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Modulation of nitric oxide synthase I transcription by Tau and Alpha-Synuclein and its relevance to Alzheimer's and Parkinson's diseases

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Modulation of Nitric Oxide Synthase I Transcription by Tau and Alpha-Synuclein and its Relevance to Alzheimer's and Parkinson's Diseases

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by Alexandra Lilly Deal and Taelor Allison Weaver

May 2016

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Degree of Bachelor of Science.

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TABLE OF CONTENTS

LIST OF FIGURES:

Figures

Tables

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ABSTRACT

Alzheimer's Disease (AD) and Parkinson's Disease (PD) are both progressive neurodegenerative disorders that affect millions of Americans and for which there are no cures. AD can significantly impair the ability to think, remember, communicate, and carry out daily activities, while PD can affect motor functions such as balance, coordination, and the ability to speak. Intracellular protein aggregation is a hallmark of both diseases, with AD being characterized by the build up of neurofibrillary tangles composed of misfolded tau protein and PD being characterized by Lewy bodies composed of alpha-synuclein. Both normal tau and alpha-synuclein can localize to the nucleus; however, their nuclear roles have not been fully elucidated. These proteins bind and stabilize alternative DNA structures, which form most readily at purine-pyrimidine repeats. The *1f* promoter of the Nitric Oxide Synthase I (*NOSI*) gene, which is misregulated in both AD and PD, contains one such repeat. This *NOSI* repeat is polymorphic and has the sequence $(TG)_mTA(TG)_n$ where m and n can vary from individual to individual. Genotyping shows that shorter dinucleotide polymorphisms are associated with AD and PD. Promoters with shorter repeats also have decreased transcriptional expression compared to promoters with larger repeats. Because tau and alpha-synuclein can bind such repeats, we hypothesize that tau and alpha-synuclein may modulate *NOSI* transcription through the $(TG)_nTA(TG)_m$ repeat. Reporter genes directed by the *NOSI 1f* promoter with and without the $(TG)_nTA(TG)_m$ repeat region were transfected into human neuroblastoma cells (SK-N-MC) and human cervical cancer cells (HeLa) that express varying levels of tau and alpha-synuclein. Promoters with the TG repeat directed approximately two-fold changes in reporter gene expression, while promoters without the TG repeat caused no change in expression. These findings suggest that tau and alpha-synuclein modulate *NOSI* expression through interaction with a dinucleotide polymorphism associated with disease development.

BACKGROUND

Both Alzheimer's Disease (AD) and Parkinson's Disease (PD) are progressive, neurodegenerative disorders which account for millions of deaths each year (Centers for Disease Control and Prevention, 2015). The severe and debilitating nature of AD and PD make it imperative that we investigate the molecular mechanisms underlying neurodegeneration. AD and PD are similar in that they are both characterized by protein misfolding which leads to protein aggregation. Misfolded tau and alpha-synuclein accumulate in potentially pathogenic intracellular neurofibrillary tangles in AD and PD, respectively (Vasudevaraju *et al.,* 2012). Understanding the normal function of tau and alpha-synuclein is critical in understanding the pathology of these neurodegenerative diseases.

In neurons, tau, short for tubulin associated factor, functions to maintain microtubule stability which is critical for cellular transport through axons (Duan *et al*., 2012). Hyperphosphorylation causes tau to dissociate from microtubules, allowing the microtubules to depolymerize. The free hyperphosphorylated tau is truncated and can then aggregate into neurofibrillary tangles (Mania *et al*., 2015; Frost *et al*., 2014; Duan *et al*., 2012 & Lee *et al*., 2001). It is unclear whether free truncated tau or the neurofibrillary tangles themselves are more toxic to the cell. Some studies suggest that neurofibrillary tangles are toxic, while others suggest that neurofibrillary tangles are a cellular protective mechanism against free truncated tau (Mania *et al*., 2015).

Alpha-synuclein is a neuron-specific protein localized primarily in the nucleus and presynaptic nerve terminals (Maroteaux *et al*., 1988). Its exact function in the cell is still unknown; however, many studies suggest significant implications of α-synuclein in synaptic transmission (Cabin *et al*., 2002). A missense mutation in the gene that encodes alpha-synuclein (SNCA) was associated with familial cases of early-onset PD. The mutation in SNCA is one

potential cause of Lewy Body formation and is thought to disrupt secondary protein structure leading to self-aggregation of alpha-synuclein (Polymeropoulos *et al*., 1997). Alpha-synuclein has since been studied in the contexts of PD pathogenesis, but the exact role of the protein in neurodegeneration has yet to be elucidated.

Much research on AD and PD has focused on the roles of tau and alpha-synuclein in the cytoplasm; however, focusing only on these tangles has failed to fully explain the mechanisms behind neurodegeneration. Therefore, our study examines the role of tau and alpha synuclein in the nucleus. Unphosphorylated tau and alpha-synuclein localize to the nucleus. Although their nuclear binding site has yet to be elucidated, several studies suggest they bind preferentially to purine-pyrimidine repeats in the DNA (Sultan *et al*., 2011; Vasudevaraju *et al*., 2012 & Liu and Gotz, 2013). Purine-pyrimidine repeats can often be found in promoter regions where transcription of the gene produces negative supercoils, which can cause the DNA in the repeat region to switch into alternative forms (Cox *et al*., 2012). Furthermore, one study showed that the tau protein is capable of binding and stabilizing a CG oligonucleotide in the Z-DNA conformation, while alpha-synuclein stabilizes the oligonucleotide in an altered B-DNA conformation (Vasudevaraju *et al*., 2012). Circular dichroism studies have detected Z-DNA in post-mortem AD brains, while DNA isolated from PD brains is far more likely to contain altered B-DNA structures, which do not match any currently crystallized forms of DNA (Vasudevaraju *et al*., 2012). Thus, tau may be responsible for changes in the DNA structure observed in AD, as well as alpha-synuclein in PD.

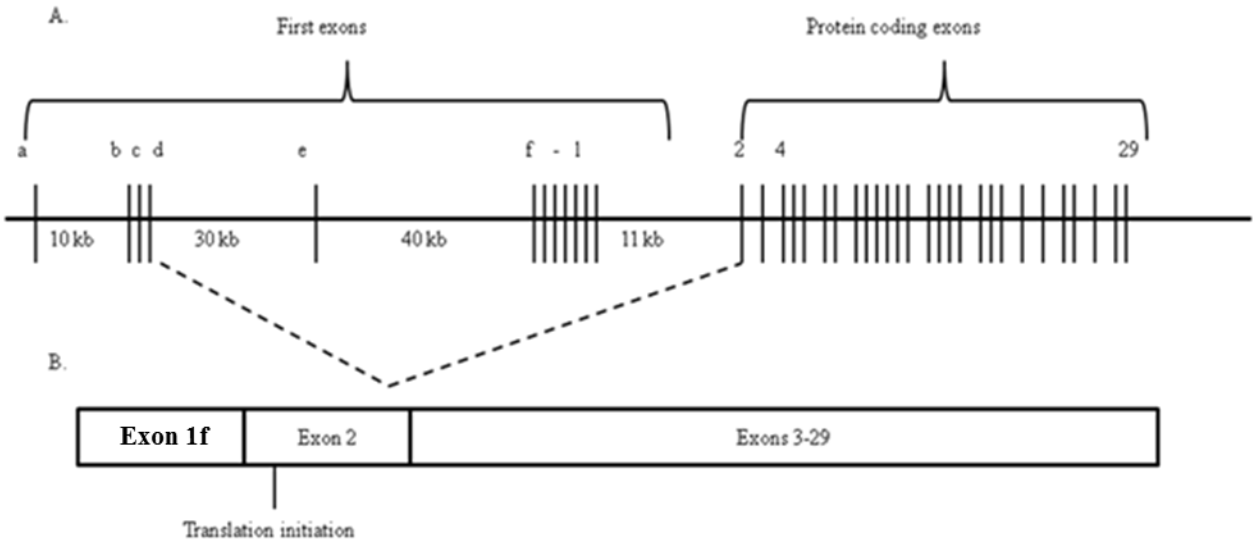
Because these changes in DNA structure are occurring in promoter regions, they are likely to affect transcription factor access to the DNA. Thus, tau and alpha-synuclein induction or stabilization of alternate DNA structures could affect transcription of the genes with purine-

pyrimidine repeats within their promoter regions (Vasudevaraju *et al*., 2012). The Nitric Oxide Synthase I gene (*NOSI*), which is misregulated in both AD and PD, contains a purine-pyrimidine repeat in its promoter region. Therefore, it is a good model to study the ability of tau and alpha-synuclein to modulate gene expression at purine-pyrimidine repeats.

Many studies associate the misregulation of *NOSI* with neurodegenerative disorders including AD and PD (Knott and Bossy-Wetzel, 2009 and Przedborski *et al*., 1996). The NOSI enzyme produces secondary messenger nitric oxide (NO), which is crucial for the regulation of a variety of body functions including controlling blood pressure, long-term memory, synapse formation, pain regulation, muscle movement, glucose regulation, and urine formation (Toda and Okamura, 2003; & Prast, 2001). The most direct link between *NOSI* and neurodegenerative disease was discovered using the MPTP mouse model. Mice treated with MPTP develop PD-like symptoms, while *NOSI*-deficient mice treated with MPTP do not develop PD symptoms (Przedborski *et al*., 1996). These findings suggest that NOSI plays a role in MPTP-induced neurotoxicity. *NOSI* transcription must be tightly regulated, as excess NO can have neurotoxic effects, while low levels can have neuroprotective effects (Sultana *et al*., 2006). Thus, transcriptional misregulation of *NOSI* likely contributes to the pathology of AD and PD, making it important to examine the *NOSI* promoter.

Transcriptional regulation of *NOSI* is highly complex but has not been extensively studied. The human *NOSI* gene, located on chromosome 12, is over 160 kb in length and consists of 29 exons (Hall *et al*., 1994). Of these 29 exons, 12 first exons (1a-l) controlled by 12 separate first promoters are alternatively spliced to exon 2, which contains the translation start site (Rife et al., 2009). Expression of the different first exons is tissue specific with first exons *1d, 1f*, and *1g* predominating in the brain (Bros et al, 2006). This study focuses on the human *1f* promoter,

which contains a polymorphic purine-pyrimidine repeat, $(TG)_mTA(TG)_n$ (Figure 2). Shorter lengths of this purine-pyrimidine repeat correlate with a higher risk of both AD and PD (Galimberti et al., 2008, Rife et al., 2009). Previous gene expression assays revealed that NOSI constructs containing the polymorphic $(TG)_mTA(TG)_n$ repeat change their expression in response to tau knockdown as well as alpha-synuclein overexpression. These studies also showed that longer repeats respond more than shorter repeats to changing levels of tau and alpha-synuclein, indicating that changes in gene expression may be mediated through the $(TG)_mTA(TG)_n$ repeat.



C.

1441	**TAGACCTGCC**	**CTGCCCTGCG**	**TGGCTACTAC**	**ATTCAGCCCT**	**ATCCAGGCTC**
1491	**CCAGAAATTG**	**TCATTGTGTG**	**TGTGTGTGTG**	**TGTGTGTGTG**	**TGTGTGTGTA**
1541	**TGTGTGTGTG**	**TGTGTGTGTG**	**TGTGTGTTTC**	**CTGATAGAAA**	**AAAAAAAATG**
1591	**GAAATTAGGT**	**TATATAAAAG**	**ATGTATGCTT**	**TGGAGCCCAG**	**AGCGGCTCTT**
1641	**TTAATGAGGG**	**TTGCG**			

Figure 1. Map of human *NOSI* gene and an example mRNA transcript. A) Schematic of the 12 first exons that are alternatively spliced to the protein coding exons 2-29. **B)** Example mRNA transcript of exon *1f* spliced to exon 2 with a marked translation start site in exon 2. **C)** Sequence of *NOSI 1f* promoter region from GenBank reference numbers 1441-1655 containing the polymorphic $(TG)_mTA(TG)_n$ repeat. The repeat is shown in pink, and the TATA box is shown in blue. Figure 1A and 1B were obtained from Logan Murphy.

Hypothesis:

In summary, the tau protein creates neurofibrillary tangles in AD neurons, while alpha-synuclein creates tangles in PD neurons. Studies have shown that both of these proteins can enter the nucleus and bind preferentially to purine-pyrimidine repeats, a common site for altered DNA structures found in AD and PD brains (Vasudevaraju *et al*. 2012). The *NOSI* gene, which is known to be misregulated in both AD and PD, contains a polymorphic thymine-guanine repeat in its *1f* promoter. Because shorter repeats are more highly correlated with AD and PD and because varying length repeats express differently in response to tau and alpha-synuclein, we hypothesize that tau and alpha-synuclein are modulating the expression of *NOSI* through this $(TG)_mTA(TG)_n$ repeat.

To test our hypothesis, we created and tested three new *NOSI* promoter deletion constructs and compared expression of their luciferase reporter gene to that of an already existing full length *NOSI 1f* promoter under high and low tau and alpha-synuclein levels. The *Full Promoter* construct contains a 54 bp $(TG)_mTA(TG)_n$ repeat while the *ΔTG* construct includes the entire promoter excluding only the $(TG)_mTA(TG)_n$ (Figure 2). If tau or alpha-synuclein act through the TG repeat, then we expect no change in expression in the *ΔTG* construct when tau or alpha-synuclein levels are changed. We also made two 5' deletion constructs of the full promoter – *Δ5' Region* and *Δ5' Region & TG*. The *Δ5' Region* construct contains the entire $(TG)_mTA(TG)_n$ repeat with no upstream sequences and the *Δ5' Region &TG* construct has no repeat or upstream sequences (Figure 2). Expression assays with construct *Δ5' Region* allow us to either rule out or consider the upstream region of the *NOSI* promoter as a potential site of regulation for tau and alpha-synuclein. Because this construct includes the $(TG)_mTA(TG)_n$ repeat, we expect *Δ5' Region* to exhibit similar expression changes to the *Full*

Promoter construct when tau and alpha-synuclein are differentially expressed. The *Δ5' Region & TG* construct will allows us to narrow down whether tau and alpha-synuclein could be regulating transcription through only the region downstream of the $(TG)_mTA(TG)_n$ repeat. We expect this construct to exhibit no expression changes when tau is knocked-down or alpha-synuclein overexpressed. However, if *ΔTG* or *Δ5' Region & TG* change expression in response to differential tau and alpha-synuclein expression, we will have to consider other regions of the *NOSI* promoter as potential sites of regulation for these proteins.

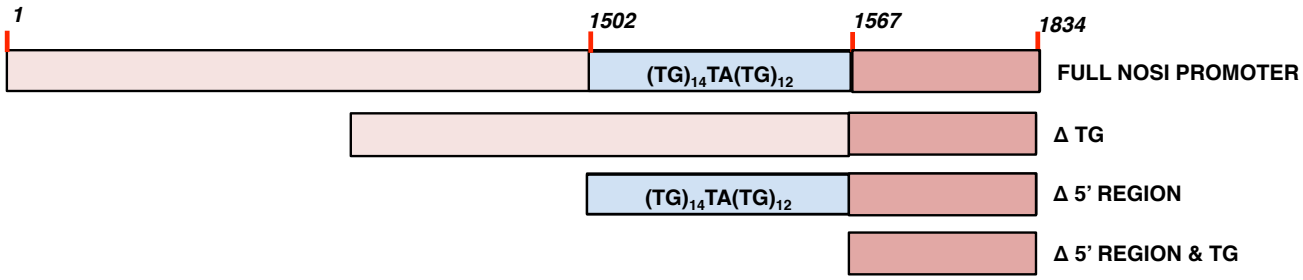


Figure 2. Schematic of the human *NOSI 1f* promoter with modified clones used in this study. The full *NOSI* promoter containing the $(TG)_{14}TA(TG)_{12}$ repeat was obtained from the Young lab at Ohio State University. Light pink bars indicate the regions upstream of the $(TG)_{14}TA(TG)_{12}$ repeat while dark pink bars indicate regions downstream of the $(TG)_{14}TA(TG)_{12}$ repeat. Red flags indicate GenBank Reference numbers where deletions were made. The *ΔTG*, *Δ5' Region,* and the *Δ5'Region & TG* constructs were cloned into a pGL3 luciferase reporter gene for this study.

METHODS:

Cloning:

PCR using the *Full Promoter* as a template was performed to obtain truncated *NOSI* promoters for cloning into pGL3 luciferase vectors. Table 1 contains the primers designed to amplify the targeted promoter regions. The *ΔTG* required three rounds of PCR to stitch nucleotides 1-1504 to nucleotides 1567-1834 (Figure 3), while constructs *Δ5' Region* and *Δ5' Region & TG* required only one round of PCR (Figure 4).

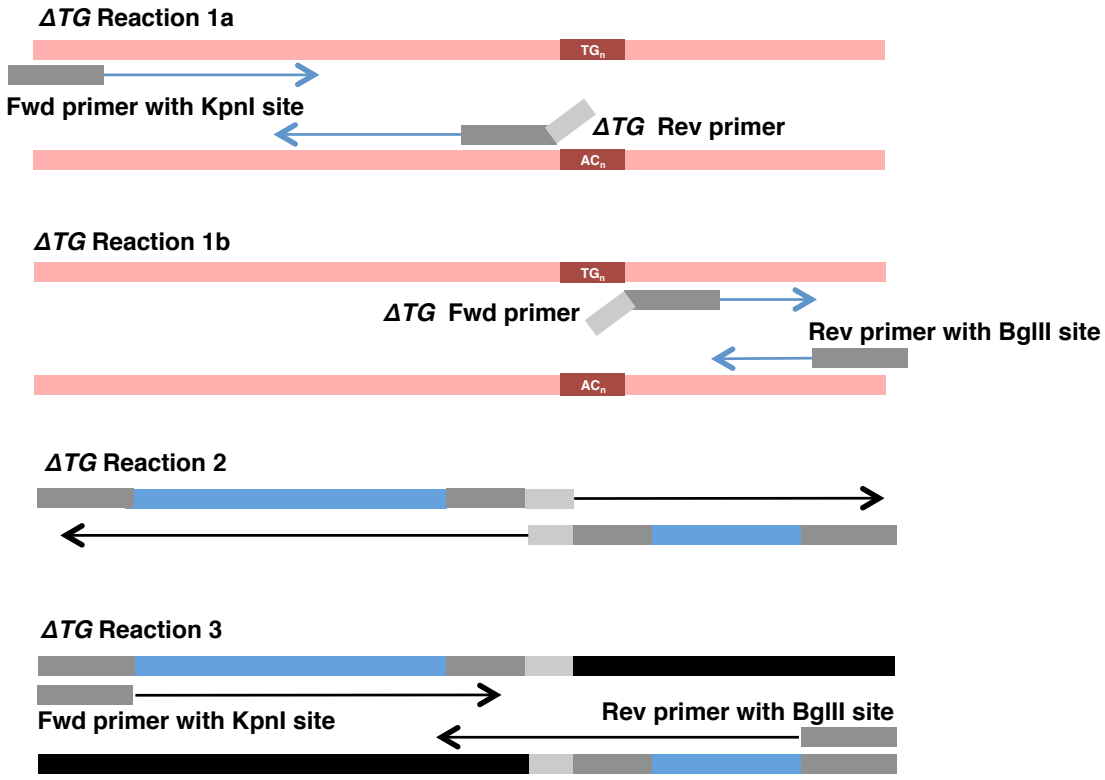


Figure 3. Schematic of PCR for the *ΔTG* construct. The pink lines represent the *NOSI Full Promoter*, and the maroon lines represent the $(TG)_{14}TA(TG)_{12}$ repeat. Reaction 1 produces the upstream and downstream region of the *ΔTG* construct that will be attached to remove the $(TG)_{14}TA(TG)_{12}$ repeat. The light gray 5' overhangs of the*ΔTG* Fwd and *ΔTG* Rev primers contain sequence from the other side of the repeat that is being removed; these overhangs also contain regions that are complementary to each other and will bind as shown in *ΔTG* Reaction 2. The forward and reverse primers containing a KpnI and BglII site, respectively, are utilized so that *ΔTG* can be cloned into pGL3.

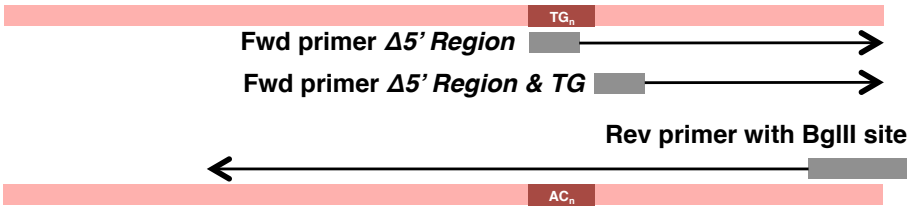


Figure 4. Schematic of PCR for constructs *Δ5' Region* and *Δ5' Region & TG.* The pink lines represent the *NOSI Full Promoter,* and the maroon lines represent the $(TG)_{14}TA(TG)_{12}$ repeat. The forward primer for *Δ5' Region* binds to the TG repeat, while the forward primer for *Δ5' Region & TG* binds directly after the TG repeat. The reverse primer binds to the downstream region of the Full Promoter, and the 5' region of the reverse primer contains a hanging BglII restriction site to allow for cloning into pGL3.

Table 1. Forward and reverse primer sequences used to clone various regions of the *NOSI* promoter. *ΔTG* requires three reactions while *Δ5' Region* and *Δ5' Region & TG* require one. KpnI sites are indicated in red, while BglII sites are indicated in blue. The dark grey forward primers both contain the same sequence, and the light grey reverse primers contain the same sequence.

Reaction/Construct	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')
ΔTG Reaction 1a	GGGGTACCAAGCTTGTGCTCCCAGAG	GAGGGTCTTTAACAGTAAAAGG
ΔTG Reaction 1b	GTCATTTTCGTGATAGAAAAAAAA	CACTCCTCGATGAATCGCGTCTAGAAG
ΔTG Reaction 2	Nested PCR using complementary products from *ΔTG* 1a and *ΔTG* 1b	
ΔTG Reaction 3	GGGGTACCAAGCTTGTGCTCCCAGAG	CACTCCTCGATGAATCGCGTCTAGAAG
Δ5' Region	CATTGTGTGTGTGTGTGTGTGTGTGTGT	CACTCCTCGATGAATCGCGTCTAGAAG
Δ5' Region & TG	TTTCCTGATAGAAAAAAAAAAATGG	CACTCCTCGATGAATCGCGTCTAGAAG
Sequencing Primer 1	CTTTATGTTTTTGGCGTCTTCCA	
Sequencing Primer 2	CTAGCAAAATAGGCTGTCCC	

Each polymerase chain reaction mixture contained 1X high fidelity PCR buffer, 0.2 mM dNTP, 2 mM $MgSO_4$, 0.2 µM forward primer, 0.2 µM reverse primer, 1.0 units platinum Taq high fidelity DNA polymerase, and 200 ng plasmid or PCR product. All PCR reactions containing primers were run at 94 °C for 2 minutes to denature, followed by 30 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds, and 68 °C for 1 minute, and then a final round of 68 °C for 10 minutes. Nested PCR reactions without primers used to clone No TG reaction 2 ran for only 15 cycles under the same conditions. Final PCR products were run on a 2% agarose gel, purified with the Invitrogen PureLink Quick Gel Extraction & PCR Purification Combo kit, and inserted into the pCR®2.1-TOPO® TA cloning plasmid vector according to the New England BioLabs

(NEB) T4 DNA Ligase protocol (Figure 5). The ligated recombinants were transformed into competent One Shot® *E. coli* cells and grown on ampicillin plates spread with 40 µL of 40 mg/mL X-Gal. Blue-White screening was used to select colonies that contain successfully inserted PCR product. White colonies were cultured in LB broth, mini-prepped, digested with EcoRI, and examined on a 1% agarose gel to determine if the correct size construct was present. Vectors of the correct size and orientation were cut out of the cloning vector with BglII and Acc65I, band isolated, purified using the Invitrogen PureLink Quick Gel Extraction & PCR Purification Combo Kit, and inserted into pGL3 via sticky-end ligation according to the NEB T4 DNA Ligase protocol (Figure 5). Recombinant pGL3 vectors were transformed into competent *E. coli* cells, mini-prepped, digested with Acc65I and BglII, and run on a 1% agarose gel to ensure that the expected constructs were successfully inserted prior to sending them for sequencing. All recombinant plasmids were sequenced at the Plant-Microbe Genomics Facility at Ohio State University. Sequencing reactions were analyzed using NCBI Blast and the 4Peaks chromatogram tool. Constructs with confirmed sequences were maxi prepped using the Invitrogen PureLink HiPure Plasmid Filter Maxi Prep Kit and DNA concentrations were calculated with Qubit Fluorometer.

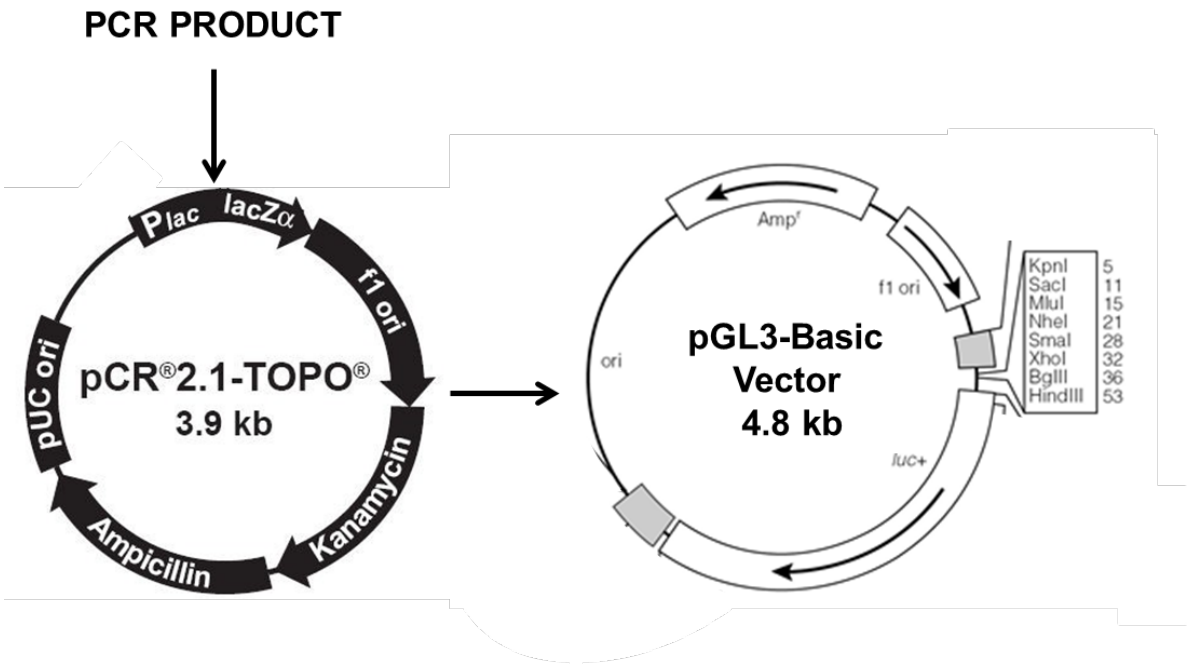


Figure 5. **Cloning strategy for *NOSI* promoter constructs.** PCR products with adenine overhangs were inserted into the pCR®2.1-TOPO® vector and ligated to its thymine overhangs. If correctly inserted, the PCR product will disrupt transcription of the lacZα gene, allowing for Blue-White selection. Correctly ligated recombinants were digested with Acc651 and BglII, and the *NOSI* promoters were ligated into the KpnI and BglII sites of the pGL3-Basic vector.

Cell Line Maintenance:

SK-N-MC cells were grown in Eagles Modified Minimal Medium (EMEM) supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum, and 100 units /mL penicillin/streptomycin. HeLa cells were grown in DMEM high glucose media with 2mM L-glutamine, 10% (v/v) fetal bovine serum, and 100 units/mL penicillin/streptomycin. SK-N-MC cells were maintained at 37 ˚C with 5% CO_2.

Transfection of Cell Lines:

1.25 x 10^6 SK-N-MC cells or 3.0 x 10^5 HeLa cells were seeded on 6-well plates 24 hours prior to transfection. Reporter gene constructs with *NOSI* promoters were transfected into SK-N-MC or HeLa cells, which endogenously express high levels of tau and low levels of alpha-synuclein. To knock down tau expression, cells were transfected with iTau (Santa Cruz), an

siRNA known to knock-down tau. An siRNA not known to knock down any human gene was used as a control. Alpha-synuclein overexpression was achieved by transfecting a pSI-WTsyn plasmid containing the alpha-synuclein gene donated from B.T. Hymen into cells. An empty pSI-WTsyn plasmid was used as a control. All cells were cotransfected with Beta-galactosidase plasmid, pNassβ, to normalize for transfection efficiency. The pGL3-Control vector with SV40 promoter and SV40 enhancer was utilized as a highly expressing positive control for luciferase assays, and the pGL3-Basic plasmid with no inserted promoter was utilized as the negative control. DNA quantities utilized in each transfection are found in Table 2. Cells with no luciferase or beta-galactosidase transfected were utilized to normalize for background light values in both assays.

Table 2. Quantities of DNA utilized in each transfection.

Construct	Tau assay	Alpha-synuclein assay
Reporter gene construct	5 μg	3.5 μg
Beta-galactosidase	3 μg	3.5 μg
iTau	1.8 X 10^{-4} μMoles	--
siRNA	1.8 X 10^{-4} μMoles	--
pSI-WTsyn	--	3.5 μg
Control alpha-synuclein	--	3.5 μg

SK-N-MC transfections were done using both the Lipofectamine 2000 and Mirus Bio Trans-IT X2 protocols, while HeLa transfections were performed only with Trans-It X2. Cells were incubated for 24 hours after transfection at 37 C with 5% CO_2. To harvest, transfected cells were rinsed with sterile PBS and spun at 5000 g for 5 minutes. Dry pellets were stored at –80 C for up to 1 week prior to assaying. These pellets were re-suspended in 300 µL 0.25 M Tris-HCl. To lyse the cells, each tube was sonicated at amplitude 60 on ice for 7 pulses no more than 1 second long. Lysed cells were spun at 13000 g for 10 minutes at 4 C. The supernatant contained the gene products of interest.

Luciferase Assay:

Luciferase was used as a reporter gene to measure gene expression directed by our cloned *NOSI* promoters. For each sample, a Berthold Detection Systems SIRIUS luminometer injected 100 µL of luciferin into a cuvette containing 350 µL of substrate and 40 µL of cell extract. The proportions for the substrate include 0.5 volume of 50mM gly-gly pH 7.8, 0.25 volume of10mg/mL ATP pH 6-8, 0.10 volume of 150 mM $MgSO_4$, and 0.15 volume of H_2O. The luminometer reports luciferase activity in relative light units (RLU).

Protein Assay:

The protein assay was used to normalize for the number of cells in each sample. One milliliter of room temperature Bio-Rad Protein Reagent Dye (1 part filtered dye to 4 parts water stock) was added to a cuvette for each sample followed by either five microliters of protein standards at 0.0, 0.125, 0.25, 0.5, 0.75, 1.0, 1.25, and 1.47 mg/mL or ten microliters of cell extract. Absorbance was measured at 595 nm.

Beta-Galactosidase Assay:

Beta-galactosidase (Bgal) assays were used to normalize for transfection efficiency. Each reaction contained 60 µL of cellular extract, 3 µL of 100X Mg solution (0.1 M $MgCl_2$, 4.5 M beta-mercaptoethanol), 66 µL of 1X ONPG (a 4 mg/mL solution of o-nitrophenyl- β -D-galactopyranoside dissolved in 0.1 M sodium phosphate), and 151 of µL 0.1 M sodium phosphate at pH 7.5 (41 mL 0.2 M Na_2HPO_4 * $2H_2O$, 9 mL 0.2 M NaH_2PO_4- * $2H_2O$, and 50 mL H_2O). Reactions were incubated at 37 °C for 60 minutes or until a faint yellow color developed. Reactions were stopped with 500 µL of 1.0 M sodium bicarbonate and measured in a spectrophotometer at 420 nm.

Data Analysis:

Expression was recorded as mean relative light units (RLU) per unit of beta-galactosidase (Bgal Unit) activity per minute ± standard deviation. Expression fold-changes were calculated between high and low tau or alpha-synuclein, and two-tailed t-tests were performed to determine significance which was assigned at $p<0.05$ in all tests.

RESULTS

Cloning:

The *ΔTG*, *Δ5' Region*, and *Δ5' Region & TG* constructs were made to examine whether the $TG_m(TA)TG_n$ repeat in the *NOSI 1f* promoter may be a binding site for tau and alpha-synuclein to regulate *NOSI* gene transcription. The constructs were first made from PCR on the *NOSI Full Promoter* template and were cloned into the pCR®2.1-TOPO® cloning vector. Insertion into the cloning vector was verified as correct by digesting plasmids with EcoRI and observing the expected 1810 bp fragment of *ΔTG*, 364 bp fragment of *Δ5' Region,* and 299 bp fragment of *Δ5' Region & TG*. Correct plasmids were cut with BglII and Acc65I, inserted into the pGL3 reporter gene vector.

When the *ΔTG* construct was inserted into pGL3, we observed the expected 4818 bp fragment from pGL3 and a smaller 1795 bp *ΔTG* promoter fragment upon digestion with BglII and Acc651 (Figure 6). The *Δ5' Region* construct was obtained in the pCR®2.1-TOPO® cloning vector from previous lab work by Kristen Grathwald (Rife *et al*., 2009). Upon digestion of *Δ5' Region* in pGL3 with Acc651 and BglII, we observed the expected 4818 bp fragment and the 349 bp fragment containing the *Δ5' Region* promoter (Figure 6). Finally, the expected 4818 bp and 284 bp fragments of the *Δ5' Region & TG* construct were observed upon digestion with Acc651 and BglII (Figure 6). The completed plasmids are 6582 bp, 5167 bp, and 5102 bp for *ΔTG, Δ5' Region, and Δ5' Region & TG,* respectively.

Once restriction enzyme digests confirmed the intended plasmid design, the constructs were sequenced by the Ohio State Plant Microbe Genome Facility. The resulting sequences were blasted against our expected sequence for each plasmid, and we determined that each plasmid

contained the correct sequence with no mutations. Sequencing output data can be found in Appendix 1.

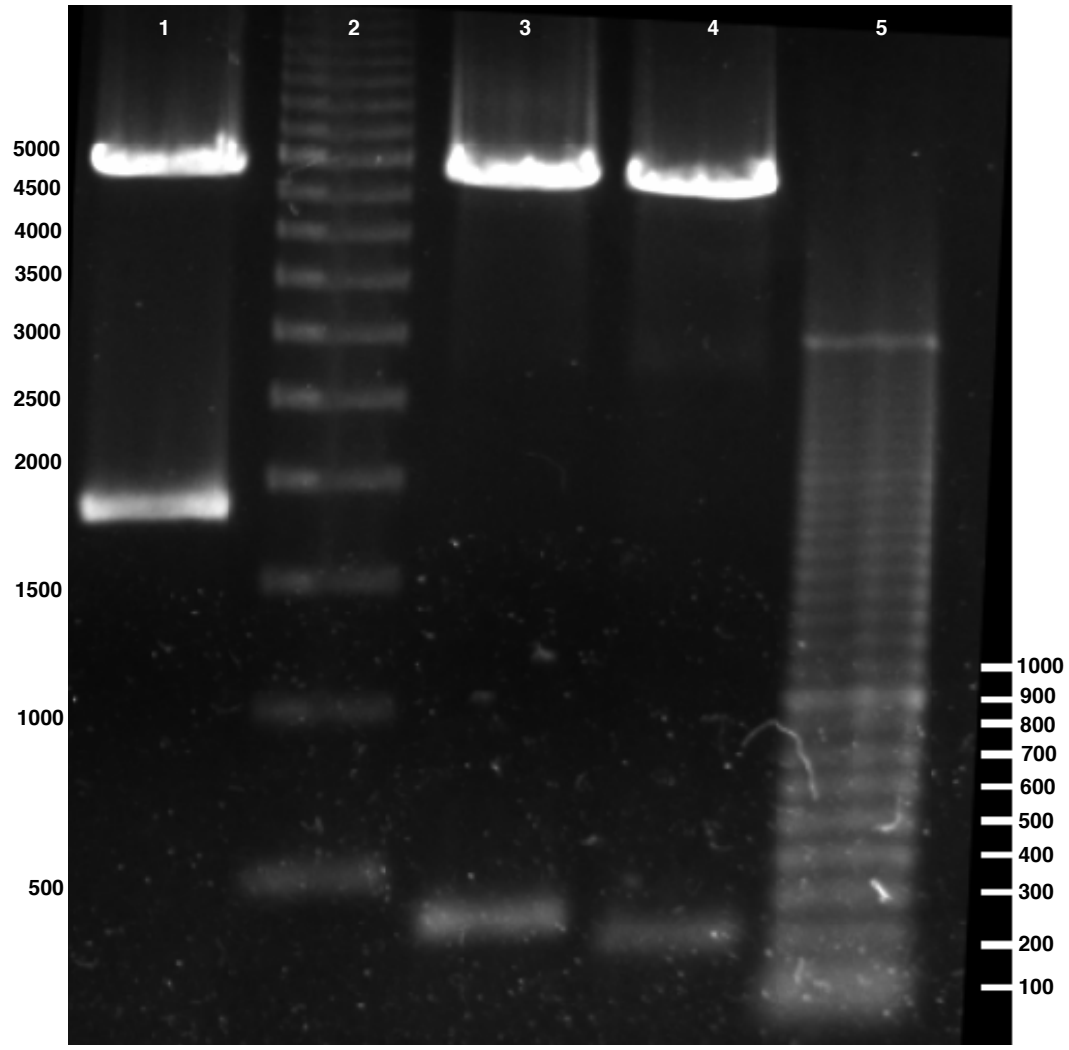


Figure 6. Gel evidence of insertion of *NOSI* promoter constructs *ΔTG*, *Δ5' Region*, and *Δ5' Region & TG* into reporter gene vector pGL3. All pGL3 vectors containing the correct *NOSI* promoter were digested with BglII and Acc65I overnight at 37 °C. Lane 1 contains the pGL3 vector and the *ΔTG* promoter. Lane 2 contains a 500 bp molecular weight ladder, whose band sizes are marked on the left side of the gel in base pairs. Lane 3 contains pGL3 vector and the *Δ5' Region* promoter, and lane 4 contains pGL3 and the *Δ5' Region & TG* promoter. Lane 5 contains a 100 bp molecular weight ladder whose band sizes in base pairs are marked on the right.

Effect of tau knock-down on *NOSI* promoter activity in SK-N-MC cells:

Constructs *Full Promoter*, *Δ5' Region*, and *Δ5' Region & TG* were transfected into SK-N-MC cells, along with either iTau or control siRNA and luciferase expression assays were performed to examine whether tau is mediating expression changes through the $(TG)_mTA(TG)_n$ repeat. Following tau knockdown, *Full Promoter* directed 8365.5 ± 540.1 RLU/Bgal Unit while this construct expressed 4535.8 ± 292.4 RLU/Bgal Unit at high tau levels (Figure 7). Therefore, tau knockdown induced a 1.84-fold increase in expression ($p = 0.014$) of a reporter gene directed by the *Full Promoter* construct. The *Δ5' Region* construct exhibited a 1.63-fold increase in expression ($p = 0.002$) with RLU/Bgal Unit values of 5461.7 ± 387.3 and 3360.5 ± 2.349 for low and high tau levels, respectively (Figure 7). Construct *Δ5' Region & TG* had a 1.01-fold change in expression ($p = 0.918$), with low tau conditions yielding 2991.2 ± 133.8 RLU/Bgal Unit and high tau conditions yielding 3009.0 ± 306.1 RLU/Bgal Unit. Two additional assays of tau knockdown with the *Full Promoter* yielded a 1.40-fold ($p = 0.057$) and a 1.82-fold ($p = 0.0003$) increase in expression. The assay yielding a 1.40-fold change was calculated using RLU values from the tau knockdown and no siRNA conditions. Subsequent experiments with *Δ5' Region* construct yielded 1.50-fold ($p = 0.050$) and 1.68-fold ($p = 0.002$) increases in expression, while the *Δ5' Region & TG* construct yielded 1.05-fold ($p = 0.984$) and 1.02-fold ($p = 0.263$) increases in expression, showing virtually no change when tau was knocked down.

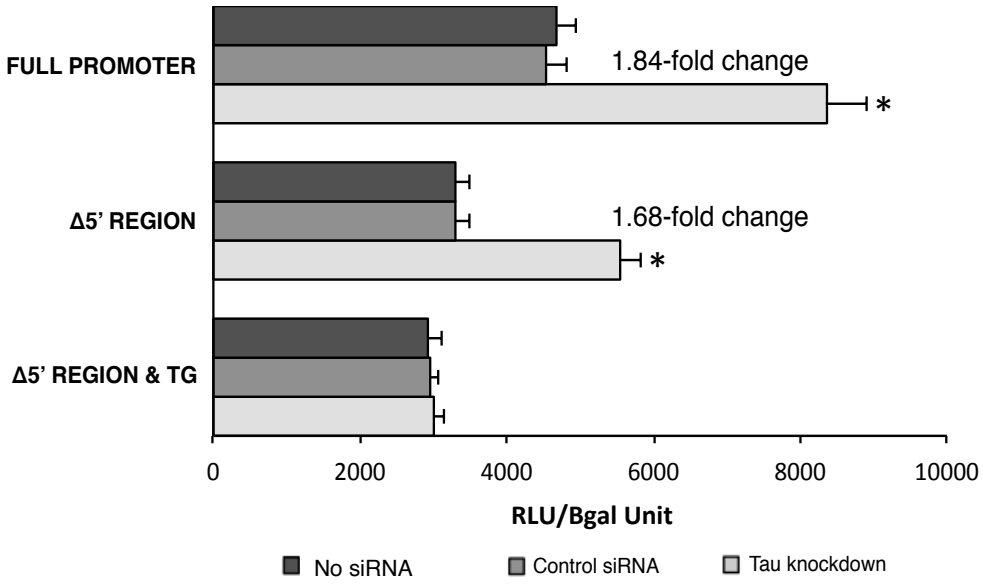


Figure 7. The effect of tau knock-down on *NOSI* promoter activity in SK-N-MC cells. *NOSI 1f* promoter reporter gene constructs were transfected into SK-N-MC cells with beta-galactosidase and either iTau or a control siRNA not known to knock down any human gene. The results of the luciferase assays were presented as relative light units (RLU) over units of beta-galactosidase activity per minute. Data represent the average values for three repeated trials. Error bars represent one standard deviation, and a * indicates constructs that were significantly different from controls in a t-test ($p < 0.05$).

Effect of alpha-synuclein overexpression on *NOSI* promoter activity in SK-N-MC cells:

The *Full Promoter*, construct *Δ5' Region*, and construct *Δ5' Region & TG* were transfected into SK-N-MC cells along with either an alpha-synuclein overexpression vector or a control vector, and luciferase expression assays were performed to examine whether alpha-synuclein is mediating expression changes through the $(TG)_mTA(TG)_n$ repeat. The *Full Promoter* had expression values of 4264.0 ± 352.5 and 2054.9 ± 243.0 RLU/Bgal Unit for conditions of low and high alpha-synuclein, respectively, demonstrating a 2.07-fold decrease in expression ($p = 0.022$) upon alpha-synuclein overexpression (Figure 8). The *Full Promoter* displayed a 2.18-fold ($p = 0.048$) and a 2.08-fold ($p = 0.012$) decrease in expression upon two subsequent trials of alpha-synuclein overexpression. The *Δ5' Region* promoter directed expression levels of 3037.0 ±

185.1 RLU/Bgal Unit under low alpha-synuclein conditions and 1437.4 ± 329.6 RLU/Bgal Unit under high alpha-synuclein conditions, resulting in a 2.11-fold decrease in expression ($p = 0.033$) (Figure 8). On the other hand, no changes in expression were observed in construct *Δ5' Region & TG*. At low alpha-synuclein levels, the *Δ5' Region & TG* construct directed 2839.1 ± 202.0 RLU/Bgal Unit, and at high alpha-synuclein levels, the *Δ5' Region & TG* promoter directed 2798.1 ± 236.7 RLU/Bgal Unit, accounting for a 1.01-fold change in expression ($p = 0.892$) (Figure 8). The *Δ5' Region & TG* promoter also directed 0.99-fold change in expression in two subsequent experiments ($p = 0.880$).

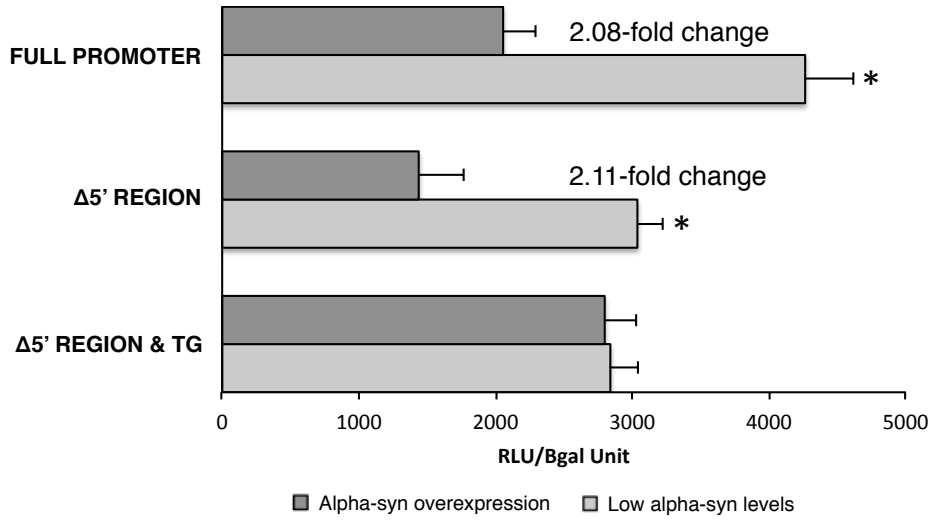


Figure 8. The effect of alpha-synuclein overexpression on *NOSI* promoter activity in SK-N-MC cells. *NOSI 1f* promoter constructs were cloned in front of luciferase in the pGL3 reporter gene vector (Promega). *NOSI* reporter gene constructs into SK-N-MC cells with beta-galactosidase and either an alpha-synuclein overexpression vector (pSI-WTsyn) or a control vector. The results of luciferase assays are presented as relative light units (RLU) over units of beta-galactosidase activity per minute. Data represent the average values for three repeated trials. Error bars represent one standard deviation, and an * indicates constructs that were significantly different from controls in a t-test ($p < 0.05$).

Effect of alpha-synuclein overexpression on *NOSI* promoter activity in HeLa cells:

To examine the role of alpha-synuclein in the regulation of the *NOSI* promoter in a different cell line, we optimized the protocol for HeLa cells and performed a single trial using. We determined that the following conditions were optimal for experiments with HeLa cells:

- Seed cells at a concentration of $3.0x10^5$ cells per well, rather than $1.25x10^6$ cells per well
- Add TransIT-X2 reagent in a 2:1 TransIT-X2:ng DNA ratio in HeLa rather than a 3:1 ratio
- Thoroughly mix the transfection reagent, DNA, and media prior to the incubation period by pipetting the entire volume up and down
- Incubate transfection reagent, DNA, and media for 15 minutes at room temperature before adding to cells
- Utilize 40 µL cell extract for luciferase assays and 60 µL cell extract for beta-galactosidase assays

We transfected only the *Full Promoter* construct and construct *Δ5' Region & TG* into HeLa cells along with either an alpha-synuclein overexpression vector or a control vector, and the constructs expressed at higher levels than in SK-N-MC. The *Full Promoter* construct displayed a 2.23-fold increase in expression ($p<0.05$) when alpha-synuclein was overexpressed (Figure 9), whereas alpha-synuclein overexpression was associated with a decrease in expression in SK-N-MC cells (Figure 8). Additionally, expression driven by the *Δ5' Region & TG* promoter construct increased 2.81-fold ($p<0.05$), despite the absence of the $(TG)_mTA(TG)_n$ repeat (Figure 9).

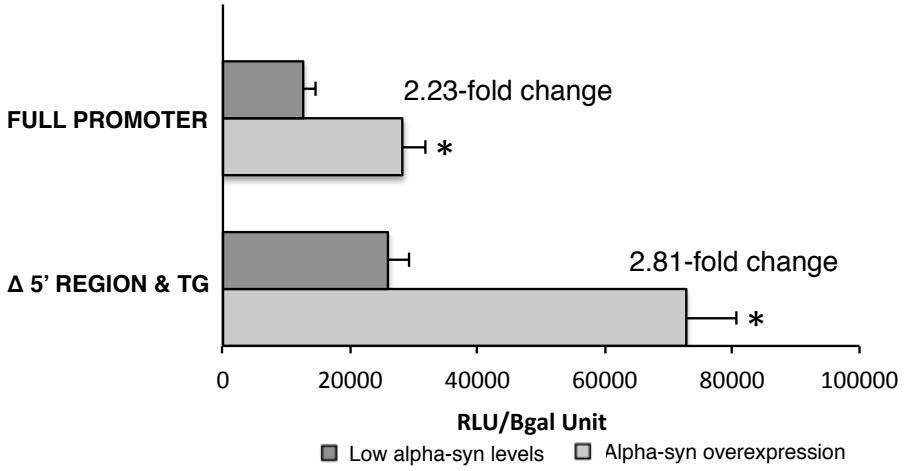


Figure 9. The effect of alpha-synuclein overexpression on *NOSI* promoter activity in HeLa cells. *NOSI 1f* promoter constructs were transfected into HeLa cells with beta-galactosidase and either an alpha-synuclein overexpression vector (pSI-WTsyn) or a control vector. The results of luciferase assays are presented as relative light units (RLU) over units of beta-galactosidase activity per minute. Data represent the average values for three repeated trials. Error bars represent one standard deviation, and an * indicates constructs that were significantly different from controls in a T-test ($p < 0.05$).

DISCUSSION

To examine whether tau and alpha-synuclein are modulating transcription of the *NOSI* gene at a polymorphic $(TG)_mTA(TG)_n$ repeat, we measured gene expression driven by variations of the *NOSI* promoter. A large portion of our project involved cloning these promoter constructs into the pGL3 luciferase reporter gene vector. The sequencing output matched our expected sequences for constructs *ΔTG, Δ5' Region*, and *Δ5'Region & TG*, so we can reasonably trust that all expression data from those constructs did not occur as a result of cloning mutations. The *ΔTG* construct was not finished in time to put into any assays, but it will be used in the next experiments. When tau was knocked down in SK-N-MC cells, the *Full Promoter* and construct *Δ5' Region*, which both contain the $(TG)_mTA(TG)_n$ repeat, displayed a significant increase in expression. However, construct *Δ5' Region & TG*, which excludes the repeat, displayed no significant change in expression in response to tau knockdown. Thus, tau appears to only affect gene expression when the $(TG)_mTA(TG)_n$ repeat is present, which is consistent with our predictions.

Furthermore, when alpha-synuclein was overexpressed in SK-N-MC cells, both the *Full Promoter* and *Δ5' Region* displayed approximately 2-fold decreases in expression. However, the *Δ5' Region & TG* construct that excludes the repeat did not exhibit a significant change in expression. Overexpressed alpha-synuclein had no effect on expression when the $(TG)_mTA(TG)_n$ repeat was deleted, suggesting that the $(TG)_mTA(TG)_n$ repeat may also be necessary for alpha-synuclein mediated transcriptional changes in SK-N-MC. However, expression levels were low for these constructs in SK-N-MC, so it is questionable whether excess alpha-synuclein could have further suppressed transcription in the *Δ5' Region & TG* construct. Therefore, we tested the constructs in HeLa cells, which are known to robustly express our *NOSI* promoter constructs.

Although HeLa cells are a non-neuronal cell line, they express multiple neuronal genes including alpha-synuclein, and are frequently used to study neurological conditions such as AD and PD (Culvenor *et al.,* 1995; *&* Liu *et al.,* 2012).

As we hoped, the *Full Promoter* and *Δ5' Region & TG* constructs expressed at higher levels in HeLa than in SK-N-MC. The *Full Promoter* exhibited a 2.23-fold increase in expression in HeLa but a 2.08-fold decrease in expression in SK-N-MC when alpha-synuclein was overexpressed. Additionally, the *Δ5' Region & TG* construct showed a significant 2.81-fold increase in expression in HeLa cells, but showed no change in expression in SK-N-MC cells when alpha-synuclein was overexpressed. While it appears that alpha-synuclein is modulating *NOSI* expression in HeLa cells, the $(TG)_nTA(TG)_m$ repeat may not be required. The differences in expression between SK-N-MC and HeLa experiments are likely due to the presence of different transcription factors in the cell lines. Alpha-synuclein could be interacting with other proteins and transcription factors, and subsequently regulating transcription through a different region in the promoter. The data from HeLa experiments will need to be repeated to confirm these results because difficulties with cell growth may have affected the results.

HeLa cells should be incubated with CO_2 levels between 5% and 10%; however, our incubator consistently displays CO_2 levels greater than 10% for unknown reasons. Elevated levels of CO_2 can induce oxidative stress (McKenna, 2009), and studies have shown that this stress increases nuclear levels of alpha-synuclein (Siddiqui *et al.,* 2012). Therefore, it is possible that this stress on our cells caused alpha-synuclein to localize to the nucleus, regardless of whether or not cells were transfected with alpha-synuclein. If alpha-synuclein is binding to DNA in the low alpha-synuclein condition, it would mimic the results of alpha-synuclein overexpression. Despite the need for further optimization, our preliminary data is promising in

that it indicates that alpha-synuclein overexpression does change the way our promoter constructs express.

Studies have implicated overexpressed alpha-synuclein in causing differential expression of genes other than *NOSI*, one of which is called *PGC1-alpha*. PGC1-alpha is a transcriptional co-activator involved in the activation of several genes implicated in mitochondrial biogenesis and cellular respiration (Siddiqui *et al.*, 2012). Misexpression of *PGC1-alpha* has been reported in the brains of sporadic PD patients (Mallajosyala *et al.*, 2009). Siddiqui *et al.* (2012) identified a CCCCTTCC consensus sequence for alpha-synuclein binding and observed decreased *PGC1-alpha* promoter activity upon alpha-synuclein overexpression. These studies implicate alpha-synuclein binding in *PGC1-alpha* expression changes; however, a mechanism has yet to be established. Interestingly, the *PGC1-alpha* promoter construct purchased from Addgene contains a 50 bp TG repeat. Currently, our lab is working to corroborate the work of Siddiqui *et al.* and show whether this TG repeat is needed for alpha-synuclein induced changes in *PGC1-alpha* transcription. Our experiment in HeLa cells revealed a 2-fold increase in *PGC1-alpha* expression with overexpressed alpha-synuclein, which is opposite the trend observed by Siddiqui *et al* and in our *NOSI* experiments with SK-N-MC. The Siddiqui *et al.* studies were performed in PC12 cells derived from a pheochromocytoma of the rat adrenal medulla, a tissue that has neuroblastic origins. Therefore, overexpressed alpha-synuclein appears to decrease expression of promoter constructs in neuronal cell lines, while increasing expression in HeLa cells. Additional studies with this construct in HeLa and SK-N-MC cells may reveal whether alpha-synuclein promotes or inhibits *PGC1-alpha* expression in these cells and whether these expression changes are mediated through a TG repeat.

Although we have not tested it, the *ΔTG* construct will provide better information on the impact of the polymorphic $(TG)_mTA(TG)_n$ repeat on *NOSI* promoter expression because it contains the entire promoter sequence, including enhancers and repressors that would normally be necessary for *NOSI* gene transcription. Although the $(TG)_mTA(TG)_n$ repeat has been correlated with AD and PD, other TG repeats in the *NOSI* promoter could be affecting gene expression even in the absence of the large repeat. For example, there are two smaller TG repeats further upstream in the *NOSI 1f* promoter: a 16 bp TG repeat starting at bp 848 and a $(TG)_5TA(TG)_2$ repeat beginning at bp 866 (Figure 10). According to the literature, tau is able to bind to purine-pyrimidine repeats as short as 13 bp (Mania *et al.*, 2016). Construct *ΔTG* may still be affected by tau and alpha-synuclein due to these smaller TG repeats. The additional upstream TG repeats could explain small differences in fold-change between the *Full Promoter* and *Δ5' Region*.

Figure 10. ***NOSI 1f*** **promoter sequence displaying smaller upstream TG repeats.** The dark grey highlighted region shows the 16 bp TG repeat beginning at bp 848 as well as the $TG_5(TA)TG_2$ repeat beginning at bp 866. The light grey highlighted region shows the polymorphic repeat that is known to be correlated with AD and PD.

Although it appears that tau and alpha-synuclein are modulating gene expression in the *NOSI* promoter, regulation may not be through direct binding. For a more definitive answer to whether tau and alpha-synuclein are binding to the $(TG)_nTA(TG)_m$ repeat, future studies will utilize ChIP-PCR. This procedure crosslinks proteins to DNA and utilizes antibodies to pull down either tau or alpha-synuclein with bound DNA. Subsequent qPCR analysis using primers specific to the region of the *NOSI* gene containing the $(TG)_nTA(TG)_m$ repeat would determine whether tau or alpha-synuclein are modulating transcription through direct binding to the repeat. ChIP-sequencing performed on a cohort of PD patients demonstrated that alpha-synuclein can

bind to the promoter region of genes such as *NEDD4*, *CDC42*, and *SLC4A5*, and that it can increase promoter activity of *SLC4A5* (Pinho, et al, 2014). We examined the aforementioned genes on the UCSC Genome Browser, but we were unable to find any TG repeats longer than a few base-pairs within 2,000 base-pairs of the transcriptional start sites. The longest purine-pyrimidine repeat we found was an 11 bp TA repeat in NEDD4, suggesting that alpha-synuclein may be altering gene expression through motifs other than a purine-pyrimidine repeat. Another ChIP-sequencing study also identified *NEDD4* and *CDC42* as major peaks on a list of 238 target genes for alpha-synuclein binding in PD (Martins *et al.,* 2011). We aligned the *NOSI* promoter sequence with *NEDD4*, *CDC42*, and *pGC1-alpha* to investigate other potential regulatory sequences, and the results can be found in Table 3. We may wish to explore these sequences in the future.

Table 3. Potential regulatory sequences identified based on alignment of *NOSI 1f* promoter with other promoters known to bind alpha-synuclein. The sequence outputs aligned with 100% identity in NCBI BLAST®.

Alignment	Potential Regulatory Sequences
NOSI to *NEDD4*	5'-CTCCAGGCCTCC-3'
	5'-CCTGGGGCTGG-3'
	5'-CGGGCTCCTGG-3'
NOSI to *CDC42*	5'-GCCTCTTCTCT-3'
NOSI to *pGC1-alpha*	5'-AGAAAGGAAGGGAAG-3'
	5'-AGGCTCCCAGAAA-3'
	5'-AAGAAAGGAAGG-3'
	5'-GAAAAGATCAAA-3'

Although the mechanism by which tau modulates expression of *NOSI* is still unclear, our study indicates that it may be acting at the $(TG)_mTA(TG)_n$ repeat. Many studies have demonstrated that tau can impact gene expression, and there are a variety of suggestions regarding its DNA-binding preferences and sequence specificity (Mania *et al*., 2016). Thus, it is important to test tau knockdown in HeLa cells. One study indicated that tau bound and bent the

DNA through electrostatic interactions at AT-rich regions in the minor groove, preferentially at sequences of 13 bp or longer (Wei *et al*., 2008). Another study indicated that tau bound to GC rich oligonucleotides in addition to AT-rich regions (Camero *et al*., 2014). Thus, tau's binding preferences may be based on hydrophobicity or general structure of the backbone as opposed to specific sequences (Camero *et al*., 2014). Because the $(TG)_mTA(TG)_n$ repeat is capable of forming alternative DNA structures, we believe that the polymorphic repeat is worth pursuing in future investigations.

The cellular mechanisms involved in neurodegeneration are complex, and animal models are critical to better understanding and treating the associated diseases. Animal models of AD and PD have focused heavily on the role of intracellular neuronal plaques and tangles; however, these rodent models have provided us with limited success in developing new clinical treatments and in demonstrating how neurofibrillary tangles contribute to disease pathology (Howell, 2015). In addition to our work discussed in this paper, we also generated a promoter construct with an extra short $(TG)_4TA(TG)_2$ repeat for future use in the lab (Appendix 2). This construct was modeled after the very short $(TG)_mTA(TG)_n$ repeat found in the *NOSI* promoter of mice and rats. If this construct responds to alpha-synuclein and tau less significantly than the human *Full Promoter* constructs, it may indicate that shorter purine-pyrimidine repeats in rodents make them less susceptible to pathological changes in gene expression. These discrepancies in gene expression could explain the shortcomings of current rodent models.

Currently, there are no cures for either Alzheimer's Disease or Parkinson's Disease, and there are limited treatments to inhibit their progression. Protein modulation of *NOSI* gene expression may only be a small piece of the complex cellular machinery that goes awry in devastating neurodegenerative diseases. However, understanding this mechanism may point to a

novel role for the proteins that have been implicated in these diseases. Furthermore, this study may further expand our knowledge about the ways in which purine-pyrimidine repeats as well as altered B-DNA structures affect gene expression in a wide variety of cellular processes.

APPENDICES

APPENDIX 1. Sequencing output from Ohio State University Plant-Microbe Genomics Facility for cloned NOSI promoter constructs in pGL3.

Table a. Sequencing results for *ΔTG* construct. The final plasmid contains the entire pGL3 vector, shown in red, a portion of the pCR2.1 cloning vector in blue, and the PCR product in green.

ΔTG Construct Reaction 1 (GL Primer 2)

NNNNNNNNNNNNNNNNNGATGCCAGCTTACTTAGATCGCAGATCTGCGCTAAGTAGCTCCTCACCCAGCGGCTGGAAACGCGGCATAAATATGTAG
GGACTGAGCCGTGAGCTCAGCGAGGATCGTGAGGGGGAATTTATTTCTTAATGTCCCTCCTTTATCCTATCATCACTGAAACGAATTAGCAGTGACGT
CCAACCCGACTGGTTTATGGGTGTGGGGAGGGAGACGTCGCAACCCTCATTAAAAGAGCCGCTCTGGGCTCCAAAGCATACATCTTTTATATAACCTA
ATTTCCATTTTTTTTTTTNTATCAGGAAAATGACACACACACACACACACACATACACACACACACACACACACACACACACAATGACAATTTCTGGG
AGCCTGGATAGGGCTGAATGTAGTAGCCACGCAGGGCAGGGCAGGTCTATTTGGGCAGAGAAGGCCCCATGGGAGGCAGGGATGGTTATTTCTGCCT
CTACATCANTGTTGGGANNGCATTCCTTAATGGGACAGTGGAACACACNNCCTCCCAGGCTGGGCAGTGCTGGGAGCCCCTCCCANGAGCCCGCANA
GGTTGCAGCCTGGGTTCTGGCCTTGGCTCTCGGGTGGACAGACNGANAAAAGCANCCTGTTCNNAAATGTTGCCTGGTTCTGGTCTTTTCTGGAGCCA
CACTCAGGCCTGGCATCTGGGGAGAGTGANNATGGANGGCACATGTTTCNTCTGTGGCCTGNGAGCCCNACNNTGTGGAGANGCCAANGNCTGGGC
TGGAGGANGAGAAGACACNGANAACNCANCTTCCCTTCNTTNTCTTAGGGGCCCTAACATATGGGTAANCATATAAACCCNACTNAAAATCTGTCTT
TTCTTGGGCAGATTNCNAAGGATTTNNCNNCTCCCNTTGCTGTTGCTGCTGCTCNCNNNNCTTGGGAAAACGGGGAAAANCATGGAANANANGACAC
NANNN

ΔTG Construct Reaction 2 (RV Primer 3)

NNNNNNNNNNNNNNNNNANNTTTCTCTATCGATAGGTACCAAGCTTGTGCTCCCAGAGAGGAAGTGCCCCTACTCTGAGCTTCTTTTGGCCTTGACCC
ACACATAGGCAGACGTGGACTCTGTTTTGAGCAGGTGAACCGCCAGCCCAGTGGAAGAGCAGCTTCCCTTCCCCTAGCTTCCTGATTTTCCCCAGTTG
CCATGGTAATTGAGGGAGCAGTGTTCTGAGAATGACACCACTGAAACCCAGCGCTCAACTTTGAAATGGTTCGGGCAAGGGACGCTGGACCCAAAAC
AGGAGGGGACCAGACTGCAAGGACTCAGCTCCAGCCTCAATTTTCTGGGACCCACCCCCCATCCAGCCTGTATGCTGGATTATTCTCATCATCATCAA
AATCACCATTATTGTTGGCAACTGTTTCCTGAGCATTTACTATATGTCTAGCACCATGTCAAGGGCTTCACCAGCATCATCTCATTGTGTATATCCTCA
CGACATCCCCAGGCCCATCCATGGCTCAGGAAACAGGCTCTGAAACTTGCCCTTGTGGCAGCCAGGAAGTGGCAGAGCCCTGGGGCTGGTGGCATTT
CTCCAGGCCTCCTTCCAGCATGTGGAAGACAGCATAGACCTCCAGGCACCCTCGTCAGCTTATTCTTTATTCCTTCCTCCCCTCCTCATCCCCCACACC
CTTCTTCCTGCCCCCAAAGTCACACACCCCCTCCCCTGCTGGATGTGCAAGACGATCTGAAAAGCATTTATTAGTGACAAAGGGGGTGGTGGTTTTCC
TCTTTGCCTCTTCTCTGCGCAACTCGAANCCTGANTGGGTTTGCTCCGCTTGGCAAGGCTGGGGANGANGAAAAGATCAAATCACCCNNAGCCAGGT
GCCAAGTGTGTGTGTGTGTGTTTGTGTGTNNNNAGNGCGCNTGTGCNCNTGNNNNNNNGTGTGTCNCTCNTTCCTTGATTTTNCCCCGTTTTCNCA
GNNTNTGTGAGCNNCNGCANNGNNACNGGANANNNAAATNCNTTGNNNCTGCNNNNANNNNNNTTTGANNCNNNNNNNNGNTTCCGTNNGTTNNN
CCCTANANNGNAGNNNCNNNNNNNTNTNNNNCTTCCNCNNNNNNNNNNNNNNNNNNNNCTNGNNGGNTCAGNNNNNATNANNNNNNNNNNCNNN
NTNNNNNANTNNNTCTGANTNNNNNNCNNNNNNNNNNNNN

ΔTG Construct Reaction 3 (Middle Primer)

NNNNNNNNNNNNCNNGTTCTGGTCTTTTCTGGAGCCNCACTCAGGCCTGGCATCTGGGGAGAGTGAAGGATGGAGGGCACATGTTCATCTGTGGCCT
GGGAGCCCCACAGGTGTGGAGAGGCCAAGGTCTGGGCTGGAGGAGGGAAGACACAGAGAACGCAGCTTCCCTTCCTTTCTTAGGGGCCCTAACATAT
GGGTAACCATATAAACCCGACTCAAAATCTGTCTTTTCCTGGGCAGATTGCAAAGGATTTTGCATCTCCCCGTTGCTGTTGCTGCTGCTCACACAGTCT
TGGGAAAACGGGGGAAAATCAAGGAAAGAGAGGACACACACACGCGCACGCGCACACGCGCACATACACACACACAAACACACACACACACACAC
TTGGCACCTGGCTCTGGGTGATTTGATCTTTTCCTCCTCCCCAGCCTTGCCAAGCGGAGCAAACCCACTCAGGCTTCNAGTTGCGCAGAGAAGAGGCA
AAGAGGAAAACCACCACCCCCTTTGTCACTAATAAATGCTTTTCAGATCGTCTTGCACATCCAGCAGGGGAGGGGGTGTGTGACTTTGGGGGCAGGA
AGAANGGTGTGGGGGATGAGGAGGGGAGGAAGGAATAAAGAATAAGCTGACGAGGGTGCCTGGAGGTCTATGCTGTCTTCNACATGCTGGAANGA
GGCCTGGAGAAATGCCACCAGCCCCNNGGCTCTGCCACTTCNTGNCTGCCACAAGGGCNAGTTTNNNAGCCTGTTTCCTGAGCCATGGATGGGCCTG
GGGGATGTCNTGAGNAT

Confirmed *ΔTG* Promoter Sequence:

CTAGCAAAATAGGCTGTCCCCAGTGCAAGTGCAGGTGCCAGAACATTTCTCTATCGATAGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTG
TGCTGGAATTCGGCTTGGGGTACCAAGCTTGTGCTCCCAGAGAGGAAGTGCCCCTACTCTGAGCTTCTTTTGGCCTTGACCCACACATAGGCAGACGT
GGACTCTGTTTTGAGCAGGTGAACCGCCAGCCCAGTGGAAGAGCAGCTTCCCTTCCCCTAGCTTCCTGATTTTCCCCAGTTGCCATGGTAATTGAGGG
AGCAGTGTTCTGAGAATGACACCACTGAAACCCAGCGCTCAACTTTGAAATGGTTCGGGCAAGGGACGCTGGACCCAAAACAGGAGGGGACCAGAC
TGCAAGGACTCAGCTCCAGCCTCAATTTTCTGGGACCCACCCCCCATCCAGCCTGTATGCTGGATTATTCTCATCATCATCAAAATCACCATTATTGTT
GGCAACTGTTTCCTGAGCATTTACTATATGTCTAGCACCATGTCAAGGGCTTCACCAGCATCATCTCATTGTGTATATCCTCACGACATCCCCAGGCCC
ATCCATGGCTCAGGAAACAGGCTCTGAAACTTGCCCTTGTGGCAGCCAGGAAGTGGCAGAGCCCTGGGGCTGGTGGCATTTCTCCAGGCCTCCTTCCA
GCATGTGGAAGACAGCATAGACCTCCAGGCACCCTCGTCAGCTTATTCTTTATTCCTTCCTCCCCTCCTCATCCCCCACACCCTTCTTCCTGCCCCCAA
AGTCACACACCCCCTCCCCTGCTGGATGTGCAAGACGATCTGAAAAGCATTTATTAGTGACAAAGGGGGTGGTGGTTTTCCTCTTTGCCTCTTCTCTGC
GCAACTCGAAGCCTGAGTGGGTTTGCTCCGCTTGGCAAGGCTGGGGAGGAGGAAAAGATCAAATCACCCAGAGCCAGGTGCCAAGTGTGTGTGTGTG
TGTGTTTGTGTGTGTGTATGTGCGCGTGTGCGCGTGCGCGTGTGTGTGTCCTCTCTTTCCTTGATTTTCCCCCGTTTTCCCAAGACTGTGTGAGCAGCAG
CAACAGCAACGGGGAGATGCAAAATCCTTTGCAATCTGCCCAGGAAAAGACAGATTTTGAGTCGGGTTTATATGGTTACCCATATGTTAGGGCCCCT
AAGAAAGGAAGGGAAGCTGCGTTCTCTGTGTCTTCCCTCCTCCAGCCCAGACCTTGGCCTCTCCACACCTGTGGGGCTCCCAGGCCACAGATGAACAT
GTGCCCTCCATCCTTCACTCTCCCCAGATGCCAGGCCTGAGTGTGGCTCCAGAAAAGACCAGAACCAGGCAACATTTCCTGAACAGGCTGCTTTTCTC
CGTCTGTCCACCCGAGAGCCAAGGCCAGAACCCAGGCTGCAACCTCTGCGGGCTCCTGGGAGGGGCTCCCAGCACTGCCCAGCCTGGGAGGCTGTGT
GTTCCACTGTCCCATTAAGGAATGCTATCCCAACACTGATGTAGAGGCAGAAATAACCATCCCTGCCTCCCATGGGGCCTTCTCTGCCCAAATAGACC
TGCCCTGCCCTGCGTGGCTACTACATTCAGCCCTATCCAGGCTCCCAGAAATTGTCATTTTCCTGATAGAAAAAAAAAAAATGGAAATTAGGTTATATA
AAAGATGTATGCTTTGGAGCCCAGAGCGGCTCTTTTAATGAGGGTTGCGACGTCTCCCTCCCCACACCCATAAACCAGTCGGGTTGGACGTCACTGCT
AATTCGTTTCAGTGATGATAGGATAAAGGAGGGACATTAAGAAATAAATTCCCCCTCACGACCCTCGCTGAGCTCACGGCTCAGTCCCTACATATTTA
TGCCGCGTTTCCAGCCGCGCTACTTAGCGCAGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAAGACGCCAAAA
ACATAAAG

Table b. Sequencing results for the *Δ5' Region* construct. The final plasmid contains the entire pGL3 vector, shown in red, a portion of the pCR2.1 cloning vector in blue, and the PCR product in green.

Δ5' Region Construct Reaction 1 (GL Primer 2)
NNNNNNNNNNNNCGGNTGCCAGCTTACTTAGATCGCAGATCTGCGCTAAGTAGCTCCTCACCCAGCGGCTGGAAACGCGGCATA AATATGTAGGGACTGAGCCGTGAGCTCAGCGAGGGTCGTGAGGGGGAATTTATTTCTTAATGTCCCTCCTTTATCCTATCATCACT GAAACGAATTAGCAGTGACGTCCAACCCGACTGGTTTATGGGTGTGGGGAGGGAGACGTCGCAACCCTCATTAAAAGAGCCGCT CTGGGCTCCAAAGCATACATCTTTTATATAACCTAATTTCCATTTTTTTTTTTCTATCAGGAAACACACACACACACACACACAC ATACACACACACACACACACACACACACACACAATGAAGCCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGT ACCTATCGATAGAGAAATGTTCTGGCACCTGCACTTGCACTGGGGACAGCCTATTTTGCTAGTTTGTTTTGTTTCGTTTTGTTTTGA TGGAGAGCGTATGTTAGTACTATCGATTCACACAAAAAACCAACACACAGATGTAATGAAAATAAAGATATTTTATTGCGGCCGC TCCAAGTACCTCCCGTACCTTAATATTACTTACTTATCANGGTAGCTTGGGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCC CTTCCCAACAGTTGCGCAGCCTGAATGGCGAANGGCAAATTGTAAGCGTTAATATTTTGNNAAAATTCGCGTTAAATTTTNGTTAA ATCAGCTCATTTTTTAACCNATAGGCCGAAATCGGCAAAATCCNTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTG TTCCAGTTTGGAACAAGAGTCCACTNTTAAAGAACGTGGANTCCAANGTCAANGGCGAAAAACCGTNTATCAGGGCGATGGNCC CACTACGTGAACCATCACCCTANTCAAGTTTTTTGGGGTCGANGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGGAGCCCC CGAATTTANAGNTTGACGGGGAAAGCNCGGCNAACNTGGNNGAAAGNNAGGGNNNAAAGCNAANGGANCGGGNNNTTAGGGC GCTTGGCAANTGTANCGGTNANCGCNNNCGCNNACTCNNNNNNNNNNNNNNCNNNTTANNNCNCNNNTACNGGGGCGNNGNNN NGNGGCNNNTTTTTNNGGGAAANTNNGNGCNNNGGNANCCCNNNNNNNGNNNNNNNNNNNNNAAANNNNNTTNNAAANNNNN NNNNCNCTCNNNNGANANNANNNNNNNNNAANAANNNNCTTNCNANTAANNTNNNNNNNNAANANNNNNNNANNNNNNNNNN NNNTNNNCNNNNNNNNCCCNNNNNTNNNNNNNTNNNGNCGNNNNNTNTNNNNGCNCNNNNNNNNNNNNT
Δ5' Region Construct Reaction 2 (RV Primer 3):
NNNNNNNNNNNANTTTCTCTATCGATAGGTACCGAGCTCGGATCCNCTAGTAACGGCCGCCAGTGTGCTGGAATTCGGCTTCATT GTGTGTGTGTGTGTGTGTGTGTGTGTGTATGTGTGTGTGTGTGTGTGTGTGTTTCCTGATAGAAAAAAAAAAATGGAAATTAGG TTATATAAAAGATGTATGCTTTGGAGCCCAGAGCGGCTCTTTTAATGAGGGTTGCGACGTCTCCCTCCCCACACCCATAAACCAGT CGGGTTGGACGTCACTGCTAATTCGTTTCAGTGATGATAGGATAAAGGAGGGACATTAAGAAATAAATNCCNCCTCACGACCCTC GCTGAGCTCACGGCTCAGTCCCNACATATTTATGCCGCGNTTCCAGCCGNTGGGTGAGGAGCTACTTAGCGCAGATCTGCGATCT AAGTAAGCTTGGCATTCCGGTACTGTTGGNAAAGNCACNATGGAAGACGCCAAANACATNAAGAAAGGCCCGGCGCCATTCTAT CCGCTGGAAGATGGAACCGCTGGAGAGCNACTGCNTAAGGCTATGAANAGATACGCCCTGGTTCCTGGAACAATTGCTTTTACAG ATGCACATATCGAGGTGGACATCACTTACGCTGANTACTTCNAAATGTCCGTTCGGTTGGCAGAAGCNNTGAAACGATATGGGCT GAATACAAATCACANAATCGTCGTATGCANNTGAAAACTCTCTTCANTTCTTTTATGCCGGTGTTGGGCGCGTTTATTTATCGGAG TTGNAGTTGCGCCCGCGAACGACATTTATAATGAANGTGAATTGCTCAACCAGTANGNGCNNNTCGCAGCCTACCGNGGTGTTCG TTTTCNNAAAGGGGTTGCNAAAATTTTTGAACGTTGCNAAAAANNCTCCCCANTCNTCCNNAA
Confirmed *Δ5' Region* Construct sequence:
CTAGCAAAATAGGCTGTCCCCAGTGCAAGTGCAGGTGCCAGAACATTTCTCTATCGATAGGTACCGAGCTCGGATCCACTAGTAA CGGCCGCCAGTGTGCTGGAATTCGGCTTCATTGTGTGTGTGTGTGTGTGTGTGTGTGTGTATGTGTGTGTGTGTGTGTGTGTT TCCTGATAGAAAAAAAAAAAATGGAAATTAGGTTATATAAAAGATGTATGCTTTGGAGCCCAGAGCGGCTCTTTTAATGAGGGTTG CGACGTCTCCCTCCCCACACCCATAAACCAGTCGGGTTGGACGTCACTGCTAATTCGTTTCAGTGATGATAGGATAAAGGAGGGA CATTAAGAAATAAATTCCCCCTCACGACCCTCGCTGAGCTCACGGCTCAGTCCCTACATATTTATGCCGCGTTTCCAGCCGCTGGG TGAGGAGCTACTTAGCGCAGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAAGACGCCAAA AACATAAAG

Table c. Sequencing results for the *Δ5' Region & TG* construct. The final plasmid contains the entire pGL3 vector, shown in red, a portion of the pCR2.1 cloning vector in blue, and the PCR product in green.

Δ5' Region & TG Construct Reaction 1 (GL Primer 2)
NNNNNNNNNNNNNNCGGNNGCCAGCTTACTTAGATCGCAGATCTGCGCTAAGTAGCTCCTCACCCAGCGGCTGGAAACGCGGCA TAAATATGTAGGGACTGAGCCGTGAGCTCAGCGAGGGTCGTGAGGGGGAATTTATTTCTTAATGTCCCTCCTTTATCCTATCATCA CTGAAACGAATTAGCAGTGACGTCCAACCCGACTGGTTTATGGGTGTGGGGAGGGAGACGTCGCAACCCTCATTAAAAGAGCCG CTCTGGGCTCCAAAGCATACATCTTTTATATAACCTAATTTCCATTTTTTTTTTTCTATCAGGAAAAAGCCGAATTCCAGCACACTG GCGGCCGTTACTAGTGGATCCGAGCTCGGTACCTATCGATAGAGAAATGTTCTGGCACCTGCACTTGCACTGGGGACAGCCTATTT TGCTAGTTTGTTTTGTTTCGTTTTGTTTTGATGGAGAGCGTATGTTAGTACTATCGATTCACACAAAAAACCAACACACAGATGTA ATGAAAATAAAGATATTTTATTGCGGCCGCTCCAAGTACCTCCCGTACCTTAATATTACTTACTTATCATGGTAGCTTGGGCTGGC GTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCAAATTGTAAGCGTTAATAT TTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATANGCCGAAATCGGCAAAATCCCTTATAAATCAA AAGAATAGACCGAGATAGGGTTGAGTGTTGNTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGNACTCCAACGTCAAAG GGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGNTTTTTTGGGGTCGAGGTGCCGTAAAG CACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCNAGAAGGAAGGNAAGA AAGCGAAGGAGCGNGCGCTAGGGCGCTGGCAGTGTAGNGGNCACGCTGCGNGTAATCACCACACCCGCCGCGCNTAANGCNCCG CTACAGGNNNNNNNGGTGNNNNTTTTCGGGGAAATNNGCGCGAACCCCNNTTNGTTATTTTNNNAANNACANTNNNTNNNNNTC CGCTCNNGNANACNANACCNNGANNAATGCNTCATANNNTGAAAAANNNNNNTNTNNNNNATNNNNNNNNCNNNNNNNNNNTC NNNTTTNNNGCATTNCNNCNGNTTTGCNNNCCNNNNNNNGGNNNAGNNAAGAAGNCNNNNNCNNNNNNNNNCANNNNGTNG
Δ5' Region & TG Construct Reaction 2 (RV Primer 3)
NNNNNNNNNNNANNTTTCTCTATCGATAGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGGCTTTTT CCTGATAGAAAAAAAAAAAATGGAAATTAGGTTATATAAAAGATGTATGCTTTGGAGCCCAGAGCGGCTCTTTTAATGAGGGTTGC GACGTCTCCCTCCCCACACCCATAAACCAGTCGGGTTGGACGTCACTGCTAATTCGTTTCAGTGATGATAGGATAAAGGAGGGAC ATTAAGAAATAAATTCCCCCTCACGACCCTCGCTGAGCTCACGGCTCAGTCCCTACATATTTATGCCGCGTTTCCAGCCGCTGGGT GAGGAGCTACTTAGCGCAGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAAGACGCCAAAA ACATAAAGAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAGAGCAACTGCATAAGGCTATGAAGAGATACG CCCTGGTTCCTGGAACAATTGCTTTTACAGATGCACATATCGAGGTGGACATCACTTACGCTGAGTACTTCGAAATGTCCGTTCGG TTGGCAGAAGCTATGAAACGATATGGGCTGAATACAAATCACAGAATCGTCGTATGCAGTGAAAACTCTCTTCAATTCTTTATGC CGGTGTTGGGCGCGTTATTTATCGGAGTTGCAGTTGCGCCCGCGAACGACATTTATAATGAACGTGAATTGCTCAACAGTATGGG CATTTCGCAGCCTACCGTGGTGTTCGTTTCCAAAAAGGGGTTGCAAAAAATTTTGAACGTGCAAAAAAAGCTCCCAATCATCCAA AAAATTATTATCATGGATTCTAAAACGGATTACCAGGGATTTCAGTCGATGTACACGTTCGTCACATCTCATCTACCTCCCGGTTT TAATGAATACGATTTTGTGCCANANTCCTTCGATAGGGACAAGACAATTGCACTGATCATGAACTCCTCTGGATCTACTGGNCTGC CTAAAGNNGTCGCTCTGCCTCATAGAANTGCCTGCGNGAGATTCTCGCATGNCANANATCCTANTTTGGCNNTNNAATCNTTCCN GNATACTGCGATTTTNANNNTNNNNCCATTNCATCACGGTTTNGNNNNTTACTACACTCGNANATTTGATATGTGGANTTNNNNN CGNNNNANNNNNAGATTGANANNANCTGTTTTNNNAGNNCNNNNNNTNNAGATCAANNNNNCNNCTNNNNNNNCNANNNTNNN NNNNTNNNNNAGNNNNNNNNNTNNNNAANNNNATTNNNNNNNNNNNNNNNNNNNNNNNNNNGNNNNNCCNNNNTN
Δ5' Region & TG Construct Promoter Sequence
CTAGCAAAATAGGCTGTCCCCAGTGCAAGTGCAGGTGCCAGAACATTTCTCTATCGATAGGTACCGAGCTCGGATCCACTAGTAA CGGCCGCCAGTGTGCTGGAATTCGGCTTTTTCCTGATAGAAAAAAAAAAATGGAAATTAGGTTATATAAAAGATGTATGCTTTGG AGCCCAGAGCGGCTCTTTTAATGAGGGTTGCGACGTCTCCCTCCCCACACCCATAAACCAGTCGGGTTGGACGTCACTGCTAATTC GTTTCAGTGATGATAGGATAAAGGAGGGACATTAAGAAATAAATTCCCCCTCACGACCCTCGCTGAGCTCACGGCTCAGTCCCTA CATATTTATGCCGCGTTTCCAGCCGCTGGGTGAGGAGCTACTTAGCGCAGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTG TTGGTAAAGCCACCATGGAAGACGCCAAAAACATAAAG

APPENDIX 2: Cloning strategy for the rodent construct containing $(TG)_4TA(TG)_2$ polymorphism.

We have already conducted cloning and sequencing for the construct containing a $(TG)_4TA(TG)_2$ repeat, modeled after the extra short polymorphism in the rodent NOSI promoter. We refer to this construct as the *Rodent* construct. The *Rodent* construct required three rounds of PCR, similar to the strategy for *ΔTG* (Figure 3). Reaction 1a uses forward primer GGGGTACCAAGCTTGTGCTCCCAGAG and reverse primer GAAACACATACACACACAATGACAATTTCTGG. Reaction 1b uses forward primer CATTGTGTGTGTATGTGTTTCCTGATAGAAA and reverse primer CACTCCTCGATGAATCGCGTCTAGAAG. Reaction 3 uses forward primer GGGGTACCAAGCTTGTGCTCCCAGAG, and reverse primer GGAAAATGACA ATTTCTGGGAG. The *Rodent* construct was cloned into pGL3 and sequenced. The final expected plasmid was 6679 bp. However, we found a discrepancy between sequencing reaction 2 and our expected *Rodent* sequence (Table d). The highlighted region of Table d shows only two cytosines (C) rather than the expected three C's. The discrepancy does not appear in the other two sequencing reactions because they do not cover that region. The chromatogram corroborates the error, but it is possible that the deletion resulted from a sequencing error rather than a cloning error. An additional sequencing reaction is needed to confirm this result. Future studies must be done to examine whether *Rodent* contains a true mutation and how this promoter directs expression in cells with and without tau and alpha-synuclein.

Table d. Sequencing results for the *Rodent* construct containing $(TG)_4TA(TG)_2$ polymorphism. The final plasmid contains the entire pGL3 vector, shown in red, a portion of the pCR2.1 cloning vector in blue, and the PCR product in green. The highlighted region contains a discrepancy between our expected sequence and sequencing reaction 2.

Rodent Construct Reaction 1 (GL Primer 2)

NNNNNNNNNNNNNNACCNGATGCCAGCTTACTTAGATNNNNNATCTGCGCTAAGTAGCTCCTCACCCAGCGGCTGGAAACGCGGCATAAATATGTAGGGACTGA
GCCGTGAGCTCAGCGAGGGTCGTGAGGGGGAATTTATTTCTTAATGTCCCTCCTTTATCCTATCATCACTGAAACGAATTAGCAGTGACGTCCAACCCGACTGGTT
TATGGGTGTGGGGAGGGAGACGTCGCAACCCTCATTAAAAGAGCCGCTCTGGGCTCCAAAGCATACATCTTTTATATAACCTAATTTCCATTTTTTTTTTTCTATC
AGGAAACACATACACACACAATGACAATTTCTGGGAGCCTGGATAGGGCTGAATGTAGTAGCCACGCAGGGCAGGGCAGGTCTATTTGGGCAGAGAAGGCCCCA
TGGGAGGCAGGGATGGTTATTTCTGCCTCTACATCAGTGTTGGGATAGCATTCCTTAATGGGACAGTGGAACACACAGCCTCCCAGGCTGGGCAGTGCTGGGAGC
CCCTCCCAGGAGCCCGCAGAGGTTGCAGCCTGGGTTCTGGCCTTGGCTCTCGGGTGGACAGACGGAGAAAAGCAGCCTGTTCAGGAAATGTTGCCTGGTTCTGGT
CTTTTCTGGAGCCACACTCAGGCCTGGCATCTGGGGAGAGTGAAGGATGGAGGGCACATGTTCATCTGTGGCCTGGGAGCCCCACAGGTGTGGAGAGGCCAAGG
TCTGGGCTGGAGGAGGGAAGACACAGAGAACGCANCTTCCCTTCCTTTCTTANGGGCCCTAACATATGGGTAACCATATAAACCCNACTCAAAATCTGTCTTTTC
CTGGGCAGATTGCAAAGGATTTTGCATCTCCCCGTTGCTGTTGCTGCTGCTCNCACAGTCTTGGAAAACGGGGAAAATCNAGGAAAGANAGGACACACATACNC
GCACGCGCACACNNNCNCATACNNNCNCNCNNNNNNNNNNNNNNNNNNNTTGGCNCCTNGNNCNNGNNANTTGNTCTTTTTCCTNNNCCNNNCTNGCCAGCGG
NNNAANNCNNNCNGNNTNNNNTNNNNNANAANNGNTANANNAACACNNNNNNTGTNCNNNNATAANNCTTTNNNTCNNNTGNCANNCNNNGNNNNGNNNNNN
ANNTNNNNNGAANNNNNNNNNNNNNNNNNNNGATNNNNNNNNNNTANNNNNNNNCNNNNNN

Rodent Construct Reaction 2 (RV Primer 3)

NNNNNNNNGNNCNNANNTTTCTCTATCGATAGGTACCAAGCTTGTGCTCCAGAGAGGAAGTGCCCCTACTCTGAGCTTCTTTTGGCCTTGACCCACACATAGGCA
GACGTGGACTCTGTTTTGAGCAGGTGAACCGCCAGCCCAGTGGAAGAGCAGCTTCCCTTCCCCTAGCTTCCTGATTTTCCCCAGTTGCCATGGTAATTGGGGGAGC
AGTGTTCTGAGAATGACACCACTGAAACCCAGCGCTCAACTTTGAAATGGTTCGGGCAAGGGACGCTGGACCCAAAACAGGAGGGGACCAGACTGCAAGGACTC
AGCTCCAGCCTCAATTTTCTGGGACCCACCCCCCATCCAGCCTGTATGCTGGATTATTCTCATCATCATCAAAATCACCATTATTGTTGGCAACTGTTTCCTGAGCA
TTTACTATATGTCTAGCACCATGTCAAGGGCTTCACCAGCATCATCTCATTGTGTATATCCTCACGACATCCCCAGGCCCATCCATGGCTCAGGAAACAGGCTCTG
AAACTTGCCCTTGTGGCAGCCAGGAAGTGGCAGAGCCCTGGGGCTGGTGGCATTTCTCCAGGCCTCCTTCCAGCATGTGGAAGACAGCATAGACCTCCAGGCACC
CTCGTCAGCTTATTCTTTATTCCTTCCTCCCCTCCTCATCCCCCACACCCTTCTTCCTGCCCCCAAAGTCACACACCCCCTCCCCTGCTGGATGTGCAAGACGATCTG
AAAAGCATTTATTAGTGACAAAGGGGGTGGTGGTTTTCCTCTTTGCCTCTTCTCTGCGCAACTCGAANCCTGAGTGGGTTTGCTCCGCTTGGCAAGGCTGGGGAGG
ANGAAAAGATCAAATCACCCANAGCCAGGTGCCAAGTGTGTGTGTGTGTGTTTGTGTGTGTGTATGTGCGCGTGTGCNCNTGNNNNTATGTGTGTCCTCTCTTT
CCNTGANTTTCCCCNNTTTCCCAANNCTNNNNNNANCNGCANCNNCNGCNNNGGGNANANNCAAANTCCTTTNCANCTNNCCAGNAAANANNATTTTNNNNNG
GTTTNNNTGNNTNCCNTATGTTAGGNCNCTANNANNNANNNNCTNNNNTNTCNGNNNNNNNTCNCTCNNNNNNACNNNNNNNNNCNANNNNNNGNNTCNGTCN
NNNNANNNNNCNNCNTNNNNNNNNNNAANNCANNNNNNNAGNNNNGNNNNNN

Rodent Construct Reaction 3 (Middle Primer)

NNNNNNNNNNNNTNCNNGTTCTGGTCTTTTCTGGAGCCNCACTCAGGCCTGGCATCTGGGGAGAGTGAAGGATGGAGGGCACATGTTCATCTGTGGCCTGGGAG
CCCCACAGGTGTGGAGAGGCCAAGGTCTGGGCTGGAGGAGGGAAGACACAGAGAACGCAGCTTCCCTTCCTTTCTTAGGGGCCCTAACATATGGGTAACCATAT
AAACCCGACTCAAAATCTGTCTTTTCCTGGGCAGATTGCAAAGGATTTTGCATCTCCCCGTTGCTGTTGCTGCTGCTCACACAGTCTTGGGAAAACGGGGGAAAAT
CAAGGAAAGAGAGGACACACATACGCGCACGCGCACACGCGCACATACACACACACAAACACACACACACACACTTGGCACCTGGCTCTGGGTGATTTGATC
TTTTCCTCCTCCCCAGCCTTGCCAAGCGGAGCAAACCCACTCAGGCTTCGAGTTGCGCAGAGAAGAGGCAAAGAGGAAAACCACCACCCCCTTTGTCACTAATAA
ATGCTTTTCAGATCGTCTTGCACATCCAGCAGGGGAGGGGGTGTGTGACTTTGGGGGCAGGAAGAAGGGTGTGGGGGATGAGGAGGGGAGGAAGGAATAAAGA
ATAAGCTGACGAGGGTGCCTGGAGGTCTATGCTGTCTTCCACATGCTGGAAGGAGGCCTGGAGAAATGCCACCAGCCCCAGGGCTCTGCCACTTCCTGGCTGCCA
CAAGGGCAAGTTTCAGANCCTGTTTCCTGAGCCATGGATGGGCCTGGGGATGTCGTGAGGATATACACAATGAGATGATGCTGGTGAAGCCCTTGACATGGTGCT
ANACATATAGTAAATGCTCAGGAAACAGTTGCCAACAATAATGGTGATTTTGATGATGATGAGAATAATCCANCATACAGGCTGGATGGGGGGTGGGTCCCANA
AAATTGANCTGGNNCTGANTCCNTGCNNNCNNGTCCCTCCTGTTTTGGGNNCAGCGTCCCNTGCCNAACNATTTCNANTTGAGCGCNGGGTTTCAGTGGTGNNNT
NNNNGANNCTNCTCCCNNNNCATGGCACTGGGGAAANCNGANCNNGGNNNNCNNNTNTCNCTGGNTGGCNNNNNNNGCTNNAANNNNNNNNNNNNNNNNNN
NNNNANNNNNNNNNNANNNNNNNNNNTNTGNNNANCNNNNNNN

Confirmed full promoter sequence for *Rodent* Construct:

CTAGCAAAATAGGCTGTCCCCAGTGCAAGTGCAGGTGCCAGAACATTTCTCTATCGATAGGTACCGAGCTCGGATCCACTAGTAA
CGGCCGCCAGTGTGCTGGAATTCGGCTTGGGGTACCAAGCTTGTGCTCCCAGAGAGGAAGTGCCCCTACTCTGAGCTTCTTTTGGC
CTTGACCCACACATAGGCAGACGTGGACTCTGTTTTGAGCAGGTGAACCGCCAGCCCAGTGGAAGAGCAGCTTCCCTTCCCCTAG
CTTCCTGATTTTCCCCAGTTGCCATGGTAATTGAGGGAGCAGTGTTCTGAGAATGACACCACTGAAACCCAGCGCTCAACTTTGAA
ATGGTTCGGGCAAGGGACGCTGGACCCAAAACAGGAGGGGACCAGACTGCAAGGACTCAGCTCCAGCCTCAATTTTCTGGGACC
CACCCCCCATCCAGCCTGTATGCTGGATTATTCTCATCATCATCAAAATCACCATTATTGTTGGCAACTGTTTCCTGAGCATTTACT
ATATGTCTAGCACCATGTCAAGGGCTTCACCAGCATCATCTCATTGTGTATATCCTCACGACATCCCCAGGCCCATCCATGGCTCA
GGAAACAGGCTCTGAAACTTGCCCTTGTGGCAGCCAGGAAGTGGCAGAGCCCTGGGGCTGGTGGCATTTCTCCAGGCCTCCTTCC
AGCATGTGGAAGACAGCATAGACCTCCAGGCACCCTCGTCAGCTTATTCTTTATTCCTTCCTCCCCTCCTCATCCCCCACACCCTTC
TTCCTGCCCCCAAAGTCACACACCCCCTCCCCTGCTGGATGTGCAAGACGATCTGAAAAGCATTTATTAGTGACAAAGGGGGTGG
TGGTTTTCCTCTTTGCCTCTTCTCTGCGCAACTCGAAGCCTGAGTGGGTTTGCTCCGCTTGGCAAGGCTGGGGAGGAGGAAAAGAT
CAAATCACCCAGAGCCAGGTGCCAAGTGTGTGTGTGTGTGTGTTTGTGTGTGTGTATGTGCGCGTGTGCCGTGCGCGTGTGTGTGT
CCTCTCTTTCCTTGATTTTCCCCCGTTTTCCCAAGACTGTGTGAGCAGCAGCAACAGCAACGGGGAGATGCAAAATCCTTTGCAAT
CTGCCCAGGAAAAGACAGATTTTGAGTCGGGTTTATATGGTTACCCATATGTTAGGGCCCCTAAGAAAGGAAGGGAAGCTGCGTT
CTCTGTGTCTTCCCTCCTCCAGCCCAGACCTTGGCCTCTCCACACCTGTGGGGCTCCCAGGCCACAGATGAACATGTGCCCTCCAT
CCTTCACTCTCCCCAGATGCCAGGCCTGAGTGTGGCTCCAGAAAAGACCAGAACCAGGCAACATTTCCTGAACAGGCTGCTTTTCT
CCGTCTGTCCACCCGAGAGCCAAGGCCAGAACCCAGGCTGCAACCTCTGCGGGCTCCTGGGAGGGGCTCCCAGCACTGCCCAGCC
TGGGAGGCTGTGTGTTCCACTGTCCCATTAAGGAATGCTATCCCAACACTGATGTAGAGGCAGAAATAACCATCCCTGCCTCCCAT
GGGGCCTTCTCTGCCCAAATAGACCTGCCCTGCCCTGCGTGGCTACTACATTCAGCCCTATCCAGGCTCCCAGAAATTGTCATTGT
GTGTGTATGTGTTTCCTGATAGAAAAAAAAAAATGGAAATTAGGTTATATAAAAGATGTATGCTTTGGAGCCCAGAGCGGCTCTT
TTAATGAGGGTTGCGACGTCTCCCTCCCCACACCCATAAACCAGTCGGGTTGGACGTCACTGCTAATTCGTTTCAGTGATGATAGG
ATAAAGGAGGGACATTAAGAAATAAATTCCCCCTCACGACCCTCGCTGAGCTCACGGCTCAGTCCCTACATATTTATGCCGCGTTT
CCAGCCGCGCTACTTAGCGCAGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAAGACGCCA
AAAACATAAAG

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