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Expression of the mutant allele of IT-15 (the HD gene) in striatum and cortex of Huntington's disease patients

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Huntington's disease (HD) is an inherited neurodegenerative disorder expressed when a trinucleotide repeat in the gene IT-15 is expanded. The mechanism by which the expanded repeat causes the expression of the disease is unknown. Possible mechanisms include alterations in the amount of the mRNA, potentially resulting from changes in gene transcription or abnormal mRNA stability. In order to determine whether the expanded IT-15 allele is present in mRNA, we isolated total RNA from the cortex and striatum of patients and controls. To distinguish the two alleles of the IT-15 transcript in HD patients, we amplified across a region containing a dimorphic single triplet deletion observed on some chromosomes and found that the relative intensity of the two PCR bands amplified from genomic DNA and those amplified from first strand cDNA from brain tissue were essentially equal. In order to determine whether the exon containing the expanded CAG repeat is present in IT-15 mRNA from HD patients, we amplified across this region and demonstrated the presence of the expanded repeat in cDNA from both striatum and cortex. Based on this evidence, we suggest that the mechanism of disease expression does not occur during transcription or in the stability of the RNA, but rather occurs during translation or postranslationally.

INTRODUCTION

Huntington's disease (HD) is an inherited neurodegenerative disorder expressed when a trinucleotide repeat in the gene IT-15 is expanded (1,2). Normal alleles have 10–30 repeats of CAG in a long open reading frame, while expanded disease alleles have 36–100 repeats (3). Earlier age of onset is highly correlated with longer repeat expansions (4–8). The mechanism by which the expanded repeat causes the expression of the disease is unknown.

Possible mechanisms include alterations in the amount of the mRNA, including changes in gene transcription, or abnormal mRNA stability. In Fragile X syndrome, a disease associated with an expansion of a CCG repeat, the expanded allele of the causative gene (FMR1) cannot be detected in mRNA (9) and the protein is undetectable as well (10), perhaps because of hypermethylation near the transcription initiation site (11). Similarly, in myotonic dystrophy (12), associated with an expansion of a CTG repeat, mRNA expression of the expanded allele is probably reduced in adults leading to decreased expression of the protein (13–15), though not all studies are in agreement (16). Another possible mechanism involving changes in the mRNA is that the repeat expansion could cause aberrant splicing resulting in the selective exclusion of the exon containing the expanded repeat (17–19).

Alternative possible mechanisms involve changes in the amount or cellular function of the protein. In this case, the expanded IT-15 CAG repeat would be transcribed and spliced normally and be present in normal amounts in the mRNA. Ambrose *et al.* (20) have shown that the mutant allele is transcribed in lymphoblasts.

In order to determine whether the expanded IT-15 allele is present in RNA from brain, we isolated RNA from the striatum and cortex, two regions pathologically involved in HD, from patients and controls. We now demonstrate that the mRNA from mutant HD allele is present at similar levels as the normal allele and that the expanded repeat occurs in the mRNA.

RESULTS

In order to distinguish the two alleles of the IT-15 transcript in HD patients, we amplified across the region of the gene containing a three base pair (GAG) insertion/deletion polymorphism present in about 10% of the population (20). We selected two HD patients who were heterozygous for this polymorphism near amino acid 2642 (21). We compared the relative intensity of the two PCR bands amplified from genomic DNA with those amplified from first strand cDNA from brain

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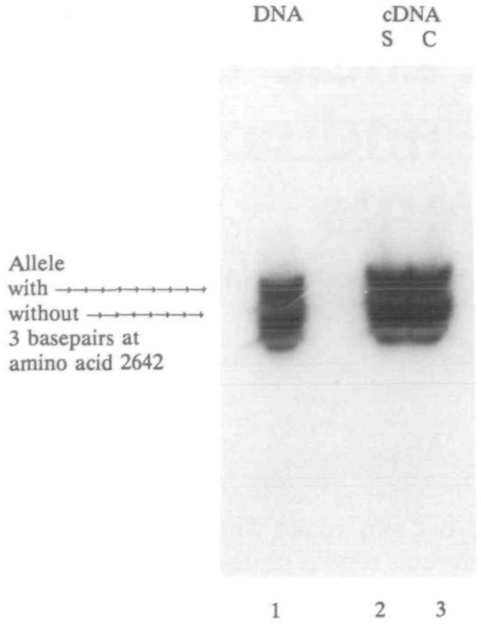

Figure 1. Autoradiogram of amplified DNA from the region containing a 3 bp insertion/triplet deletion glutamate 2642 (20) in the IT-15 gene, from an individual affected with HD. The DNA product in Lane 1 was amplified using a genomic DNA template. The DNA in Lanes 2 and 3 was amplified using first strand cDNA synthesized from RNA isolated from the striatum and cortex, respectively. The two bands correspond to the different alternative alleles in a heterozygous individual.

Table 1. The relative ratios of the alternative alleles with and without the 3 bp at amino acid 2642 of IT-15 in genomic DNA and cDNA from the striatum and cortex of two HD patients

Case	repeat length	age of onset	Relative ratios		
			genomic DNA	cDNA striatum	cDNA cortex
HD54	46	31	0 97	1.03	1.00
HD61	39	51	1.02	0.99	0.98

tissue. The two alleles of the IT-15 locus in HD patients amplify from genomic DNA equally (Fig. 1, Lane 1; Table 1). The two alleles are also amplified with essentially equal intensities from cDNA obtained from either striatum or cortex (Fig. 1, Lanes 2 and 3) in both patients (Table 1). The ratio of intensities of the two alleles was approximately 1:1 (range 0.93–1.07) during the log phase of the PCR (measured after 15 and 20 cycles) and at the end stage of the reaction (measured at 25 and 30 cycles). Samples of RNA treated with DNase-free RNase prior to reverse transcription and PCR, and RNA samples not treated with reverse transcriptase failed to yield any detectable amplified product after 30 cycles and prolonged exposure to X-ray film. Our results are consistent with those of Ambrose *et al.* (20).

In order to determine whether the exon near the 5′ end of the gene containing the expanded CAG repeat is present in IT-15 mRNA from HD patients, we used oligonucleotides on either side of the repeat. Primers were selected which amplified either cDNA or genomic DNA. Unlike primers which flank the single amino acid deletion, amplification of the expanded allele is less efficient even in genomic DNA where the ratio

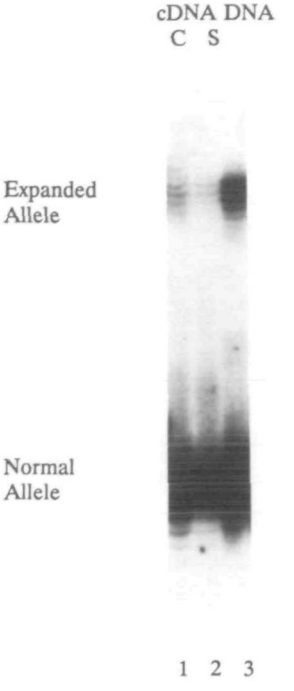

Figure 2. Autoradiogram of amplified DNA from the region containing the expanded triplet repeat in the IT-15 gene, from an individual affected with HD. The DNA in Lanes 1 and 2 was amplified using first strand cDNA synthesized from RNA isolated from the cortex and striatum, respectively. The DNA product in Lane 3 was amplified using a genomic DNA template.

is known to be 1:1 (Fig. 2, Lane 3). Furthermore, the amplification of first strand cDNA requires going through an additional step using reverse transcriptase to traverse the long CAG repeat. This additional step may well result in there being less cDNA including the entire expanded repeat than there is mRNA (16,22). Nevertheless, PCR across this region clearly demonstrated the presence of the expanded repeat in cDNA from both striatum and cortex (Fig. 2, Lanes 1 and 2) in both patients examined. Samples of RNA treated with DNase-free RNase prior to reverse transcription and PCR, and RNA samples not treated with reverse transcriptase failed to yield any detectable amplified product after 40 cycles and prolonged exposure to X-ray film.

DISCUSSION

In this study, we have used PCR techniques to distinguish the allele with the expanded CAG repeat of IT-15 from the normal allele in patients with HD. In the first experiment, we chose patients who were heterozygous for the insertion/deletion of the single GAG triplet at amino acid 2642. We have not identified which of the two alleles is the normal and which is abnormal for each of these patients, but we are able to demonstrate that the mutated allele is present at a level comparable to the normal allele in total RNA from the cortex and striatum of HD patients. While PCR is an inherently non-quantitative technique, in this situation one can be relatively confident that the PCR should proceed similarly for the two alleles since they use identical primers and have nearly the same length product. The equivalence of the two competing

reactions is confirmed by our observation that the two bands are of equal intensity in normal controls who are also heterozygous for this glutamate deletion (data not shown). Our results are consistent with those of Ambrose *et al.* (20) and extend them to brain tissues.

The next issue we addressed was whether the expanded CAG repeat is present in mRNA transcribed from the DNA strand with the expansion. Because of the difficulty of polymerization across the long CG rich repeat in both the reverse transcriptase and polymerase chain reactions, it is very difficult to make quantitative conclusions regarding this issue (12,16,23). As seen in Figure 2, the PCR across the expanded repeat yields considerably less product than PCR across the normal repeat even using genomic DNAs as the template. It is likely that reverse transcriptase is at least as vulnerable as *Taq* polymerase since reverse transcriptase acts at a lower temperature, at which secondary structure might be more persistent. While we cannot rule out the possibility that some of the mRNA molecules are spliced so as to delete this exon, it is clear that at least an appreciable quantity of message with the expanded allele is indeed present.

We and others have previously shown that the IT-15 message is expressed widely in both peripheral and central nervous systems as well as in most if not all tissues throughout the body of normal rats and humans (24,25). Within the brain, it is expressed at a slightly lower level in the basal ganglia compared to other regions. The overall level of the IT-15 message is not substantially altered in basal ganglia of HD patients compared to controls (24). The data in the present study indicate that the mutated allele is present in similar quantities compared to the normal allele and strongly suggest that the expanded CAG repeat is indeed present in the IT-15 mRNA in HD patients. Additionally, studies using antibodies to the IT-15 gene product have demonstrated similar amount of the protein from tissue from both patients and controls (26,27). It is conceivable that the expanded triplet repeat prevents translation of the protein, although it is not clear how this would lead to dominant inheritance with complete penetrance (28). Our data are consistent with the possibility that the expanded CAG repeat is translated into an abnormally long glutamine repeat which may have a dominant effect at the protein level, either as a dominant negative, or gain of function mutation. One recent hypothesis suggests the possibility of formation by a transglutaminase enzyme activity of peptidase resistant and thus toxic epsilon (γ-glutamyl) lysine isodipeptides (29). Alternatively it is possible that, as has been proposed for the androgen receptor in spinal and bulbar muscular atrophy (30), there is an abnormality in interactions between the IT-15 protein and other cellular proteins (31).

MATERIALS AND METHODS

Samples of cortex and striatum were obtained from the brains of two HD patients. Tissue was stored at −80°C after dissection within 16 h of death. Genomic DNA was extracted by standard SDS−phenol−chloroform procedures (32). Total RNA was isolated by centrifugation in cesium chloride (33). The isolated RNA was converted to cDNA by RNase H^- reverse transcriptase (BRL) using oligo-dT as a primer according to the manufacturer's instructions. The cDNA was amplified with two sets of primers: one pair flanking the expanding CAG repeat (1) and one flanking a single amino acid insertion/deletion at glutamate 2642 (20). In both cases, the PCR products do not cross a boundary between an intron and an exon. In each pair, one primer was end-labelled with ^{32}P using T4 kinase in buffer provided by the supplier (New England Biolabs).

PCR for the primers flanking the CAG repeat, HD-1 (5′-AAA CTC ACG GTC GGT GCA GCG GCT CCT CAG-3′) and HD-2 (5′-ATG AAG GCC TTC GAG TCC CTC AAG TCC TTC-3′), was carried out in 10 µl reactions containing 200–1000 ng of genomic DNA, 10% DMSO, 200 µM dATP, TTP and dCTP, 100 µM dGTP and 100 µM deaza-dGTP, 1 µM of each primer, 0.05 µM radioactively labelled primer, 1×*Taq* polymerase buffer (10 mM Tris pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 5 µM EDTA, 0.01% gelatin) and 3 U *Taq* polymerase (Boehringer Mannheim). This reaction produced a product 250–390 bp long. The template DNA was denatured at 99°C for 1 min, then quick chilled on dry ice before the rest of the components were added. Using a Techne thermal cycler, 40 cycles of 95°C, 20 s; 54°C, 20 s; 72°C, 40 s were performed. The set of primers flanking the amino acid deletion (GCT GGG GAA CAG CAT CAC ACC) and (CCT GGA GTT GAC TGG AGA CGT) were used to initiate 30 µl reactions using *Taq* polymerase (Boehringer Mannheim) in buffers as provided by supplier. After 5 min denaturation, 30 cycles of 95°C, 60°C, 72°C for 30 s each were performed. This reaction produced a product 111 or 114 bp long. In one experiment, the reaction was stopped for less than 2 min after 15, 20, and 25 cycles while a 5 µl aliquot was withdrawn.

The amplified DNA fragments were separated on denaturing polyacrylamide gels under standard conditions (32). The gels were dried and exposed to X-ray film. The length was determined by comparison of the PCR products to radioactively-end-labeled fragments of ϕX174 digested with *Hae*III. Band intensities were quantified with an Amersham RAS1000 Image Analysis System.

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