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Review

Triplet repeat disorders: discussion of molecular mechanisms

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Abstract. Comparison of the growing number of disorders known to be associated with triplet repeat expansions reveals both common features and a diversity of molecular pathways. Despite significant progress towards the characterization of proteins coded by the mutant genes, the complex nature of these disorders requires identification of all molecular components of the triplet repeat pathways. In this brief review we will discuss recent progress in determining the molecular mechanisms of disorders with unstable trinucleotide mutations.

Key words. Fragile X syndrome; myotonic dystrophy; Friedreich's ataxia; polyglutamine disorders; molecular pathogenesis.

Introduction

The fast-growing group of triplet repeat disorders includes several neurological and neuromuscular diseases associated with a greatly increased number of repeats of a specific trinucleotide sequence. Trinucleotide repeats are normally present in the genome and can affect the expression of certain genes when they are located within or close to those genes. Under certain circumstances, the number of triplet repeat units increases beyond a certain level, at which point the triplet repeat expansion becomes a mutation that induces disease. How triplet repeat instability causes amplification of repetitive units is currently unknown and a subject of intensive investigation (reviewed in [1]).

The triplet repeat disorders known at this time share some common features and differ in other respects. All triplet repeat expansions associated with known disorders are characterized by mitotic and meiotic instability (reviewed in [2, 3]). The number of repeats is always higher in an affected population versus unaffected. Genetic anticipation is seen in all disorders, with the probable exception of spinocerebellar ataxia type 6 (SCA6), which has not been well studied. Based on the similarities and specific features of the mutations, the triplet repeat disorders can be divided into two subgroups, namely type I and type II (reviewed in [2, 3]). The type I group includes diseases associated with neuronal loss in the brain, brainstem and spinal cord. The members of this group are Huntington disease (HD), spinal and bulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA), Machado-Joseph disease/spinocerebellar ataxia type 3 (MJD/SCA3), and the spinocerebellar ataxias types 1, 2 and 6 (table 1). All members of this subgroup carry CAG triplet repeat expansions in the coding regions of their various genes, resulting in the synthesis of polyglutamine tracks in mutant proteins (reviewed in [4]). Although all these diseases are associated with damage of different regions of the brain, they have shared features that suggest a common mechanism for their pathways. All the type I

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disorders are autosomal dominant, except SBMA, which is X-linked. Although proteins coded from mutant genes are widely expressed, each disease is characterized by a specific pattern of neuron loss. There is a correlation between size of repeat and severity of phenotype: with longer repeats the disease has an earlier onset and greater severity.

The type II group includes fragile X syndrome (FraX), Friedreich's ataxia (FRDA) and myotonic dystrophy (DM). Diseases in this subgroup are characterized by a complexity of symptoms involving many tissues. DM is associated with skeletal muscle weakness, myopathy and myotonia [5]. In addition, patients with the DM mutation have abnormalities in heart, brain, testes, skin and eyes [5]. FraX is primarily a brain disorder, but it also associated with testicular abnormalities and involves specific facial features (reviewed in [6]). FRDA is a progressive neurodegenerative disorder involving both the central and peripheral nervous systems [7]. It is an autosomal recessive disorder characterized by progressive ataxia, hypertrophic cardiomyopathy, optic atrophy and diabetes [7].

There are different sequences of triplet repeats involved in these disorders (table 1). For instance, FraX is associated with CGG triplet repeats [8–10] and DM with CTG repeats [11–16]. Until recently it was thought that unstable triplet repeats could be associated only with GC-rich structures, since the majority of known triplet repeat disorders was associated with CAG, CTG or CGG repeats. However, discovery of the GAA repeat in FRDA [17] showed that this suggestion is incorrect. Apparently any repeated trinucleotide sequence can become unstable under certain conditions.

Disorders in the type II group are each characterized by a specific location of the triplet repeat (table 1). The expanding triplet repeats are located in the 5′ untranslated region (UTR) of the FMR1 gene in FraX [8–10], in the 3′ UTR of DMPK in DM [11–16] and in the first intron of the frataxin gene in FRDA [17]. These observations show that triplet repeats might be located in any position in relationship to the gene. The sequence and location of the repeat are probably responsible for the unique mechanism of pathogenesis.

A significant feature distinguishing the two types of disorders is the threshold of the length of expansion (table 1). In type I disorders the length of repeat in affected individuals mostly ranges from 40 to 70, whereas in disorders of type II the size of the repeat ranges from 50 up to several hundred and even thousand of repeats. In SCA6, the size of expanded alleles is within the normal size range of alleles in other CAG repeat diseases (table 1). Within the type II group, FRDA differs from FraX and DM in this way: in FraX and DM large expansions occur from unstable alleles with intermediate sizes, but in FRDA large expansions are transmitted by asymptomatic carriers.

Molecular analysis of triplet repeat expansions suggests that the mechanism of each mutation is directed by the sequence of the repeat and its location in relationship to a gene. Below we will discuss experimental data related to the molecular pathways mediated by triplet repeat expansions.

FraX: disruption of RNA processing

FraX is a dominant X-linked disorder with a frequency of 1:4000 in males and 1:6000 in females. The most significant features of patients with FraX are delayed development, mental retardation and macroorchidism (reviewed in [6]). The FraX syndrome is caused by the

Table 1. General molecular features of triplet repeat disorders showing the sequence of the triplet repeat, its position in the gene and a possible mechanism of disease.

Disease	Repeat	Location of repeats	Possible mechanism of mutation	Result of mutation
SBMA	$(CAG)_{40-62}$	ORF	gain of function	translation of mutant proteins, containing extra poly(Gln) tracks, leading to cell death
HD	$(CAG)_{36-121}$	ORF	gain of function	
DRPLA	$(CAG)_{49-88}$	ORF	gain of function	
MJD/SCA3	$(CAG)_{68-79}$	ORF	gain of function	
SCA1	$(CAG)_{41-81}$	ORF	gain of function	
SCA2	$(CAG)_{35-59}$	ORF	gain of function	
SCA6	$(CAG)_{21-27}$	ORF	gain of function	
FraX	$(CGG)_{60\rightarrow 2000}$	5′ UTR	loss of function	absence of FMRP1; disruption of RNA metabolism (?)
DM	$(CTG)_{50\rightarrow 2000}$	3′ UTR	gain of function at DNA or RNA level	1. Disruption of RNA processing. 2. silencing of transcription in DMPK region
FRDA	$(GAA)_{200\rightarrow 900}$	intron	loss of function	reduction of frataxin in mitochondria leading to altered iron metabolism

Repeat length for FraX and DM includes premutation.

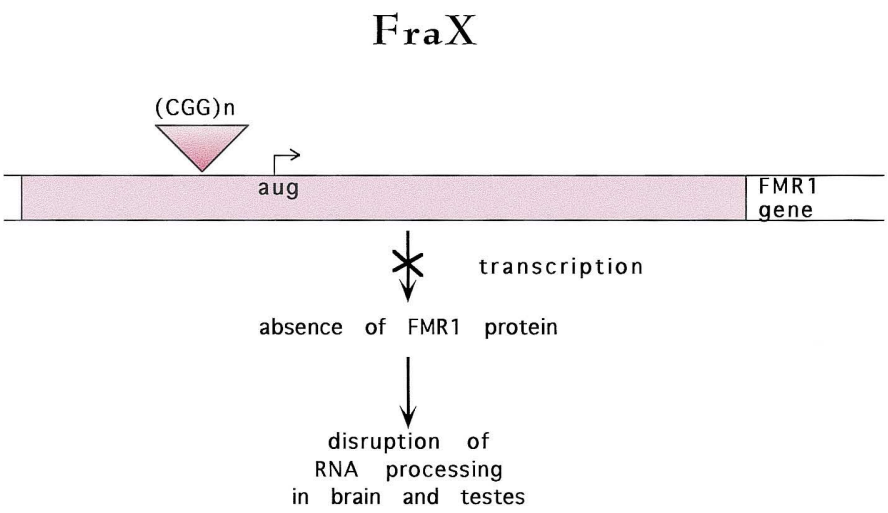

Figure 1. Model for fragile X syndrome. CGG expansion in the FraX gene results in hypermethylation of the FraX promoter and GC island, leading to silencing of the FraX gene. The protein product of the FraX gene, FMRP, is an RNA-binding protein. The absence of this protein might result in disruption of RNA metabolism in the brain and testes.

expansion of CGG repeats in the 5′ UTR of the *FMR1* gene [8–10]. The normal allele of the *FMR1* gene contains 6–54 repeats [8–10], and the premutation allele carries 43–200 repeats. Normal alleles are stable in transmission, but premutation alleles when transmitted by females increase in size by more than 200 repeats. An increased number of CGG repeats results in alteration of the methylation status of the repeat and the *FMR1* promoter [18], which leads to the silencing of transcription of the *FMR1* gene [19]. Thus, the molecular basis for FraX is loss of FMRP protein induced by expanded CGG repeats and abnormal methylation of a GC island in the 5′ region of *FMR1* gene [18, 19] (fig. 1). The significance of the absence of FMRP protein in disease development was confirmed by analysis of *Fmr1* knockout mice [20]. Mice lacking the *Fmr1* gene have reduced learning abilities, difficulty in handling changes in spatial information and enlarged testes similar to FraX patients [20].

After absence of the FMRP protein in patients with FraX was proven, the next important question was, how does the absence of this protein induce the clinical phenotype? The answer to this question requires understanding of the physiological function of FMRP in normal cells and in the cells of FraX patients. Functions of many novel proteins can be predicted based on their homology to other known proteins. Although analysis of FMRP sequence did not show significant homology to other proteins, it did reveal the presence of conserved motifs (two KH domains and an RGG box) that usually occur in proteins interacting with RNA and involved in RNA processing [21]. Identification of RNA binding motifs in the FMRP structure suggested that FMRP is probably a novel RNA-binding protein. Characterization of this protein required solution of several problems: (i) What is the binding specificity of FMRP—can it bind to any RNA or only to specific subclasses of RNA? (ii) What are the native RNA targets for FMRP in vivo? and (iii) What is a function of this protein in vivo?

Studies of the RNA-binding specificity of FMRP showed that it binds to poly(G) and poly(U) in vitro [21]. A point mutation in the second KH domain affected FMRP binding specificity to poly(U) under high-salt conditions, but not under low-salt conditions [22]. Since a primary defect in FraX is associated with brain dysfunction, it is reasonable to suggest that FMRP is required for regulation of RNA processing in brain. In agreement with this suggestion, FMRP binds to 4% of brain messenger RNAs (mRNAs), including its own mRNA [23]. It remains to be determined whether FMRP binds with specific RNAs in vivo. Based on FMRP intracellular localization, it has been suggested that FMRP is a cytoplasmic protein [24]. However, experiments with FMRP transfections indicated that it is also located in nuclei [24] and the nucleolus [6]. It was therefore suggested that FMRP shuttles between the cytoplasm and the nucleus. Detection of FMRP in both cytoplasm and nuclei raises the question of what the physiological role of FMRP in both compartments is. Analysis of FMRP localization in cytoplasm indicated that FMRP is associated with ribosomes, suggesting

possible involvement of this protein in translation [25]. Sequence analysis of FMRP revealed a nuclear localization signal (NLS) and a nuclear export signal (NES) [26]. The NLS element of FMRP is functionally similar to a signal identified in REV, a regulatory protein of human immunodeficiency virus type I that mediates the export of viral RNA from the nucleus to cytoplasm. It has been proposed that FMRP is predominantly a cytoplasmic protein that migrates from cytoplasm to nuclei because of the NLS element, and binds in nuclei to specific RNAs and proteins forming ribonuclear proteins. Because of the presence of the NES signal, FMRP could leave the nucleus and migrate to the cytoplasm where it binds to ribosomes. A significant amount of FMRP was found in the nucleolus [6], suggesting that FMRP might participate in the transport of ribosomal RNAs.

The most important question regarding FraX molecular pathology is identification of the precise function of FMRP in brain. If FMRP participates in the translation and transport of a broad spectrum of RNAs, then deletion of this protein would result in lethality. However, a complete absence of FMRP is not lethal [20], suggesting that FMRP probably has homologs that could compensate for its absence. Such a compensatory function could theoretically be performed by two human homologs of FMRP, FXR1P and FXR2P [27], both of which can interact with FMRP. FXR1P and FXR2P show high levels of homology to FMRP (60%) and have similar structures; the presence of both NLS and NES signals indicates the possibility of shuttling between cytoplasm and nucleus. Identification of other homologs of FMRP and precise investigation of the role of FMRP and its homologs in RNA processing will help us to understand why the absence of this protein induces mental retardation in FraX patients.

Two distinct mechanisms for DM mediated by RNA-binding proteins and a homeodomain protein

Reduction of DMPK is not sufficient for disease induction

DM is associated with CTG triplet repeat expansion in the 3′ UTR of the *DMPK* gene [11–16]. This location of CTG triplet repeats suggests that they could not directly affect structure of a protein translated from the mutant gene. Theoretically, CTG repeats could affect DMPK expression indirectly by affecting DMPK transcription, RNA processing or DMPK translation. Numerous studies on DMPK expression in DM yielded interesting but sometimes contradictory data. Although in a majority of patients DMPK was found to be reduced [28, and reviewed in 29], some cases of DMPK activation have also been described [30]. Studies on the mechanism of regulation of DMPK expression also revealed confusing data. It was shown that CTG repeats affect chromatin structure [31], but it seems that the repeat does not affect DMPK transcription, because mutant transcripts were detected by reverse transcription-polymerase chain reaction and Northern analysis [30]. Some studies demonstrated that a DNA region containing long CTG repeats could be hypermethylated [32], affecting transcription in the region of CTG repeats, but others found no effect of CTG repeats on DNA methylation [33]. Several reports described abnormalities in DMPK RNA processing. First it was demonstrated that DM patients show some abnormalities with DMPK polyadenylation because poly(A) DMPK levels were reduced more significantly compared with total RNA [34, 35]. In addition, abnormal accumulation of DMPK transcripts was found in nuclei of DM patients [36, 37]. One study described foci of DMPK transcripts in nuclei of DM cells, although transport of DMPK mRNA was apparently not hindered, because it was detected in cytoplasm [38]. It has been suggested that long CUG repeats may result in accumulation of DMPK RNA in nuclei, resulting in reduction of DMPK protein. However, generation of *DMPK* knockout mice showed that although DMPK is important for muscle function, even complete absence of DMPK is not sufficient to induce myotonia and other DM symptoms [39, 40]. These data showed that although CTG triplet repeats remain a major element in DM disease, alteration of DMPK expression is only a part of DM pathogenesis.

DM latest mouse models

The absence of the complete DM phenotype in mice lacking DMPK [39, 40] suggests a specific function for CTG repeats. Understanding of the role of CTG repeats in DM pathogenesis requires synthesis of long stable CTG triplet repeats, which has only recently become technically possible [41]. The use of long CTG repeats in mouse models and experimental systems with cultured cells will come in time. Two transgenic mouse models have been generated so far, with transgenes containing 55 and 162 CTG repeats [42, 43]. Although in both of these cases CTG triplet repeats showed mild instability, there is no overt disease phenotype. One important finding is that the presence of a portion of the 3′ UTR of DMPK interrupted with 162 CTG repeats was not sufficient for disease induction, at least in mice [42]. There are several possible explanations for the absence of phenotype in these mice: (i) transcription of CTG repeats into CUG repeats in RNA might be necessary; (ii) CTG repeats probably require other sequences from *DMPK* gene, therefore a portion of the 3′ UTR is not sufficient to induce the disease; and (iii) the length of the triplet repeat in mice should probably

be longer. Another transgenic model was generated to carry a long portion of genomic DNA containing the *DMPK* gene with 55 CTG repeats, flanked by the *DMR-N9* and *DMAHP* genes, which are normally neighbors of *DMPK* in the human genome [43]. Again, the CTG repeats in these mice showed mild instability but did not induce the disease phenotype. Although these mice have triplet repeats in the true *DMPK* environment that are expressed into CUG repeats, the length of repeat was close to the normal size. Several lines of evidence suggest that CTG repeats should be expressed into RNA CUG repeats to induce the phenotype. If this is the case, long CTG repeats placed under an appropriate promoter should result in the DM phenotype. On the other hand, it is very likely that CTG repeats must be placed into the *DMPK* gene to induce the phenotype. Thus, knock-in models where a *DMPK* gene containing long CTG repeats is overexpressed in *Dmpk* knockout mice could be a close model for DM disease.

Role of DMAHP in DM pathogenesis

Since it was demonstrated that the absence of DMPK is not sufficient for disease, it was suggested that CTG repeats could affect other genes. Currently two hypotheses have been proposed to explain the mechanism for DM. One hypothesis is based on the effect of CTG repeats on the transcription of genes adjacent to *DMPK*. Sequence analysis of the DMPK region revealed a novel homeodomain gene (*DMAHP*) located downstream of *DMPK* [44]. CTG repeats in the 3′ UTR of the *DMPK* gene are located only ~ 1 kb upstream of the 5′ end of the *DMAHP* gene [44]. The close proximity of CTG repeats to the regulatory regions of *DMAHP* could affect transcription of this gene by a scenario similar to that seen in FraX (reviewed in [45]). If the mechanism of DM mutation is similar to FraX, then hypermethylation of the GC island in the *DMPK* gene should repress DMAHP transcription. So far, data related to the methylation status of the *DMPK* gene in DM patients and data on expression of DMAHP are conflicting and require verification [36, 46, 47]. Some reports describe reduction of DMAHP in DM disease [46, 47], but others provide evidence that DMAHP is not affected in DM disease [36]. This situation is very similar to the initial studies on DMPK expression. It is probable that DMAHP, like DMPK, is affected in DM patients but that alteration of its expression is not significant. Knockout mice for the *Dmahp* gene will obviously indicate whether reduction of DMAHP is crucial for DM pathogenesis. It is possible that DMAHP is responsible for some symptoms in DM patients. To understand the role of DMAHP in DM disease, its function in normal cells must first be determined. Currently, DMAHP should be placed together with DMPK, insulin receptor, CUGBP1, cardiac troponin T (cTnT) and probably others in the growing group of genes possibly affected by CTG/CUG repeats in DM patients.

RNA mechanism for DM—the role of CUG triplet repeats

Another hypothesis suggests that the DM mutation could be manifested at the RNA level. Hoffman proposed that mutant DMPK mRNA could affect RNA metabolism in trans [35]. This group found not only reduction of DMPK mRNA in DM patients, but demonstrated that RNA for insulin receptor was also reduced [35, 48]. We further suggested that the negative effect of mutant DMPK mRNA could be mediated by specific RNA-binding proteins [49, 50] (fig. 2). It has been hypothesized that these proteins are involved in the processing of RNAs important for cell development in skeletal muscle, heart, brain and testes. In DM patients, expansion of CUG repeats results in the accumulation of RNA binding sites for specific CUG-binding proteins. Overexpression of RNA binding sites could result in sequestration of these proteins with a resulting effect on RNA metabolism (fig. 2). In agreement with this hypothesis, a protein, CUGBP1, specifically bound to RNA CUG sequences, was identified [49, 51]. Although it was hypothesized that CUGBP1 expression should be reduced due to sequestration by CUG repeats, RNA-binding activity of this protein was in fact activated in DM cells [49, 52]. Activation of its activity and expression was consistent with accumulation of CUG triplet repeats [53]. The mechanism affecting CUGBP1 expression is unknown; however, it is clear that its expression is dependent on the number of CUG repeats. Activation of CUGBP1 in DM disease results in alteration of RNA processing of CUGBP1-dependent RNAs. For instance, it has been demonstrated that CUGBP1 binds via CUG repeats to mRNA encoding cTnT and regulates its splicing [54]. cTnT splicing was found altered in DM hearts [54], consistent with alteration of CUGBP1 [49, 52, 53]. In addition to splicing, CUGBP1 is involved in translation of a transcription factor, C/EBPβ, that plays an important role in cell proliferation and differentiation [53]. C/EBPβ mRNA directs synthesis of three isoforms, full-length and truncated: LAP and LIP [55]. The CUGBP1 binding site is located close to the first initiation codon. We found that activation of CUGBP1 in DM patients results in alteration of the ratio between the full-length and truncated isoforms of C/EBPβ. CUGBP1 also binds to DMPK mRNA [49] and tropomyosin α [unpublished data]. The RNA metabolism of these RNAs could be also affected in DM patients. Altered RNA metabolism of specific

RNAs in different tissues may induce symptoms seen in DM patients. For instance, abnormal splicing of cTnT in DM hearts might be associated with development of cardiac abnormalities. It has been demonstrated that point mutations in cTnT induce hypertrophic cardiomyopathy [56]; therefore, it is reasonable to suggest that alteration of cTnT structure due to abnormalities in splicing might potentially induce cardiac disease. It is also possible that CUGBP1 regulates not only cTnT but also other cardiac RNAs, such as tropomyosin α. Another possibility is that other RNA-binding proteins homologous to CUGBP1 are involved in the regulation of RNA metabolism in heart. We recently identified a second member of a family of CUG-binding proteins, ETR-3, that is abundant in human heart [57]. This protein exhibits binding activity similar to CUGBP1 [57]. We therefore suggest that these two proteins might regulate different RNAs in different tissues: ETR-3 could function in the heart and CUGBP1 in skeletal muscle or brain. Other CUG-binding proteins also could exist. Two brain proteins with RNA CUG-binding activity were identified as candidates for regulation of RNA processing in brain of DM patients [58]. Proteins with CUG-binding activity might be involved in the development of skeletal muscle myotonia and myopathy. One study demonstrated inhibition of differentiation after overexpression of the 3′ UTR of DMPK [59]. This effect could be corrected by deletion of the DMPK 3′ UTR [60]. Other studies implicated DMPK in muscle differentiation, showing that overexpression of DMPK leads to the alteration of expression of some RNAs required for muscle function [61]. A disease mechanism involving RNA does not contradict the abnormalities of DMPK expression seen in DM disease. CUGBP1 or other CUGBP1-like proteins could regulate expression of DMPK, resulting in alteration of DMPK expression in DM patients. Moreover, specific binding of CUGBP1 to the 3′ UTR of DMPK has been

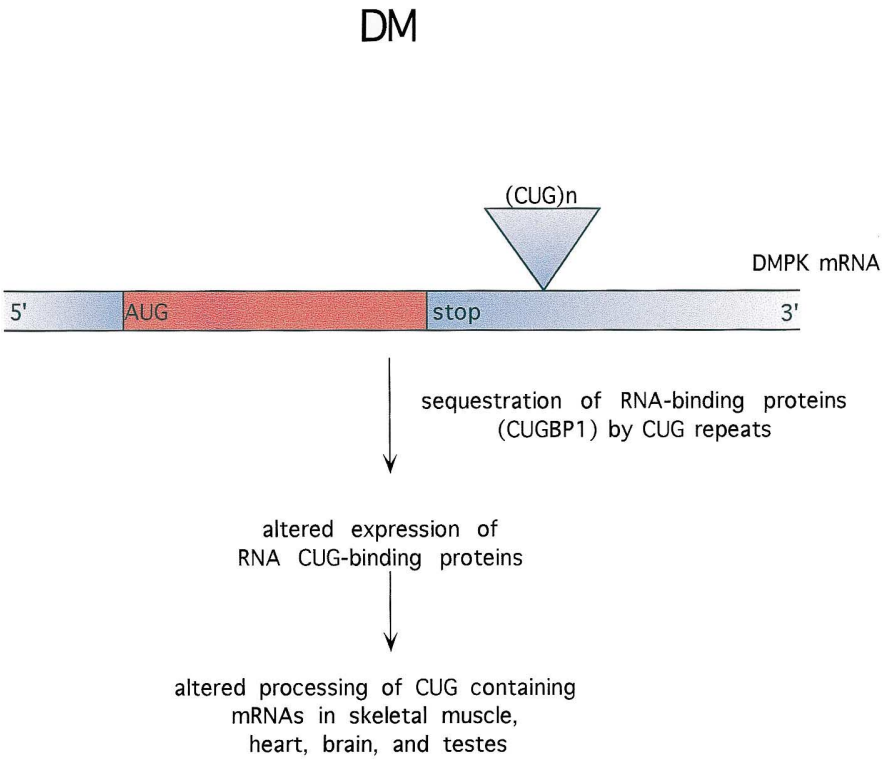

Figure 2. An RNA model for DM. CTG triplet repeat expansion in the DMPK gene is transcribed into CUG repeats in DMPK mRNA. CUG triplet repeats are potential binding sites for specific RNA-binding proteins. An increase in the number of RNA-binding sites in DM patients will affect specific RNA-binding proteins, such as CUGBP1. Alteration of the expression of RNA-binding proteins affects the RNA metabolism of several mRNAs and pre-mRNAs containing CUG repeats in the regulatory regions. In agreement with this suggestion, processing of some mRNAs containing CUG repeats in the regulatory regions such as DMPK, cTnT and C/EBPβ are affected in DM patients. The RNA metabolism of other mRNAs in skeletal muscle, brain, heart and testes could also be altered. Alteration of RNA metabolism in affected tissues might induce the development of clinical symptoms (myotonia, cardiac abnormalities, mental retardation and others).

demonstrated [49]. We found that splicing of DMPK was affected near the CUGBP1 binding site [53]. Alterations of DMPK expression, including reduction or induction of DMPK protein, alterations of splicing or accumulation in nuclei of unprocessed DMPK RNA, should affect the pathway involving DMPK.

DMPK and its substrates

A role for DMPK has been implicated in muscle function because its deletion results in development of muscle weakness and myopathy [39, 40]. In addition, overexpression of DMPK in cultured cells leads to alteration of expression of muscle-specific mRNAs that are required for muscle differentiation [61]. These results provide a background for the studies of DMPK protein and specifically to the identification of all components in the DMPK-mediated pathway. Since DMPK exhibits kinase activity, it was suggested that any perturbations in its expression or its activity could potentially affect other molecules involved in the DMPK-mediated pathway. There are two general approaches in the DMPK studies: (i) identification of native substrates for DMPK; and (ii) analysis of DMPK function by comparison with other known kinases. The search for potential substrates for DMPK was initially based on the knowledge that DM is associated with defects in muscle relaxation-contraction [5]; therefore, ion channels could potentially be the best native substrates for DMPK. In agreement with this suggestion, two ion channels, voltage-dependent calcium release channel and sodium channel, were proposed as substrates for DMPK. It has been demonstrated that DMPK is able to phosphorylate in vitro the β subunit of the voltage-dependent Ca^{2+} release channel [62]. Consistent with this observation, knockout mice for DMPK showed abnormalities of Ca^{2+} homeostasis [63]. Another possible substrate for DMPK is a voltage-gated sodium channel [64]. Cotransfection of DMPK and this channel resulted in the alteration of current regulated by sodium channels [64]. Although these data demonstrated possible involvement of ion channels in the DMPK pathway, analysis of protein-protein interaction by a two-hybrid system identified other proteins interacting with DMPK. One of them is a novel protein, DMAP, which shows homology to D1 snRNP [65]. The function of this protein is unknown, although its tissue distribution shows that it is abundant in skeletal muscle and heart, similar to DMPK. D1 snRNP is a small RNA-binding protein that is involved, together with other proteins, in the formation of spliceosomes [66]. Because of the high homology between D1 snRNP and DMAP, the function of DMAP is predicted to be in splicing or other stages of RNA metabolism. We found that DMPK interacts in vitro with CUGBP1, suggesting possible involvement of DMPK in CUGBP1 regulation via phosphorylation [52]. A splicing function for CUGBP1 has been demonstrated [54]. Identification of two RNA-binding proteins, CUBPB1 and DMAP, specifically interacting with DMPK, suggests the role of DMPK in the regulation of specific RNA-binding proteins.

It has been also suggested that DMPK is involved in the stress-responsive system, because it interacts with a novel protein showing homology to small heat shock protein, MKBP [67]. mRNA coding this protein is highly expressed in skeletal muscle and heart. It was shown that MKBP is localized to the neuromuscular junction where DMPK was predominantly localized [68]. In addition, MKBP expression was increased in DM muscle. This protein differs from other heat shock proteins because it is not activated by heat. DMPK phosphorylates MKBP insignificantly, but MKBP rather activates kinase activity of DMPK and protects it from heat-induced inactivation. It was hypothesized that MKBP could act as a molecular chaperone specific for DMPK that stabilizes and protects its kinase activity. Therefore, activation of MKBP could be part of a feedback mechanism trying to compensate for the reduction of DMPK [67].

DMPK function could be studied based on homology to other kinases. Recently, several Rho-binding kinases homologous to DMPK were identified. These kinases have homology to DMPK not only in the position of the kinase domain but throughout the molecule [69]. It has been suggested that signals from the Rho family of small guanosine triphosphate (GTP)-binding proteins lead to inhibition of myosin phosphatase activity, altering the level of myosin regulatory light chain phosphorylation, which is important for muscle contraction [70]. In agreement with this suggestion, it was shown that Rho-binding Ser-Thr kinase can phosphorylate and inhibit the activity of a myosin phosphatase in vitro [71]. This model was supported by identification of DMPK-homologous proteins from *Caenorhabditis elegans* that are important for skeletal muscle organization [72]. These data suggest that DMPK could phosphorylate myosin phosphatase in vivo [72]. Current data suggest that DMPK might have several substrates in vivo. Ion channels, as well as proteins important for muscle function, are considered the best native substrates for DMPK. Identification of RNA-binding proteins interacting with DMPK in vivo suggests another pathway for DMPK in RNA metabolism. Obviously, all molecules interacting with DMPK as well as other components of DMPK pathway must be isolated and studied. Functional study of DMPK protein will allow us to determine the physiological role of this kinase and its activators and substrates.

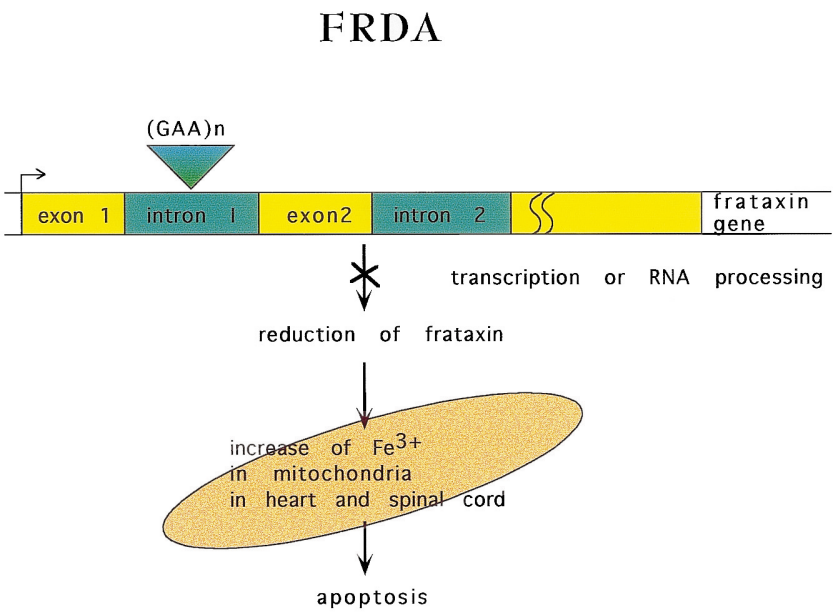

Figure 3. Friedreich's ataxia is a disease of iron transport. GAA expansion in the first intron of the frataxin gene leads to the reduction of protein expression. Frataxin is a mitochondrial protein that is associated with transport of iron. Reduction of frataxin induces the accumulation of iron in mitochondria and probably disrupts mitochondrial function.

Since DMPK represents only a part of the DM mechanism, future studies of DM pathogenesis should be focused not only on DMPK but also on understanding the role of CTG and CUG repeats. Obviously, functional studies of CUG-binding proteins, as well as proteins interacting with the 3′ UTR of DMPK, will give us an information about the role of the 3′ UTR and the biological significance of CTG repeats in the 3′ UTR. So far, several potential RNA targets for CUG-binding proteins have been isolated, including DMPK, cTnT and C/EBPβ. It is reasonable to suggest that CUG-binding proteins could regulate other RNAs in different tissues, including skeletal muscle, brain and testes. Identification of CUGBP1-dependent RNAs will explain the role of CUGBP1 in DM pathology. In addition, other RNA-binding proteins, interacting with the 3′ UTR around the CUG repeat, could be involved in the disease. It is also important to determine the mechanisms of regulating expression of insulin receptor and DMAHP. If it is proven that both insulin receptor and DMAHP are affected in DM patients due to disruption of RNA metabolism, then DM could be considered an RNA disorder. However, if DMAHP is regulated at the DNA level via disruption of transcription, then DM could be in part due to an RNA mechanism and in part due to a DNA mechanism. In this case, the contribution of each mechanism must be evaluated.

FRDA: a disease of iron transport

The recessive inheritance of FRDA suggested that a genetic mutation in this disease would lead to the loss of function of the mutant protein. It has been demonstrated that the majority of FRDA patients have expanded GAA repeats in the first intron of a novel gene encoding frataxin [17]. A few compound heterozygous patients have one allele of the mutant gene with GAA expansion and another allele with point mutations [17]. The identification of patients with point mutations in frataxin confirms the role of this protein in the pathogenesis of FRDA and argues in support of the loss-of-function mechanism. Some studies of the genomic region affected in patients with FRDA showed that the gene encoding frataxin is located in close proximity to another gene encoding STM7 [73]. Analysis of some splicing variants for STM7 showed that frataxin and STM7 could be synthesized from a single gene [73]. If these data are confirmed, then a mutation causing FRDA could occur in the *STM7* gene. STM7 protein has high homology to human placental phosphatidylinositol-4-phosphate 5-kinase type II (PtdInsP 5-kinase), which regulates the synthesis of phosphatidylinositol-4,5-biphosphate, a critical component in signaling pathways involved in cell proliferation, differentiation, motility and other processes [73].

Expression studies of frataxin indicated that frataxin mRNA was almost undetectable in patients with

FRDA [17, 74] (fig. 3). The mechanism of reduction of frataxin is unknown, but it is possible that GAA expansion in the first intron interferes with transcription or RNA processing of frataxin.

Although frataxin was identified as a novel gene with unknown function, its homologs were identified in *C. elegans*, *Saccharomyces cerevisiae* [17, 75, 76], mouse [75] and rat. It has been demonstrated that frataxin mRNA is expressed at high level in tissues with a high metabolic rate such as liver, kidney, brown fat and heart [75]. In addition, it was found that mouse and yeast frataxin contain a potential mitochondrial sequence [75]. The yeast homolog of frataxin (YFH1) encodes a mitochondrial protein involved in iron homeostasis and respiratory function [76, 77]. Moreover, mitochondrial dysfunction in FRDA was predicted even before gene identification, based on the observations that patients with FRDA have reduced activity for several enzymes of the mitochondrial matrix [78]. Later, when the frataxin gene was discovered, phylogenic analysis showed that frataxin should be a mitochondrial protein [79]. All these data suggest that human frataxin is a mitochondrial protein.

After identification of frataxin as mitochondrial protein, a major question was how reduction of frataxin in mitochondria induces the disease. It is known that mitochondria are places for aerobic energy metabolism. Heart muscle, neurons and pancreatic islet cells respire aerobically. These tissues are rich in mitochondria and are usually affected in mitochondrial diseases [80]. How important is reduction of frataxin for mitochondria? It has been demonstrated that deletion of the C-terminal domain in frataxin is critical for maintenance of mitochondrial DNA [77]. The authors suggested that reduction of frataxin leads to the disruption of mitochondrial function. In this case, abnormalities in the function of mitochondria could be considered a primary defect in FRDA and the reduction of frataxin a secondary defect [77].

A yeast homolog of frataxin was shown to be involved in the regulation of respiratory function and iron homeostasis in mitochondria [76]. Moreover, it was demonstrated that deletion of this protein results in the accumulation of mitochondrial iron at the expense of cytosolic iron. Iron is an essential component of mitochondrial metabolism, present in the heme of the cytochromes and in FeS center proteins. Iron must be imported into mitochondria, but the iron concentration must be very well regulated, because an excess of iron results in damage to mitochondrial DNA. Iron deposits have been found in the myocardium of FRDA patients [81], and deletion of the yeast homolog of frataxin also results in severe accumulation of iron [82]. Several hypotheses were formulated regarding how an increase of iron in mitochondria might affect cell function. First, it could result in hypersensitivity to oxidative stress [77]. Second, it is known that iron at elevated concentrations induces the formation of free radicals that are toxic to cells [83]. The increase of radicals due to elevated iron could result in nonspecific oxidative damage of heme-containing proteins [84]. Therefore, understanding the regulation of iron transport to mitochondria and the role of frataxin in this process should be studied.

Polyglutamine disorders and cell death

Disorders within the type 1 group are associated with CAG triplet repeat expansions that are translated into a polyglutamine tract, resulting in the formation of proteins containing an excess of polyglutamines (fig. 4). The fact that all disorders in this group are characterized by neurodegeneration that is associated with expansion of polyglutamines suggests that molecular pathways in these disorders might overlap. On the other hand, each of these disorders shows specific, distinctive defects in the brain, suggesting unique molecular features in each pathway. Obviously, the polyglutamine sequence must be placed into specific proteins, which are presumably required for certain phenotypes. Comparison of protein sequences expressed from the genes responsible for CAG triplet repeat disorders did not show any homology, suggesting that specificity of each pathway could be determined by protein sequences around polyglutamines. Another interesting feature of these disorders is that the primary defect in CAG triplet repeat disorders is neurodegeneration, but the proteins are widely expressed in different tissues. This suggests that under disease conditions, the mutant protein probably functions normally in all tissues except brain. In agreement with dominant inheritance of these disorders, it was suggested that polyglutamine tracts result in a gain-of-function of mutant proteins via alteration of their properties. To understand the nature of alterations in the mutant proteins in the presence of polyglutamine tracts, several important questions must be answered: (i) What is the function of these proteins in normal cells? (ii) What is the role of a peptide-containing polyglutamines, and (iii) Why must polyglutamines be placed into specific sequences to induce the disease? We will briefly discuss current data related to each of these questions.

Polyglutamine-containing proteins and their partners

The first step in the functional studies of polyglutamine-containing proteins required their identification and structural analysis. These experiments showed that in most disorders, polyglutamines were located in novel proteins (ataxin-1, MJD, DRPLA and huntingtin) (re-

viewed in [4]). These proteins show no homology to other proteins, and it is therefore impossible to predict their function based only on sequence information. However, there are two exceptions to this: SBMA and SCA6. The protein responsible for SBMA is a well-studied androgen receptor [85]. SCA6 is associated with CAG expansion in an α subunit of voltage-dependent calcium channel [86], which plays an important role in muscle relaxation/contraction. Thus, it is expected that molecular mechanisms for SBMA and SCA6 will be clarified sooner compared with other disorders.

It has been suggested that expansion of polyglutamines could affect a protein's function via alteration of its interaction with other proteins. In addition, despite the wide expression throughout the body, the specificity of mutant proteins could be mediated by interaction with brain-specific partners. Therefore, several studies were focused on the identification of brain-specific proteins interacting in vivo with ataxin-1, huntingtin and others. It has been demonstrated that a glucolytic enzyme, GAPDH, interacts with polyglutamine peptides of different lengths in vitro [87]. This result was confirmed by studies in a two-hybrid system, indicating binding between GAPDH and ataxin-1, androgen receptor, DRPLA protein and huntingtin. Nevertheless, whether this binding is dependent on the number of polyglutamines remains to be seen [87, 88]. Moreover, the physiological significance of such an interaction in vivo must be studied in detail. GAPDH is a multifunctional protein; therefore, several cellular processes could be affected due to this binding. There are also some questions and concerns about this hypothesis. GAPDH is an abundant protein, but polyglutamine-containing proteins are expressed at relatively low levels. It is important to understand the stoichiometric ratio in the complexes between GAPDH and polyglutamine-containing proteins. In addition, GAPDH is highly expressed in many tissues, and it is therefore difficult to explain why the defect is restricted only to the brain. One possible explanation is that GAPDH is involved, together with other proteins, in the complex pathway mediated by polyglutamines. Therefore, the search for other brain-specific proteins interacting with polyglutamine-containing proteins has continued.

Two-hybrid screening revealed a novel protein interacting with huntingtin (HAP-1) which is selectively expressed in the brain [89]. This protein is colocalized with neuronal nitric oxide synthase (nNOS), suggesting a possible relationship between these two proteins [90]. It has been shown that huntingtin also interacts with calmodulin [91] and ubiquitin [92]. We discuss below the potential role of interaction between huntingtin and ubiquitin.

To explain the involvement of ataxin-1 in the development of ataxia and Purkinje degeneration, a leucine-rich acidic nuclear protein (LANP) specifically interacting

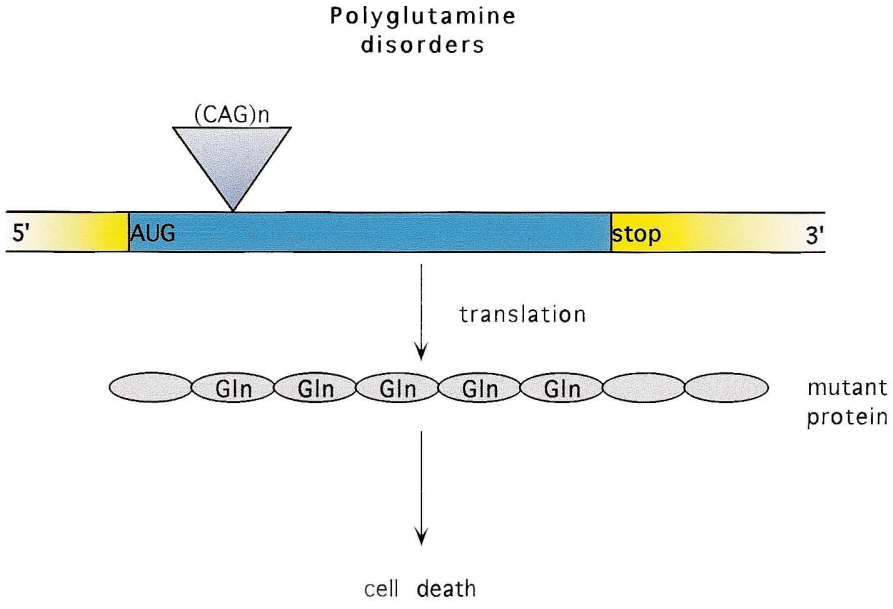

Figure 4. Model for polyglutamine disorders. CAG expansions in the neurodegenerative disorders are translated into polyglutamine tracks. Alteration of protein structure based on the insertion of excess polyglutamines induces degeneration of neurons.

with ataxin-1 was identified [93]. Although the function of LANP is unknown, it is expressed predominantly in Purkinje cells, the primary site of the SCA1 defect. Interaction between ataxin-1 and LANP is modulated by a number of polyglutamines and becomes stronger with increased size of the polyglutamine tract. It suggests that polyglutamines in the mutant ataxin-1 might result in sequestration of LANP protein and/or other nuclear factors [93] that could result in disruption of nuclear function of proteins dependent on ataxin-1 and LANP. In agreement with this suggestion, a protein of nuclear matrix, PML (promyelocytic leukemia protein), showed altered distribution in SCA1 patients [94].

Mouse models

Functional studies of CAG triplet repeat genes and their proteins are still underway. However, the important question whether expansion of polyglutamines is necessary for the development of disease could only be answered by the generation of mouse models.

To study the mechanism for SCA1, two types of mice were generated with either a normal allele containing 30 repeats or a mutant allele containing 82 repeats (reviewed in [95]), under regulation of a murine Purkinje-specific promoter. The transgenic line expressing the expanded SCA1 allele developed adult-onset ataxia and Purkinje degeneration. Since mice containing the transgene with 30 CAG repeats did not develop pathology, it was concluded that expansion of polyglutamines beyond 30 within ataxin-1 is necessary for disease induction.

Transgenic mice overexpressing a truncated huntingtin containing 44 polyglutamines also developed a phenotype (reviewed in [95]). These data indicate that expansion of polyglutamines is a key element in development of neuronal degeneration. This conclusion was supported by generation of a mouse line where huntingtin mRNA containing CAG repeats was expressed, but no protein was translated due to mutation in the construct [96]. These mice were phenotypically normal, showing that CAG repeat disorders are probably mediated by mutant proteins rather than by mutant RNA [96]. In addition, Ikeda et al. generated a mouse line where only polyglutamines were expressed [97]. The molecular abnormalities, including apoptotic response, in these mice were similar to abnormalities seen with overexpression of mutant proteins containing long polyglutamine tracts. These data support the conclusion that unstable CAG expansion acts not on the RNA level, but on the protein level. At the same time, transgenic mice overexpressing androgen receptor with polyglutamines did not have a phenotype, probably due to a relatively low level of transgene expression (reviewed in [95]).

Misfolding of mutant proteins and apoptosis

Although analysis of transgenic models for CAG diseases indicated the significant role of polyglutamines, it is unknown how polyglutamines induce the disease. The first suggestion was that polyglutamines affect interaction of protein with other proteins such as GAPDH, HAP-1, LANP and others. Another hypothesis is that the presence of polyglutamines results in misfolding of mutant proteins. This suggestion is based on the data of analyses of subcellular localization of mutant proteins in normal controls and in affected individuals. It was shown that patterns of ataxin-1 distribution in control and disease conditions, as well as in transgenic models for disease, were different [98]. Although the level of ataxin-1 did not change in affected populations, a mutant protein formed nuclear aggregates [98]. The same effect was observed with huntingtin protein [99]. When cells were transfected with the full-length or truncated huntingtin with 15 CAG repeats, protein was distributed in the cytoplasm [100]. However, when the number of glutamines was increased, protein was detected in perinuclear aggregates [100]. Similar aggregates were found in other serious degenerative diseases, including SCA3 [101], DRPLA [102], Alzheimer, Parkinson and prion disorders [103]. Although the formation of aggregates is well documented, recent studies show that nuclear localization of the mutant ataxin-1 [104] or huntingtin [105] is sufficient for the induction of disease.

Understanding of the role of intranuclear inclusions requires the identification of their origin. It has been suggested that nuclear aggregates could contain a mutant protein that could not be processed due to protein misfolding. It is possible that the long glutamine tract destabilizes the protein conformation in proportion to the size of the glutamine expansion. In agreement with this suggestion, it was found that both ataxin-1 and huntingtin form conjugates with ubiquitin molecules [92, 98]. Formation of ubiquitin conjugates is a first step in a protein degradation pathway mediated by ubiquitin-proteasome [106]. Then, ubiquitinated proteins are hydrolyzed by the 26S proteasome. Immunostaining of brain tissue from SCA1 patients and transgenic mice showed that the 26S proteasome is located in nuclei close to the sites of ataxin-1 protein aggregates [98]. Abnormalities in the function of proteasomes are associated with increased expression of chaperones. Chaperones (some stress-response and heat-shock proteins) interact with misfolded proteins, suppressing their aggregation. The human chaperone, HDJ-2/HSDJ, was also located within nuclei where ataxin-1 aggregates are present [98]. Overproduction of this chaperone resulted in reduction of misfolded ataxin-1 [98]. It has been suggested that accumulation of improperly processed proteins results in activation of programmed cell death

or apoptosis. Similar conclusions were made from studies of Machado-Joseph disease (MJD). It was demonstrated that expression of constructs containing truncated MJD with polyglutamines in the open reading frame results in cell death [97]. Moreover, there was direct correlation between the size of the repeats and the number of dead cells. Expanded proteins formed aggregates, suggesting that they interact with each other or form insoluble aggregates. A similar effect was seen not only in cultured cells transfected with expanded proteins but also in mice [97]. It is interesting to note that the apoptotic effect of truncated constructs or even polyglutamines alone was higher than that of the full-length proteins [97].

The evidence for induction of apoptosis by expression of polyglutamine-containing proteins is supported by accumulation of truncated mutant proteins under disease conditions. It has been shown that truncated huntingtin is accumulated in the brains of patients with HD [107]. The same effects were demonstrated for almost all neurological disorders associated with polyglutamine expansions. Since these proteins are subject to proteolysis, which could be a key event in the molecular pathology, it was important to identify proteases participating in the cleavage of polyglutamine-containing proteins. To date, 10 mammalian caspases, or cysteine proteases involved in apoptotic death, have been identified, which have been classified into three major subfamilies: ICE-like, activator and effector caspases [108]. It was shown that huntingtin, ataxin-3 and atrophin-1 are substrates for one or more caspases [109]. Interaction of different proteins with caspases suggests that mechanisms of neurodegeneration in these disorders are similar. However, it does not explain the specificity of clinical features for each disorder. It is possible that specific neuronal loss in each disorder is mediated by a specific caspase. Other factors important for apoptosis should also be investigated. For instance, apoptosis could be associated not only with polyglutamines but also with mutant proteins. Several studies with nullizygous mice for the huntingtin gene demonstrated that mice die with features of increased apoptosis [110–112]. It also remains to be investigated whether apoptosis results in cell dysfunction, or whether cell dysfunction leads to apoptosis. Apoptosis, programmed cell death, seems to play a role in elimination of defective cells. From this point of view, cell dysfunction should cause apoptosis. In agreement with this suggestion, detailed studies of SCA1 mice models showed that cell dysfunction preceded Purkinje cell death [95]. It is therefore possible that cell death in CAG repeat diseases is secondary to cell dysfunction induced by unknown mechanisms. These investigations are very important for the development of therapy of these disorders. If cell death is a primary effect resulting from the presence of polyglutamines, then one possibility for therapy could be inhibition of caspases. However, if apoptosis is a secondary effect, then other steps in polyglutamine pathways must be investigated.

There are also other hypotheses to explain the formation of protein aggregates: (i) polyglutamine sequences are highly insoluble because they form hydrogen-bonded polar zippers [113], and polypeptides with polyglutamines could form multimeric but not covalently bonded aggregates; and (ii) polyglutamines could be a substrate for transglutaminase [114]. Polyglutamines could, in the presence of active enzyme, become cross-linked with polypeptides containing lysyl groups to form covalently bonded aggregates.

In agreement with the first hypothesis, ataxin-1 was found to be able to self-associate [115]. However, this self-association was due to interaction in a region outside of the polyglutamines. As a confirmation of the second hypothesis, it has also been demonstrated that polyglutamine peptides containing 2–18 amino acids are substrates for transglutaminase [116]. It remains to be investigated whether polyglutamine-containing proteins interact with transglutaminase in vivo.

Small polyglutamine expansion in the α 1A-voltage-dependent calcium channel

The search for mutations in other neurodegenerative disorders offers other possible mechanisms for pathogenesis. Recently a new type of SCA was identified, SCA6, which is a disease characterized by spinocerebellar degeneration. The mutation responsible for SCA6 was found to be a CAG repeat in the α subunit of the voltage-dependent calcium channel [86], which is important for normal Purkinje cell function and survival. Although this disease is associated with CAG repeats, as are other type I disorders, an important feature is that the size of expanded alleles in SCA6 is within the normal size range of alleles in other CAG repeat diseases. Patients affected with SCA6 have 21–27 CAG repeats, but in all normal individuals an allele size did not exceed 16 units [86]. This represents the smallest known size difference between normal and expanded alleles. Because of the small size of the repeats, SCA6 mutant alleles are more stable than those in other CAG disorders, and they do not show transmission instability [86]. It is unknown how such small expansions could induce the disease. Although it was suggested that this mutation leads to gain of function of the mutant protein by a scenario similar to other CAG repeat disorders that induce cell death, it is difficult to explain how an excess of only a few glutamines could induce the phenotype. Presumably other factors in addition to CAG repeats are important in this disease.

Conclusions

Molecular studies of pathogenesis associated with trinucleotide expansions showed that although each triplet repeat disorder contains a mutation in a single gene, the mechanism for each disorder is complex and requires identification of all components of the triplet repeat pathway. Current molecular studies have revealed not only specific features for each disease, but also some similarities between them. It is interesting that both FraX and DM seem to be associated with alterations of RNA metabolism. In FraX, a protein coded by a mutant gene, FMRP, is an RNA-binding protein. Its absence should result in the disruption of RNA metabolism for specific brain mRNAs. In DM patients, expanded CUG repeats affect the expression of RNA-binding proteins that result in alterations of RNA metabolism in several tissues. Mental retardation seen in both disorders could be due to disruption of RNA processing of the same mRNAs in brain. Another parallel could be drawn between polyglutamine disorders and FRDA. Polyglutamine diseases are characterized by the involvement of an apoptotic response to the polyglutamine expansion. Accumulation of the excess of iron in mitochondria in FRDA patients could potentially induce apoptosis. Apoptosis is programmed cell death that is associated with activation of many genes. These genes are activated to balance the number of healthy and sick cells. If the same genes are affected by different mutations, then it is possible that their downstream pathways overlap. Identification of all components of molecular pathways for triplet repeat disorders is required for understanding disease pathogenesis and developing possible therapy.

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