MQP-BIO-DSA-4105

LOCALIZATION OF THE INFβ-1B INTERACTING SITE ON THE NCOA7-AS PROMOTER REGION

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

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April 25, 2013

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ABSTRACT

When treated with interferon β-1b, gene NCOA7 expresses a new variant that begins from an alternative start site NCAO7-AS with strong antioxidant properties. Segments of three different lengths of the upstream region of the gene were inserted into phRLnull. After testing and confirming their structure, the plasmids were transfected into mammalian cells. A luciferase assay was performed to determine which segments showed an increase in activity when treated with interferon. While each ligated plasmid acted as a strong promoter for *Renilla* luciferase, no significant difference in signal due to INFβ-1b treatment was observed.

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ACKNOWLEDGEMENTS

First, I would like to thank Dr. Michael Volkert for allowing me to work in his laboratory and assist with his ongoing research, as well as for working closely with me over the course of the project. I would also like to thank Lijian Yu for assisting me with laboratory procedures that were new to me along the way, and offering suggestions for troubleshooting experiments. Finally, I would like to thank Dr. David Adams for initiating this project and assisting me with draft editing during the project.

BACKGROUND

Reactive Oxygen Species

Damage to cellular components at a molecular level can occur in a number of ways. A common form of damage that occurs to biomolecules, such as nucleic acids, proteins, and lipids is oxidative damage, resulting from reactive oxygen species (ROS). ROS are partially reduced species of molecular oxygen that are formed as byproducts of metabolic reactions that occur naturally within cells (Imlay, 2003). ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxide radicals ($OH^\cdot$) as seen in **Figure 1** below.

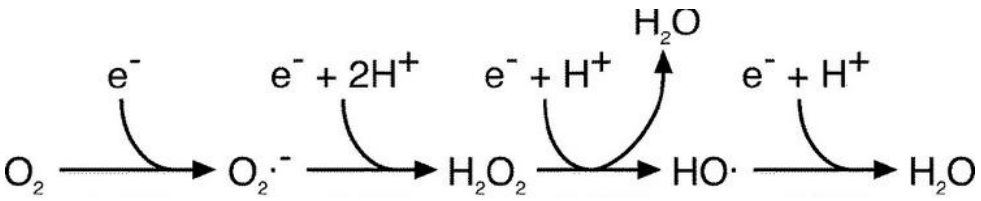

Figure 1: The Process of Reducing of Molecular Oxygen to Water. ROS molecules are generated at each internal step (Imlay, 2003).

ROS have a greater chance of causing injury to an organism than molecular oxygen due to being greater electron acceptors, thus making them better oxidants (Imlay, 2003). Of the three ROS mentioned the hydroxyl radical is the most reactive, and will react with the first molecule it encounters (Imlay, 2003). The other two are less reactive due to hydrogen peroxide's stable O-O bond and superoxide's negative charge preventing it from interacting with other negatively charged molecules (Imlay, 2003).

Formation of Superoxide and Hydrogen Peroxide

Studies have revealed that superoxide and hydrogen peroxide are produced in the mitochondria of a cell in physiological conditions at the NADH dehydrogenase in the electron transport chain (for review see Turrens, 2004). At this location ROS are generated at the NADH binding site by a reduced flavin molecule when the radical is passed from the reduced flavin molecule to molecular oxygen instead of the Q-site of the enzyme, as per usual function as seen in **Figure 2** (Esterházy et al., 2008). It has also been shown that several other flavin molecules as well as succinate dehydrogenase and fumarate dehydrogenase also can generate ROS in this was (Imlay, 2003).

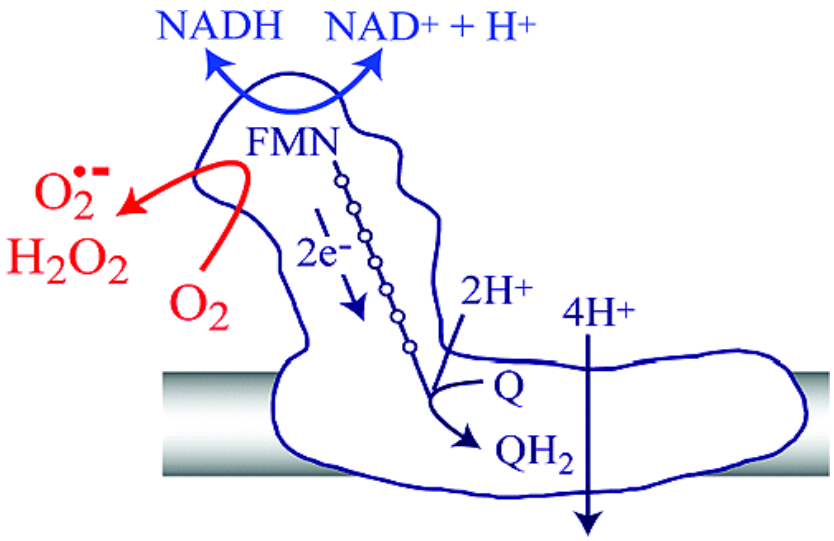

Figure 2: The Electron Transport Chain's Complex I (NADH Dehydrogenase.) Reactive oxygen species are generated from reduced flavin molecule giving electrons to molecular oxygen. Normal pathway in blue, ROS generating pathway in red (Esterházy et al., 2008).

Hydroxyl Radical Formation

Hydroxyl radicals can be generated in a few different processes. The primary way that hydroxyl radicals are formed is through Fenton chemistry (Tashjian, 2009). Ferrous iron ions can react with hydrogen peroxide to generate ferric iron, a hydroxyl ion, and a hydroxyl radical. The reaction must be carried out with free iron generated by reducing

ferric iron (Fe^{3+}) to ferrous ion (Fe^{2+}) (Imlay, 2003). Molecules such as superoxide, cysteine, and $FADH_2$ can accomplish the reduction of Fe^{3+} to Fe^{2+} (Imlay, 2003).

Cellular Damage by ROS

As each of the reactive oxygen species have unique structures and levels of stability each species creates different types of damage in the cell. As stated before, the hydroxyl radical is the most dangerous of the ROS species as it will react with the first molecule it encounters (Imlay, 2003). As such the hydroxyl radical can create a large amount of damage in the cell. The most notable areas hydroxyl radicals can affect are the lipid membrane and nucleic acids (Tashjian, 2009). When hydroxyl radicals interact with the methylene groups between the cis double bonds of polyunsaturated acids in the cell membrane, carbon centered radicals are formed (Marnett, 1999).

The production of the carbon centered radicals can create a chain reaction of lipid peroxidation from a single hydroxyl radical creating vast damage to the membrane (Tashjian, 2009). Along with damage to the membrane, lipid peroxidation creates products that are hazardous to the cell. For instance, the product 4-hydroxynenal (HNE) has the ability to modify cysteine, lysine, and histidine residues which can lead to vital proteins becoming inactive, and could eventually lead to disruptions in DNA, protein synthesis, and gene regulation (Tashjian, 2009). Another dangerous product is Malondialdehyde which interacts with DNA to cause G to T transitions, as well as C to T and A to G transversions less frequently (Marnett, 1999).

As superoxide and hydrogen peroxide are less reactive than hydroxyl radicals, they are less dangerous to the cell and primarily are hazardous for their parts in creating

hydroxyl radicals (see formation of Hydroxyl radicals). Superoxide has the ability to react with iron-sulfur clusters causing them to be inactivated by binding to ferrous iron in the enzyme's active site to form a reduced ferrous ion and hydrogen peroxide which then creates a hydroxyl radical (Tashjian, 2009).

Hydrogen peroxide causes damage to the cell by oxidizing methionine residues, cysteine residues, ferric iron, and iron sulfur clusters (Imlay, 2003). When the sulfur atoms within cysteine clusters are oxidized by hydrogen peroxide disulfide bridges can be formed within the protein. This alteration can cause cysteine residues to lose catalytic activity and severely alter protein structure, possibly leaving them inert (Tashjian, 2009).

Damage to DNA is the most damaging effect caused by ROS. By oxidizing nucleotides, transversions and frameshift mutations can occur in the cell and create truncated proteins, create premature transcription stops for essential proteins, changes in the cell cycle, or changes in gene regulation. Such effects can lead to a variety of negative health effects leading to oxidative stress and damage being linked to such detrimental effects and conditions such as aging, cancer, muscular dystrophy, Alzheimer's, Parkinson's, ALS, and many other forms of human pathogenesis (Tashjian, 2009; Yu et al., submitted for publication).

Oxidative Damage Prevention

Because some of the enzymes that create ROS within the body are vital, such as NADH dehydrogenase, mechanisms are set in place by the cell to limit ROS formation or limit damage by previously formed ROS. The most common mechanism is the use of antioxidant enzymes which have the ability to convert ROS into oxygen and water

molecules (Tashjian, 2009). These enzymes such as superoxide dismutase, catalase, and Selenium-dependent GSH peroxidase only work on superoxide and hydrogen peroxide and do not affect hydroxyl radicals (Tashjian, 2009).

Oxidative Resistance Genes

In addition to antioxidant proteins, eukaryotic cells are equipped with genes that allow for the translation of a family of oxidative resistance proteins. These are called the OXR family of genes due to homologous structures within each of the genes. Currently the mechanism of how the OXR family proteins prevent oxidative damage is not fully understood, but studies have shown that the expression of these proteins can protect cells from oxidative damage (Elliot & Volkert, 2004; Volkert et al., 2000; Yu et al., submitted for publication).

OXR1

One of the genes within the OXR gene family is *OXR1*. The *OXR1* gene was discovered in a screen devised to identify human genes that confer resistance to oxidative damage to a repair-defective spontaneous mutator strain of *E. coli* and was selected as a gene of particular interest (Volkert, et al., 2000). This strain of bacteria has mutant *mutM* and *mutY* genes causing the bacteria to develop a synergistic increase in GC→TA transversion mutagenesis as the bacterial cells needs these genes to correct damage from the predominant DNA oxidative lesion 8-oxoguanine (8-oxoG). Buildup of 8-oxoG will result in GC→TA transversions and the amount of DNA oxidative DNA occurring within the cell can be monitored by the *lacZ* cc104 allele. This allele reverts to Lac+ and a blue

product only in the presence of GC→TA transversions such as those caused by buildup of 8-oxoG in this bacterial strain. Cells naturally produce ROS which cause buildup of 8-oxoG as part of their natural metabolism to generate the necessary mutations to turn colonies blue through activation of the *LacZ* gene. When the human OXR1 gene was inserted into this bacterial strain, the gene suppressed oxidative DNA damage to the cells, indicated by the colonies turning blue at a lower rate than control cells without the OXR1 gene, suggesting that the gene conferred oxidative resistance (Volkert, et al., 2000).

Further study revealed that yeast deficient in OXR1 are more vulnerable to damage by hydrogen peroxide, showing that the protein helps to prevent damage done by oxidative stress (Volkert, et al., 2000). Oxidative resistance was shown to be restored by expressing the human OXR1 gene in the yeast OXR1 deletion mutant, suggesting the oxidation resistance function is also functional in the human protein (Volkert, et al., 2000).

Later study revealed that *OXR1* can be induced by both heat and oxidative stress and is localized to mitochondria within yeast and humans (Elliot & Volkert, 2004). This localization is most likely due to the large amount of reactive oxygen species that is created by the metabolic processes of the mitochondria.

Homologues of the *OXR1* gene were found within many species of Eukaryotes but not in Prokaryotes. The most conserved region between OXR1 homologues is the carboxyl-terminal half of the protein which may help give the protein its oxidative damage resistance properties (Volkert, et al., 2000).

NCOA7

Another gene within the oxidative resistance gene family is the gene nuclear receptor coactivator protein 7 (NCOA7). *NCOA7* is a very close homologue to the *OXR1* gene, as seen in **Figure 3**. This close homology between genes was likely as a result of a duplication event (Durand et al., 2007). Study of *NCOA7* in a repair-defective spontaneous mutator strain of *E. coli* showed that the addition of the human NCOA7 gene was sufficient to lower rates of spontaneous oxidative mutations. Unlike the OXR1 protein however, the NCOA7 protein is localized to the nucleus and cytoplasm, but is stimulated to migrate to the nucleus when cells are treated with estradiol, as NCOA7 is an estrogen receptor associated protein (Durand et al., 2007).

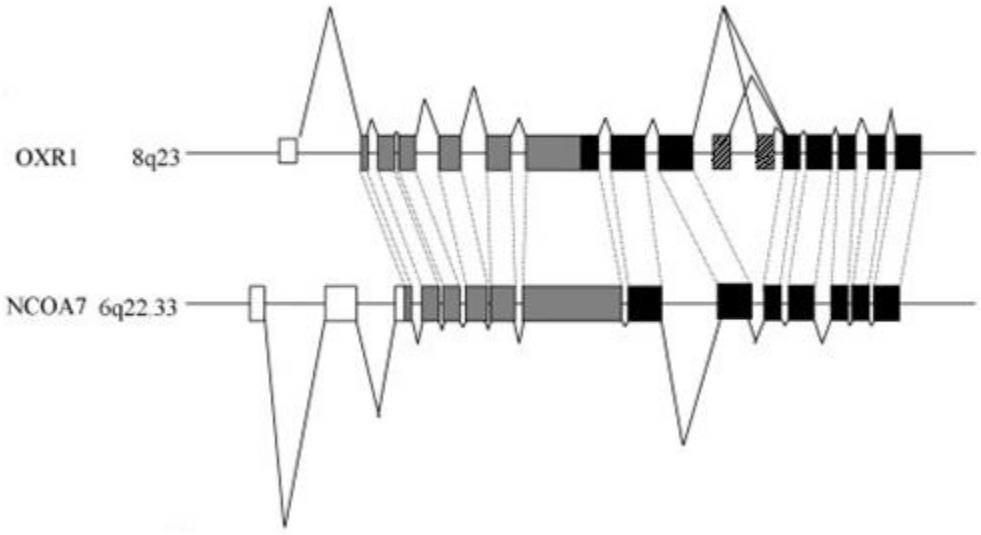

Figure 3: Comparison of OXR1 and NCOA7 Structure. Exon and intron structures of both genes are shown. Black boxes are exons that comprise the minimal OXR domain, grey boxes are areas of similarity between the two genes, and white boxes are regions that are unique to either gene. The striped exons are exons 10 and 11, which are also unique to OXR1. The length of the lines connecting exons is an indication of the relative size of the intron (Durand et al., 2007).

While OXR1 is induced to be expressed during oxidative stress, NCOA7 is expressed constitutively within the cell. This expression pattern along with NCOA7's

different localization and its estrogen stimulated association with the estrogen receptor suggest that NCOA7 may serve to prevent a different cellular function of oxidative damage protection than OXR1, likely oxidative by-products of estrogen metabolite-mediated DNA damage (Durand et al., 2007).

Structurally *NCOA7* differs from *OXR1* in that it does not contain any homologous exons to exons 10 and 11 of *OXR1*. Each gene also contains exons that are unique in upstream region of the minimal OXR domain. The minimal OXR domain is a conserved region of the OXR1 and NCOA7 proteins that has been shown, using truncated proteins, to be required to confer antioxidant activity (Durand et al., 2007).

NCOA7-AS

Analysis of peripheral blood mononuclear cells gathered from muscular dystrophy patients and human fetal brain cells led to the discovery of an alternative start variant of NCOA7 known as NCOA7-AS. NCOA7-AS differs from the full length protein as its transcription begins at an alternate start intron, intron 10a, which is not included within the full length protein as seen in **Figure 4**. By spicing so, the NCOA7-AS protein does not contain *NCOA7* exon 9. This exon, a part of the minimal OXR domain, is considered essential for oxidative damage resistance activity in full length NCOA7, however the NCOA7-AS protein has shown to confer oxidative damage resistance comparable to the full length protein (Yu et al., submitted for publication). NCOA7-AS does contain NCOA7's C-terminal TLDc domain, the last five exons of NCOA7, making it a member of the OXR family (Yu et al., submitted for publication).

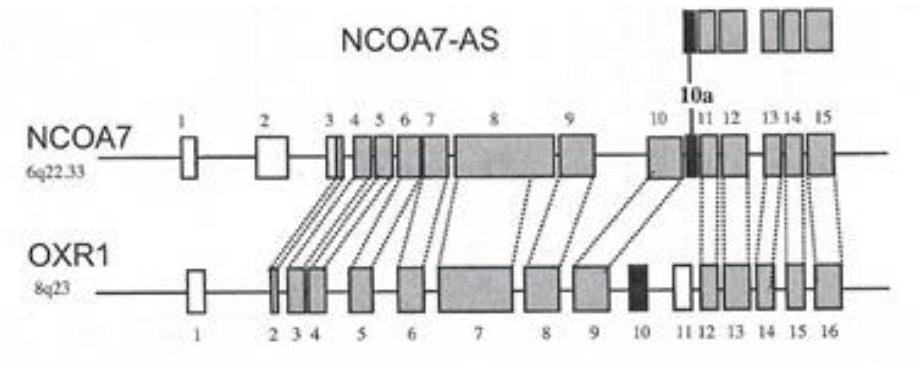

Figure 4: Comparison of the Structures of the NCOA7, NCOA7-AS, and OXR1 Genes (Yu et al., submitted for publication)

Unlike NCOA7, NCOA7-AS is not produced constitutively within the cells and instead transcription is induced by the compound interferonβ-1b (INF), a drug commonly used to treat the relapsing-remitting form of the autoimmune disease muscular sclerosis (MS). The exact role of NCOA7-AS in the treatment of MS is not confirmed, but it likely that tissue inflammation seen in MS could be caused by an increase of local ROS. Thus activation of the NCOA7-AS production by INF, long associated for its ability to reduce inflammation, may also reduce oxidative damage and lower the rate of neuronal cell body loss (Yu et al., submitted for publication).

Gene Induction by Interferon

The induction of genes such as NCOA7-AS by INF involves an interaction of the compound with two cell surface receptor chains known as interferon alpha receptor 1(INFAR1) and interferon alpha receptor 2 (INFAR2). Once IFN has bound to the cell receptors, they phosphorylate themselves using specific tyrosine amino acid residues which lie within the cytoplasmic domain of each of the receptor chains. The receptor chains then activate a signaling pathway by phosphorylating Janus Kinase 1(JAK1) and

Tyrosine Kinase 2 (Tyk2) which then phosphorylates the Signal Transducers and Activators of Transcription (STATs), known as STAT1 and STAT2, which bind with Interferon Response Factor 9 (INF9) as a complex of several proteins to response elements in the gene's promoter region. Such elements include the STAT response element, interferon-stimulated response elements (ISRE), and the IRF site (Stark and Darnell, 2012; Stark et al., 1998).

Experiments were done in HT1080 cells where the JAK1, STAT2, or the IFNAR1 receptor genes were inactive in order to determine if the JAK-STAT signaling pathway was necessary for the production of NCOA7-AS protein. Each mutation eliminated the ability of INF to induce NCOA7-AS production, confirming the JAK-STAT pathway was necessary for INF induction of NCOA7-AS (Yu et al., submitted for publication).

NCOA7-AS Promoter Region

NCOA7-AS was identified as an interferon induced gene. Examination of the DNA sequence upstream of exon 10a identifies a possible promoter region of NCOA7-AS (**Figure 5**). This region contains a sequence which is very close to that of two other INF inducible genes known as OAS1a and OAS1b. Within this homologous region lay the STAT, ISRE, and IRF regions which are suspected as being the essential elements needed for NCOA7-AS induction by INF (Yu et al., submitted for publication).

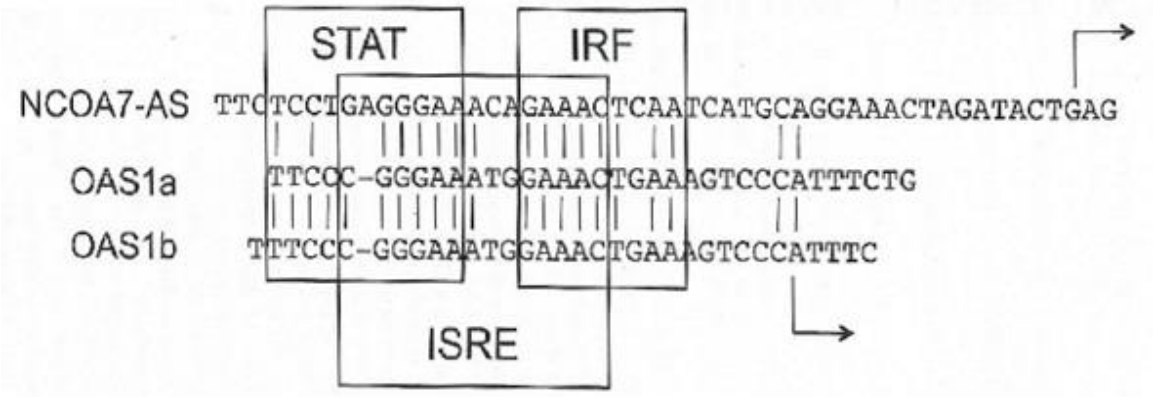

Figure 5: Comparison of the NCOA7-AS Promoter Region to INF Inducible Genes OAS1 and OAS2 (Yu et al., submitted for publication).

While it is known that the JAK-STAT signaling pathway is necessary for INF induction of NCOA7-AS production, the exact portion of the upstream regulatory area of the gene needed for induction has not been verified. As such it is currently unknown whether or not induction can be carried out with only the promoter region elements shown above, or if activator or enhancer sequences upstream are involved in induction in some way as well. A possible way of determining what portion of the promoter region is necessary for INF induction is use of the Dual-Glo® luciferase Assay System, after fusion of predicted promoter sequences to a promoter-less luciferase coding sequence.

Dual-Glo® Luciferase Assay System

The Dual-Glo® Luciferase Assay System is a product of the company Promega that is used to perform high throughput analysis of mammalian cells that contain genes for both firefly and *Renilla* luciferase within a 96- or 384-well plate format (Promega, 2013). The assay works by using one type of luciferase as a transfection control and comparing the expression of the experimental luciferase to the expression of control (Promega, 2013).

The assay makes use of two substrates, each used to activate expression of one type of luciferase creating luminescence which can be read by a plate reader. The first substrate, the Dual-Glo® Luciferase substrate, causes expression of the 61 kDa firefly luciferase protein, while also inducing cell lysis. The expression of firefly luciferase becomes stable after ten minutes and remains relatively stable for two hours (Promega, 2013). The second substrate, the Dual-Glo® Stop and Glo® Substrate, is then added with the Dual-Glo® Stop and Glo® Buffer to quench firefly luminescence 10,000-fold and generate the expression of the 36 kDa *Renilla* luciferase protein. The luminescence of *Renilla* luciferase becomes stable after ten minutes and remains relatively stable for two hours as well (Promega, 2013).

In order to test which portion of the NCOA7-AS promoter region is necessary for induction by INF, three inserts containing different sized portions of the upstream promoter sequence of NCOA7-AS were ligated into the promoter region of a Renilla luciferase plasmid which is co-transfected into mammalian cells along with a firefly luciferase plasmid so that expression can be normalized. Once transfected, cells will be treated with interferon to test if the promoter is induced resulting in increased Renilla luciferase luminescence. There are several different plasmids that are used within the experiment to create experimental conditions and ensure the assay is working accurately.

PhRLnull

The phRLnull control plasmid is a 3320 base pair plasmid which contains a polyclonal site upstream of the start site of its *Renilla* luciferase gene as well as an ampicillin resistance gene. The phRLnull plasmid contains no promoter or enhancer

elements of its own and is designed to allow for regulatory elements to be cloned into the plasmid in order to drive expression of the luciferase protein (Promega, 2012). In this experiment phRLnull is used as the vector which contains each of the inserts. Its native, promoter-less, form is used as a negative control for the assay.

PhRL-TK

The phRL-TK plasmid is a 4045 base pair plasmid that contains an ampicillin resistance gene as well as a herpes simplex virus thymidine kinase (HSV-TK) promoter for its *Renilla* luciferase gene. This promoter creates low to moderate luciferase expression in the assay (Promega, 2012). PhRL-TK transfected cells are used as a positive control. A greater luminescence in these cells than the control phRLnull plasmid transfected cells shows that the transfection has worked, and that the presence of the promoter results in increased *Renilla* luciferase activity.

PGL3

The pGL3 control vector is a firefly (*Photinus pyralis*) luciferase containing plasmid that uses a SV40 promoter to activate gene expression (Promega, 2012). Within the experiment pGL3 is co-transfected with each experimental plasmid to create firefly luciferase activity and to serve as a measure of transfection efficiency. Firefly luciferase luminescence acts as a marker of transfection and allows the ratio of each luciferase luminescence to be normalized for each well in the 96-well plate.

PROJECT PURPOSE

As discussed in the Background, INFβ-1b interaction with the *NCOA7-AS* promoter region induces the transcription of the *NCOA7-AS* gene. However it is currently unknown how much of the promoter region is necessary for the maximal amount of transcription induction by INFβ-1b. The primary purpose of this project is to make use of the Dual-Glo® Luciferase Assay System to determine how much of the upstream portion of the NCOA7-AS promoter region is necessary in order to interact with INFβ-1b to produce the NCOA7-AS protein. The narrowing down of the necessary region will allow later experiments to use directed mutagenesis to determine how mutations in various regions affect NCOA7-AS production.

METHODS

E. coli Strains, Plasmids, and Primers

Strain Number	Cell Type and Plasmid Inserted
MV 7051	Q cells + pGL3 vector
MV 7052	Q cells + pGL3 vector
MV 7053	Q cells + phRL-TK vector
MV 7054	Q cells + phRL-TK vector
MV 7055	Q cells + phRLnull vector
MV 7056	Q cells + phRLnull vector
MV 7057	DH5α + pMV 1562
MV 7058	DH5α + pMV 1563
MV 7059	DH5α + pMV 1564
MV 7060	DH5α + pMV 1565
MV 7061	DH5α + pMV 1566
MV 7062	DH5α + pMV 1567
MV 7063	DH5α + pMV 1568
MV 7064	DH5α + pMV 1569
MV 7065	DH5α + pMV 1570
MV 7066	DH5α + pMV 1571
MV 7067	DH5α + pMV 1572
MV 7068	DH5α + pMV 1573

Table 1: Table of Cell Lines used in this Experiment

Plasmid Name	Vector	Insert	Mutation
pMV 1562	pRLnull vector, AMP^r	568 bp NCOA7-AS promoter region insert.	Deletion of A within insert at plasmid base pair 275.
pMV 1563	pRLnull vector, AMP^r	568 bp NCOA7-AS promoter region insert.	No mutations
pMV 1564	pRLnull vector, AMP^r	568 bp NCOA7-AS promoter region insert.	A-C transversion at plasmid base pair 277, A-G transition at bp 278, and G-A transition 344.
pMV 1565	pRLnull vector, AMP^r	568 bp NCOA7-AS promoter region insert	T-C transition at plasmid base pair 38 and G-A

			transition at bp 521.
pMV 1566	pRLnull vector, AMP^r	133 bp NCOA7-AS promoter region insert.	A-G transition within insert at plasmid base pair 96.
pMV 1567	pRLnull vector, AMP^r	133 bp NCOA7-AS promoter region insert.	Inserted sequence not shown on chromatogram. Mutation status unknown.
pMV 1568	pRLnull vector, AMP^r	133 bp NCOA7-AS promoter region insert.	No mutations
pMV 1569	pRLnull vector, AMP^r	133 bp NCOA7-AS promoter region insert.	No mutations
pMV 1570	pRLnull vector, AMP^r	54 bp NCOA7-AS promoter region insert	No mutation
pMV 1571	pRLnull vector, AMP^r	54 bp NCOA7-AS promoter region insert	Sequencing was only successful in one direction. Mutation status unknown.
pMV 1572	pRLnull vector, AMP^r	54 bp NCOA7-AS promoter region insert	No mutation
pMV 1573	pRLnull vector, AMP^r	54 bp NCOA7-AS promoter region insert	No mutation

Table 2: Table of Plasmids Created in this Experiment

Primer Number	Primer Name	Sequence (5'-3')	Use
651	NCOA7 AS-up	GCGCCTCGAGGCCTGGCAGGAAG TGTGTTTGC	Created 133 bp insert with both primers 652 and 659. Contains Xho1 restriction site.
652	NCOA7 A-Sdn	CGCGGGCGCCCTGTTTTCTCAGTA TCTAGTTTCC	Created 133 bp insert. Contains Nar1 restriction site. Replaced by primer 659.
653	AS-TOP	TCGATTCTCAGGGAAACAGAAAC AGAAACTCAATCATGCAGGAAAC TAGATACTGAG	Oligonucleotide sequences were annealed together to create 54 base pair insert.
654	AS-BOT	CTAGCTCAGTATCTAGTTTCCTGC ATGATTGAGTTTCTGTTTCCCTCA GGAGAA	
655	pRLinser t-F	CATGCAGGAAACTAGATACTG	Tested plasmids for insert ligation through PCR.
656	pRLinser		

	t-Rev	CAATAAGGTCTGGTATAATACAC CGC	
657	657	GCGCCTCGAGTATAAAACTACAT GTAAGATGAC	Created 568 bp insert
658	658	GCGCACTAGTCTGTTTTCTCAGTA TCTAGTTTCC	
659	Pncoa7	CGCGACTAGTCTGTTTTCTCAGTA TCTAGTTTCC	Replaced primer 652 to create 133 bp insert. Contains Spe1 restriction site.
660	pRLseqf	GCCTTTTTACGGTTCCTGGCC	Sequencing primers
661	pRLseqR	GTAACCTTGATACTTACCTGC	

Table 3: Table of Primers Used in This Experiment

General Processes

Purification of plasmids

All plasmids were purified from *E. coli* cells grown overnight in a culture of LB+ Ampicillin (AMP) broth. These cells were pelleted in a centrifuge and then the plasmids were extracted using Qiagen's QIAprep Spin miniprep kit.

Calculation of Cellular DNA Concentration

Concentration of DNA was calculated using the Nanodrop 1000 spectrophotometer using nanodrop software. After blanking the machine with 10% Elution buffer, 1.5-2µl of each sample was loaded onto the machine. Concentration data for each sample was then reported in ng/µl.

Creation of Inserts

Three inserts, each containing the STAT, IRSE, and INF sites, as well as different amounts of the upstream region of the NCOA7-AS promoter region, were created in order to learn what size of the upstream promoter region was necessary for the induction of NCOA7-AS by interferonβ-1b (See **Figure 6**). The 568 and 133 base pair inserts were

created through the use of a PCR reaction while the 54 base pair insert was developed using annealing two oligonucleotide sequences in a thermocycler.

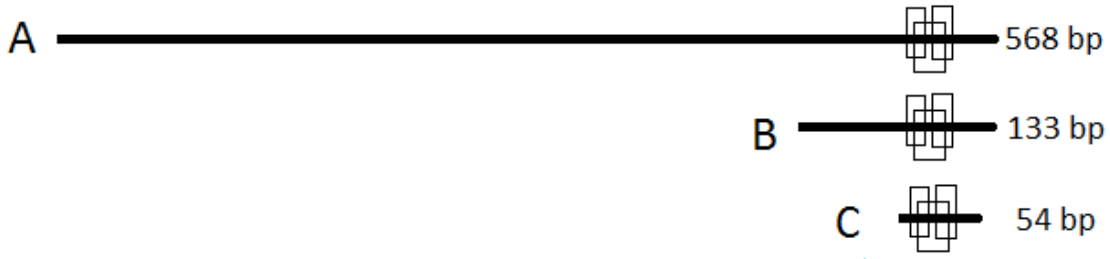

Figure 6: Pictorial Representation of PCR-Generated Inserts. Location of the STAT, ISRE, and IRF sequences on each insert are Indicated by boxes. Sequence A is 568 bp long, Sequence B is 133 bp long, and Sequence C is 54 bp long.

PCR Insert Creation

The 596 insert was developed by performing PCR reaction with primers 657 (GCGCCTCGAGTATAAAACTACATGTAAGATGAC) and 658 (GCGCACTAGTCTGTTTTCTCAGTATCTAGTTTCC) to create a product which contained the suspected promoter boxes of NOA7-AS as well as about 500 base pairs of the region's upstream sequence (see Figure 6A). The PCR product was purified using Qiagen's QIAquick PCR purification kit and analyzed by gel electrophoresis to determine if correct product was formed.

The 133 base pair insert was created and analyzed in the same process as the 596 base pair insert using primers 651 (GCGCCTCGAGGCCTGGCAGGAAGTGTGTTTGC) and 659 (CGCGACTAGTCTGTTTTCTCAGTATCTAGTTTCC) creating a product containing the suspected promoter region along with around 75 base pairs of the surrounding

sequence. Originally primer 652 (CGCGGGCGCCCTGTTTTCTCAGTATCTAGTTTCC) was used to generate the insert along with primer 651. This primer was replaced as it contains a Nar1 restriction site which was determined to be inefficient in cutting the phRLnull vector.

Restriction Digest of PhRLnull and Inserts

Both PCR product inserts contain an upstream Xho1 and a downstream Spe1 restriction site which correspond to the same sites within the polycloning domain of the phRLnull plasmid. To prepare the inserts and plasmids for ligation, inserts and plasmids were digested with Xho1 and Spe1 in New England Biolabs' reaction buffer 4 and 0.1 mg/ml BSA for two hours at 37ºC. At an hour and a half 4µl of the plasmid digestion was run through gel electrophoresis to determine if the complete digestion was achieved. If digestion was not seen an additional sample was run every half hour until digestion was achieved. When digestion was achieved the enzymes were heat inactivated by incubation in a 65ºC water bath for twenty minutes. Samples were then purified using the Qiagen's QIAquick PCR purification kit to remove unwanted segments.

Plasmid Dephosphorylation

A portion of the digested plasmids had their 5' sticky ends dephosphorylated using Antarctic Phosphatase to prevent the plasmid from re-annealing on itself to increase ligation efficiency. 1/10 volume of 10x Antarctic Phosphatase buffer was mixed with digested plasmids and then 1µl Antarctic phosphatase was mixed into the solution. Cells

were incubated at 37ºC for 15 minutes and then the enzyme was heat inactivated at 65ºC for 5 minutes.

Annealing of 54 base pair insert

The 54 base pair insert was created by annealing the two oligonucleotides, primers 657 (GCGCCTCGAGTATAAAACTACATGTAAGATGAC) and 658 (GCGCACTAGTCTGTTTTCTCAGTATCTAGTTTCC) together in the thermocycler using a preset program that heated the DNA to 90°C for 1 min then dropping 0.1°C/second until it reaches 4°C, then held at 4°C until the DNA was removed. The annealed 54 bp oligonucleotide insert contains the predicted promoter region only with no additional surrounding sequences. The ends of the insert contain the sticky end sequences for the Xho1 and Spe1 to allow for ligation of the insert to the cut phRLnull plasmid.

DNA Ligations

Both insert digests were ligated with dephosphorylated digested plasmids using New England Biolab's Quick Ligase kit. The 54 base pair annealed oligonucleotide insert was ligated to digested plasmids that were not dephosphorylated as the 5' phosphate was necessary. The digests were ligated at room temperature for 5 minutes and then stored on ice.

Generation of Experimental Plasmids and Cell Lines

With the inserts ligated into plasmids, transformations were performed to create stable *E. coli* cell lines containing each insert.

Making Competent Cells

DH5α E. coli cells were made competent for transformation in Dr. Volkert's lab through use of the Inoue method of creating competent cells. Cells were grown overnight in LB media and diluted 1:20 LB media. Cells were grown to have A_{600} of about 0.7 and placed on ice. Cells were then spun at 2000g for 10 minutes at 4ºC and the pellet was resuspended in 16 ml 4ºC transformation buffer. Cells were spun at 2000g for ten minutes at 4ºC once again and the pellet was resuspended in 4ml of 4ºC transformation buffer with 0.3ml sterile DMSO. Cells were then incubated on ice for ten minutes and then the cell suspension was separated into 100µl aliquots. Aliquots were then flash frozen in liquid nitrogen and stored at -80ºC until ready for use.

Transformation of Ligated Plasmids

When used competent cells were thawed on ice for thirty minutes and mixed with either no DNA or 5µl product of a ligation reaction. Cells were allowed to incubate on ice for thirty minutes and were then heat shocked at 42ºC for thirty seconds. Cells were returned to the ice bath for two minutes and then were suspended in 250µl of LB medium and were placed in 37ºC rotating incubator for one hour. Cells were then plated on LB+ AMP plates and incubated overnight at 37ºC.

Plasmid Identification

In the morning plates were examined for positive transformants. If growth on experimental plates was greater than control, single colonies were selected from

experimental plates and used to inoculate culture tubes of LB+ AMP broth which were incubated overnight at 37ºC. Remaining cell suspension was used to streak for single colonies on LB+AMP plates.

Plasmids were purified from cell suspension using Qiagen's QIAprep Spin miniprep kit and were tested presence of the insert using three different methods. First each plasmid was tested for the presence of a specifically sized PCR products using primers 655 (CATGCAGGAAACTAGATACTG) and 656 (CAATAAGGTCTGGTATAATACACCGC).

The second method was to test for inclusion of the insert by restriction digestion. Digestions of plasmids with 568 base pair insert were carried out as before with Spe1 and Xho1. The other two inserts were digested with Bsu361 and Bgl1 and New England Biolabs' reaction buffer 3 and 0.1 mg/ml BSA for two hours at 37ºC. The restriction enzymes were then heat inactivated for twenty minutes at 65ºC.

Finally plasmids which showed appropriate cutting patterns were sent to Sequegen with primers 660 (GCCTTTTTACGGTTCCTGGCC) and 661 (GTAACCTTGATACTTACCTGC) and the resulting chromatograms were analyzed using Finch T.V. and a BLAST alignment search to compare the expected sequence of the phRLnull plasmid with the sequence obtained from each insert. Plasmids containing inserts lacking mutations were selected for use in the luciferase assays.

Plasmids that were sent for sequencing were given a plasmid number. The cell strain from which each plasmid was derived was selected from the single colony streaking plate and grown overnight at 37ºC in LB+AMP broth. The next day the cell

suspensions were mixed with sterile DMSO (10% final concentration) and were stored at -80ºC.

Determining the INF- Interacting site on the NCOA7-AS Promoter Region

Plasmids with mutation-free inserts were used in the Dual-Glo® luciferase assay to determine the amount of the upstream sequence needed for induction by Interferon β-1b.

Splitting of HT1080 cells

Human HT1080 fibrosarcoma cells were grown by Dr. Volkert's lab from frozen stock. Once cells in culture flask reached a cell density of around 80% cell confluence, the cells were washed and suspended by draining the media, washing with PBS and then trypsinizing the cells for 5 minutes. Once trypsinized the cells were resuspended in fresh media, the cells were split into at least 35 wells in a 96- well plate along with a daughter flask and allowed to grow overnight.

Transfection of Vectors

Six wells in the 96-well plate were co-transfected with pGL3 along with either pMV 1572, pMV 1568, pMV 1563, phRLTK, or phRLnull using Invitrogen's Lipofectamine® 2000 reagent. 0.4µg of each plasmid was mixed with 40µl of Opti-MEM® I Reduced Serum Media. These mixtures were mixed with 2.4µl of Lipofectamine in 40µl of Opti-MEM® I Media, after a five minute incubation. After a 20 minute incubation at room temperature 10µl of each Lipofectamine mixture was added to

six wells on each plate in the format seen as **Figure 7**. After two and a half hours the media in each well was replaced.

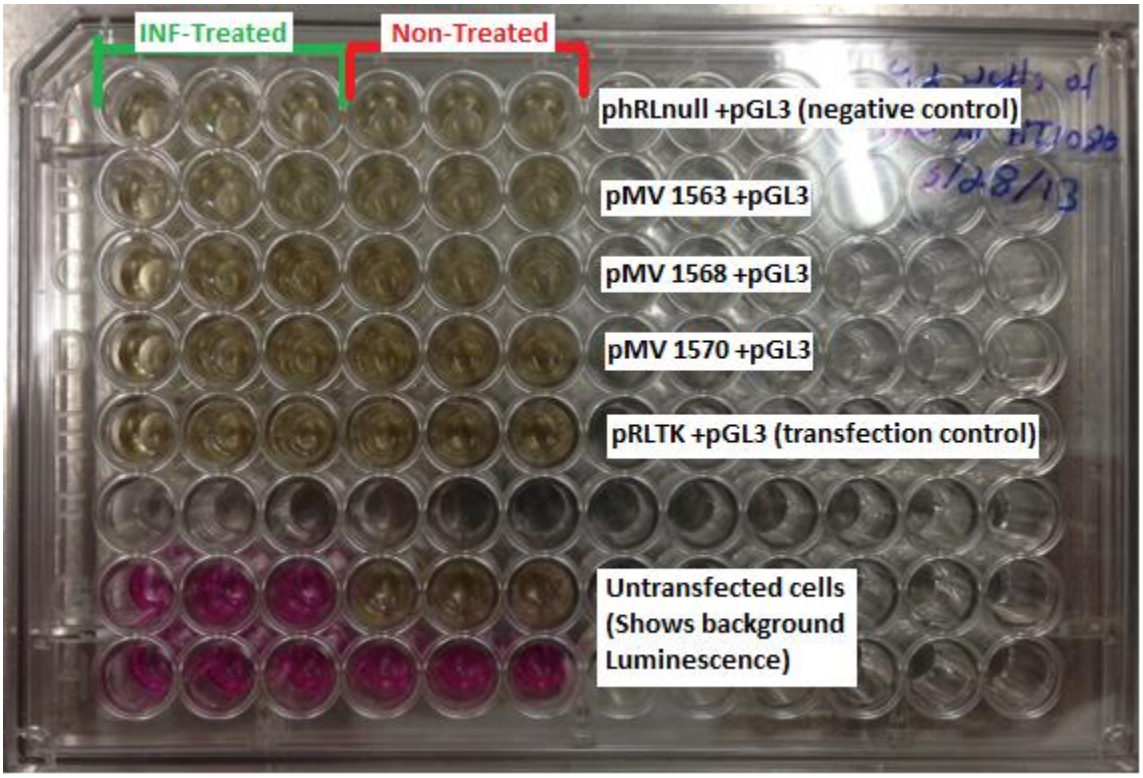

Figure 7: Experimental setup for Dual Luciferase Assay

Interferon Treatment

The first three wells of each row were treated with 2µl of INF that was diluted from 1.32 mg/ml to 7.46×10^{-5} µg/µl in early experiments or 1.91×10^{-3} µg/µl in the final test after tests to determine if increased interferon concentration was needed for induction. Interferon was allowed to incubate at 37ºC for four hours and then assay was performed.

The Dual- Glo® Luciferase Assay

The volume of media in each well was brought to 75µl and then each cell was treated with 75µl of the Dual-Glo® Luciferase substrate to induce the expression of firefly luciferase. After a ten minute incubation, luminescence was recorded using a ten

second exposure time for each well to ensure an accurate measure of luminescence. After firefly luminescence was recorded, the Dual-Glo® Stop and Glo® Reagent was mixed by diluting the Dual-Glo® Stop and Glo® Substrate in Dual-Glo® Stop and Glo® Buffer to 1:100 the needed volume of reagent. Once mixed 75µl of the Reagent was added to each well to squelch firefly luminescence and induce *Renilla* luciferase luminescence. After 10 minutes Renilla luciferase luminescence was read in the plate reader with a ten second exposure time.

Data Analysis

All luminescence readings were compared to non-transfected control wells to determine levels of background luminescence and check for possible contamination. The recorded Renilla luminescence from each well was divided by the firefly luminescence of that well to normalize the data. The mean for each type transfection ±INF treatment was calculated along with standard deviation.

RESULTS

The purpose of this project was to determine what portion of the *NCOA7-AS* promoter region was necessary for gene induction by INFβ-1b treatment using Promega's Dual-Glo® Luciferase Assay. This was accomplished by creating three phRLnull plasmid strains that each contained the suspected *NCOA7-AS* promoter region sequence as well as varying amounts of the upstream sequence of the promoter within the plasmid's luciferase promoter region. These plasmids were transfected into human HT1080 cells. It was suspected that if the necessary promoter region was within the transfected cells then treatment of the cells with INFβ-1b would induce stronger *Renilla* luciferase luminescence than untreated cells of the same transfection.

The three inserts were created using primers designed to test different portions of the upstream region of the *NCOA7-AS* gene. The smallest insert was designed to test only the suspected promoter region, the 133 bp promoter was designed to test the suspected promoter and the immediate upstream region, and the 568 bp promoter was designed to search a large upstream area to maximize the chance that the promoter region would be contain all the necessary regulatory sequences and show INF induction. By observing a response to INF in any of the promoter-luciferase fusions estimations can be made about how much of the region is necessary for INF induction of *NCOA7-AS* and guide future experiments to narrow down the region and determine which elements are critical for function .

The two large experimental plasmids were developed by using primers to generate the insert using PCR. The smallest insert was produced by synthesizing two

complimentary oligonucleotides containing the promoter sequence and sequences required for annealing to the vector's restriction sites. Inserts were then digested with Spe1 and Xho1 along with the phRLnull plasmids in order to create complimentary sticky ends. Inserts and cut plasmids were ligated together and transformed into DH5α *E. coli* cells and grown on LB+AMP plates to see if ligation was successful. If colonies on the experimental plate outnumbered colonies on a control plate with no insert, colonies from the experimental plate were inoculated in LB+AMP broth to create a cell stock of each plasmid.

Plasmids were tested for presence of insert using PCR with primers 655 (CATGCAGGAAACTAGATACTG) and 656 (CAATAAGGTCTGGTATAATACACCGC) to generate a 583 bp band when tested on the large 568 bp insert containing plasmid. None of these PCR reactions generated the expected product however as the internal plasmid sequence primer, primer hsdfdh, did not match the internal sequence of the plasmid due to an incorrect plasmid sequence being given to Dr. Volkert's lab by Promega. As such four successful transformants from the 568 bp insert plate and were sequenced by Sequegen to find out if the insert successfully made it into any of the plasmids. One plasmid that was sequenced, pMV 1563, was shown to have the expected sequence with no mutations, see **Figure 8**, and was selected to be used in the luciferase assay.

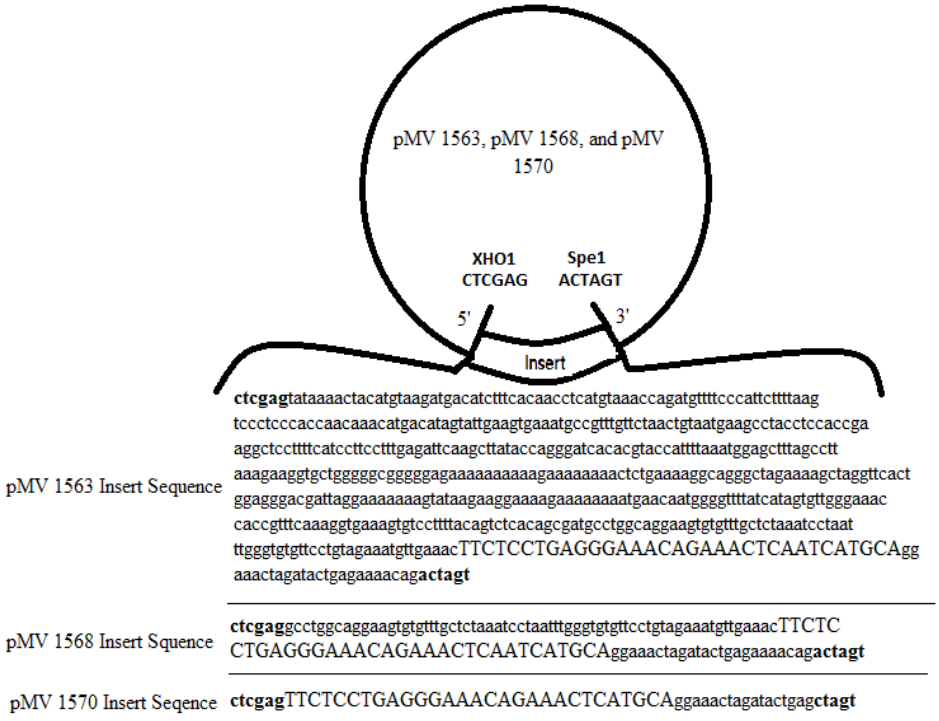

Figure 8: Insert Sequences of Experimental Vectors. Restriction enzyme sequences are shown in bold. Predicted NCOA7-AS promoter region sequence shown in capitalized sequence.

The next two plasmids were checked for insert inclusion by a double digestion with the restriction enzymes Bgl1 and Bsu361 as the sequence given to Dr. Volkert's lab suggested phRLnull had a single Bgl1 site and the addition of an insert would create a single Bsu361 site within the plasmid. The predicted products of these digests were 2316 bp and 1103 bp for the 133 bp insert plasmids 2,335 bp and 1,037 bp for the 54 bp inserted plasmid. As the plasmid sequence which was used to generate this test was incorrect, additional cuts by both enzymes were found to be present present in the phRLnull plasmids leading to smaller bands, as seen in **Figure 9**. Following this result four of each type of plasmid were sequenced and pMV 1568 and pMV 1570 were shown

to have the correct, mutation free insert sequence, seen in Figure 8, and were selected to be used in the luciferase assay.

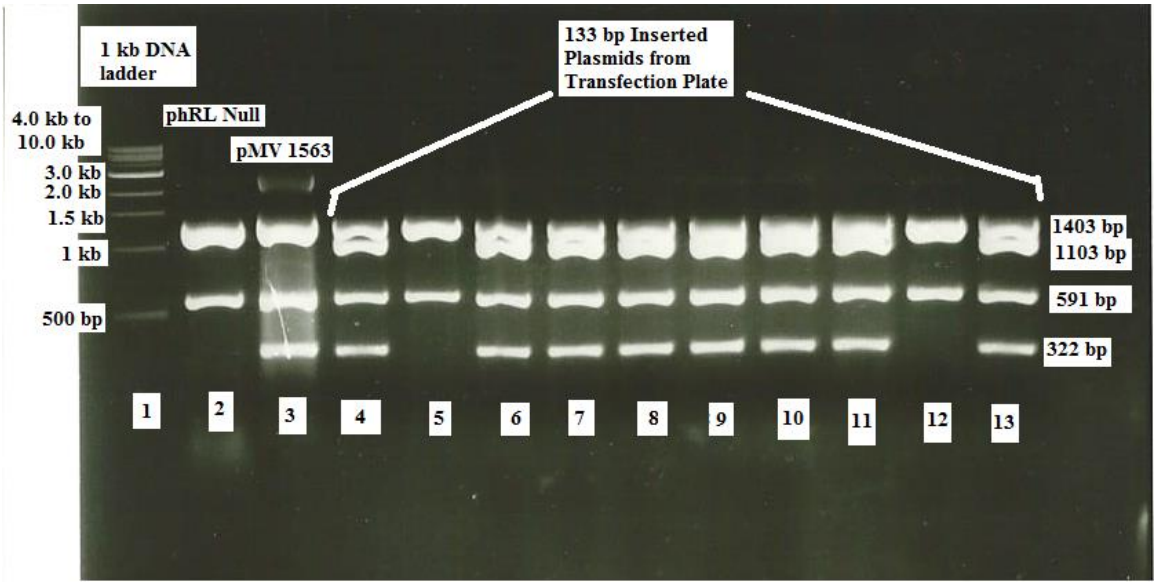

Figure 9: Gel of Bsu361 and Bgl1 Digested 133 bp Inserted Plasmids. PhRLnull acts as a negative control and pMV 1563 shows a positive reaction. Tested plasmids in lanes 5 and 6 were determined to be negative for insert. All other plasmids show positive insertion of 133 bp insert.

Thus PMV 1563 contains the correct 568 bp promoter, pMV 1568 contains the correct 133 bp promoter, and pMV 1570 contains the correct 54 bp promoter. These three plasmids, along with phRLnull and phRLTK, were each co-transfected with pGL3 into six wells of HT1080 cells grown in a 96-well plate as seen in Figure 7. Three days after the transfection, three wells of each transfection were treated with 2µl interferon beta at a concentration of 7.46×10^{-5} µg/µl and allowed to incubate for four hours.

After the four hour incubation each well was treated first with the Dual-Glo® Luciferase substrate to allow firefly luciferase luminescence (encoded by pGL3) to be read and then treated with the Dual-Glo® Stop and Glo® Luciferase substrate to allow the *Renilla* Luciferase to be read. The *Renilla* luciferase luminescence reading was divided by the firefly luciferase luminescence reading in order to normalize the results for

each well to account for difference in transfection efficiency. The normalized luminescence was multiplied by 100. This data is shown in Table 4.

	(+) INF			(-) INF		
Well #	1	2	3	4	5	6
phRLnull	4.04	5.51	6.25	6.9	5.36	5.5
pMV 1563	68.49	75	89.36	62	76.92	81.25
pMV 1568	34.88	47.54	28.89	45.76	31.91	28.89
pMV1568	16.13	22.62	22.39	19.75	18.03	19.23
pRLTK	7.69	12.12	10.53	11.43	8.57	9.76

Table 4: Normalized Luciferase Assay Data. Data shown is (*Renilla* luciferase luminescence/ firefly luciferase luminescence) *100.

The normalized values of each transfection ± INF treatment were averaged and their standard deviations were calculated for each. This allowed each set of numbers to be compared to see if any cells carrying promoter-luciferase fusions showed an increase in Renilla luciferase luminescence when treated with INF. As **Figure 10** shows, no increase in luciferase luminescence due to INF treatment was seen in any of the cells tested. However each insert acted as constitutive promoter for luciferase activity, since luciferase expression levels of phRL plasmids were greater than that seen with the promoter-less phRLnull plasmids (Figure 4).

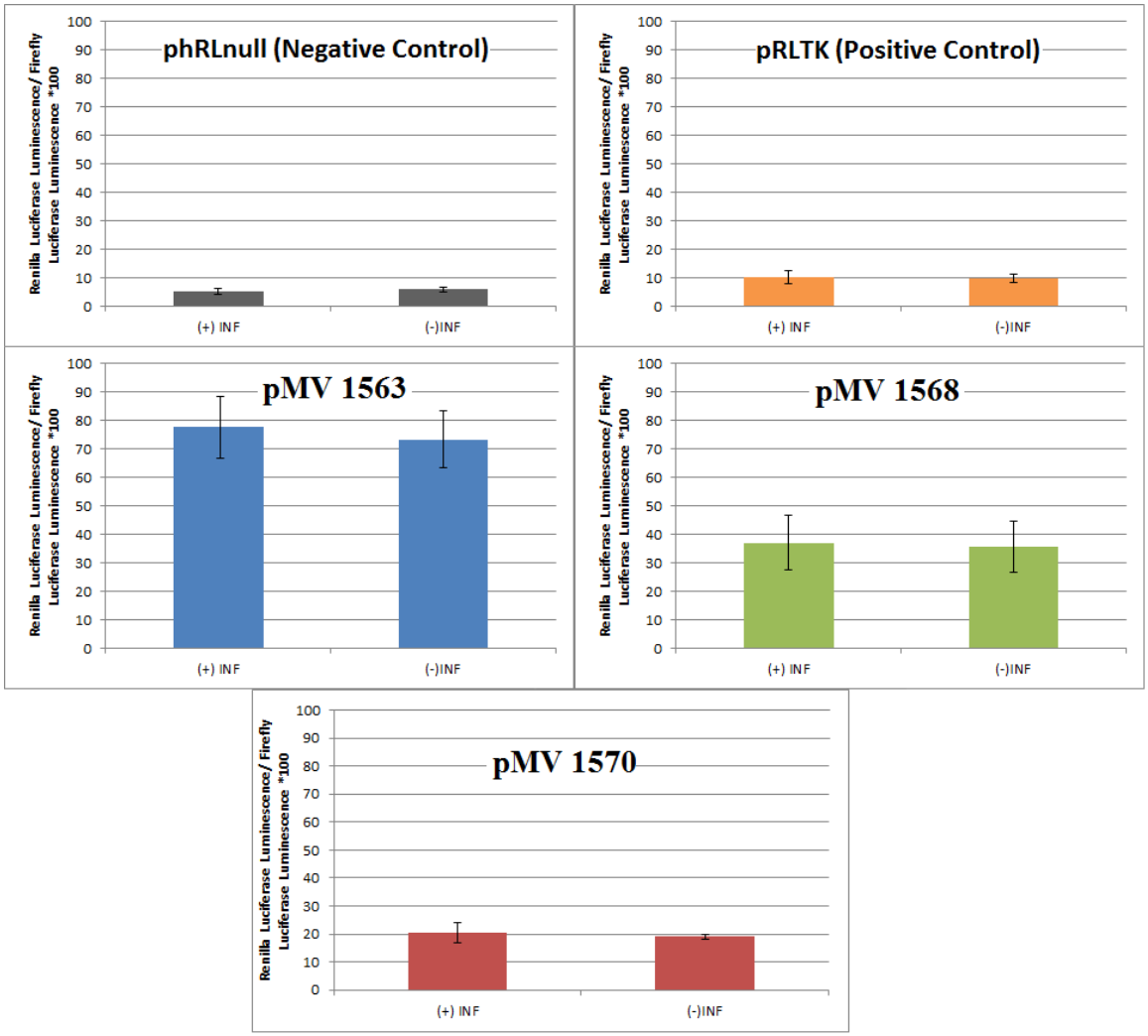

Figure 10: Averaged Normalized Reponses ±INF Treatment. The average normalized ratio for each type of transfection ±INF treatment is shown by bar. Error bars indicate standard deviation.

In order to determine if the concentration of interferon was insufficient a small experiment was conducted to determine if a higher concentration was needed to induce the promoter. Eight wells were co-transfected, four with pMV 1563 and pGL3, and four with phRLnull and pGL3. After three days one well of each transfection was treated with the same concentration of INF used in the previous experiment (Table 4), one well of each plasmid bearing cell line was treated with a concentration that was ten times greater, one well of each cell line was treated with twenty-five times the original concentration, and one of each was left untreated. After four hours the luciferase assay

was performed on the wells giving the data seen below in Table 5. The data suggested that increasing the INF concentration may yield an increase in promoter activity as seen in the doubling in signal between untreated and twenty-fives times concentrated INF treated pMV1563 transfected cells.

Treatment	Untreated	1x INF	10X INF	25x INF
Well #	1	2	3	4
phRLnull	1.42	6.02	4.82	4.42
pMV 1563	25.71	36.36	48.72	58

Table 5: Normalized INF Concentration Test. Data shown is (*Renilla* luciferase luminescence/ firefly luciferase luminescence) *100.

A full scale experiment using interferon beta that was $1.91x10^{-3}$ µg/µl was performed in an otherwise identical way as the previous experiment to yield the normalized *Renilla* Luciferase ratios seen in Table 6.

	(+) 25x INF			(-) INF		
Well #	1	2	3	4	5	6
phRLnull	3.57	8.33	9.08	10.25	8.16	5.357
pMV 1563	71.43	100	94.74	93.75	88.89	93.33
pMV 1568	56	76.19	84.21	105	100	65.38
pMV1568	16.67	22.86	53.33	40	43.75	23.33
pRLTK	20	27.27	30	33.33	33.33	27.27

Table 6: Normalized Ratios for Increased Luciferase Concentration Experiment. Data shown is (*Renilla* luciferase luminescence/ firefly luciferase luminescence) *100.

The values ± INF treatment were averaged as before, as seen in **Figure 11**, showing no difference between INF treated and untreated cells as before. This suggests that INF was unable to induce the Renilla luciferase luminescence over the levels that the promoter constitutively expresses, leaving the results about INF induction inconclusive. A notable item in this experiment was the large signal within the non-transfected cells which was unseen in other experiments (data not shown). This suggests that the cells had

low transfection rates making the cells unable to bring luminescence over baseline levels, and may have been contaminated bringing the results of this experiment into question.

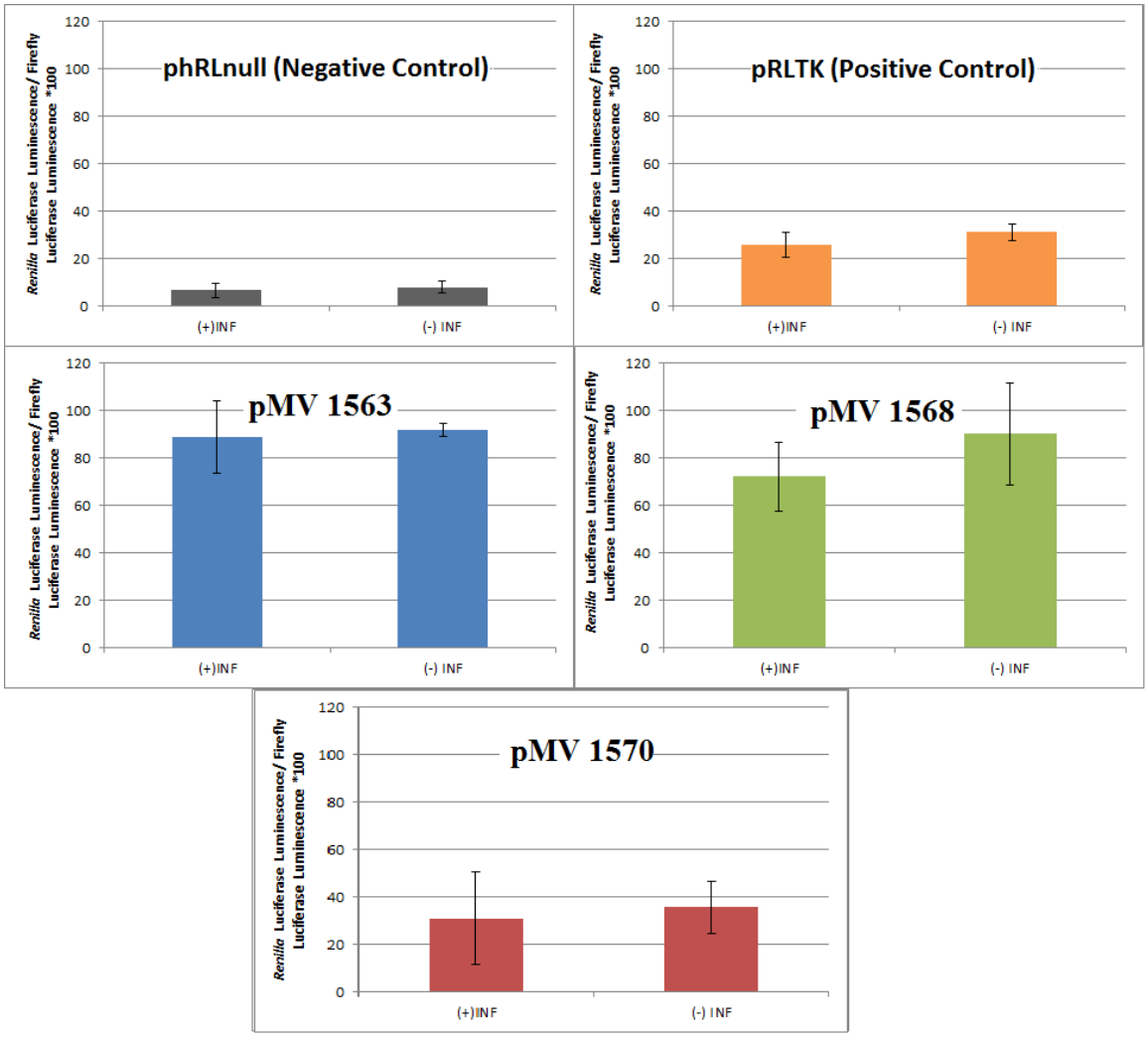

Figure 11: Averaged Normalized Reponses ± 25x INF Treatment. The average normalized ratio for each type of transfection ±INF treatment is shown by bar. Error bars indicate standard deviation.

DISCUSSION

It is known through the experiments conducted by Yu et al. that JAK-STAT signaling is required for induction of NCOA7-AS by interferon beta because mutant derivatives of HT1080 cells with inactivating mutations JAK1, STAT2, or IFNAR2 receptor genes lost the ability to be induced by INF. (Yu et al., submitted for publication). These regulatory elements are known to bind to a conserved sequence that is similar to the sequence found in the region present within all three of the promoters I constructed.

This region plus increasing upstream regions were present in the three different promoters. The NCOA7-AS gene is known to respond to interferon beta and shows little or basal level expression in the absence of IFN and a strong induction of mRNA within four hours of treatment with INF. As such it was expected that a similar result would occur in one or more of the promoters. The amount of upstream DNA necessary for this induction was presumed to depend on whether any less characterized JAK-STAT sequences were also required for induction.

Instead of the expected results, we found that all the promoters showed were essentially equally active with or without INF treatment. The IFN used in the experiment was deemed active, since induction of mRNA of the native gene could still be achieved in HT1080 cells with the same stock of IFN. Thus it appears that all promoters were constitutive when introduced into the luciferase plasmid, rather than inducible. This suggests there may be more at work in the induction of NCOA7-AS then we currently realize.

There are a number of things that these results suggest. One possibility is that, as the gene is eukaryotic, the promoter may have different activity when placed into a plasmid. For full repression of the promoter to take effect chromatin structure and proper positioning of histones may be required. Chromatin structure has been shown as important to a class of inducible promoters that are known to be associated with closely spaced nucleosomes. One well characterized promoter of this type is the promoter of the gene *PHO5*. *PHO5* is a gene in yeast which encodes an acid phosphatase and which is induced and repressed based on the level of intercellular phosphate (Adkins & Tyler, 2006). The induction of *PHO5* causes several nucleosomes, the repeating unit of chromosomal DNA that is wrapped around a histone, to be dissociated from the gene's promoter region. When *PHO5* is repressed the nucleosome is reformed with new histones (Adkins & Tyler, 2006). If the histone chaperone SPT6, which mediates nucleosome reassembly, is mutated however, the activated PHO5 gene, along with other genes with this type of promoter, will be transcribed even in the absence of transcription factors suggesting that the histone presence is essential for gene regulation (Adkins & Tyler, 2006; Rando & Ahmad, 2007).

As the NCOA7-AS promoter region is not a well characterized, it is possible that the promoter may be associated with closely spaced nucleosomes. If this is the case, inserting the promoter region into a plasmid may prevent essential gene regulation by histones, leaving the promoter region constitutively active. This potential explanation should be considered when guiding future experiments to refine the parameters in which the promoter region is analyzed and the experimental constructs are developed.

The experiment performed with the twenty-five times increased concentration of INF was interesting as the results in the large scale experiment did not match those of the small test assay performed earlier. Though this could be due to lack of multiple trials, as in usual testing there are fairly large standard deviations between plasmids of the same transfection and treatment, there is a possibility of experimental error. The evidence of this was that the wells of non-transfected HT1080 cells that were tested reported much higher levels of luminescence than previously observed in any of the previous trials. As these wells are assayed to show the levels of background luminescence, it is possible that contamination of the wells or the luciferase assay reagents occurred leading the results of that assay to be inconclusive. As such it is suggested that the assay be carried out again with new reagents and cells to determine if the concentration of INFβ-1b could induce a greater luminescence signal over non-treated cells.

Another possibility to consider would be to extend the incubation times of INFβ-1b treated cells for longer periods of time before reading the luciferase to see if there is an increase in luminescence in cells that were treated with INFβ-1b for a longer period of time. Northern blots should also be performed post assay to determine if the mRNA levels of luciferase have increased due to induction by INFβ-1b.

If either of these methods of manipulating the assay do not give the expected increase in INF response through treatment, analysis through the use of the luciferase assay should be abandoned as the promoter likely is unable to retain its chromatin structure which may be necessary for regulation of the *NCOA7-AS* gene by INF. Future studies should find a way to recreate our promoter sequences to a reporter gene in a chromatin structure and treat similarly with INF to see if activation is dependent on

chromatin structure, as well learning possibly learning something about why chromatin structure may cause such different expression patterns than plasmid circular structure.

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