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REVIEW ARTICLE
Friedreich Ataxia: From GAA Triplet–Repeat Expansion to Frataxin Deficiency

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Introduction

Friedreich ataxia (FRDA [MIM 229300]), the most prevalent inherited ataxia, is an autosomal recessive disease with onset in early childhood followed by an unremitting course that terminates with death in the fourth or fifth decade of life. It occurs at a frequency of 1–2 per 50,000 individuals. FRDA is characterized clinically by progressive gait and limb ataxia; signs of upper motoneuron dysfunction including dysarthria, areflexia, and loss of the senses of position and vibration; cardiomyopathy; diabetes mellitus; and secondary skeletal abnormalities. The majority (>95%) of patients with FRDA are homozygous for large expansions of a GAA triplet–repeat sequence (66–1800 triplets) located within the first intron of the gene *X25*, which encodes the protein frataxin (Campuzano et al. 1996). The expansion causes a severe reduction in the levels of frataxin, a 210–amino acid protein that is targeted to mitochondrial matrix and that appears to play a crucial role in iron homeostasis. The severity of the disease is directly correlated with the length of the expansion. A very small minority of patients are compound heterozygotes for the GAA expansion and for point mutations within the *X25* gene. Chamberlain and coworkers have recently summarized all point mutations described to date (Pook et al. 2000).

In this review, we will discuss recent developments that have increased our understanding of the effect of the GAA expansion on transcription and replication. Approaches used to investigate the unusual conformational properties of the GAA expansion and the unique behavior of the expansion in cultured cells will be described. Insights into the biogenesis of frataxin, including processing of the precursor protein and import into the mitochondria, will be summarized. We will describe advances made in the construction of mouse models for the disease. Finally, we will discuss important advances that have been made in deciphering the structure and function of frataxin, and we will conclude with prospects for therapy.

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GAA Triplet–Repeat Expansion as an Impediment to Transcription

RT-PCR and RNAse protection studies of RNA isolated from lymphoblast cell lines of patients with FRDA who were homozygous for the GAA expansion clearly demonstrated that frataxin RNA levels were severely reduced as a consequence of the expansion mutation. Several groups have demonstrated that the GAA-repeat expansion interferes with transcription. Bidichandani et al. (1998) reported a length- and orientation-dependent interference with in vitro transcription of GAA-repeat sequences, from patients with FRDA, consisting of 45, 79, or 100 GAA repeats that were cloned downstream of a bacteriophage T7 or a cytomegalovirus promoter. When the template strand contained the GAA sequence, which corresponds to the normal orientation of the GAA sequence within the *X25* gene, the reduction was more pronounced than when the reverse complement of the expansion, or $(TTC)_n$, was present on the template strand.

Ohshima et al. (1998) examined the effect of the presence of GAA repeats—ranging in number from 9 to 270 and cloned from patients with FRDA into the vector pSPL3—on splicing, gene expression, and replication. RNAse protection products corresponding to the spliced exon 1 + 2 transcript and to exon 1– and exon 2–containing transcripts showed that increasing numbers of GAA repeats lead to a reduction in the amount of mature transcript containing both exon 1 and 2 of the HIV gp120 reporter gene containing GAA repeats . However, the initiation of transcription and splicing were unaffected, confirming that the repeats interfered with elongation of transcripts rather than with their initiation or splicing. Lower levels of GAA-repeat–containing plasmids were also recovered compared with the levels of control plasmids, suggesting an inhibitory effect on rep-

lication. Again, the effects on transcription and replication were much more prominent when $(GAA)_n$ was in the template strand.

Grabczyk and Usdin (2000*a*) studied the effect of GAA tracts, in the absence of any other frataxin gene sequences, on in vitro transcription by bacteriophage T7 polymerase. A reduction in the amount of GAA-containing transcripts was seen, an effect that was exacerbated by both tract length and superhelicity. One novel observation made by this group was that the transcripts are truncated at the distal end of the GAA tract, which accounts for the slightly different model for the mode of action of the repeats (fig. 1*C*).

The length-dependent reduction in in vitro (Bidichandani et al. 1998; Grabczyk and Usdin 2000*a*) and in vivo (Ohshima et al. 1998) transcription of the templates used in the above studies is consistent with the increase in severity—assessed as an earlier age at onset—of FRDA that occurs with an increased number of GAA repeats. The orientation-dependent effect (Bidichandani et al. 1998; Ohshima et al. 1998; Grabczyk and Usdin 2000*a*) is consistent with the working model for the mode of action of the expansion mutation (see below).

Unusual DNA Structure Formed by GAA Expansion

The location of the GAA expansion in the first intron of the frataxin gene raised immediate questions concerning the resulting molecular underpinnings leading to pathogenesis. Several mechanisms could be envisioned for the reduction in transcript levels, including abnormal splicing of the heteronuclear RNA, disruption of an enhancer element, or creation of a transcriptional blockade. No evidence for aberrantly spliced products was apparent when RNAse protection studies were conducted with probes that spanned exons 1 and 2, although this method could not exclude short-lived transcripts originating from the allele with the expansion (Bidichandani et al. 1998). Enhancer activity was not detected when a fragment containing the wild-type GAA repeat and flanking sequence was cloned into an SV40 promoter-driven luciferase expression vector and assayed for enhancer function in fibroblast cells (Bidichandani et al. 1998). Tissue-specific enhancer activity could not, of course, be ruled out by this assay. Bidichandani et al. (1998) first demonstrated that the FRDA expansion was likely to be associated with an unusual DNA structure (Cavadini et al. 2000*b*). PCR-amplified linear fragments including ~1.5 kb of intron 1 sequence in the context of the normal (9 GAA repeats) and expanded (up to 350 repeats) alleles were probed with osmium tetroxide, hydroxylamine, and diethylpyrocarbonate, chemicals typically used for the probing of unusual DNA structures such as DNA triplexes and cruciform DNAs. These studies suggested the presence of a non–B DNA structure that was hypothesized to most likely represent an intramolecular triplex (fig. 1*A*). Recently, in keeping with the finding that triplex DNA structures are prone to increased mutagenesis, this group has found evidence for enhanced mutagenesis (point mutations) in the sequence immediately flanking the expanded GAA repeats (Bidichandani et al. 1999).

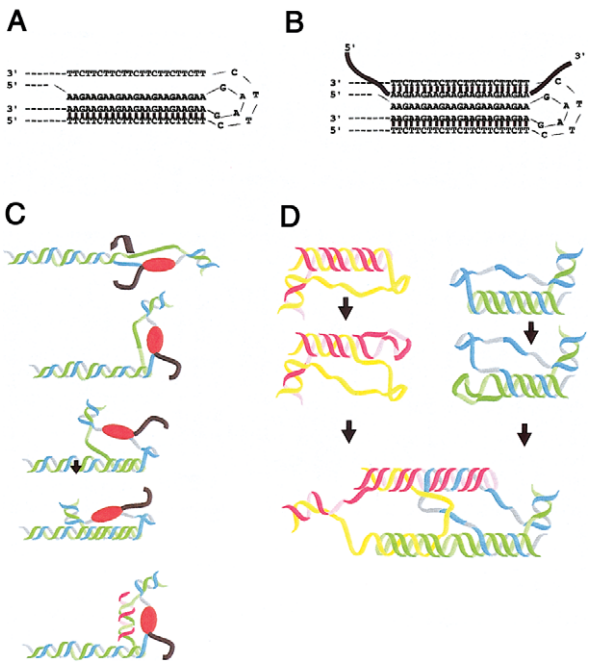

Figure 1 Proposed models for the formation of non–B DNA conformations by GAA-repeat sequences. *A*, Intramolecular RRY triplex, formed when the purine-rich strand dissociates from its complementary strand and pairs by Hoogsteen bonding in an antiparallel orientation with the major groove (only one DNA isoform is shown). *B*, The triplex shown in *A*, paired with the GAA-containing transcript, thereby further stabilizing the triplex (Bidichandani et al. 1998; Ohshima et al. 1998). *C*, A variation in the intramolecular triplex model, in which the wave of negative supercoiling induced by RNA polymerase induces triplex formation, leading to a pause at the promoter distal end of the structure (Grabczyk and Usdin 2000*a*). Binding of a triplex-forming oligonucleotide (shown in red) to the nontemplate strand can alleviate the transcriptional block by preventing formation of the triplex (Grabczyk and Usdin 2000*b*). *D*, Association of two triplexes to form "sticky DNA." A strand-exchange model is depicted, in which two circular plasmid molecules associate with each other at the RRY triplex regions. The Y strands from the RRY triplexes reassociate with the R looped region from the second triplex and reconfigure to form hybrid RRY triplexes (Sakamoto et al. 1999).

Wells, Pandolfo, and colleagues proposed a similar intramolecular triplex model based on their investigations with GAA repeats in an in vivo transcription assay (Bidichandani et al. 1998; fig. 1*A*, *B*). Further elegant studies by this group have shed more light on the molecular nature of the transcriptional block posed by the formation of a non–B DNA structure at the GAA-repeat

expansion (Sakamoto et al. 1999). They noted that, during standard agarose gel electrophoresis of linearized $(GAA)_n$-containing plasmid DNA, a portion of the DNA migrated anomalously, with an apparent molecular weight that was 2.4-fold greater than expected. Detailed investigations indicated that the extent of retardation was a function of location of the repeat within the linear DNA and that negative supercoiling promoted its formation. The slowly migrating DNA is P1-nuclease sensitive and thermostable in the absence of EDTA, and its formation is favored at neutral pH. The authors postulate that the GAA-rich sequences self-associate (see fig. 1*D*) to form a novel DNA structure that they refer to as "sticky DNA." A $(GAAGGA)_{65}$-repeat sequence present in the first intron of the *X25* gene of some individuals did not display the properties of sticky DNA, suggesting an intrinsic preference for uninterrupted $(GAA)_n$ sequences to form this structure (Ohshima et al. 1999).

In a collaborative effort between the Pearson, Sinden, and Gao laboratories, a detailed analysis of individual GAA and TTC repeats was performed using the methods of nuclear magnetic resonance, UV melting, and gel electrophoresis, as well as chemical and enzymatic probing (LeProust et al. 2000). This study highlighted the structural diversity of these dynamically unstable repeats: Together, the GAA and TTC repeats were able to form various structures, including parallel-stranded duplexes, antiparallel (Watson-Crick) duplexes, and pyrimidine-purine-pyrimidine (YRY) triplexes. Unlike the CNG repeats, hairpin formation by either the GAA or the TTC repeats was not readily detected. However, the GAA repeat—but not the TTC repeat—was capable of forming a metastable structure. Most interesting was the observation of a very stable parallel duplex between the complementary strands under physiological conditions (the common Watson-Crick B-DNA duplexes are antiparallel). Intriguingly, the parallel duplex could be formed only between GAA strands and TTC strands containing a 5′-hydroxyl (rather than a 5′-phosphate). It may not be a coincidence that the 5′ ends of Okazaki fragments, produced during semiconservative DNA replication, are transiently dephosphorylated to expose a 5′-hydroxyl group (Pohjanpelto and Holtta 1996). Perhaps the transient state of the 5′ ends during DNA replication could facilitate the formation of alternative local DNA structures, which may then serve as mutagenic intermediates in the expansion process.

Studies using a single oligonucleotide composed of both GAA and TTC repeats (Gacy et al. 1998; Mariappan et al. 1999) demonstrated triplex formation in the parallel orientation. However, triplex formation by these designed oligonucleotides was forced by the juxtaposition of the two repeats in a single DNA chain. Formation of the purine-purine-pyrimidine (RRY) triplex by this single oligonucleotide (Gacy et al. 1998; Mariappan et al. 1999) contrasted with the YRY triplex formed by either plasmid-borne $(GAA)_n \cdot (TTC)_n$ inserts (Hanvey et al. 1988) or the mixture of individual GAA and TTC strands (LeProust et al. 2000). On the basis of their oligonucleotide studies, Gacy et al. (1998) proposed an alternative model for intramolecular triplex formation in the expansion process.

Germline and Somatic Variation in GAA Expansion Length

The GAA expansion shows intergenerational variation in length, with evidence for changes in the prezygotic and postzygotic stages. Studies have shown that the expanded alleles seen in patients arose from a small pool of uninterrupted "large normal" alleles referred to as "premutations." Interruptions within the pure GAA triplet repeats impeded these large normal alleles from expanding into disease-causing alleles. De Michele et al. (1998) have noted that premutation alleles can undergo large expansions in a single generation. Expanded GAA repeats can expand or contract when transmitted through the female germline. In contrast, contractions are favored in male transmission. This is attributed to postzygotic mechanisms, because shorter expansions are seen in sperm DNA when compared with lymphocyte DNA (prezygotic mechanism). However, evidence for postzygotic variation in repeat number has also been suggested, because the degree of repeat contraction in the sperm is greater than that actually seen in intergenerational transmission and because the overall length of expanded alleles is shorter in homozygous versus heterozygous carriers.

We have noted reversion of the expanded GAA triplet repeat to a normal size in a patient with an atypically mild phenotype. This contraction was seen in two independent peripheral blood samples (Bidichandani et al. 1999). It is unclear whether somatic mosaicism for the fully contracted allele is responsible for the milder phenotype.

In contrast with other triplet-repeat expansions, significant somatic variation has been seen for the GAA expansion (Bidichandani et al. 1999). Pronounced length variation was detected in serially passaged lymphoblastoid cells from individuals who were homozygous or heterozygous for the expansion (Bidichandani et al. 1999). Contractions and expansions were observed with equal frequency and magnitude. Hellenbroich et al. (2001) used small-pool PCR to examine the expansion length in leukocytes and found mosaicism for the length of the repeat, with differences in length that were up to 1,000 triplets per genome. A significant correlation was found between the size of the largest

allele and the range of mosaicism (Hellenbroich et al. 2001).

Biogenesis of Frataxin

The FRDA gene encodes a precursor form of frataxin (Babcock et al. 1997; Wilson and Roof 1997). Positively charged presequences with the general features of mitochondrial matrix–targeting signals are present at the N-termini of all known frataxin precursors from yeast, mammals, and other eukaryotes (Gibson et al. 1996; Canizares et al. 2000). Human frataxin (GenBank accession number Q16595) has been detected at the matrix side of the inner mitochondrial membrane, by immunoelectron microscopy (Campuzano et al. 1997), and yeast frataxin (Yfh1p [GenBank accession number Q07540]) has been found to behave like a soluble matrix protein upon fractionation of mitochondria (Branda et al. 1999*a;* Geissler et al. 2000). Koenig and colleagues first noted that, whereas the precursors of most mitochondrial matrix proteins are subjected to a single proteolytic event upon import, frataxin is processed to its mature form in two steps, at least one of which was catalyzed by the general mitochondrial-processing peptidase (MPP) (Koutnikova et al. 1998). The implication that some unique requirements might underlie mitochondrial import and the processing of frataxin drew immediate attention, especially in light of the fact that a reduction in the levels of—and not a loss of function of—frataxin is the most frequent cause of FRDA (Campuzano et al. 1996, 1997). Important strides in the definition of the main steps and molecular components involved in the biogenesis of frataxin have since been made, primarily through studies of the yeast and human proteins.

Mitochondrial Import and Processing of Frataxin

The mitochondrial targeting signals of yeast and human frataxin consist of 51 and 55 amino acids, respectively (Adamec et al. 2000; Cavadini et al. 2000*a*; Gordon et al. 2001). During or immediately after translocation, the targeting signal of Yfh1p is cleaved twice by MPP, between residues 20 and 21 and residues 51 and 52, generating an intermediate and a mature form (Knight et al. 1998; Branda et al. 1999*a*; Adamec et al. 2000; Gordon et al. 2001). This pattern of processing is unusual, given that most precursors are cleaved only once by MPP to generate the mature form (Neupert 1997). In addition to MPP, two components of the mitochondrial protein import machinery, Tim44 and Ssq1, are also required for efficient production of the intermediate (Geissler et al. 2000) and mature form (Knight et al. 1998; Voisine et al. 2000) of Yfh1p, possibly by influencing protein folding and making the two cleavage sites more accessible to MPP.

Additional requirements appear to influence the maturation of mammalian frataxin. Koenig and colleagues first observed cleavage of the mouse frataxin precursor to a product with an apparent MW of 21 kDa, larger than the form of frataxin detected in normal mouse or human tissues (~18 kDa) (Koutnikova et al. 1998), which is also the form that is reduced in FRDA patients (Campuzano et al. 1997). It was later shown that both a ~21 kDa and a ~18 kDa form are produced by sequential cleavage of the human frataxin precursor by MPP, and the two cleavage sites were mapped between residues 41–42 and 55–56 (Branda et al. 1999*a*; Cavadini et al. 2000*a*). The second cleavage was shown to be very slow, however, limiting the overall rate at which the mature form was produced by purified MPP or within mitochondria (Cavadini et al. 2000*a*). These kinetics may explain why the second cleavage was not detected in another study of human frataxin biogenesis (Gordon et al. 1999). Given that either rat or yeast MPP was used in all of these studies, the observed kinetics may also reflect a lack of species specificity between the peptidase and its substrate.

These observations collectively have raised the interesting possibility that the steady-state levels of mammalian frataxin may be regulated through specific interactions with MPP. In this respect, the fact that mouse frataxin was found to interact with the β and not the α subunit of MPP (Koutnikova et al. 1998) is intriguing, given our current knowledge that α-MPP is responsible for substrate binding, whereas β-MPP is primarily involved in catalysis (reviewed by Adamec et al. [2001]).

Variable Effects of FRDA Point Mutations on the Biogenesis of Human Frataxin

Interaction between the mouse frataxin precursor and β-MPP is inhibited by two of the most common FRDA point mutations, I154F and G130V (Koutnikova et al. 1998). The I154F mutation was further found to affect the processing of human frataxin in COS cells (Koutnikova et al. 1998) but not in vitro (Gordon et al. 1999). In more recent studies, the W173G mutation inhibited the second processing step both in vitro and in yeast, whereas the G130V mutation had no obvious effect on frataxin processing (Cavadini et al. 2000*a,* 2000*b*).

Although MPP often cleaves at position -2 or -3 from an arginine residue (Gavel and von Heijne 1990), both the overall secondary structure of the presequence as well as structural elements in the mature portion of the precursor molecule are important determinants for substrate recognition by this peptidase (reviewed by Adamec et al. [2001]). Thus, it is not surprising that

FRDA point mutations far from the actual MPP cleavage sites—especially mutations such as I154F or W173G, which are predicted to cause major changes in the protein fold (Dhe-Paganon et al. 2000)—can affect recognition and/or processing of frataxin by MPP. One caveat is that heterologous experimental systems may not precisely reproduce the consequences of FRDA point mutations in human mitochondria. This possibility is especially suggested by the diversity of effects associated with the I154F or G130V mutation in COS cells, yeast, or in vitro assays (Koutnikova et al. 1998; Gordon et al. 1999; Cavadini et al. 2000*a,* 2000*b*). Thus, studies of protein import in human cells or in isolated mitochondria, as well as processing experiments using purified human MPP, will be required to establish with certainty the functional effects of these and other point mutations.

Possible Role of the Mitochondrial Import Machinery in FRDA

The studies summarized above have revealed unique requirements for the efficient import and processing of frataxin. Although some caution may be necessary when extrapolating from model systems to humans, this information suggests that certain components of the mitochondrial protein-import machinery may influence the pathogenesis and clinical variability of FRDA. For instance, there may exist genetic variants of MPP associated with reduced rates of frataxin processing, which would presumably lower the threshold for phenotypic expression of expanded FRDA alleles. In addition, given that both translocation and processing of the frataxin precursor are ATP-dependent (Geissler et al. 2000; Voisine et al. 2000), respiratory chain defects such as those associated with FRDA (Rotig et al. 1997; Lodi et al. 1999; Bradley et al. 2000) might easily trigger a vicious cycle in which frataxin deficiency would initially cause a decrease in ATP production, and this would, in turn, impair protein import and exacerbate frataxin deficiency. The genetic and biochemical information necessary to begin to address these possibilities in patients is now available. The identification of genetic modifiers and synergistic pathogenic mechanisms and the defining of strategies for the treatment of FRDA will be significant goals of these studies.

Role of Frataxin in Iron Homeostasis

The first insights into the function of frataxin came from studies in yeast, which showed that a lack of Yfh1p impairs iron export from the mitochondrion (Radisky et al. 1999), which leads to cytoplasmic iron depletion, induction of plasma membrane proteins involved in iron uptake, and mitochondrial iron overload (Babcock et al. 1997; Foury and Cazzalini 1997; Radisky et al. 1999). Iron-induced oxidative damage was postulated to be the most probable cause underlying the loss of mtDNA and iron-sulfur proteins characteristic of Yfh1p-deficient yeast (yfh1Δ) (Babcock et al. 1997; Foury and Cazzalini 1997).

A direct involvement of Yfh1p in the biosynthesis of iron-sulfur clusters has been proposed more recently (Foury 1999; Lutz et al. 2001). This view has been challenged by another study showing that a lack of Yfh1p does not result in any significant loss of iron-sulfur–containing enzyme activities if mitochondrial iron accumulation and, hence, oxidative damage to iron-sulfur proteins can be prevented (Chen and Kaplan 2000). Differences in the approaches used to assess the functionality of iron-sulfur enzymes may explain these contrasting results. In any case, it is clear that the maintenance of functional mitochondrial iron-sulfur enzymes, whether directly or indirectly, is a critical aspect of the role played by Yfh1p in global cellular iron homeostasis (Foury and Talibi 2000) and in mitochondrial respiratory function (Babcock et al. 1997; Foury and Cazzalini 1997; Koutnikova et al. 1997; Wilson and Roof 1997; Branda et al. 1999*b*).

Iron-Induced Oxidative Damage and the Pathogenesis of FRDA

A large body of studies has addressed the important questions of whether frataxin plays a role in iron homeostasis in humans and what the implications are for the pathogenesis of FRDA. Chimeric Yfh1p-human frataxin and full-length human frataxin constructs allow partial and full complementation of yfh1p mutants, respectively, including the ability to maintain normal mitochondrial iron levels and aconitase activity (Wilson and Roof 1997; Cavadini et al. 2000*b*). Conversely, expression in yfh1Δ yeast of a full-length frataxin construct containing the W173G mutation resulted in increased mitochondrial iron levels and loss of respiratory function (Cavadini et al. 2000*b*) consistent with the severe clinical presentation associated with the W173G mutation (Cossee et al. 1999). These basic studies have therefore demonstrated that human frataxin participates in iron homeostasis, at least in the context of yeast mitochondria.

Beginning with an early report of iron deposits in hearts of patients with FRDA (Lamarche et al. 1980), pathological and clinical studies have shown that the consequences of frataxin defects are indeed similar between the yeast model and mammalian cells. Granular iron deposits suggestive of mitochondrial iron overload, as well as signs of oxidative damage including multiple iron-sulfur enzyme defects and loss of mtDNA, have

been observed in hearts from these patients (Rotig et al. 1997; Bradley et al. 2000), and increased levels of mitochondrial iron and hypersensitivity to oxidative stress have been detected in the patients' cultured cells (Delatycki et al. 1999; Wong et al. 1999). Elevated serum levels of the transferrin receptor, indicative of limited cytoplasmic iron supplies (Wilson et al. 2000) and of malondialdehyde, a marker of lipid peroxidation (Emond et al. 2000), have also been noted in patients.

The pathogenic importance of oxidative damage in FRDA is further underscored by two studies in which administration of the antioxidant idebenone led to a reduction in myocardial hypertrophy in 3 patients (Rustin et al. 1999) and to a decrease in urine levels of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative DNA damage, in 33 patients (Schulz et al. 2000). In addition, conditional frataxin knockout mice exhibit multiple iron-sulfur enzyme deficiencies and mitochondrial iron accumulation (Puccio et al. 2001) (see below).

Together, these findings indicate that frataxin controls a critical iron pool within mitochondria. This function is clearly important for maintenance of overall cellular iron homeostasis and for protection from iron-induced oxidative damage.

Mice with Complete or Tissue-Specific Deficiency of Frataxin

The first attempt to create a mouse model for FRDA involved the creation of a homozygous deletion of exon 4 of *Frda* (Cossee et al. 2000). The deletion is predicted to create a truncated protein that lacks more than half of the frataxin protein sequence, including the most conserved domain of the protein (Cossee et al. 2000). This mutation is embryonically lethal a few days after implantation, suggesting that frataxin is essential during development. Both apoptotic and necrotic features were noted in the *Frda* null embryos. Notably, there is no evidence for accumulation of iron. FRDA patients appear to tolerate frataxin deficiency during development. One possible explanation is that humans are aided by compensatory mechanisms for the handling of frataxin deficiency. The other, more likely, possibility stems from the observation that the vast majority of patients with FRDA are homozygous for GAA expansions. As noted above, the expansion appears to pose a transcriptional block, and it is quite possible that just enough frataxin is produced in patients with FRDA to allow normal development, with the deficiency posing problems gradually (Cossee et al. 2000). Indeed, low levels of frataxin are expressed in lymphoblast lines from patients with FRDA who have expansions, and this expression is directly correlated with the size of the expansion (Campuzano et al. 1997).

Koenig and coworkers recently generated two conditional mouse models for FRDA through creation of an exon 4 deletion in striated muscle tissue or in neural and cardiac muscle tissue, through use of a Cre transgene under the control of tissue-specific promoters (Puccio et al. 2001). Frataxin is absent in skeletal and cardiac tissue of the conditional line referred to as the "MCK mutant," in which the Cre transgene, which removes exon 4 by driving recombination at *lox p* sites surrounding the exon, is under the control of the muscle creatine kinase promoter. These mice start to lose weight at age ~7 wks and have a life expectancy of 76 ± 10 d, with a 29% weight reduction at death. The NSE mutant, in which the Cre transgene is driven by the neuron-specific enolase promoter, has a shorter life expectancy of 24 ± 9 days, with a 41% weight reduction at death. The NSE mutants have a progressive neurological phenotype with ataxia, hunched stance, and loss of the sense of position. Frataxin is absent in skeletal and cardiac tissue. Cardiac hypertrophy, the predominant cause of death in patients with FRDA, is seen in both mutants. Time-dependent iron accumulation was noted in the hearts of MCK mutants but, importantly, the iron deposits followed—and, therefore, were apparently not responsible for—the cardiac pathology. Multiple Fe-S enzyme deficiencies similar to those in patients were noted and were seen to occur in parallel with the pathology in the CNS and heart of the mutant lines. These lines recapitulate the major biochemical and pathophysiological features of the human disease and will be very useful not only for an understanding of the relationship between Fe-S enzyme deficiency and the accumulation of iron but also for the provision of useful models for the testing of therapeutic strategies based on antioxidants and/or iron chelators.

Mechanistic and Structural Studies of Frataxin

A continuous supply of iron is needed in mitochondria, primarily for the biosynthesis of heme and iron-sulfur clusters, both of which require ferrous iron in vitro and, most likely, in living cells as well (reviewed by Ferreira et al. [1995] and Muhlenhoff and Lill [2000]). Iron transport across the inner mitochondrial membrane is an energy-dependent process with an absolute requirement for reducing equivalents provided by the respiratory chain (reviewed by Gattermann et al. [1993]), indicating that iron must cross the inner membrane in the ferrous form. Under aerobic conditions at physiologic pH, however, Fe^{2+} is rapidly oxidized by dioxygen to Fe^{3+}, a reaction that leads to a substantial decrease in iron solubility (from $\leqslant 10^{-8}$ M to $\leqslant 10^{-18}$ M) (Williams 1982) and that generates superoxide radical (Halliwell 1978). In addition, Fe^{2+} can interact with hydrogen peroxide, a by-product of respiration, to give hydroxyl radical, which is the most reactive oxygen species known

(Gutteridge et al. 1981). Thus, it is essential for the mitochondrion to maintain a supply of Fe^{2+} but at the same time prevent its participation in radical reactions.

Work by Isaya and colleagues has revealed that the involvement of Yfh1p in iron export may reflect a more general role of this protein as an iron ligand in the mitochondrial matrix. To elucidate the mechanism of action of frataxin, they have expressed the mature form of Yfh1p in *Escherichia coli* and analyzed the ability of the isolated protein to bind iron in vitro (Adamec et al. 2000). Titration of isolated Yfh1p with increasing concentrations of a ferrous iron salt under aerobic conditions induced stepwise assembly of a regular spherical multimer with a molecular weight of $>10^6$ and the ability to sequester >3,000 atoms of iron. A high–molecular-weight (>600,000) form of Yfh1p was also detected in yeast mitochondria, and when yeast cells were grown in the presence of ^{55}Fe, immunoprecipitates of this form were found to contain >16 atoms of ^{55}Fe per molecule of Yfh1p (Adamec et al. 2000). On the basis of these findings, these investigators have proposed that the function of Yfh1p is to bind iron and to keep it in a soluble and bioavailable form. Interestingly, Flatmark and Romslo (1975) reported >25 years ago that a pool of "non-heme non–iron-sulfur" iron could be recovered from the matrix of isolated rat liver mitochondria in a large hydrophilic complex with molecular weight $>10^6$. They suggested that a macromolecule distinct from ferritin might be responsible for the binding of iron in a soluble and stable form in the mitochondrial matrix.

Indeed, a more recent study has revealed that isolated Yfh1p binds Fe^{2+} and keeps it in reduced and readily available form in aqueous solution at physiologic pH (J. Adamec, E.E. Arias, A. Mangravita, G.C. Ferreira, G. Isaya, unpublished data). The duration of this effect ranges from several minutes to >1 h, depending on the experimental conditions. During this time, Yfh1p can protect other biomolecules from iron-induced oxidative damage and can provide Fe^{2+} ions for heme synthesis in the presence of ferrochelatase and protoporphyrin IX. The iron that remains bound to Yfh1p is otherwise progressively oxidized and stored in a soluble form within the assembled protein.

These findings suggest that the function of frataxin is to bind Fe^{2+} and to decrease the rate of Fe^{2+} oxidation to Fe^{3+} (fig. 2*A*). In this manner, frataxin could promote iron export to the cytoplasm or its utilization by the iron-sulfur cluster or heme biosynthetic pathways, preventing iron participation in radical reactions. Such properties could explain the activation of oxidative phosphorylation associated with frataxin overexpression in cultured cells (Ristow et al. 2000), assuming that respiration is normally limited by the levels of redox-active Fe^{2+} and/or the rates of iron-sulfur cluster or heme biosyntheses. Moreover, through its modulation

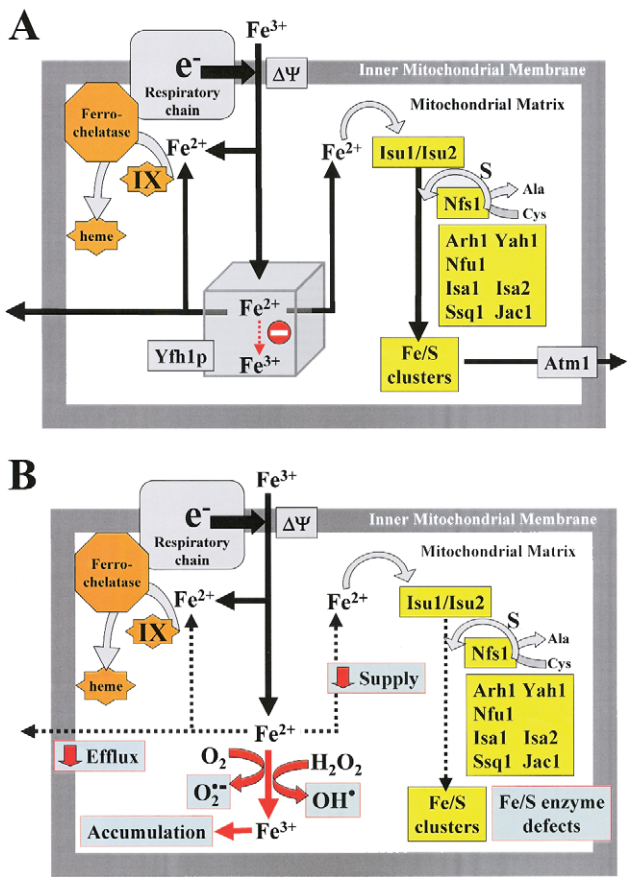

Figure 2 *A*, Proposed function of frataxin in normal mitochondria. *B*, Consequences of frataxin deficiency. Ferrochelatase catalyzes the final step of the heme biosynthetic pathway, which involves the insertion of Fe^{2+} into protoporphyrin IX (*IX*). This enzyme is localized to the matrix side of the inner mitochondrial membrane and is believed to be able to bind Fe^{2+} immediately after its reduction by the respiratory chain (Taketani et al. 1986). The initial step in the iron-sulfur (Fe/S) cluster biosynthetic pathway involves Fe^{2+} binding by the Isu1 and Isu2 proteins, which serve as scaffolds for cluster assembly (Muhlenhoff and Lill 2000). These proteins are localized to the mitochondrial matrix and may depend on frataxin for a sufficient supply of Fe^{2+}. Both decreased biosynthesis and oxidative damage may contribute to Fe/S enzyme defects. $\Delta\Psi$ = mitochondrial inner-membrane potential.

of iron oxidation, frataxin could provide a means of storing ferric iron in soluble and possibly useful form, similar to the iron stored in ferritin (reviewed by Harrison and Arosio [1996]). Conversely, in the case of frataxin deficiency, iron would no longer be efficiently exported or utilized, causing immediate oxidative damage and progressive iron accumulation (fig. 2*B*). This sequence of events is consistent with the phenotype of conditional frataxin-deficient mice, in which loss of iron-sulfur enzyme activities is an early effect, whereas mitochondrial iron deposition is time-dependent (Puccio et al. 2001). The proposed model may also explain the early embryonic lethality, without iron accumula-

tion, induced by deletion of the mouse frataxin gene (Cossee et al. 2000). It is possible that in the absence of frataxin a significant fraction of the iron entering the mitochondrion may be neither used nor detoxified. This condition may result in embryonic cell death before detectable levels of iron can accumulate. Interestingly, early embryonic lethality is also associated with inactivation of the mouse *H-ferritin* gene (Ferreira et al. 2000), suggesting that both ferritin and frataxin are critically required for iron bioavailability and iron storage during development.

The three-dimensional structures of human and bacterial frataxin have revealed a novel fold characterized by a highly conserved negatively charged surface (Cho et al. 2000; Dhe-Paganon et al. 2000; Musco et al. 2000). The negatively charged surface is similar to the anionic surface involved in the iron-storage mechanism of ferritin (Dhe-Paganon et al. 2000), and human frataxin may utilize a second, uncharged surface to assemble with itself and form a negatively charged iron-storage cavity similar to that of *Listeria innocua* ferritin (Musco et al. 2000). Indeed, high–molecular-weight forms of frataxin can be detected by gel filtration or other nondenaturing approaches in human and mouse tissues as well as in yeast cells expressing the human protein (Airoldi et al. 2000; P. Cavadini and G. Isaya, unpublished data). On the other hand, in testing a recombinant form of human frataxin, Pastore and colleagues did not detect any iron-binding or self-assembly activity (Musco et al. 2000). This suggests that, whereas Yfh1p is capable of undergoing self-assembly in vitro, human frataxin may require molecular chaperones or other factors that remain to be investigated.

Conclusions and Prospects for Therapy

Since the discovery of the FRDA gene (Campuzano et al. 1996) and the seminal observation that frataxin mediates cellular iron homeostasis in yeast (Babcock et al. 1997), the function of frataxin has been studied by genetic and biochemical means in a variety of systems, providing insights into the importance of mitochondrial iron metabolism and into the risks associated with defects in this process. Evidence of increased oxidative stress in patients with FRDA and of the promising effects of idebenone (Rustin et al. 1999; Schulz et al. 2000) has provided a strong rationale for the development of treatments that may inactivate reactive radicals or mobilize iron from mitochondria, thereby correcting the consequences of frataxin deficiency. Inhibition of the formation of the abnormal DNA structure formed by the GAA expansions represents an alternative strategy that could potentially ameliorate frataxin deficiency. Grabzyck and Usdin (2000*b*) have recently shown that oligodeoxyribonucleotides designed to block particular types of triplex formation lead to specific and concentration-dependent increases in full-length transcript upon in vitro transcription with bacteriophage T7 RNA polymerase. These studies, which were conducted with artificial GAA tracts, are a promising avenue for further exploration. Additional studies of GAA expansions, within the molecular and cellular context of the *X25* gene as seen in FRDA patients, will be essential in order to engender further support for this novel avenue of treatment.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html (for Yfh1p [accession number Q07540] and human frataxin [accession number Q16595])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for FRDA [MIM 229300])

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