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Ajay Premkumar 4.13.2010

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Mapping the Core Promoter Element of UOL Gene Encoded by Herpes Simplex Virus

By
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An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
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2010

Abstract

Mapping the Core Promoter Element of UOL Gene Encoded by Herpes Simplex Virus

By Ajay Premkumar

The activity of a gene promoter directly influences the level of gene expression. The activity of a promoter is highly regulated on both the epigenetic and DNA sequence levels, and regulatory mechanisms can be very complex. In order to understand the regulation of expression for a particular gene, identifying the key promoter elements in the promoter of that gene is an important step. The UOL ("Upstream of Latency-associated-transcript") gene of the Herpes Simplex Virus 1 (HSV-1) is expressed in acute infections and as a late gene in infected neuronal cells after reactivation of the latent HSV-1. The goal of my research is to map the core active region, and the key promoter elements of the UOL promoter. In this paper, I describe the use of luciferase assays to determine localized promoter regions of interest for the UOL gene in various cell lines. Previous studies have mapped the UOL promoter to a certain region and our results indicate different subregions within that region that may show enhancing and silencing activity, respectively. These results provide a basis for future analyses that will help determine the specific transcription factors and their binding sites of the UOL promoter. These transcription factors and binding sites could be future drug targets against the HSV-1 reactivation virulent phenotype.

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Table of Contents

Introduction

Herpes Simplex Virus type 1 (HSV-1) is a ubiquitous neurotropic DNA virus. HSV-1 is a prevalent virus pathogen infecting approximately 60-90% of the adult world population (Whitley & Roizman, 2001; Stevens, 1989). Other studies have shown that at least 80% of the world's adult population is sera-positive for HSV-1, making HSV-1 a significant health threat (Nahmias *et al.,* 1990*)*.

HSV-1 establishes latency in sensory neurons of its infected host and associates with the host throughout life (Stevens & Cook, 1971). During infection, HSV-1 travels by retrograde axonal transport to the nuclei of the sensory ganglia, where viral replication occurs briefly. Then, either viral replication leads to neuronal destruction or in the case of neuronal survival – life long viral latency is established in the neuronal cell nucleus (Morishige et al., 2006; Naito et al., 2005). Specifically, the virus will enter its latent state in the nuclei of sensory neurons of the trigeminal ganglion (Roizman & Sears, 1996). Once latency is established, the virus may reactivate spontaneously or as a result of external stimuli, such as emotional stress, trauma, or ultraviolet radiation (Whitley, 2001).

Viral reactivation usually causes only bothersome and uncomfortable symptoms including cold sores and fever blisters. However, during primary infection or viral reactivation, the virus can induce encephalitis in the infected host, which can cause significant morbidity and mortality (Wolf, 1950). In fact,

recurrent ocular HSV-1 remains the leading cause of infectious corneal blindness in industrialized nations (Nesburn, 1983).

HSV-1 is a large enveloped virus containing ~152 Kbp of linear double stranded DNA encoding more than 80 known genes (McGeoch et al., 1986; Perry and McGeoch, 1988). New genes are being constantly uncovered in the HSV-1 viral genome. The HSV-1 life cycle is tightly regulated and interference at any steps of the life cycle may result in mutant viral phenotypes, both in vivo and in vitro. Interestingly, the only actively transcribed gene during neuronal latency of the virus is the latency associated transcript (*LAT*) gene (Naito, 2005).In animal models, progress has been made in identifying other viral genomic sequences that contribute to virulence. Specific regions containing genomic material encoding for viral envelope glycoproteins and gene products which may affect apoptosis of the host cell or immune functions of the host have been proven to contribute to virulence (Enquist, 1999; Mettenleiter, 2000). Also, DNA sequences located between {nucleotides 107,920 and 126,160 (Thompson et al., 1983), 115,824 to 119,624 (Becker et al., 1986); 108,680 and 120,992 (Rosen et al., 1986); 4560 and 7600; 110,960 and 118,560; and 118,560 and 121,1600 (Javier et al., 1986) on the HSV-1 genome have all been shown to contribute to virulence (Naito et al., 2005). Interestingly, the regions mentioned above are mainly found within one contiguous region representing less than 1/10 of the viral genome: the HpaI P fragment (see Figure 1 B). This region also contains the LAT genomic sequence.

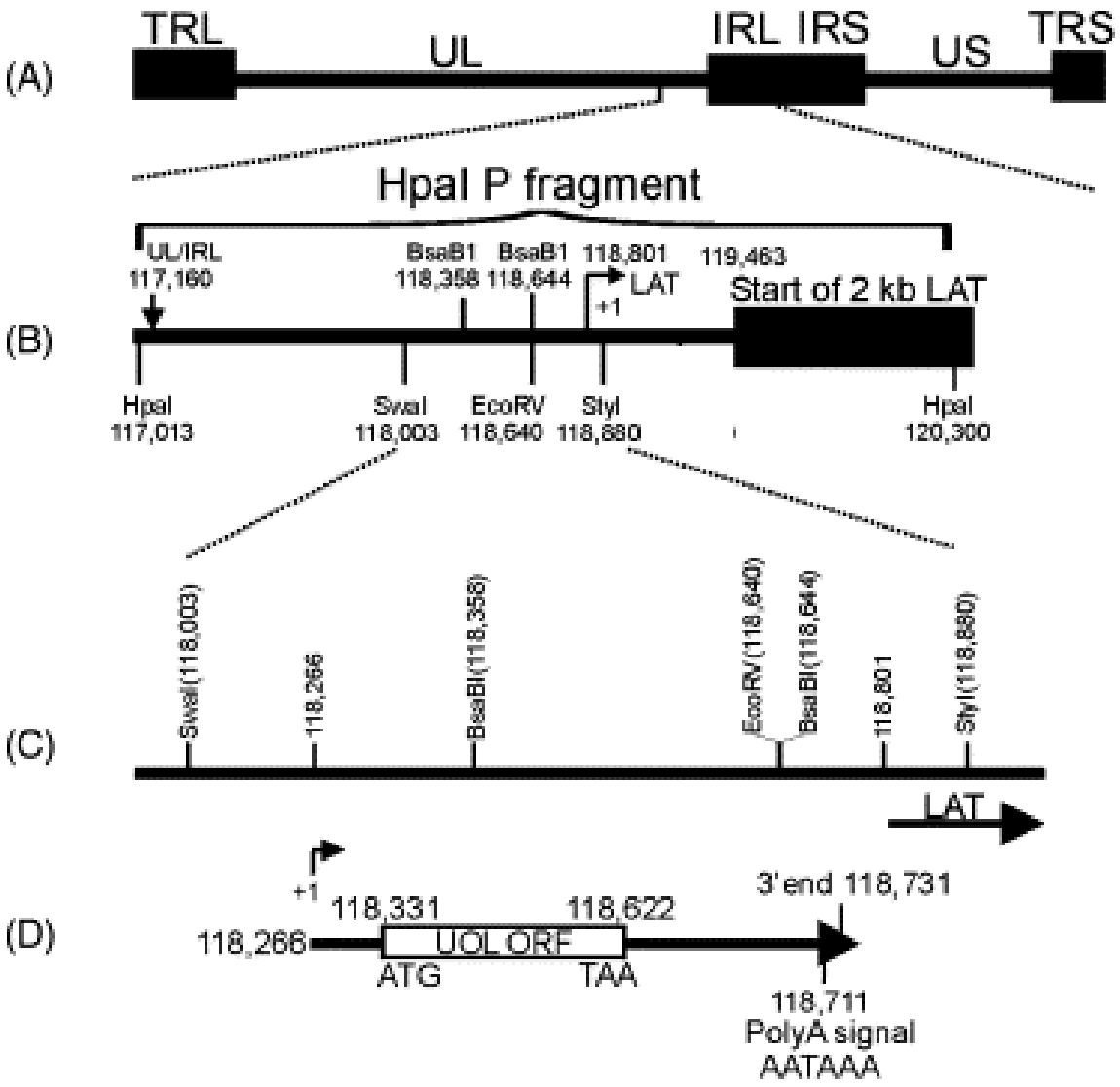


Figure 1: Relative Location of UOL to the HSV-1 Viral Genome
(Naito et al., 2005)

(A) Genomic Structure of wildtype HSV-1, showing terminal and internal long repeats and short repeats (TRL and IRL, and TRS and IRS, respectively); and long and short unique regions (UL and US, respectively)

(B) Expanded HpaI P fragment, containing junction of UL and IRL. Numbers indicate genomic nucleotide positions for 17syn+ HSV-1 strain

(C) Further expansion of region upstream of LAT transcript

(D) UOL mRNA; UOL transcript begins at nt 118,266 and UOL open reading frame ends at nt 118,731

As seen in Figure 1 B above, the UOL ("Upstream Of LAT") transcript falls in the same region of the HSV-1 genome that has been shown to contribute to virulence. The UOL transcript contains 466 nucleotides, a polyadenylated tail, and one ORF which encodes a 96 amino acid protein with a predicted molecular mass of 11 kDA. The 5' end of the UOL transcript starts at nucleotide 118,266 and the 3' end of the UOL transcript ends at nucleotide 118,731 based on the published 17syn+ genomic sequence. A UOL protein was detected, via Western Blotting, in sera from mice infected with wild-type HSV-1 and it has been shown that UOL is expressed as a late gene in infected cell culture (Naito et al., 2005).

Studies have shown that deletion of the UOL gene in the HSV viral genome results in an attenuated virulence phenotype in the infected host (Naito et al., 2005). Further studies have indicated that UOL is exclusively expressed in infected CNS neuronal cells, that it is expressed in the sense direction of LAT, and has the kinetics of a late gene during the lytic cycle of infection (Naito et al., 2005). Therefore, the regulation of UOL expression may play a key role in virus-induced virulence.

Gene promoters are very important genetic factors. The term 'promoter' is historically used to describe a region in the genome upstream of a gene transcription start site. However, DNA sequences downstream of the start transcription site may also affect transcription. Promoters affect gene transcription initiation, specificity, and rate. Promoter regions consist of several domains that are tightly and specifically regulated by multiple factors. Often,

promoters can be extremely complex and involved in an array of activities. For example, the UOL gene and promoter is found upstream of the LAT start site and was once considered a part of the LAT promoter.

Reporter vectors are essential for the quantitative analysis of the regulation of gene expression by promoter regions. The sensitivity of the Luciferase reporter assay makes it an ideal method for monitoring promoter activity; using Luciferase reporter vectors can lead to fine mapping the core active region of a promoter. As mentioned above, the core active region of a promoter can determine whether a gene is switched on or off, or expressed at different levels at different times. Mapping the core domain of the promoter for the UOL gene could potentially lead to new developments and treatments of HSV induced disease.

Although the location of the UOL promoter has been suggested (Zhu *et al.,* 1999), it has not been defined clearly. In previous studies, the macro core promoter of the UOL gene has been mapped to the region between nucleotide 117,800 and 118,330, based upon the published 17syn+ HSV-1 genome (McGeoch *et al.,* 1988; Perry & McGeoch, 1988). I have attempted to further fine map the UOL core promoter to a more defined and manageable region.

Materials & Methods

Overview

Three overlapping segments of the putative UOL promoter region were amplified by PCR and directionally cloned into the pGL4 Luciferase vector, a basic promoterless plasmid containing a Luciferase gene. The resulting plasmid was co-transfected into Rabbit Skin and Neuro 2A cells with a proper normalizing control (pRLSV40) and cells were harvested 48 hours after transfection. Cell lysates were prepared by specifications of the Promega Luminescence Reporter Gene Assay System and Luciferase activity was measured accordingly. High levels of Luciferase activity would indicate that the core promoter region lies within that generated PCR fragment.

Preparation of plasmid constructs

After preparation of HSV-1 genomic DNA, this DNA was used as a template for PCR amplification. Three overlapping PCR products were created within the regions of the previously reported UOL promoter region (Zhu, *et al.*, 1999), with a *KpnI* restriction site at the 5′ end and a *SacI* restriction site at the 3′ end. These PCR products were designated A, B, and C, and are 530 bp, 290 bp, and 80 bp, respectively. Mutant A contains what could be a TATA box, while the other two do not. Comparison among the mutants allowed for the identification of active promoter regions.

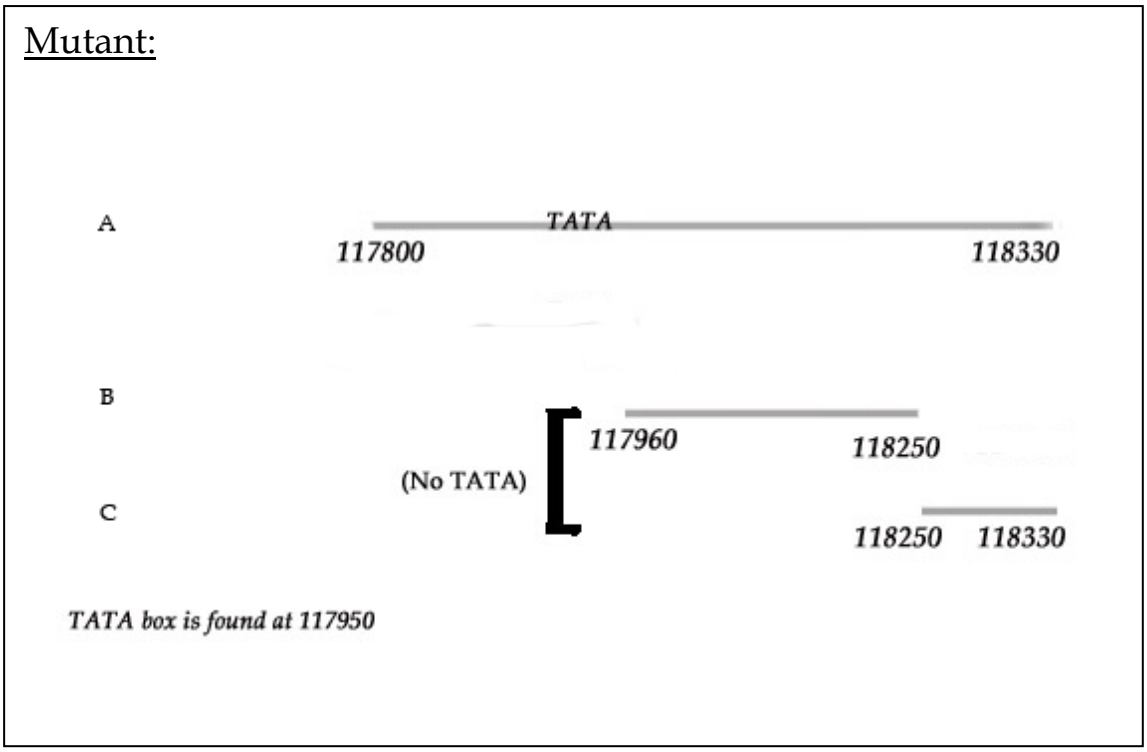


Figure 2: Segments of Template to be Amplified via PCR
Segment A contains the known RNA polymerase binding site called the TATA box, the other two segments do not

This method generated several promoter fragments that contain restriction sites compatible with that of the polylinker of the pGL4 basic vector (see Figure 3). This vector includes firefly luciferase cDNA located immediately downstream of the promoter insert.

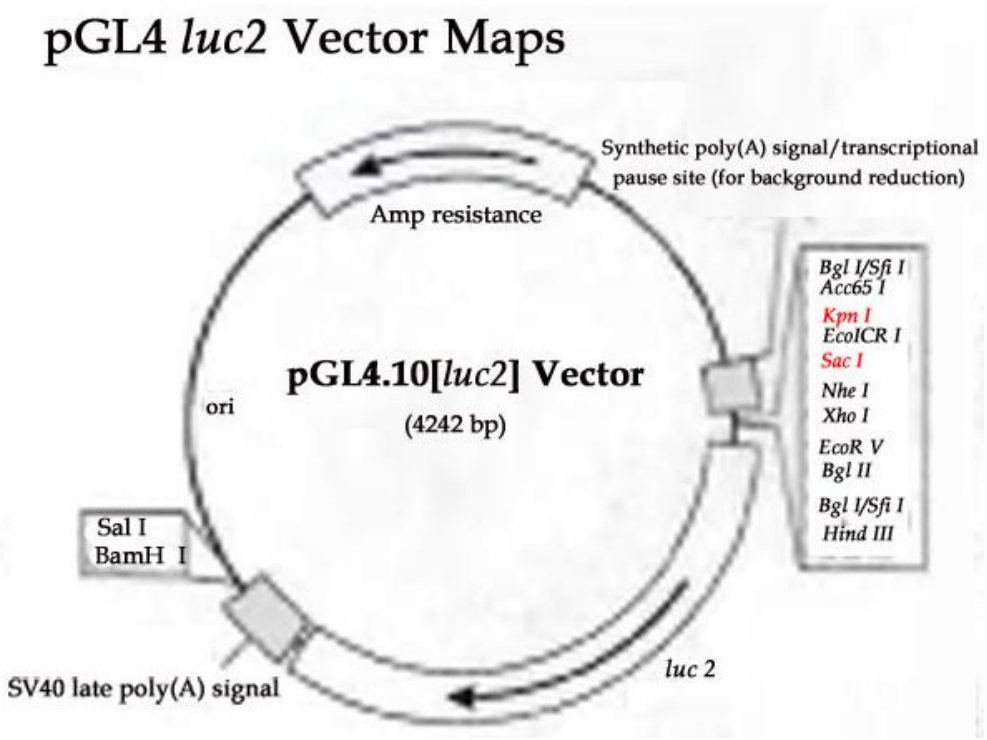


Figure 3: pGL4 *luc2* Vector
Luciferase gene downstream from multiple cloning site, KpnI and SacI restriction sites in red

Once amplified by PCR, the promoter fragments were digested with *KpnI* and *SacI*, separated on an agarose gel, purified, and ligated with T4 Ligase into the vector. Restriction enzyme digestion was performed using 2 µL of 10X buffer, 0.5 µL of each of the two restriction enzymes, 8 µL of the PCR product DNA, 0.2 µL of 100X BSA, and 9.3 µL of dH_2O to equal a total volume of 20 µL. The agarose gels used to separate the PCR products and restriction enzyme digestion products were typically 1.8% agarose, but varied between 1.5% and 2% depending on the length of the specific PCR product being tested. Purification of the post restriction enzyme digested DNA after gel electrophoresis was

performed in accordance to Qiagen's Gel Extraction Kit. After successful ligation, the plasmids were amplified by transformation into DH5α competent E. coli cells and RR-1 cells according to standard procedures, as follows.

For transformation into DH5α cells: 5 µL of the plasmid DNA was gently mixed with 100 µL of competent cells and left to incubate on ice for 30 minutes, followed by heat-shocking the cells at 42˚C for 1 minute and 15 seconds. The suspension was then re-stabilized for 2-5 minutes on ice, after which it was mixed with 0.9 mL of pre-warmed SOC medium and left to incubate at 37 ˚C in a shaker for 45 minutes. SOC medium is similar to LB medium but contains even more nutrients to provide an environment where the relatively fragile recombinant cell could grow. After this incubation period, the cells were pelleted by centrifugation, resuspended, and then plated onto agar plates, which were then left to incubate overnight at 37˚C. Colonies were selected and inoculated by suspension in 3 mL LB-medium with 3 µL of 1000X ampicillin to further induce specific growth.

Due to the high GC content of HSV-1 DNA and in the UOL core promoter region, RR-1 E. Coli cells were used to stabilize the plasmid inserts longer than 200 bp. For transformation into RR-1 cells: One mL of LB medium was inoculated with RR1 cell stock and grown at 25˚C. The next day, all the inoculant was added to 25 mL of LB and put on a shaker at room temperature. Several hours later, the culture was chilled on ice for 10 minutes, and then cells were spun at 6000 G for 10 minutes at 4˚C. Pellets were then resuspended in 12.5 mL sterile 0.1 M $CaCl_2$,

then put on ice for 15 minutes, spun again at the specifications above, and then resuspended in 1 mL cold $CaCl_2$. Subsequently, 250 µL of culture was used for each ligation mixture. After the ligation mixture was added, the mixture was kept on ice for 30 minutes, followed by heat-shocking the cells at 42˚C for 1 minute and 15 seconds. The mixture was then restabilized briefly and given 250 µL of LB and placed in a shaker at room temperature for one hour. The cells were grown at room temperature and transformation was done promptly. Cells were plated on agar plates with ampicillin resistance and were grown at room temperature for 2 – 3 days. Colonies were selected and inoculated by suspension in 3 mL LB-medium with 3 µL of 1000X ampicillin to further induce specific growth.

Plasmid DNA was then extracted from 3 mL of bacterial culture medium using the Fermentas or Qiagen Miniprep kit according to the manufacturer's instructions and quantified by absorbance measurements at *260 nm*. Clones were verified by *KpnI* and *SacI* restriction enzyme digestion followed by agarose gel electrophoresis. A positive clone was further confirmed by sequencing, and this clone was produced in large amounts using Qiagen's Miniprep/Plasmid Purification kit. After these clones were successfully created, RS-1 and Neuro 2A cells were transfected and the Luciferase activity was assessed to indicate promoter regions of interest.

Cells

Cell cultures were processed in the lab and were used throughout the experiment. Particular cell types used were Neuro2A (Mouse neuroblastoma cells), and RS 1 (rabbit skin cells).

Transfection Procedure

The transfection agent Lipofectin was used according to the manufacture's protocols for performing transfections of the luciferase-based vector pGL4. For transient transfection of the various cells, 2 µg of plasmid DNA and 2 µg of the normalization control (pRLSV40) was added to 100 µL of OptiMEM medium containing 5 µL of Lipofectin (already mixed for at least 45 minutes) and the solutions were incubated for 15 minutes at room temperature. Afterwards, an additional 800 µL of OptiMEM medium was added under gentle stirring. Cells that were transfected were plated at a density of 7,500-30,000 cells per well in 24-well plates and grown for 24 hours. This procedure should have yielded approximately 70-80% confluence. The plates were then washed with PBS and incubated in a cell-specific transfection solution (EMEM for RS-1 cells and a specific 10% serum EMEM solution for the Neuro 2A cells) for 5-24 hours at 37 ˚C. Transfected cells were then washed with PBS and incubated with complete medium. Subsequently, these cells were left to incubate for an additional 24-48 hours at 37 ˚C and then monitored for promoter activity using the luciferase reporter assay.

Assessment of Luciferase activity

Luciferase activity in transfected cells was monitored using the Luciferase Assay System (LAS) made by Promega. The LAS was comprised of D-luciferase, ATP and coenzyme A. Coenzyme A functioned to enhance the catalytic reaction leading to the emission of the bioluminescent signal and to render this signal stable for up to 1 hour. Cells were washed extensively and lysed by adding 4 volumes (v/v) of distilled water and one volume of Passive Lysis Buffer 5X to yield a sufficient volume of the solution to the monitor. The lysates were collected in an Eppendorf centrifuge tube, vortexed, and centrifuged at 10,000 rpm for 15 minutes to remove cell debris. The emitted luminescence from the dual-reporter vector was then measured in the Microbeta luminometer by mixing 20 µL of the Passive Lysis Buffer lysate with 50 µL Luciferase Assay Reagent for measurement of firefly luciferase activity, and then injection of 50 µL of Stop & Glo reagent for measurement of *renilla* luciferase activity (for normalization of results).

Results & Discussion

PCR amplification of promoter fragments

As previously stated, three overlapping segments of the putative UOL promoter region were amplified by PCR and directionally cloned into the pGL4 Luciferase vector. Originally, however, five promoter segments were targets for PCR amplification, cloning, and testing. Below in Figure 4 are these five

promoter regions within the known UOL promoter, of which only the three in red were successfully cloned and tested. The poor success rate of PCR amplification was probably due to the high GC content of the HSV-1 viral genome. The GC content of the HSV-1 virus is upwards of 68%; in perspective, the GC content of the host (human) genome is a mere 41% (Brown et al., 2007). It is believed that the high GC content within this promoter target region could lead to temporary hair-pin loops impacting the DNA's secondary structure and rendering PCR less effective in amplification.

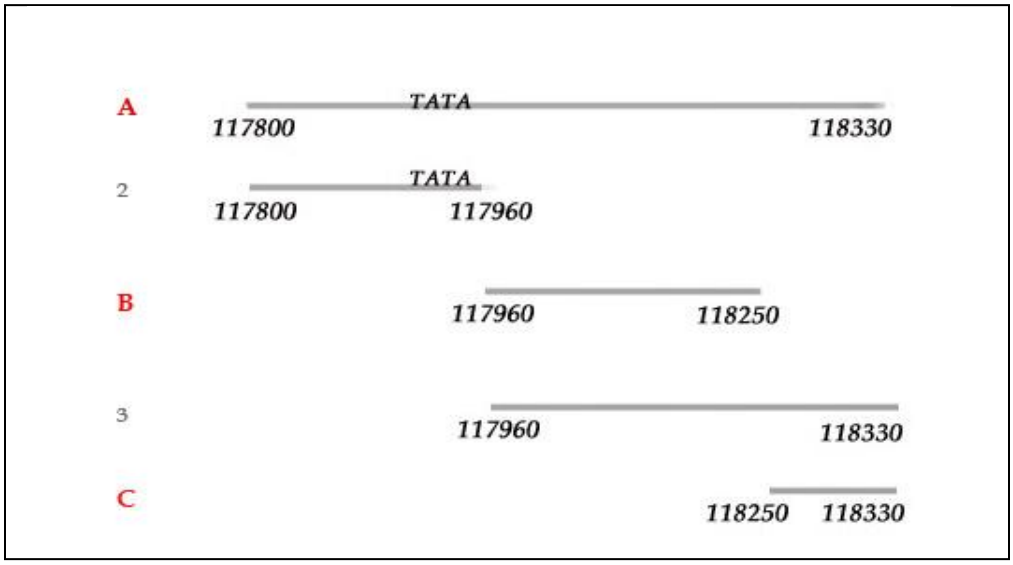
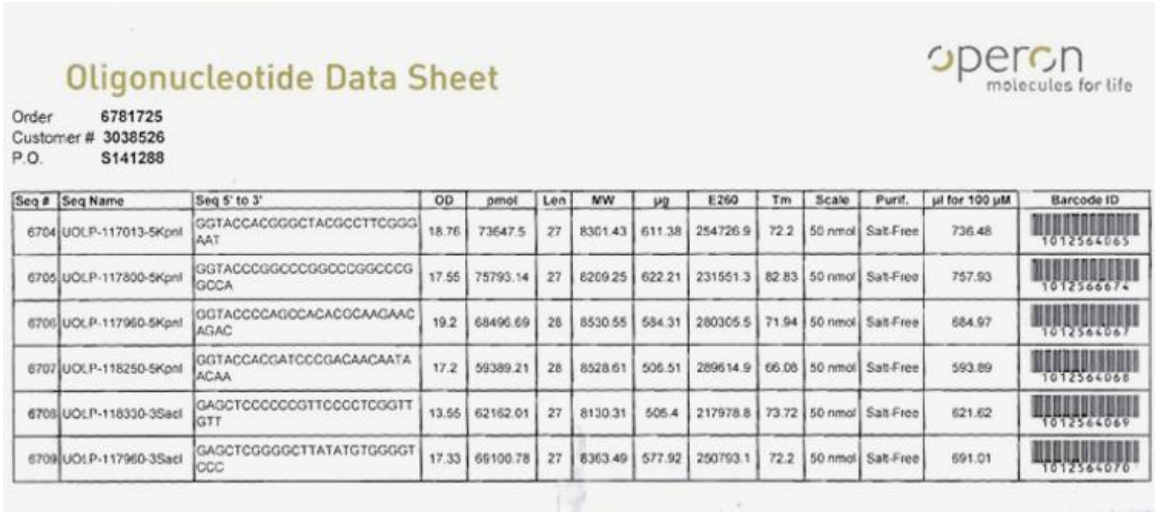


Figure 4: Target Promoter Fragments
Note that only three of these fragments (A, B, and C) were successfully cloned into the pGL4 Basic vector and subsequently tested for Luciferase activity

Figure 5 below shows the order information of the various primers used in PCR amplification and isolation of the promoter inserts.



Oligonucleotide Data Sheet

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Order 6781725
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P.O. S141288

Seq #	Seq Name	Seq 5' to 3'	OD	pmol	Len	MW	µg	E260	Tm	Scale	Purif.	µl for 100 µM	Barcode ID
6704	UOLP-117013-5KpnI	GGTACCACGGGCTACGCCTTCGGGAAT	18.76	73647.5	27	8301.43	611.38	254726.9	72.2	50 nmol	Salt-Free	736.48	1012564065
6705	UOLP-117800-5KpnI	GGTACCCGGCCCGGCCCGGCCCGGCCA	17.55	75793.14	27	8209.25	622.21	231551.3	82.83	50 nmol	Salt-Free	757.93	1012566674
6706	UOLP-117960-5KpnI	GGTACCCCAGCCACACGCAAGAACAGAC	19.2	68496.69	28	8530.55	584.31	280305.5	71.94	50 nmol	Salt-Free	684.97	1012564067
6707	UOLP-118250-5KpnI	GGTACCACGATCCCGACAACAATAACAA	17.2	59389.21	28	8528.61	506.51	289614.9	66.08	50 nmol	Salt-Free	593.89	1012564068
6708	UOLP-118330-3SacI	GAGCTCCCCCCGTTCCCCTCGGTTGTT	13.55	62162.01	27	8130.31	505.4	217978.8	73.72	50 nmol	Salt-Free	621.62	1012564069
6709	UOLP-117960-3SacI	GAGCTCGGGGCTTATATGTGGGGTCCC	17.33	69100.78	27	8363.49	577.92	250793.1	72.2	50 nmol	Salt-Free	691.01	1012564070

UOLP-117800-5KpnI atGGTACCCGGCCCGGCCCGGCCCGGCCA
UOLP-117960-5KpnI atGGTACCCCAGCCACACGCAAGAACAGAC
UOLP-118250-5KpnI atGGTACCACGATCCCGACAACAATAACAA
UOLP-117960-3SacI atGAGCTCGGGGCTTATATGTGGGGTCCC
UOLP-118330-3SacI atGAGCTCCCCCCGTTCCCCTCGGTTGTT

Subsequent primers to further help isolate the promoter fragments of interest:

UOLP-118351 – 3 SacI atGAGCTCCTGAGCTCTTCCACTTCCCGTCCTTCCAT
UOLP-118267 – 3 SacI atGAGCTCATGAGCTCATTGTTGTCGGGATGTGCGG
UOLP-117967 – 5KpnI atGGTACCATGGTACCCACGCAAGAACAGACACGCAG

Figure 5: Information on specific primers ordered for PCR amplification of promoter inserts
Note that KpnI and SacI restriction sites are used at the 5' and 3' end of all inserts, respectively

The primers used were chosen to be 21 bp long and cross-checked for accuracy by assessing their GC content and performing a BLAST search to find similar regions in the genome that could be targeted other than the insert's target sequence. Then, PCR amplification of the promoter mutants was conducted with 28 cycles, an annealing temperature of 58 ˚C, and an extension period of 1 minute and 30 seconds, and using a PCR mixture of 5 µL 10x PCR buffer, 1 µL dNTP, 1.5

µL 50mM $MgCl_2$, 1 µL each of forward and reverse primers and 1X template, 0.2 µL of Platinum Taq DNA polymerase, and 39.3 µL of dH_20 yielded results for the "3" and "C" mutants (see figure 9). However, different methods were required to extract the "2" mutant. The PCR amplification to isolate the "2" mutant was conducted with 32 cycles, an annealing temperature of 55 ˚C, and an extension period of 2 minutes, and involved 10 µL of 1X template and consequently 29.3 µL of dH_20. The "A" mutant was isolated using the standard Invitrogen PCR master mix, with 35 cycles, an annealing temperature of 52 ˚C, an extension period of 1 minute and 30 seconds, and using 0.5 µL each of 100 pM forward and reverse primers, 3 µL of raw 1X DNA template, 1.25 µL of DMSO, and 2.5 µL of glycerol to reach a final volume of 25 µL. The "B" mutant was similarly isolated, using the same PCR mixture, but different conditions. The B mutant needed the lower annealing temperature of 48˚C. As seen firsthand through numerous PCR reactions, certain fragments required lower annealing temperatures for less-specific binding, to be isolated. Also, the addition of glycerol at 10% of the total volume and DMSO at 5% of the total volume showed significant improvements in PCR amplification of the high GC regions.

Figure 6 below shows the agarose gel results of PCR amplification that resulted in the successful isolation of the "2" mutant.

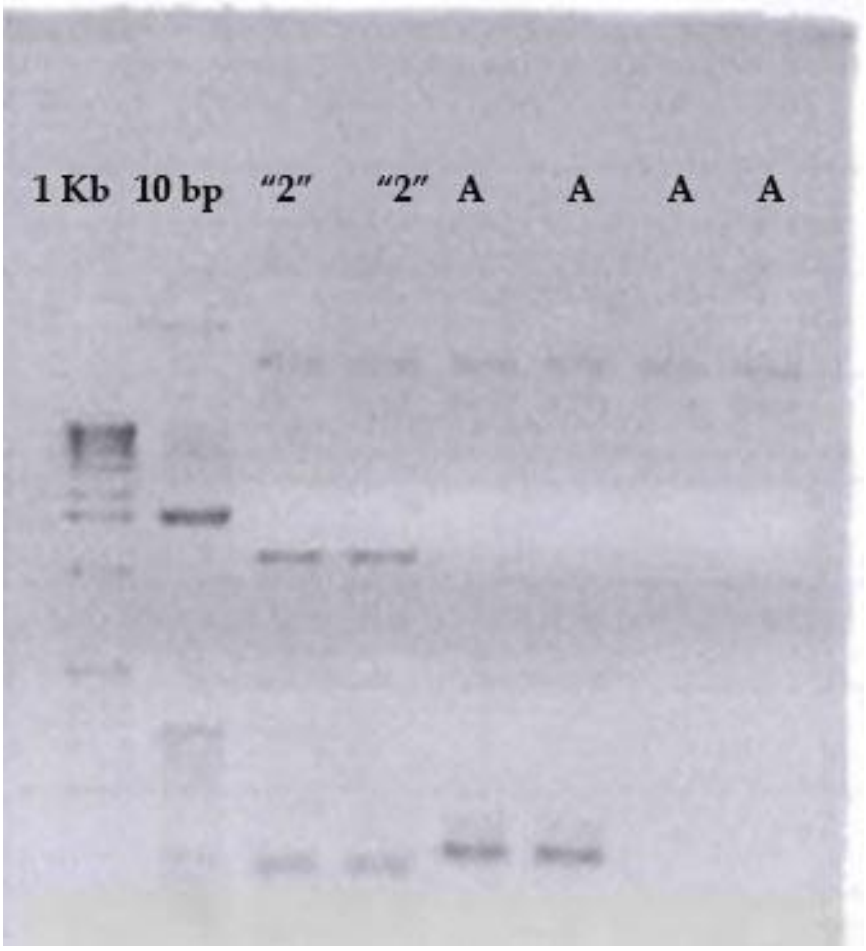


Figure 6: Gel picture of "2" and A mutants after PCR
Only the "2" mutant was successfully isolated. 1 Kb and 10 bp markers are shown on the left.

Figures 7, 8, and 9 below show the agarose gel results of PCR amplification that resulted in the successful isolation of the "A," "B," "C," and "3" mutants.

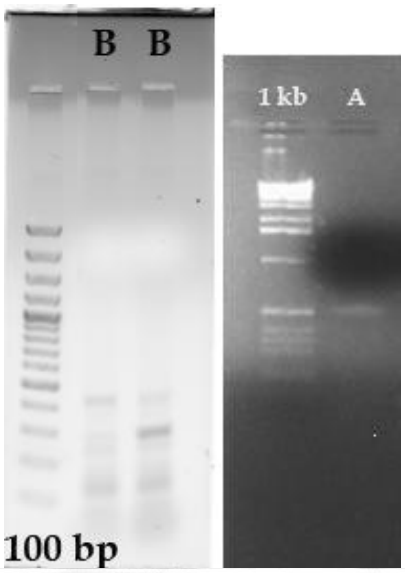


Figure 7& 8: Gel picture of the "B" and "A" mutants after PCR
100 bp and 1 Kb marker is shown on the left.

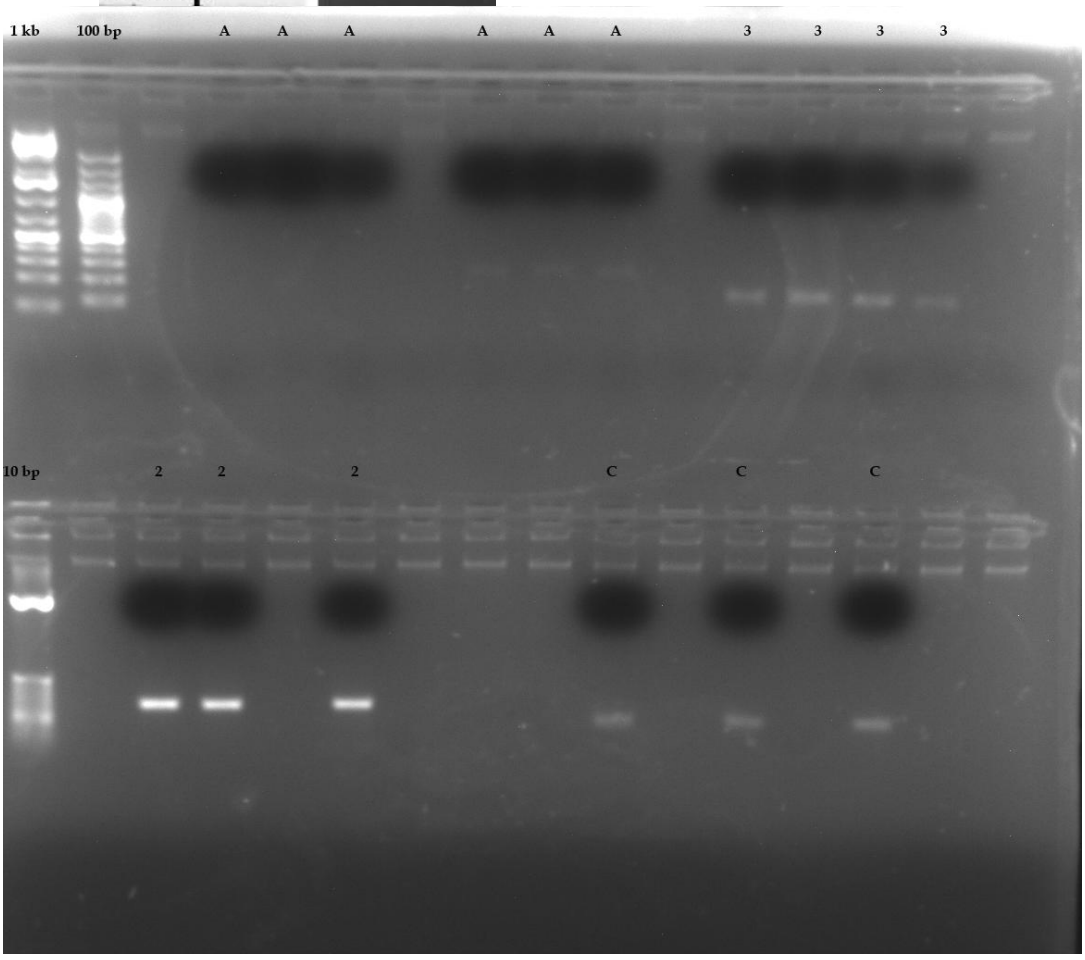


Figure 9: Gel picture of A, "3", "2," and C mutants after PCR
Only the "3," 2," and C mutants were successfully isolated. 1 Kb, 100 bp, and 10 bp markers are shown on the left. Note that "2" mutant is run with same conditions as earlier but with addition of glycerol and DMSO

Ligation and Verification via Restriction Enzyme Digestion

Although all of the above promoter fragments were successfully isolated (and many others not listed here weren't), not all of them were successfully cloned into the pGL4 reporter vector. After isolating the mutants, three of them (A, B, and C) were successfully ligated into the pGL4 vector. Both the mutant and vector DNA was cut using *KpnI* and *SacI* restriction enzymes in a mixture also containing 10x restriction enzyme buffer (Promega Buffer "J") and dH_20. Figure 10 shows the successful double cut of the restriction enzymes on the vector, before preparation of the insert DNA and ligation of the two.

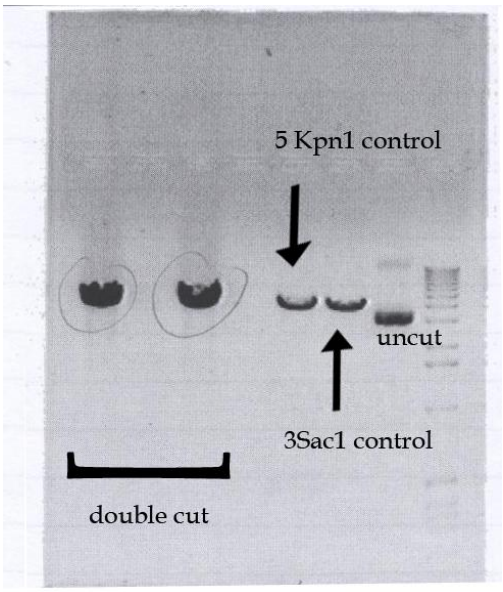


Figure 10: Gel picture of pGL4 vector with restriction enzyme cuts
Double cuts of vector were successful, now ready for insert ligation

After ligation, each plasmid was verified by restriction enzyme digestion. The results are below.

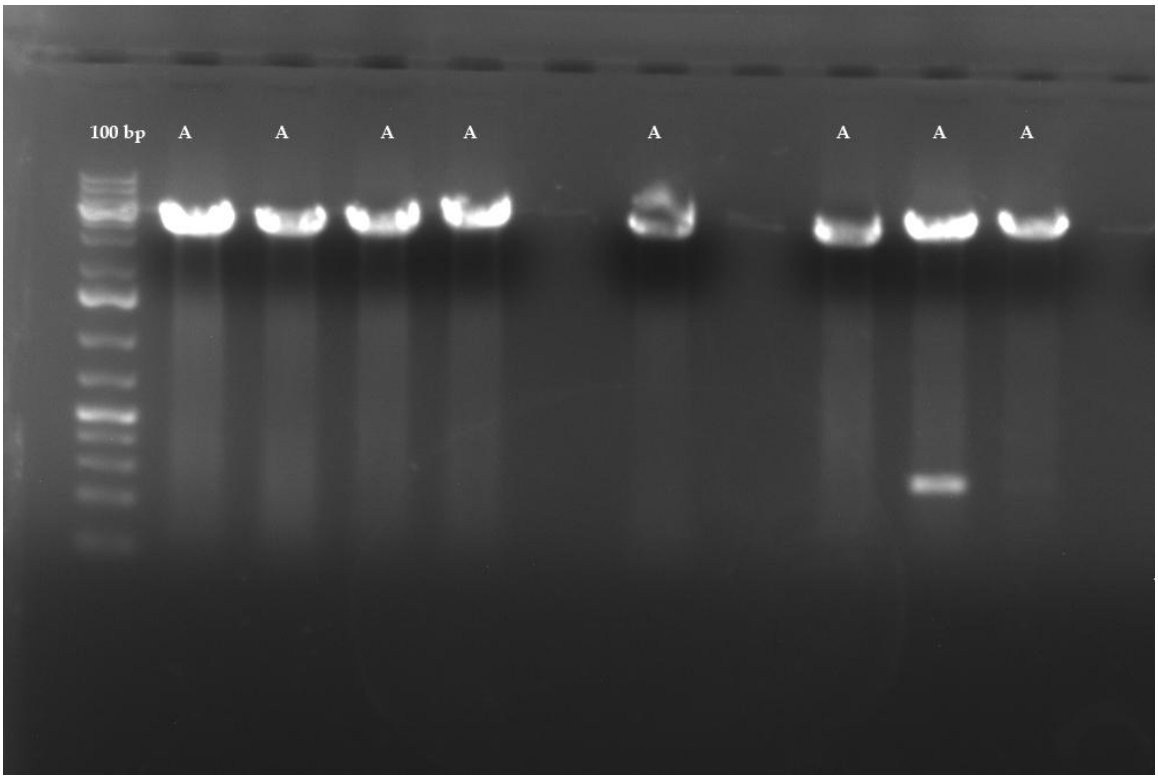


Figure 11: RE verification of ligation; plasmid w/ insert A
Band second from right shows the correct insert A, all other lanes just show plasmid without insert. Test was done after plasmid transformation into DH5a cells

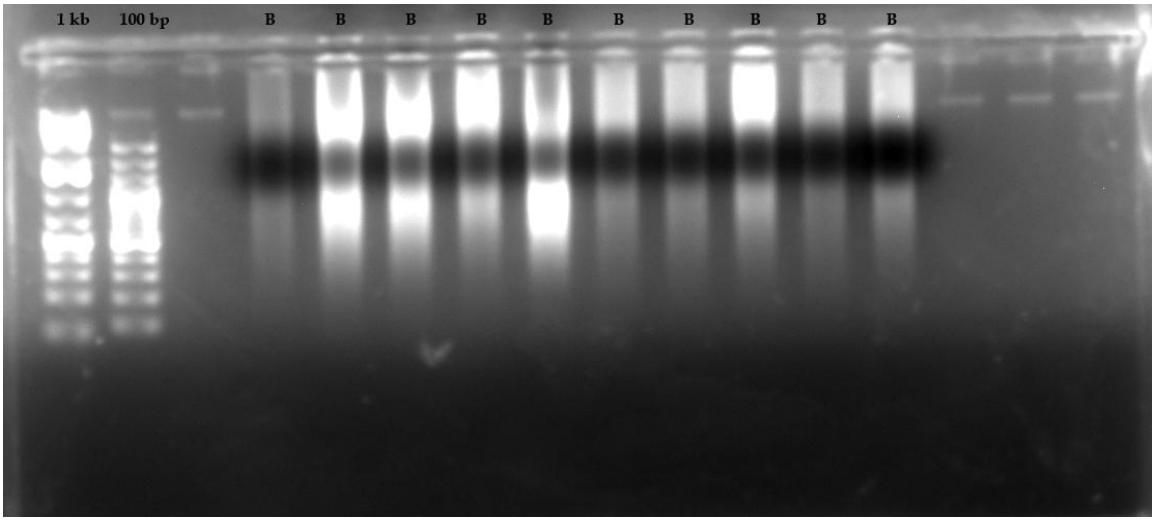


Figure 12: RE verification of ligation; plasmid w/ insert B
Lanes two, three and four were subsequently re-confirmed via PCR for the insert of plasmid B. All other lanes just show plasmid without insert. Test was done after plasmid transformation into RR-1 cells.

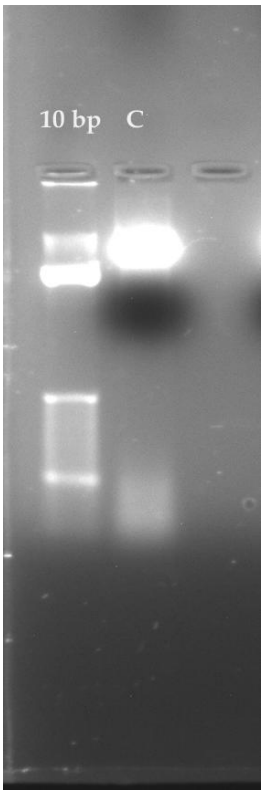


Figure 13: RE verification of ligation; plasmid w/ insert C
Lane one shows the correct insert C. Test was done after plasmid transformation into DH5a cells

As noted in the figures above, the promoter fragment B was transformed into RR-1 cells instead of DH5α cells. This insight that transformation efficiency is higher for inserting DNA with high GC content in RR-1 cells was provided by Dr. Perng. This could be potentially due to the large plasmid copy number in DH5α cells, and the resultant possibility of chromosomal homologous recombination between plasmids in these cells. In contrast, RR-1 cells have a low copy number of plasmids and this problem is avoided.

Luciferase Assays

Luciferase assays are reporter assays widely used to study gene expression and cellular physiology. The term "dual reporter assays" refers to the simultaneous expression and measurement of two individual reporter enzymes

(in the case of this experiment – firefly and *renilla* luciferases). *Renilla* luciferase is derived from the sea pansy (*Renilla reniformis)* and as its name implies, firefly luciferase is derived from the firefly (*Photinus pyralis)*. In this experiment, firefly luciferase activity is related to the pGL4 vector as shown in Figure 3. *Renilla* luciferase activity, co-transfected with a known vector (pRLSV40) then provides an internal control that served as a baseline activity level with which to normalize the firefly luciferase results. Mentioned earlier, the Stop & Glo reagent serves to quench the firefly luciferase reaction and produce stable signal from the *renilla* luciferase. Thus, the results below are plotted as the ratio between pGL4 and pRLSV40 activity.

The plasmids verified by restriction enzyme digestion were co-transfected with pRLSV40 into RS1 or Neuro 2A cells. The figures below indicate the transfection efficiency by use of a vector with a green fluorescent protein (GFP). Each top image represents the cells in a portion of one well, the second image uses a fluorescent filter to show the location and amount of GFP in this same region, and the third image superposes the first two onto each other. The transfection efficiency was between 40-50%.

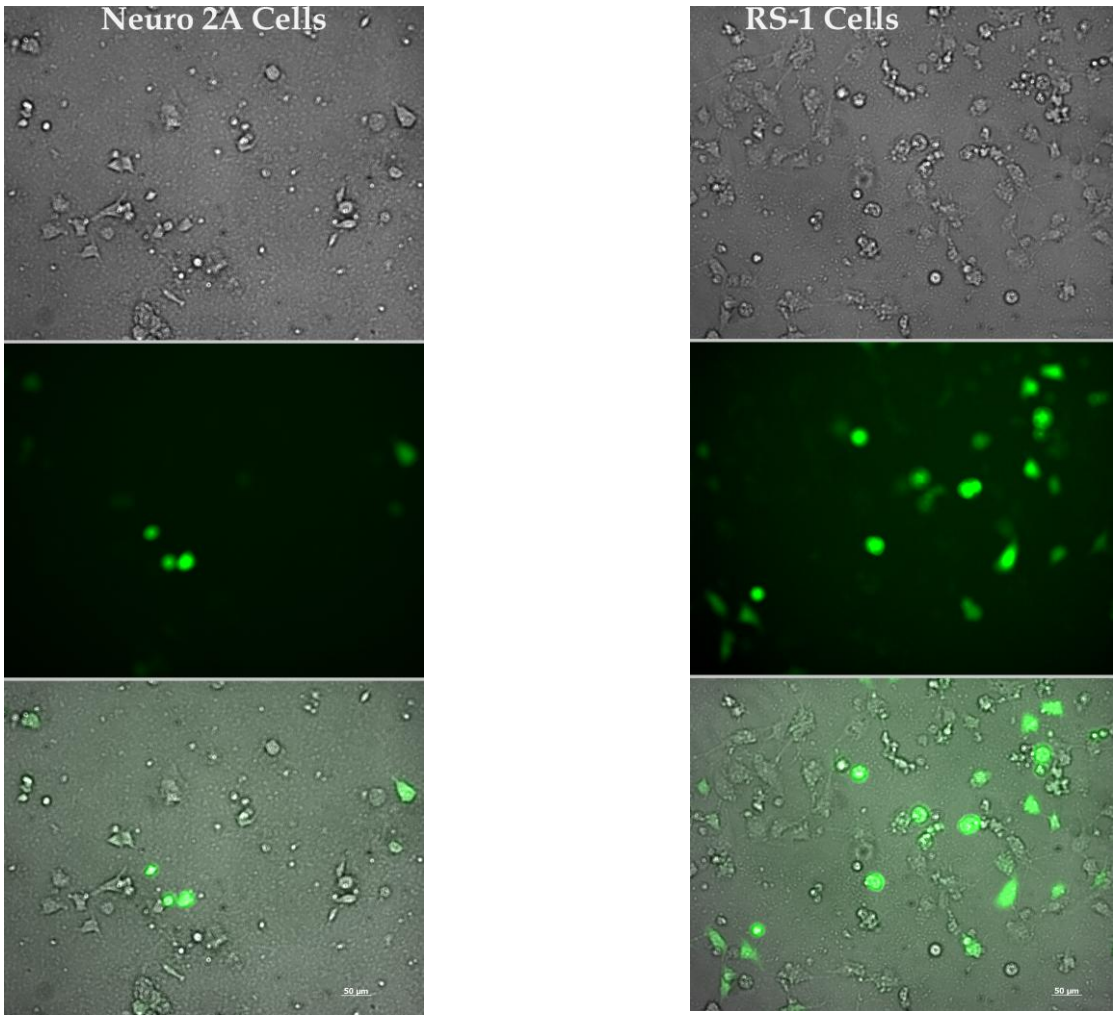
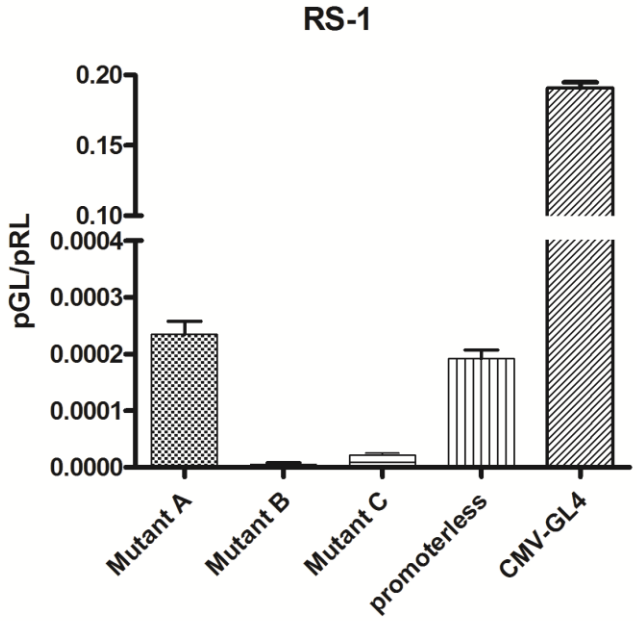


Figure 15: Transfection Efficiency into RS-1 and Neuro 2A cell lines
GFP used to transfect cells and determine the transfection efficiency. Note the lower than normal (50-70%) cell confluency in both images.

As seen in Figure 3, the luciferase gene is downstream of the cloning region containing *KpnI* and *SacI* restriction sites in the pGL4 Basic Vector. Without a proper promoter embedded in the PCR insert, no luciferase expression would be detected once the vector was transfected into RS1 or Neuro2A cells. This negative control is seen by the level of activity associated with the promoterless vector in Figure 15. Consequently, a high ratio of firefly luciferase

to *renilla* luciferase activity and a high fold of induction relative to the promoterless vector are indicators of the presence and proper orientation of a functional promoter. The results of the Luciferase assays are below.



Fold of induction compared to promoterless
Mutant A: 1.229 fold
Mutant B: 0.026 fold
Mutant C: 0.111 fold
pGL/CMV: 999.9 fold

Figure 15: Luciferase Activity of Promoter Segments in Rabbit Skin Cells
Controls used are a promoterless vector and a known strong promoter (CMV-GL4).

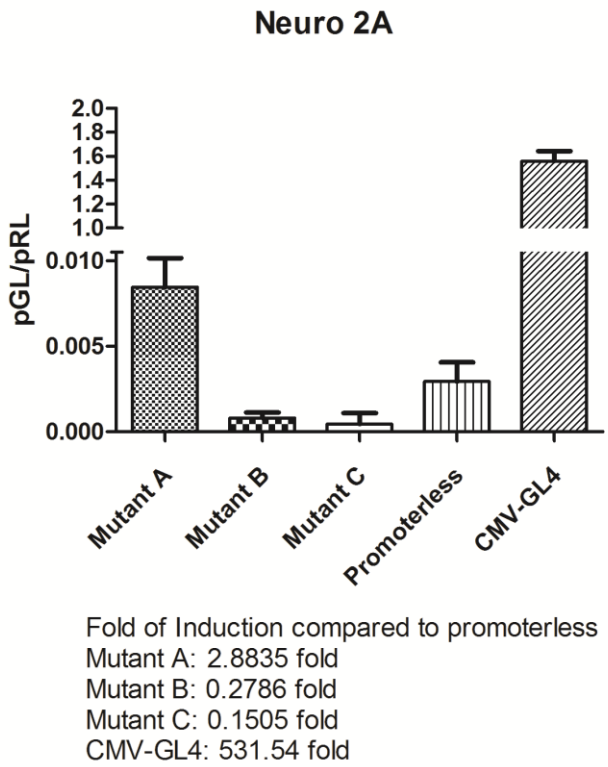


Figure 16: Luciferase Activity of Promoter Segments in Neuro 2A cells
Controls used are a promoterless vector and a known strong promoter (CMV-GL4). Notice the difference in folds of induction for A and B between neuronal cells and rabbit skin cells

The luciferase activity driven by the A region is the highest in both cell lines, and relatively higher in the Neuro 2A cell line. Because Neuro 2A is a neuronal cell line and HSV-1 infects neuronal cells, these results are indicative of cell line specificity in the UOL promoter. Similarly, mutant B's activity is also indicative of cell line specificity. The cell line specificity and differential promoter activity will be the subject of future investigation in the lab.

Much to our surprise, mutant C and mutant B both show a decrease in activity relative to the promoterless vector, which indicates repressive qualities. Mutant C also shows relatively greater repressive qualities than mutant B in the neuronal cell line. Interestingly, mutant C contains a potential GCF transcription factor binding site at nt 118285. GCF is a known repressor of transcription that

targets GC rich areas, however further tests are necessary to confirm its presence in regulating the UOL promoter (Kageyama, 1989).

From the results in Figures 15 and 16 we can also conclude that the region contained with Mutant A upstream of the Mutant B start site, from nt 117800 to nt 117960 (using the 17syn+ HSV-1 genomic sequence), contains positive promoter activity. Although this activity could be due to specific enhancer elements or transcription factor binding sites, it may also be due to the possible TATA box located at nt 117950. The proximity of this TATA box to UOL's transcription start site (nt 118,266) suggests that it could be the TATA box for the UOL gene.

This same segment within Mutant A also contains potential SP-1 transcription factor binding sites at nt 117824, nt 117907, and nt 117933. SP-1 is a mammalian gene-specific transcription factor that has been shown to bind and help activate several genes during the HSV-1 lytic cycle (Jones, 1985). Interestingly, SP-1 phosphorylation has also been linked to the decrease of "intermediate early" and "early" gene expression, late in the HSV-1 lytic cycle (Kim, 2002). This is particularly relevant to the UOL promoter as the UOL gene, besides expression during acute infection, is only expressed as a late gene during viral reactivation. Although the implications of an SP-1 binding site in the UOL promoter are far-reaching, further studies are required for its presence to be proven.

To confirm the presence of a repressive binding site in mutant C or the presence of an SP-1 binding site in mutant A, it would be necessary to do several follow up experiments. Follow-up projects to test for transcription factor presence and binding sites would include electrophoresis shift assays and DNase footprinting assays.

Unfortunately, many unforeseen problems arose during this experiment. Some of the unexpected difficulties in determining the level of gene expression in the transfected cells were low transfection efficiency and susceptibility of cell lines. Thus, numerous repeated experiments were required. At times, the transfection efficiency was no more than 30-40%, about half of the expected efficiency of 70-80% mentioned above. Also, due to the high GC content of the HSV-1 genome, PCR amplification proved troublesome. Nevertheless, the demonstration of promoter activity in Mutant A and a lack of activity in mutants B and C has further fine-tuned our map of the UOL promoter and helped provide a solid foundation upon which future tests should be conducted.

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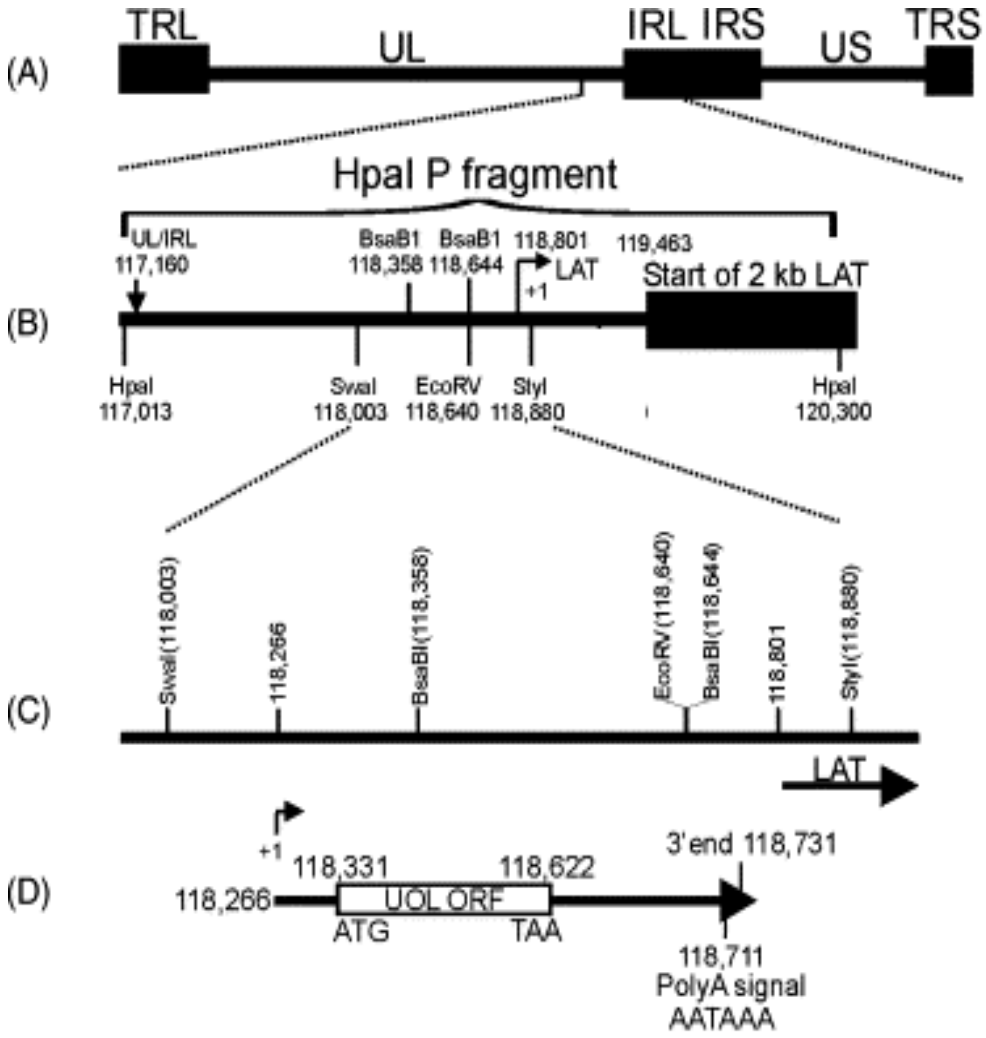


Figure 1: Relative Location of UOL to the HSV-1 Viral Genome
(Naito et al., 2005)

(E) Genomic Structure of wildtype HSV-1, showing terminal and internal long repeats and short repeats (TRL and IRL, and TRS and IRS, respectively); and long and short unique regions (UL and US, respectively)

(F) Expanded HpaI P fragment, containing junction of UL and IRL. Numbers indicate genomic nucleotide positions for 17syn+ HSV-1 strain

(G) Further expansion of region upstream of LAT transcript

(H) UOL mRNA; UOL transcript begins at nt 118,266 and UOL open reading frame starts at nt 118,731

Mutant:

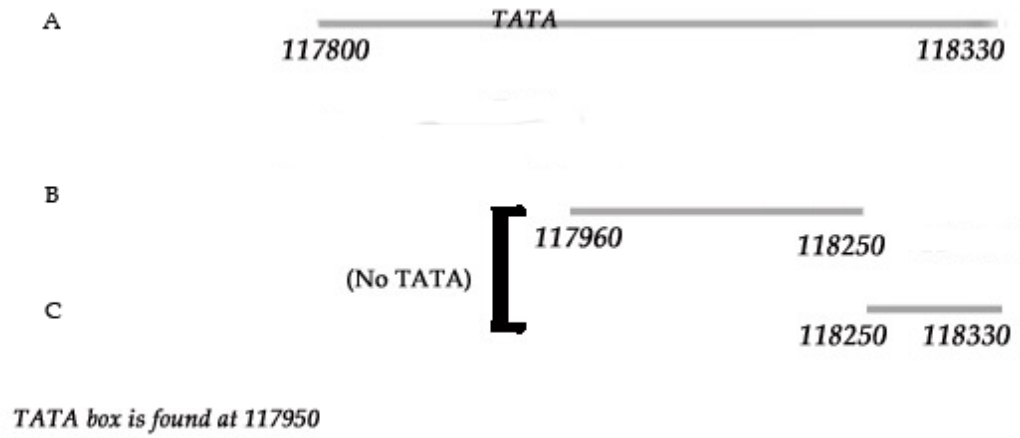


Figure 2: Segments of Template to be Amplified via PCR
Segment A contain the known RNA polymerase binding site called the TATA box, the other two segments do not

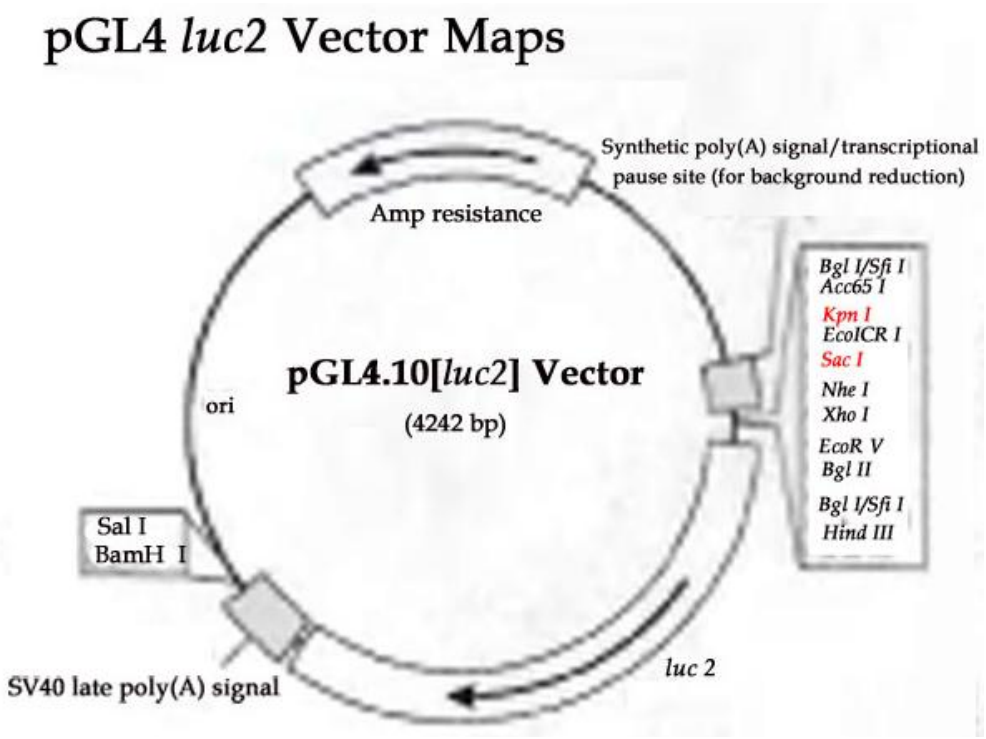


Figure 3: pGL4 *luc*2 Vector
Luciferase gene downstream from multiple cloning site, KpnI and SacI restriction sites in red

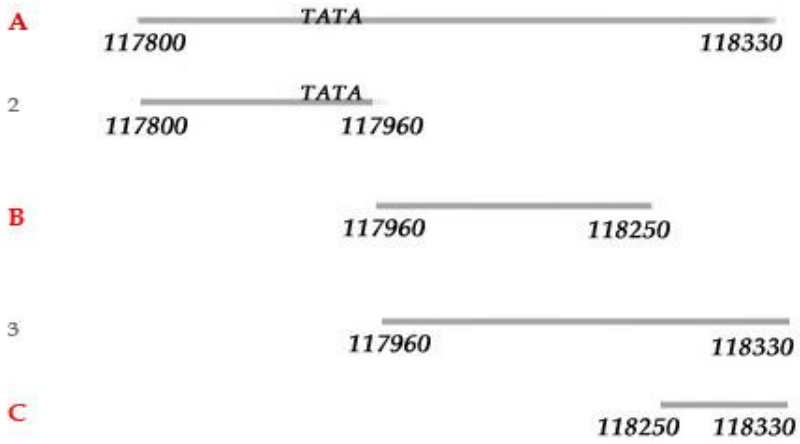
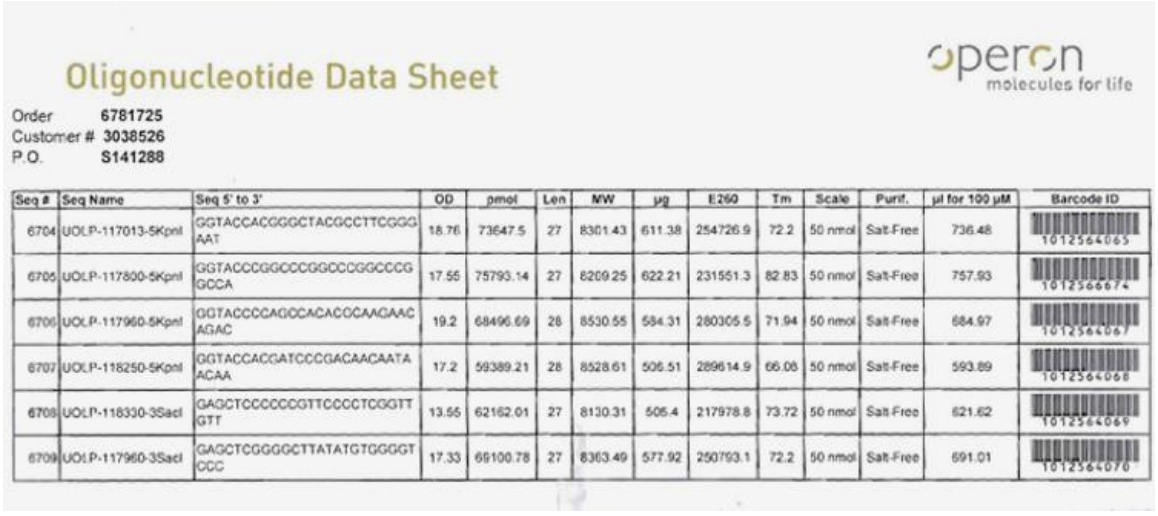


Figure 4: Target Promoter Fragments
Note that only three of these fragments (A, B, and C) were successfully cloned into the pGL4 Basic vector and subsequently tested for Luciferase activity



Oligonucleotide Data Sheet

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Customer # 3038526
P.O. S141288

Seq #	Seq Name	Seq 5' to 3'	OD	pmol	Len	MW	µg	E260	Tm	Scale	Purif.	µl for 100 µM	Barcode ID
6704	UOLP-117013-5KpnI	GGTACCACGGGCTACGCCTTCGGGAAT	18.76	73647.5	27	8301.43	611.38	254726.9	72.2	50 nmol	Salt-Free	736.48	1012564065
6705	UOLP-117800-5KpnI	GGTACCCGGCCCGGCCCGGCCCGGCCA	17.55	75793.14	27	8209.25	622.21	231551.3	82.83	50 nmol	Salt-Free	757.93	1012566674
6706	UOLP-117960-5KpnI	GGTACCCCAGCCACACGCAAGAACAGAC	19.2	68496.69	28	8530.55	584.31	280305.5	71.94	50 nmol	Salt-Free	684.97	1012564067
6707	UOLP-118250-5KpnI	GGTACCACGATCCCGACAACAATAACAA	17.2	59389.21	28	8528.61	506.51	289614.9	66.08	50 nmol	Salt-Free	593.89	1012564068
6708	UOLP-118330-3SacI	GAGCTCCCCCCGTTCCCCTCGGTTGTT	13.55	62162.01	27	8130.31	505.4	217978.8	73.72	50 nmol	Salt-Free	621.62	1012564069
6709	UOLP-117960-3SacI	GAGCTCGGGGCTTATATGTGGGGTCCC	17.33	69100.78	27	8363.49	577.92	250793.1	72.2	50 nmol	Salt-Free	691.01	1012564070

UOLP-117800-5KpnI atGGTACCCGGCCCGGCCCGGCCCGGCCA
UOLP-117960-5KpnI atGGTACCCCAGCCACACGCAAGAACAGAC
UOLP-118250-5KpnI atGGTACCACGATCCCGACAACAATAACAA
UOLP-117960-3SacI atGAGCTCGGGGCTTATATGTGGGGTCCC
UOLP-118330-3SacI atGAGCTCCCCCCGTTCCCCTCGGTTGTT

Subsequent primers to further help isolate the promoter fragments of interest:

UOLP-118351 – 3 SacI atGAGCTCCTGAGCTCTTCCACTTCCCGTCCTTCCAT
UOLP-118267 – 3 SacI atGAGCTCATGAGCTCATTGTTGTCGGGATGTGCGG
UOLP-117967 – 5KpnI
atGGTACCATGGTACCCACG
CAAGAACAG

Figure 5: Information on specific primers ordered for PCR amplification of promoter inserts

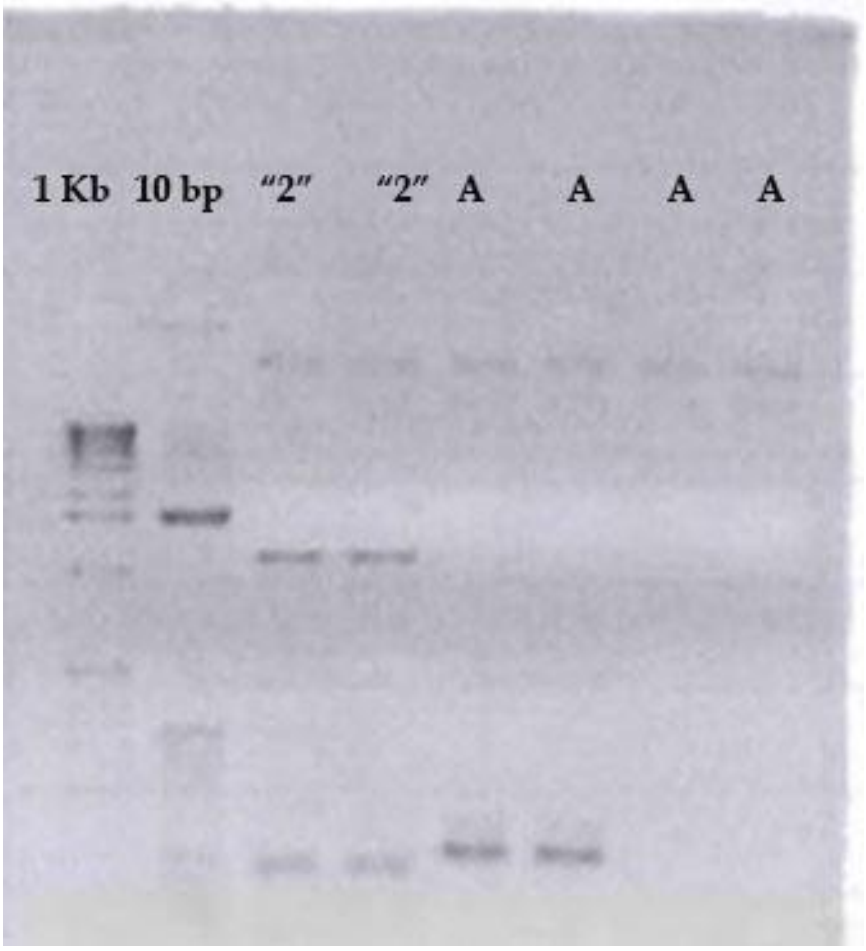


Figure 6: Gel picture of "2" and A mutants after PCR
Only the "2" mutant was successfully isolated. 1 Kb and 10 bp markers are shown on the left.

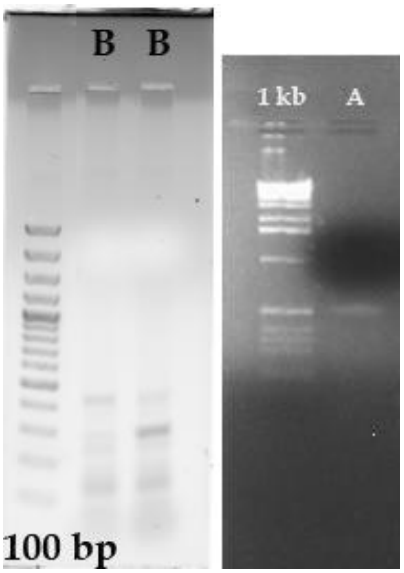


Figure 7& 8: Gel picture of the "B" and "A" mutants after PCR
100 bp and 1 Kb marker is shown on the left.

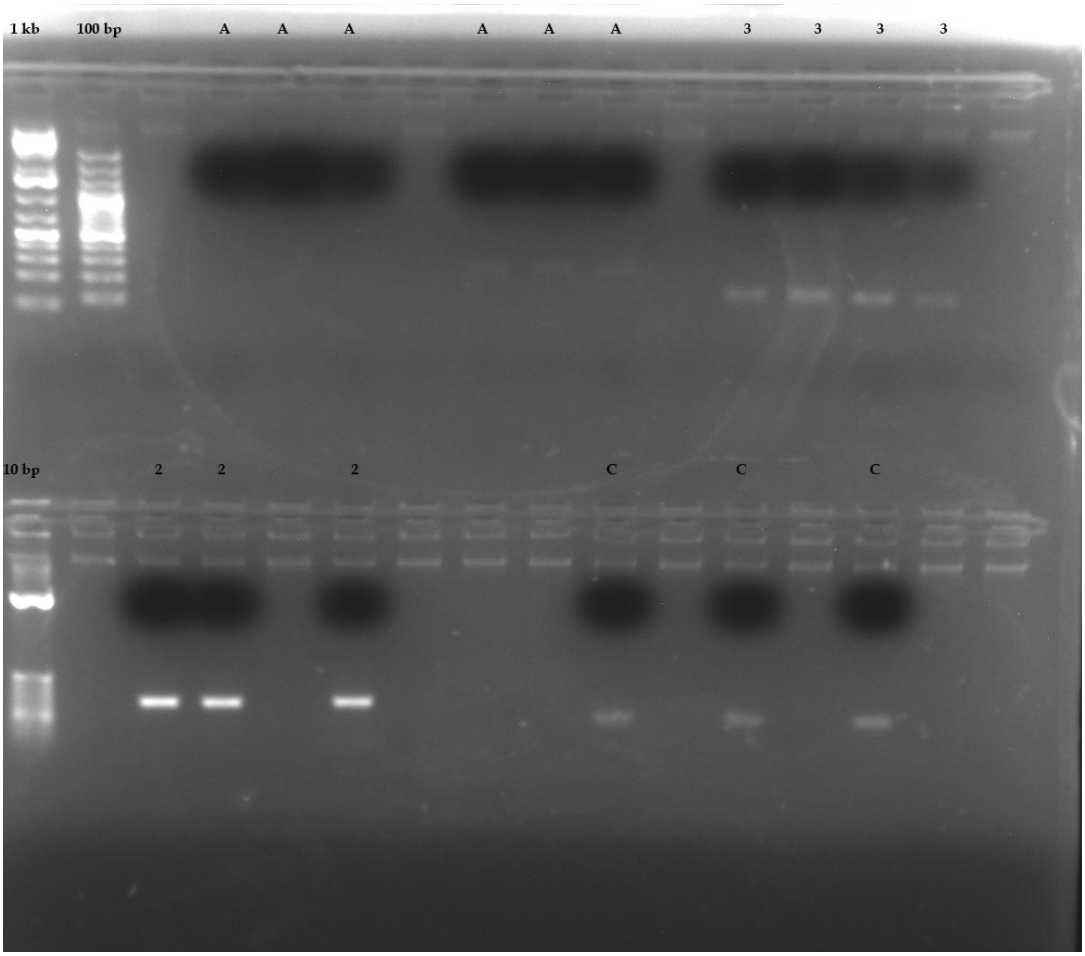


Figure 9: Gel picture of A, "3", "2," and C mutants after PCR
Only the "3," 2," and C mutants were successfully isolated. 1 Kb, 100 bp, and 10 bp markers are shown on the left. Note that "2" mutant is run with same conditions as earlier but with addition of glycerol and DMSO

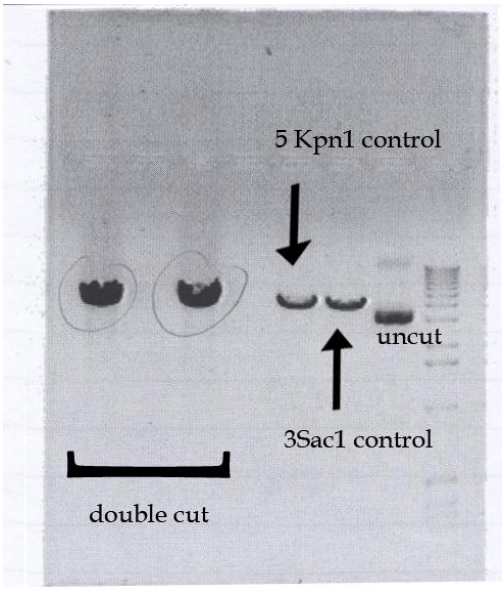


Figure 10: Gel picture of pGL4 vector with restriction enzyme cuts
Double cuts of vector were successful, now ready for insert ligation

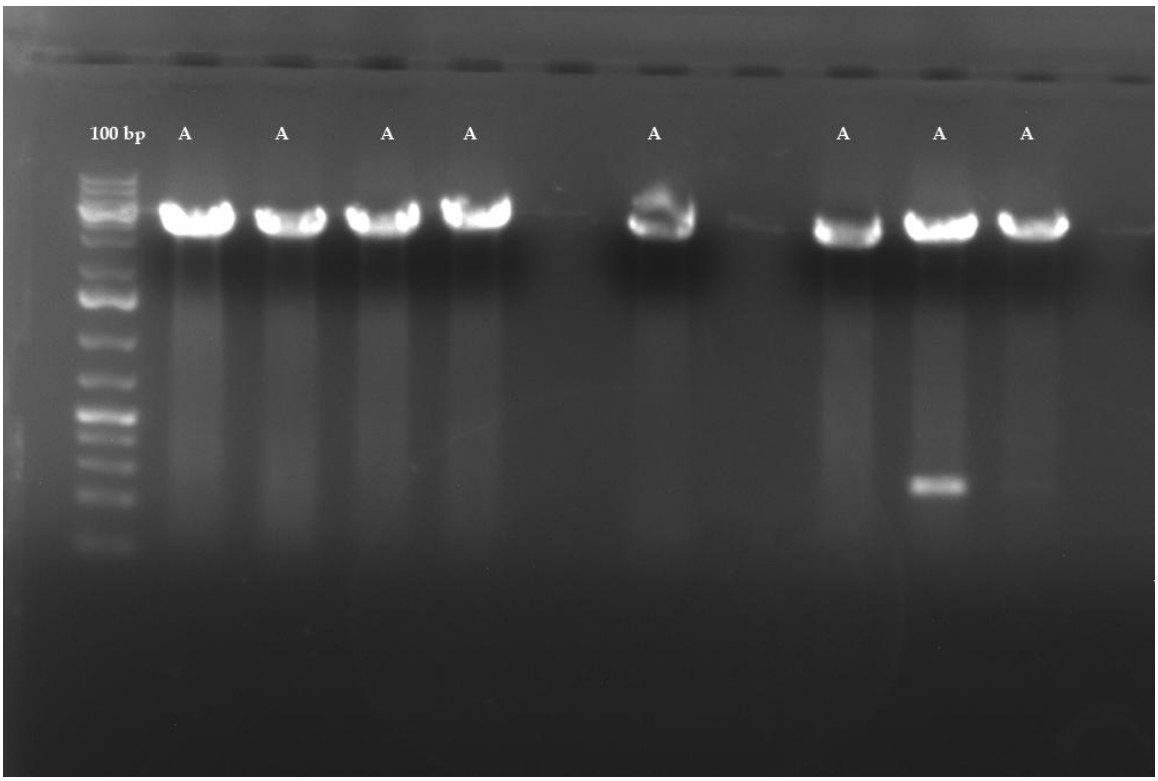


Figure 11: RE verification of ligation; plasmid w/ insert A
Band second from right shows the correct insert A, all other lanes just show plasmid without insert. Test was done after plasmid transformation into DH5a cells

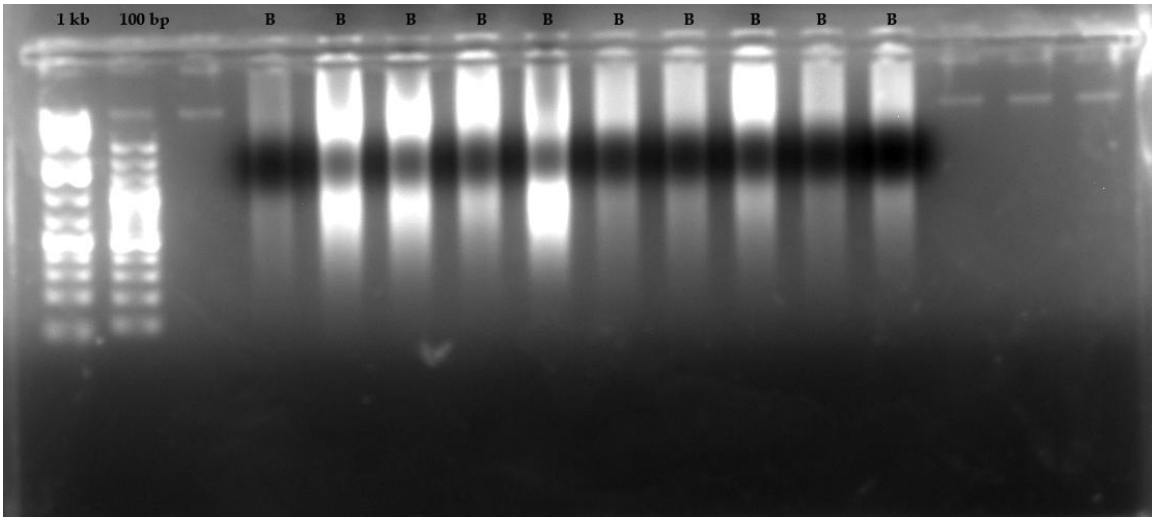


Figure 12: RE verification of ligation; plasmid w/ insert B
Lanes two, three and four were subsequently re-confirmed via PCR for the insert of plasmid B. All other lanes just show plasmid without insert. Test was done after plasmid transformation into RR-1 cells.

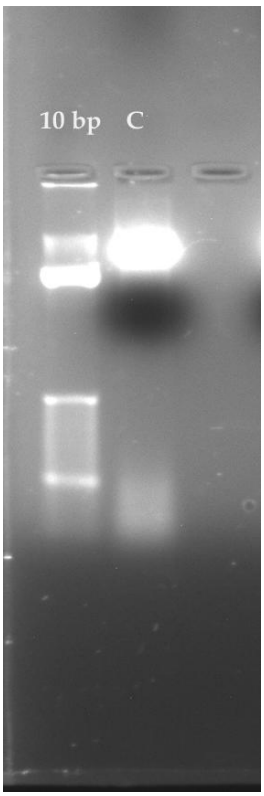
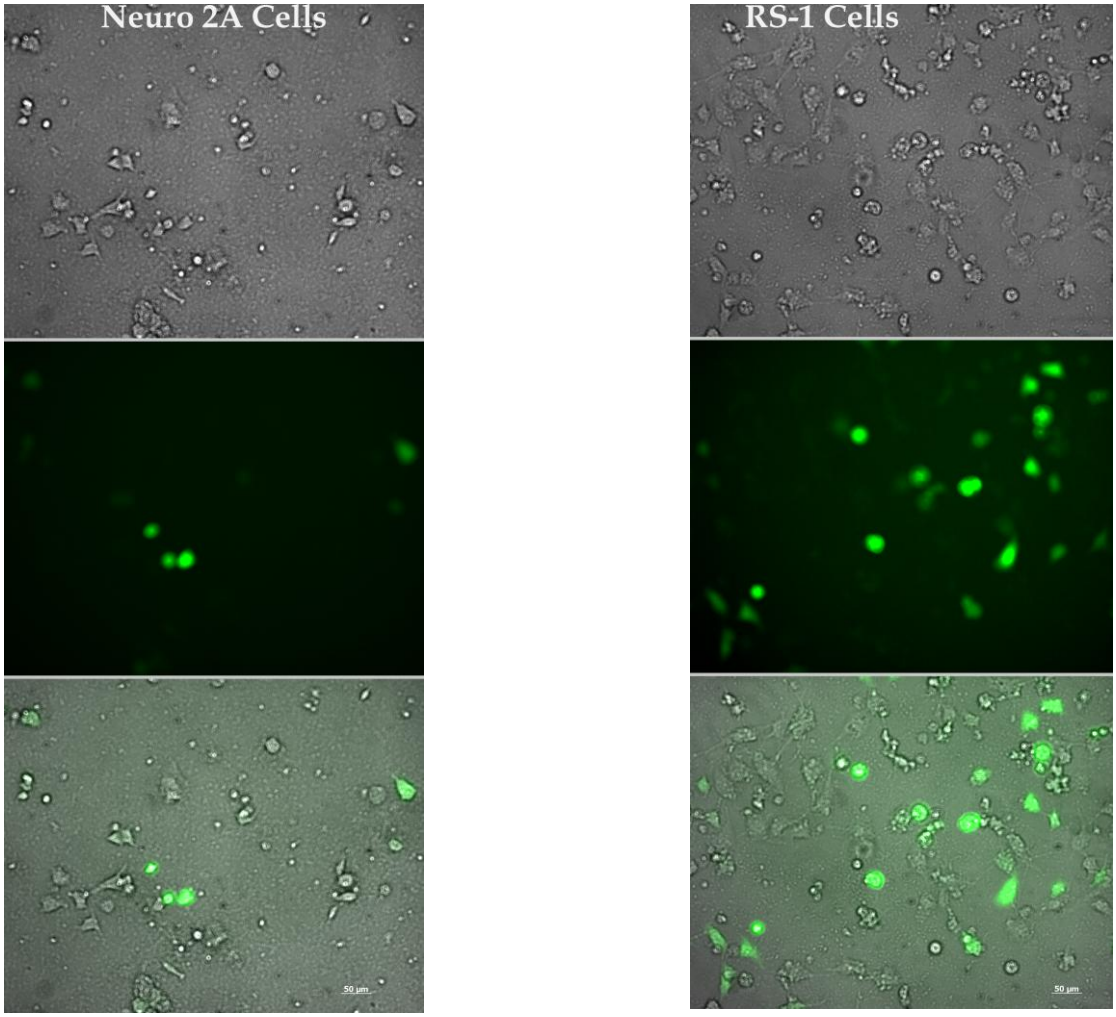


Figure 13: RE verification of ligation; plasmid w/ insert C
Lane one shows the correct insert C. Test was done after plasmid transformation into DH5a cells



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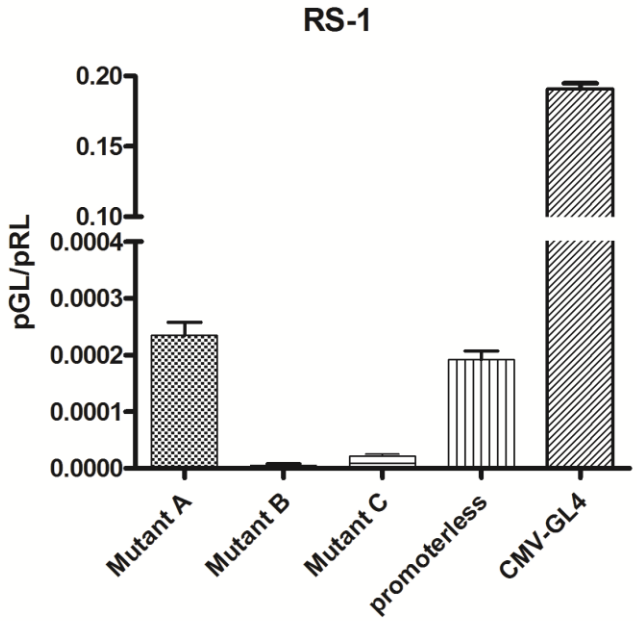

Fold of induction compared to promoterless
Mutant A: 1.229 fold
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pGL/CMV: 999.9 fold

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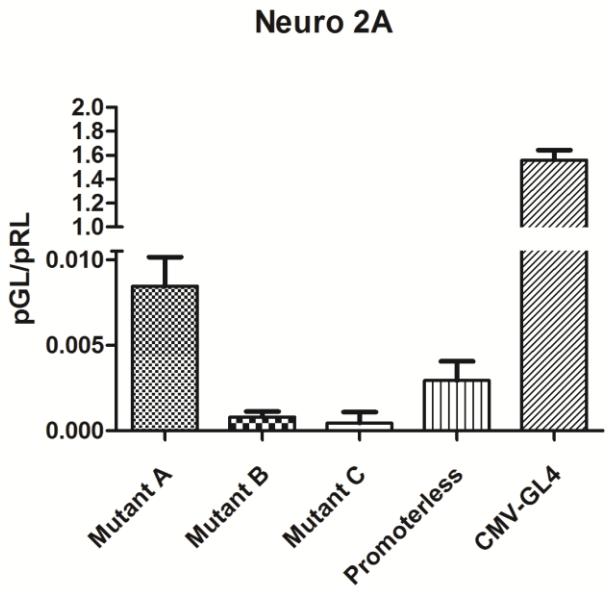
Controls used are a promoterless vector and a known strong promoter (CMV-GL4).



Fold of Induction compared to promoterless
Mutant A: 2.8835 fold
Mutant B: 0.2786 fold
Mutant C: 0.1505 fold
CMV-GL4: 531.54 fold

Figure 16: Luciferase Activity of Promoter Segments in Neuro 2A cells
Controls used are a promoterless vector and a known strong promoter (CMV-GL4). Notice the difference in folds of induction for A and B between neuronal cells and rabbit skin cells