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Mitotic stability and meiotic variability of the $(CAG)_n$ repeat in the Huntington disease gene

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The gene causing Huntingtons's disease, an autosomal dominantly inherited, neurodegenerative disorder, has been identified recently. The corresponding mutation is involving an expansion in the number of $(CAG)_n$ repeats in the coding region of the Huntington's disease gene on chromosome 4. In this report, we demonstrate the length variation of the repeat in 513 non-HD chromosomes from normal individuals and HD patients showing 23 alleles with 11 to 33 repeats. Analyzing the inheritance of the $(CAG)_n$ stretch we found meiotic instability for HD alleles ($[CAG]_{40}$ to $[CAG]_{75}$) with a mutation frequency of approximately 0.7, while in 431 meioses of normal alleles only two expansions were identified. The risk of expansion during spermatogenesis is enhanced compared to oogenesis explaining juvenile onset by transmission from affected fathers. Further, the number of $(CAG)_n$ copies in an affected individual in relation to the sex of the transmitting parent was evaluated and no significant differences were found. No mosaicism or differences in the repeat lengths were observed in the DNA from different tissues including brain and lymphocytes of two HD patients indicating mitotic stability of the mutation. Therefore, the determination of the repeat number in the DNA of blood lymphocytes is probably representative of all tissues in a patient.

INTRODUCTION

Huntington's disease (HD) is an autosomal dominant genetic disorder estimated to affect approximately 1 in 10,000 individuals (1). It is characterized by progressive neurodegeneration resulting in choreic movements, cognitive decline, and dementia as a result of neuronal cell death, particularly in the nucleus caudate and putamen of the basal ganglia. The disorder shows complete penetrance but variable age of onset. The beginning of symptoms (age of onset) is partially influenced by the sex and the age at onset of the affected parent (2), with earlier onset in children of male transmitting parents. The HD gene was localized to the short arm of chromosome 4 in the 4p16.3 region by linkage of RFLPs (restriction fragment length polymorphisms) (3).

Recently, the mutation was identified as an unstable $(CAG)_n$ trinucleotide repeat in the 5'coding region of the Huntington's disease gene (4). PCR-based analyses of this region in unaffected individuals have shown numbers of repeats vary between 11 and 34 copies (4,5). The elongation of the $(CAG)_n$ sequence to more than approximately 40 copies is presumably causing the disease. An inverse correlation between age of onset and number of repeats is highly probable (4,5). Transcripts of the HD gene of about 11 kb were discovered in lymphoblastoid cell lines, liver, spleen, kidney, muscle, and brain, but the structure and function of the encoded protein still remain unknown.

To date, besides HD in four other human genetic diseases trinucleotide repeat expansion mutations have been identified. Fragile X syndrome (6), spinal and bulbar muscular atrophy (7), myotonic dystrophy (8), and spinocerebellar ataxia type 1 (9) are caused by amplification of polymorphic exonic trinucleotide sequences beyond the normal size range.

Genetic anticipation, a phenomenon in which the severity of an inherited disease increases in successive generations of an affected family, is associated with progressive amplification of the respective trinucleotid repeat and is strictly maternal in fragile X syndrome (10). Anticipation was also observed in pedigrees with myotonic dystrophy (DM). This disease is variable both in severity and age of onset, depending on the trinucleotide repeat number.

The triplet repeat expansions identified in these disorders may exhibit remarkable variation both within cells of a single individual (particularly in fra X and DM) and between members of a sibship. To investigate the stability of the $(CAG)_n$ repeat in the HD gene we analyzed 431 normal and 54 HD meioses. The length variation of the trinucleotide was determined by PCR analysis and allele quantitation by sequencing gel analysis (11), a highly informative method of direct diagnosis. This assay allows us to determine exactly the number of repeats in genomic DNA from normal and HD individuals, to study allele frequency in control individuals and to provide data concerning meiotic variability of HD alleles. The mitotic stability of the $(CAG)_n$ sequence on HD chromosomes was examined for different tissues including brain and blood lymphocytes from two patients. In the investigated German population alleles with greater than 39

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repeats revealed a high mutation frequency during meiosis. Furthermore, we could confirm, that the risk of expansion of the HD repeat is obviously higher in spermatogenesis than in oogenesis.

RESULTS

Polymorphism of the $(CAG)_n$ repeat in normal chromosomes

To assess length variation within the $(CAG)_n$ repeat, a PCR-based assay was employed (11). The analysis of 513 control chromosomes 4 from unrelated German individuals revealed 23 alleles (Table 1) ranging in size from 11 to 33 with a median of 20 $(CAG)_n$ copies and frequencies between 0.189 ($[CAG]_{17}$) and 0.002 ($[CAG]_{12}$ and $[CAG]_{28}$). Comparing the frequency of 309 normal alleles in HD patients to that of 204 control chromosomes no significant differences were found (Table 1). An overall heterozygote frequency of about 85% is found in this control population.

Polymorphisms of the $(CAG)_n$ repeat in HD chromosomes

In determining the copy number of the polymorphic repeat in 352 HD patients, 40 to 75 $(CAG)_n$ triplets were found (5). The evaluation of the $(CAG)_n$ copy number in 314 unrelated HD patients revealed approximately 60% of the alleles in the range between 41 and 45 trinucleotide repeats (Table 2). Comparison of the number of $(CAG)_n$ repeats in 70 patients resulting from paternal transmission with 69 patients resulting from maternal transmission, showed no significant difference (Table 3).

Variation of the $(CAG)_n$ repeat on HD chromosomes: meiotic instability and paternal anticipation

Following the HD allele in 38 pedigrees during 54 meioses an overall relation of stable to altered copy number of 15:39 was discovered (28% stable:72% unstable). Furthermore, weak sex specific differences in the stability of the HD repeat could be observed. Analyzing maternal inheritance 15 out of 22 HD chromosomes revealed instability (68%). For paternal inheritance 24 out of 32 HD alleles showed length variations (75% instability), not significantly different from maternal inheritance.

Two types of changes were discovered: on the one hand, small alterations resulting in addition or reduction of 1 to 3 repeats and on the other hand, expansion of more than three triplets (Table 4).

In about 18% of meioses progressive expansions of 4 to 28 repeat units were observed, occuring exclusively during male transmission (Table 4). In one family the progressive meiotic expansion during male transmission results in juvenile age of onset. The father ($[CAG]_{45}$) was affected between the fourth and fifth decade of life, whereas the offspring ($[CAG]_{73}$) developed first symptoms before the age of twenty. Excessive amplification of the HD repeat has not been observed during maternal transmission. The obvious sex specific differences (Wilcoxon rank sum test, $p<0.05$) in the amplification of the HD repeat may be important for genetic counselling.

The other mutation type, a slight expansion or reduction ($+/-[CAG]_{1-3}$) of the HD repeat, was identified in both sexes with equal frequency. During 22 meioses (41%) slight elongation of the trinucleotide sequence has occured. A reduction of the repeat was found in 7 out of 54 HD meioses (13%) (Table 4). During maternal transmission the $(CAG)_n$ sequence was shortened by 3 triplets in two cases, resulting possibly in a delayed age of onset. It would be interesting to find a reduction to less than 40 trinucleotides to confirm an exact threshold value for disease manifestation.

In contrast to HD alleles, $(CAG)_n$ repeats on control chromosomes 4 are highly stable. In 431 non-HD meioses

Table 1. Distribution of $(CAG)_n$ repeat copies on non-HD chromosomes

CAG	C	%	Non-HD	%	All	%	95%-Confidence
11	2	0.98	2	0.65	4	0.78	[0.002; 0.020]
12	–	–	1	0.32	1	0.19	[0.000; 0.011]
13	2	0.98	5	1.62	7	1.36	[0.006; 0.028]
14	2	0.98	2	0.65	4	0.78	[0.002; 0.020]
15	4	1.96	5	1.62	9	1.75	[0.008; 0.033]
16	6	2.94	2	0.65	8	1.56	[0.007; 0.031]
17	41	20.01	56	18.12	97	18.91	[0.156; 0.226]
18	28	13.73	44	14.24	72	14.04	[0.111; 0.173]
19	17	8.33	27	8.74	44	8.57	[0.063; 0.113]
20	22	10.78	47	15.21	69	13.45	[0.106; 0.167]
21	30	14.71	50	16.18	80	15.59	[0.126; 0.190]
22	14	6.86	26	8.41	40	7.79	[0.056; 0.105]
23	11	5.39	17	5.50	28	5.46	[0.037; 0.078]
24	6	2.94	9	2.91	15	2.92	[0.016; 0.048]
25	8	3.92	2	0.65	10	1.95	[0.009; 0.036]
26	–	–	3	0.97	3	0.56	[0.001; 0.037]
27	3	1.47	3	0.97	6	1.17	[0.004; 0.025]
28	–	–	1	0.32	1	0.19	[0.000; 0.011]
29	1	0.49	2	0.65	3	0.56	[0.001; 0.037]
30	2	0.98	2	0.65	4	0.78	[0.002; 0.020]
31	2	0.98	1	0.32	3	0.56	[0.001; 0.037]
32	2	0.98	1	0.32	3	0.56	[0.001; 0.037]
33	1	0.49	1	0.32	2	0.39	[0.001; 0.034]
N	**204**		**309**		**513**		

The $(CAG)_n$ number varies from 11 to 33 with a median value of 20 trinucleotide copies. The number of chromosomes of control persons (C), non-HD alleles of patients (NON-HD), the overall number of chromosomes (ALL) and the 95%-confidence intervals for each $(CAG)_n$ repeat length are given. N indicates the number of chromosomes for the investigated groups.

investigated in 26 control and 43 HD families two cases of repeat variation (+$[CAG]_2$; +$[CAG]_1$) in non-HD pedigrees were found (Figure 1).

Mitotic stability of the HD $(CAG)_n$ repeat

To estimate the stability of the repetitive $(CAG)_n$ element during mitosis, we investigated genomic DNA from different tissues including brain compartments and blood lymphocytes of two male HD patients by PCR amplification. No differences in the repeat length or mosaicism in the analyzed tissues were observed (Figure 2). In calculating the fragment sizes, the strongest signal was taken, the 2 to 5 shadow bands appearing in PCR products are not included. In one patient a single $[CAG]_{45}$ HD allele was identified in the DNA of peripheral blood lymphocytes, nucleus caudate and putamen, hippocampus, and white matter (Figure 2a), indicating mitotic stability of the HD gene. The 'smear' in the cortex PCR presumably results from a degradation of genomic DNA and not from mosaicism of the tissue, as ascertained by additional PCR amplifications for unrelated loci (data not shown). The second patient revealed uniform alleles ($[CAG]_{17/41}$) in the DNA of skeletal and heart muscle, liver, kidney, lymphocytes, parietal and temporal lobes, thalamus, hippocampal gyrus, nucleus caudate and striatum (Figure 2b). These results may exclude a progressive expansion of the potential polyglutamine stretch in the nucleus caudate as a pathogenic mechanism. Also, it has to be considered, that the caudate is not

Table 2. $(CAG)_n$ copy number in 314 unrelated HD patients

Repeat	Number	%
40	21	6.69
41	35	11.15
42	36	11.46
43	34	10.83
44	39	12.42
45	38	12.10
46	28	8.91
47	22	7.00
48	16	5.10
49	7	2.23
50	6	1.91
51	5	1.59
52	9	2.87
53	3	0.96
54	2	0.64
55	4	1.27
57	2	0.64
58	3	0.96
61	1	0.32
63	1	0.32
73	1	0.32
75	1	0.32

The mean value of allele sizes representing the expanded HD chromosomes is 44 repeats, with a range of 40 to 75 $(CAG)_n$. The number and percentage of HD chromosomes for the identified repeat lengths are listed.

Table 3. Number of $(CAG)_n$ repeats and inheritance

Repeats	Father	Mother
40	4	1
41	4	2
42	2	5
43	8	5
44	5	10
45	11	7
46	10	11
47	13	8
48	1	4
49	2	3
50	–	3
51	2	2
52	–	2
53	1	–
54	3	–
55	1	1
56	1	–
57	–	1
60	1	2
61	–	2
62	1	–
Chromosomes	70	69

The number and $(CAG)_n$ length of HD chromosomes in affected offsprings inheriting the disease from the father or from the mother is given for 40 to 62 $(CAG)_n$ repeats.

Table 4. Instability of HD alleles during meioses

Repeat Variation	Number of Meioses Maternal	Paternal
−3	2	–
−2	–	1
−1	2	2
0	7	8
+1	6	3
+2	2	6
+3	3	2
+4	–	3
+5	–	1
+7	–	1
+8	–	2
+9	–	1
+10	–	1
+28	–	1

The expansion or reduction (variation) of the $(CAG)_n$ repeat length during parental meioses in dependence of the sex of the transmitting parent is shown.

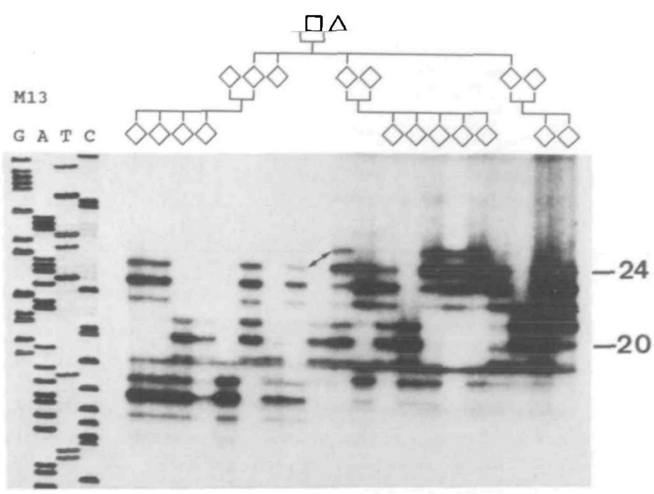
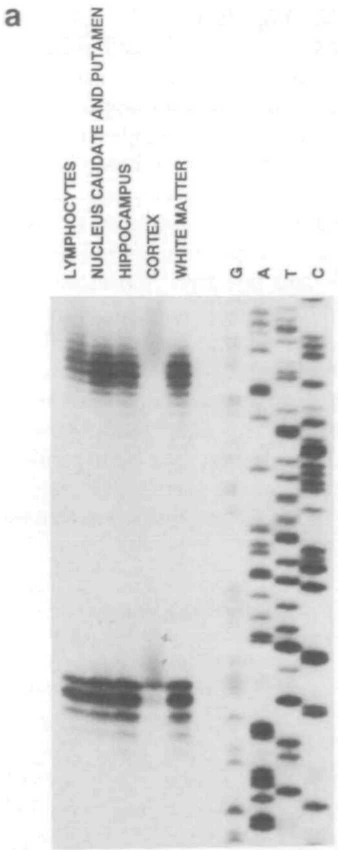


Figure 1. PCR amplification of DNA from one control family. An elongation of the $(CAG)_n$ repeat by two copies during male meiosis is marked by arrows.



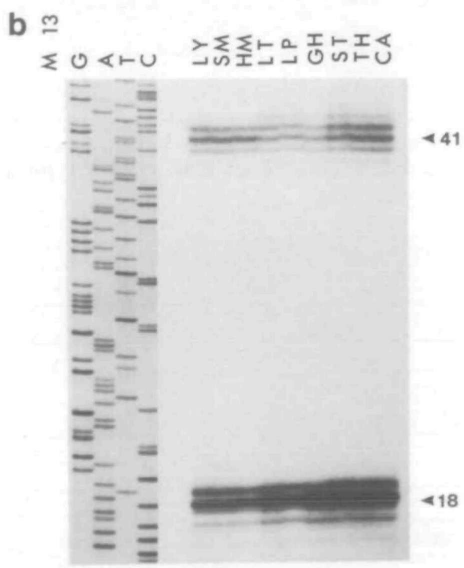


Figure 2. Repeat number in different tissues of two male HD patients. a) DNA was extracted from blood lymphocytes, nucleus caudate + putamen, hippocampus, cortex, and white matter and amplified by PCR. The repeat sizes ($[CAG]_{22}$ = normal allele; $[CAG]_{45}$ = HD allele) were determined by comparison to an M13 sequencing ladder (G A T C). b) DNA from blood lymphozytes (LY), skeletal muscle (SM), heart muscle (HM), lobus temporalis (LT), lobus parietalis (LP), gyrus hippocampalis (GH), striatum (ST), thalamus (TH), and caudatum (CA) was amplified and showed repeat sizes of $[CAG]_{18}$ and $[CAG]_{41}$ in all examined tissues.

a homogeneous tissue but is composed of glial cells and different neuronal types not all of which die in the brains of individuals with HD.

DISCUSSION

In the past two years, trinucleotide repeat expansions causing genetic diseases have been identified in at least five genes. Regarding the genetics of HD, we found differences and conformities in copy number and repeat stability in comparison to other trinucleotide diseases.

Amplification of the $(CAG)_n$ trinucleotide repeat causing Huntington's disease

For HD chromosomes we found 40 to 75 $(CAG)_n$ copies in contrast to 11 to 33 repeats on control chromosomes. Comparison to the $(CAG)_n$ repeat unit number of the German population to those published recently (12,13,14) revealed no overlap between the normal and expanded allele size distribution in our individuals, but a clear gap of 6 $(CAG)_n$ repeats. Possibly, this is a consequence of PCR primers used in our assay, creating smaller amplification products which do not include the entire prolin rich region 3′ to the $(CAG)_n$ repeat. A polymorphism in the number of prolin residues may be responsible for divergent results in PCR reactions with different primer sequences.

In HD, as in spinocerebellar ataxia type 1 (SCA1) and spinal and bulbar muscular atrophy (SBMA), the amplification, causing pathological reactions, is about twice the normal copy number, while in fragile X syndrome (fra X) and myotonic dystrophy (DM) thousands of repeats may be added. Such excessive repeat amplification is obviously not present in our HD patients. Elongation of the sequence to more than approximately 100 $(CAG)_n$ copies may result possibly in prenatal death of the affected individual. Consequences of these mutations may result in altered gene expression (fra X), altered stability of the message (DM) or gain of function as proposed for HD, SCA1 and SBMA (4,15).

Mitotic stability of the HD mutation

Variations in the number of repeats in single individuals have been observed as well in fra X as in DM due to mitotic instability of the mutations. We investigated the mitotic integrity of the HD allele in two patients. In contrast to the fra X and DM mutations, no heterogeneity of the HD repeat could be demonstrated in different tissues of single patients, suggesting high stability of this trinucleotide repeat. For one of our patients we could analyze the $(CAG)_n$ repeat in tissues deriving from the three primary germ layers (endoderm:liver; ectoderm:brain; mesoderm:blood, muscle, kidney). The overall homogeneity of the $(CAG)_n$ copy number in this individual excludes the possibility of mosaicism in the embryo. This result is confirmed by the stability of the repeat in brain and lymphocyte DNA of a second patient. Our data implicate the integrity of the $(CAG)_n$ repeat and therefore, the analysis of lymphocyte DNA from affected and at-risk individuals is sufficient and yields exact results in DNA diagnosis.

Anticipation and meiotic variability

The risk of an affected offspring in these disorders may depend upon the sex of the transmitting parent. Expansion of the HD allele associated with decreased age of onset occurs preferentially during male transmission (significant in Wilcoxon test with an error rate $p<5\%$), while large amplifications as documented for fra X and DM appear to be predominantly the result of female meiotic events (16). Recently, heterogeneous expansions of the $(CAG)_n$ repeat in the sperm DNA of HD males hes been described (12). The amplification of the $(CAG)_n$ repeat can be regarded as the genetic explanation for decreased age of onset in HD pedigrees. The biological mechanism of anticipation phenomena is unknown. In fra X and DM amplification of the repeat length is related to the phenotype, while in HD the age of onset is correlated with the $(CAG)_n$ copy number (5). This specific effect of a mutation may depend of the function of the particular gene product.

Reduction of repetitive sequences

A reduction of the mutated allele was observed in some HD pedigrees. In 13% of HD meioses the $(CAG)_n$ stretch was reduced by one to three tripletts. Caused by the low case number, no differences in dependence to the sex of the transmitting parent could be shown. The loss of three tripletts was observed in two female, but not in male meioses. This variation type was reported previously for two of the described disorders. In at least four DM families, transmission of the affected chromosome accompanied by a decrease in length of the triplet repeat to normal alleles was reported (17,18), as well as small reductions (16). In fra X, a reduction of the repeat length from a full to a premutation state in maternal inheritance can occur (6). Whether the reduction to lower repeat numbers in HD is preferentially an event during female meiosis has to be investigated in extensive family studies. No case of triplet repeat reversion of an affected allele to the normal size range in HD pedigrees has been reported until now. Such reversal of mutations is only detectable in combining linkage analysis with flanking DNA markers and direct proof of the expanded repeat.

Elongation of trinucleotide sequences

The risk of progressive expansion of mutated alleles may be enhanced with higher numbers of repeats in the ancestor. In this study, we found ten expansions of more than three trinucleotide units. The transmitting males showed 42 to 51 $(CAG)_n$ copies, their offsprings 46 to 73 repeats (data not shown). Our results provide no evidence of an increasing risk for elongation and early age of onset in progeny of males with $[CAG]_{>50}$. Either this is due to the limited number of meioses or no strong correlation is present. This would be supported by one premutation case. To the allele of the father ($[CAG]_{35}$) 8 triplets are added in the offspring ($[CAG]_{43}$) (5). Furthermore, in five families expansion as well as reduction of the HD allele was established. These examples may reflect an enhanced mutation frequency in some HD families, involving possibly further gene products or replication factors.

So far, unstable trinucleotide repeats are reported only in human DNA correlated with genetic diseases. Autosomal dominant and X-linked traits are identified, but recessive inheritance of this mutation type associated with loss of transcription as seen in the fra X syndrome is also imaginable. One of the many questions is, why these unstable sequences have not been identified in any other species. The isolation of corresponding genes from related organisms, and the identification and variability of these triplet regions will be of great interest.

MATERIALS AND METHODS

DNA samples from HD patients and control individuals were studied. From peripheral lymphocytes genomic DNA was isolated by standard methods. For preparing high molecular weight DNA from different brain compartments, skeletal and heart muscle, liver and kidney, excised tissues were immediately placed in liquid nitrogen. About 100 mg brain material was digested with proteinase K in 1% Sarcosyl, 0.1 mM EDTA and incubated at 55°C for several hours. The DNA was extracted three times using phenol−chloroform (1:1) and recovered by ethanol precipitation. Approximately 100 ng of DNA was analysed by PCR with oligodeoxyribonucleotide primers (Hu3: 5'-GGCGGCTGAGGAAGCTG-AGGA-3' and Hu4: 5'-ATGGCGACCCTGGAAAAGCTGATGAA) specific for a part of the published IT 15 cDNA sequence. Amplification and polyacrylamide gel electrophoresis was performed as described (11). Alleles were identified by sizing relative to an M13 sequencing ladder.

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