A Well Known Truncation of INI-1

An Honors Thesis
By

Rebekah Sook

Ruhl Laboratory

Department of Biochemistry and Molecular Biology

05/06/2016

Abstract

Atypical Teratoid Rhabdoid Tumor (AT/RT) is a rare type of pediatric brain tumor that most commonly affects children under the age of 3. Being that the people who are primarily affected by this disease are so young, current treatments such as chemotherapy and radiation are not very effective at treating this cancer giving a bleak survival rate of less than 10%. More than 95% of patients with AT/RT are missing or severely lacking a protein from the SWI/SNF complex, INI-1, although the function of this protein is still unknown. There are several common truncations of INI-1 that are associated with AT/RT. Among the common truncations is the C-terminal fraction, INI-1 (186-385). The goal of this project is to successfully clone this 200 amino acid truncation into a vector so that the causation of AT/RT may be better understood. In order to clone, I first had to amplify the DNA using PCR, purify the product, perform restriction digests on both my gene of interest and the cloning vector, and successfully complete ligation and transformation.

Acknowledgements

I would like to express my appreciation to the faculty and staff who have helped to advise and guide me for the past three years as I worked on this project: Dr. Donald Ruhl, Ms. Judy Hall, and Dr. Jessica Matts. I want to extend a special thanks to Dr. Ruhl who graciously allowed me to work in his lab as such a young undergraduate. For the past few years he has served as my mentor teaching me, supporting me, and never giving up on me or allowing me to give up on myself. Additionally, I would like to thank Dustin Steele, a graduate student who has helped me in countless ways and undoubtedly contributed to my success. Finally, I would like to thank Leah Underwood and Elizabeth McMurray who I worked very closely with and who assisted me in some of the experiments.

Introduction

Central Nervous System (CNS) Atypical Teratoid/ Rhabdoid Tumor (AT/RT) is a rare type of pediatric cancer in which tumors form on the brain and spinal cord. These tumors account for 1-2% of all pediatric brain tumors. This fast growing malignant tumor often proves deadly, having a depressing survival rate of less than 10% using current treatment options such as chemo-radiation and surgical removal. While the majority of people who have AT/RT are under the age of three years old, it can occur in older children and adults as well [1, 4]. By gaining a better understanding of this disease, and more importantly what some possible reasons are for it, better treatment options could be produced, and in long term, maybe even a cure.

This disease has only been classified within the past 15-20 years. It is commonly misdiagnosed as Primitive Neuroectodermal Tumor (PNET), the most common type of pediatric brain tumors. With similar symptoms and appearance, immunohistochemistry can be done on removed sample of tissue to determine if it is AT/RT. Immunohistochemistry works by using antibodies to check for certain types of antigens in a sample. AT/RT can be determined by the lack of INI-1 protein [3].

It has been observed that greater than 95% of all AT/RT cases lack all or part of the INI-1 protein. This data establishes a potential link between INI-1 and AT/RT, and is the only strong correlation found thus far. The INI-1 protein works in a complex or group of proteins known as SWI/SNF [2]. Specifically, this protein complex is a chromatin remodeling complex which preforms multiple biological functions such as roles in genetic expression and DNA replication

and repair. Although this protein complex has been studied extensively, little is known about INI-1. While the function, location within the complex, and appearance of this protein is still unknown, INI-1 has been observed to be absent, or seriously truncated in tumor cells among children with AT/RT, while all of SWI/SNF's other proteins remain present. This information establishes a correlation between missing INI-1 proteins and AT/RT.

Rationale for Study and Experimental Goal

While the observation has been made that INI-1 is absent of severely truncated in the majority of cases of AT/RT, it is not known whether or not the loss/ truncation of INI-1 causes the disease to occur and the tumors to grow, or if the disease causes the loss/truncation of INI-1. This cannot simply be "checked" as this would be an invasive procedure which is unnecessary in healthy individuals. So, by cloning known truncations, and using a full length INI-1 and an absent INI-1 as controls, we can eventually insert them into AT/RT tumor cells to see how the tumor and/or the INI-1 is effected, this question, along with other, can be answered.

Goal: My goal is to successfully clone the well-known truncation of INI-1 (186-385) into a cloning vector, pBABE, so that it can be inserted into tumor cells in the future.

Methods

After thorough research on the topic of Atypical Teratoid/ Rhabdoid Tumor (AT/RT), and the protein INI-1, I chose a common truncation of amino acids 186-385 to work with. Using the National Center for Biotechnology Information (NCBI) website, I determined the nucleic acid base pairs which code for the INI-1 protein. I was then able to use the codons of these base pairs to translate this sequence into the amino acid sequence of full-length INI-1. From here, I cut out the amino acids 1-185, leaving me with my chosen truncation, amino acids 186-385 (*Figure 1; Appendix 1*).

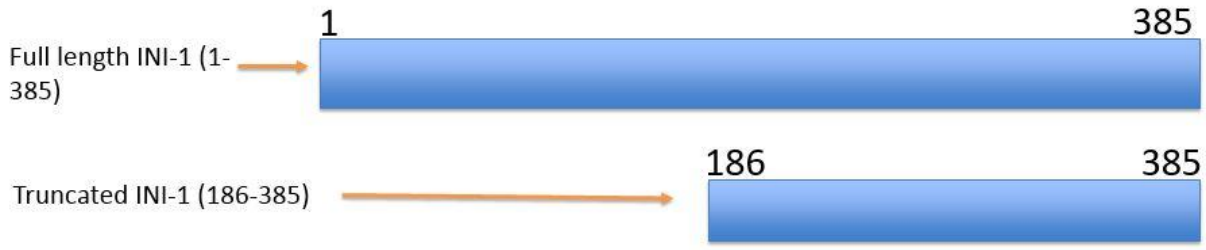


Figure 1: Depiction of chosen truncation [INI-1(186-385)] as compared to full-length INI-1

Primer Design and PCR

With the amino acid sequence for the truncation (186-385), I was able to use bioinformatics to design primers which would cut out my desired truncation from the full length sequence. A primer is a strand of nucleic acid that serves as a starting point for DNA synthesis. Primers used in laboratory techniques, such as polymerase chain reaction (PCR), are usually short, chemically synthesized oligonucleotides which are hybridized to target DNA, which is then copied by the polymerase. My primers consisted of the primer itself (18 base pairs shown in

red) and 3 nucleotides (shown in yellow) followed by a restriction site (6 base pairs shown in blue). My primers are as follows: Primer F, 5'-CGGGAATTCCTGGTCCCCATCCGGCTG-3', which comes from the first 18 base pairs of the gene and has an EcoR1 restriction site, and Primer R, 5'-CGGGGATCCCCAGGCCGGGGCCGTGTT-3', which comes from the last 18 base pairs and has a BamH1 restriction site. The purpose of adding the restriction site onto the primer is for future use when cleaning up and digesting the protein. The gene of interest [INI-1 (186-385)] does not have the specific sequence of these restriction sites, which ensures that the truncated gene will not be cut into smaller pieces. By adding the restriction site sequences to the 5' end of the primers, it is possible to cut the gene of interest out without losing any of the gene. After I had completed the design, I ordered the primers *(For full primer design, see Appendix 1).*

10X TAQ buffer, nanopure H2O, 10 mmol dNTPs, the two primers mentioned above, 0.1 M MgCl2, and the DNA template for INI-1 were used in the PCR mixture. PCR was then run to cut the DNA and produce the fragment. This process, developed in 1983 by Kary Mullis, enables researches to produce millions of copies of a specific DNA sequence, a previously tedious process, in approximately 2 hours. Specific primers, the primer R and primer F mentioned above, are used to replicated target DNA. This reaction employs heat cycles to denature, apply primers, and essentially clone the desired DNA sequence.

Once PCR was complete, I inserted the amplified sample of truncated INI-1 and a Kb+ ladder into a 1.5% agarose gel made in TAE buffer and 2 µL of Ethidium Bromide (EtBr). BPB loading dye and nanopure water were added to the samples for loading and visibility purposes. This addition did not affect the length of the fragment. Gel electrophoresis, a method for the analysis and separation of DNA and other macromolecules and their fragments, was run for approximately half an hour (when the loading dye was visibly half way down the gel). By using

a Kb+ ladder as a guide, gel electrophoresis was used to determine the length of the DNA fragment obtained through PCR, which was expected to be 600 amino acids in length causing to show up just below the 650 line in the KB+ ladder (*Figure 2).*

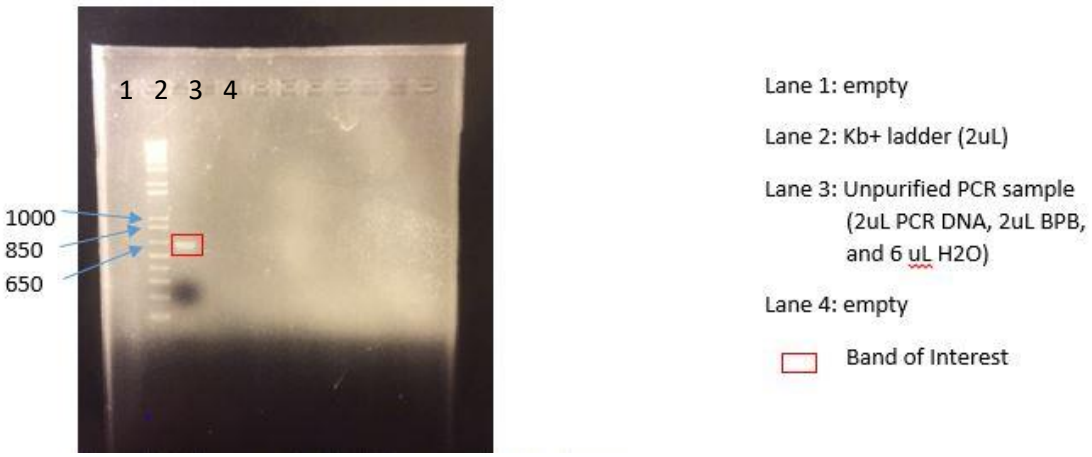


Figure 2: 1.5% Agarose gel made with TAE and EtBR.
This gel was run at 140V for approximately 40 minutes.

Isolation of PCR product

The PCR amplified DNA (450 uL) was cleaned and isolated using phenol extraction and ethanol precipitation. An equal volume of phenol was added to the PCR sample. This mixture was vortexed for 15 seconds, then placed in a centrifuge, balanced, and spun at 13,000 rotations per minute (rpm) for five minutes. The top layer was removed via micropipette and placed into a clean microfuge tube. Next, equal volumes of pre-made ½ phenol, ½ chloroform stock was added to the tube. This mixture was vortexed for 10 seconds and then placed in a balanced centrifuge. The tube was spun at 13,000 rpm for 5 minutes. The top layer was taken and placed in a clean microfuge tube via micropipette. An equal amount of chloroform was added and the mixture was vortexed for 10 seconds. This was again centrifuged at 13,000 rpm for 5 minutes. The top layer was removed and placed into a 1.5 mL microfuge tube. 1/10 of the total volume of

3 M NaOA was measured and added to the tube. Once this was mixed, 2.5 volumes of 95% EtOH was added and mixed by inversion 8 times. The entire mixture was centrifuged at 13,000 rpm for 15 minutes. A pellet was formed in the bottom of the tube. The supernatant was removed. 500 uL of 70% ethanol was added. This mixture was centrifuges at 13,000 rpm for 10 minutes. The supernatant was removed and it was again centrifuged at 13,000rpm for 1 minute. Remaining EtOH was removed from the tube and 30 uL of sterile water was added to re-suspend the pellet. Isolated DNA was NanoDropped and ran on a 1.5% agarose gel made with TAE. The NanoDropped sample was a concentration of 593.6 ng/uL with a 260/280 of 1.63 meaning that the sample was concentrated enough, and pure enough to go on. The gel showed the purified PCR to be just below the 650 bp mark in the Kb+ latter and therefore, it was assumed that the band was just as expected in the purified PCR sample, right at 600 bp.

Restriction Digest

Restriction Digests are done in preparation of traditional cloning. Before I began my restriction digest I tested the enzymes to ensure that they were cutting properly, and tested the vector which was selected to clone into. The restriction enzymes which I used were EcoR1 and BamH1 (*Figure 3; Figure 4*). Restriction enzymes are used to cut DNA. They can yield two different types of cuts which would yield either sticky ends (*as shown in figure 2 and 3*) or blunt ends. They cut at a 6 amino acid restriction site which is specific to each enzyme. The chosen vector, which I will clone into is pBABEpuro. This is a vector which is engineered with multiple restriction sites as well as an Ampicillin Resistance gene (*Figure 5*).

Figure3: EcoR1 restriction site showing cut sites; Image was derived using Google Images [5].

Figure 4: BamH1 restriction site showing cut sites; Image was derived using Google Images [5].

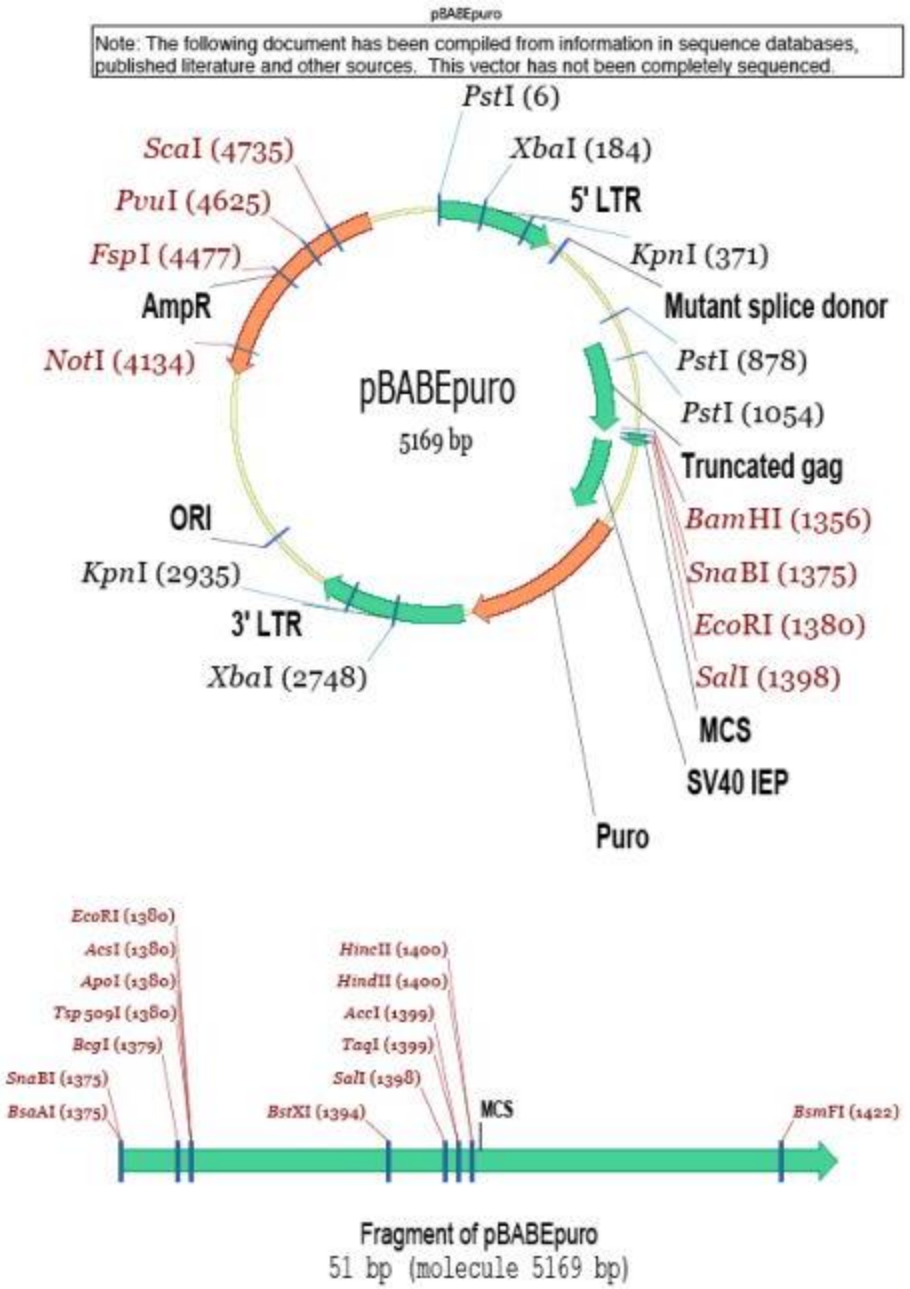


Figure 5: pBABEpuro vector; Figure was derived using Google Images [6].

Restriction digest was performed on both the amplified, truncated INI-1 (187-385) and the vector (pBABE). 10 uL of INI-1 was placed in tube one with 1.5 uL of BSA, 1.5 uL of 10X buffer 4, 1.0 uL of Bam-HI, and 1.0 uL of EcoR1. Tube 2 consisted of 10 uL of pBABE diluted

to 100 ng/uL, 1.5 uL of 10X Buffer 4, 1.0 uL of BamH1, and 1.0 uL of EcoR1. The entire procedure was done on ice, and ezymes were kept on ice throughout this experiment. Both tubes were incubated in a hot water bath at 37˚C for one hour and heat killed for 20 minutes at 80˚C to inactivate the BamH1 enzyme.

The vector, pBABE was not cleaned like the PCR sample, after it was heat inactivated and NanoDropped, it was stored in the -20˚C freezer until needed for ligation. The NanoDrop showed that the digested pBABE had a 260/ 280 value of 0.72. While this is low, I suspect it is due to the proteins still in solution as it was not cleaned up. INI-1 was cleaned up using gel filtration.

Agarose Gel Purification

In gel filtration, the agarose gel cannot use EtBr as a stain because it is too harsh and will denature DNA. Since in gel purification the DNA band of interest is cut out in order to harvest the DNA, SYBR safe will be used as the staining agent for the gel in place of EtBr. A 1% agarose gel using TAE and 3 uL of SYBR safe was used for this procedure (*Figure 7*). In order to have a large well for the band of interest since the band will be harvested from the gel, tape was used on the comb (*Figure 6*).

Figure 6: Example of comb for gel purification gel-mold

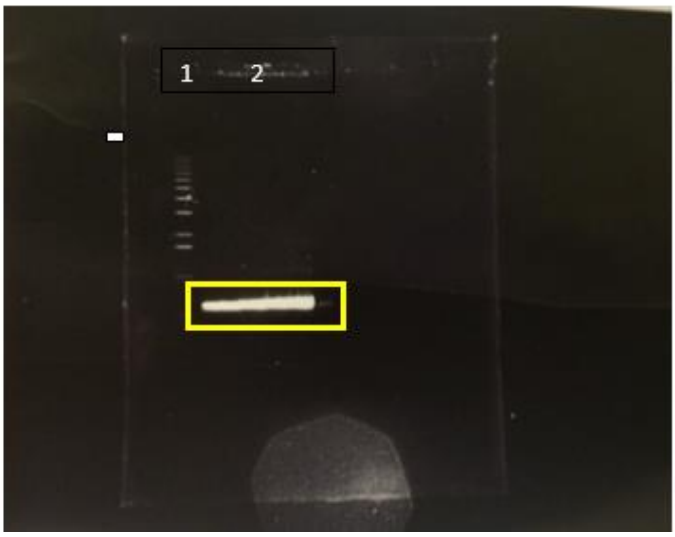


Figure 7:1% agarose gel made with TAE and SYBR Safe; Gel was run at 140V for 40 minutes

The band containing INI-1 was carefully cut under UV light (*Figure 7*). The band was then sliced into small pieces and placed into the gel purification apparatus (*Figure 8*). Once the cut band was placed into the blue insert, the cap was placed on, and the entire apparatus was place into the centrifuge using an empty microfuge tube as a balance. This was spun for 15 minutes at 3,000 rpm. The low rpm was to ensure that the gel remained in the top compartment and did not escape into the tube through the filter. The 1.5 mL centrifuge tube was removed, capped, and labeled "supernatant 1." A new tube was placed at the bottom of the apparatus and 50 mL of TAE was added to the top blue compartment. This was once again spun down at 3,000 rpm for 15 minutes

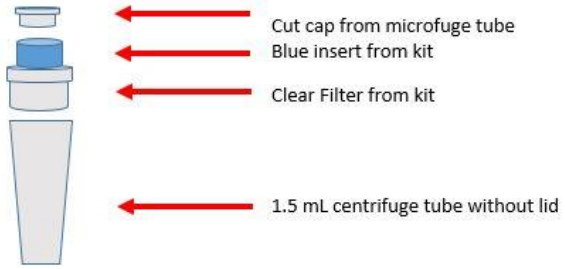


Figure 8: Gel Purification apparatus

to ensure that all the DNA was extracted from the gel. This tube was capped and labeled "supernatant 2." The NanoDropped Supernatant 1 yielded 28.4 ng/uL. Gel-purified pBABE which had been previously successful in cloning was also NanoDropped to be used in ligation and was measured at 28.4 ng/uL. The tubes were stored at -20˚C to await ligation.

Ligation

Ligation is a temperature sensitive cycle which employs the enzyme ligase to glue cut DNA back together. This enzyme is manipulated and used in the lab in cloning as it helps to glue a gene of interest into a vector.

Using the NEBio Ligation Calculator, dilutions for ligations were calculated. Tube contents can be seen in Table 1.

	Tube 1	Tube 2	Tube 3	Tube 4
pBABE	2.0	1.0	3.0	2.0
INI-1	2.0	3.0	1.0	---
Autoclaved Water	4.0	4.0	4.0	6.0
Ratio (vector:insert)	1:1	1:3	3:1	control

Table 1: Ligation dilutions and tube contents. All amounts are given in uL.

All tubes were tapped to mix and placed in water bath to incubate at 65˚C for 5 minutes. After incubation, tubes were cooled at room temperature for 5 minutes. 10X ligation buffer was warmed to 45˚C for a few minutes to ensure that it all was in solution. 1 uL of 10X ligation buffer and 1 uL of T4 DNA Ligase were added to each tube. Tubes were tapped to mix and sat at room temperature overnight. All tubes were then stored at 4˚C until transformation.

Transformation

Transformation is a very temperature sensitive processes which is used to introduce vectors into competent bacteria cells. Competent cells are E. coli cells which are able to take up exogenous DNA (7). They are very important in transformation because they can be manipulated by cooling and heat shocking which causes them to take up DNA which can then be plated on agar plates.

	T1	T2	T3	T4	Control
Ligated	5.0	5.0	5.0	5.0	1 ul conc. pBABE+4ul Autoclaved Water
DH5α (Competent Cells)	20.	20.	20.	20.	20.

Table 2: Transformation tubes (number correlate to the tubes previously listed in table 1). Amounts listed in the table are measured in uL.

Transformation of all 5 tubes listed in Table 2 (T1, T2, T3, T4, and Control) were iced for 30 minutes, heated for one minute at 42˚C (heat-shock), and then iced for 3 minutes. 200 uL of Luria Broth (LB) was added to each tube and mixed by flicking. Tubes were plated on Agar + Ampicillin (AMP) plates labeled to correspond to each tube using aseptic technique. Ampicillin was added to the plates to ensure that bacteria who did not take up the vector could not survive and thrive. Bacteria which did contain the vector had a resistance to the antibiotic ampicillin because of the AMP resistant gene in pBABE. This allowed competent bacteria which took in the vector to able to make colonies. By using antibiotic and the engineered antibiotic resistance gene in the cloning vector, successful transformations can be differentiated from unsuccessful transformations allowing me to know which transformation could contain my gene of interest. Plates were incubated upside down at 37˚C for 24 hours.

After 24 hours colonies were assessed and counted. As expected, the positive control with consisted only of uncut pBABE and water yielded a lawn of colonies, and the negative ligation control yielded few colonies. This showed that the transformation was successful and that there was a little background of either uncut pBABE or rejoined pBABE which did not contain the gene of interest. However the other plates contained very few colonies (*Figure 9*).

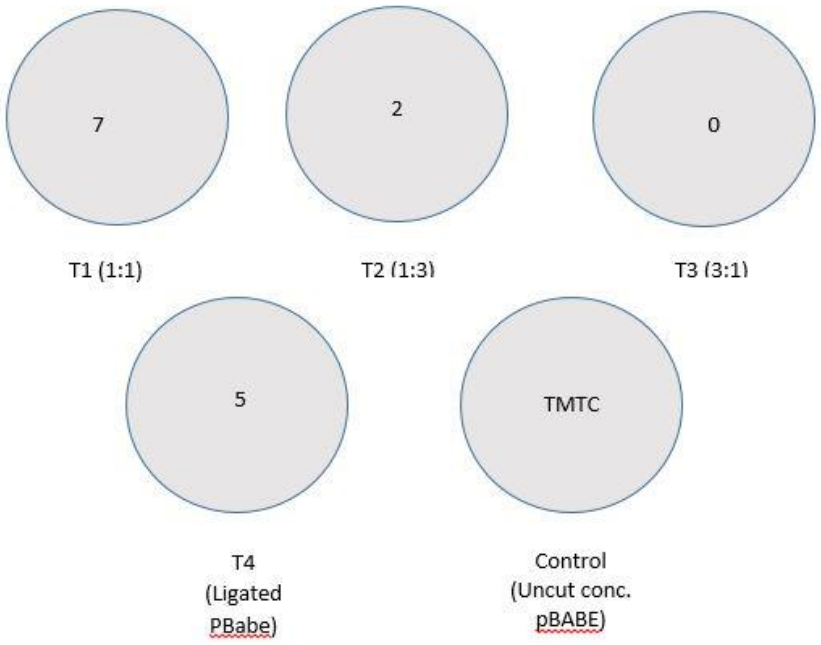


Figure 9: Agar+AMP plates after 24 hours T4 plate served as the negative control and served as "background," meaning that it showed the number of colonies which could simply be ligated pBABE on the other plates, having no insert. The control plate served as the positive control which showed that the transformation was successful.

With so few colonies, the colony growth on plates T1, T21, and T3 did not meet the expectations that I had. I thought that I would have more than one plate which produced more colonies than that of the negative control since the negative control account for any "background," or pBABE that does not contain the gene of interest (my INI-1 truncation). This was only the case on one plate which contained 7 colonies, giving reason to believe that 2 may contain the gene of interest. Despite my unexpected results, I continued on, harvesting all 9

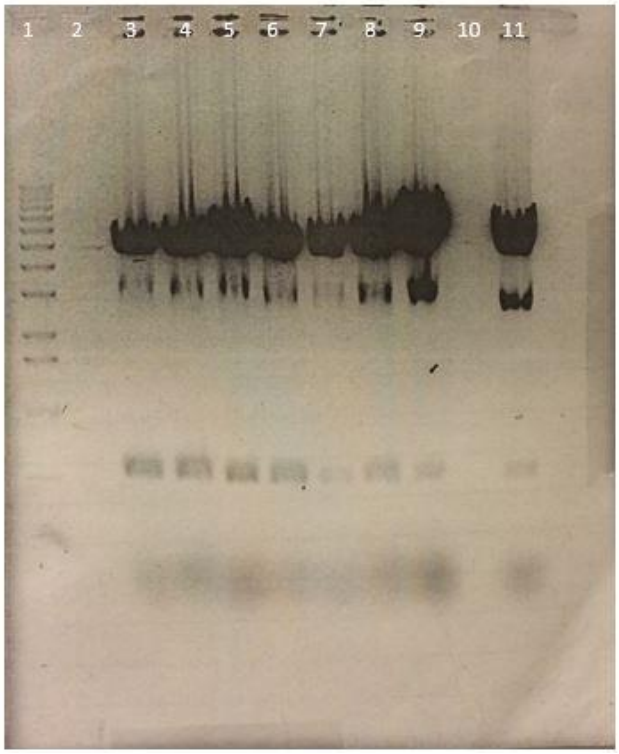
possible colonies. The colonies were stuck with clean, autoclaved micropipette tips under ascetic conditions, and placed into tubes each containing 5mL of LB+AMP. Tubes were labeled 1-9, and placed in an incubator, shaking at 37˚C for 24 hours. When tubes were removed from the incubator, 1.5 mL from each tube was transferred into clearly labeled microfuge tubes and spun down in the centrifuge at 4,000 rpm for 4 minutes. A pellet of bacteria formed in the bottom. Supernatant, which did not contain the DNA, was removed.

100 uL of solution 1 was added to each tube to resuspend the pellets. 200 uL of solution 2 was added next and the tubes were mixed by inversion. Solution 3 was then added (150uL) and mixed immediately by inversion. This solution was placed in a balanced centrifuge and spun at 13,000 rpm for 10 minutes. This was done so that the protein and chromosomal DNA would form a pellet at the bottom, leaving the plasmid in the supernatant. Isopropanol was added to the remaining supernatant, along with glycoblue. This solution was centrifuged to form a pellet of the desired DNA. The supernatant was removed and the wanted DNA was suspended using 50 uL of autoclaved water.

10 uL of each of the newly made 9 microfuge tubes (containing wanted DNA suspended in water) were taken and digested using previously listed Restriction Digest method. The digested DNA was run on an agarose gel (*Figure 10*).

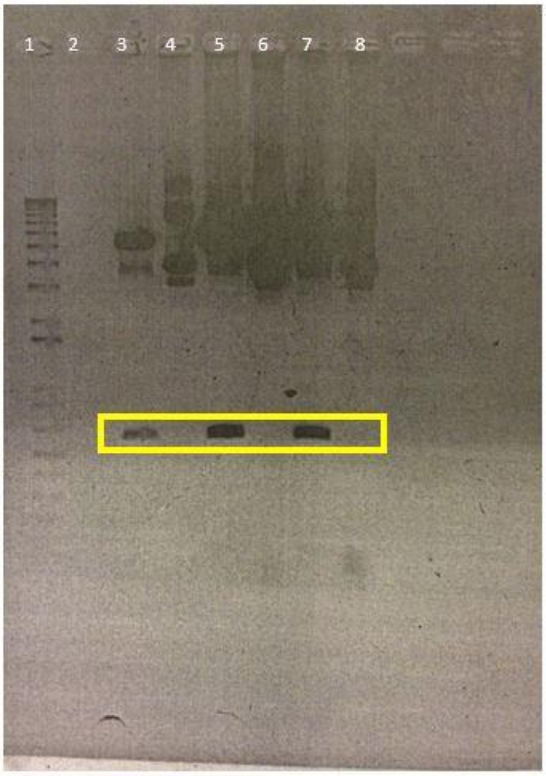
The gel was unusual in that lanes contained 3 bands. I had expected a successful cloning lane to have 2 bands and an unsuccessful lane to contain one band. After further analysis, I thought that this third band might be a successful clone that was not cut by the restriction enzyme. 8 of the 9 lanes contained a band that appeared to be in the correct spot. The only one that did not show this band was tube 8. Since it was impossible to determine if this was the correct band because I was unable to fully decipher the Kb+ latter because it was so faint, I ran a

second gel. This time, during the restriction digest I used excess enzymes and used uncut controls in the gel in addition to the newly digested DNA (*Figure 11*). This revealed that one of the three bands on the previous gel could very well be a successful clone.



Lane 1: Kb+ ladder
Lane 2: Empty Lane
Lane 3: Tube 1
Lane 4: Tube 2
Lane 5: Tube 3
Lane 6: Tube 4
Lane 7: Tube 5
Lane 8: Tube 6
Lane 9: Tube 7
Lane 10: Tube 8
Lane 11: Tube 9

Figure 10: 1.5% Agarose gel made with TAE and run at 140V for 40 minutes. Ladder was faint and gel was smeared. I assume that this occured in the staining process



Lane 1: Kb+ ladder

Lane 2: Empty lane

Lane 3: Tube 1

Lane 4: Tube 1 uncut control

Lane 5: Tube 2

Lane 6: Tube 2 uncut control

Lane 7: Tube 3

Lane 8: Tube 3 uncut control

Bands of interest

Figure 11: 1.5% Agarose gel made with TAE and run at 140V for 40 minutes

With the conformation from the second gel that the lowest band may contain a clone of interest, tubes 1, 2, 3, 4, 5, 6, 7, and 9 were sent off for sequencing.

Results

Of the 8 tubes that were sent off for sequencing, one contained the successful clone (*for sequence see appendix*). Tube 1, the tube containing successful clone, was transformed into liquid media, grown up at 37˚C overnight, and saved as a cryovial for future use.

Discussion and Future Directions

AT/RT is a devastating disease which all too often takes the lives of children before they have even had a chance to live them. It has been found that the severe truncation or lack in INI-1

plays a major role in this rare form of pediatric brain tumors. INI-1's role is still unclear, and it is not known whether or not the lack of a functional protein causes the disease, or if the disease causes INI-1 to be absent or severely truncated. By successfully cloning a common truncation of INI-1, we are one step closer to determining this. Using my clone, along with other clones of known truncations and the full length clone, they can be inserted into cells, providing a starting place to figure out more about this protein and this disease which will hopefully, years down the line, lead to lives being saved.

The Ruhl laboratory has now successfully cloned three known truncations of INI-1 as well as the full-length INI-1. These clones should now be inserted into AT/RT tumor cells and changes should be observed. Several assays should be done including growth and proliferation assays and biochemical assays such as an apoptosis assay.

I expect that when growth and proliferation assays are done in a side by side comparison of a full-length INI-1 control, a vector only control (contains no INI-1), and my truncation clone, the cells which contain no INI-1 will continue to grow and proliferate at a high rate similar to that seen in patients who have AT/RT. I believe that the protein which will have the full-length INI-1reintroduced will have a slowing or even halting of the growth and proliferation of the tumor cell. I hypothesize that the cells which have my truncated INI-1 introduced to them will continue to grow and proliferate similar to that of the vector only cell because the tumor suppressing part of the protein is on the front half of the protein. While INI-1's shape and function is not known, I believe this based on the trend that patients with this specific truncation generally have a severe case of AT/RT similar to that of someone who is completely missing INI-1.

This will hopefully help to determine whether or not the lack of or truncation of INI-1 causes the tumors, or if the tumors causes the truncation and absence. Additionally, this might help determine what part of the protein is responsible for specific functions since the three truncations we currently have are all different and would in turn offer a different location of the gene present. For example, maybe having the front half of the protein would lead to a normalcy and the person would not have tumors, but if you are missing the front portion, it would cause the same degree of tumors as if the protein were completely absent. Much more research needs to be done on INI-1, its truncations, and its correlation with AT/RT.

Bibliography

1. Cellular Classification and Tumor Biology of Childhood Central Nervous System Atypical Teratoid/Rhabdoid Tumor." *National Cancer Institute*. N.p., 2 Apr. 2013. *Google Scholars*. Web. 18 Nov. 2013. http://www.cancer.gov/types/brain/hp/child-cns-atrt-treatment-pdq.
2. Modena, Piergiorgio, Iacopo Sardi, Monica Brenca, Laura Giunti, and Anna Maria Buccoliero. "Case Report: long-term survival of an infant syndromic patient afftected by atypical teratoid-rhabdoid tumor." *BMC Cancer* 13.100 (2013). *Pub Med*. Web. 10 Sept. 2013.
3. Ginn, Kevin F., and Amar Gajjar. "Atypical teratoid rhabdoid tumor: current therapy and future directions." *frontiers in oncology* 2.114 (2012): 1-13. *Pub Med*. Web. 15 Jan. 2013. <www.frontiersin,org>.
4. "Atypical Teratoid / Rhabdoid Tumor (ATRT) ." *St. Jude's Children's Research Hospital*. St. Jude's Children's Research Hospital, n.d. Web. 29 Apr. 2016.
5. *Sigma-Aldrich*. MilliporeSigma, n.d. Web. 29 Apr. 2016. <http://www.sigmaaldrich.com/catalog/product/sigma/r0260?lang=en®ion=US> . *Addgene*. Addgene, n.d. Web. 29 Apr. 2016. <https://www.addgene.org/1764/>.
6. *Biology Online Dictionary*. Biology Online, n.d. Web. 29 Apr. 2016. <http://www.biology-online.org/dictionary/Competent_cell>.

Appendices

Appendix 1:

Primer Design INI-1 (186-385)

DNA Sequence: INI-1 (186-385)

tgctggtccc catccggctg
gacatggaga tcgatgggca gaagctgcga gacgccttca cctggaacat gaatgagaag
ttgatgacgc ctgagatgtt ttcagaaatc ctctgtgacg atctggattt gaacccgctg
acgtttgtgc cagccatcgc ctctgccatc agacagcaga tcgagtccta ccccacggac
agcatcctgg aggaccagtc agaccagcgc gtcatcatca agctgaacat ccatgtggga
aacatttccc tggtggacca gtttgagtgg gacatgtcag agaaggagaa ctcaccagag
aagtttgccc tgaagctgtg ctcggagctg gggttgggcg gggagtttgt caccaccatc
gcatacagca tccggggaca gctgagctgg catcagaaga cctacgcctt cagcgagaac
cctctgccca cagtggagat tgccatccgg aacacgggcg atgcggacca gtggtgccca
ctgctggaga ctctgacaga cgctgagatg gagaagaaga tccgcgacca ggacaggaac
acgaggcgga tgaggcgtct tgccaacacg gccccggcct gg

Amino Acids: INI-1 (186-385)

LVPIRLDMEIDGQKLRDAFTWNMNEKLMTPEMF
SEILCDDLDLNPLTFVPAIASAIRQQIESYPTDSILEDQSDQRVIIKLNIHVGNISLV
DQFEWDMSEKENSPEKFALKLCSELGLGGEFVTTIAYSIRGQLSWHQKTYAFSENPLP
TVEIAIRNTGDADQWCPLLETLTDAEMEKKIRDQDRNTRRMRRLANTAPAW

Primer (without Restriction Sites):

F: ACG ACC AGG GGT AGG CCG

R: TTG TGC CGG GGC CGG ACC

Primer (With Restriction Sites):

Appendix 2:

INI-1 (186-385) Clone Sequence:

Primer: pBABE-F plasmid clone Sequenced: #1

AAAGTTCGGTCGGATCCTGGTCCCATCCGGCTGGACATGGAGATCGATGGGCAGAAGCTGCGAGACGCCTTCACC
TGGAACATGAATGAGAAGTTGATGACGCCTGAGATGTTTTCAGAAATCCTCTGTGACGATCTGGATTTGAACCCGC
TGACGTTTGTGCCAGCCATCGCCTCTGCCATCAGACAGCAGATCGAGTCCTACCCCACGGACAGCATCCTGGAGGA
CCAGTCAGACCAGCGCGTCATCATCAAGCTGAACATCCATGTGGGAAACATTTCCCTGGTGGACCAGTTTGAGTG
GGACATGTCAGAGAAGGAGAACTCACCAGAGAAGTTTGCCCTGAAGCTGTGCTCGGAGCTGGGGTTGGGCGGG
GAGTTTGTCACCACCATCGCATACAGCATCCGGGGACAGCTGAGCTGGCATCAGAAGACCTACGCCTTCAGCGAG
AACCCTCTGCCCACAGTGGAGATTGCCATCCGGAACACGGGCGATGCGGACCAGTGGTGCCCACTGCTGGAGACT
CTGACAGACGCTGAGATGGAGAAGAAGATCCGCGACCAGGACAGGAACACGAGGCGGATGAGGCGTCTTGCCA
ACACGGCCCCGGCCTGGTAAGAATTCGGGAGATCTCCCATCGATGGGGTCGACCCTGTGGAATGTGTGTCAGTTA
GGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGG
TGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTC
CCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTT
TTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCC
TAGGCTTTTGCAAAAGCTTACCATGACCGAGTACAAGCCCACGGTGCGCCTCGCCACCCGCGACGACGTCCCCAG
GGCCGTACGCCACCCTCGCGCCGCGTTCGCGACTACCCCGCCACGCGCCACACCGTCGATCCGGGACCGCCACATC
GAGCGTCCCCGGAGCTGCAAGACTCTTTCCTCCACGCGCGTCGGCTCGACATCGGCAGTGTGTCCGGACAGACGG
GTCTCCGGGTGGAGCGGCTCTGGACACACCCCCGGAGAAGAA

Primer: pBABE-R plasmid clone Sequenced: #1

GTGAAAGGACTCGATGGTGACTCCCGATTCTTACCAGGCCGGGGCCGTGTTGGCAAGACGCCTCATCCGCCTCGT
GTTCCTGTCCTGGTCGCGGATCTTCTTCTCCATCTCAGCGTCTGTCAGAGTCTCCAGCAGTGGGCACCACTGGTCCG
CATCGCCCGTGTTCCGGATGGCAATCTCCACTGTGGGCAGAGGGTTCTCGCTGAAGGCGTAGGTCTTCTGATGCCA
GCTCAGCTGTCCCCGGATGCTGTATGCGATGGTGGTGACAAACTCCCCGCCCAACCCCAGCTCCGAGCACAGCTTC
AGGGCAAACTTCTCTGGTGAGTTCTCCTTCTCTGACATGTCCCACTCAAACTGGTCCACCAGGGAAATGTTTCCCAC
ATGGATGTTCAGCTTGATGATGACGCGCTGGTCTGACTGGTCCTCCAGGATGCTGTCCGTGGGGTAGGACTCGAT
CTGCTGTCTGATGGCAGAGGCGATGGCTGGCACAAACGTCAGCGGGTTCAAATCCAGATCGTCACAGAGGATTTC
TGAAAACATCTCAGGCGTCATCAACTTCTCATTCATGTTCCAGGTGAAGGCGTCTCGCAGCTTCTGCCCATCGATCT
CCATGTCCAGCCGGATGGGGACCAGGGATCCGGCCGGCGCCTAGAGAAGGAGTGAGGGCTGGATAAAGGGAGG
ATCGAGGCGGGGTCGAACGAGGAGGTTCAAGGGGGAGAGACGGGGCGGATGGAGGAAGAGGAGGCGGAGGC
TTAGGGTGTACAAAGGGCTTGACCCAGGGAGGGGGGTCAAAAGCCAAGGCTTCCCAGGTCACGATGTATGGGAC
CTGGTCTGGGTGTCCATGCGGGCCAGGTGAAAAGACCTTGATCTTAACCTGGGTGATGAGGTCTCGGTTAAAGGT
GCCGTCTCGCGGCCATCCGACGTTAAAGGTTGGCCATTCTGCAGAGCAGAAGGTAACCCAACGTCTCTTCTTGACA
TCTACCGACTGGTTGTGAGCGATCCGCTCGACATCTTTCCAGTGACCTAACGTCAACTTAAGGGAGTGGTAACAGT
CTGGCCCTATTTTCAGAACAATACAGGAAAACACAGGTCAGACGAGACAACACAGACGATGCTGCAGCGCTGCAG
CAGAACAAGAACGCGCGGCTCGATCCAACCGAAGCAAATTCGACGAGCGGAAGCTGTTTTAGCTTCTCGATCTCC
TAACCCGGAAGCCCACAGAC