DNA sequence-specific polyamides alleviate transcription inhibition associated with long GAA·TTC repeats in Friedreich's ataxia

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The DNA abnormality found in 98% of Friedreich's ataxia (FRDA) patients is the unstable hyperexpansion of a GAA·TTC triplet repeat in the first intron of the frataxin gene. Expanded GAA·TTC repeats result in decreased transcription and reduced levels of frataxin protein in affected individuals. β-Alanine-linked pyrrole–imidazole polyamides bind GAA·TTC tracts with high affinity and disrupt the intramolecular DNA·DNA-associated region of the sticky-DNA conformation formed by long GAA·TTC repeats. Fluorescent polyamide-Bodipy conjugates localize in the nucleus of a lymphoid cell line derived from a FRDA patient. The synthetic ligands increase transcription of the frataxin gene in cell culture, resulting in increased levels of frataxin protein. DNA microarray analyses indicate that a limited number of genes are significantly affected in FRDA cells. Polyamides may increase transcription by altering the DNA conformation of genes harboring long GAA·TTC repeats or by chromatin opening.

gene regulation | triplet repeat expansion | frataxin

The neurodegenerative disease Friedreich's ataxia (FRDA) is caused by hyperexpansion of GAA·TTC repeats in the first intron of a nuclear gene that encodes the essential mitochondrial protein frataxin (1–4). Normal frataxin alleles have 6–34 repeats, whereas FRDA patient alleles have 66–1,700 repeats. Intronic GAA·TTC repeats interfere with gene transcription (5–7). Longer repeats cause a more profound frataxin deficiency and are associated with earlier onset and increased severity of the disease (4). Biochemical studies have documented that expanded GAA·TTC repeats adopt unusual non-B DNA structures, such as triplexes, containing two purine GAA strands along with one pyrimidine TTC strand, flanking a single-stranded pyrimidine region (5, 8) as well as intramolecular "sticky" DNA (6, 9–12). Long $(GAA{\cdot}TTC)_n$ repeat sequences form sticky DNA with two separate long $(GAA{\cdot}TTC)_n$ repeating tracts associated within a single closed plasmid DNA. The interaction of the two tracts requires the repeats oriented in the direct repeat orientation, negative supercoiling and the presence of divalent metal ions to stabilize the DNA·DNA-associated region (8–10). We have demonstrated the capacity of sticky DNA to form both *in vitro* (10, 13) and *in vivo* (11–13). Triplexes and/or sticky DNA block elongation by RNA polymerase II (5). Another study using artificial transgenes has shown that expanded GAA·TTC repeats induce repressive heterochromatin *in vivo* in a manner reminiscent of position-effect variegation (14). This effect was augmented by coexpression of heterochromatin protein 1. Here, we address whether minor-groove DNA-binding small molecules can alleviate transcription repression of the frataxin gene.

Molecules that reverse triplex/sticky DNA and/or heterochromatin formation in the frataxin gene may increase successful elongation through expanded GAA·TTC repeats, thereby relieving the deficiency in frataxin mRNA and protein in FRDA cells (11, 14, 15). Pyrrole–imidazole polyamides are cell-permeable small molecules that can be programmed to recognize a broad repertoire of DNA sequences (16). Two classes of polyamides are well established: hairpin polyamides bind mixed-sequence DNA with high affinity and specificity (16, 17), and linear β-alanine-linked polyamides are available for targeting purine tracts of DNA, such as GAGAA·TTCTC repeats (18, 19). β-Alanine-linked polyamides have been shown to bind GAGAA repeats in *Drosophila* satellite DNA both *in vitro* and in cytological chromosome spreads (19). These molecules induce chromatin opening and reverse heterochromatin-mediated position-effect gene silencing when administered to *Drosophila* embryos (19, 20).

Structural studies indicate that β-alanine-linked polyamides bind the minor groove of canonical B DNA (21). Given the high affinity of β-alanine-linked polyamides for purine tracts (18), these molecules might act as a thermodynamic "sink" and lock GAA·TTC repeats into double-stranded B DNA. Such an event would disfavor duplex unpairing, which is necessary for formation of FRDA triplexes and sticky DNA. Although single polyamides bound within coding regions of genes do not appear to block transcription elongation (22–24), we cannot be certain that multiple polyamides have the potential to relieve transcription repression at expanded GAA·TTC repeats. Alternatively, polyamides may relieve heterochromatin-mediated repression by opening the chromatin domain containing the frataxin gene (20).

Results and Discussion

Targeting GAA·TTC Repeat DNA with High-Affinity Ligands. We synthesized two β-alanine-linked polyamides of different length, FA1 (ImPyβImPyβImβDp, where Py is pyrrole, Im is imidazole, β is β-alanine, and Dp is dimethylaminopropylamine) to target the 9-bp site 5′-AAGAAGAAG-3′ and FA3 (ImPyβImPyβImPyβImβDp) to target the 12-bp site 5′-AAGAAGAAGAAG-3′ (Fig. 1*A*). Quantitative DNase I footprinting (25) demonstrates that FA1 binds to a radiolabeled PCR product containing a $(GAA{\cdot}TTC)_6$ sequence with an apparent dissociation constant (K_d) of 0.1 nM (Fig. 2*A* and Table 1). FA3 exhibits a K_d of ≈3 pM in footprinting experiments performed at low DNA concentrations (Fig. 6*A*, which is published as supporting information on the PNAS web site, and Table 1); however, this value may be an underestimation of the affinity of this molecule for GAA·TTC repeat DNA, because our K_d measurements are limited by a minimum DNA concentration of ≈2 pM in the binding reaction. Two mismatch controls, FA2 (ImPyβImImβPyβDp) (Fig. 1*A*) and FA4

Conflict of interest statement: No conflicts declared.

Abbreviations: FRDA, Friedreich's ataxia; qRT-PCR, quantitative RT-PCR.

Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE5040).

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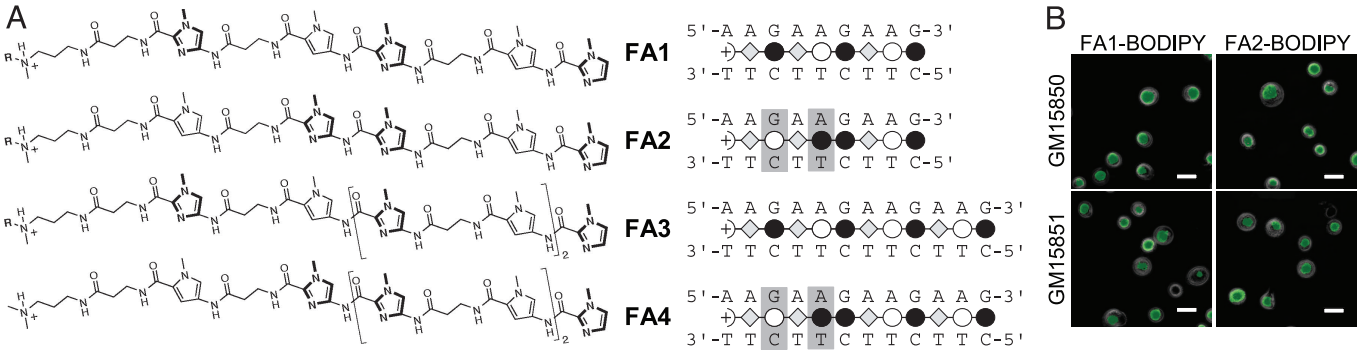

Fig. 1. Polyamide structures, binding models, and nuclear localization in cell culture. (*A*) Structures of polyamides FA1, FA2, FA3, and FA4 (R, methyl) and their Bodipy conjugates (R, *N*-propylbutanamide-linked Bodipy FL). Polyamide structures are represented schematically as binding models. Filled and open circles are Im and Py rings, respectively; diamonds are β-alanine; and the semicircle with plus sign is dimethylaminopropylamine. Linear polyamides bind in a carboxyl-to-amino-terminal orientation with respect to the 5′-to-3′ sequence of their DNA target site (18). Mismatches formed with polyamides FA2 and FA4 are indicated with shaded boxes. (*B*) Deconvolution microscopy of unfixed lymphoid cells (GM15850, derived from an FRDA patient, and GM15851, derived from a healthy sibling) incubated with Bodipy conjugates of each of the indicated polyamides at 2 μM concentration in culture medium for 16 h before visualization, as described (44). (Scale bars, 10 μm.)

(ImPyβImPyβImImβPyβDp), have binding affinities for the $(GAA{\cdot}TTC)_6$ target sequence that are three orders of magnitude lower than those of the match polyamides (Figs. 2*B* and 6*B* and Table 1). FA1 is also able to bind extended regions of GAA·TTC repeats [$(GAA{\cdot}TTC)_{33}$] with no loss in affinity, with several molecules of FA1 bound per DNA molecule (Fig. 7, which is published as supporting information on the PNAS web site). As another test for sequence specificity, footprinting experiments with FA1 and FA3 and a radiolabeled DNA fragment containing a mismatch DNA sequence (5′-. . . AGGAG-GAGGTGGAGGAGGA. . . -3′) were performed. Neither FA1 nor FA3 bound this DNA sequence at polyamide concentrations up to 100 nM (Fig. 8, which is published as supporting information on the PNAS web site). FA1 binds other polypurine DNA tracts with single G-to-A transitions, maintaining similar or slightly reduced binding affinities compared with the 5′-AAGAAGAAG-3′ site. A sequence harboring two transitions (A-to-G and G-to-A), giving rise to 5′-AAAAGGAAG-3′, increases the K_d of FA1 by more than three orders of magnitude. Truncation of FA1 to ImPyβImβDp increases its K_d on 5′-AAGAAGAAG-3′ by more than four orders of magnitude (P.B.D. and J.W.P., unpublished data).

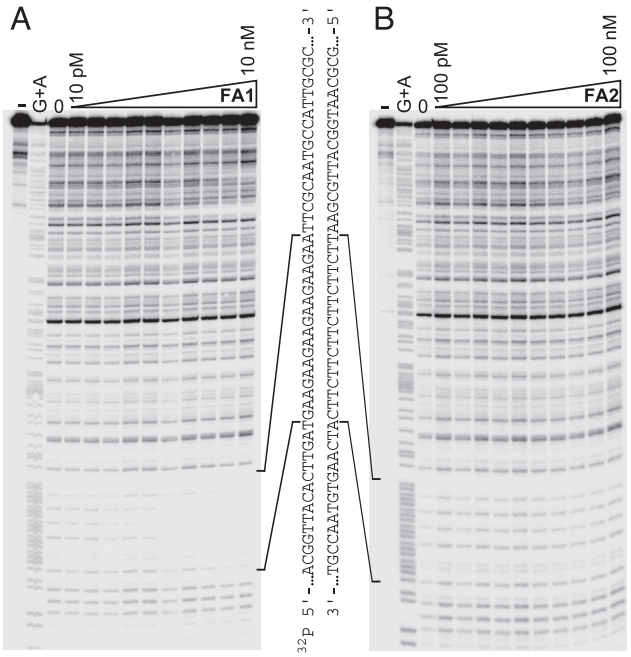

Fig. 2. DNA-binding properties of the polyamides. Quantitative DNase I footprint analysis for polyamide binding to a radiolabeled PCR product containing a $(GAA{\cdot}TTC)_6$ repeat sequence, labeled on the purine strand. DNA (at ≈20 pM) and polyamide were allowed to equilibrate for 16 h, with the indicated ranges of polyamide concentrations, before DNase digestion and gel analysis (25). The PhosphorImage of each gel is shown, with undigested DNA in the lane marked ''−''; a G+A sequencing reaction of the same DNA is shown, along with DNase-treated DNA in the absence of polyamide (in the lane marked ''0''). (*A*) FA1 targeting 5′-AAGAAGAAG-3′. (*B*) FA2, mismatch control for FA1. An excerpt of the DNA sequence cloned in pCR2.1 DNA, used to generate the PCR product for footprinting reactions is referenced between *A* and *B*.

Nuclear Localization of Fluorescent Polyamides. Fluorescent versions of the match polyamides FA1 and FA3 and mismatch polyamide FA2 were synthesized, where the dye Bodipy FL (C_5) was attached at the carboxyl terminus of the polyamide (Fig. 1*A*). Quantitative DNase I footprinting demonstrated that polyamides FA1- and FA3-Bodipy exhibit 13- to 20-fold losses in binding affinity for $(GAA{\cdot}TTC)_6$ DNA, compared with the parent polyamides (for FA1-Bodipy, $K_d = 1.3$ nM) (Fig. 9, which is published as supporting information on the PNAS web site; for FA3-Bodipy, $K_d = 0.04$ nM). Epstein–Barr virus-transformed lymphoblast cell lines from an FRDA patient (line GM15850) and from an unaffected sibling (line GM15851) were obtained from the NIGMS Human Genetic Cell Repository (Coriell Institute, Camden, NJ). Both the match FA1-Bodipy and mismatch FA2-Bodipy conjugates localize in the nu-

Table 1. Polyamides designed to target GAA·TTC repeats in the frataxin gene

Polyamide sequence	$(GAA{\cdot}TTC)_n$ repeat no. in target site, n	Binding affinity (K_d, nM)*
FA1: ImPyβImPyβImβDp	3	0.11 ± 0.02
FA2: ImPyβIm<u>Im</u>βPyβDp	3	>100
FA3: ImPyβImPyβImPyβImβDp	4	0.003 ± 0.001
FA4: ImPyβImPyβIm<u>Im</u>βPyβDp	4	2.0 ± 0.4

Mismatch amino acids are underlined. Im, imidazole; Py, pyrrole; β, β-alanine; Dp, dimethylaminopropylamine.

*Binding affinities (mean values of the K_d from a minimum of two determinations, and standard deviations) determined by quantitative DNase I footprinting, as in Fig. 2.

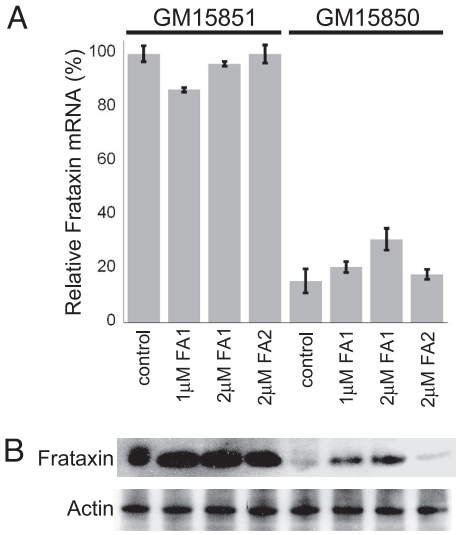

Fig. 3. Polyamide FA1 increases the levels of frataxin mRNA and protein in an FRDA lymphoid cell line. (*A*) Measurement of frataxin mRNA levels, relative to that of GAPDH, in cell lines derived from an unaffected individual (GM15851) and an FRDA patient (GM15850) by qRT-PCR (44). Polyamides FA1 and FA2 were included in the cell culture medium at the indicated concentrations, and frataxin and GAPDH mRNA were determined after 7 days, with media and polyamide replenished on days 3 and 5. Error bars are derived from the percent error of the average and standard deviation of the change in cycle threshold between frataxin and GAPDH for triplicate experiments, with triplicate qRT-PCR determinations for each experiment. (*B*) Effects of polyamides on frataxin protein in cultured lymphoid cells. FRDA or control cells were incubated as in *A* before Western blot analysis with antibody to human frataxin or actin. Equivalent amounts of total-cell-extract protein were loaded in each lane.

cleus of live, unfixed normal and FRDA lymphoid cells after 16-h incubation in culture medium, as determined by deconvolution microscopy (Fig. 1*B*). The Bodipy-conjugate of the longer polyamide FA3 also localizes in the nucleus of the FRDA cells.

GAA·TTC-Specific Polyamides Increase Frataxin mRNA and Protein. To assess whether polyamides alleviate transcription inhibition caused by expanded GAA·TTC repeats in the frataxin gene, we used real-time quantitative (q)RT-PCR to monitor frataxin mRNA levels in the lymphoid cell lines described above, with the levels of GAPDH mRNA as an internal control for each RNA sample. No differences in GAPDH mRNA levels were found between the two cell lines. As expected, the FRDA cell line had a markedly lower level of frataxin mRNA compared with the cell line from the normal individual (Fig. 3*A*; 13 ± 6%, for 20 determinations, Table 2, which is published as supporting information on the PNAS web site). We incubated the FRDA and control cells with various concentrations of each of the polyamides for various lengths of time and found that only polyamide FA1 increased frataxin mRNA levels after 7-days incubation in culture medium. No changes in frataxin mRNA levels were observed on shorter incubation times. Over the concentrations of 1–8 μM, we find that polyamides are not cytotoxic to the lymphoid cell lines (as determined by trypan blue exclusion and measurements of ATP levels) and do not affect cell growth rates. Importantly, the level of frataxin mRNA in the FRDA GM15850 cell line was increased 2.5-fold by incubation with polyamide FA1 (at 2 μM, Fig. 3*A*). The average fold increase observed with 2 μM FA1 in the FRDA cell line is 2.2 ± 0.6 (in 20 experiments), resulting in an average of ≈27% of the level of frataxin mRNA found in the normal cell line (Table 2). We note that polyamide concentrations greater than the K_d for *in vitro* binding are required for increasing frataxin transcription, presumably because of the large number of potential polyamide-binding sites in the human genome and availability of these sites in the cell nucleus. Despite the fact that FA1 binds the 9-bp 5′-AAGAA-GAAG-3′ repeat with high affinity, the sequence landscape of all possible high-affinity sites for this class of β-linked oligomers has not yet been fully characterized (26). Neither higher concentrations of FA1 nor longer incubation times increased frataxin transcription above the levels observed at 2 μM on 7-day incubations. Polyamide FA1 did not increase frataxin mRNA levels in the cell line derived from the normal individual. Similar incubations with the mismatch polyamide FA2 were without significant effect in either cell line. The levels of GAPDH mRNA were not changed by polyamide treatment in either cell line.

We next examined the effect of removal of polyamide FA1 from the culture medium on frataxin transcription. After induction of frataxin mRNA synthesis by FA1 (7 days at 2 μM), transfer of the cells to fresh medium lacking polyamide caused frataxin mRNA levels to decrease to their original levels after 96 h (data not shown). Thus, polyamides must be continuously present to maintain active transcription of FRDA frataxin alleles. The finding that incubation periods of 7 days or more are necessary to observe increases in frataxin mRNA suggests that multiple rounds of DNA replication are necessary for the compound to alter either the DNA or chromatin structure of expanded frataxin alleles, leading to active transcription, and removal of the polyamide causes the frataxin gene to readopt its inactive DNA or chromatin conformation. To test whether cellular proliferation is required for frataxin gene activation, we serum-starved FRDA and control cells, leading to cell cycle arrest (confirmed by fluorescence activated cell sorting, data not shown), and then incubated the arrested cells with polyamide FA1 for 7 days before qRT-PCR analysis. We find no increase in frataxin mRNA with polyamide FA1 under these conditions, suggesting that cell division is a requirement for up-regulation of transcription by the polyamide.

It was curious that the highest-affinity compound, FA3, did not increase frataxin mRNA levels, because the fluorescent version of this molecule localized in the nucleus of FRDA lymphoid cells. Previous studies have established that nuclear localization is sensitive to polyamide composition and structure and, especially, the nature of the carboxyl terminus (27, 28); therefore, the nonfluorescent version of FA3 may not enter the nucleus. To test this hypothesis, we monitored the levels of frataxin mRNA after incubation with FA3-Bodipy, and found an ≈2- to 3-fold increase in relative levels of frataxin mRNA (compared with GAPDH) after 2- to 4-day incubations (data not shown). Thus, FA3 may not have the optimum chemical properties for nuclear localization or DNA binding in the context of cellular chromatin. In contrast, FA1-Bodipy did not increase frataxin mRNA levels in experiments where positive effects were found with FA1.

Because the primary transcripts from FRDA frataxin genes contain long stretches of GAA repeat RNA sequence, it is conceivable that this RNA will not be correctly processed into mature frataxin mRNA, and frataxin protein will not be produced. To test whether polyamide FA1 leads to increased levels of frataxin protein in treated lymphoid cells, total-cell extracts from polyamide-treated (1–2 μM for 7 days), and untreated GM15851 control and GM15850 FRDA cells were subjected to SDS/PAGE and the corresponding blots probed with anti-frataxin or anti-actin antibodies (Fig. 3*B*). An ≈2- to 3-fold increase in frataxin protein is observed with FA1 in the FRDA cells, which correlates well with the observed increase in frataxin mRNA (Fig. 3*A*).

Effects of Polyamides on Global Gene Expression. DNA microarray analyses were performed with RNA isolated from GM15850 FRDA and GM15851 normal lymphoid cells that were either untreated or treated with polyamides FA1 (at 1 and 2 μM) or FA2 (at 2 μM) for 7 days on Affymetrix Human Genome U133 Plus 2.0 GeneChips. FA1 was found to affect the mRNA levels for a limited number of genes in the FRDA cell line (at $P \leq 0.005$, 51 genes

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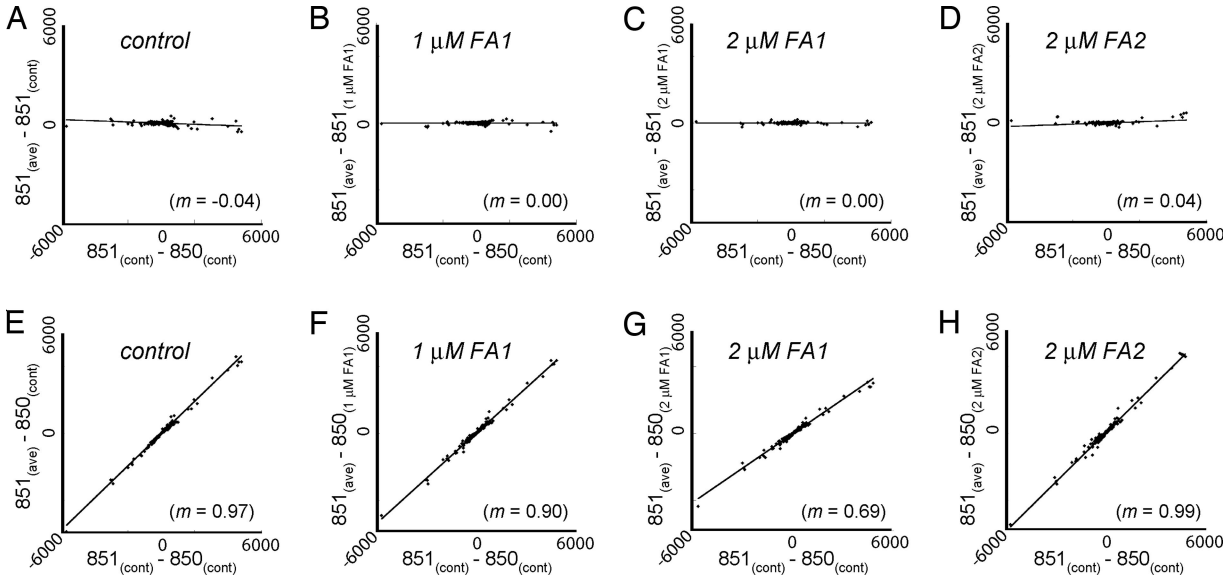

Fig. 4. Microarray analysis of polyamide effects on global gene expression. Correlation of significant genes called from a class comparison of all arrays (n = 632 genes at $P \leq 0.005$). Cells were cultured with and without the addition of FA1 at 1 μM or 2 μM or FA2 at 2 μM for 7 days before RNA extraction. Each graph represents the difference between the geometric mean of intensities (from an average of the logged robust multichip average data for each condition) (47) of untreated GM15851 cells (denoted 851 in the graphs) and untreated GM15850 cells (denoted 850) plotted versus the difference between the average from all GM15851 geometric means of intensities and each of the individual cell types and conditions. Thus, the untreated controls for GM15851 and GM15850 cells should give slopes for the least-squares-fit line approaching 0 and 1, respectively. Graphs for normal GM15851 cells are shown for the untreated control (*A*) and cells treated with FA1 at 1 μM (*B*) and 2 μM (*C*) and FA2 at 2 μM (*D*). Graphs for FRDA GM15850 cells are shown for the untreated control (*E*) and cells treated with FA1 at 1 μM (*F*) and 2 μM (*G*) and FA2 at 2 μM (*H*).

affected by 1 μM FA1, 16 genes affected by 2 μM FA1) (Table 3, which is published as supporting information on the PNAS web site), and only 2 genes in the normal cell line (Table 3). Although more genes were called affected by FA1 at 1 μM than at 2 μM, this difference is largely due to genes whose mRNA levels change by $<\approx$25% in either direction. At 2 μM FA1, 15 genes were increased in expression by >50%, and 1 gene was decreased by 45%. At 1 μM FA1, only 3 genes had comparable changes in their mRNA levels. For GM15851 cells, 2 genes were up-regulated by FA1, and no genes were down-regulated. For the frataxin gene, untreated GM15850 cells showed 17% of the frataxin mRNA found in untreated GM15851 cells, and incubation with FA1 at 2 μM increases frataxin mRNA by 2.5-fold, bringing the frataxin mRNA level in GM15850 cells to 42% of that found in GM15851 cells. These values are comparable with those obtained by qRT-PCR (Fig. 3*A*). Transcript levels for frataxin were not changed by FA2 in either cell line, and FA2 affected only a small number of genes in either cell line (at $P \leq 0.005$, 3 genes affected by 2 μM FA2 in GM15850 cells, and 1 gene affected in GM15851 cells) (Table 3).

To examine the overall changes in gene-expression profiles in treated and untreated populations of FRDA and control cells, we first determined the genes whose expression was called significantly different among all conditions of untreated and treated GM15851 and GM15850 cells. At a P value of $\leq$0.005, this class comparison generated a total of 632 genes. We then generated a correlation graph of the difference in geometric means of intensities for these genes between untreated and treated cells for each experimental condition (Fig. 4). Neither the match FA1 nor mismatch FA2 polyamide affected the profile of GM15851 cells (slope of the correlation between conditions = -0.04 to $+0.04$, Fig. 4 *A–D*), whereas FA1-treated GM15850 FRDA cells (at 2 μM) have a gene-expression profile that approaches that of untreated GM15851 cells compared with untreated FRDA cells (Fig. 4, compare *G* with *E*; slope = 0.69 compared with 0.97, respectively). This effect is seen to a lesser degree at 1 μM FA1 (Fig. 4*F*), but was not seen with the mismatch polyamide FA2 (Fig. 4*H*). These changes in gene expression in the affected cell line may be a consequence of changes in frataxin protein levels (29, 30), or some could be direct effects of the polyamide, as suggested by the occurrence of FA1 binding sites in up-regulated genes (Table 2). Taken together, these data lead us to conclude that polyamide FA1 increases frataxin gene expression and, perhaps, downstream targets of frataxin, but this molecule has a limited effect on global gene expression. A search of the GenBank database reveals that most regions of the GAA·TTC DNA sequence [$(GAA{\cdot}TTC)_6$ or longer] are present in nontranscribed repetitive DNA elements (including Alu sequences) in the human genome (31). Polyamide FA1 has no effects on lymphoid cell morphology, metabolism, or growth in culture.

Influence of Polyamides on Sticky-DNA Conformation. The capacity of sequence-specific polyamides to disrupt the intramolecular sticky-DNA structure formed by GAA·TTC repeat tracts was investigated. Plasmids harboring the sticky-DNA structure are visualized by gel electrophoresis after restriction endonuclease cleavage (Fig. 5*A*). Linear DNA is indicative of disruption of the sticky-DNA structure by a polyamide, whereas the cleaved sticky-DNA band that migrates with a much slower mobility reveals no influence of the ligand. Plasmid pRW4886, which contains two tracts of $(GAA{\cdot}TTC)_{176}$ in a direct-repeat orientation (10), was incubated with each of the polyamides FA1, FA2, FA3, and FA4 at concentrations ranging from 0 to 50 μM. The polyamide-bound DNA was then digested with EcoNI and electrophoresed on 1% agarose gels to determine the amount of the EcoNI-cleaved sticky-DNA-retarded band present (Fig. 5*B*). Incubation of pRW4886 DNA with FA3 shifted the equilibrium from a maximum amount of sticky DNA to a complete loss of the EcoNI-cleaved sticky-DNA-retarded band at a concentration of 50 nM. FA4, which is a mismatch of FA3 and has a K_d value $\approx$1,000 times higher than FA3 (Table 1), did not affect the stability of sticky DNA in pRW4886 until an FA4 concentration of 5 μM. For FA1, a 1 μM concentration was needed to dissociate the DNA·DNA structure-forming

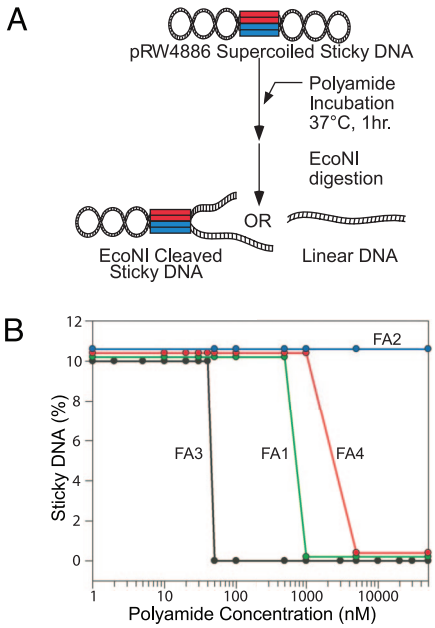

Fig. 5. Effect of polyamide binding to plasmid DNA on sticky-DNA stability. (*A*) Illustration showing the assay for influence of polyamides on sticky-DNA conformation. (*B*) The capacity of a polyamide to disrupt the DNA·DNA-associated region in the sticky-DNA structure was revealed by the formation of linearized pRW4886 rather than the EcoNI-cleaved sticky DNA, which showed the absence of a perturbing influence of the ligands. The polyamides that had higher K_d values required higher concentrations to observe the disruption of the DNA·DNA-associated region of sticky DNA. The polyamides used were FA1 (green), FA2 (blue), FA3 (black), and FA4 (red).

region. The mismatched polyamide FA2, having the highest K_d value of all of the polyamides tested, showed no effect on sticky-DNA stability, even at a 100 μM concentration. Thus, the binding affinities of the polyamides for the GAA·TTC sequence had an intimate relationship with the concentration needed to shift the equilibrium from the DNA·DNA-associated structure to the duplex conformation (Fig. 5*B*). The absence of the EcoNI-cleaved sticky-DNA-retarded band demonstrated the capacity of the sequence-specific polyamide binding to shift the non-B–B-DNA equilibrium toward a conventional DNA duplex conformation in supercoiled plasmids. Because sticky DNA inhibits transcription (5, 32), and because the polyamides destabilize this conformation by shifting the structural equilibrium to duplex B DNA, increases in frataxin mRNA observed with polyamide FA1 may be due to this structural transition, although other mechanisms, such as heterochromatin desilencing, must be considered (19).

Conclusions

Increased frataxin transcription with GAA·TTC-specific polyamides may be due to a change in DNA structure, as suggested by reversal of sticky DNA, which then allows for transcription through expanded GAA·TTC-repeat DNA, or by reversing heterochromatin caused by expanded GAA·TTC repeats (14). Polyamides designed to target the related sequence GAGAA·TTCTC repeat DNA, which is found in *Drosophila* satellite V, have been shown to alter gene expression in developing embryos by opening the chromatin domains containing these repeated sequences (14, 19) and to displace heterochromatin protein 1 (HP1) and the other chromosomal proteins D1 and topoisomerase II (33). Interestingly, in the transgene study reported by Festenstein and colleagues (14), HP1 was found to promote gene silencing by expanded GAA·TTC-repeat DNA. Thus, polyamides targeting GAA·TTC-repeat DNA may increase transcription of genes that harbor these sequences by displacement of similar repressor proteins and reversal of inactive heterochromatin to a more active chromatin structure.

Materials and Methods

Polyamide Synthesis and Characterization. Polyamides were synthesized by solid-phase methods (18, 34) and their identity and purity verified by MALDI-TOF MS and analytical HPLC. Fluorescent conjugates were prepared by coupling Bodipy FL C5 (Molecular Probes) to the carboxyl terminus (27). Binding affinities for match and mismatch sites were determined by quantitative DNase I footprinting (25). A plasmid harboring six GAA·TTC repeats was constructed by cloning the oligonucleotide 5′-GCCTTACGGTTACACTTGATGAAGAAGAAGAAGAAGAATTCGCAATGCCATTGCGCTATGA-3′·3′-ACGGAATGCCAATGTGAACTACTTCTTCTTCTTCTTCTTAAGCGTTACGGTAACGCGATAC-5′ in the pCR2.1 TOPO vector (Invitrogen), and a 251-bp singly end-labeled PCR product was generated from this plasmid with the following oligonucleotides: 5′-GAAAGACCCGTGTGTAAAGCC-3′ and 5′-CTCGATATCTGCAGAATTGCC-3′, where the second oligonucleotide was labeled with γ-[^{32}]ATP and polynucleotide kinase, by using standard procedures, to generate a PCR product labeled on the GAA strand. A 204-bp singly end-labeled PCR product was derived from plasmid pMP142 DNA, containing 33 GAA·TTC repeats (5), with the following oligonucleotides: 5′-GGCCAACATGGTGAAACC-3′ and 5′-GTAGCTGGGATTACAGGCGC-3′. The first oligonucleotide shown was radiolabeled as above to generate a PCR product labeled on the GAA strand. A 150-bp PCR product containing a $(GGA{\cdot}TCC)_3$ mismatch sequence was derived from the erbB2 (Her2-neu) promoter in human genomic DNA with the following oligonucleotides: 5′-CTTGTTGGAATGCAGTTGGA-3′ and 5′-GGTTTCTCCGGTCCCAAT-3′, with the first oligonucleotide radiolabeled.

Cell Culture. Epstein–Barr virus-transformed lymphoblast cell lines GM15850 from a FRDA patient (alleles with 650 and 1,030 GAA·TTC repeats in the frataxin gene, from the Coriell Cell Repository) and GM15851 from an unaffected sibling (normal range of repeats) were propagated in RPMI medium 1640 with 2 mM L-glutamine and 15% FBS at 37°C in 5% CO_2. Cell growth and morphology were monitored by phase-contrast microscopy and viability by trypan blue exclusion and an ATP assay (ApoSENSOR; BioVision). Polyamides were added directly to the culture medium in PBS, and incubations were for the times indicated in the text and figure legends. Nuclear localization of the polyamides was verified by deconvolution microscopy, as described (35).

Real-Time qRT-PCR. Real-time qRT-PCR analysis was performed essentially as described in ref. 45, by using the following primers for the frataxin gene: 5′-CAGAGGAAACGCTGGACTCT-3′ and 5′-AGCCAGATTTGCTTGTTTGG-3′. RNA was standardized by quantification of GAPDH mRNA (37), and all values are expressed relative to GAPDH. qRT-PCR was performed by using iScript One-Step RT-PCR kit with SYBR green (Bio-Rad). Statistical analysis was performed on three independent qRT-PCR experiments for each RNA sample, and triplicate cell incubations were performed.

Western Blot Analysis. Total-cell extracts were used for SDS/PAGE and Western blotting with antibodies to human frataxin (Chemicon) or actin (Santa Cruz Biotechnology) as a control for cell number and protein loading. Signals were detected by chemiluminescence after probing the blot with HRP-conjugated secondary antibody (Supersignal West; Pierce). To quantify the relative levels of proteins, autoradiograms (within the linear response range of x-ray film) were converted into digital images and the signals quantified by using Molecular Dynamics ImageQuant software.

DNA Microarrays. FRDA and control lymphoid cells were incubated with polyamides FA1 (at 1 or 2 μM) or FA2 (at 2 μM), or in the absence of polyamide, in triplicate for 7 days before RNA purification and microarray analysis at the California Institute of Technology microarray facility. Affymetrix U133A Plus 2.0 GeneChips were hybridized in groups of eight for each of the three replicates. Raw GeneChip data were normalized with RMAExpress (38), and the normalized data were filtered to remove probe sets called absent on 24 of 24 chips from class comparisons. The Affymetrix probe set-level data were imported to BRB Arraytools (Version 3.3.0 Beta 3a), selecting the U133 chips used in the experiment and leaving all filters off. For class comparisons between groups of arrays, unpaired samples were used, and the random variance model was selected, with the univariate significance threshold set to 0.005. The restrictions for the univariate test were maintained as the default values of 10 for the maximum number of false discovered genes, 0.1 for the maximum proportion of false discoveries, and a 90% confidence level. Because of poor data correlation in one set of replicates, class comparisons were performed by using all chips for the control group versus two of the three replicates for the treatment group (five groups are the minimum number required for class comparisons). Microarray data (accession no. GSE5040) have been deposited at Gene Expression Omnibus.

Effects of Polyamides on Sticky DNA. Plasmid pRW4886, which contains two tracts of $(GAA{\cdot}TTC)_{176}$ in a direct repeat orientation (10) and forms sticky DNA (13), was treated with polyamides at concentrations of 0–50 μM at 37°C for 1 h. Restriction digestion with EcoNI after the polyamide incubation enabled visualization of the presence or absence of an EcoNI-cleaved sticky-DNA band that runs with decreased mobility compared with the linearized plasmid on 1% agarose gels (13). Quantitation was by densitometric analysis using FluorChem (Version 3.04; Alpha Innotech).

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