

Enhancing mRNA Stability through the Addition of Stabilizing Untranslated Regions

by

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**Submitted to the Department of the Mechanical Engineering
In Partial Fulfillment of the Requirements for the Degree of**

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ABSTRACT

Messenger-RNA (mRNA) therapy, in which mRNA is introduced to cells or tissues to cause a transient expression of specific genes, has applications ranging from tissue engineering to neural regeneration. This transient nature of mRNA, however, limits the duration of gene upregulation, and potentially reduces the efficacy of mRNA therapy.

In this paper, various mRNA constructs were studied to identify stable designs which could be used for long-lasting mRNA therapies. Specifically, the GFP gene was prepared with four different hemoglobin untranslated regions (UTR's), which are known for their stabilization effects on mRNA. Each mRNA template was created through the digestion and ligation of a cDNA template, reversed transcribed from total RNA, and transfected into fibroblasts. The resulting fluorescence was measured as a surrogate for translation efficiency and duration. It was determined that the human beta-globin B UTR resulted in a fluorescence level that was tenfolds brighter than human alpha-globin 1 (HBA1), which was the least effective stabilizing untranslated region. This highlights the importance of UTR selection for future mRNA therapies.

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1. Introduction

Neurons from the peripheral nervous system (PNS) can exhibit partial functional regeneration after injury, while injured neurons from the central nervous system (CNS) do not recover. This difference in regeneration capacity is due to two main factors: injured CNS neurons lack the ability to activate the required intracellular factors for enhancing neurite growth, and the extracellular environment in the injured CNS releases inhibitory factors which prevent neurite regrowth. An approach to solve the first issue is to artificially enhance the expression of proteins known to encourage neurite outgrowth by transfecting neurons with the mRNA encoding these proteins. Furthermore, this strategy holds additional promise: the tips of developing neurons contain ribosomes that can locally translate mRNA to achieve high levels of growth-promoting proteins at the site of injury. mRNA is also safer to use relative to DNA because it does not interfere with the genome and therefore mitigates the risk of cancer associated with this therapy. However, the main drawback of mRNA is its instability and its shorter half-life relative to that of DNA, resulting in only a transient expression of regeneration genes.

The main goal of this project is to focus on the 5' and 3' untranslated regions (UTR) of the mRNA in order to stabilize the mRNA coding strand itself. A range of different stabilizing 5' and 3' untranslated regions will be digested with their corresponding restriction enzymes, and subsequently ligated to destabilized GFP. These newly-formed genes will be screened for marked enhancement of green fluorescent proteins (GFP) expression stability in fibroblasts. By measuring the time-course of fluorescence in fibroblasts, we can identify the untranslated regions which provide the highest stability of protein expression. By maximizing the half-life of the mRNA, the required therapeutic dosage, and associated costs and risks, will be minimized for this therapy.

2. Background

2.1 Untranslated Regions Selection

Most mRNAs have half-lives that last from several minutes to several hours, as they are destroyed by intracellular exo- and endonucleases. However, mRNAs from globins have been

known to have half-lives that last from 16 hours to 48 hours, which is useful for mRNA therapy. Specifically, the 3' UTR is known to play a large role in mRNA stabilization, as a variant in the α - globin gene 3' UTR is known to cause nondeletional α - thalassemia¹. The mutation leads to the ribosome reading through the stop codon, causing disruption to the sequences or structures within the 3'UTR of the α - globin mRNA, and mRNA destabilization

In addition, one study of α - and β - globin gene stabilization shows that they have different mechanisms of stabilization due to the level of expression when exposed to the α – variant. α - globin was shown to have less than 2 percent expression which is in contrast to the 40 percent expression of β - globin. Also, the α - globin in mRNA is destabilized when ribosomes read 4 codons into the 3' UTR, whereas β - globin destabilization occurs only if the ribosome reads further into the 3'UTR.² These two observations indicate that the α - and β - globins have different mechanisms of 3' UTR stability, and therefore, one globin might have a larger role in stability or have a longer half-life compared to the other globin.

The poly-A tail, found at the end of the mRNA, is another crucial factor in mRNA stability. This tail prevents the mRNA from degrading in the cytoplasm, promotes efficient translation of the mRNA, as well as keeping the mRNA stable during the exportation from the nucleus to cytoplasm. Once transcribed, the poly-A tail gradually shortens due to exonuclease activity. Once the tail has degraded, the mRNA is rapidly destroyed.

To try and extend the life of mRNA, a poly(thio-adenosine) tail was also investigated, due to the non-hydrolysable sulfur bond that might increase stability. A poly-U polymerase was found to efficiently add 1-thio-adenosine to the tail for this purpose.

In summary, four different globins 3' UTR were used for this experiment, as well as a variant poly-A tail: β - globin (HBB), α -1- globin (HBA1), α - 2 – globin (HBA2), a sextuplet repeat of α - 2 – globin (polyHBA2), and HBB with a poly A tail, capped by a short run of 1-thio-adenosines (thioHBB).³

¹ JE Russell and SA Liebhaber, "The stability of human beta-globin mRNA is dependent on structural determinants positioned within its 3' untranslated region," *Blood* 87 (1996): 5314-5323.

² Ibid. 5314-5323.

³ Ibid. 5314-5323.

2.2 Use of Pseudouridine

Pseudouridines are used in the mRNA template, rather than uridines, as this results in more translation and a lower innate-immune response⁴. This enhancement is due to the fact that uridine activates RNA-dependent protein kinase (PKR), which is known to phosphorylate translation factor 2-alpha, and thus inhibit translation. Pseudouridines are known to evade PKR, and thus there is more translation, and less immune response. Although both uridine and pseudouridine both have some suppressive effect on translation, there was a noticeable larger suppression of protein synthesis in unmodified or uridine-containing mRNA compared to pseudouridine containing mRNA.

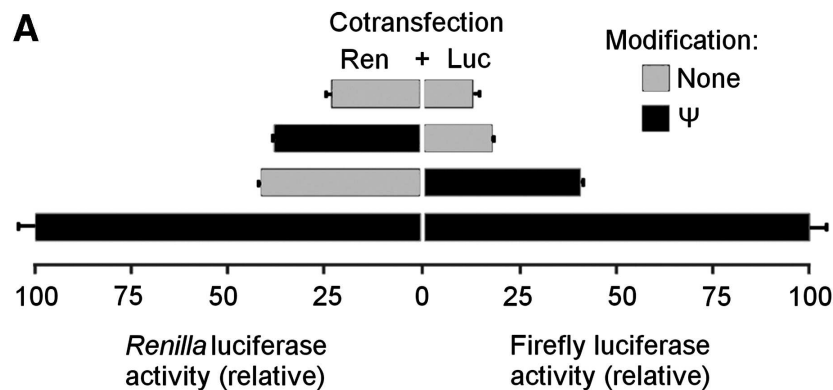


Figure 1: mRNA encoding *Renilla* luciferase and Firefly luciferase were transfected with and without pseudouridine modification and then mixed. The above shows that with both modified mRNA, there was more luciferase activity than in transfection with unmodified mRNA.⁵

The above figure from Kariko's study shows that when both mRNA were modified with pseudouridine, there was relatively more activity than when both mRNA were unmodified with uridine. Additional experiments also showed that the beneficial effects of pseudouridine is independent of cap and poly(A) tail, which are known to help stabilize mRNA further.

⁴ Katalin Kariko and Bart Anderson, "Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation," *Nucleic Acids Research* (2010): 1-9.

⁵ Ibid. 3.

In addition, mRNA is known to induce an immune response from the body, thus making it less effective for gene replacement therapy. However, by using pseudouridine, most of the toll-like receptors that activate cells of the innate immune system are inactivated. This inactivation is due to the relation between uridine and RIG-I, in which type I interferon secretion occurs when mRNA contains 5' triphosphate. However, when using modified mRNA, this replacement inactivates the secretion of interferon and therefore diminished its immunogenicity in vivo.⁶

3. Preparation of mRNA Template and mRNA Transfection into Fibroblast Cells

3.1 Creating the DNA Template

From a total-cell lysate from rat hippocampal neurons, the total RNA was extracted and isolated from the DNA using the Qiagen RNEasy Mini Kit. The extracted total RNA was then reverse transcribed in order to obtain the cDNA that will serve as the template for the mRNA.

Table 1. Reverse Transcription Protocol

<u>Reverse Transcription</u>	<u>Final Concentration</u>	<u>Per 20uL Reaction</u>	<u>Temperatures</u>
mRNA Template		10	65C-45min
5uM ivT Reverse Primers	0.25uM	1	90C-5min
10mM dNTP Mix	200uM	0.4	4C-forever
MonsterScript Reverse Transcriptase	1:20	1	
5x MonsterScript Buffer	1x	4	
H2O		3.6	
		20	uL Total

5' and 3' UTR's were isolated from the cDNA mixture by choosing the complimentary primers based on HBB, HBA1, HBA2 and pHBA2 5' and 3' UTR genetic sequences. Also, the coding sequence (CDS) for destabilized Green-Fluorescent Protein (dsGFP) and Green-Fluorescent Protein (GFP) were obtained from Clontech. Using the following PCR protocol, the UTR's and CDS were amplified twice—first time to isolate the UTR and CDS from other DNA sequence and second time to increase the concentration of UTR and CDS.

⁶ Katalin Kariko, "Incorporation of Pseudouridine Into mRNA Yields Superior Nonimmunogenic Vector With Increased Translational Capacity and Biological Stability," *The American Society of Gene Therapy* 16 (2008): 1833-1840

Table 2. PCR Protocol for HBB 5-UTR

	<u>Final</u> <u>Concentration</u>	<u>Per 25uL</u> <u>Reaction</u>	<u>Temperatures</u>
HBB 5-UTR Kz			
5x HF Buffer	1X	5	95C - 5min
10mM dNTP Mix	200uM	0.75	98C - 15sec
5uM HBB 5-UTR Forward Primer	.5uM	2.5	68C - 30sec
5uM HBB 5-UTR Reverse Primer 2	.5uM	2.5	68C - 2min
RT Reaction		1	4C - forever
KAPA HiFi Hot-Start DNA Polymerase	0.5U	0.5	
H2O	H2O	12.75	
		25	uL Total

Gel electrophoresis was used to isolate the UTR and CDS from other extraneous DNA, RNA and protein. The gel was made up of 1.5% agarose, along with 3mL of TAE buffer and 4μL of Ethyl Bromide. TriDye 2-Log DNA Ladder was used as the standardized ladder to determine the relative length of UTR. Afterwards, the correct band was extracted using the QiaQuick Gel Extraction Kit. This process was repeated to obtain the following UTR's: 5' HBB UTR, 5' HBA1 UTR, 5' HBA2 UTR, 3' HBB UTR, 3' HBA1 UTR, 3' HBA2 UTR, 3' pHBA2 UTR, GFP, and dsGFP.

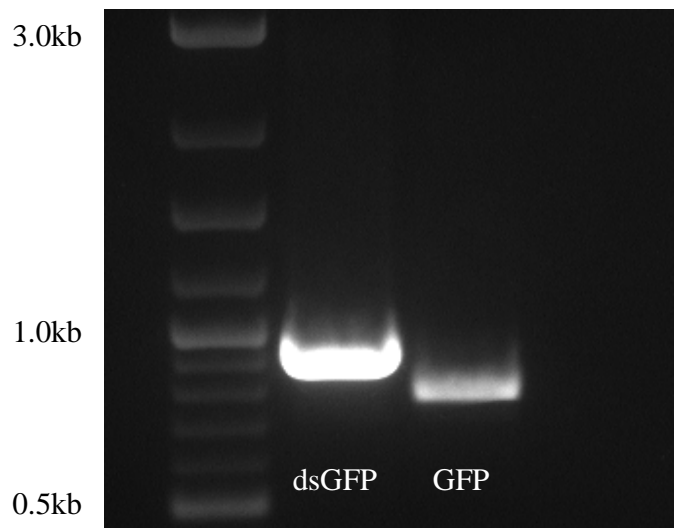


Figure 2: In order to determine that the gene we obtained was dsGFP, we knew that dsGFP had a length of 0.925 kb whereas GFP had a length of 0.755 kb. This gel indicates that we have obtained the right gene for this study.

Afterwards, the 5' UTR and 3' UTR are digested with their restriction enzymes--Nhe I and Age I, respectively. GFP and dsGFP were digested with both Nhe I and Age I as well with the following digestion protocol:

Table 3. Restriction Enzyme Digestion Protocol

<u>Digestion</u>	<u>Final Concentration</u>	<u>per 50uL Reaction</u>	<u>Temperatures</u>
10X NEBuffer 1	1X	5	37C - 1hr
100X BSA	1X	0.5	65C - 20 min
CDS or HBB UTR PCR Product	0.25-1.5ug	30	
AgeI (restriction enzyme)	2.5U	0.5	
KpnI-HF (restriction enzyme)	2.5U	0.5	
H2O		13.5	
		50	uL Total

The digested products are purified using the QiaQuick Gel Extraction Kit before being ligated together using the following ligation protocol:

Table 4. Ligation Protocol

<u>Ligation</u>	<u>Final Concentration</u>	<u>per 50uL Reaction</u>	<u>Temperatures</u>
10X E.coli DNA Ligase Buffer	1X	5	16C - 18hr
Digested CDS Kz PCR Product	10-50nM	15	65C - 20min
Digested HBB 5-UTR kz PCR Product	50nM	5	1.5% Gel - 150ml
Digested 3-UTR PCR Product	25nM	5	Elute in 30uL
E.coli DNA Ligase	5U	2.5	
H2O		17.5	
		50	uL Total

The following DNA template was created after the ligation step, purified, and amplified using the PCR protocol:

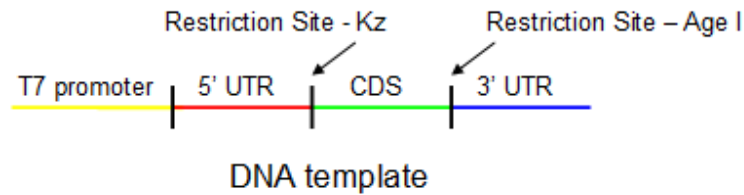


Figure 3: The DNA template consists of a T7 promoter, allowing for transcription to take place. The 5'UTR and 3'UTR are ligated onto the coding sequence with different restriction site – Nhe I and Age I, respectively. In order to ensure that the UTRs are effective, we used the coding sequence of dsGFP so that the only way to stabilize dsGFP would be through the UTRs and not through its own stabilizing UTRs.

As a result, the following cDNA templates were created:

- 5' HBB UTR – dsGFP – 3' HBB UTR
- 5' HBA1 UTR – dsGFP – 3' HBA1 UTR
- 5' HBA2 UTR – dsGFP – 3' HBA2 UTR
- 5' HBA2 UTR – dsGFP – 3' pHBA2 UTR

After the DNA templates were created, the templates were inserted into a bacterial plasmid, which are then transfected into e.coli bacteria. Colonies were grown overnight, and only the e.coli colonies that took up the plasmid successfully will be able to grow on the kanamycin-treated LB agar plate. The colonies are then grown in 100ml flasks and plasmids are removed using a Qiagen Maxi-kit. The plasmids are then quantitated and diluted to 1ug/6uL and run on a gel in order to ensure the correct DNA template was obtained by looking at its relative length along the ladder. Then the sample is sent for sequencing in order to further assure that the right sequence of cDNA template was obtained.

3.2 Creating the mRNA Template

The DNA template from the previous step will be used to create the mRNA template. The first step is to transcribe the mRNA from the DNA template using this protocol:

Table 5. In Vitro Transcription Protocol

in vitro Transcription	<u>per 20uL Reaction</u>	<u>per 50uL Reaction</u>	Settings:
Rnase-free water	0.8	2	37C for 2hs
mScript 10x Transcription Buffer	2	5	
NTP Solution*	7.2	18	
100mM DTT	2	5	
mScript T7 Enzyme Mix	2	5	
T7 Template	6	15	
Total	20	50	

*The NTP solution used here consists of adenine, cytosine, thymine, and pseudo-uridine rather than uridine due to pseudouridine contribution to mRNA stability, higher protein expression, and decreased immunogenicity.

1uL of DNA digestion enzyme per 20 uL reaction is added and held at 37C for 15 minutes. This is to ensure that all the DNA templates are digested within the solution. In order to purify the mRNA, an RNeasy RNA extraction kit is used to remove DNA template and proteins that may remain within the solution. The RNA is then quantitated in order to determine the amount of mRNA gained from the IVT transcription. A sample of the uncap/untail mRNA is stored in order to compare later with the cap/tail mRNA. Afterwards the 5' cap and 3' tail is added to the mRNA in order to stabilize the mRNA as well as prevent the mRNA from degrading. The 5' cap is added using the following protocol:

mRNA is denatured at 65C for 10 minutes prior to capping

Table 6. 5' Capping mRNA Protocol

5' Capping	<u>per 100uL Reaction</u>	Settings:
10x ScriptCap Capping buffer	10	37 degrees 1hr 65C for 10min
20mM GTP	5	
20mM SAM	1	
ScriptGuard	2.5	
mScript Capping Enzyme	4	
mScript 2'-O-Methyltransferase	4	
Heat Denatured RNA	73.5	
Total	100	

Solution of mRNA is denatured at 65C for 10 prior to tailing

Table 7. PolyA Tailing mRNA Protocol

<u>PolyA Tailing</u>	<u>per 123.5 uL</u> <u>Reaction</u>	<u>Settings:</u>
5' Capping Product	100	37 degrees 40 minutes (EXACTLY) hold at 4C (for 0s!)
ScriptGuard Rnase Inhibitor	0.5	
mScript 10X Tailiong Buffer	12	
20mM ATP	6	
mScript Poly(A) Polymerase	5	
Total	123.5	

It is important to determine the tailing time for each mRNA in order to ensure that each mRNA tail is the same length compared to the uncap/untail mRNA. By keeping the mRNA tail the same length, this will ensure that the stability of the mRNA is entirely due to the 3' UTR, rather than the tail length of the mRNA. The completed mRNA is then purified using the RNeasy kit and quantitated to determine the amount of mRNA gained from the procedure. The mRNA is then diluted to the smallest concentration of the different type of mRNAs obtained and superase inhibitor is added to each mRNA in order to prevent degradation. The following mRNA template is created as a result:

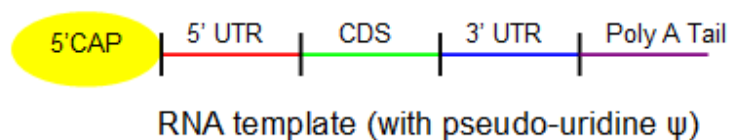


Figure 4. RNA Template is similar to DNA template in terms of 5'UTR, Coding Sequence, and 3'UTR. The only difference is the addition 5' CAP to prevent degradation when the mRNA travels into the cytoplasm of the cell. Also, the Poly A Tail adds stability to the mRNA.

Five different mRNAs were created using the above protocol:

- 5' CAP – 5' HBB UTR -dsGFP- 3' HBB UTR – Poly A Tail
- 5' CAP – 5' HBA1 UTR -dsGFP- 3' HBA1 UTR – Poly A Tail
- 5' CAP – 5' HBA2 UTR -dsGFP- 3' HBA2 UTR – Poly A Tail

5' CAP – 5' HBA2 UTR -dsGFP- 3' pHBA2 UTR – Poly A Tail

5' CAP – 5' HBB UTR -dsGFP- 3' HBB UTR – Poly A Tail + Poly thio-A Tail

The last mRNA (thioHBB) was created in order to determine if the sulfur bonds in the Poly thio-A Tail contribute more to the stability of the mRNA. An RNA gel is created in order to see if the cap/tail length is the same across all RNA. Also, the RNA gel also shows whether the quantitation is correct so that equal amount of mRNA can be used to transfect cells each time.

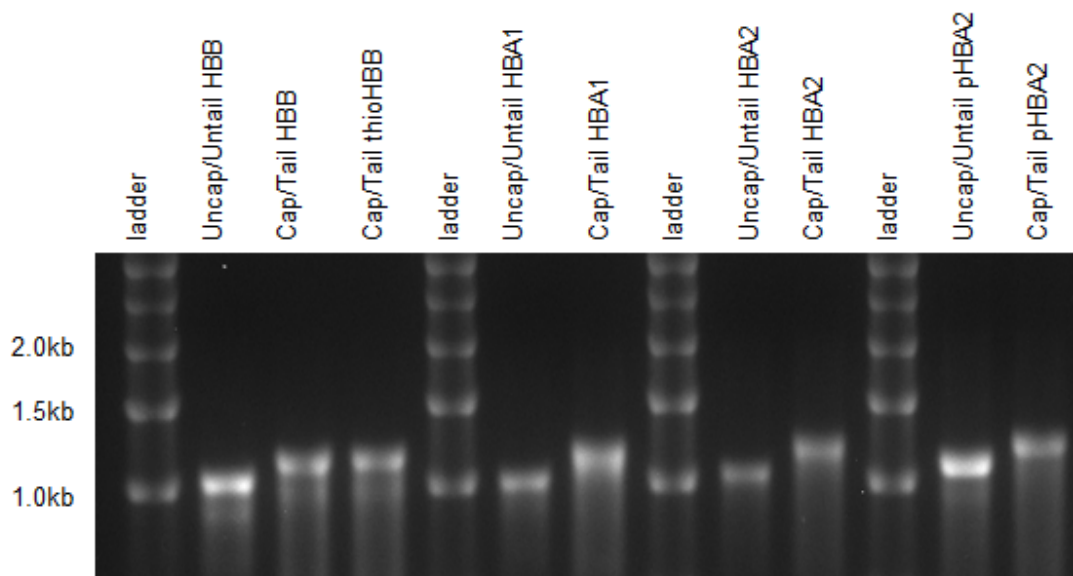


Figure 5. This RNA gels compares the uncap/untail mRNA with the cap/tail mRNA. The gap between each uncap/untail mRNA and cap/tail mRNA is about the same, showing that the tail length for each mRNA is approximately the same length and thus tail will not influence the stability within this experiment. Also, the cap/tail mRNA are about equal brightness, which shows that the mRNA are diluted to the same concentration so that concentration will not play a role in fluorescence level as well.

3.3 mRNA Transfection into Cells

Once fibroblasts were plated into 6-well plates and incubated overnight, mRNA is prepared by mixing with OptiMEM (cell media) and RNAiMax (binds the RNA to lipid, which cells will intake through endocytosis). Therefore, once the solution is prepared according to Table 9:

Table 9. mRNA Transfection Solution and Protocol

OptiMEM	100	uL/ug RNA
RNA	1	Ug
RNAiMax	5	uL/ug RNA
NeuroMAG	3.5	uL/ug RNA

IMMEDIATELY pipette RNAiMax up and down when added to optimem

Set a timer for 15 minutes since added to the first sample

The solution is pipetted into each well. Afterwards, cells are monitored every 6-8 hours for the first 48 hours and then cells are measured every 24 hours afterwards. The expression of dsGFP was measured using epifluorescence. At each timepoint, five images are randomly taken per well. After 152 hours, each cell's fluorescent intensity is measured and averaged in order to determine the average amount of fluorescent per hour. The highest level of fluorescence will indicate the highest level of stability provided by the 3'UTR for the mRNA.

4. Results and Data Analysis

By monitoring the fibroblasts over 152 hours, cells transfected with mRNA with various 3'UTR's showed significantly different fluorescence levels. Also, all RNA reached a peak in fluorescent level at about 40 hours before decreasing, once the mRNA had all been translated and expressed in protein:

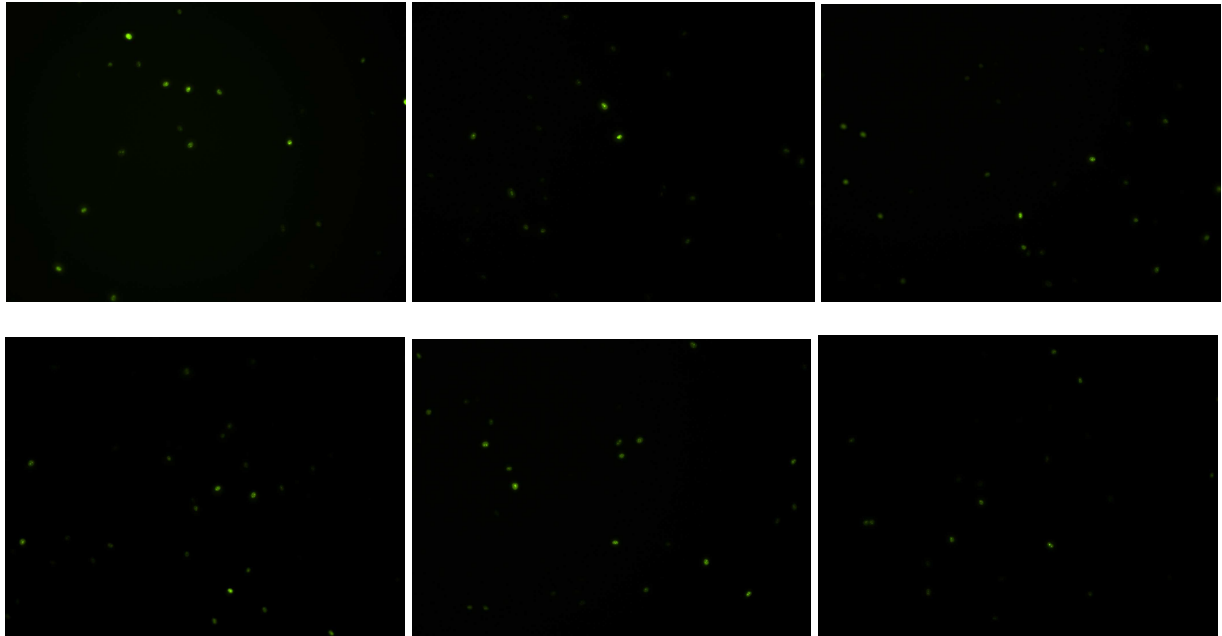


Figure 6. These images of fibroblasts expressing GFP with HBB UTR illustrate the brightness time lapse over 56 hours. Each image is at a different exposure due to the exponential increase in fluorescence. HBB at time exposure of 250ms after 10 hours (top right), HBB at time exposure of 60ms after 18 hours (top middle), HBB at time exposure of 60ms after 26 hours (top left), HBB at time exposure of 40ms after 32 hours (bottom left), HBB at time exposure of 50ms after 48 hours (bottom middle), HBB at time exposure of 50ms after 56 hours (bottom right).

HBB has the highest level of fluorescent from the brightness of the cell nuclei as well as the shortened camera exposure. ThioHBB resulted in less translation, which suggests that the poly U tail did not add more stability and might actually decrease the stability of mRNA. HBA2 and pHBA2 has the same level of stability, showing that the HBA2 repeats did not enhance the mRNA stability. Finally HBA1 had the lowest level of stability of all the 5 mRNAs.

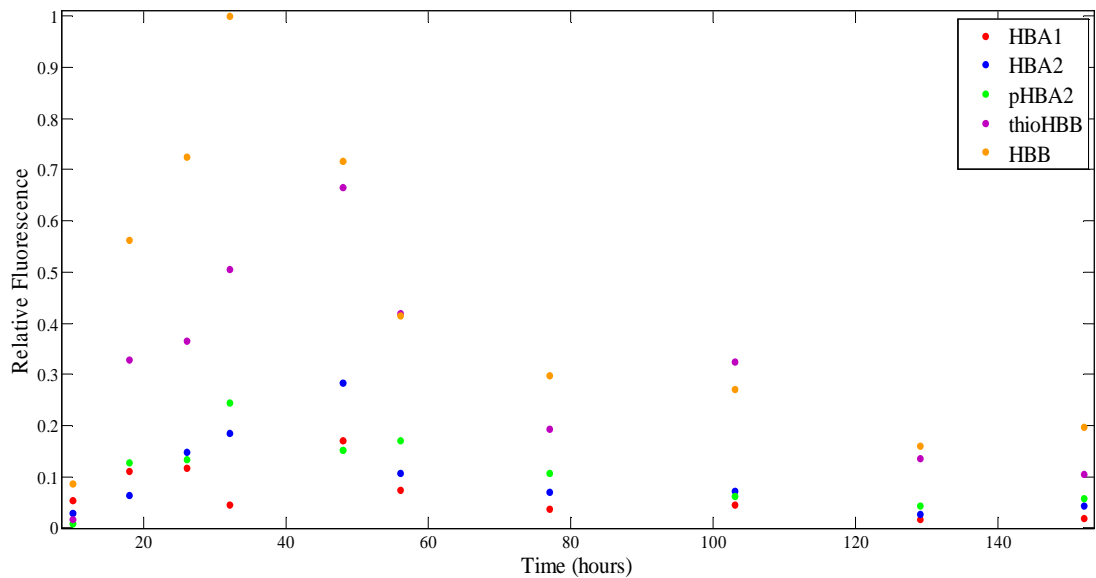


Figure 7. This graph measures the relative fluorescence of each mRNA with time in hours. HBB has the highest fluorescence level and HBA1 has the lowest fluorescence level.

Overall, the trend and images show that HBB results in the most fluorescence and therefore is likely the most effective in terms of mRNA stability, whereas HBA1 is the least fluorescent and therefore the least effective in terms of mRNA stability.

The curves can be described by considering the half-life of mRNA and half-life of the protein. The transfection process can be modeled as seen with figure 8 and the following equations can be used to describe this process:

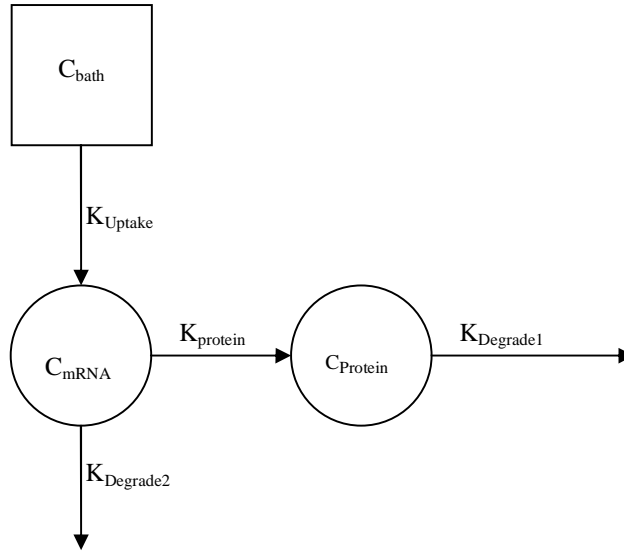


Figure 8. This representation shows the relationship of the concentration of the bath (media), concentration of mRNA, and the concentration of protein and their relationship to one another.

$$\frac{dC_m}{dt} = k_u C_B - k_{d2} C_m \quad (1)$$

$$\frac{dC_p}{dt} = k_p C_m - k_{d1} C_p \quad (2)$$

Where C_m is the concentration of mRNA, C_p is the concentration of protein, C_B is the concentration of bath, k_u is the rate of uptake, k_p is the rate of mRNA to protein, k_{d1} is the rate of protein degradation, and k_{d2} is the rate of mRNA degradation.

However, equation (1) can be simplified since C_B is only crucial within the first four hours of the experiment, and since the experiment was done over 152 hours, C_B becomes negligible and therefore equation (1) is now:

$$\frac{dC_m}{dt} = -k_{d2} C_m \quad (3)$$

By solving for equation (2) and (3) with the following boundary conditions:

$$C_m = C_m^0 \text{ at } t = 0,$$

$$C_p = 0 \text{ at } t = 0,$$

Then the solution is:

$$C_m(t) = e^{-k_{d2}t} \quad (4)$$

$$C_p(t) = \left(\frac{k_p}{k_{d1} - k_{d2}} \right) \cdot (e^{-k_{d2}t} - e^{-k_{d1}t}) \quad (5)$$

When this model was fitted with the data for HBB and pHBA2, the resulting plot appeared:

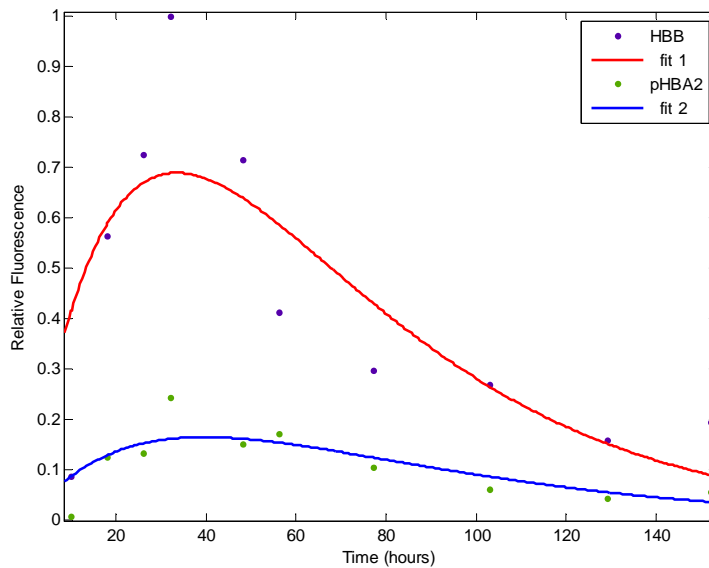


Figure 9. HBB and pHBA2 data fitted with the curves as predicted from the suggested model above.

The model does not fully predict the observed protein expression dynamics. In particular, it is unable to recapitulate the sharp peaks in expression suggested by the data. There are some limitations to the model due to the assumptions made in order to solve the differential equations. One assumption made was that the mRNA intake was instantaneous and therefore mRNA

concentration was modeled as an exponential decay curve. In reality, the mRNA is added to the cells at time $t=0$ hours, and requires several hours to be uptaken to the cell, several more hours to be released from endosomes, and then several more hours for protein translation to be completed. The first two factors would cause a non-instantaneous upswing in mRNA concentration. The last factor would add a simple delay to the protein expression curve. Taking all three of these factors into account would allow the model to more accurately recapitulate the peaks seen in the data. The protein decay segment in the latter part of the experiment fits the simple model accurately.

5. Further Experiments

Currently, growth factors for neurons are being created through a similar method in the procedure section of this study. GAP, CAP, CSTAT3, and ATF3 coding sequences will be ligated with 5' HBB UTR and 3' HBB UTR due to its high stability effect on mRNA. These genes will then be transfected into neurons and cell growth will be measured by taking images and measuring the length of neurites.

6. Discussion

HBB has the highest fluorescent level of all the mRNA, followed by thioHBB. HBA2 and pHBA2 had the next highest fluorescent level, and finally HBA1 had the lowest fluorescent level. This result indicates that HBB a very effective stabilizing untranslated region, resulting in ten times the fluorescent intensity compared to the HBA1 fluorescent level. Based on the images of the experiment, HBB required a significantly shorter exposure time compared to other mRNA and was more evenly expressed in many cells compared to other mRNA-transfected cells. However, there are limitations to this result, since the experiment was only completed once, the standard deviation was quite large. Also, the cells were manually clicked upon for measurement and therefore there was scope for human error as well. Finally, the number of cells within each well was assumed to be roughly equal. Nevertheless, since HBB was so fluorescent compared to other mRNA, these errors are negligible in terms of relative brightness compared to one another.

Improvements to this experimental design could be done in terms of the data analysis and measurements to be taken. Rather than manually clicking on each cell, by writing a program that automatically identifies a cell will make the data collection more accurate. Also, by taking the number of fluorescent nuclei over the total number of nuclei within the well will give a more accurate comparison across all 6 wells. Finally, another suggestion is to plate the same number

of cells per well and to take the total fluorescent signal per well. However, even with these experimental limitations, HBB resulted in significantly higher protein expression. This result highlights the importance of UTR selection for mRNA stability. In the next step, these mRNA-encoding growth factors will be transfected into neurons to encourage neurite outgrowth. Therefore, by maximizing the half-life of mRNA, not only will this knowledge help with the next portion of the experiment, but it will help lower mRNA therapeutic dosage as well as mRNA risk associated with mRNA therapy.

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