERRUPTED EXPANDED POLYGLUTAMINE TRACTS

The role of interruptions of the $(CAG)_n$ sequences has frequently been analysed in connection with length instability, whereas little is known about the role of purity in the corresponding protein products. It has

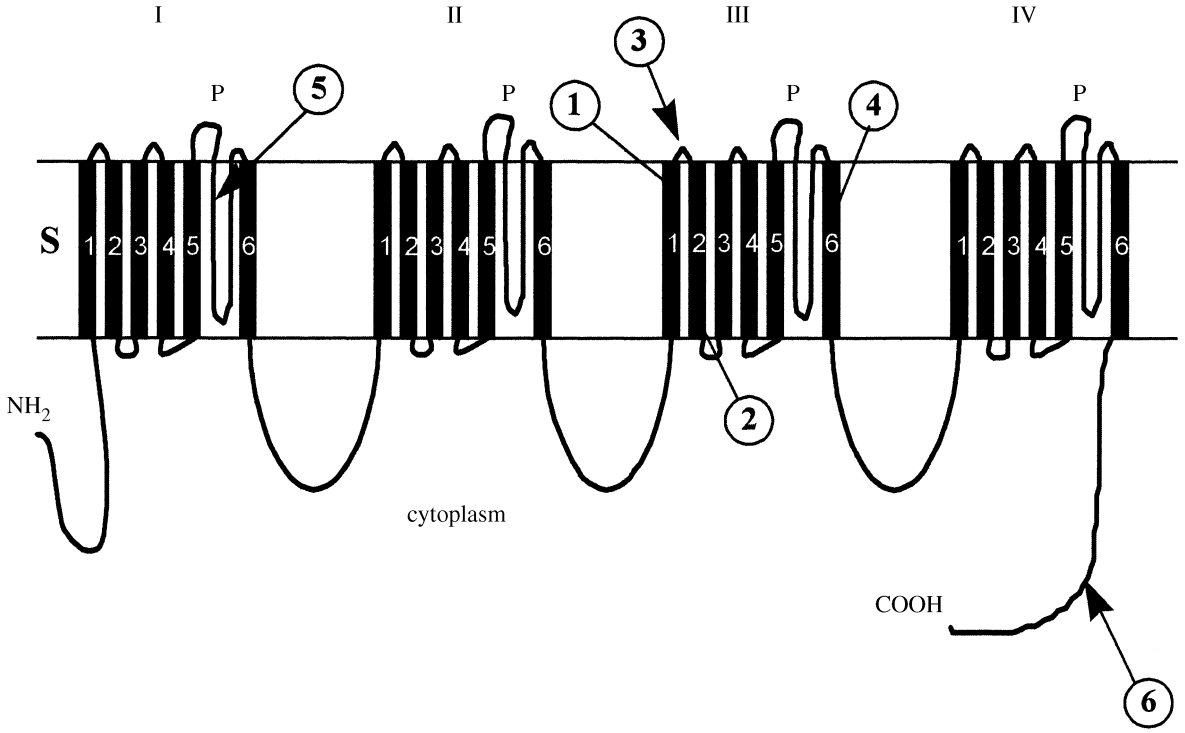

Figure 3. Structure model of the α_{1A} subunit of Ca^{2+} channel type P/Q. The protein possesses four domains (I–IV) containing six hydrophobic α-helical transmembrane regions (S1–S6) connected by hydrophilic links. The P segments connecting S5 and S6 of each domain form the pore of the channel. The location of the mutations responsible for the EA2/SCA6 phenotype described in table 3 are reported.

recently been shown, both *in vivo* and *in vitro*, that expanded polyglutamine stretches lead to the formation of intranuclear inclusions containing insoluble ubiquitinated aggregates of the protein (see, for example, Davis *et al*. 1997; Di Figlia *et al*. 1997; Paulson *et al*. 1997; Scherzinger *et al*. 1997). Several lines of evidence suggest that the intranuclear inclusions have a role in the pathogenesis of these disorders, rather than simply being a by-product: (i) the inclusions have been found *post mortem* in specifically affected tissues, but not in those unaffected by the disorder (Di Figlia *et al*. 1997; Paulson *et al*. 1997), (ii) the inclusions have been found in transgenic animals before the onset of symptoms (Davis *et al*. 1997), (iii) inclusions have a greater frequency in juvenile cases characterized by large numbers of glutamine units and a more severe phenotype (Di Figlia *et al*. 1997), and (iv) transgenic mice, which express a hypoxanthine–guanine phosphoribosyltransferase (normally not containing polyglutamine) engineered with the insertion of a large polyglutamine tract, show intranuclear inclusions and have a progressive neurological deficit (Ordway *et al*. 1997). Little is known about the process underlying the formation of intranuclear inclusions. A first step is likely to be the proteolytic cleavage of the expanded polyglutamine tract. The cleaved sequence seems to have the role of recruiting the full-length protein, independently of its number of glutamine units, into insoluble aggregates (Paulson *et al*. 1997).

The question is whether a 'non-pure' expanded polyglutamine sequence can have the same pathogenetic role. The serendipitous finding of two members of an Italian family (figure 2) carrying an unusual SCA1 allele with 45 repeat units, i.e. well within the range of expansions,

Table 2. *Main clinical features in the three disorders due to CACNA1A mutations*

symptoms	FHM	EA2	SCA6
migraine with hemiplegic aura	+	–	–
episodes of ataxia–vertigo	+/–	+	+/–
permanent nystagmus	+/–	+	+
permanent progressive ataxia	+/–	+/–	+/–
cerebellar atrophy	+/–	+/–	+/–

seems to provide a first clue in addressing this question. The family was ascertained not on the basis of an ataxic phenotype, but during a random population analysis. Subject II-1 (figure 2), 24 years old and in good health, carried a 45-repeat allele whose sequence showed two CAT–CAG–CAT interruptions (figure 2). At the protein level, this implies a similar arrangement of histidine residues within the polyglutamine stretch. The analysis of the subject's parents showed that the father, 66 years old and in good health, carried the same allele. Published data (table 1) on 314 SCA1 patients, molecularly tested, show that the age at onset is rarely more than 60 years, even when the number of CAG repeats is smaller than 45, and is never above 65. Had the interrupted allele had the same effect of pure $(CAG)_n$ stretches, then the oldest carrier of the unusual pattern would most probably have been already affected at his age. This would suggest that interrupted alleles have, at least, a delaying effect on age at onset, if not a non-pathogenetic role altogether. A similar pattern of interruptions in an expanded SCA1 allele, with 44 repeats, has been reported by Quan *et al*.

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Table 3. *Mutations at CACNA1A gene causing EA2/SCA6 phenotype*

mutation	effect on protein	domain/segment	source
1. del C (exon 22)	premature stop–truncated protein	III S1	Ophoff *et al.* (1996)
2. G→A (5′ splice junction intron 24)	aberrant splicing–truncated protein	III S3	Ophoff *et al.* (1996)
3. C→T (exon 23)	Arg→Stop	III (S1/S2)	Yue *et al.* (1998)
4. T→C (exon 28)	Phe→Ser	III S6	Trettel *et al.* (1998)
5. G→A (exon 6)	Gly→Arg	I P	Yue *et al.* (1997)
6. $(CAG)_n$ expansion	$(Gln)_{\leqslant 20} \rightarrow (Gln)_{\geqslant 30}$	C-terminal tail	Zhuchenko *et al.* (1997) Jodice *et al.* (1997*b*)

(1995) in a family ascertained through a young member affected with an early-onset (at age two years) cerebellar ataxia. Both the child and her healthy 33-year-old father carried the same allele. The young age of both subjects and the presence of an early-onset ataxic phenotype (although incongruous with the relatively low number of repeats) could well be compatible with a pathogenetic role of the unusual allele. The present family, instead, provides stronger support for the hypothesis that expanded polyglutamine stretches interrupted by histidine residues have a low or null pathogenetic potential. The presence of histidine residues might prevent or decrease the formation of intranuclear inclusions by altering the structure of the polyglutamine stretches, which, according to Perutz *et al.* (1994), form β-strands, tending to aggregate by linking to one another through hydrogen bonds between their main-chain and the side-chain amides. Alternatively, histidine residues could decrease the probability of polyglutamine being cleaved from the protein, or they might lower the affinity of polyglutamine for transglutaminase. This enzyme, according to Green (1993) and Kahlem *et al.* (1996), favours aggregation by linking glutamine residues to the ε-amino group of lysine residues of other proteins, by means of isopeptide bonds. A closer study of the role of purity in expanded polyglutamines would be extremely important not only for a better evaluation of the predictive and diagnostic value of tests based on $(CAG)_n$ expansion, but also for the analysis of the processes involved in the pathogenesis of neurodegenerative disorders due to expanded polyglutamine tracts.

4. ROLE OF THE SMALL $(CAG)_n$ EXPANSIONS AT THE *CACNA1A* GENE

In expanded polyglutamine disorders, the mutated $(CAG)_n$ stretches typically have a number of units ranging from 35 to over 100. Several lines of evidence indicate that the mutation confers on proteins a gain of function (Quigley *et al.* 1992; Zeitlin *et al.* 1995; White *et al.* 1997). So far the only exception seems to be SCA6. This disorder is caused by small expansions (20–30 units) of the CAG repeat sequence (4–20 units) at the *CACNA1A* gene, which codes for the α_{1A} subunit of the voltage-gated calcium channel type P/Q (figure 3). A complete sequence analysis of the gene-coding region in a patient with 23 CAG repeats showed that the expansion was the only detectable mutation (Jodice *et al.* 1997*b*). In terms of the type of mutation and associated phenotype, SCA6 was thought (Zhuchenko *et al.* 1997) to differ from the other two disorders owing to point mutations at the same gene, i.e. familial hemiplegic migraine (FHM) and episodic ataxia type 2 (EA2) (Ophoff *et al.* 1996). Zhuchenko *et al.* (1997) described the SCA6 phenotype as a permanent and progressive ataxia differing from EA2, which is instead characterized by episodes of ataxia and/or vertigo and mild interictal cerebellar signs. Both disorders differ in turn from FHM, whose landmark is migraine with hemiplegic aura, even if its phenotype can also include mild cerebellar signs (Joutel *et al.* 1994).

Recent evidence, however, suggests that EA2 and SCA6 should be considered as expressions of the same disorder. This conclusion is supported by the observation of an unstable allele containing 20 or 25 CAG repeats in two branches of the same family (Jodice *et al.* 1997*b*). Patients with 25 repeats had a severe progressive ataxia similar to SCA6, whereas patients with 20 repeats had the typical features of EA2. Moreover, families with EA2 caused by point mutations were reported as also including patients with a permanent progressive ataxia (Yue *et al.* 1997; Trettel *et al.* 1998). EA2 and SCA6 therefore seem to have an identical, although highly variable, phenotype (table 2), which ranges from short episodes with mild interictal signs to severe progressive ataxia with cerebellar atrophy. Both extremes of this spectrum have been found in association with either point mutations or small $(CAG)_n$ expansions.

Several mutations are now known to be associated with the SCA6/EA2 phenotype. These also include mutations producing truncated proteins that are probably unable to exert any function at all. Should a common pathogenetic mechanism for all the EA2/SCA6 mutations be postulated, this would imply that the small $(CAG)_n$ expansions differ from all the other polyglutamine disorders by inducing a loss rather than a gain of function of the corresponding protein. In fact, the EA2/SCA6 mutations reported so far (table 3, figure 3) include (i) the deletion of a highly conserved nucleotide in codon 1266, which alters the reading frame for a substantial portion of the coding region and leads to a premature stop signal at codon 1294; (ii) a single nucleotide substitution that abolishes the 5′ splice site of intron 24, causing an aberrant splicing; (iii) a nonsense mutation creating a premature stop signal at codon 1279 (exon 23). All these defects are probably producing truncated proteins that should severely impair the formation or the functioning of the channels, either through a mechanism of haploinsufficiency or by interfering with channel assembly in the cell membrane. Defective channel activity can also be

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postulated for the two substitutions of a highly conserved amino-acid residue located in the P and S6 segments. These regions are, in fact, responsible for ion-binding and channel-gating activity, respectively (Yue *et al*. 1997). The change from a non-polar amino-acid residue (Phe) to a polar one (Ser) in III S6, and from a non-charged residue (Gly) to a charged one (Arg) in IP, might well interfere with the role of these segments. As far as the small CAG repeat expansion is concerned (whose length is well within the normal ranges of other CAG repeat tracts), it should be noted that (i) the sequence is translated into a polyglutamine stretch only in some of the isoforms (Zhuchenko *et al*. 1997); (ii) the α_{1A} subunit is expressed in brain and kidney (Ophoff *et al*. 1996), but little is known about the ratio of the different isoforms in different cell types; and (iii) the polyglutamine tract, when present, is located in the intracytoplasmic C-terminal part of the protein, which is known to be involved in the tonic inhibition of the channel opening probability (Wei *et al*. 1994). On the basis of this evidence, a loss of function for the expanded alleles could be explained, at a pre-translational level, by their altering the RNA stability or by their interfering with the translation process. In both cases the end result will be a decrease in protein synthesis acting through a mechanism of haploinsufficiency. Alternatively, at a post-translational level, expanded polyglutamine stretches could cause a defective functioning of the channel by interfering with the role of the C-terminal tail of the protein.

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