Dynamic mutation in Dutch Huntington’s disease patients: increased paternal repeat instability extending to within the normal size range

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Abstract
Analysis of the distribution of normal and expanded alleles of the polymorphic (CAG), repeat in the IT15 gene in the Dutch population confirmed the presence of an expanded repeat on all Huntington’s disease (HD) chromosomes. Our results show that the size distributions of normal and affected alleles overlap. Normal alleles range from 11 to 37 repeats and HD alleles contain 37 to 84 repeats. A clear correlation is found between age at onset and repeat length, but the spread of the age at onset in the major repeat range producing characteristic HD is too wide to be of diagnostic value. In the available parent-offspring pairs, maternal HD alleles show a moderate instability with a slight preponderance of size increase over size decrease. Paternal alleles have a bimodal distribution: the majority (69%) behave similarly to the maternal alleles, while the remainder (31%) show a dramatic expansion, the degree of which appears proportional to the initial size. This is shown in three out of four juvenile patients, who have repeats of 71, 74, and 84 copies, respectively, originating from expanded paternal HD alleles in the previous generation. Two sporadic cases are caused by expansion of ‘large’ normal paternal alleles of 32 and 34 repeats, respectively, to 46 copies. This not only confirms the diagnosis of HD in two de novo cases, but it also underlines the increased paternal instability. In addition paternal repeat instability was once detected within the normal range in two sibs who inherited 21 and 22 repeats, respectively, on the same paternal chromosome. In two Dutch HD families the segregation of the expanded (CAG), repeat was found. Analysis of the (CAG), repeat in our previously reported recombinants confirmed their disease status. (J Med Genet 1993;30:996–1002)

Huntington’s disease (HD) is a progressive neurodegenerative disorder with an autosomal dominant pattern of inheritance. The characteristics of this devastating disorder include motor disturbances, personality changes, and dementia. The symptoms are caused by selective cell death in the basal ganglia, predominantly in the caudate nucleus and putamen, while other areas, including the globus pallidus and cerebral cortex, are only mildly affected. HD affects approximately 1 in 10,000 subjects in most populations of European origin. The onset of the disease usually occurs in the third to fifth decade of life and progresses for 10 to 20 years until death. Occasionally (±5%), HD has a juvenile onset (before the age of 20 years), typically presenting with more severe symptoms and rigidity. In 70% the gene is inherited from the father. The biochemical basis for the neuronal death in HD is unknown and consequently there is no effective treatment for this disorder.

Using linkage analysis the genetic defect responsible for HD was localised to chromosome 4p16.3 in 1983.2 Recently, after 10 years of intensive worldwide search, the molecular defect causing HD has been identified.3 It consists of an expansion of a polymorphic (CAG), repeat in the 5' part of the IT15 gene. The gene spans about 210 kb of genomic DNA and encodes a protein with a predicted size of ~348 kDa, designated ‘huntingtin’. It is expressed in a wide variety of tissues and shows no relation to any known gene.

An initial study showed that the (CAG), repeat has at least 17 different alleles in the normal population, which vary between 11 and 34 copies of the repeat unit. Analysis of the (CAG), repeat in 75 independent HD families showed that they all had one allele in the normal range and one expanded allele, which ranged from 42 to over 66 copies. Furthermore, in two families with isolated cases of HD, analysis of the (CAG), repeat showed the potential of repeats in the high normal range (33 and ~36 copies) to expand to within the affected range, thus causing de novo HD.3

One of the striking characteristics of HD is its highly variable age at onset. While most patients have an onset in midlife, cases have been reported with ages at onset ranging from 2 to over 80 years.4 A higher age at onset was found in females5 and a preferential paternal transmission was found in juvenile cases.6 Both age at onset and sex of the affected parent seem to influence age at onset in offspring.7 In the initial study of the molecular defect, a rough correlation was observed between repeat length and age at onset, which was more pronounced in juvenile patients. However, the diagnostic value of the number of repeats in
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PREDICTION of the age at onset remained to be established.

Analysis of the distribution of the length of the (CAG)ₙ₀ repeat in the Dutch population confirmed the presence of an expanded repeat on all HD chromosomes. Since expanded trinucleotide repeats tend to be unstable in transmission, we studied the behaviour of the (CAG)ₙ₀ repeat in successive generations in all available affected parent-offspring pairs. Furthermore, we have analysed the (CAG)ₙ₀ repeat in two Dutch families with sporadic cases of HD.

Materials and methods

PATIENTS

All HD patients from families participating in the presymptomatic DNA testing service and from five families of our mapping panel were tested. To establish allelic variation of the (CAG)ₙ₀ repeat in the normal population we included 95 unrelated spouses or normal parents of affected HD patients. Diagnosis of HD in these families was confirmed as previously described. The age at which choreic movements became manifest was designated as the age at onset of HD in these patients. Four juvenile patients with an age at onset of 10, 10, 7, and 6 years, respectively, were included in this study.

PCR ASSAY

Genomic DNA was isolated from venous blood or cultured EBV transformed cell lines as previously described. For PCR analysis of the (CAG)ₙ₀ repeat the published primers were used. The PCR assay was modified as follows. PCR was performed in a reaction volume of 25 µl using 100 ng of genomic DNA, 100 pmol of each primer, 10 mmol/l Tris-HCl (pH 8.3), 5 mmol/l KCl, 2 mmol/l MgCl₂, 200 µmol/l of each dNTP (with a 1:1 ratio of dGTP and 7-deaza-dGTP), 10% DMSO, 20 µCi of [³²P]-dCTP (Amersham), and 1.25 U of AmpliTaq (Cetus). After heating to 95°C for 10 minutes, 40 cycles of one minute at 95°C, one minute at 65°C, two minutes at 72°C were performed in a DNA Thermal Cycler (Perkin Elmer Cetus). The addition of 7-deaza-dGTP proved essential to generate an interpretable signal and low background.

PCR products were mixed with an equal volume of 95% formamide loading buffer, denatured for five minutes at 95°C, and separated on 6% denaturing polyacrylamide gels. Autoradiography of fixed and dried gels was one to four days at room temperature. Since the band below the upper band of the PCR product was in most cases the major band using these primers and PCR conditions, this band was taken to estimate allele sizes relative to

![Figure 1](http://jmg.bmj.com/10.1136/jmg.30.12.996) Distribution of the allele sizes of the (CAG)ₙ₀ repeat in the Dutch population. Normal (open bars) and HD (filled bars) alleles, presented as the number of (CAG)ₙ₀ repeats, are depicted on the x axis. The number of each allele is depicted on the y axis. The number of normal alleles consists of combined data from 95 normal subjects and the normal alleles of 180 HD patients, yielding 370 normal chromosomes. The total number of HD alleles is 182. The large Dutch HD family is not included in these data.
to M13 sequencing ladders, not incubated with 7-deaza-dGTP. Incubation of the marker with 7-deaza-dGTP did not alter its electrophoretic mobility. The proximity of markers proved essential for accurate size determination.

Results

VARIABILITY OF THE (CAG)$_n$ REPEAT IN THE DUTCH POPULATION

Analysis of the polymorphic (CAG)$_n$ repeat in the IT15 gene in the DNA of 95 normal Dutch subjects showed the presence of 18 alleles ranging from 11 to 37 copies (fig 1) with a heterozygosity frequency of 87%.

We determined the number of (CAG)$_n$ repeat units of both alleles in 181 patients from 80 unrelated families. In 180 patients one normal and one expanded allele were found, while one subject was homozygous for two expanded alleles. The distribution of the normal alleles in the HD patients was similar to the distribution in the normal chromosomes analysed, although three additional alleles of 12, 28, and 30 units were detected increasing the total number of normal alleles to 21 (fig 1). Expanded alleles ranged from 37 to 84 units (fig 1). In our population 29% of the expanded alleles fell in the range of 37 to 41. The majority of alleles contained 42 to 47 units (59%), while 12% consisted of more than 48 units. The distribution of allele sizes did not change significantly when the mean allele size per family was used instead of every patient in the family. In fig 2 the largest normal allele (37 repeats), found in an unaffected spouse, was electrophoresed next to the smallest expanded repeat (37 repeats), indicating an overlap between normal and affected allele sizes.

To investigate whether it is possible to find expanded alleles in chorionic villi (CV), the number of (CAG)$_n$ repeats in the DNA of two CV samples from two different families was analysed (data not shown). In the first case the DNA of the CV contained 39 units, while the at risk mother had 41 units. The second CV sample showed the same number of repeats as the at risk parent, that is, 42 copies. The fetuses had both been given an increased risk for developing HD previously on the basis of haplotype analysis.

INSTABILITY OF THE (CAG)$_n$ REPEAT

The size of the expanded allele varies within families both between and within sibships. While it has an overall tendency to increase in length, contractions are observed as well.

For those cases where affected parent-offspring pairs were available for analysis, the data were divided according to parental sex (fig 4). In six out of 13 maternal cases (46%) the size of the repeat increased upon transmission from parent to offspring, in two cases (15%) the repeat number decreased, and in five cases (38%) no change was observed. This is only a minor deviation from the line that is obtained when alleles are transmitted unchanged (fig 4). Paternal alleles show clear bimodal behaviour: in 11 out of 16 (69%) paternal transmissions the repeat stability is comparable to the maternal transmission: moderately unstable with five (45%) small increases, three (27%) decreases, and three unaltered cases (27%). In addition, five alleles (31%) were significantly elongated. Interestingly, the degree of the expansion itself seems to be proportional to the initial size of the paternal allele (fig 4). Three of these were expanded HD alleles giving rise to juvenile HD in the second generation, while the remaining two were long normal alleles causing de novo HD (see above). In two parent-offspring pairs a large expansion of the repeat, from 44 to 71 and from 47 to 84 copies, was found. In one case, with a 74 copy repeat, the elongation of the repeat in transmission from father to child could not be directly verified in the father as his DNA was not available. How-

SPORADIC CASES

The mutation rate in HD was thought to be very low. Using molecular analysis, however, the expansion of normal alleles to the HD range was found in two families. We analysed the size of the (CAG)$_n$ repeat in two Dutch families with apparently sporadic HD, in which extensive family research did not show any previous cases of HD. Fig 3 shows the results of haplotyping and PCR analysis of the (CAG)$_n$ repeat in these cases. In family 1 (fig 3A) both parents have two alleles in the normal range, of which the father has one allele in the high normal range, that is, 32 copies. The proband with clear symptoms of HD shows an expanded repeat of 46 units on the chromosome with the paternal haplotype (fig 3C). Similarly, an increase from 34 to 46 copies was observed in transmission from the father to the affected offspring in family 2 (fig 3C). In the latter case, but not in the former, the affected chromosome carried the major HD haplotype reported by MacDonald et al. Non-paternity was excluded in both cases.
Figure 3  Haplotyping and PCR analysis of the (CAG)_n repeat in two families with sporadic HD. Diamonds are used to protect confidentiality; closed symbols indicate affected subjects. A, C: M13 sequencing ladders; bp: basepairs. Haplotype analysis has been performed with polymorphic markers in 4p16.3 in family 1 (A) and family 2 (B). PCR analysis (C) shows alleles in the normal range in parents and alleles in the expanded range in the affected offspring in both families. Note the large normal alleles in both fathers.

ever, his sister had a repeat size of 45 copies. Since both of these sibs had received the HD allele from their mother, the repeat size in the father was probably not very different from 45 copies. The uncertainty of the paternal allele size is indicated in fig 3 by a dotted horizontal line of several units width.

Strikingly, and further substantiating the increased paternal instability, in one family a difference in repeat number was found between two sibs, occurring in one of the two normal paternal chromosomes. One sib had 21 copies and the other 22. The father was dead, so his repeat number could not be determined, though the availability of other family members allowed unambiguous determination of the haplotypes. The haplotype in question occurred only twice in four sibs, but all four parental haplotypes were detected and non-paternity was excluded in this family (data not shown for reasons of confidentiality).

Segregation of the (CAG)_n repeat in HD
with occasional changes is shown in two Dutch HD families (fig 5). In family 1 subject I.1 has transmitted his affected chromosome to his two children. In II.1 the number of repeats, which was 40 in the father, has decreased to 38, while in the other child, II.2, the size of the repeat did not change. In family 2 transmission from I.2 to II.1 did not change the size of the repeat.

CORRELATION BETWEEN THE NUMBER OF REPEAT UNITS AND AGE AT ONSET
The observation of very large alleles in two juvenile patients corroborated the suggested correlation between repeat size and age at onset. Four Dutch juvenile patients, with an age at onset of 10, 10, 7, and 6 years, respectively, had very large alleles of 59, 71, 74, and 84 repeats, respectively. When the age at onset in our patient population is plotted against the number of repeat units a clear correlation can be seen (fig 6). Only when the HD allele elongates to more than 55 repeats is a significant decrease in age at onset observed.

RECOMBINATION EVENTS
Seven recombination events in our HD patient population have been described previously, two of which were used to define the proximal border of the HD candidate region. In all cases the precise localisation of the gene or examination of repeat size or both has confirmed the disease status of these subjects. One particularly interesting case concerns a person who had reached the age of 72 without developing HD, while having inherited the complete affected haplotype. A double crossover, gene conversion, or a delayed age at onset had been considered to explain the genotype-phenotype inconsistency. Eventually, analysis of the (CAG)n repeat showed an expanded allele of 39 units and recently was reported to be showing the first symptoms of HD at the age of 74 years.

Discussion
The size and distribution of the normal alleles of the polymorphic (CAG)n repeat in the IT15 gene in normal Dutch chromosomes are similar to those found recently. Furthermore, all HD patients in the Dutch population show one allele in the normal range and one expanded allele, while one homozygous patient has two expanded alleles. This confirms that expansion of this (CAG)n repeat is the major or single cause of Huntington's disease. However, the distribution of the affected alleles in our population is somewhat different from that reported by the Huntington's Disease Collaborative Research Group (HDCRG). Expanded repeats as short as 37 units were found and the majority of the Dutch HD alleles fall in the 42 to 47 range (50% compared to the published 41%), while previously reported results show a predominance of alleles larger than 48 copies (59% v 12% in our population). This could be either because of statistical variation or, alternatively, may reflect population differences in susceptibility to expansion of HD chromosomes.

While new mutations to HD were thought to be rare, MacDonald et al have argued that multiple ancestor haplotypes exist in the population, which is supported by the molecular findings, and one haplotype seemed more prone to repeat expansion than others. In one of our two families with sporadic HD a large normal allele of 34 units has expanded to 46 units on a paternal chromosome with this particular haplotype. The other de novo expansion, while not on this haplotype, was also on a paternal chromosome and of similar magnitude (32 to 46 units). The phenomenon of founder chromosomes, that is, chromosomes with a specific haplotype which are prone to repeat expansion, has also been described for the fragile X syndrome and myotonic dystrophy. Since these diseases are also caused by expansion of a trinucleotide repeat, the mutational mechanism could be comparable.

Most remarkably we observed an overlap of large normal alleles and small affected alleles. The largest normal allele as well as the smallest
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Figure 5  PCR analysis of the (CAG)_n repeat in two Dutch HD families. Offspring are shown as diamonds and birth order has been changed for confidentiality. Alleles in numbers of repeat units are shown below each subject. A: M13 sequencing ladders. The lowest allele visible in the lane of sample I.1 of family 1 is the result of leakage of an adjacent lane, containing a homozygous 16/16 sample. The alleles of I.1 are marked with arrows.

Figure 6  Correlation between age at onset and repeat length. Age at onset in Dutch HD patients is plotted against the number of repeat units in the expanded (CAG)_n repeat. A negative correlation is found; correlation coefficient (r) = −0.7243.
complementing normal subunits become of critical importance.

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