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Molecular Characterization of the Human *RAII* Promoter

By

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Thesis

Submitted in partial fulfillment of the requirements for Honors in Biology at the
University of Mary Washington.

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This Thesis by Rose Irene Salzberg is accepted in its present form as satisfying the thesis requirement for Honors in Biology.

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ABSTRACT

Smith-Magenis syndrome (SMS) is a type of mental retardation disorder associated with a deletion of DNA from chromosome 17p11.2. It affects approximately 18,000 individuals in the US alone. Major phenotypes are associated with a mutation in the Retinoic Acid-Inducible gene-1 (*RAII*) and include: developmental delay, repetitive behaviors, short stature, and hoarse voice. Studying the molecular mechanisms that lead to SMS will help promote earlier detection of the disorder and will help develop a better treatment plan for patients. The purpose of this project was to characterize the promoter of *RAII*. Studying the promoter of *RAII* will help determine the normal expression of the gene providing clues to its normal function and providing insight into why the lack of *RAII* leads to SMS.

The method used for characterizing promoter activity was the reporter gene assay. The goal of this assay was to determine if previously cloned putative promoter fragments contained any promoter activity. The putative *RAII* promoter was cloned in front of the firefly luciferase reporter gene in the pGL3 plasmid and transiently transfected into the mammalian cell line HEK293. The results we obtained suggest that the putative promoter fragments did not contain any promoter activity.

In order to identify new putative promoter fragments, 5'RLM-RACE was used on RNA isolated from HEK293 cells. The purpose of the procedure was to identify the 5' end of the *RAII* transcript. If successfully cloned, the sequence of this fragment could be used to align with the human genome sequence and locate potential promoter elements. These studies are currently underway.

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INTRODUCTION

Smith-Magenis Syndrome. Smith-Magenis syndrome (SMS) is a type of mental retardation disorder associated with a deletion of DNA from chromosome 17 or a mutation of Retinoic Acid Inducible gene 1 (*RAI1*). The birth incidence rate is approximately 1/25,000 births. However, a higher incidence rate is suspected due to both under-diagnosis and misdiagnosis of SMS [1, 2]. Smith-Magenis syndrome was first discovered in 1986 by a group of researchers in Denver, Colorado [3]. Among those researchers was Dr. Elsea who has since then continued her work with SMS at the Medical College of Virginia in Richmond, VA. I did this project in collaboration with Dr. Elsea and the members of her lab.

Physiological Significance. There are several phenotypes associated with SMS, some more common than others. The most common phenotypes can be divided into two groups: major developmental/behavioral phenotypes and physical phenotypes. The most common developmental/behavioral phenotypes include developmental delay, repetitive behaviors, self-injury, sleep disturbances, and hyperactivity. An example of a repetitive behavior often seen with patients with SMS is self-hugging. The sleep disturbances seen are due to an inverted circadian rhythm which causes increased wakefulness at night. The most common physical phenotypes include short stature, hoarse voice, dental anomalies, and oral/motor dysfunction. Examples of oral/motor dysfunction would include decrease in tongue strength and an increase in drooling. Less common phenotypes include seizures, heart defects, immune function abnormalities and renal/urinary tract abnormalities [4] [5]. In one study involving multiple patients with

SMS, it was found that certain phenotypes become more prominent with age. Some of those phenotypes are hoarse voice, frontal prominence (large forehead), and coarsening of facial features [6].

Causes of SMS. SMS was initially thought to be caused by a large scale alteration in the expression of genes in the p arm of chromosome 17. There are two possible mechanisms by which large scale alterations can occur. These methods are parental imprinting and deletion of DNA. Parental imprinting is a type of epigenetic inheritance that results in only one of the two inherited alleles being expressed. However, in an experiment conducted in 1991, it was found that there were no differences in phenotypes between both maternally and paternally derived chromosomes in SMS patients [6]. This evidence ruled out the possibility of parental imprinting as a mechanism for producing SMS [7]. Large scale deletions of DNA can occur in two ways. Deletion may occur by unequal crossing over between repeated sequences producing an unbalanced DNA rearrangement. Deletions can also occur spontaneously in the gonads of the parents therefore not affecting the parents but affecting their children [8].

Studies of SMS patients have defined the SMS critical interval as the common region on the p arm of chromosome 17 in which deletions causing the phenotypes associated with SMS occur. The most common deletion is ~3.5Mb, found in about 75% of patients [9]. Differences in phenotype and severity may be due to the differences in deletion sizes and other genetic factors [7]. Smaller sized deletions are not as common but have allowed for the refinement of the SMS critical interval. In 2003, a group of researchers compressed the critical interval from 3.5 Mb to 950kb by using fluorescent *in situ* hybridization (FISH), analysis [1]. FISH analysis uses fluorescent labeling of

specific DNA sequences in order to determine if they are deleted or not. If the portion of DNA that the probe binds to is not deleted then it will fluoresce. If the portion of DNA is deleted then there will be no fluorescence. In 2005, a different group of researchers further compressed the critical interval to 650kb, also by using FISH [10].

Within the 650kb critical interval, there are several candidate SMS genes (Table 1). DNA from patients that appear to have SMS but do not have a deletion were sequenced to see if any of these candidate genes were mutated. All of these patients were shown to have a mutation in Retinoic Acid Inducible gene 1 (*RAII*). A mutation of the *RAII* gene produces a nonfunctional or truncated protein which results in haploinsufficiency [11] meaning that half of the amount of protein is not enough to produce a normal phenotype. The majority of the features seen with SMS patients are now attributed to haploinsufficiency of *RAII* because *RAII* mutations or deletions have been found in all patients. Other deletions on chromosome 17p11.2 may cause some of the less common phenotypes and contribute to phenotypic variability of SMS.

Table 1: Official names of genes found in the critical region on chromosome 17.

Gene short-hand	Official name of gene
<i>RASD1</i>	RAS dexamethasone-induced 1
<i>PEMT</i>	Phosphatidylethanolamine N-methyltransferase
<i>SMCR5</i>	Smith-Magenis syndrome chromosome region, candidate 5
<i>RAII</i>	Retinoic acid induced 1
<i>SREBF1</i>	Sterol regulatory element binding transcription factor 1
<i>TOMIL2</i>	Target of myb 1-like 2
<i>ATPAF2</i>	ATP synthase mitochondrial F1 complex assembly factor 2
<i>DRG2</i>	Developmentally regulated GTP binding protein 2
<i>MYO15A</i>	Myosin XVA
<i>LLGL1</i>	Lethal giant larvae homolog 1
<i>FLII</i>	Friend leukemia virus integration 1

<i>SMCR7</i>	Smith-Magenis syndrome chromosome region, candidate 7
<i>TOP3A</i>	Topoisomerase (DNA) III alpha
<i>SMCR8</i>	Smith-Magenis syndrome chromosome region, candidate 8
<i>SHMT1</i>	Serine hydroxymethyltransferase 1

In addition to its role in SMS, other functions of *RAII* have been identified. *RAI-I* may also play a role in schizophrenia and the age of onset of spinocerebellar ataxia [12]. These two roles of *RAII*, along with the role of *RAII* in SMS, indicate that *RAII* has a major effect on normal brain functioning. Furthermore, *RAII* has been shown to have some anti-viral properties [13] [14].

In this project, the *RAII* promoter was studied. Studying the promoter of *RAII* will help determine the normal expression of the gene thereby providing clues to its normal function. Furthermore, an understanding of the normal expression of *RAII* may provide insight into why the lack of *RAII* leads to SMS.

Eukaryotic Gene Expression. In order for an organism to express a functional protein from a gene, the specific DNA that encodes the protein must first be transcribed into mRNA and then the mRNA must be translated into a functional protein. In eukaryotes, there are four main steps to transcription. They are initiation, elongation, termination, and post-RNA processing. Initiation is when RNA polymerase II binds to the promoter region of the DNA. Elongation is the process of forming the RNA transcript. Termination involves the release of the RNA transcript and the RNA polymerase from the DNA template. Pre-RNA processing refers to further modification of the RNA transcript after or during transcription that is necessary for translation of the RNA into a protein [8].

This project will focus on the *RAII* promoter and the initiation of transcription. A promoter is a section of DNA important in initiation of transcription because it serves as a recognition sequence for the attachment of the preinitiation complex. The preinitiation complex that binds to the promoter region includes general transcription factors (GTFs), coactivators, corepressors, and RNA polymerase II [8]. There are four specific portions of the promoter that are of importance in the binding of GTFs and coactivators/corepressors. They include the TATA box, the initiator element, the downstream core promoter element, and the TFIIB recognition element. The TATA box is the binding site of the TATA binding protein (TBP). The initiator element directly overlaps the transcription start site and performs the same functions as the TATA box including helping with the formation of the preinitiation complex. The downstream core promoter element is a seven nucleotide sequence found about 30bp downstream of the transcription start site. This element is particularly important for TATA-less promoters. The TFIIB recognition element is a sequence specifically for the binding of TFIIB. Coactivators and corepressors serve to either activate or repress transcription by binding to certain places on the DNA [15].

There are two general types of promoters found within cells. They include promoters with a TATA-box and promoters that don't possess a TATA-box. Although TBP usually binds to the TATA sequence, it can bind to the DNA in TATA-less promoters. Mechanisms for binding include activator proteins to keep it in place, direct binding to the DNA, or the interaction of TBP with other initiatory proteins [14]. Upon examination of the *RAII* sequence, it was found that the promoter of *RAII* is a TATA-less promoter. These types of promoters usually contain more transcription initiation

sites and multiple binding sites for the transcription factor stimulating protein 1 (Sp1). The function of Sp1 is to help in the formation of the preinitiation complex. Also associated with TATA-less promoters is a sequence with high G/C content. TATA-less promoters do not contain a TATA box, but require other transcription binding sites in order to initiate transcription. The downstream core promoter element is also important in the binding of the preinitiation complex in TATA-less promoters. It acts in conjunction with the initiator element to bind the TFIID complex of transcription factors [16]. The actual method of transcription initiation for TATA-less promoters varies for different TATA-less promoters. A major goal of this project is to determine which of these elements are present in the human *RAII* promoter and their function in the expression of the *RAII* protein.

Summary. The purpose of this project is to characterize the *RAII* promoter. The main methods used for characterizing the *RAII* promoter are the reporter gene assay and 5'RLM-RACE. The goal of the reporter gene assay is to determine if any *RAII* fragments contain promoter activity. The goal of 5'RLM-RACE is to determine the 5' end of the *RAII* transcript. Studying the *RAII* promoter will help determine the normal mechanisms for expression which may lead to insights into its role in causing Smith-Magenis syndrome.

METHODS

Cloning the 2.3kb *RAII* putative promoter construct. The purpose of cloning the 2.3kb *RAII* fragment was to clone the putative *RAII* promoter containing the upstream response element. The predicted length of the longer putative promoter construct was 2.3kb. In order to clone the longer fragment of *RAII*, PCR (polymerase chain reaction) was used to amplify the fragment from genomic DNA. PCR was followed by gel electrophoresis in order to visualize the PCR product.

PCR. The reaction mix used for the PCR reaction was: 1.0 μ L of HEK genomic DNA, 2.5 μ L of 10X PCR buffer, 1.5 μ L of 25mM MgCl₂, 0.5 μ L of 10mM dNTP, 1.0 μ L (10 μ M) the forward primer SHE 599F, 1.0 μ L (10 μ M) of the reverse primer DZ2R, 17.25 μ L of dH₂O, and 0.25 μ L (5U/ μ L) of Taq DNA polymerase. The primers were designed by Dr. Elsea and Dr. Zies for the *RAII* sequence from the publicly available Human Genome Project. The forward primer sequence for SHE 599F was 5'TAAGTAGTTTGCCCAAAGCCACCC. The reverse primer sequence for DZ2R was 5'ACACCACACAAAGCAAGGACC. The amplification conditions for the PCR reaction were 94°C for 5 minutes, 40 cycles of amplification at 94°C for 1 minute, 60°C for 1 minute, 72°C for 3 minutes, and final extension at 72°C for 10 minutes.

Gel Electrophoresis. A 1% agarose gel was prepared by combining 600mg agarose in 60mL of 1X TAE buffer. The mixture was heated for approximately one minute in a microwave until all the agarose dissolved. GelRed solution was added to the agarose gel before it hardened at 1:10,000 for visualization of the DNA. Loading dye was added to

the samples at ¼ of the volume of the sample before they were applied to the gel. A 100bp DNA ladder was used as the marker and was added to lane one for every gel run. For each sample, 15-20µL was added to the other wells. The gel ran for one hour at 100 volts.

Development of shorter *RAII* putative promoter construct. A member of Dr. Elsea's lab was able to successfully clone a small fragment of *RAII*. I was given this fragment in order to perform further experiments on it. With this previously cloned fragment, I developed a shorter fragment in order to remove potentially problematic ATG sequences. It was cloned into a TOPO vector and then moved to the plasmid pGL3 (Promega, Inc.). The methods used for developing the shorter plasmid were: restriction digest with the restriction enzyme *NcoI*, gel electrophoresis (as previously described), gel extraction, ligation, transformation, and plasmid purification.

Restriction Digest with NcoI. The following components were mixed in a microcentrifuge tube: 10.5µL of dH₂O, 2.0µL of RE 10X buffer, 2.0µL of acetylated BSA at 10µg/µL, and 5.0µL of DNA at 1µg/µL. The components were mixed by pipetting and then 0.5µL of the restriction enzyme *NcoI* at 10U/µL (Promega, Inc) was added bringing the total volume of the reaction to 20.0µL. The reaction was incubated for one hour in a 37°C water bath.

Gel Extraction. This method was performed as described in the gel extraction kit from Qiagen, Inc. After gel electrophoresis, the correct DNA band was removed from the

agarose gel using a scalpel and then weighed in a pre-weighed 1.5mL microcentrifuge tube. In order to dissolve the gel, 3 volumes of Buffer QG were added to the gel slice and then the gel slice was incubated in a 50°C water bath for 10 minutes with intermittent vortexing to aid in dissolving the gel. After the gel slice had dissolved, one volume of isopropanol was added to the mixture. DNA was bound to the spin column matrix by adding the mixture to a spin column, placing it inside a 2mL collection tube and centrifuging for 1 minute at 13,000rpm. The bound DNA was washed by adding 0.5mL of Buffer QG and centrifuging for 1 minute at 13,000rpm and then adding 0.75mL of Buffer PE and centrifuging for an additional 1 minute at 13,000rpm. The flow-through was discarded at each step and the spin column was centrifuged for an additional minute to dry the column. The spin column was placed in a sterile 1.5mL microcentrifuge tube for elution. The DNA was eluted by adding 50µL of elution buffer to the center of the spin column followed by centrifugation for 1 minute.

Ligation. The following components were mixed in a 1.5mL microcentrifuge tube: 1µL of gel extracted DNA, 1µL of 10X ligation buffer, 7µL of dH₂O, and 1µL of ligase at 10U/µL (Promega, Inc.). The reaction was incubated at 15°C overnight in the thermocycler.

Transformation. Competent DH5a cells (Invitrogen, Inc) from the -80°C freezer were thawed on ice before use. Then 1µl of the ligation reaction from the previous step was added to the cells and gently mixed with the tip of a pipette. The cells were incubated on ice for 30 minutes followed by heat shock for 20 seconds in a 42°C water bath. The cells

were then placed back on ice for 2 minutes. Preheated LB broth (950 μ L) was added to the cells and placed on a shaker for 45 minutes in a 37°C incubator. The cells were then centrifuged for a few seconds in order to pellet the cells. The majority of the supernatant was removed (~825 μ L). The cells were resuspended in the remaining media and 100 μ L was plated on LB plus ampicillin plates using sterile technique. The plates were incubated overnight at 37°C.

Plasmid Purification. This method was performed as described in the plasmid DNA miniprep kit from Qiagen, Inc. Bacterial colonies were selected from the LB-Amp plates from the transformation and grown in 3mL of liquid LB-Amp overnight. The 3mL bacterial cultures were pelleted using a microcentrifuge and the supernatant was removed. The pellet was resuspended using 250 μ L of resuspension buffer (Buffer P1). Following addition of Buffer P1, 250 μ L of lysis buffer (Buffer P2) was added and then mixed by inverting the tube. Then, 350 μ L of neutralization buffer (Buffer N3) was added and mixed by inverting the tube. The tubes were centrifuged for 10 minutes at 15,682 RCF. In order to bind the DNA, the supernatant was applied to the spin column and then centrifuged for 30-60 seconds. The DNA was washed by adding 0.5mL of Buffer PB to the spin column and centrifuging for 1 minute and then adding 0.75mL of Buffer PE and centrifuging for 1 minute at 15,682 RCF. The flow-through was discarded at each step and the spin column was centrifuged for an additional minute in order to dry the column. In order to elute the DNA, 50 μ L of elution buffer was added to the center of the spin column that was placed in a 1.5mL microcentrifuge tube. The column was incubated for 1 minute at room temperature and then centrifuged for 1 minute at 15,682

RCF. The concentration of the new plasmid (*RAII* short) was quantified by measuring the absorbance of the solution at 260nm and 280nm.

Luciferase Reporter Gene Assay. The luciferase reporter gene assay was performed on *RAII* long plasmid and *RAII* short plasmid in order to detect activity of the *RAII* promoter using a luminometer. The reporter gene constructs were transfected into HEK293 cells. After 24 hours, the cells were lysed and mixed with appropriate reporter gene substrates. The reporter gene activity was detected using the luminometer located at the Medical College of Virginia (MCV).

Transient Transfection. Three plasmids of interest were transfected into the mammalian cell line HEK293. The *RAII* long (original *RAII* plasmid) and *RAII* short plasmids were both cotransfected with the control plasmid pSV- β gal. Before beginning the assay, HEK293 cells were grown in 500 μ L of medium (DMEM+10%FBS without antibiotics) per well of a 12-well plate in order for them to be 90-95% confluent (about $0.5-2 \times 10^5$ cells) at the time of transfection. For each plasmid sample, 1.6 μ g of DNA was mixed with 100 μ L DMEM with no serum. Separately, 4 μ L of Lipofectamine 2000 (Invitrogen, Inc.) was mixed with 100 μ L DMEM and incubated for 5 minutes. After the 5 minute incubation, the diluted DNA was added to the diluted Lipofectamine 2000 bringing the total volume to 200 μ L. The solution was mixed and incubated for 20 minutes at room temperature. Then, the 200 μ L Lipofectamine/DNA complexes were added dropwise to each well containing cells. The contents were mixed by gently rocking it back and forth before placing it in a 37°C CO₂ incubator overnight.

Luciferase Cell Culture Lysis. The transfected cells were lysed using 1X reporter lysis buffer (Promega). First, the growth medium from the cells was removed using a pipette. Then the cells were washed with 200 μ L PBS (phosphate buffered saline) followed by the addition of 150 μ L of 1X lysis buffer to each well ensuring that the bottom of each well was covered. The lysed cells were scraped off the bottom of the well using a scraper and transferred to a 1.5mL microcentrifuge tube on ice using a pipette. Each tube was vortexed for 10-15 seconds and then centrifuged at 12,000 RCF for 15 seconds at room temperature. The supernatant was transferred to a new tube and the cell lysates were stored at -80°C.

Reporter Gene Assay. First, 20 μ L of each prepared cell lysate was added to two wells of a solid white 96-well plate. Then 100 μ L of steady-glo luciferase (Promega, Inc.) was added to half of the wells and 70 μ L of a 1:100 dilution of β -gal substrate (Applied Biosystems) and buffer mix was added to the other half of the wells. After incubating for 30 minutes, 100 μ L of the β -gal accelerator was added to the designated β -gal wells and activity was measured using the luminometer (Wallac 1420). Within those 30 minutes, luciferase activity of the steady-glo luciferase samples was measured using the luminometer. Each sample was run in duplicate.

β -galactosidase Reporter Gene Assay. The β -gal reporter gene assay was performed in place of the luciferase reporter gene assay in order to work out technical difficulties with the transient transfection assay and to use equipment available at UMW. The β -gal

reporter gene assay uses color change, instead of light, as an indicator of promoter activity. Transient transfection was performed 24 hours before beginning the β -gal reporter gene assay.

Preparation of Lysates. Both 6-well and 12-well plates were used for growing cells for this assay. The growth medium was carefully removed from the cells using a pipette. The cells were then washed twice with 1X PBS. For the 6-well plate, 300 μ L of 1X RLB (reporter lysis buffer) was added to each well. For the 12-well plate, 200 μ L of 1X RLB was added to each well. After ensuring complete coverage of the cells by the lysis buffer, the cells were incubated for 15 minutes at room temperature rocking the dish every 3 minutes. The lysed cells were then scraped to the lower edge of the plate, transferred to a clean 1.5mL microcentrifuge tube using a pipette, and immediately placed on ice. Then the cells were vortexed for 10-15 seconds and centrifuged in a microcentrifuge at 4°C for 2 minutes at 15,682 RCF. The supernatant was transferred to a new tube and stored at -80°C until ready to proceed to the standard assay.

Standard Assay. For the assay, 150 μ L of each cell lysate was transferred to a new 1.5mL microcentrifuge tube followed by the addition of 150 μ L of Assay 2X Buffer to each tube. The samples were mixed by vortexing them for a few seconds and then incubated at 37°C for 30 minutes or until the reaction turned a faint yellow color. In order to stop the reactions, 500 μ L of 1M sodium carbonate was added to each tube. The reactions were vortexed briefly before their absorbance was read at 420nm.

Generation of Standard Curve. A 1:10,000 dilution stock of β -galactosidase was prepared in 1X RLB (reporter lysis buffer). Using this stock, seven β -gal standards were made as shown in the following table.

Table 2. β -gal standards for the β -gal reporter gene assay.

β -galactosidase Standard (milliunits)	Volume of 1:10,000 stock (μ L)	Volume of 1X RLB (μ L)
0	0	150
1.0	10	140
2.0	20	130
3.0	30	120
4.0	40	110
5.0	50	100
6.0	60	90

The total volume for each standard used in the assay was 150 μ L.

Using the same procedure as for the standard assay, 150 μ L of Assay 2X Buffer was added to each standard and incubated for 37°C for 30 minutes. The reactions were stopped by adding 500 μ L of 1M sodium carbonate. The reactions were vortexed briefly before their absorbance was read at 420nm.

5'RLM-RACE. 5'RLM-RACE (Ambion, Inc.), or RNA Ligase Mediated Rapid Amplification of cDNA Ends, was used in order to determine the 5' end of the *RAII* transcript. RNA was isolated from HEK293 cells for this procedure.

Cell Lysis and RNA Isolation. This method was performed as described in the RNAqueous kit by Ambion, Inc. HEK cells were grown to confluency in a 100mm culture dish. The growth medium was removed from the dish and the cells were washed with a 5mL of PBS. Then, 1400 μ L of lysis buffer was added to the dish. The cells were scraped using a scraper and two 700 μ L aliquots were collected in 1.5mL microcentrifuge

tubes. The cell lysates were vortexed and stored at -80°C until RNA isolation was performed. An equal volume of 64% ethanol was added to the lysates and mixed. The lysate/ethanol mixture was added to the filter cartridge in a collection tube and then centrifuged for 15 seconds-1 minute at 13,000 RCF. The previous two steps were repeated until all the sample had been drawn through the filter cartridge. In order to wash the DNA, 700 μL of wash solution #1 was applied to the filter cartridge and then centrifuged. As an additional wash step, 500 μL of wash solution #2/3 was applied to the filter cartridge and centrifuged followed by the addition and centrifugation of another 500 μL of wash solution #2/3. In order to dry the column, the sample was centrifuged again. The flow-through was discarded after each step. The first step for eluting the RNA was to pipet 40-60 μL of preheated elution solution (about $70-80^{\circ}\text{C}$) to the center of the filter placed in a 1.5mL microcentrifuge tube and then centrifuge for 30 seconds at room temperature (13,000 RCF). Then a second aliquot (10-60 μL) of preheated elution solution was added to the center of the filter and re-spun for 30 seconds. To complete RNA elution, a third 10-60 μL aliquot of elution solution was added and centrifuged in the same manner as the first step. The RNA samples were stored at -80°C until further use.

CIP and Termination. The purpose of treating the RNA with Calf Intestine Alkaline Phosphatase (CIP) is to remove the 5' phosphate from degraded RNA. The following components were mixed for the treatment with CIP step: 10 μg (standard reaction) or 1 μg (small reaction) of RNA (in X μL), 2 μL of 10X CIP Buffer, 2 μL of CIP, and nuclease-free water up to 20 μL . The reaction was then mixed, centrifuged briefly, and incubated

for one hour at 37°C. In order to terminate the CIP reaction, the following components were added to the CIP reaction: 15µL of ammonium acetate solution, 115µL of nuclease-free water, and 150µL of acid phenol:chloroform. The reaction was vortexed thoroughly and centrifuged for 5 minutes at 15,000 RCF in a microcentrifuge. The top layer (aqueous layer) was transferred to a new tube. Then 150µL of chloroform was added to the sample, vortexed thoroughly, and centrifuged for 5 minutes at 15,000 RCF in a microcentrifuge. The top layer (aqueous layer) was transferred to a new tube.

Precipitation and Resuspension of RNA. According to the protocol from Ambion, Inc., the first step in precipitation of RNA was to add 150µL of isopropanol to the sample, vortex thoroughly, and chill on ice for 10 minutes. Then the sample was centrifuged for 20 minutes at maximum speed in a microcentrifuge. The pellet was washed by adding 0.5mL cold 70% ethanol and then centrifuged for 5 minutes at 15,000 RCF. The ethanol was removed carefully and the pellet was dried. The resuspension step differed for the standard reaction and the small reaction. Both of these reactions were performed and are differentiated below. For the standard reaction, the pellet was resuspended in 11µL of Nuclease-free water. For the small reaction, the pellet was resuspended in 4µL of 1X TAP Buffer.

Treatment with TAP. The purpose of the fifth step, treatment of RNA with Tobacco Acid Pyrophosphatase (TAP), is to remove the 5' cap from the HEK RNA. For this step, the following components were mixed for the standard reaction: 5µL of CIP'd RNA, 1µL of 10X TAP buffer, 2µL of TAP, 2µL of nuclease-free water. The following components

were mixed for the small reaction: 4 μ L of CIP treated RNA and 1 μ L of TAP. The reactions were then mixed, centrifuged briefly, and incubated for one hour at 37°C.

Ligation of 5'RACE Adapter. The purpose of the ligation reaction is to ligate the 5'RACE adapter oligonucleotide to the 5' end of the RNA. For the standard reaction of this step, the following components were mixed: 2 μ L of CIP/TAP treated RNA, 1 μ L of the 5'RACE adapter (3 μ g/ μ L), 1 μ L of 10X RNA ligase buffer, 2 μ L of T4 RNA Ligase (2.5U/ μ L), and 4 μ L of nuclease-free water. The following components were mixed for the small reaction: 5 μ L of CIP/TAP treated RNA, 1 μ L of the 5'RACE adapter, 1 μ L of 10X RNA ligase buffer, 2 μ L of T4 RNA ligase (2.5U/ μ L), and 1 μ L of nuclease-free water. The reactions were mixed gently, centrifuged briefly, and incubated for one hour at 37°C.

Reverse Transcription. For the reverse transcription reaction, the following components were assembled in a 1.5mL microcentrifuge tube on ice: 2 μ L of the ligated RNA, 4 μ L 10 μ M dNTP mix, 2 μ L of 50 μ M random decamers, 2 μ L of 10X RT buffer, 1 μ L of 10U/ μ L RNase inhibitor, 1 μ L of M-MLV reverse transcriptase, and 8 μ L of nuclease-free water. The reaction was mixed gently, centrifuged briefly, and incubated for one hour at 42°C.

Nested PCR for 5'RLM-RACE. The PCR for the 5'RLM-RACE consisted of two PCR reactions: an outer PCR followed by an inner PCR. For the outer PCR, the following components were mixed in a PCR tube: 1 μ L of the reverse transcription reaction from

the previous step, 5 μ L of 10X PCR buffer, 4 μ L of 10 μ M dNTP mix, 2 μ L of the 5'RACE gene specific outer primer at 10 μ M (p40-5'TGCGGGTTATAATAGTCCTTGGCG), 2 μ L of the 5'RACE outer primer, 36 μ L of nuclease-free water, and 0.25 μ L of 5U/ μ L thermostable DNA polymerase. The cycling profile for the PCR reaction was 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and final extension at 72°C for 7 minutes. For the inner PCR reaction, the following components were mixed in a PCR tube: 2 μ L of the outer PCR product, 5 μ L of 10X PCR buffer, 4 μ L of dNTP mix, 2 μ L of the 5'RACE gene specific inner primer at 10 μ M (p39-5'TTCTCTAGGCGTGATGTTTCCTGC), 2 μ L of the 5'RACE inner primer, 35 μ L nuclease-free water, and 0.25 μ L of 5U/ μ L thermostable DNA polymerase. The cycling profile for the inner PCR was the same as for the outer PCR. Inner PCR was followed by gel analysis of the PCR products using gel electrophoresis as previously mentioned.

TOPO Cloning. The TOPO cloning reaction was used in order to insert the PCR product into a vector suitable for sequencing (Invitrogen, Inc.). The following components were added together and mixed gently: 2 μ L of fresh PCR product, 1 μ L of salt solution, 2 μ L of water, and 1 μ L of the TOPO vector. The reaction was incubated for 5 minutes at room temperature and was placed on ice before proceeding to transformation. The TOPO clone was transformed using the method of transformation previously mentioned but used One Shot® TOP10 chemically competent cells. Following transformation, the plasmid was purified using the plasmid purification method previously mentioned. After plasmid purification, the plasmid was sent off to AGGT, Inc. for sequencing.

RESULTS

Cloning the 2.3kb RAI1 Fragment. The purpose of cloning a longer *RAI1* fragment was to include the potential retinoic acid response element 1 as well as additional DNA that may serve as part of the *RAI1* promoter. The primers used for the PCR reaction were the forward primer SHE599 and the reverse primer DZ2. Primer SHE599 is located upstream of the putative retinoic acid response element 1 and primer DZ2 is located downstream of *RAI1* exon 1 (Figure 1A). Figure 1B shows a representative 1% agarose gel of the PCR reactions. The experimental reactions are in lanes 2 and 3. Multiple bands were seen for each sample with the largest molecular weight band at about 1000bp. This was not the result we were expecting since we were expecting a band at 2.3kb.

Development of a Shorter RAI1 Plasmid. The *RAI1* fragment I received from MCV was thought to contain several ATGs, or start codons, within intron 1. These potential start codons might pose a problem for the translation of the luciferase reporter gene when performing the reporter gene assay. Therefore, the restriction enzyme *NcoI* was used to cut out *RAI1* intron 1. The restriction sites for *NcoI* are immediately upstream of intron 1 and at the translation start site for pGL3 (Figure 2A). Figure 2B shows a representative 1% agarose gel of the *NcoI* digest. The top band from the restriction digest is about 6550bp. The bottom band from the restriction digest is 326bp and represents the piece of DNA cut out from pGL3 (*RAI1* intron 1). Figure 2C shows a representative 1% agarose gel of the *RAI1* long plasmid (lane 2) and the *RAI1* short plasmid (lane 3) after restriction digest with *NcoI*. Since the *RAI1* short plasmid was already digested with *NcoI*, there

was only one band present at about 6550bp. The long *RAII* plasmid had two bands present with the smaller band being the fragment cut out by *NcoI*.

RAII Promoter Constructs. All *RAII* promoter constructs were inserted in front of the luciferase gene in the pGL3 plasmid. The *RAII* long promoter construct, containing the *RAII* intron 1, is shown in Figure 3A and the *RAII* short promoter construct, without *RAII* intron 1, is shown in Figure 3B. The control plasmid pGL3, in which there was no insertion of the putative *RAII* promoter in front of the luciferase gene, is shown in Figure 3C.

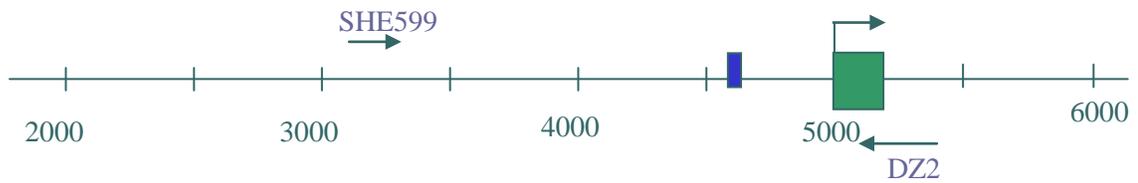
Representative Data from Initial Reporter Gene Assay. The *RAII* long plasmid, *RAII* short plasmid, and pGL3 were cotransfected with the control plasmid pSV- β -Gal. The *RAII* long and *RAII* short plasmids were run in quadruplicate whereas the pGL3 plasmid was run in triplicate. In addition, each sample was run in duplicate for the luciferase substrate and for the β -gal substrate (Table 3). For the *RAII* long plasmid and the *RAII* short plasmid, the β -gal numbers were higher than the luciferase numbers.

β -gal Reporter Gene Assay. The β -gal assay was run in quadruplicate with two different concentrations of DNA: 0 μ g of DNA as a control and 1 μ g of DNA as the experimental. Assay results were read on a spectrophotometer and the absorbance numbers at 420nm for each concentration of DNA. As shown in Table 4, the absorbance numbers for 1 μ g of β -gal plasmid were higher than the absorbance numbers for no β -gal plasmid.

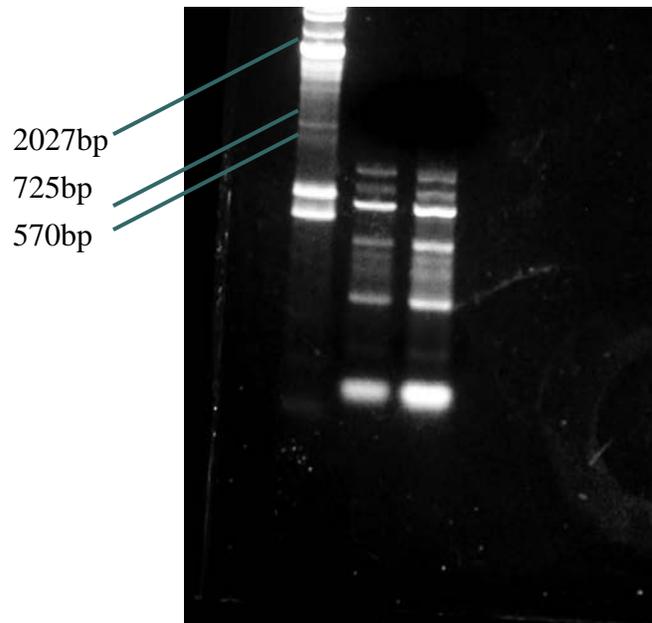
Reporter Gene Assay Showing No Activity. The reporter gene assay was run with different concentrations of *RAII* short and *RAII* long plasmids (1 μ g vs. 2 μ g). Each combination of concentration/plasmid was run in duplicate. The light unit values for each plasmid/concentration combination are shown in Table 5. In general, the β -gal numbers were higher than the luciferase numbers. In addition, both the β -gal and luciferase numbers were consistent across the board indicating the transfection was working.

Reporter Gene Assay with DZ23. The reporter gene assay was run in duplicate with the plasmid DZ23, which has known promoter activity, the *RAII* long plasmid, *RAII* short plasmid, and pSV- β -gal. Table 6 shows the light unit data from each of the plasmids for the luciferase substrate samples and the B-gal substrate samples. The data shows that plasmid DZ23 has luciferase activity whereas the other three plasmids do not have any luciferase activity.

5'RLM-RACE. The 5'RLM-RACE protocol was used in order to amplify the 5' end of RNA from *RAII*. A representative 1% agarose gel from one of the two 5'RLM-RACE procedures conducted, is shown in Figure 5. Lane 2 is from the outer PCR and lanes 3 and 4 are from the inner PCR. The bands are all less than 25bp strongly indicating they were excess primers and that I did not successfully amplify the 5' end of the *RAII* transcript.



A)



B)

Figure 1. Cloning the 2.3kb *RAI1* fragment. A) Position of the primers used for cloning the 2.3kb fragment. The blue box represents the retinoic acid response element 1 and the green box represents *RAI1* exon 1. The green arrow represents the transcription start site for *RAI1*. SHE599 was the forward primer used in PCR located upstream of the Retinoic Acid Response Element 1. DZ2 was the reverse primer used located directly downstream of *RAI1* exon 1. B) A representative agarose gel showing the PCR products. Lane 1 is the DNA ladder whereas lanes 2 and 3 are the PCR product samples.

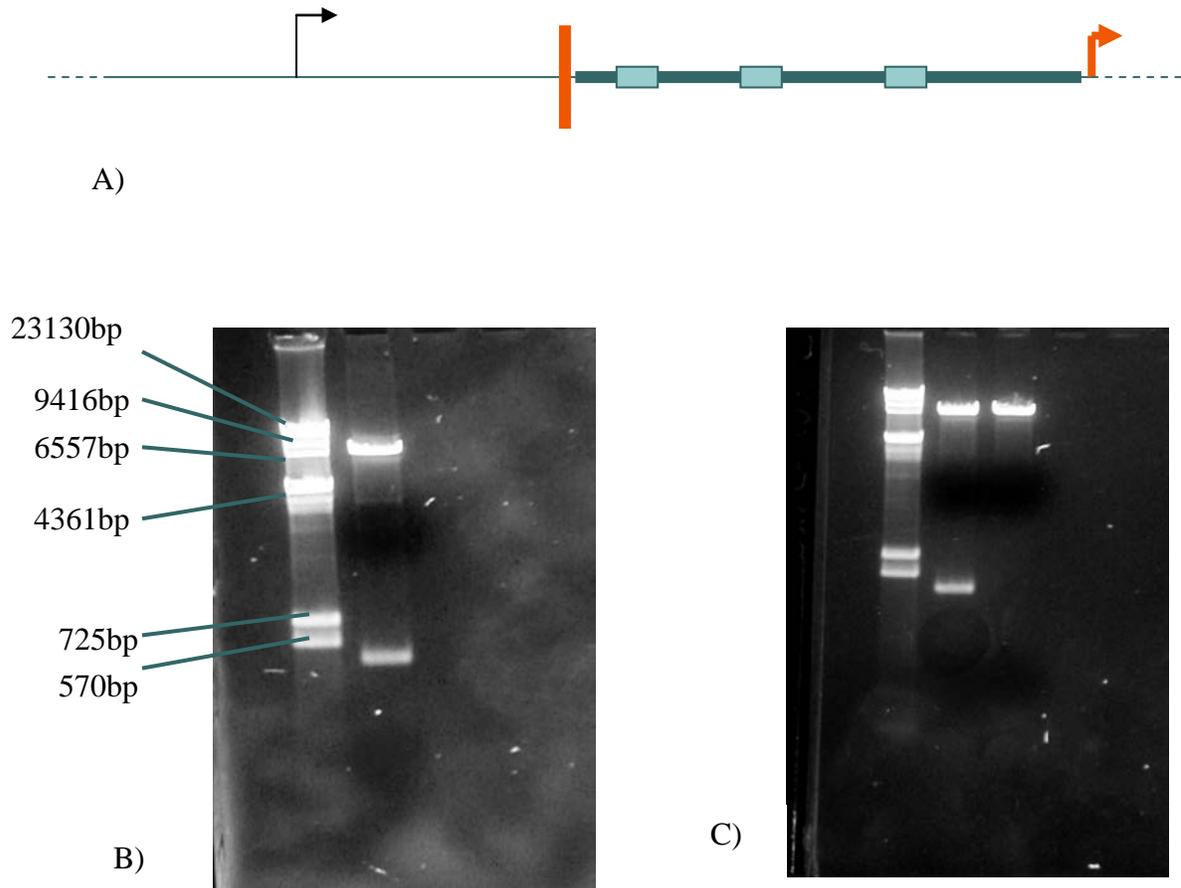


Figure 2. Development of Shorter *RAI1* Plasmid. A) *NcoI* restriction sites. *NcoI* has two restriction sites represented by the red line and the red arrow. One restriction site cuts the DNA at the start of *RAI1* intron 1 (indicated by the thick blue line) and the second restriction site cuts the DNA at the translation start site for pGL3 (the red arrow). The dashed lines represent pGL3. The blue boxes within intron 1 represent possible ATGs or start codons for translation of *RAI1*. The black arrow represents the transcription start site for *RAI1*. B) A representative gel following restriction digest with *NcoI*. Lane 1 is the DNA ladder and lane 2 is the sample. The bottom band of the sample lane is the DNA fragment removed from *NcoI* and is 326bp in length. C) A representative gel of the restriction digests of *RAI1* short and *RAI1* long plasmids. Lane 1 is the DNA ladder. Lane 2 is the restriction digest with *NcoI* of the *RAI1* long plasmid. Lane 3 is the restriction digest of *RAI1* short plasmid.

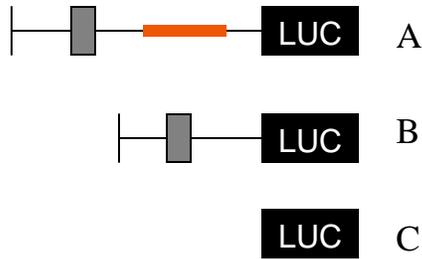


Figure 3. *RAI1* Promoter Constructs. The gray box represents the putative *RAI1* promoter. The black box with LUC represents the luciferase gene. A) The red line represents the sequence of DNA that was excised using the restriction enzyme *Nco*I. This fragment is the *RAI1* long fragment. B) This fragment is the *RAI1* short fragment which has the red line removed. C) This fragment represents the control plasmid pGL3 with no *RAI1* promoter fragment inserted in front of the luciferase gene.

Table 3. Representative data of luciferase reporter gene assay using *RAI1* long plasmid, *RAI1* short plasmid, and pGL3.

	<i>RAI1</i> long plasmid				<i>RAI1</i> short plasmid				pGL3 plasmid		
LUC	18	21	21	24	174	269	494	130	55	209	72
	32	30	28	19	167	247	453	157	40	192	67
β-gal	611	401	323	3432	322	387	1583	288	180	495	88
	700	406	325	3526	391	474	38	27	35	487	33

All three plasmids were cotransfected with pSV- β gal. Quadruplets were run of the *RAI1*-long plasmid and the *RAI1*-short plasmid. The pGL3 plasmid was run in triplicate. The first two rows represent duplicate samples that had the luciferase substrate added (LUC). The last two rows represent duplicate samples that had the β -gal substrate added. All numbers shown are in light units (LU).

Table 4. Representative data from the β -gal reporter gene assay.

Amount of DNA	Absorbance at 420nm
0 μ g	0.141
	0.153
	0.157
	0.154
1 μ g	0.269
	0.264
	0.299
	0.219

The assay was done in quadruplet for each concentration of DNA.

Table 5. Raw data of the reporter gene assay using different concentrations of the *RAII* short and *RAII* long plasmids.

	1 μ g <i>RAII</i> -long (LU)		2 μ g <i>RAII</i> -long (LU)	1 μ g <i>RAII</i> -short (LU)		2 μ g <i>RAII</i> -short (LU)	
LUC	84	86	73	58	68	87	49
β-gal	3599	3870	2863	1710	1549	2499	2201

Duplicates were run for each concentration/plasmid combination.

Table 6. Raw data of reporter gene assay using plasmid DZ23 as a positive control

	β-gal		DZ23		<i>RAII</i> short		<i>RAII</i> long	
LUC	71	85	1139	1042	76	86	61	70
β-gal	3277	4000	3224	2991	3653	3364	3387	4354

. The reporter gene assay was also performed on β -gal plasmid and both *RAII* plasmid constructs. All numbers shown are in light units. Duplicates were run for each plasmid.

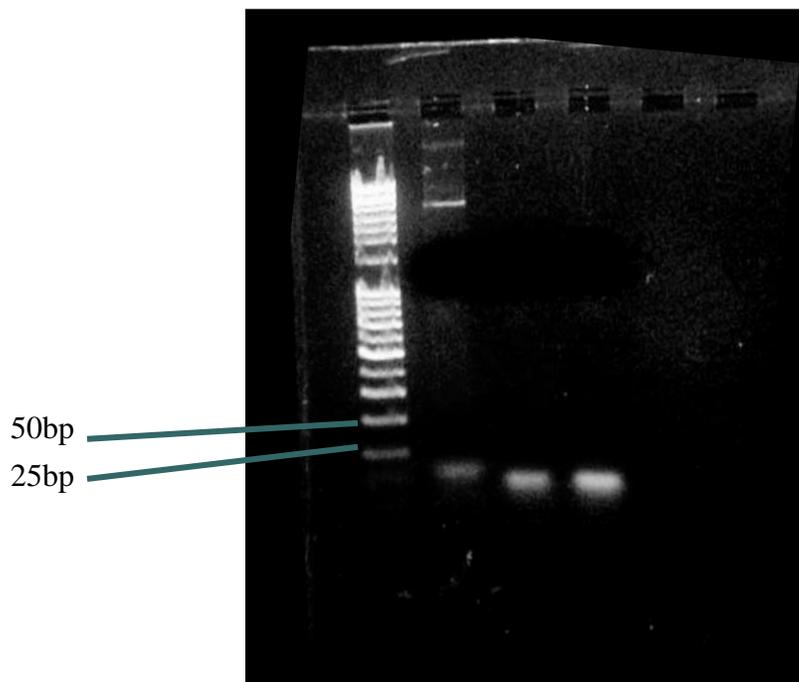


Figure 5. Representative 1% agarose gel of 5'RLM-RACE. Lane 1 represents the DNA ladder. Lane 2 represents the PCR product resulting from the outer PCR. Lanes 3 and 4 represent PCR products resulting from the inner PCR

DISCUSSION

Cloning the 2.3kb RAI1 Fragment. As shown in Figure 1A, the expected length of the *RAI1* fragment from PCR was 2.3kb. Several attempts to clone the 2.3kb fragment were conducted, varying PCR conditions each time such as the concentration of MgCl₂ and the temperature cycling profile. However, there were no bands present at 2.3kb for any of the attempts. Figure 1B shows the best result that was obtained from the PCR. The lack of bands at 2.3kb indicates that the primers did not work in cloning the longer *RAI1* fragment. These data, along with additional attempts using alternative primers (not shown), suggest that the published sequence of the *RAI1* upstream region from the human genome project may be inaccurate.

Reporter Gene Assay. The restriction digest with *NcoI* was successful in cutting out the *RAI1* intron 1. Removing *RAI1* intron 1 from the *RAI1* promoter construct would eliminate the hypothetical possibility that the start codons present in intron 1 would interfere with translation of the luciferase gene. This would increase the chances of the reporter gene assay being successful.

The results from the first reporter gene assay were somewhat discouraging. In general, the β -gal numbers were larger than the numbers for the luciferase samples which was to be expected (Table 3). However, we were expecting numbers in the 1000s and 10000s for both the luciferase and β -gal samples. The luciferase sample numbers were generally higher for *RAI1* short plasmid than for the *RAI1* long plasmid. This indicated that there was a possibility that the *NcoI* restriction digest did make a difference in the amount of luciferase protein produced. The highest luciferase number was only 494 light units for *RAI1* short plasmid and 32 light units for the *RAI1* long plasmid. The low

luciferase numbers were attributed to a problem with the transient transfection assay efficiency.

In order to determine transfection efficiency and to use equipment available to at UMW, the β -gal reporter gene assay, that was spectrophotometric rather than luminescent, was performed. This assay involved transfection with pSV- β -gal, cell lysis, and measurement of promoter activity by measuring a color change in a spectrophotometer. The β -gal reporter gene assay showed higher absorbance numbers when pSV- β -gal plasmid was transfected versus when no pSV- β -gal was transfected (Table 3). This indicated that the cells were transiently transfected, and allowed for the elimination of transfection as a possible source of error. Since the transfection was working, this suggested to us that the low luciferase numbers observed in the first luciferase reporter gene assay were from background noise.

Since the conditions for the transfection assay were worked out, another luciferase reporter gene assay was performed (Table 4). Once again, the β -gal numbers were larger than the luciferase numbers, as expected. When comparing the first reporter gene assay (Table 2) to the current attempt, the β -gal numbers were improved indicating that the transfection was working well. Furthermore, the amount of plasmid transfected (1 μ g vs. 2 μ g) did not have an effect on the degree of luminescence in the luciferase samples. These results further led us to believe that the luciferase activity that was detected was due to background noise. The presence of low luciferase activity indicated one of two possibilities. There was either no promoter activity in the *RAII* promoter constructs or the luciferase part of the assay wasn't working.

In order to determine which of these possibilities was correct, the luciferase reporter gene assay was performed with plasmid DZ23 which has known promoter activity. In addition, the assay was performed with *RAII* long plasmid, *RAII* short plasmid, and β -gal plasmid (as a control). Plasmid DZ23 had significantly higher luminescence compared to the other three plasmids. This confirmed that DZ23 has promoter activity, but that the two *RAII* promoter constructs do not. Therefore, the *RAII* fragments we used did not contain the *RAII* promoter as we initially thought. One reason why we did not have any promoter activity in the *RAII* promoter constructs is that the sequence for the location of the 5' end of the *RAII* gene obtained from the Human Genome Project (from which these constructs were derived) may be inaccurate. If the sequence for the *RAII* promoter is incorrect, this would also explain why there was difficulty in amplifying the 2.3kb fragment.

5'RLM-RACE. The 5' end of the *RAII* sequence was originally obtained through the Human Genome Project. However, upon investigation of the origin of the sequence, it was discovered that the methods used to identify the 5' end weren't as accurate as 5'RLM-RACE [12]. In order to determine the true end of the *RAII* transcript, 5'RLM-RACE, a more precise method of determining the 5' end, was used. The 5'RLM-RACE protocol is also the first step in trying to determine the location of the *RAII* promoter experimentally. The purpose of 5'RLM-RACE was to amplify the 5' end of the RNA of *RAII*. The sequence determined from 5'RLM-RACE could then be aligned with the Human Genome sequence in order to develop primers to amplify the region of DNA directly upstream of the 5' end. In theory, the *RAII* promoter would be located upstream of the 5' end of the RNA of *RAII* allowing for the *RAII* promoter to be successfully

cloned. The results from the 5'RLM-RACE indicate that the bands present are just remains of the primers used. Experiments to date were unsuccessful in amplifying the 5' end of *RAI1*. Time constraints prevent troubleshooting this lengthy process.

Summary. The purpose of this project was to characterize the *RAI1* promoter using the reporter gene assay. In addition to performing the reporter gene assay, I attempted to clone the 2.3kb fragment of *RAI1*. Unfortunately, the attempts I made at cloning the 2.3kb fragment were unsuccessful so that portion of the project had to be abandoned. I then switched to working with a fragment of *RAI1* previously cloned by a member of Dr. Elsea's laboratory. I used this fragment in order to develop a shorter *RAI1* promoter construct for use in the reporter gene assay. From the results of the multiple reporter gene assays that I performed, I concluded that the *RAI1* promoter constructs I had did not contain any promoter activity. Therefore, the *RAI1* fragments that I had did not contain the *RAI1* promoter indicating that the sequence for the *RAI1* promoter is incorrect. Since the fragment I was working off of was developed from the sequence of *RAI1* from the Human Genome Project, I turned to looking at determining the location of the *RAI1* promoter experimentally. The first step in determining the promoter experimentally was 5'RLM-RACE which amplifies the 5' end of the RNA of *RAI1*. Both trials of 5'RLM-RACE were unsuccessful in amplifying the 5' end.

The experiments that I have performed throughout the course of this project have established a stepping stone for the next person that takes over my project. Due to the work I did with the reporter gene assay, we now know that the *RAI1* fragments previously cloned did not contain the *RAI1* promoter. Because of that finding, we can now move on to finding the actual location of the *RAI1* promoter.

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