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The GAA Triplet-Repeat Expansion in Friedreich Ataxia Interferes with Transcription and May Be Associated with an Unusual DNA Structure

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Summary

Friedreich ataxia (FRDA), an autosomal recessive, neurodegenerative disease is the most common inherited ataxia. The vast majority of patients are homozygous for an abnormal expansion of a polymorphic GAA triplet repeat in the first intron of the *X25* gene, which encodes a mitochondrial protein, frataxin. Cellular degeneration in FRDA may be caused by mitochondrial dysfunction, possibly due to abnormal iron accumulation, as observed in yeast cells deficient for a frataxin homologue. Using RNase protection assays, we have shown that patients homozygous for the expansion have a marked deficiency of mature *X25* mRNA. The mechanism(s) by which the intronic GAA triplet expansion results in this reduction of *X25* mRNA is presently unknown. No evidence was found for abnormal splicing of the expanded intron 1. Using cloned repeat sequences from FRDA patients, we show that the GAA repeat per se interferes with in vitro transcription in a length-dependent manner, with both prokaryotic and eukaryotic enzymes. This interference was most pronounced in the physiological orientation of transcription, when synthesis of the GAA-rich transcript was attempted. These results are consistent with the observed negative correlation between triplet-repeat length and the age at onset of disease. Using in vitro chemical probing strategies, we also show that the GAA triplet repeat adopts an unusual DNA structure, demonstrated by hyperreactivity to osmium tetroxide, hydroxylamine, and diethyl pyrocarbonate. These results raise the possibility that the GAA triplet-repeat expansion may result in an unusual yet stable DNA structure that interferes with transcription, ultimately leading to a cellular deficiency of frataxin.

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Introduction

Friedreich ataxia (FRDA) is the most common inherited ataxia, with an estimated prevalence of 1/50,000–2/50,000. Clinical features include progressive gait and limb ataxia, areflexia, loss of position sense, dysarthria, and positive Babinski sign (Harding 1981). In addition to the neurodegenerative phenotype, most patients have hypertrophic cardiomyopathy, and nearly a third have either diabetes mellitus or a lesser degree of carbohydrate intolerance (Finocchiaro et al. 1988). Most patients develop symptoms in their early teens, are wheelchair bound by their late 20s, and usually die in their mid 30s. For a recessive disease, the range of phenotypic severity displayed by FRDA patients is unusually broad, including exceptionally delayed age at onset (De Michele et al. 1994), retention of deep-tendon reflexes (Palau et al. 1995), and atypically gradual progression of disease seen in Acadians (Barbeau et al. 1984) and some Caucasians (Bidichandani et al. 1997).

The vast majority (>95%) of FRDA patients are known to be homozygous for an unstable GAA triplet-repeat expansion in the first intron of the *X25* gene, which normally encodes a 210-amino-acid protein, frataxin (Campuzano et al. 1996). FRDA is associated with alleles bearing 66–1,700 or more GAA triplets, whereas normal individuals have 7–38 repeats (Campuzano et al. 1996; Dürr et al. 1996; Epplen et al. 1997). GAA triplet-repeat expansions arise by hyperexpansion of long normal alleles (Cossée et al. 1997*b;* Montermini et al. 1997*a*), somewhat similar to the intergenerational expansion of premutation alleles seen in the other triplet-repeat diseases (Bidichandani and Patel 1996). A strong negative correlation was demonstrated between the age at onset and the size of the GAA triplet-repeat expansion in the smaller of the two expanded alleles (Dürr et al. 1996; Filla et al. 1996; Montermini et al. 1997*b*). The remaining patients are compound heterozygotes for the expansion and another pathological mutation in the *X25* gene (Campuzano et al. 1996; Filla et al. 1996; Bidichandani et al. 1997; Cossée et al. 1997*a*). Most point mutations generate null alleles, although elsewhere we have demonstrated that the G130V missense mutation

generates a mild loss-of-function FRDA allele associated with an atypically mild phenotype (Bidichandani et al. 1997). No molecular explanation has been causally related to the retention of deep-tendon reflexes seen in some patients or to the slow disease progression in Acadian FRDA patients (Montermini et al. 1997*b*).

Frataxin is a mitochondrial protein thought to regulate iron homeostasis. A yeast strain deficient for a homologous protein exhibits both excess mitochondrial iron accumulation and defective oxidative metabolism as a result of mitochondrial dysfunction (Babcock et al. 1997). Human frataxin is likely to serve a similar function, for the following reasons: its localization to the mitochondria, the demonstration of abnormal myocardial iron deposits in FRDA patients (Lamarche et al. 1993), and the pathological involvement of postmitotic tissues largely dependent on efficient oxidative metabolism (Harding 1993).

We present experiments performed in an effort to understand how the GAA triplet-repeat expansion in intron 1 of *X25* leads to FRDA. Our demonstration of a severe deficiency of *X25* steady-state mRNA levels in a series of patients homozygous for the GAA triplet-repeat expansion is consistent with the observed autosomal recessive inheritance. A single patient known to carry an expansion smaller than that seen in the average FRDA patient was shown to have a less severe deficiency, supporting the association of smaller expansions with milder disease. Using cloned repeats within the appropriate context of the *X25* intron 1 sequence derived from FRDA patients, we demonstrate that the GAA repeat produces a length- and orientation-dependent deficiency of in vitro transcription, directed by both prokaryotic and eukaryotic RNA polymerases under physiological conditions. Furthermore, using chemical probes, we demonstrate that the homopurine•homopyrimidine (pur•pyr) sequence resulting from the GAA triplet repeat, flanked by its natural intronic sequence, adopts an unusual DNA structure in vitro under physiological conditions.

Material and Methods

Antisense Riboprobe Preparation and RNase Protection Assay

For synthesis of antisense riboprobes, two regions of the *X25* cDNA, one containing partial sequence from exons 1 and 2 spliced together (+58 to +252 [Campuzano et al. 1996]; FRDA-riboX2) and the other containing partial sequence of exon 4 and complete sequence of exon 5b spliced together (+344 to +642 [Campuzano et al. 1996]; FRDA-riboA1), were subcloned into the pMOS*Blue* T-vector (Amersham). One microgram of *Eco*RI-linearized plasmid was used as a template for in vitro transcription using T7 RNA polymerase and the Ambion Maxiscript kit in a reaction containing 3.125 μM [α-^{32}P] UTP (Amersham). Full-length labeled antisense transcripts were purified after preparative denaturing PAGE. A radiolabeled human GAPDH antisense riboprobe (pTRI-GAPDH human; Ambion) was generated as a control. RNase protection assays were performed by use of the RPAII Ribonuclease protection assay kit (Ambion), according to the manufacturer's recommendations. Exposure to x-ray film was for 6 d at −70°C with intensifying screens. Sizes of the protected fragments were estimated by use of a sequence ladder that had been coelectrophoresed.

Cloning of GAA/TTC Repeat Sequences

Individual FRDA alleles containing GAA triplet-repeat sequences were amplified from two patients and from a heterozygous carrier. PCR was performed with primers (GAA-104F and GAA-629R) to amplify the GAA triplet repeat, including 251 bp and 248 bp of upstream and downstream intron 1 flanking sequence, respectively (Filla et al. 1996; GenBank accession number U43748). Both the normal allele from the heterozygous carrier (9 repeats) and the smaller of the two expansions from two patients (240 and 350 repeats) were gel purified and directly subcloned into the pCR3.1 eukaryotic TA cloning vector (Invitrogen). In order to minimize the chances of rearrangements mediated by the repeat sequences, bacterial cultures (TOP10F′; Invitrogen) were routinely grown by orbital shaking at room temperature. The pCR3.1 vector contains the cytomegalovirus (CMV) immediate-early promoter and the T7 promoter upstream of the multiple cloning site, for eukaryotic and prokaryotic gene expression, respectively. Inserts of various sizes were obtained, and, after direct sequencing of the plasmid vectors, four TTC repeats (reverse orientation) were selected for further analysis: $(TTC)_n$, where n = 9, 44, 75, and 100. Minor variations in the sequence of otherwise homogeneous TTC tracts were seen, but these did not alter the pur•pyr nature of the repeats. The exact sequences of the TTC tracts were as follows: $(TTC)_9$ = $(TTC)_9$; $(TTC)_{44}$ = $(TTC)_{35}$ $T(TTC)_3TCC(TTC)_5$; $(TTC)_{75}$ = $(TTC)_{66}T(TTC)_3TCC$ $(TTC)_5$; and $(TTC)_{100}$ = $(TTC)_{87}TCC(TTC)_3T(TTC)_3$ $TCC(TTC)_5$. Since all recombinants contained inserts in the reverse orientation, the four sequenced TTC inserts were flipped to obtain GAA inserts. This was done by PCR amplifying each of the four TTC repeats (again with GAA-104F and GAA-629R) and recloning them into the pCR3.1 vector. This resulted in inserts of either orientation. Minor changes in repeat length (with no mutations in the flanking sequence) led to the selection of four GAA repeats for further analysis: $(GAA)_n$, where n = 9, 46, 79, and 100. The exact sequences of the GAA

tracts were as follows: $(GAA)_9$ = $(GAA)_9$; $(GAA)_{46}$ = $(GAA)_5GGA(GAA)_3A(GAA)_{37}$; $(GAA)_{79}$ = $(GAA)_5GGA(GAA)_3A(GAA)_{70}$; and $(GAA)_{100}$ = $(GAA)_5GGA(GAA)_3A(GAA)_3GGA(GAA)_{87}$. Identical sequence interruptions were seen in the otherwise pure GAA/TTC sequence of four of five expanded TTC clones generated from the GAA-240 allele (including two not used for experiments in this paper), and these are distinct from those reported by Montermini et al. (1997*a*). All experiments were performed with the same plasmid maxipreps as were used for sequencing.

In Vitro Transcription Experiments

In vitro transcription of GAA/TTC repeat-containing constructs was performed with both T7 RNA polymerase (MAXIscript kit; Ambion) and HeLa nuclear extract (*In Vitro* Transcription system; Promega). Transcription using T7 RNA polymerase was performed as follows: 0.1 μg of *Xba*I-linearized plasmid was transcribed in a 20-μl volume by use of 0.5 mM of each of the three nonlimiting cold ribonucleotides, 10 units of T7 RNA polymerase in a buffer supplying 6 mM $MgCl_2$, and pH 7.5. For synthesis of the GAA-rich and UUC-rich transcripts, [α-^{32}P] rGTP and [α-^{32}P] rCTP (Amersham) were used at a final concentration of 2.4 μM each. Reactions were supplemented with 25 μM cold rGTP and rCTP, respectively, to facilitate the generation of full-length transcript. After incubation at 37°C for 1 h, DNA templates were digested by RNase-free DNase I (Ambion).

Transcription using HeLa nuclear extract (Promega) was performed exactly according to the manufacturer's recommendations. In brief, 0.1 μg *Xba*I-linearized plasmid was transcribed in a 25-μl volume by use of 0.4 mM of each of the three nonlimiting cold ribonucleotides, 8 units transcription-competent HeLa nuclear extract (Promega) in a buffer supplying 3 mM $MgCl_2$, and pH 7.9. For synthesis of the GAA-rich and UUC-rich transcripts, [α-^{32}P] rGTP and [α-^{32}P] rCTP (Amersham) were used at a final concentration of 1.0 μM each. Reactions were supplemented with 16 μM cold rGTP and rCTP, respectively, to facilitate the generation of full-length transcript. Reactions were performed at 30°C for 1 h, and the products were phenol-extracted and ethanol-precipitated.

The expected full-length runoff transcript sizes generated with the above-listed $(TTC)_n$ and $(GAA)_n$ constructs, by use of T7, are 671, 776, 869, and 944 nt and 671, 779, 881, and 944 nt, respectively. For transcripts expressed from the CMV promoter, the corresponding sizes are 689, 794, 887, and 962 nt and 689, 797, 899, and 962 nt, respectively. Products were denatured in formamide and were resolved by denaturing PAGE. Densitometric analysis was performed by use of the Collage™ image-analysis software package (Fotodyne). Densitometric readings were divided by the exact number of G's and C's expected in the full-length GAA-rich and UUC-rich transcripts, respectively, to normalize the results for the changes in transcript length (densitometric units/nt).

Chemical Probing of Repeats

Partial *X25* intron 1 sequences containing normal and expanded GAA repeats were amplified, gel purified (Qiaex II; Qiagen), and radiolabeled prior to chemical analyses. The GAA repeats were flanked by 0.4 kb and 1.1 kb upstream and downstream intron 1 sequence, respectively (Campuzano et al. 1996). Two expanded alleles, containing 240 and 350 GAA triplets, and six normal alleles isolated from unrelated heterozygous carriers were analyzed. As controls, 590 bp of the murine *Rapsyn* gene and 822 bp of the *Escherichia coli* β-galactosidase gene (Hanley and Merlie 1991) were similarly analyzed. DNA fragments were end labeled by use of [γ-^{32}P] ATP and either were purified by use of nucleotide-removal columns (Qiagen) or subsequently were digested with *Bam*HI (TTC strand labeled) or *Sma*I (GAA strand labeled), and gel-purified. Osmium tetroxide (0.025% in the presence of 3% pyridine for 1–2 h at 37°C; Aldrich) reactions and DEPC (1%–6% for 15 min at 37°C; Sigma) reactions were performed in TM10-7.4 buffer (10 mM Tris-HCl pH 7.4 and 10 mM $MgCl_2$). Osmium tetroxide reactions were also performed in five additional buffers, at pH 7.0–8.0 (Sigma). Hydroxylamine hydrochloride (2.3 M for 1–2 h at 37°C; Sigma) reactions were performed at pH 6, with diethylamine (Sigma) in the presence of 10 mM $MgCl_2$. Chemical modifications were followed by cleavage with piperidine (10% for 30 min at 90°C; Sigma) and resolution of cleavage products on 1% denaturing agarose gels in alkaline electrophoresis buffer (50 mN NaOH and 1 mM EDTA). Gels were treated with 7% trichloroacetic acid prior to drying and autoradiography.

Results

Severe Deficiency of Normally Spliced X25 *mRNA in FRDA Patients*

To investigate levels of *X25* mRNA in FRDA patients homozygous for the GAA triplet-repeat expansion, we performed RNase protection assays using two probes targeted to different regions of the mature *X25* transcript. Both probes, one recognizing the junction between exons 1 and 2 (FRDA-riboX2; fig. 1) and the other recognizing exons 4 and 5b (FRDA-riboA1; data not shown), revealed a striking reduction in steady-state *X25* mRNA levels in lymphoblasts from a series of FRDA patients. One patient (P63) however, revealed levels significantly greater than those seen in the other patients

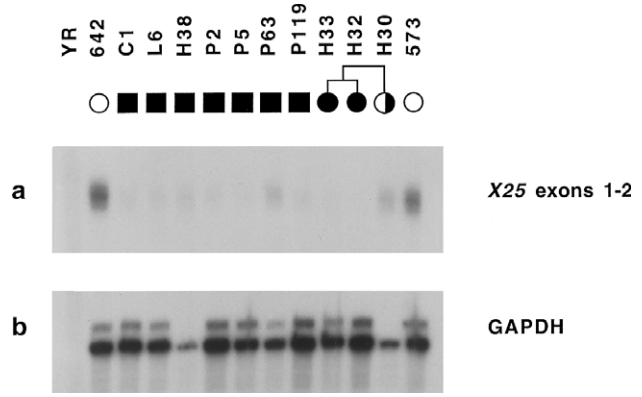

Figure 1 RNase protection assay of mature *X25* transcript, showing a marked deficiency in FRDA patients. RNase protection assays were performed by use of FRDA-riboX2 (*a*) and a control human GAPDH riboprobe (*b*). Total lymphoblast RNA from control individuals (*unblackened circles*), patients (*blackened symbols*), and a single carrier (*half-blackened circle*) were analyzed as indicated above the respective lanes. "YR" indicates the use of yeast RNA as a control. The FRDA-riboX2 probe detected a single protected fragment of 273 nt, corresponding to the spliced product of exons 1 and 2 (for exact cDNA positions, see Material and Methods). Except for P63, who showed a less severe deficiency of residual transcript, all FRDA patients show a marked reduction of mature *X25* mRNA. No other protected bands were visible.

tested in the same experiment (fig. 1). Assessment of the length of GAA repeat expansion by long-range PCR (Campuzano et al. 1996) revealed that all patients except P63 had 500–950 repeats in both their FRDA alleles. Patient P63 carried one expanded allele that was in the same size range as that seen in the other patients (530 triplets), whereas his other allele had a much smaller expansion, containing 240 repeats.

In addition to mRNA quantitation, the riboprobe FRDA-riboX2 was specifically designed to investigate the splicing status of intron 1. If the abnormally expanded intron 1 were to be misspliced, individual exon 1–only and exon 2–only protected fragments would be expected. However, only the normally spliced product of exons 1 and 2 was detected, indicating that the abnormal expansion of intron 1 in FRDA does not significantly interfere with *X25* mRNA processing. It should be noted that this is not a formal exclusion of missplicing, since it is possible for the anomalously spliced isoform(s) to be unstable and therefore undetectable in this assay.

Prokaryotic and Eukaryotic In Vitro Transcription of GAA Triplet Repeats: Length- and Orientation-Dependent Deficiency

Using PCR products generated from FRDA patients and normal controls, we were able to clone four lengths each of $(GAA)_n$ (n = 9, 45, 79, and 100) and $(TTC)_n$ (n = 9, 44, 75, and 100) repeats into a suitable expression vector that would allow in vitro transcription using both prokaryotic (T7) and eukaryotic (CMV immediate early) promoters (fig. 2*A*). The exact sequence of these repeats were determined by direct sequencing of the plasmid vectors (see Material and Methods). In addition to the actual triplet-repeat sequence, these inserts also contain ~250 bp of intron 1 flanking sequence on either side, creating a natural sequence context as compared with the analysis of pure synthetic trinucleotide-repeat elements. Using these constructs, we sought to investigate the relative efficiencies of transcription of various lengths of GAA and TTC triplet repeats, in an attempt to determine whether the GAA expansion posed a particular hindrance.

Figure 2*A* shows a diagrammatic representation of the repeat-containing constructs and the two promoters used, as well as the site of linearization permitting runoff in vitro transcription. As seen in figure 2*B* and *C*, a deficiency was seen in the transcription of expanded GAA repeats, by use of both the prokaryotic T7 promoter and the eukaryotic CMV promoter. It is important to note that these assays involve the incorporation of radiolabeled rNTPs and that the increase in length of the transcript therefore results in an underestimation of the actual deficiency. Normalization for the changes in transcript length was therefore performed (see Material and Methods) and was used to plot the graphs in figure 2*B* and *C*. These transcription experiments were performed twice, on two independent occasions, yielding identical results.

There were four significant features displayed by the observed transcriptional deficiency. First, it was observed at physiological pH and magnesium-ion concentration. Second, it was length dependent, becoming significant only after a threshold was reached—that is, when the two larger GAA repeat lengths (79 and 100) were transcribed. Third, the effect on transcription was orientation specific, wherein the synthesis of GAA-rich transcript—that is, the physiological direction of transcription—was clearly more severely affected. Synthesis of UUC-rich mRNA was either relatively unchanged (fig. 2*C*) or more mildly affected (fig. 2*B*) over the lengths used in these experiments. Minor variation in the profiles of transcription deficiency observed in figure 2*B* and *C* possibly reflect the differing dynamics of the prokaryotic and eukaryotic systems used. The increase in band intensity seen with synthesis of longer UUC RNA by use of HeLa nuclear extract (fig. 2*C*) most likely reflects the increased incorporation of radiolabel, because of increase in transcript length, which is not seen after normalization. Fourth, the reduction in transcription was observed with use of linear templates and is therefore independent of DNA template superhelicity. Taken together, these results are consistent with the observed

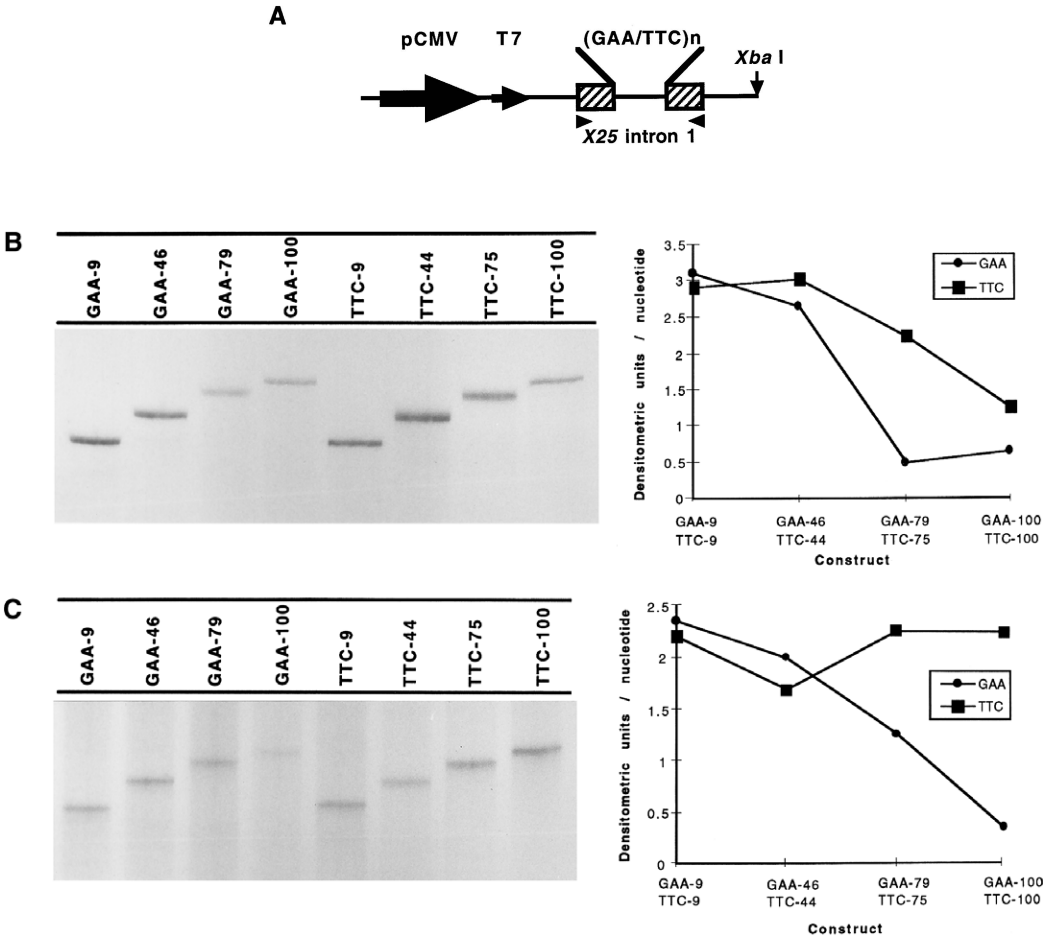

Figure 2 Expansion of GAA triplet repeat interfering with transcription. *A,* Diagrammatic representation of recombinant constructs containing GAA/TTC triplet repeats (not drawn to scale). A partial sequence of *X25* intron 1 (*hatched boxes*) is shown containing *n* copies of either the GAA (correct orientation) triplet repeat or the TTC (reverse orientation) triplet repeat. Intron 1 sequences were amplified by PCR primers (*arrowheads*) and inserted in the TA-cloning site of pCR3.1 (Invitrogen). The CMV immediate early promoter (pCMV) and the T7 promoters are indicated by large and small arrows, respectively. Plasmids were linearized at the unique *Xba*I site, to permit runoff in vitro transcription. For the exact sequence of repeats and PCR primers used, see Material and Methods. *B,* In vitro transcription using T7 RNA polymerase and the indicated GAA/TTC repeat–bearing constructs as templates. Products of transcription were resolved by denaturing PAGE and relative band intensities analyzed by densitometry. Densitometric readings were normalized for length of the full-length transcript and were plotted as densitometric units/nucleotide. A marked reduction is seen in the accumulation of full-length transcript when the GAA repeat length is increased from 46 to 79 triplets. A less severe reduction is seen with comparable increases in TTC repeat length. *C,* In vitro transcription using HeLa nuclear extract and the indicated GAA/TTC repeat–bearing constructs as templates. Products of transcription were resolved and analyzed as in *B*. A marked reduction is seen in the accumulation of full-length transcript when the GAA repeat length is increased from 46 to 100 triplets. No reduction is seen with comparable increases in TTC repeat length. The increase in band intensities seen with longer TTC clones is thought to reflect the length-dependent increase in incorporation of radiolabel, since this was not seen after normalization. Truncated transcripts were not detected.

length-dependent deficiency of normally spliced mature *X25* mRNA in FRDA patients' lymphoblasts.

The GAA Triplet Repeat within Its Natural Flanking Intronic Sequence: Adoption of an Unusual DNA Structure In Vitro

Pur•pyr sequences such as the GAA triplet repeat are known to form intramolecular triplexes in supercoiled plasmids (see Discussion). However, since reduced synthesis of the GAA-rich mRNA was observed after transcription of linearized DNA templates, we sought to investigate whether the GAA repeat adopts an unusual DNA structure without a source of supercoiling. With osmium tetroxide, hydroxylamine, and DEPC used as chemical probes for the detection of such unusual DNA structures, linear DNA molecules representing normal and expanded alleles, each flanked by intron 1 sequence (~0.4 kb upstream and ~1.1 kb downstream of the GAA

repeat sequence, respectively), were assayed in the presence of physiological pH and cationic conditions.

The above-mentioned chemical probes have been widely used for the detection and finer characterization of unusual DNA structures, including intramolecular DNA triplexes, left-handed Z DNA, and cruciforms, where they detect unpaired regions or regions of altered DNA conformation (Wells et al. 1988). Neither chemical is found to modify regular right-handed B DNA, although their reactivity with specific bases is significantly enhanced if unpaired or altered in conformation; osmium tetroxide reacts with pyrimidines (mainly thymine), hydroxylamine with cytosines, and DEPC with purines (adenine > guanine). The chemically modified bases are then readily detected after cleavage with piperidine, as in standard Maxam-Gilbert sequencing reactions. Most investigators resolve their reaction products on sequencing gels, to determine exact base positions of the hyperreactive sites. In our experiments, we used relatively long DNA repeat sequences (1.5–3.0-kb fragments containing 9–350 GAA repeats), and therefore resolution was performed on denaturing (alkaline) agarose gels, resulting in thicker or fuzzier cleavage bands (possibly representing a collection of bands differing only slightly in length), allowing only approximate mapping of hyperreactive sites.

As seen in figure 3*A,* the normal, $(GAA)_9$, and the expanded $(GAA)_{240}$, DNA molecules show cleavage after being probed with osmium tetroxide and DEPC, indicating that the DNA in this region adopts a non–B DNA structure. Another expanded allele, consisting of 350 GAA repeats, and five additional alleles with normal repeat lengths, isolated from unrelated heterozygous carriers, were also shown to be similarly hyperreactive by use of these chemical probes (data not shown). Probing of the $(GAA)_9$ repeat with hydroxylamine produced the same cleavage pattern as was seen with osmium tetroxide (fig. 3*B*). The single cleavage product seen with osmium tetroxide and with hydroxylamine and the smaller of the two products seen with DEPC mapped to the homopyrimidine and homopurine strands of the GAA sequence, respectively. Identical analyses with unrelated control sequences, originating from the murine *Rapsyn* gene and from the *E. coli* β-galactosidase gene, did not reveal any hyperreactive sites (data not shown).

A 38-bp A-rich sequence, $(A)_6(TAA)_4AT(A)_5T(A)_6T(A)_4T$, was revealed on examination of the flanking intronic sequence in the region of the second (larger) DEPC cleavage product (located ~150 bp 3′ of the GAA repeat; GenBank accession number U43748). For better localization of the observed hyperreactive sites and for confirmation of the location of the larger DEPC cleavage product, portions of the flanking intronic sequence were removed by digestion with restriction enzymes prior to chemical probing (see Material and Methods). As seen in figure 3*C,* the osmium tetroxide hyperreactive site mapped to the homopyrimidine strand of the GAA repeat. Prior to being probed with DEPC, the radiolabeled DNA template was cleaved at the unique *Sma*I site, separating the GAA repeat from the A-rich sequence (fig. 3*F*), and the DNA fragment containing the former (purine strand labeled) was gel purified. This time only one DEPC cleavage product was generated (with use of two different concentrations of DEPC), which mapped to the homopurine strand of the GAA repeat (fig. 3*D*). Significantly, the pyrimidine-specific and purine-specific chemical probes did not react with bases on the opposite strands (fig. 3*C* and *D*). Hyperreactivity to chemical probes was tested and detected at pH 6.0–8.0. Hydroxylamine treatment was performed at pH 6.0 (see Material and Methods), which showed a cleavage pattern identical to that seen with osmium tetroxide in buffers at pH 7.0–8.0 (fig. 3*B* and *E*).

As mentioned above, it was not possible to map the exact base locations of the hyperreactive sites within the GAA repeat. However, the well-known base specificities of the chemical probes allow us to surmise that osmium tetroxide, hydroxylamine, and DEPC detected unpaired T's, C's, and A's in the respective complementary strands of the GAA repeat sequence (Wells et al. 1988). These results (summarized in fig. 3*F*) indicate that the region of the GAA repeat (flanked by the natural intron 1 sequence) adopts an unusual, non–B DNA structure in the absence of any source of supercoiling under physiological conditions (see Discussion). Although these results are consistent with the formation of an intramolecular DNA triplex, the approximations involved in our experiments do not permit such a conclusive determination. Consequently, these results are also consistent with other unusual structures, such as slipped DNA (Pearson and Sinden 1996), heteronomous DNA (Evans and Efstratiadis 1986), and distortions of duplex DNA that are seen at duplex-triplex junctions flanking intermolecular DNA triplexes (Hartman et al. 1992). It is, however, not consistent with the J DNA conformation observed with the $(GA)_{18}$ sequence at mildly alkaline pH (Htun and Dahlberg 1988).

Discussion

We have shown that FRDA patients homozygous for the GAA expansion have a severe deficiency of mature *X25* mRNA, with no evidence of abnormal splicing of the expanded intron 1. This is expected to lead to a deficiency of frataxin, and affected tissues could suffer the same mitochondrial dysfunction seen in yeast strains deficient in a frataxin homologue (Babcock et al. 1997). We have also shown, under physiological conditions, that the GAA repeat results in a length-dependent deficiency of transcription in vitro, using both prokaryotic

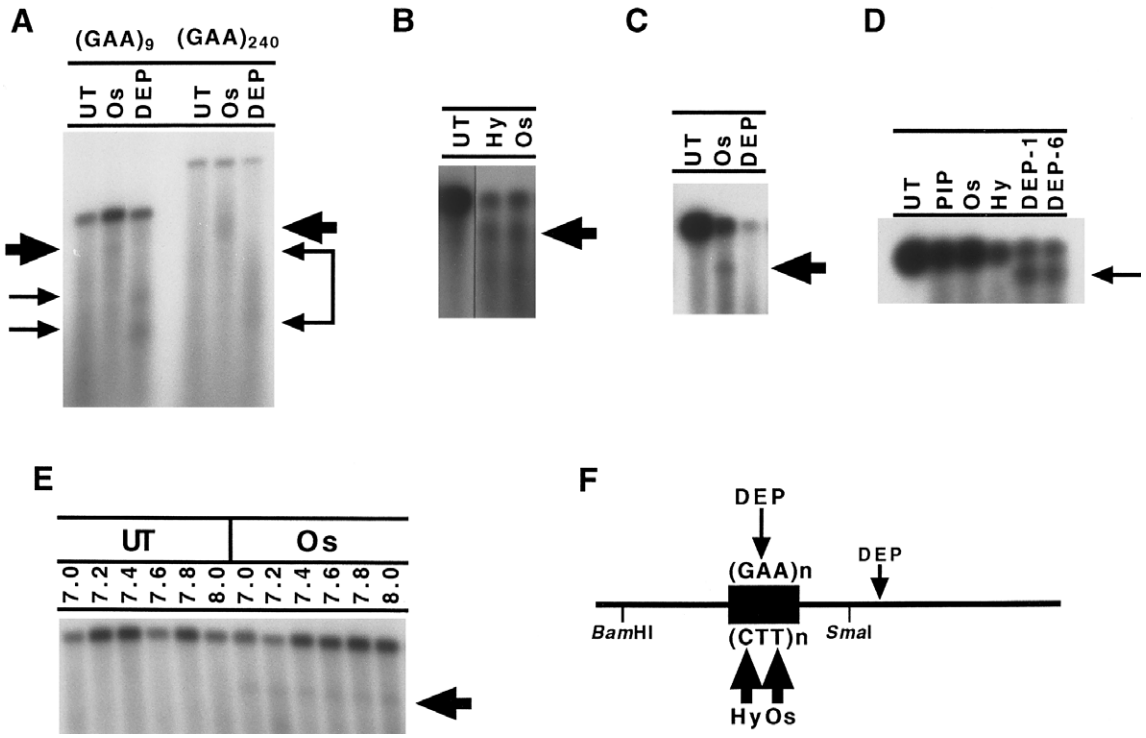

Figure 3 Adoption of an unusual DNA structure in vitro by the GAA triplet repeat within *X25* intron 1. Chemical probing of the $(GAA)_9$ repeat is shown in *A–E,* and that of the $(GAA)_{240}$ repeat is shown in *A*. DNA sequences were analyzed with both strands labeled (*A, B,* and *E*) and with either the pyrimidine-rich (*Bam*HI-cleaved [*C*]) or the purine-rich (*Sma*I-cleaved [*D*]) strands labeled. The repeats were analyzed within the natural context of their flanking *X25* intron 1 sequence. Reaction products were resolved on denaturing (alkaline) agarose gels. Chemical treatment was performed with osmium tetroxide (Os), hydroxylamine (Hy), and DEPC (DEP). "DEP-1" and "DEP-6" denote samples treated with 1% DEPC and 6% DEPC, respectively. "UT" and "PIP" refer to untreated DNA molecules and to molecules treated with piperidine without any prior chemical modification, respectively. In *E,* the numbers (7–8) indicate the pH of the reaction buffers used. Arrows represent the presence of cleavage products. Thick and thin arrows indicate cleavage with a pyrimidine-specific chemical (Os or Hy) and a purine-specific chemical (DEPC), respectively. As seen in *A,* the $(GAA)_{240}$ repeat was cleaved with both osmium tetroxide and DEPC (seen as a smear), indicating that both the GAA and TTC strands are unpaired or altered in conformation (non–B DNA). The $(GAA)_9$ repeat similarly shows cleavage with osmium tetroxide (*A–C* and *E*), DEPC (*A* and *D*), and hydroxylamine (*B*), indicating that the normal (non-FRDA) GAA triplet-repeat length also adopts an unusual DNA structure. Notably, the pyrimidine-specific chemical (C) and the purine-specific chemical (*D*) do not react with bases on opposite strands. Hydroxylamine (at pH 6) and osmium tetroxide (at pH 7–8) produce identical cleavage products (*B* and *E*). DEPC reacts with the purine strand of the GAA repeat and with an A-rich sequence element situated ~150 bp downstream of the GAA repeat (*A* and *F;* see text). The results of the various chemical probings are summarized in *F.* The GAA repeat (*blackened box*) is shown within the region of the *X25* intron 1 analyzed (*horizontal line*). The positions of the unique *Bam*HI and *Sma*I restriction sites are indicated. Thick and thin arrows are used to indicate pyrimidine- and purine-specific cleavages, respectively. The smaller thin arrow indicates the cleavage at the A-rich sequence located downstream of the GAA repeat and the *Sma*I site. The positions of the arrows within the GAA repeat are arbitrary and do not reflect sublocalization to either the center or either end of the repeat tract (see text).

and eukaryotic systems, and that the GAA trinucleotide repeat adopts an unusual DNA structure. In the course of the probing of the GAA repeat sequence, an A-rich sequence element was discovered, in the vicinity of the GAA repeat, that also adopts an unusual structure in vitro. In addition to the expanded GAA repeats, the unusual DNA structure was also detected in normal repeat-bearing alleles, which are apparently transcribed normally. Our hypothesis is that the unusual DNA structure formed by the expanded allele is likely to be more stable—and that it would thus pose a more formidable block to transcription.

We have determined, using both prokaryotic and eukaryotic RNA polymerases in vitro, that the threshold for the transcriptional deficiency is <79 GAA repeats. Because of the inherent instability and recombinogenic nature of the GAA triplet repeat within plasmids in *E. coli,* the largest stable repeat cloned consisted of 100 GAA triplets. However, the $(GAA)_{79}$ and $(GAA)_{100}$ repeat alleles used in our transcription experiments are both larger than the smallest expansion associated with FRDA (i.e., GAA_{66}). Using a series of cloned repeats, we have successfully demonstrated the biologically significant negative correlation between increase in GAA re-

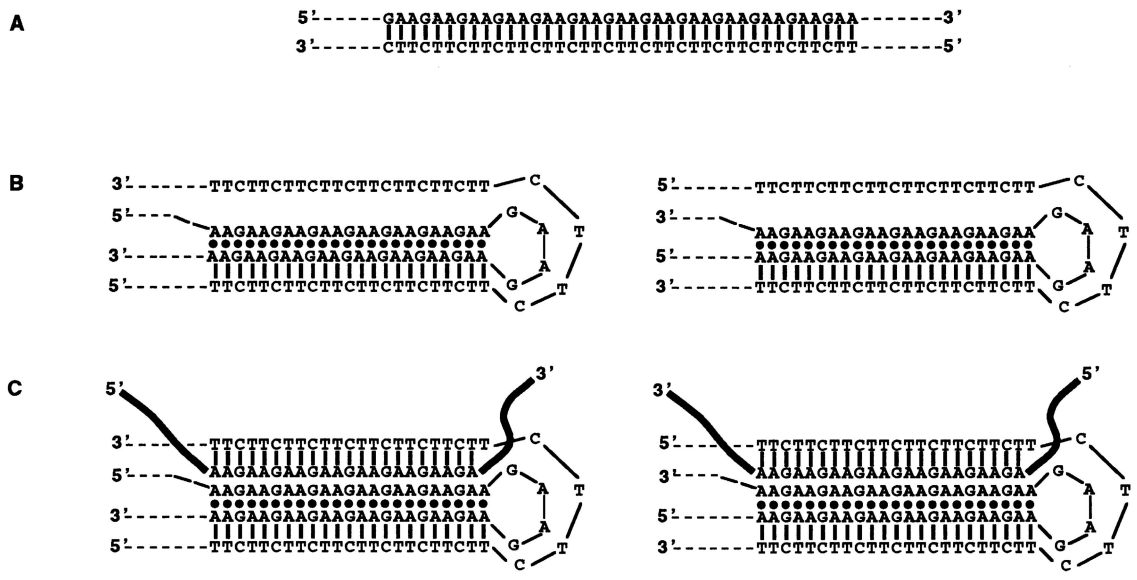

Figure 4 Possible non–B DNA structures adopted by the pur•pyr tract, resulting from the GAA repeat sequence. Although FRDA alleles may possess hundreds of GAA triplets, only 13 or 17 repeats are shown in these models, for the sake of simplicity. *A,* Pur•pyr tract, shown as a linear duplex with normal Watson-Crick base pairing indicated (*vertical bars*). *B,* Intramolecular pur-pur•pyr triplex, which could result if the 5′ or 3′ half of the purine-rich strand dissociates from its complementary duplex strand and intercalates in an antiparallel orientation, in the major groove of the 3′ or 5′ half of the pur•pyr tract, by reverse Hoogsteen pairing (•). These are the so-called r5 (*left*) and r3 (*right*) configurations, respectively. Although pyr-pur•pyr intramolecular triplex (i.e., H DNA) formation is also theoretically possible, this does not form at physiological pH and in the presence of divalent cations and therefore has not been considered. *C,* Pur-pur•pyr intramolecular triplex, after formation of the GAA-containing *X25* pre-mRNA (*unbroken line*). This could pair with the single-stranded half of the homopyrimidine strand via Watson-Crick bonds (*vertical bars*), further stabilizing the triplex structure. These triplex models are compatible with physiological conditions and could theoretically form in vivo.

peat length and accumulation of full-length transcript. This length dependency is consistent with (*a*) the observation of a milder deficiency of *X25* mRNA in lymphoblasts isolated from a patient with a smaller-than-average expansion and, more significantly, (*b*) the inverse correlation between age at onset and GAA repeat length (Dürr et al. 1996; Filla et al. 1996; Montermini et al. 1997*b*). The deficiency of in vitro transcription seen in our heterologous system implies that the reduction of *X25* mRNA in FRDA is likely to be transcriptional rather than posttranscriptional. The length-dependent deficiency further implies an acquisition of additional stability by longer GAA sequences, possibly via some higher-order structure. Although we have only compared transcription of GAA repeats with TTC repeats of similar length, it would be interesting to also generate comparative data with other similarly expanded sequences.

Why is the transcriptional block apparent or more severe when synthesis of GAA-rich transcript is attempted? A similar observation has also been made by Grabczyk and Fishman (1995), who coined the term "transcriptional diode" to describe the unidirectional transcriptional blockade observed with a 0.5-kb sequence containing three interspersed pur•pyr tracts, immediately upstream of the rat *GAP-43* gene. Pur•pyr sequences form triplexes in supercoiled DNA (Frank-Kamenetskii and Mirkin 1995), structures that can efficiently block transcriptional elongation (Escude et al. 1996; Giovannangeli et al. 1996). Given that transcription itself induces a local wave of negative supercoiling behind the polymerase (Wu et al. 1988), Grabczyk and Fishman (1995) speculated that a transcription-induced triplex was being formed. The unidirectionality was attributed to the differential stabilities, at physiological pH, of the pur-pur•pyr triplex, predicted to form when the purine-rich strand is transcribed, versus the pyr-pur•pyr triplex, in the opposite direction (Frank-Kamenetskii and Mirkin 1995). Our observation that the GAA repeat already maintains an unusual DNA structure prior to transcription would argue against this hypothesis. However, it is possible that transcription may serve to further stabilize the already altered DNA structure or perhaps even induce a novel structure.

The newly synthesized transcript could also help explain the function of the GAA repeat as a transcriptional diode. If it is assumed that the GAA repeat (fig. 4*A*) forms a pur-pur•pyr intramolecular DNA triplex (fig. 4*B*), which is known to be stable at physiological pH, the newly synthesized GAA-rich mRNA (as seen in vivo) could form a hybrid RNA-DNA duplex with the re-

sulting free TTC single strand (fig. 4*C*), further stabilizing the unusual DNA structure (Reaban and Griffin 1990, 1991; Stavnezer 1991). On the other hand, transcription of the TTC strand would not be able to serve this function, since the analogous situation would require the formation of a pyr-pur•pyr triplex, unlikely to form at physiological pH and divalent cationic concentration (used in our experiments). This would appear to be the most likely mechanism; however, the possibility that the unusual DNA structure is not a triplex cannot be totally disregarded.

Ohshima et al. (1996*a*) have predicted another scenario, whereby the GAA-rich *X25* mRNA forms an antiparallel (pur-pur•pyr) intermolecular RNA-DNA•DNA triplex (formed via A-A•T and G-G•C triplets). This would also explain the unidirectional block to transcription, since the parallel counterpart would be unstable at physiological pH and cationic concentration (Frank-Kamenetskii and Mirkin 1995). However, this model may be unlikely, for the following reasons. First, systematic analyses have shown that, in dramatic contrast to the ability of RNA to be involved in pyr-pur•pyr triplexes, a stable pur-pur•pyr triplex could not form with RNA in any of the three strands (Skoog and Maher 1993; Semerad and Maher 1994). Second, using electrophoretic-mobility-shift assays (EMSA), we were unable to demonstrate the formation of parallel or antiparallel intermolecular DNA triplexes, using a cloned $(GAA)_{11}$ repeat sequence (at pH 7.4 in the presence of 10 mM $MgCl_2$; authors' unpublished results). This appears to be a peculiarity of the GAA triplet repeat per se, since we could readily demonstrate antiparallel triplex formation with four other random pur•pyr sequences of similar length that were from the promoter regions of the *PMP22* gene, using EMSAs and footprinting analyses (S. I. Bidichandani and P. I. Patel, unpublished results).

Our chemical probing experiments demonstrated the presence of an unusual DNA structure in linear DNA molecules. There seems to be a sharp divide in the literature regarding the role of superhelicity in the etiology of non–B DNA structures adopted by pur•pyr sequences. Although some investigators have certainly shown that linear molecules containing pur•pyr sequences adopt such structures (Hentschel 1982; Htun et al. 1984; Evans and Efstratiadis 1986; McCarthy and Heywood 1987), others have maintained that supercoiling is a prerequisite (Hanvey et al. 1988; Kohwi and Kohwi-Shigematsu 1988; Voloshin et al. 1988; Bernués et al. 1989). They have used widely varying parameters, differing in pur•pyr sequence composition and length, pH, and ionic conditions, and have used chemical or enzymatic probes. Evans and Efstratiadis (1986) showed a positive correlation between cleavage of a linear pur•pyr sequence and increasing length, with $(GA)_{38}$ being clearly more reactive than $(GA)_{20}$ or $(GA)_{10}$. A similar positive correlation was also seen with lower monovalent cationic concentrations. Htun and Dahlberg (1989) showed that DNAs that are relaxed or that show low negative supercoiling form a different triplex conformer, compared to what occurs when negative supercoiling is moderately high. Another fundamental difference between the molecules used in our experiments and those used by others is that we have a considerable length of natural *X25* intron 1 sequence flanking the GAA repeat. Both the composition (Kang et al. 1992) and length (Evans and Efstratiadis 1986) of flanking sequences have been shown to play significant roles in the kinetics of non–B DNA structure formation. Modest alterations mediated by restriction digestion did not alter the cleavage pattern in our experiments.

The 38-bp A-rich sequence (79% A; 100% A/T) situated ~150 bp downstream of the GAA repeat (on the GAA strand) was also found to be hyperreactive to DEPC treatment. This sequence represents the 3′ end of the right monomer unit of the *Alu* repeat within which the GAA repeat is located. Most *Alu* elements end in a 10–48 bp A-rich sequence often interrupted by sporadic C's and G's (Kariya et al. 1987), and so this 38-bp A/T sequence may be somewhat unique. The reactivity of this sequence is analogous to that seen at the A/T-rich sequence situated immediately downstream of the chicken embryonic myosin heavy-chain gene (McCarthy and Heywood 1987). This latter sequence was sensitive to S1 nuclease at pH 4.5 and 7.5, in both supercoiled and linear plasmid DNA. These structures may also be related to the open helical structure adopted by the TAA triplet repeat, although this was not detected in linear DNA molecules or at physiological magnesium concentrations (Ohshima et al. 1996*b*). The significance of any structure adopted by the 38-bp A-rich sequence is presently unknown. This sequence is not likely to play a significant role in the inhibition of transcription, since pure GAA repeats (without any flanking intron 1 sequence) have previously, in the early stages of this project (Bidichandani et al. 1996), been found to display the same unidirectional transcriptional blockade. Furthermore, we have determined that this A/T-rich sequence is polymorphic, with a deletion of $(TAA)_2$ within the $(TAA)_4$ tract close to its 5′ end. This was seen in 3 of 36 normal chromosomes and in 0 of 20 expanded chromosomes. This polymorphism was also independently observed by Montermini et al. (1997*a*).

The causal relationship between the unusual DNA structure and the transcriptional deficiency remains to be formally proved. With all the data taken together, it would be expected that the stable unusual DNA structure arising from larger GAA expansions would interfere with transcriptional elongation. Triplexes are known to effectively block transcriptional elongation (Escude et al.

1996; Giovannangeli et al. 1996). However, the absence of any truncated products in our gels leaves open the possibility that there may be direct or indirect interference with transcriptional initiation. Situated within the first intron of *X25,* the expansion could directly influence the assembly of the eukaryotic transcriptional machinery. On the other hand, several genes are known to contain transcriptional enhancers within their first introns. The GAA triplet repeat maps within an *Alu* repeat element, and there is growing evidence indicating transcriptional regulatory roles for such sequences, within several genes (Britten 1996). It is possible that the *Alu* repeat containing the normal polymorphic GAA triplet repeat serves as an enhancer, which is disrupted by the expansion seen in FRDA alleles. However, this possibility has been tentatively ruled out by testing this segment for enhancer activity in transient-transfection experiments using a mammalian fibroblast cell line (authors' unpublished data).

Our findings raise the possibility that the GAA triplet repeat in the *X25* gene (and, by inference, also in other genomic locations) may adopt non–B DNA structures in vivo. Although it remains to be proved, this "epigenetic" modification could be the ultimate molecular basis of FRDA. If so, altering the stability of such a structure could help alleviate the transcriptional block and serve as a potential therapeutic strategy for FRDA. Moreover, the naturally occurring spectrum of GAA repeat–bearing alleles seen in FRDA chromosomes could serve as a valuable resource for studying the effect of pur•pyr sequences on DNA structure, recombination, and replication in vivo.

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