Letters to the Editor

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Two Novel Single–Base-Pair Substitutions Adjacent to the CAG Repeat in the Huntington Disease Gene (IT15): Implications for Diagnostic Testing

To the Editor:

The CAG-expansion mutation that causes Huntington disease (HD) was first identified in 1993 (Huntington's Disease Collaborative Research Group 1993). The standard PCR assay used by clinical laboratories to determine repeat length amplifies only the CAG repeat (Andrew et al. 1994; The ACMG/ASHG Huntington Disease Genetic Testing Working Group 1998). The adjacent CCG repeat varies in length by 7–12 triplets (Andrew et al. 1994), and the CCT repeat following the CCG repeat can be either two (common) or three (rare) triplets in length (Pecheux et al. 1995). A PCR assay that amplifies across all three repeats (referred to as the "CAG+CCG assay"), taking advantage of the common CCG repeat–length polymorphism, remains valuable for the detection of a second allele in cases in which only a single allele is detected by the CAG-only method (Goldberg et al. 1993; The ACMG/ASHG Huntington Disease Genetic Testing Working Group 1998). By use of a third assay, which determines the combined length of the CCG and CCT repeats (referred to as the "CCG-only assay"; Andrew et al. 1994), CAG-repeat length can be calculated. Previously, an apparently rare mutation was identified, in which the CAA triplet immediately following the CAG repeat is absent, leading to failure of the standard PCR assay for repeat length (Gellera et al. 1996). We now report two additional single-base substitutions that can lead to assay failure or errors in the calculation of CAG-repeat length.

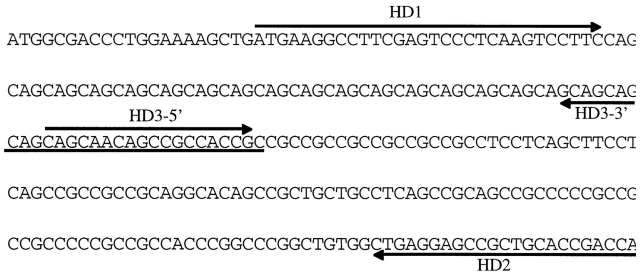
In the first case, a 51-year-old man with a 14-year history of a progressive syndrome typical of HD was referred for testing for the HD expansion mutation. His father had died, at age 56 years, of a myocardial infarction, and an extensive review of the pedigree revealed no affected relatives. After informed consent was obtained, DNA was extracted from blood (Gentra). The CAG-only assay (fig. 1*A*) yielded a single peak, indicating a CAG-repeat length of 19 triplets. The CCG-only assay (fig. 1*A*) generated two peaks, indicating the presence of alleles containing 7 and 10 CCG triplets. The CAG+CCG assay yielded a single peak consistent with a CAG-repeat length of 19 or 20 triplets. A new 5′ primer was synthesized that was identical to HD1, except for the absence of the 3′ terminal C. By use of this primer and primer HD2, the normal length CAG repeat of 20 triplets and an expanded repeat of 41 triplets were detected.

To establish the reason for the failure of the original HD1 primer to amplify the expanded repeat, genomic DNA was reamplified by use of primers HD7-5′ (5′-GGACGGCCGCTAGGTTC-3′) and HD7-3′ (5′-CGG-CTGAGGAAGCTGAGGAGG-3′) and a PCR protocol similar to the original CAG-only assay. PCR products were cloned into pCRII (Invitrogen), and sequence was obtained from three independent clones containing the expanded allele. Each clone had an expanded CAG repeat of 41 triplets, as predicted by the assay with the shortened HD1 primer, that was adjacent to a CCG repeat of 7 triplets. The sequence also revealed the presence of a C→G substitution of the base immediately preceding the CAG repeat (fig. 1*B*).

In the second case, an unaffected spouse of a patient with HD was tested for HD repeat lengths, after informed consent was obtained, as part of a presymptomatic testing protocol for her child. The CAG repeats determined by the CAG-only assay were 17 and 28 triplets in length. The CCG-only assay yielded a single peak, suggesting two CCG alleles of seven triplets each. The CAG+CCG assay indicated the presence of an allele of (CAG)17, as expected, and a second allele of (CAG)30, two triplets longer than was predicted by the other assays. To account for this discrepancy, genomic DNA was amplified, and the products were cloned into pCRII, as described above. Interpretable sequences were obtained from 11 clones. Eight clones contained a normal allele with, as expected, 7 CCG triplets and either 16 (three clones) or 17 (five clones) CAG triplets. Three clones contained a second allele with either 26 (one clone) or 27 (two clones) CAG triplets. In all three of these clones, the CCG repeat consisted of 12 consecutive CCG triplets without the CCA triplet that normally precedes the CCG repeat (fig. 1*B*).

The sequence of the regions adjacent to the CAG re-

A.



B.

Normal:	TTC (CAG)n CAA CAG CCG CCA (CCG)n
Gellera et al:	TTC (CAG)n CAG CAG CCG CCA (CCG)n
Case 1:	TTG (CAG)n CAA CAG CCG CCA (CCG)n
Case 2:	TTC (CAG)n CAA CAG CCG CCG (CCG)n

Figure 1 *A,* Repeat region of the HD gene (GenBank accession number L12392, base pairs 316–585) and primers used for amplification of the CAG repeat (HD1 and HD3-3′), the CCG repeat (HD3-5′ and HD2), and the combined CAG/CCG repeat (HD1 and HD2). For the CAG-only protocol, 500 ng genomic DNA was incubated at 99°C for 3 min; 400 nM each of primer HD1 and fluorescently tagged primer HD3-3′, 2.5 U *Taq* polymerase, and buffer containing 50 mM Tris-HCl (pH 8.3), 50 mM KCL, 200 μM each dNTP, 1.5 mM $MgCl_2$, and 8× MasterAmp PCR enhancer (Epicenter Technologies) were added, followed by denaturation at 95°C for 5 min; 33 cycles of 95°C for 45 s, 67°C for 45 s, and 72°C for 1 min; and an extension at 72°C for 7 min. The CCG-only protocol was as described above, except that annealing was at 60°C and the buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM $MgCl_2$, .01% gelatin, 10% dimethyl sulfoxide, 1.5 U *Taq* polymerase, 100 μM dGTP, 100 μM deaza-dGTP, and 200 μM each of dATP, dTTP, and dCTP. The CAG+CCG protocol was as described elsewhere (Huntington's Disease Collaborative Research Group 1993; Stine et al. 1993), modified for automated fluorescent analysis. All assays are ±1 triplet. *B,* Three substitutions identified in the repeat region of the HD gene.

peat provides an explanation for the PCR results in these two cases. In the first case, the C→G substitution falls precisely at the 3′ terminal base of the HD1 PCR primer, apparently preventing efficient annealing of this primer and, hence, synthesis of a product. The substitution results in a change from phenylalanine to leucine in the encoded protein. The clinical phenotype of the first case is typical of HD, and this substitution of one neutral hydrophobic amino acid for another possibly has no consequences on phenotype. Among the 1,236 subjects that we have tested for HD repeat length, this is the only case for which the HD1 primer has failed consistently, suggesting that this C→G substitution is a rare mutation.

The A→G polymorphism in the second case is silent, because both codons encode proline. The absence of the CCA codon in the second case presumably led to the misannealing of primer HD3-5′, which caused the false finding of a CCG repeat of seven triplets. In 26 other subjects tested with all three assays (CAG-only, CCG-only, and CAG+CCG), we did not detect a repeat-length discrepancy of two triplets, suggesting that absence of the CCA repeat is a relatively uncommon variant. The actual (CCG)12 repeat probably indicates the common (CCG)10 variant coupled with an A→G substitution that converts the adjacent CCACCG sequence into CCG-CCG. However, other combinations of deletions and insertions also could have resulted in this change. The variation in length among the cloned PCR products of the HD region of the second case may reflect either repeat-length instability during plasmid replication in bacteria or somatic variation of the repeat in leukocytes from the subject.

These two variants have implications for the determination of the repeat length in the HD gene. The first case demonstrates that test results indicative of CAG repeat–length homozygosity may be incorrect, particularly if the standard primer, HD1, is used. For cases in which both the CAG-only and the CAG+CCG assays detect a single repeat, a reasonable next step would be to repeat the assays, with the HD1-short primer. If this assay or other PCR assays using alternative primers fail to reveal a second allele, then a search, by Southern blot analysis, for an expanded allele would be prudent. Similarly, the second case suggests the use of an alternative primer in those cases for which CCG-repeat length is important but for which the standard assays of repeat length yield discrepant results. More broadly, these cases illustrate the pitfalls inherent in PCR-based assays of genetic mutations.

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