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Construction of the hemagglutinin (HA) epitope tags for Subunit H of Complex I in *Escherichia coli*

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ABSTRACT

Complex I is a member of the respiratory chain in both bacteria and human mitochondria. Studies have shown that many human mitochondria disorders are related to the poor assembly of Complex I, rather than simple enzymatic malfunction [1,2,3,4]. For the purpose of analyzing the assembly of the thirteen subunits (A-N) found in the *Escherichia coli* homolog of the Complex I enzyme, antibodies are needed to recognize the individual subunits. Since the H subunit is the only subunit without an antibody, high-affinity antibodies are needed to be produced from the HA epitope tags. The tags were introduced separately by the polymerase chain reaction (PCR) method in two different plasmids, one containing genes G-H, and the other containing H-I-J-K. DNA sequencing indicated high confidence in HA complexes. After growth of cell cultures, an induced expression of these genes by arabinose verified expression of the protein with induced tag. Then, a western blot was done to test both constructs ability to yield single proteins, of the correct size, that were recognized by the HA-antibody. Therefore, these constructs can be further tested for suitability in the analysis of Complex I assembly.

1. INTRODUCTION

Many genetic disorders and degenerative diseases have been associated with mitochondrial malfunction. About one in 5,000 humans have deficiencies in mitochondrial metabolism that contributes to such disorders [1]. These illnesses progress systemically, but eventually affect the whole body. Medication and physical therapy might alleviate the symptoms like muscle weakness, nervous system dysfunction and developmental disability, but currently there is no cure for such disorders. Within the electron transport chain are integrated proteins in charge of catalyzing pivotal pathways. One of the biggest enzymes is the respiratory Complex I [8,9]. The Complex I in *E. Coli* has 13 protein subunits (A-B-CD-E-F-G-H-I-J-K-L-M-N) where seven are homologs of the Complex I subunits coded by mitochondrial DNA in mammals [10,11, 12]. A key subunit in the assembly process is H, which is a membrane-bound subunit that forms extensive interactions with subunits in the peripheral arm that are associated with diseases [13,14,7,15]. We currently have antibodies against all the subunits except H. To study the assembly of Complex I it will be necessary to have antibodies against each of the subunits. Therefore, this project was undertaken to attach HA epitope tags to subunit H to track its path in the assembly process. In this study, using a bacterial model of Complex I that contains all the essential components found in the human version, the N and C terminal HA tags for the H subunit can be constructed in the expression vector pBAD33 [6,7]. An epitope tag can be introduced into the terminus of a protein by including its DNA sequence in the primer used to clone the gene by polymerase chain reaction (PCR). To facilitate the joining of the tagged gene with other genes for Complex I, adjacent genes G-H and H-I-J-K were cloned. In this way, a variety of assembly experiments can be carried

out in future projects.

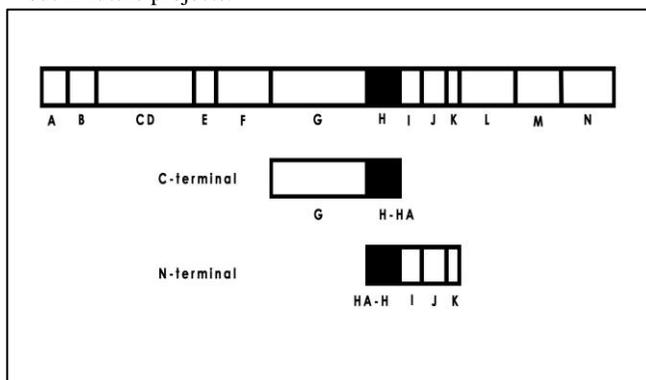


Figure 1. PCR clone design for HA tags

The HA tags were introduced to the H subunit at the C-terminal producing G-HHA and at the N-terminal producing HAH-K.

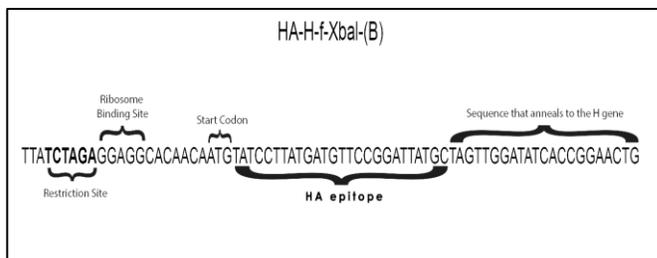


Figure 2a. PCR primer for HAH-K construct

HA-H-f-XbaI-(B) was used to attach the HA epitope to the H subunit at the N-terminus; notice the start codon compared to the stop codon on the C-terminus.

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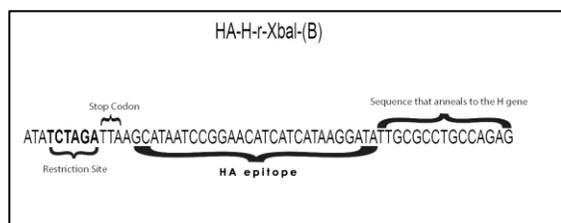


Figure 2b. PCR primer for G-HHA construct HA-H-r-XbaI-(B) was used to attach the HA epitope H subunit at the C-terminus.

2. MATERIALS AND METHODS

HA tags induction via polymerase chain reaction

The PCR vesicle contained the 9.5 μ L of sterile, nucleus free water, 12.5 μ L Q5 High-Fidelity 2X Master Mix (New England BioLabs, CA), 1 μ L of HA-H-f-XbaI-(B) (Eurofind MWG Operon, KY), 1 μ L of A-K pBAD33-r, and 2 μ L pBAD400 [16]. PCR was performed at 98 °C for 30 secs, 25 PCR cycles were then carried out (98 °C for 5-10 s/ 69 °C for 10-30 s/ 71 °C for 2 min), and a final extension step was performed at 71 °C for 2 min. PCR products were purified with QIAquick PCR Purification Kit (QIAGEN, Germany) and were evaluated by gel electrophoresis with ethidium bromide agarose gel (50 mL TAE (242g Tris base, 57.1 acetic acid+100mL of 500mM EDTA [pH=7]), .45g Agarose ,2 μ L LiBr₂)

Isolation of mitochondria membranes

Competent cells (XL1) were used to incorporate both plasmids and streaked on chloramphenicol plates (150 μ L and 300 μ L) for 24 hr. Plates were incubated at 37 °C overnight and colonies were re-streaked for cell harvest. Cells (500 mL) were harvested in 5% glucose (1 mL: 500 mL LB medium) or 3% arabinose (1 mL: 500 mL of LB medium) medium for approximately 5 hr (cultures were grown to an optical density of no more than 1.0 A₆₀₀. and pelleted by centrifugation at 6000 x g for 15 min at 4 °C. Cell pellets were resuspended in an isotonic buffer (5.33g 50M MES, 31.25mL mL 100% glycerol , 5mL 1M MgSO₄ [pH=6]) and homogenized by Tissue Grinder Potter-Elvehjem. Cell membranes were broken by French Press. Unbroken cells and nuclei were pelleted (600 x g, 15 min, 4 °C) and resulting supernatant was centrifuged (10,000 x g, 1 hr, 4 °C). Pellet was resuspended with 1mL isotonic buffer and stored at -80 °C.

Transformation with competent cell XL1

Ligation product (1.5 μ L) was electroporated, inoculated in LB medium (1L distilled water, 10g peptone from casein, 5g yeast, 5g NaCl), incubated for 1hr, and streaked on CM plates for 24 hr.

Detection of HA tags via western blot

Protein concentration was determined by DC protein assay kit (BioRad, CA). Loaded 12% SDS Mini-PROTEAN TGX gels (BioRad, CA) were ran at 150 V for 1 hr and transferred to polyvinlidene difluoride membrane at 50 V for 75 min in transfer buffer (12.12g 25mM Tris, 57.6g 192mM Glycin,

800mL Methanol). After blocking with milk solution (2.5 milk powder, 50 mL tris-buffered saline solution) and washing with tris-buffered saline solution (4.84g 20mM Tris, 58.4g 500mM NaCl, 1.5L H₂O [pH=7.5]), membranes were incubated overnight in primary anti-mouse antibody (Santa Cruz Biotechnology, CA). After washing with TBS, membranes were incubated in secondary goat anti-mouse antibody (Thermo Scientific, MA) for 1 hr. Western blots were developed by SuperSignal West Dura Luminol/Enhancer Solution (Thermo Scientific, MA) and WesternSure Pen (LI-COR Bioscience, NE).

DNA sequencing by Lone Star Labs of Houston TX

Plasmid DNA were prepared by QIAGEN Plasmid Midi Kit (QIAGEN, Germany) for sequencing. The DNA concentrations for HAH-K pBAD33 (.035 A) were calculated to be 17.5 μ L and 19.0 μ L for G-HHA pBAD33 (.038 A) at A₂₆₀, after a dilution of 100. Sequence data of HA-HK pBAD33 was confirmed with the use of seven primers (pBAD3, H-62, H-200, H-350, I-140, J-80, K-20) that covered the four genes. The DNA sequence data of G-HHA pBAD33 was confirmed with the use of 11 primers (pBAD3, G-120, G-260, G-390, G-520, G-660, G-790, G-900, H-62, H-200, H-350) that covered the two genes.

Ligation of PCR product and expression vector pBAD33

Restriction endonuclease Sbf1-HF and XbaI were used to digest the receiving plasmid pBAD33 prior to ligation to ensure enzymatic activity. The ligation reaction contained 1 μ L T4 DNA ligase (New England BioLabs, CA), 2 μ L H₂O, 2 μ L 10X Reaction Ligation Buffer (New England BioLabs, CA), 10 μ L HA-HK PCR product and 5 μ L pBAD33 [17]

Gel Extraction of expression vector pBAD33

Expression vector was digested (30 μ L of sterile, nucleus free water, 3 μ L of CutSmart buffer (New England BioLabs, CA), 4 μ L of pBAD33 [17], 1 μ L of XbaI (New England BioLabs, CA), 1 μ L of SacI-HF (New England BioLabs, CA)) for 2 hours by first enzyme and for 2 additional hours by second enzyme in 37 °C water bath and heat deactivated for 20 min at 65 °C. One microliter was removed from tube and checked by gel electrophoresis. Undigested plasmid was loaded in an adjacent lane. In a successful digestion, the super-coiled band will disappear, and a single band should be seen at the full-length size. After successfully digestion of both restriction enzymes, digestion was loaded into agarose gel and ran for 50 min at 135 V. Gel was sliced over UV light and purified by QIAquick Gel Extraction Kit (QIAGEN, Germany).

3. RESULTS

Introduction of HA tags through PCR at N-terminal

The H-K fragment was copied from pBA400, the template plasmid. The HA tag was introduced into H-K fragment through PCR. The HAH-K fragment was double digested to confirm correct base pair size of 7.8 kb (5.3 kb from

pBAD33, 2.4 kb from H-K, and 0.1 kb from HA tag). The HAH-K PCR product and pBAD33 were ligated to create the HA-HK pBAD33 plasmid. GeneJET Plasmid Miniprep Kit (Thermo Scientific, MA) was used to purify plasmid DNA in six colonies, which were double digested with restriction endonucleases SacI-HF and XbaI, and checked by gel electrophoresis.

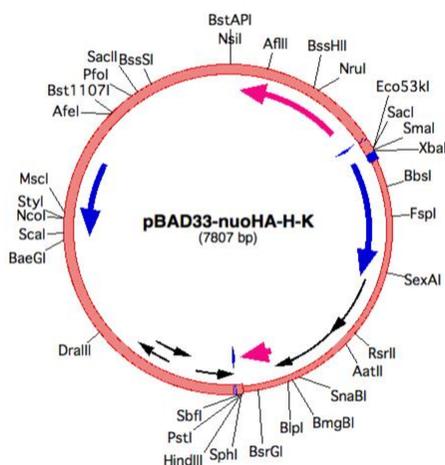


Figure 3. HAH-K pBAD33 construct
SacI and XbaI restriction sites were chosen.

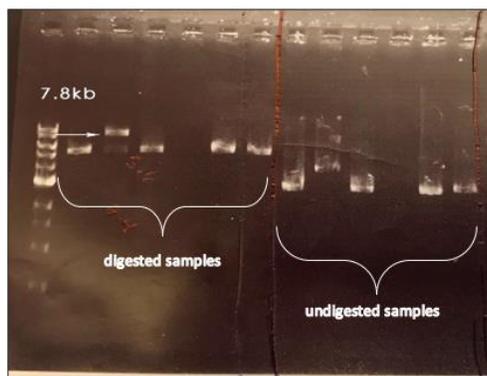


Figure 4. Screening of six PCR samples for HA-HK pBAD33 digested with XbaI and SacI-HF.

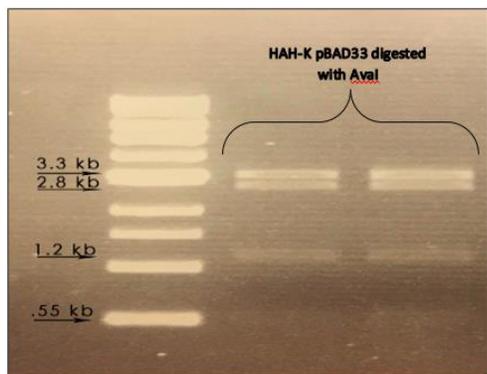


Figure 5. Confirmation of HAH-K pBAD33 with restriction enzyme AvaI.
Expected four distinct bands (.55, 1.2, 2.8, 3.3) were observed.

To confirm correct base pair size for HAH-K pBAD33, double digested of the second digested sample was performed by restriction endonuclease AvaI.

Introduction of HA tags through PCR at C-terminal

The G-H fragment was copied from pBA400, the template plasmid. HA tag was introduced into G-H fragment through PCR. G-HHA fragment was double digested to confirm correct base pair size of 9.1 kb (3.8 kb from G-HHA and 5.4 kb from pBAD33). The G-HHA PCR product and gel extracted pBAD33 fragment were ligated to create the HA-HK pBAD33 plasmid. GeneJET Plasmid Miniprep Kit (Thermo Scientific, MA) was used to purify plasmid DNA in five colonies, which were double digested with restriction endonucleases SacI-HF and XbaI, and checked by gel electrophoresis.

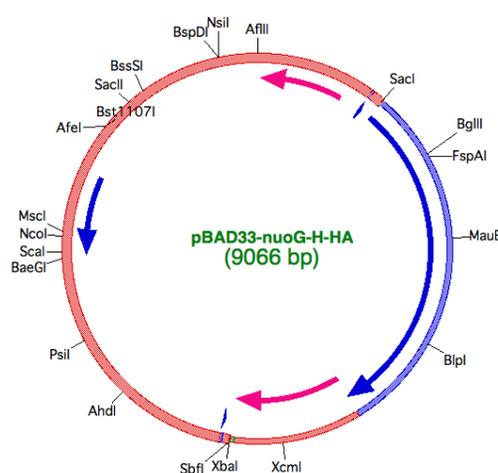


Figure 6. G-HHA pBAD33 construct
SacI and XbaI restriction sites were chosen.

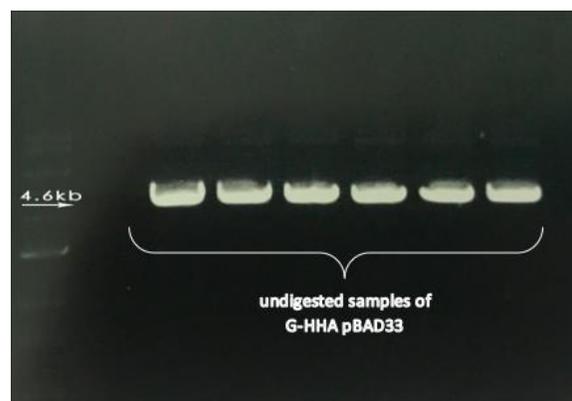


Figure 7. Screening of six samples for G-HHA pBAD33.
Correct band sizes of 4.6 kb were observed for all six samples.
Co-migration with a band of 4.6 kb is expected for a supercoiled plasmid of 9.1 kb, because samples are undigested.



Figure 8. Confirmation of G-HHA pBAD33 with XbaI and SacI-HF.

Expected band sizes of 9.1 kb (3.8 kb from G-HHA and 5.4 kb from pBAD33) were observed.

The DNA sequence data of HA-HK pBAD33 and G-HHA pBAD33 were confirmed by DNA sequencing by Lone Star Labs of Houston TX.

Detection of HA-tags through western blot

To determine if the tagged H subunits were made successfully, western blots were performed from cells harvested in medium induced with and without arabinose. The presence of the HA tag was detected by the HA antibody. Both plasmids showed expression of the tagged H subunit after induction with arabinose, and the H subunit was detected on western blots.

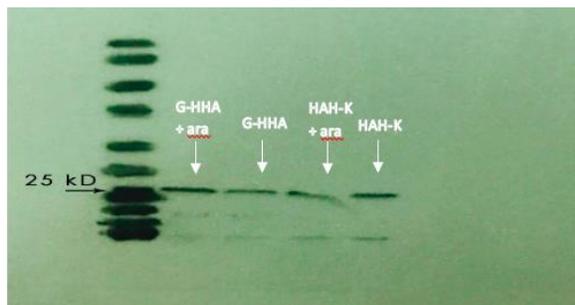


Figure 9. Western Blot data of HAH-K and G-HHA with arabinose induction.

Expression of the plasmids with and without arabinose were observed.

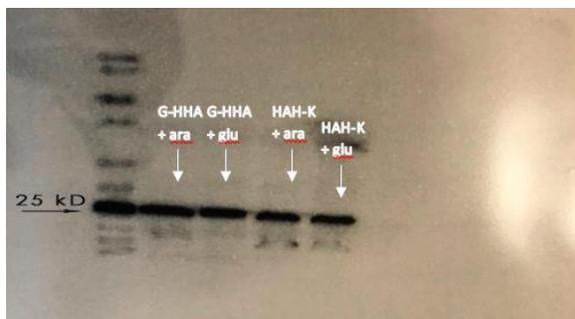


Figure 10. Western Blot data of HAH-K and G-HHA with and glucose induction.

Expression of the plasmids with arabinose or glucose were observed.

4. DISCUSSION

Both plasmids showed expression of the tagged H subunits after induction with arabinose and glucose. The use of glucose to suppress transcription of genes was not observed; however, arabinose was able to activate the genes. It is unlikely that the tag interfered with this process, but re-cloning of the plasmid to eliminate the possibility of mutations elsewhere is an alternative. From the western blot, the H-HA complex is recognizable by the HA antibody therefore creating an indirect antibody for the H subunit. Currently, all clinical mutations occur at junctions of subunits. Due to the location of the H subunit on the protein, its junctions with the G and I subunits will give key insights on how the peripheral arm and the membrane-bound proteins interact with each other during assembly. The next step is to begin mutagenesis on the H subunit and incorporate them to all the genes in complex I and track assembly of the whole protein in pBAD33. Further experiments are needed to analyze how different mutations in the H subunit will behave during assembly of Complex I. Therefore, tags for both termini were constructed to allow for more flexibility.

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6. ACKNOWLEDGEMENTS

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