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Phenotypic Characterization of Individuals with 30–40 CAG Repeats in the Huntington Disease (HD) Gene Reveals HD Cases with 36 Repeats and Apparently Normal Elderly Individuals with 36–39 Repeats

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Summary

Abnormal CAG expansions in the IT-15 gene are associated with Huntington disease (HD). In the diagnostic setting it is necessary to define the limits of the CAG size ranges on normal and HD-associated chromosomes. Most large analyses that defined the limits of the normal and pathological size ranges employed PCR assays, which included the CAG repeats and a CCG repeat tract that was thought to be invariant. Many of these experiments found an overlap between the normal and disease size ranges. Subsequent findings that the CCG repeats vary by 8 trinucleotide lengths suggested that the limits of the normal and disease size ranges should be reevaluated with assays that exclude the CCG polymorphism. Since patients with between 30 and 40 repeats are rare, a consortium was assembled to collect such individuals. All 178 samples were reanalyzed in Cambridge by using assays specific for the CAG repeats. We have optimized methods for reliable sizing of CAG repeats and show cases that demonstrate the dangers of using PCR assays that include both the CAG and CCG polymorphisms. Seven HD patients had 36 repeats, which confirms that this allele is associated with disease. Individuals without apparent symptoms or signs of HD were found at 36 repeats (aged 74, 78, 79, and 87 years), 37 repeats (aged 69 years), 38 repeats (aged 69 and 90 years), and 39 repeats (aged 67, 90, and 95 years). The detailed case histories of an exceptional case from this series will be presented: a 95-year-old man with 39 repeats who did not have classical features of HD. The apparently healthy survival into old age of some individuals with 36–39 repeats suggests that the HD mutation may not always be fully penetrant.

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Introduction

Huntington disease (HD) is a member of a group of neurodegenerative disorders that are associated with abnormal expansions of CAG repeats in the coding regions of particular genes (reviewed by Ross [1995]). These

diseases share a number of features. In each case the CAG repeats code for a stretch of glutamines. The CAG repeats in these genes are normally polymorphic but show low mutation rates on chromosomes in the general population (Kremer et al. 1995). However, longer repeat tracts associated with disease chromosomes show high mutation rates and a predisposition to increase in length from one generation to the next, particularly when the disease is transmitted through males. Since the age at onset of these diseases correlates inversely with the CAG size expansion, this leads to a tendency for the disease to present at earlier ages in successive generations, a phenomenon called "anticipation."

At present, it is not clear how the polyglutamine expansions mediate the neuronal cell death that seems to be a feature of these diseases. Current understanding suggests that these mutations act at least partly in a gain-of-function manner leading to selective neurotoxicity (Ross 1995).

The limits of the CAG size ranges on normal and HD-associated chromosomes cannot be reliably determined from the literature, since most of the initial large surveys were performed using PCR assays with primers that flanked both the CAG repeats and the adjacent CCG repeats, which were thought to be invariant (Andrew et al. 1993; Duyao et al. 1993; Huntington's Disease Collaborative Research Group 1993; Snell et al. 1993). These CCG repeats were subsequently shown to be polymorphic and vary by ≥ 8 repeats, necessitating reexamination of the data, which suggested either close approximation (Andrew et al. 1993; Duyao et al. 1993) or overlap (Snell et al. 1993) in the size ranges of normal and disease chromosomes. An important exception is the study by Kremer et al. (1994), where the smallest HD chromosomes were measured with an assay that excluded the CCG repeats and were found to have 36 CAG repeats, agreeing with similar measurements by Rubinsztein et al. (1994*b*). Thirty six CAG repeats is equivalent to 38 glutamines, since the CAG repeats are followed by CAACAG (Huntington's Disease Collaborative Research Group 1993)—we have followed the convention of defining the CAG length according to the number of uninterrupted CAG repeats, irrespective of the PCR assay used, because this is the nomenclature that has been adopted by most laboratories. Although an HD gene with 35 repeats was reported by Barron et al. (1993) in an apparently affected individual, the clinical details of this case were not presented.

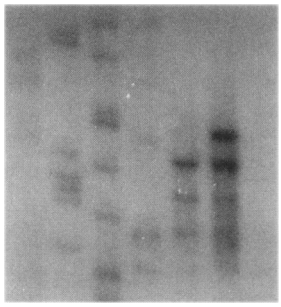
There have been some suggestions that the penetrance of the CAG mutation in HD may not be absolute. In other words, there have been limited reports of individuals who have remained without symptoms of HD with specific CAG repeat expansions in the disease size range (Legius et al. 1994; Rubinsztein et al. 1994*b*). This possibility was also theoretically suggested by data from earlier studies, which showed that the contribution of CAG length to age at onset was only ~20% in cases with ≤ 52 repeats (Duyao et al. 1993). Since the ability to predict age at onset decreases with repeat number and the confidence limits are particularly wide with small HD expansions (Andrew et al. 1993), we believed that some individuals may never show signs of HD despite living into old age, even though they have expansions that had been shown to cause disease in others.

It is also difficult to determine the limits of normal and HD CAG size ranges from the literature, because of the possibility of interlaboratory variation in sizing methodologies. Many of the studies that have used similar methods have reported very different HD and normal size ranges. These probably resulted from difficulties in determining allele sizes in large repeats (>30 CAGs), where there is more PCR stutter and possibly somatic mosaicism. It is much easier to size alleles accurately with <30 repeats. Furthermore, different laboratories may have used different size-standards. Thus, what one lab may call 36 repeats may range from 34 to 38 repeats in other laboratories.

From a diagnostic viewpoint it is important to know what the limits of the normal and disease CAG size ranges are in HD. These size ranges need to be determined by using reliable reference standards. Documentation of the extent of genotype-phenotype variability in individuals with <40 CAG repeats is a first step toward investigation of factors that may modify the penetrance of this mutation. To address this question, laboratories in the United Kingdom, United States, Canada, and Europe were enlisted in a collaborative study, where DNA samples from HD and normal individuals who have between 30 and 40 CAG repeats were sent to Cambridge for analysis. A collaborative study was essential, since it was clear that alleles in this size range were rare. All samples were analyzed in one laboratory so that there was internal consistency in the sizing.

Material and Methods

All laboratories were sent a standard questionnaire and letter requesting DNA and clinical information on any individuals with 30–40 CAG repeats in the HD gene, and genomic DNA was sent to Cambridge from 178 individuals. No samples were from transformed cell lines. All cases reported in this study have had DNA analyzed in the Cambridge laboratory. Sizing of CAG repeats was initially performed using primers HD1 and HD3 (Warner et al. 1993) under conditions described by Rubinsztein et al. (1994*a*). This method uses alpha-dCTP ^{32}P incorporation. Sizes in cases with <40 repeats (in individuals who were either symptomatic or asymptomatic and aged >65 years) were checked using the same method except labeling was performed with end-



1 2

Figure 1 Autoradiograph of a gel, comparing the band patterns of a 36-CAG repeat clone amplified using primers HD1 and HD3 (Warner et al. 1993) labeled either with alpha-dCTP ^{32}P incorporation (lane 2) or with end-labeled HD1 (lane 1). A sequence ladder (pGEM-3ZF[+]; Promega) is shown alongside for size comparison.

labeled HD1. This gave a better and less-ambiguous definition of the darkest band relative to the other bands.

Sizes of alleles were compared with those of a clone containing 36 repeats, a gift from Dr. Jon Warner (Edinburgh). Note that the PCR product of the clone shows a different pattern when labeled with alpha-dCTP compared to end-labeled HD1 (fig. 1). The top dark band of the clone amplified with alpha-dCTP incorporation migrates 3 bp higher than it should, while the same clone amplified with the end-labeled primer showed a single dominant band that migrated in the correct position. This discrepancy was not generally observed when PCR products of genomic DNA from HD cases were compared. However, alpha-labeling occasionally resulted in sizing alleles with $\geq$39 repeats with 1 extra repeat. This cloned standard and an identical sequence ladder were included in all gels. The sizes were also shown to conform to various sized clones of 30, 35, and 40 repeats (from E.A. and M.R.H.).

Finally, sizes of HD cases with $\leq$37 repeats and asymptomatic cases with $\geq$35 repeats were confirmed by comparing the sizes of the CAG repeats with those obtained using PCR assays of the CCG repeats alone (HD4F and HD5R) (Rubinsztein et al. 1993) and a PCR that includes primers HD1 and Hu3 (Reiss et al. 1993), which flank both the CAG and CCG repeats. PCR conditions with HD1 and Hu3 were as described by Rubinsztein et al. (1994*a*) except that each 12.5-μl reaction contained 200 ng primer and 1.0 mM $MgCl_2$, and Hu3 was end labeled. The combination of the these results with those obtained using primers that specifically flank only the CAG repeats allows one to determine the phase of the CAG repeats relative to the CCG repeats. The PCR that includes both the CAG and CCG repeats gives very clean definition of the darkest band relative to the stutter bands (fig. 2), the CCG repeat PCR is unambiguous (Rubinsztein et al 1993), and the small normal alleles can be sized easily by using PCR reactions that include only the CAG repeats. These three reactions allow for further definitive confirmation of the CAG repeat length on disease chromosomes. There was no inconsistency observed in the cases we examined in this way.

All symptomatic cases with $\leq$37 CAG repeats were examined for expansions in the SCA1, SCA3, and dentatorubral-pallidoluysian atrophy genes, except for one case where there was insufficient DNA. PCR reactions were performed at these loci as described elsewhere (Chung et al. 1993; Li et al. 1993; Kawaguchi et al. 1994). SCA1 repeats that were close to the affected size range were examined for interruptions by using *Sfa*N1 restriction digestion (Chung et al. 1993).

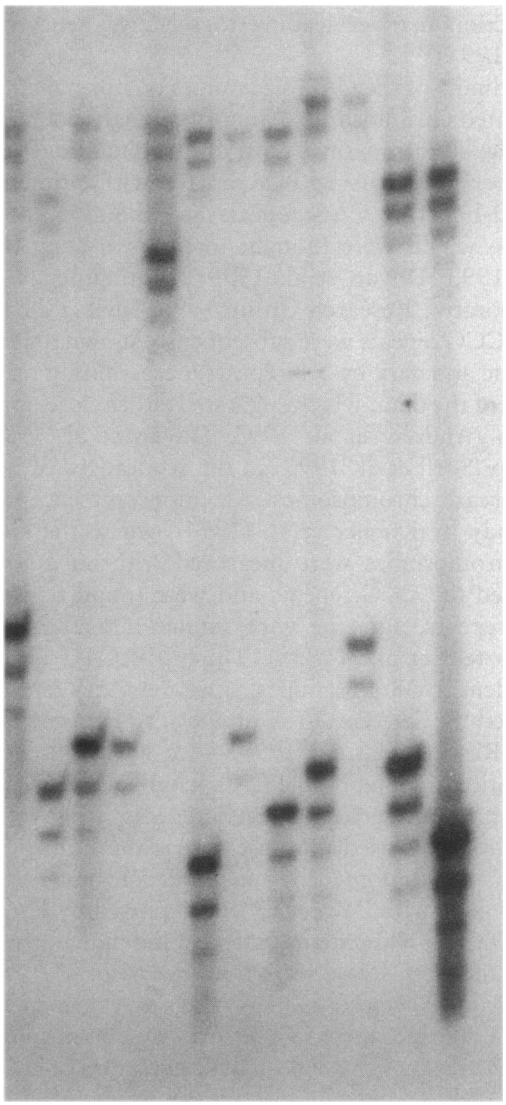


Figure 2 Autoradiograph of a gel, showing the clear differentiation of the darkest band from stutter bands in HD alleles when amplified using HD1 and Hu3 as primers.

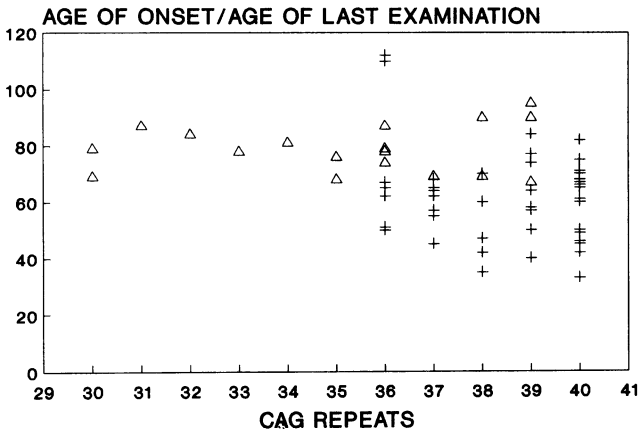


Figure 3 CAG repeat sizes in asymptomatic cases (△) >65 years of age, with age at last examination indicated, and symptomatic cases (+), with age at onset shown. Two cases with 36 repeats with unknown ages at onset are indicated as having an age at onset of >100 years. Symptomatic cases with ≥37 repeats, whose ages at onset are not known, are not included.

One individual with 39 CAG repeats in the HD gene, who did not have the clinical syndrome of HD, died at the age of 95 years. Postmortem examination of the brain was performed using standard techniques, including immunohistochemical examination for glial fibrillary acidic protein in reactive astrocytes (Hedreen and Folstein 1995).

Results

CAG repeat sizes in the HD gene were determined in all 178 cases that were sent for this study. Of the cases submitted for analysis in Cambridge, 45% showed concordance for CAG repeat length, 25% were within 1 repeat length of the Cambridge results, and 29% differed from the Cambridge results by >1 repeat. It should be noted that this study was not designed as a quality-assurance exercise, and the participating laboratories were not asked to provide precise typings. Furthermore, it is likely that some of the laboratories sent samples that were typed only with assays that include the CCG polymorphism. Thus, these results are likely to be an overestimate of the interlaboratory variation in CAG sizing.

Details of the age at which some unaffected cases were last investigated and information of the ages of onset of symptoms of some of the affected cases were not available. However, efforts were made to obtain these data, especially for asymptomatic cases with ≥36 repeats. Figure 3 shows repeat sizes of symptomatic cases plotted against age at onset of symptoms and also indicates asymptomatic cases >65 years of age. The presence of seven cases with HD with 36 repeats confirms previous suggestions that this allele size is associated with disease (Kremer et al. 1994; Rubinsztein et al. 1994*b*). None of the symptomatic cases with ≤37 repeats had abnormal expansions in the SCA1, SCA3, and dentatorubral-pallidoluysian atrophy genes. In one case (36 repeats) there was insufficient DNA for analysis.

It is difficult to define the upper border of the normal size range at this locus. However, individuals without a clinical diagnosis of HD were found at 36 repeats (aged 74, 78, 79, and 87 years), 37 repeats (aged 69 years), 38 repeats (aged 69 and 90 years), and 39 repeats (aged 67, 90, and 95 years). Of the individuals aged 90 years, the person with 39 repeats has just entered a nursing home because of mental deterioration that began at the age of 80 years, but the other individual (38 repeats) has normal mental and physical health and lives independently.

The case with 39 repeats who died age 95 is valuable, since he was seen on two occasions by an experienced clinician (S.E.F.), and his brain was examined neuropathologically by a neuropathologist who has examined many HD cases (J.C.H.). He came from a large HD family and was first seen in the context of a linkage study (Folstein et al. 1985) at age 89 years. He had the "affected" marker pattern. Because of his advanced age and normal examination, he was counted as a recombinant. He was wheelchair bound, because of severe arthritis in his hips. At this stage, he had no features suggestive of HD except "restless movements" of his hands and left foot. These were not choreic but were similar to those seen in very early HD. On neurological examination, he was alert, conversed well, and appeared to have above-average intelligence. His minimental score was 29 of 30. His lateral eye movements were smooth, and his vertical gaze was limited, as expected for age. His saccades were not slow, facial praxis was normal, and rapid tongue movements were extremely good. His speech was clear and had a normal rhythm. There was no arrhythmia on repeated syllables and no attenuation. Cerebellar function was normal. The clinician concluded that this man was unlikely to have HD. He was seen again at age 95 years, a month before he died. At this stage, he was almost totally blind and deaf. He had been in a nursing home from the age of 91 years. He did not have any involuntary movements at this examination, and such movements had not been noticed by his caregivers. Although he had some mental deterioration, cognitive and neurological function were difficult to assess, in view of his blindness and deafness. However, he could perform serial sevens perfectly. His saccades appeared to be normal, and he could perform finger-nose tests and do finger-thumb tapping. He was not aphasic, and his speech was clear and comprehensible without any dysrhythmias or dysarthrias. He had developed delirium when medically ill, but even when he was medically

Table 1

CAG/CCG Haplotypes in Cases with Symptoms of HD and ≤37 Repeats and in Unaffected Individuals >65 Years of Age

	CCG_5	CCC_7	CCG_8	CCG_9	CCG_{10}
CAG_{35}		N (76)			N (68)
CAG_{36}		N (78), N (79), N (74), N (87), A, A		A	A, A, A
CAG_{37}	A	N (69), A, A, A, A			
CAG_{38}		N (69), N (90)			
CAG_{39}		N (95), N (90), N (67)			

NOTE.—HD cases with ≤37 repeats are indicated as "A." Unaffected individuals >65 years of age are indicated as "N," and their ages are given (in years) in parentheses. CAG lengths refer specifically to these lengths, independent of the CCG alleles. The two cases that would be wrongly diagnosed if a PCR assay that included both the CAG and the CCG repeats were used are underlined.

stable he had fixed delusions that people were trying to kill him. These could not be attributed to HD, in the absence of any other neurological or cognitive signs. It is common for delusions formed during a delirium to remain fixed for some time after the delirium clears.

Postmortem examination of the brain was performed (J.C.H.). The brain weighed 1,290 g. No abnormalities were detected macroscopically. It was noted that there was no significant atrophy of the cerebral cortex, and the head of the caudate nucleus retained a convex outline at the lateral ventricle. Microscopic evaluation showed mild, patchy, perivascular neuronal loss within the striatum, particularly in the dorsal regions. These changes were considered to be different from those characteristic of HD or aging and possibly due to chronic hypertension. Changes characteristic of early HD in glial fibrillary acidic protein immunohistochemical preparations (Hedreen and Folstein 1995) were not seen. Accordingly, this man was not considered to have a pathological diagnosis of HD.

CCG repeat sizes and their phases relative to the CAG repeats were determined on the large chromosomes in affected individuals with ≤37 repeats and in asymptomatic individuals with ≥35 repeats (table 1). Insufficient data were available to allow us to rule out any possible effect the CCG repeats may have had on the penetrance of the mutation. However, all of the aged asymptomatic cases with 36–39 repeats had 7 CCG repeats (table 1), the allele associated with >90% of HD chromosomes (Andrew et al. 1994; Rubinsztein et al. 1994*a*). Four different CCG alleles were seen to be associated with chromosomes with 30–39 CAG repeats. These data illustrate that assays that employ only primers spanning both the CAG and CCG polymorphisms can lead to misdiagnosis of individuals, if one accepts that the smallest HD chromosomes have 36 repeats and the largest normal chromosomes have 35 repeats. Two cases that exemplify this point are shown in table 1. Similar cases have been observed in other studies (E. Almqvist and M. R. Hayden, unpublished data).

One symptomatic individual was found with 37 HD repeats and 40 repeats in the SCA1 gene. This individual had no family history of either disease. Since 40 SCA1 repeats seem to be associated with symptomatic and normal cases (Burright et al. 1995) and may represent an overlap at this locus, we were intrigued as to the possibility that this case may be a double heterozygote for these two diseases. The SCA1 CAG repeats are generally interrupted once or twice by CAT trinucleotides (histidine codons) on normal chromosomes but are uninterrupted on disease chromosomes (Chung et al. 1993). This case probably represents a normal SCA1 chromosome, since *Sfa*N1 restriction analysis revealed interrupted repeats. A similar large normal SCA1 allele was recently reported by Quan et al. (1995).

Discussion

The aim of this study was to determine the lower limit of the disease-associated repeats in HD and the upper limit of CAG repeat sizes in normal individuals. Since seven cases with clinical diagnoses of HD were found with 36 repeats, we can confidently associate this allele with the disease.

The presence of six normally functioning individuals aged 75–90+ years with CAG expansions in the HD size range (36–39 repeats) suggests that some people with small HD expansions may survive without symptoms into old age. At present, it is not clear whether these individuals would develop HD if they lived long enough. This question is further complicated by our lack of understanding of how HD manifests after the age of 80 years, although there are data that suggest that the presence of dementia is less pronounced in late-onset HD (Quarrell and Harper 1991; Britton et al. 1995). This is largely due to the comparative paucity of such cases. Although the man with 39 repeats who lived to

age 95 years did not have classical features of HD, he did have "restlessness of his hands," which was noted when he was 86 years old. However, involuntary movements were absent when this man was reexamined at age 95 years. The perivascular pattern of cell loss in this case was not typical of HD; however, the dorsal predominance of this loss is a feature of this disease (Vonsattel et al. 1985).

It seems that some individuals with 36–39 CAG repeats may be spared the incapacitating physical and psychological symptoms of HD into their 80s and 90s. Since CAG repeat sizes are generally determined by using lymphocyte DNA, we cannot exclude the possibility that some of these individuals may have somatic mosaicism that has resulted in repeat lengths of <36 in the target tissues for HD.

No cases of HD have been reported with ≤35 repeats. Thus, individuals with 30–35 repeats are unlikely to get HD. However, the possibility of repeat expansion into the pathogenic size range in their children should be considered in the clinical scenario (Goldberg et al. 1995).

The presence of apparently normal alleles of 35 repeats and disease alleles of 36 repeats highlights the importance of standardized and reproducible sizing of alleles with between 30 and 40 repeats. In certain rare cases, an inaccuracy of 1 repeat can lead to an incorrect diagnosis. In this size range, we believe that it is helpful to use cloned size standard(s), preferably corresponding to different CAG sizes in this range. Our experience with such a standard suggests that they should be used with caution, since alpha-dCTP incorporation can lead to prominent bands of incorrect mobility. We found that the use of end-labeled primer allows for the clearest delineation of the darkest band in an expanded allele, relative to the other stutter bands. Furthermore, the data we have presented show the dangers of sizing such alleles with PCR assays that include the polymorphic CCG repeats.

In conclusion, this large collection of individuals with 30–40 CAG repeats in the HD gene has allowed for a uniform sizing of these cases. We believe that the lower limit of the CAG size range in HD cases extends to 36 repeats. The apparently healthy survival into old age of cases with 36–39 repeats suggests that the HD mutation may not be fully penetrant in rare cases. Potential genetic or environmental factors that modulate the penetrance and age at onset of HD may serve as opportunities for therapeutic intervention.

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