Mutated Frataxin in Determining the Mechanisms of the Iron-Sulfur Cluster Assembly

An essay submitted in partial fulfillment of

the requirements for graduation from the

Honors College at the College of Charleston

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Biochemistry

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Advisor: Pamela Riggs-Gelasco Ph.D.

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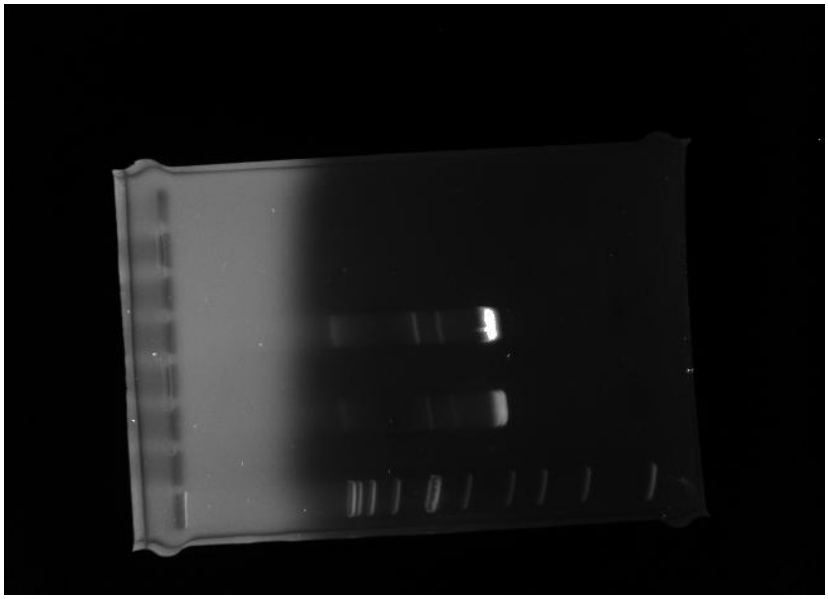

Fig. 1.
The first gel electrophoresis of the extracted DNA demonstrates a clear separation and band of DNA containing the D102H mutation of the frataxin gene.

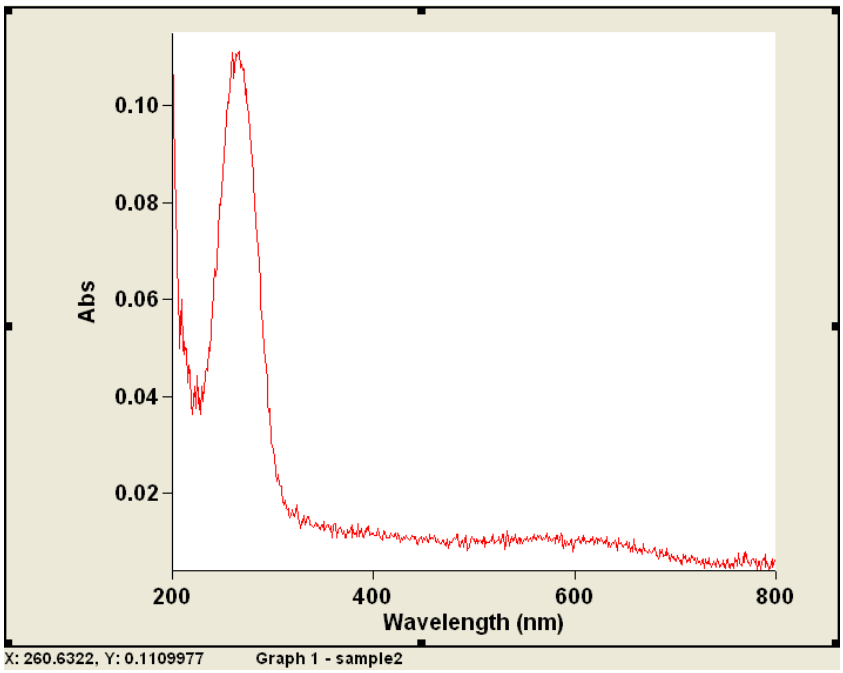

Fig. 2.
The concentration absorbance associated with the DNA extracted in figure 1.

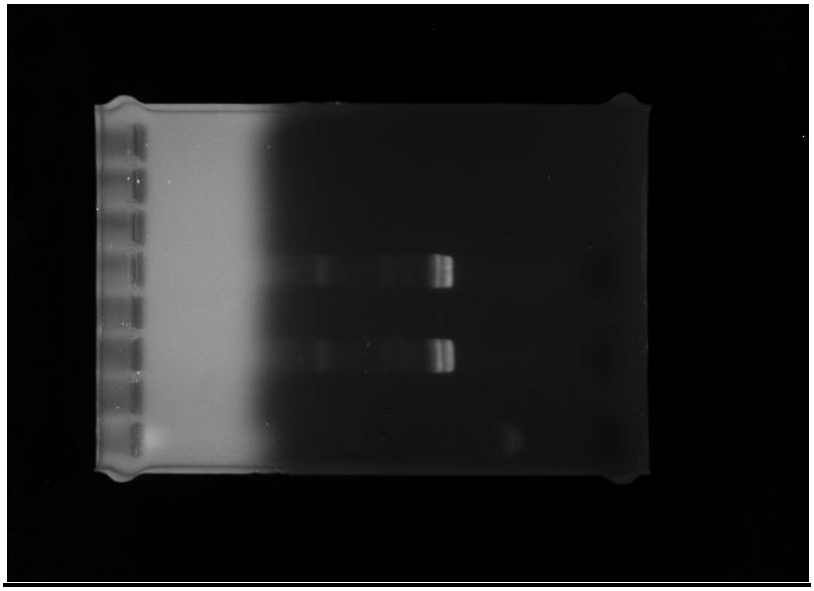

Fig. 3.
Second attempt of the first batch of DNA and its electrophoresis gel.

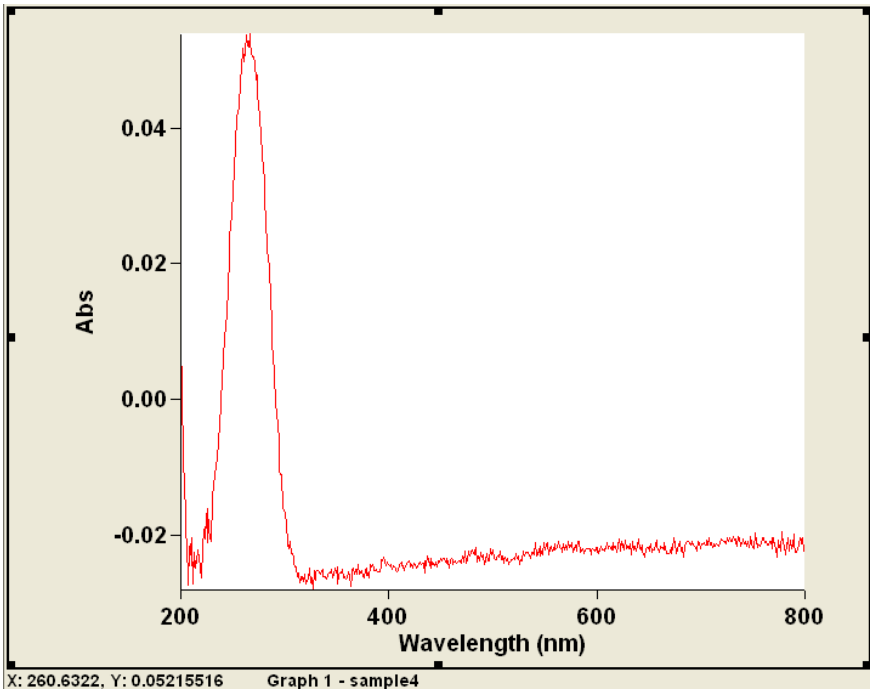

Fig. 4.
A concentration absorbance associated with figure 1.

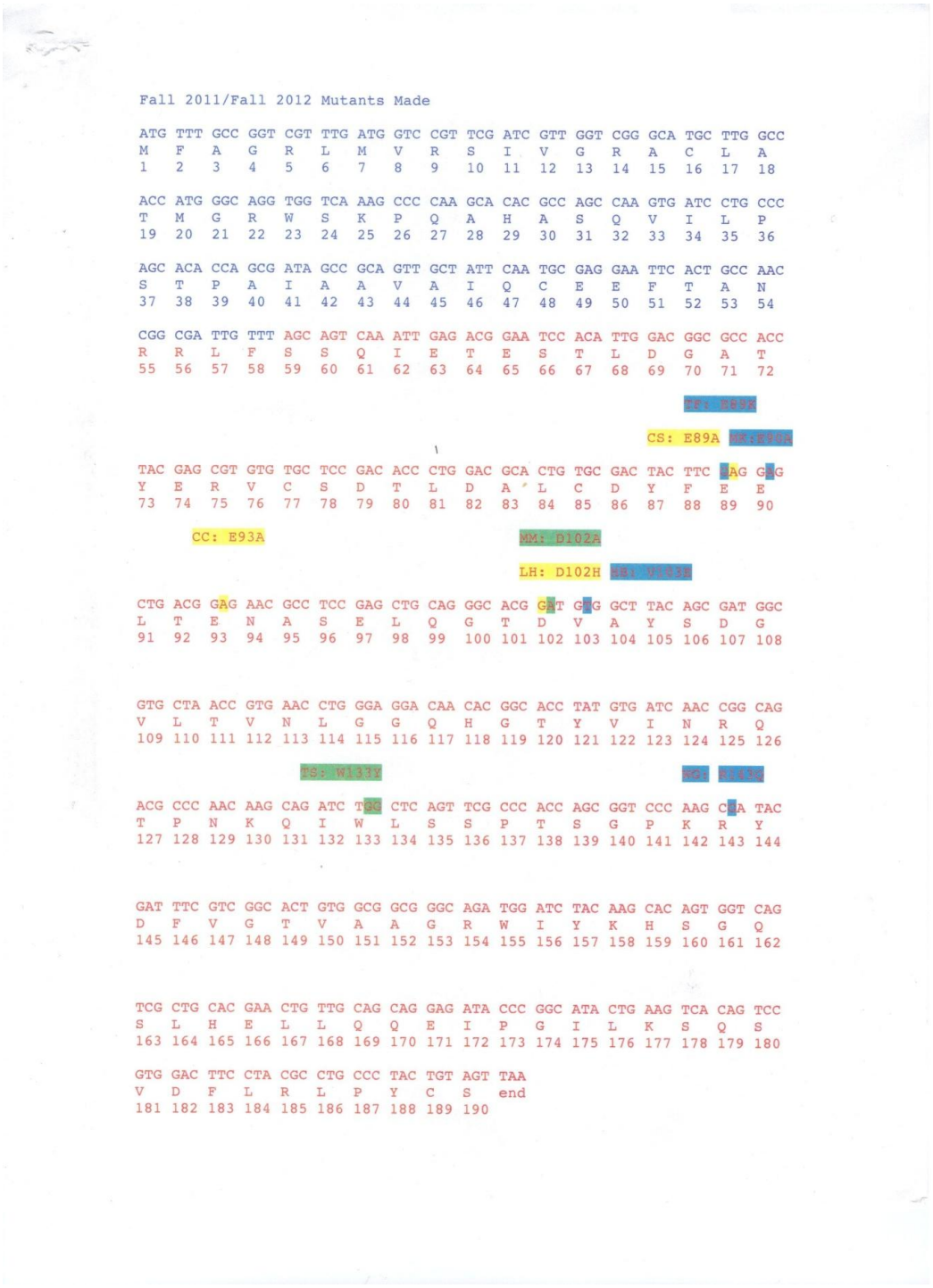

Fig. 5.

A sequence of the protein frataxin and the various site-directed mutations that were made to it by the current and former members of the research group.

```
                     10        20        30        40        50        60        70        80
             ....*....|....*....|....*....|....*....|....*....|....*....|....*....|....*....|
2GA5_A     1 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MESSTDGGVVPQEVLNLPLEKYHEEADDY
gi  9910697 1 MFAGRLMVRSIVGRACLATMGRWSKPGAHASQVILPSTPAIAAVAIQCEEFTANRRLFSSQIETESTLDGATYERVCSDT
1EKG_A     1 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GSHMGSLDETTYERLAEET

                      90       100       110       120       130       140       150       160
             ....*....|....*....|....*....|....*....|....*....|....*....|....*....|....*....|
2GA5_A        LDHLLDSLEELSEAHPDCI~PDVELSHGVMTLEI~PA~FGTYVINKQPPNKQIWLASPLSGPNRPDLLN~~~~GEWVSLR
gi  9910697   LDALCDYFEELTENASELGGTDVAYSDGVLTVNLGGQ~HGTYVINRQTPNKQIWLSSPTSGPKRYDFVGTVAAGRWIYKH
1EKG_A        LDSLAEFFEDLADKPYTFEDYDVSFGSGVLTVKL~GGDLGTYVINKQTPNKQIWLSSPSSGPKRYDQTG~~~~KNWVYSH

                     170       180       190
             ....*....|....*....|....*....|...
2GA5_A        NGTKLTDLLTEEVEKAISKSQ~~~~~~~~~~~~ 123
gi  9910697   SGQSLHELLQQEIPGILKSQSVDFLRLPYCS~~ 190
1EKG_A        DGVSLHELLAAELTKALKTKLDLSSLAYSGKDA 127
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2GA5_A        Homo sapien
gi  9910697   Yeast
1EKG_A        Drosophila melanogaster
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Fig. 6.

A protein alignment of the frataxin gene in Homo sapien, yeast, and Drosophila melanogaster. Highlighted portions are the conserved amino acids across multiple species.

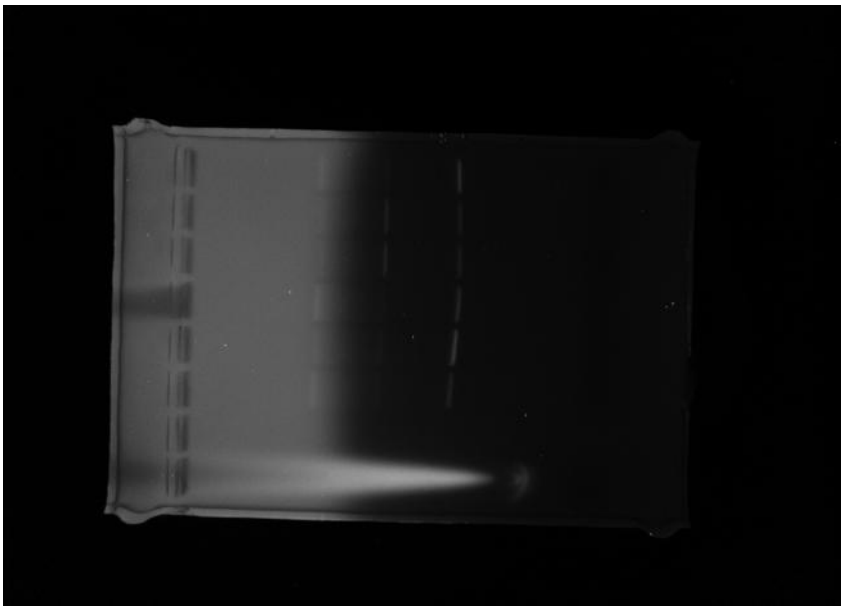

Fig. 7.
An electrophoresis gel of the six samples of the second trial

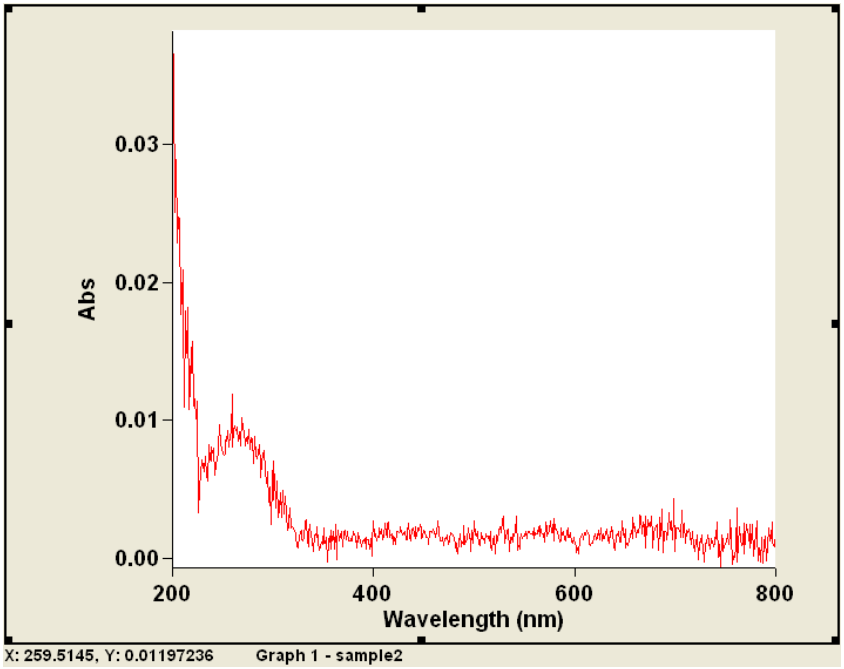

Fig. 8.
An concentration absorbance associated with Fig 7.

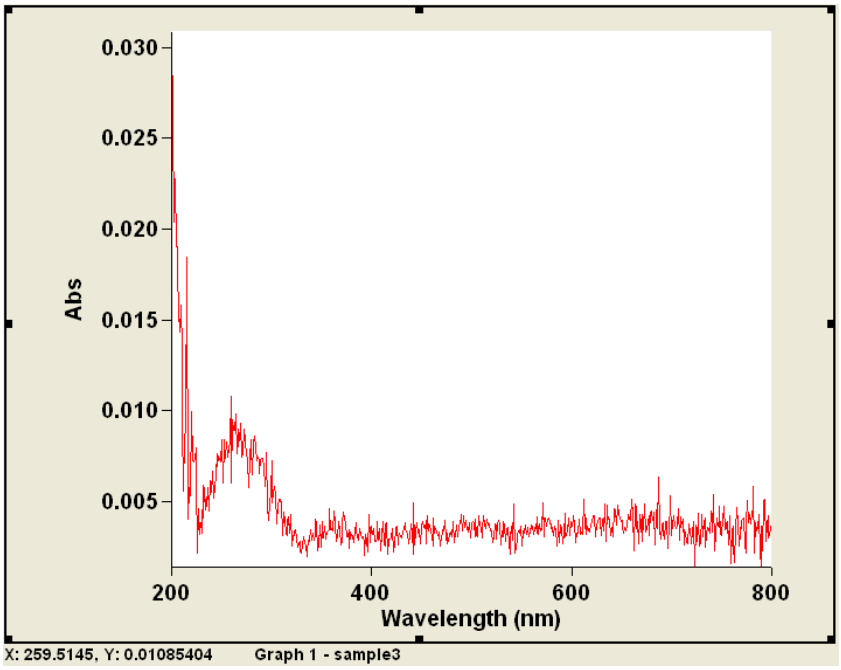

Fig. 9.

A concentration absorbance associated with Fig 7.

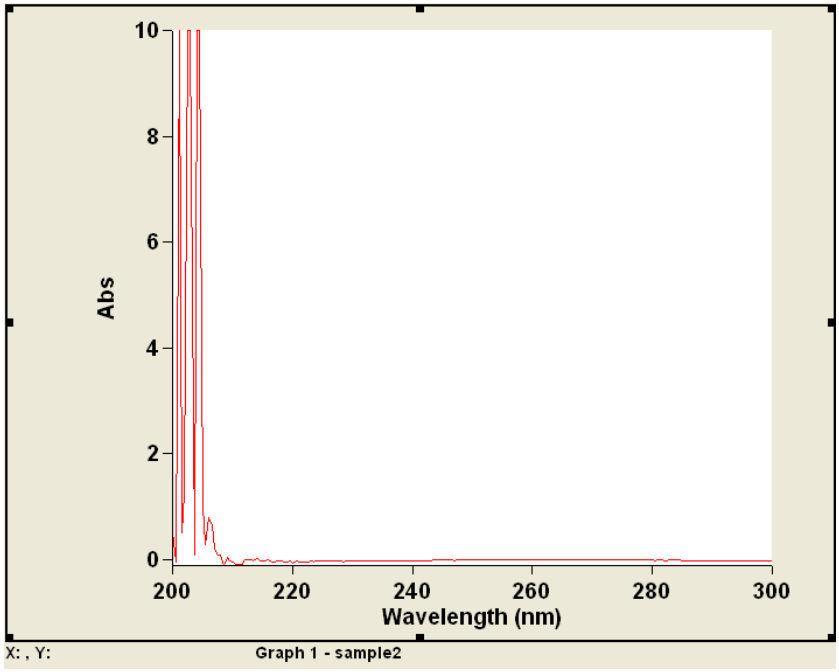

Fig. 10.

A concentration absorbance of the final extraction with the QUIAGEN midi kit of the D102H mutation of frataxin.

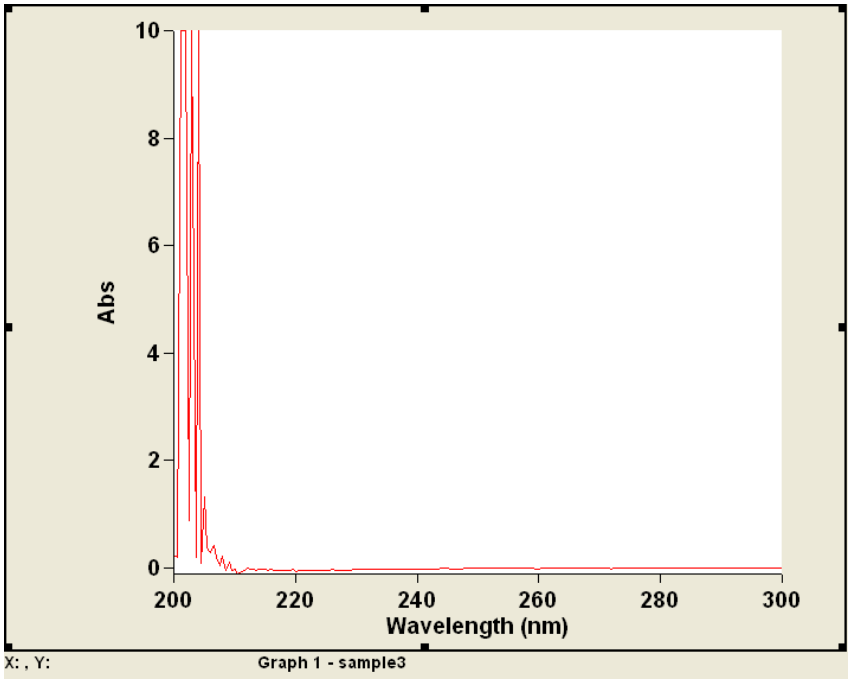

Fig. 11.
A concentration absorbance of the final extraction with the QUIAGEN midi kit of the E93A mutation of frataxin.

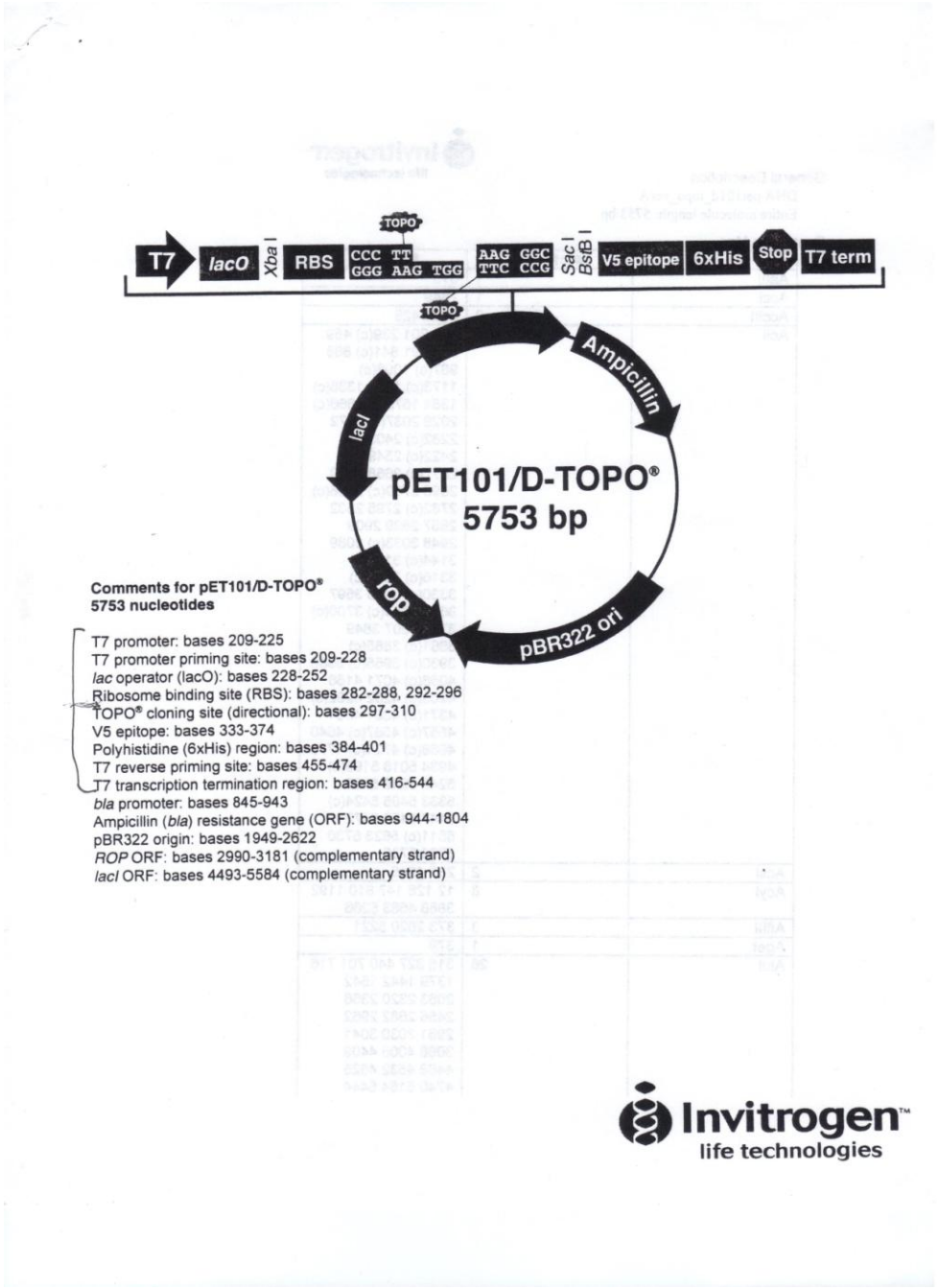

Fig. 12.
A visual representation of the donated frataxin plasmid containing the targeted genes and ampicillin resistance.

Abstract

Friedreich's Ataxia is a neurodegenerative disease that results in dysarthria, muscle weakness, spasticity in the lower limbs, scoliosis, bladder dysfunction, loss of lower limb reflexes, and loss of position and vibration senses[6]. The cause of this disease is generally associated with reduced levels of the protein frataxin. The deficiency in the mitochondrial iron-binding protein frataxin results in diminished activity of various mitochondrial iron-sulfur proteins and influence energy production in cells[5]. It is known that frataxin is involved in Fe-S cluster assembly and that it interacts with other scaffold or partner proteins such as Isu1 and IsdI. To further understand the mechanism of frataxin and its role in iron homeostasis, we identified conserved amino acids of the protein that are thought to participate in binding iron and set out to mutate these residues to evaluate changes to frataxin activity. We report here our attempts to generate the frataxin mutant D102H.

Introduction

Frataxin is a protein encoded by the FXN gene in humans that assists in the assembly of iron-sulfur clusters in the mitochondrion. For this reason, frataxin levels are the highest in the heart, spinal cord, pancreas, liver, and muscles where cells are postmitotic and highly dependent upon mitochondrial respiration[1]. Studies have shown that frataxin is a crucial protein in iron binding and directly influences energy production in the mitochondrion[3]. Therefore, it is reasonable that frataxin levels are the highest in the mitochondrion of a cell. Studies suggest that frataxin participates in iron storage or acts as an iron chaperone in the mitochondria[5]. The protein has a weak binding affinity for iron. This weak affinity allows frataxin to bind iron when in the iron-rich environment of the mitochondria and readily gives up the iron when in a low iron environment.

A reduction in the expression of the frataxin protein contributes to the development of Friedreich's Ataxia (FA), a fatal inheritable neurodegenerative disease transmitted as an autosomal recessive trait that causes progressive damage to the human nervous system[1]. This disruption of the nervous system leads to gait disturbance, speech impediments, heart diseases, and diabetes. This has made Friedreich's Ataxia the most prominent hereditary ataxia where 1 in 30,000 inherits the disease. Reduced expression of the protein frataxin can be caused by a nuclear mutation within the FXN gene or inherited[1]. Such mutations may affect the expression of the gene by silencing or inhibit intron splicing in the gene due to the extended GAA repeats within the gene[2]. Normal frataxin genes possess less than 30 GAA repeats whereas mutated frataxin gene possess more than more than 1700 GAA repeats. Shortage of frataxin appears to have an impact on the activity of the iron-sulfur cluster assembly therefore inhibiting energy production in the mitochondrion. The same frataxin/Yfh 1 deficiency in mitochondrial systems

causes an accumulation of iron in the mitochondrion and disrupts the ability of the mitochondrion to transport electrons via various ferrous species: Fe/S cluster (ISC)-assembly, ferredoxin Yah 1, ferredoxin reductase Arh 13. The same iron accumulation can cause an iron overload and eventually damage the mitochondria. A similar accumulation of iron in the mitochondria occurred in yeasts with disrupted YFH 1, a yeast frataxin homologue[1], and damage to the mitochondrial DNA and respiratory activities was observed.

Currently, there are several methods in treating Friedreich's Ataxia. The three most prominent treatment strategies are to reverse gene silence, iron chelation, and antioxidant treatment. However, most of these treatments are only temporary and do not offer a permanent solution for the ataxia. A possible solution includes using a synthetic version of the protein frataxin.

Even though the protein frataxin is highly related to mitochondria functions, the actual mechanism of the protein is unclear. An understanding of the mechanisms is crucial in developing a therapy. Studies have shown that frataxin is involved in various metabolic processes in the human body and may provide insight into other diseases such as Parkinson's disease, heptological, and hematological diseases. Parkinson's disease is also associated with iron regulation dysfuntion and therefore possesses potential ties to frataxin. Studies have also shown that certain conserved residues in frataxin participate in the formation of the multiprotein complex of Yfh1 (frataxin), and potential partner proteins Isu1 and Isd11. In order to gain more understanding on the mechanism of the frataxin protein we altered specific conserved iron binding residues in the protein frataxin. This can be achieved by designing mutations that are targeted for the conserved iron binding residues. Results should show an influence in iron binding abilities in the altered frataxin.

Materials and Methods

Electroporation of the wild type frataxin plasmid

LB plates and liquid LB media were prepared according to standard protocols. Plates were supplemented with 100 mg/L ampicillin. BioRad 0.2 cm gap electroporation cuvettes were chilled on ice. One microliter of plasmid pET101/D-TOPO (5753 bp) (donated by the Stemmler laboratory at Wayne State University Fig. 12) was added to 40 µL of DH5α eletrocompetent cells and mixed gently followed by incubation on ice for 2 minutes. A BioRad electropulser was used to shock the cells using the EC2 setting for the 0.2 cm gap in the cuvette. Immediately following the pulse, 1 mL of LB media was added and the cells were allowed to recover for 1 hour while shaking at 37 degrees Celsius. A 50 µL aliquot of cells without the plasmid was pulsed as a negative control. 100 µL of the mixture was plated on the LB/Ampicillin plates and the remaining 940 µL was centrifuged for 5 minutes to pellet the cells. The pellet was resupended 100 µL to concentrate the cells and this 100 µL was plated. All plates grew overnight at 37 degrees Celcius.

Agar gel electrophoresis

A 1% agar gel was prepared by melting 0.5mL of agarose into 200mL of Tris-Acetone-EDTA buffer. A 50 mL aliquot was cooled and 5µL of Ethidium Bromide (EtBr) was added to the mixture. CAUTION: EtBr is a known carcinogen and gloves should be worn. The pET101/D-TOPO WT frataxin plasmid was digested with either Bse R1 or ScaI-HF or both (New England BioLabs). To prepare the single digestion of the DNA, 5µL of buffer (1µL BSA), 10 µL of DNA, and 2µL of enzyme were mixed and enough water was added to make a 50 µL

reaction. For the double cut experiment, 2 µL of each enzyme was used. The microcentrifuge tubes were incubated at 37 degrees Celsius for 2 hours then the enzymes were deactivated by heat shocking the tubes at 65 degrese Celsius for 15 minutes. An uncut DNA control was 10µL of DNA in 40µL of de-nucleotide water. Five µL of BromoBlue dye (BioRad) was added prior to loading the samples in the lanes of the prepared 1% agarose gel. The gels were run on a BioRad Power Pac 300 at 100 volts for about 2 hours. The gel was imaged with a BioRad Gel Doc™ EZ Imager.

Primer design

To design primers that are needed for a site-directed mutagenesis, we used the published DNA sequence for the protein frataxin. The program BLAST was used to find the sequence of frataxin in yeast and Drosophila melanogaster. Sequence alignments helped identify conserved residues, shown marked in pink highlighter in Fig 6. The conserved regions then reveal the iron binding locations, which are conserved polar residues such as glutamic acid, aspartic acid, and arginine. These residues were targeted in our study. Of the 190 amino acid residues, the 102nd aspartic acid residue was selected for mutation to histidine, D102H. Using Integrated DNA Technologies' primer design program two different primers were selected for the sense and antisense strand. It is important to ensure that both of the primers possess the same melting points so that the primers will operate at the same optimal temperature during site-directed mutagenesis.

g304c - Sense Strand Sequence
5'-GCT GCA GGG CAC GCA TGT GGC TTA CAG-3'

g304c - Antisense Strand Sequence
5'-GTC TAA GCC ACA TGC GTG CCC TGC AGC-3'

STRATAGENE QuikChange II site-directed mutagenesis kit

A mutagenesis kit from STRATEGENE will be used for the D102H mutation of frataxin. The sense and antisense primers obtained from the primer design are constructed to target a specific mutation site. When the double strand DNA separates with the aid of the reaction buffer and increased temperature, DNA base pairs become exposed. The primers will search for their compliment on the sense or the antisense strand of the DNA. Once bound, the primer will mark the specific site for replication. The primers will insert the desired mutation into the replicated DNA. Polymerase chain reaction will continue to replicate until the cycle is broken. This will generate ample amount of DNA for reinsertion. The concentration of the primers and template is crucial for optimal mutation. The primers each needed to be measured out to as close to 125 ng as possible. Due to the unknown characteristics of the DNA template, 5 ng and 50 ng of DNA templates were utilized for mutation.

Control Reaction
5 µl of 10x reaction buffer
2 µl (10 ng) of pWhitescript 4.5-kb control template (5 µg/µl)
1.25 (125 ng) of oligonucleotide control primer #1 [34-mer (100 µg/µl)]
1.25 (125 ng) of oligonucleotide control primer #2 [34-mer (100 µg/µl)]
1 µl of dNTP mix
38.5 µl ddH_2O (for a final volume of 50 µl)

Sample Reaction
5 µl of 10x reaction buffer
X µl (5-50 ng) of dsDNA template
X µl (125 ng) of oligonucleotide primer g304c
X µl (125 ng) of oligonucleotide primer g304c_antisense
1 µl of dNTP mix
ddH_2O to a final volume of 50 µl

Once the control and sample reactions are prepared, 1 µl of Pfu Ultra HF DNA polymerase was added to each mixture. The cycling parameters for the polymerase chain reaction were chosen based on the suggested table provided with the kit. We followed the suggested protocol for site-directed mutagenesis with no alterations.

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12–18	95°C	30 seconds
		55°C	1 minute
		68°C	1 minute/kb of plasmid length

The segment 2 portion of the PCR will vary base on the length of the plasmid. The sample plasmid required an approximate reaction time of 7 minutes where approximately 5 kb of plasmid is present. 1 µL of Dpn I restriction enzyme was added to the PCR tubes once the reaction is complete to digest parental WT (Wild Type) DNA.

QUIAGEN® plasmid miniprep kit

A miniprep kit from QIAGEN, QUAprep® spin miniprep kit, was utilized to extract the DNA from the E. coli cells. Once the cells have grown in the ampicilin LB broths, the broth was centrifuged to form a cell pellet. The pellet was resuspended in 150 µL of P1 buffer. 250 µL of P2 lysis buffer was added to the solution and mixed thoroughly to ensure complete cell lysis. The lysis process was not allowed to proceed for more than 5 minutes to avoid DNA damage from the buffer. 350 µL of N3 buffer was then added to the mixture and immediately mixed thoroughly by inverting the tubes. This will stop cell lysis and neutralize the lysis buffer. The content was spun for 10 minutes at 13,000 rpm to separate the DNA from the waste material generated by the lysis buffer. The supernatant was carefully separated from the pellet and then transferred to a miniprep spin column. The column is centrifuged at 12,000 rpm for 60 seconds. The DNA will attach to the spin column and other products of cell lysis will be discarded via flow-through. The DNA will then be washed with 500 µL of PB buffer. Wash the column again with 750 µL of PE buffer and discard the flow-through. The column is spun again to remove any residue wash buffer. The DNA is eluted from the spin column with 50 µL of buffer EB (10mM Tris·Cl, pH 8.5) and use a clean Eppendorf tube to catch the flow-through.

QUIAGEN® plasmid midi kit

Before starting, RNase A was added to Buffer P1, mixed, and stored at 2-8 °C, LyseBlue® reagent was also added to Buffer P1 at a ratio of 1:1000 for visual confirmation of cell lysis. Buffer P3 was also prechilled at 4 degrees Celsius. The overnight bacteria culture were harvested by centrifuging at 6000g for 15 minutes and resuspended the bacterial pellet in 4 mL

Buffer P1. 4 mL P2 lysis buffer was added to the mixture and mix thoroughly by vigorously inverting 4-6 times. The mixture is then incubated at room temperature for 5 minutes to thoroughly lyse the cell. This cell lysis was also not allowed to proceed for more than 5 minutes to avoid DNA damage. 4 mL prechilled P3 neutralizing buffer is added to the lysis mixture to halt the cell lysis. The mixture is inverted several times to ensure the complete neutralization of the lysis buffer. The mixture incubates on ice for 15 minutes until the blue color dissipates. The tubes are centrifuged at >20,000g for 30 minutes at 4 degrees Celsius. QUIAGEN-tips are equilibrated with 4 mL of QBT buffer and allow the buffer to flow through. The supernatants are applied to QUIAGEN-tips and allow the mixture to drain via gravity flow. The DNA samples are washed with 2 mL of QC buffer 10 times. Once the DNA is washed, they are eluted with 5 mL of QF buffer each into clean 50 mL BD Falcon tubes. The DNA is precipitated with 3.5 mL of room temperature isopropanol and centrifuge at 15,000g for 30 min at 4 degrees Celsius. The supernatant is carefully discarded via decanting and the pellet air-dried for 5-10 minutes. The pellet is resuspended in TE Buffer pH 8.5.

Results / Discussion

Our project, in collaboration with Dr Tim Stemmler's research group, investigated the effects of the mutation of conserved amino acids in FXN gene on the iron binding affinity of frataxin. This is achieved via a site-directed mutagenesis, which will replace an amino acid in the FXN gene by changing the 304^{th} base pair in the FXN sequence from guanine to cytosine. Making this change will alter the 102^{nd} amino acid in the protein sequence from aspartic acid to histidine. Fig 5 shows the sequence of the FXN gene and mutations that were made to the gene. All of these mutations are conserved regions across three species that are believed to code for iron binding sites in frataxin are crucial in frataxin's role as an iron chaperon.

Once the complete sequence of the FXN gene was obtained, protein alignment was used in Fig 6 to mark the conserved sites across the three species. The 102^{nd} residue is an aspartic acid within the conserved region and appears to be a good candidate for an iron binding site. The aspartic acid was replaced with histidine via site-directed mutagenesis of single base pair. This change is believed to greatly affect the iron binding ability of frataxin. The mutated plasmid was reinserted back into the E. coli cells and amplified. The DNA was extracted from the cells using a QUIAGEN mini kit and sent to Medical University of South Carolina for sequencing. To process the sequence of the DNA, the laboratory requires the concentration of the DNA within the buffer and the electrophoresis gel of the samples to determine the approximate weight of the sample. The electrophoresis gel is shown in Fig 1 and clearly shows the presence of DNA in both samples that were submitted to the sequencing lab. A concentration was obtained of the submitted samples and this can be seen in Fig 2 and Fig 4. For obtaining the concentration of the DNA, the DNA was diluted 200 times with deionized-H_2O. The concentration of the DNA was calculated from the absorbance and was determined to be 1.09187 µg/µl and 0.50523 µg/µl. An

accurate concentration and gel allows for precise sequencing. However, there were no significant peaks were observed during sequencing. More samples were sent to the sequencing laboratory to minimize human error. Another gel was obtained to ensure a more accurate sequence; this can be seen in Fig 3. Unfortunately, the sample also came back with no significant peaks and no sequences were obtained. Since the DNA sample was obtained using a miniprep kit, so not much was left of the 50 µl sample after sending in two 15 µl samples for sequencing.

Another batch of sample was brewed and more cautious in sterile techniques were used during the procedure. However, instead of preparing only two 50 µl, six 50 µl samples were prepared. The concentration and gel were obtained for these. The electrophoresis gel can be observed in Fig 7 with the six samples plus the ladder. In this gel, the ladder appears to have degraded a bit. However, this should not have affected the determination of the plasmid weight because all six samples were relatively the same to each other. Two of the six samples were selected for sequencing based on the concentration strength observed from the gel. The concentration absorbance for both samples were obtained and is shown in Fig 8 and Fig 9. The samples were again diluted 200 times with deionized-H_2O and the absorbance at 260 nm were measured. The DNA concentrations were calculated out to be 0.1186194 µg/µl and 0.01076068 µg/µl. The samples were sent to the DNA sequencing lab at MUSC. Again, sequencing showed no significant peaks.

The cause of the lack of sequencing of the DNA is largely unclear. After careful evaluation and consultation with the DNA sequencing technician, the sources were narrowed down to a few possible causes. One of the main causes was thought to be that the concentration that was submitted was inaccurate. This caused the technician to use an excess amount of buffer and reagent for sequencing which can cause the lack of significant peaks in the results.

It was believed that DNA is present within the mutation sample D102H. Therefore, the sample was added to the rest of samples. All of the mutated plasmid sample combined for examination includes E89K, E89A, E93A, E90A, W113Y, D102H, and R143Q. All mutations are also shown in Fig 5. E89K designates a mutation of the 89th glutamic acid to lysine. E89A designates a mutation of the 89th glutamic acid to alanine. E93A designates a mutation of the 93rd glutamic acid to alanine. E90A designates a mutation of the 90th glutamic acid to alanine. W113Y designates a mutation of the 113rd tryptophan to tyrosine. R143Q designates a mutation of the 143rd arginine to glutamine. Each of these plasmids was electroporated back into DH5a E. coli cells for amplification via cell division. DNA were extracted using a QUIAGEN midi kit, which was supposed to generate 5 mL of DNA sample. However, during last centrifuge of the procedure to separate the DNA, the 15 mL BD Falcon tubes bursted and all of the DNA samples were lost. The bursting of the Falcon tubes were caused by the use of inappropriate tubes that were not able to withstand the 20,000 g that was needed to separate the DNA from the solution. In the second attempt, more suitable 50 mL Falcon tubes were used for centrifuge. However, it is important to note that when lysing the E. coli cells, the mixture did not produce a blue color of the LyseBlue reagent even though the cell appeared to be lysed by its gooey appearance. It is also important to note that the cells did not reproduce the same smell of the lysis of the cells as the previous extraction. This evidence may be important in determining the nature of the problem. While the rest of the procedure progressed relatively smoothly, DNA pellets were not visually observed. Even with the more suitable 50 mL Falcon tubes that were supposed to be able to handle up to 25,000 g, the Falcon tubes were warped but still intact. Even though DNA pellets were not visually observed in the Falcon tubes, the procedure was carried out according to kit instructions. Upon further investigation, it has come to our understanding that pure DNA may

not be visually visible and that conclusions cannot be made except for analysis by concentration absorbance via Spectrophotometer. Unfortunately, a DNA concentration absorbance was obtained of two of the six samples DNA that were extracted and neither displayed an absorbance peak indicating the lack of DNA within our sample. Prior to analysis with the spectrophotometer, a buffer zero was obtained and the raw DNA was diluted 25 times with TAE buffer. The D102H sample concentration can be seen in Fig 10 and the E93A sample concentration in Fig 11. The project could not be continued due to time constraints. However, if the extractions were successful, then we would reinsert the DNA back into another type of E. coli cells that will over-express iron-bound frataxin when exposed to ultraviolet lights. The results of this study should further our understanding of the mechanisms of frataxin.

Conclusion

The pET101/D-TOPO strand of plasmid is incorporated into DH5a E. coli cells via electroporation. The FXN genes are amplified via cell division. Then DNA was extracted using a QUIAGEN miniprep kit. Using BLAST, a complete DNA and amino acid sequence was obtained for the FXN gene in Homo sapiens. Also using BLAST, complete amino acid sequences were obtained for yeast and Drosophila melanogaster. These sequences were used for protein alignment to determine the conserved regions in the protein. The conserved sites are the regions of the gene that are most likely to contain critical iron binding residues. The 102nd aspartic acid was chosen for mutation to histidine. This was achieved via a site-directed mutagensis of the 304th base pair from guanine to cytocine. Even though the mutated strand of the FXN gene could not be sequenced by the DNA sequencing laboratory at the Medical University of South Carolina. We were confident that the mutated plasmid was present. Along with the original mutation of the study, five other mutated samples of frataxin were examined. However, the extractions of the six mutated samples were not successful due to equipment failure and the majority of the samples were lost.

Even though our attempts at furthering the understandings of the mechanisms of frataxin and the iron cluster assembly were inconclusive, our attempts should be documented to ensure the success of future research on this protein. The future of this project includes the expression of all of the mutated samples and the development of therapeutic strategies for treating Friedreich's Ataxia. Studies in this field can also be associated with other diseases with iron regulation dysfunction such as Parkinson's disease.

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