CLONING AND GENERATION OF A MURINE MODEL OF GLYCEROL-3-PHOSPHATE DEHYDROGENASE 1-LIKE GENE, A CAUSE OF THE BRUGADA SYNDROME?

by

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Cardiovascular disease (CVD) is a major public health concern. It is the Nation's leading killer for both men and women of all racial and ethnic groups. CVD is responsible for about 1 million deaths each year in the United States. Health-related behaviors such as smoking, lack of physical activity and poor nutritional habits, as well as, many genetic factors contribute to its high incidence. Many of the genetic factors have been linked to high cholesterol, high blood pressure, obesity, diabetes and cardiac arrhythmias leading to stroke or sudden cardiac death. CVDs associated with ventricular arrhythmias are most severe. Among these is the Brugada syndrome also known as Sudden Unexpected Death Syndrome or SUDS. In 1992, the Brugada syndrome was classified as a distinct clinical heart condition characterized by an apparent right bundle branch block and ST segment elevation in the right precordial electrocardiogram (ECG) leads V_1-V_3. It is the most common cause of sudden cardiac death in South Asian men who are less than 50 years of age and have no underlying cardiac disease. Currently the only effective treatment for the disease is the Implantable Cardioverter Defibrillator (ICD) surgically placed in the patient's chest. The genetic basis for the Brugada syndrome has been linked to mutations in the SCN5A gene that codes for the alpha-subunit of the cardiac sodium channel. Recently, a missense mutation has been discovered in a novel gene that causes the Brugada syndrome. The novel gene is named the Glycerol-3-phosphate Dehydrogenase 1-Like (GPD1L) gene. Preliminary studies suggest a direct relationship between the GPD1L mutation and a decrease in cellular sodium current. Transgenic murine models are useful tools for understanding the molecular function of novel genes. Transgenic constructs of the wild type and mutant GPD1L gene were generated and used for the production of transgenic mice. The mice were produced by pronuclear injection at the University of Pittsburgh Transgenic facility. These mice will provide

an *in vivo* approach to study the GPD1L gene and create the first Brugada syndrome mouse model for cardiovascular disease studies.

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LIST OF ABBREVIATIONS

bp – Base Pairs

cM – centimorgan; 1,000,000 base pairs

CIP - Calf Intestinal Phosphatase

DS – Double Stranded

ECG – Electrocardiogram

ETBR – Ethidium Bromide staining

GPD1L – Glycerol-3-Phosphate Dehydrogenase 1-Like

HGH – Human Growth Hormone

ICD - Implantable Cardioverter Defibrillator

MCS – Multiple Cloning Site

MHC – Myosin Heavy Chain Promoter

MUT – *pBluescriptGPD1L* _A280V Mutant

MUT1 – pBK-*CMVGPD1L*_A280V mutant

MUT2 - *α-MHCpBluescript II SK+GPD1L_A280V* mutant

PCR – Polymerase Chain Reaction

RE – Restriction Endonuclease

SCD – Sudden Cardiac Death

SCN5A – Sodium Channel Na1.5; Alpha Subunit

SS – Single Stranded

in-vivo **-** living system

WT – *pBluescriptGPD1L* _A280A wildtype

WT1 – *pBK-CMVGPD1L*_A280A wildtype

WT2 - α-*MHCpBluescript II SK+GPD1L_A280A* wildtype

PREFACE

A special thanks to Dr. Barry London, PhD; MD Chief of Cardiology and Director of CVI at UPMC for the opportunity to perform this project in his laboratory. I would also like to thank Kenny Rahl, BS for his help in performing the Southern Blot analysis for this study. Lastly I would like to thank and acknowledge the involvement of the University of Pittsburgh Transgenic Facility for their participation in producing the transgenic mice.

1.0 INTRODUCTION

The Brugada syndrome is a rare familial autosomal dominant cardiovascular disorder with variable phenotypic expression (1, 4, and 12). In 1992 it was first clinically described as a syndrome of recurrent arrhythmias and sudden cardiac death (SCD). The Brugada Syndrome is associated with apparent cardiac right bungle branch block and ST segment elevation in the precordial electrocardiogram (ECG) leads V_1-V_3 (4). About 20-50% of patients have a family history of SCD (4, 12). In Southeast Asia the Brugada syndrome is the most common cause of SCD in young adults with otherwise normal hearts (7). The disease is more prevalent in men than in women (1). Currently the only effective treatment for the Brugada syndrome is an implantable Cardioverter Defibrillator (ICD) (5, 6), although the effectiveness of this treatment is controversial (8). In the past, many drug therapies have been used with no long term efficiency in preventing SCD and some are unfortunately related to an acquired form of the Brugada syndrome (9, 13). With the occurrence of sudden cardiac death being the only apparent presence of the syndrome in many patients, there is need for more research on the detection and prevention of the Brugada Syndrome.

The search for better medical interventions for the Brugada syndrome can begin with the identification of causative and deleterious genetic mutations. Until now the only genetic cause of Brugada syndrome has been linked to more than eighty reported mutations in the gene *SCN5A*. This gene encodes the α-subunit of the cardiac sodium channel (1, 2, 3, 12, and 14). However,

mutations in the *SCN5A* gene account for only 20% of current Brugada syndrome cases (9). In 2002 a new gene locus was mapped to a 0.9 cM region on chromosome 3p24 (LOD score > 4) in a large multigeneration family (16). It was classified as a distinct autosomal dominant form of the Brugada syndrome that is age and sex dependent (16). Through direct sequencing of selected candidate genes located in the 3p24 region, a mutation in the glycerol-3-phosphate dehydrogenase 1-like (GPD1L) gene was identified. The mutation is a single base change (C/T) that creates an amino acid substitution of alanine to valine in a conserved amino acid (A280V) in exon 6 of the gene. GPD1L is the second described gene that mutations lead to the Brugada syndrome.

Preliminary cell physiological studies using whole cell patch clamp technique, show a 61.4% reduction in peak sodium current in cells expressing the mutant GPD1L gene versus cells expressing the wild type gene. Northern blot analysis showed tissue-specific RNA expression of GPD1L in human tissues with the highest gene expression in heart tissue as seen in Figure 1 below. However, the function of the GPD1L protein still remains unknown. Exploration and understanding the cellular function and molecular interaction of GPD1L with the SCN5A gene will require a direct in vivo approach. The creation of transgenic models will provide an animal model for understanding the genetic basis of GPD1L mutation in the Brugada syndrome. This thesis describes the design and creation of two transgenic mouse lines specifically for GPD1L over expression studies. These transgenic mice will enhance current research capabilities and aid in finding new avenues for the effective treatment of the Brugada syndrome.

Transgenic mice are commonly used as *in-vivo* models to study Human diseases (10, 11, 15, and 16). Since the early 1980's, transgenic models engineered with the various molecular genetic techniques can produce an ideal study model with incomparable molecular and

cellular research capabilities (10, 11, 15, and 16). Currently there is no mouse model for the study of the Brugada syndrome. Therefore the introduction of a transgenic Brugada Syndrome mouse will provide an essential *in vivo* model system to better understand the role of *GPD1L* mutations in the Brugada syndrome and to characterize its function in modulating the cardiac sodium channel.

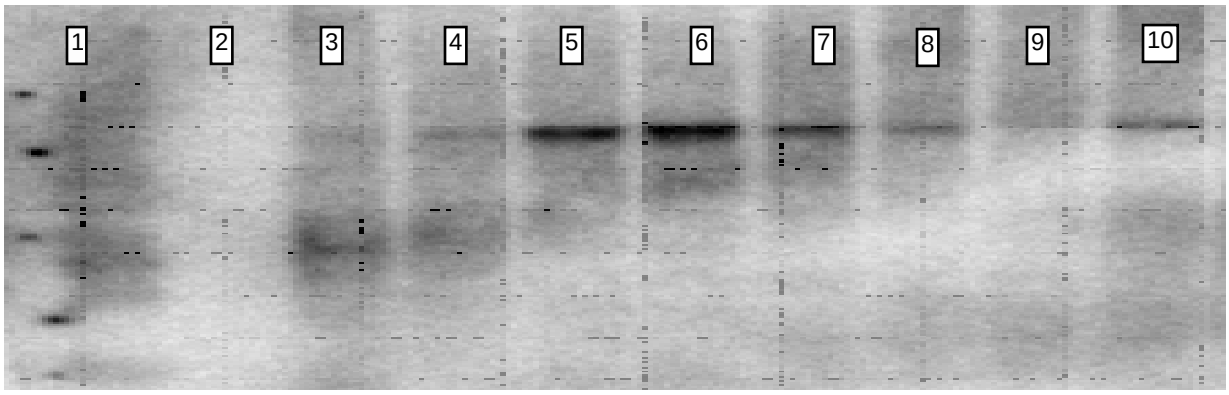


Figure 1 Tissue specific expression of GPD1L gene.

Northern Blot analysis GPD1L gene expression in Human tissues was performed using a pre-hybridized blot from Clontech and a ^{32}P-dCTP labeled cDNA probe of 346 base pairs in length. The probe aligned to the 3' end of the Human GPD1L mRNA sequence between nucleotide 955 to 1299. The lanes of the blot are labeled 1-10 and are as follows; 1 Markers, 2 blank, 3 Brain, 4 Placenta, 5 Skeletal muscle, 6 Heart, 7 Kidney, 8 Liver, 9 Spleen and 10 Colon.

2.0 BACKGROUND

2.1 TRANSGENIC MOUSE MODELS

A transgenic mouse is a genetically engineered mouse that contains foreign DNA in one or many of its cell types (18). The foreign DNA is usually in the form of a DNA construct produced by molecular cloning techniques. Molecular cloning in this study produced a wild type and mutant GPD1L construct. The construct is then used to express the gene of interest by randomly inserting it into the mouse genome using a method known as pronuclear injection. This method works by first injecting the recombinant DNA, or transgene as it is now called, into the pronucleus of a sperm head located within a fertilized egg (10, 11, and 16). The transgene can randomly integrate anywhere within the genome and do it multiple times (18). The pronuclei of the egg fuse to form a diploid zygote. The zygote divides to form a mouse embryo. The developing embryos are injected into a surrogate mother where they mature and produce genetically altered mice containing the specific transgene. The pronuclear injection method has been the most commonly used method for transgenic model production since the early 1980's (10). One reason for the popularity of the pronuclear injection method is that it can be used to characterize a specific promoter and its ability to direct tissue-specific gene expression. Usually this is done to test the efficiency or specificity of a promoter by detecting the presence of a transgenic reporter gene. Another use is to study the effects of over expressing the host

endogenous gene. Comparative DNA genotyping using polymorphisms found in related genes can be used with over expressed mice to find molecular mechanisms of possible pathways that may be too subtle given the normal expression levels of the gene (18). Or reveal allelic interaction crucial for organ or tissue specific function. The novel *GPD1L* gene plays an important pathological role in the Brugada syndrome and with over expression models there will be a powerful tool to understand what that role is.

The success rate for the pronuclear injection method can vary greatly. Many studies have shown that only a small fraction, perhaps only one in ten of transgenic lines produced actually expresses the transgene that has randomly inserted into their genome. Recently a new technique invented to improve the pronuclear injection method seems to be more reliable and greatly increases the number of successful transgenic lines by doubling the pronuclei injected into each zygote (11). The technique works by injecting three gene specific recombinants into both the male and the female pronuclei of the egg instead on just one. There was an estimated 60% increase in the yield of viable transgenic lines. This could prove to be very advantageous to researchers in the future.

There are other factors that affect how a transgene can insert into the host genome and not be genetically expressed. The estimated numbers of produced transgenic lines showing transgene expression depends greatly on the phenotype and the lethality of the transgene at the prenatal level (10, 17, and 18). Also the integration site of the transgene can disrupt the chromosomal DNA structure of the host genome and introduce insertion mutations that affect the vivacity of the transgenic mouse line (16). However, there are a few investigators who have used these mutations as molecular markers for characterizing mutant mouse lines. The insertions in this case are recessive and in no way harmful to the host (16).

2.2 MOLECULAR CLONING

Cloning is a molecular technique that involves three main components. The first component is the fragment of targeted DNA. This can be as small as a synthesized oligo- nucleotide of a few base pairs or as large as an entire gene containing several kilobases. The main goal of cloning will be to make many copies of this DNA fragment for study. The second component is the vector. Common vectors used for cloning are plasmids, cosmids and viruses. The purpose of the vector is to act as a carrier molecule that will protect the DNA fragment and enter the host cell to create many copies of the inserted DNA. Vectors are unique because they allow selective cloning. Most vectors are chosen for selective advantages such as antibiotic resistance, blue and white colony screening because of the presence of the Lac-Z gene, lethality to host cells because they contain a lethal gene and they can contain a radioactive or fluorescent marker. In the cloning process the DNA fragment is inserted into the vector with the use of endonucleases called *Restriction Enzymes* (RE). Restriction enzymes recognize specific DNA sequences and cleave the DNA at these sequences. DNA fragments that are cleaved and inserted into a vector are called recombinant DNAs. There are many types of restriction enzymes but the most commonly used are Type 1 or Type 2. Type 2 enzymes are used more often because they are site- specific endonucleases that cut directly at or very close to the recognition site. Type 1 enzymes usually cut far from the recognition site and are not as useful for cloning. The restriction enzymes create a *double-stranded* (DS) break that can be blunt ended or contain a single-stranded *single-stranded* (SS) overhang of a few bases. Complementary overhanging strands are ligated to one another to create a recombinant fragment of DNA. Likewise two blunt ended pieces can also be joined to form a recombinant fragment of DNA. Circular DNA molecules like plasmids and viruses can be linearized with a single enzymatic digest.

The third component is the host cell. The host cells used in molecular cloning are mostly bacterial cells like *Escherichia coli, Bacillus* and *streptococcus.* Some eukaryotic cells like yeast cells are often used. Mammalian cells like HEK cells are used but more for metabolic and other biochemical methodology. Host cells used for molecular cloning are called competent cells. Competent cells are cells conditioned to accept foreign DNA through cell surface pores hundreds of times greater when exposed to environmental changes like heat or electrical shock. In the presence of heat the cellular pores are forced open and the plasmid DNA is free to move into the host cell in a process called transformation. After transformation, through the selective advantage, only bacteria containing transformed plasmid will be able to grow under the specific selective growth condition. Plasmid DNA will be replicated in the cells at an exponential rate. Characterization of the plasmid will determine the presence of the cloned DNA fragment. The flow charts below illustrate the molecular cloning process in figure 2 and the production of the transgenic constructs in figure 3.

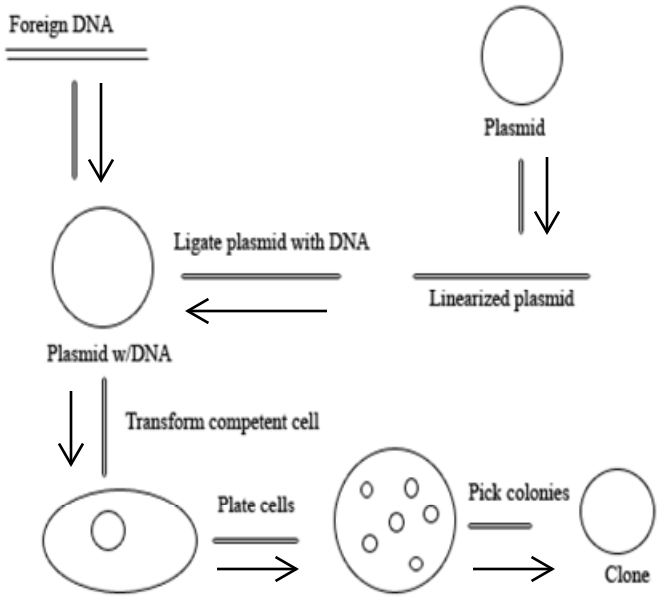


Figure 2 Diagram of Molecular cloning.

A fragment of foreign DNA is ligated to a linearized vector. In this case the vector is a plasmid. Restriction enzymes are used to create the compatible ends for ligation. The plasmid/foreign DNA are incorporated into a host cell (perhaps *E. coli*) through a process known as transformation. The transformed cells are grown and plated. The selective properties of the plasmid (antibiotic resistance, i.e.) allow only the cells that contain the plasmid/foreign DNA to grow. The colonies are picked and the clone is harvested.

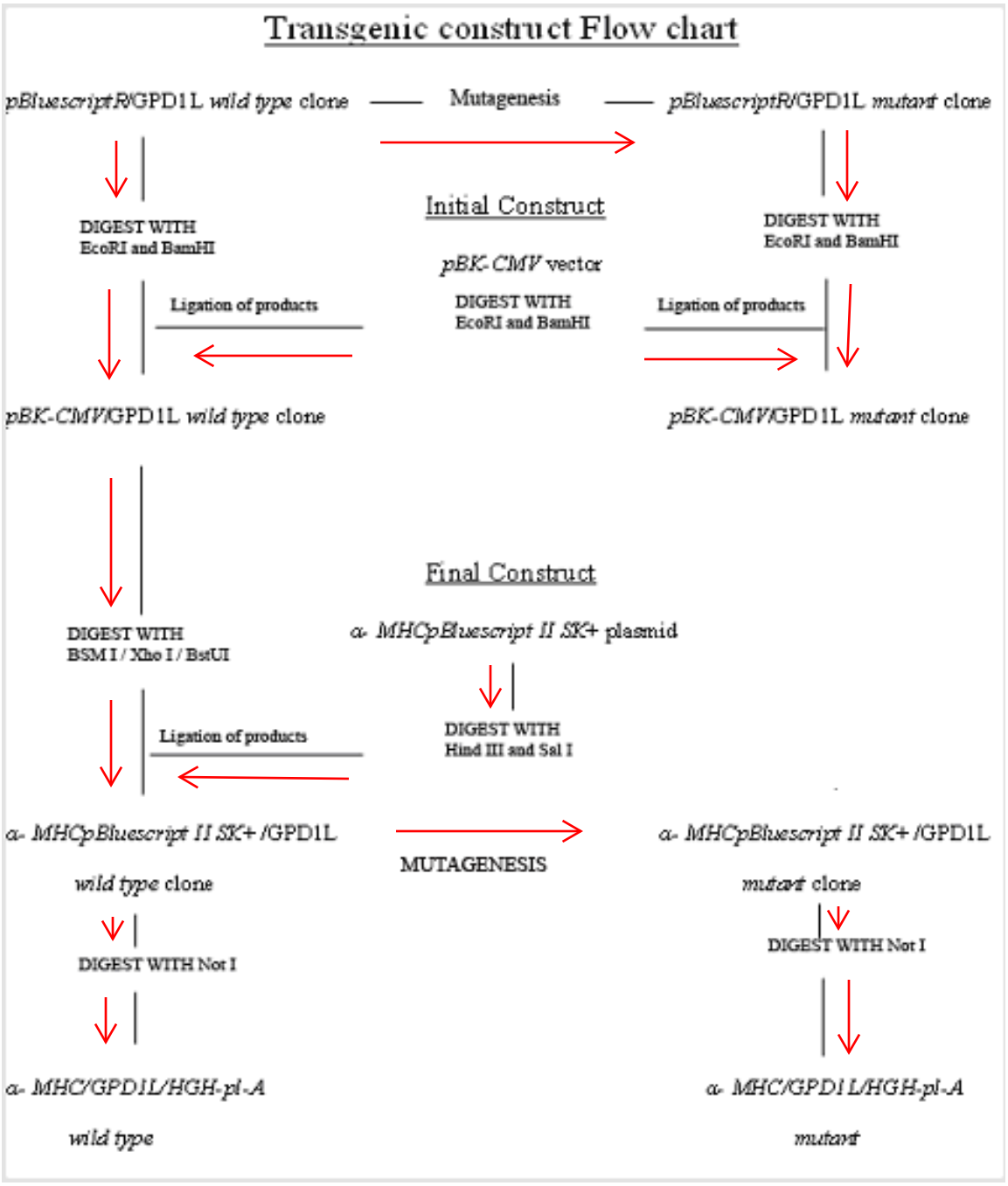


Figure 3 Transgenic construct flow chart summarizing the cloning process.

The mutant clone is produced by mutagenesis of the wild type clone. The initial construct is produced with an *EcoRI* and *BamHI* digest of the GPD1L clone and the pBK-CMV vector. The final construct is produced with a *Bsm I/Xho I/ BstUI* digest of the pBK-CMV/GPD1L clone and a *HindIII/Sal I* digest of the *α-MHCpBluescript II SK+* plasmid. The final mutant clone is produced by mutagenesis of the wild type final clone.

3.0 MATERIAL AND METHODS

3.1 PLASMID VECTORS

1. The *GPD1L* cDNA clone (~6900 bp) was purchased from Invitrogen (ID# 4820730, cat# FL1002). The clone was created by inserting the GPD1L cDNA transcript (3900 bp) into the *EcoRI* and *BamHI* restriction site located in the multiple cloning site (MCS) of the *pBluescriptR* plasmid (2998 bp) (Stratagene cat# 212206). This construct was referred to as *pBluescriptGPD1L* _A280A wildtype *(*WT). A plasmid stock solution was made. The antibiotic used for clone selection was *Ampicillin.*

2. The pBK-*CMV* plasmid (4518 bp) (Stratagene cat# 212207). A working stock of the pBK-CMV vector was made. The antibiotic used for clone selection of the pBK-CMV was *Kanamycin* (MPBiomedical cat# 194531).

3. The *α-MHCpBluescript II SK+* plasmid (9.6 kb) below in figure 4. This vector was constructed by inserting the α-Myosin Heavy Chain (MHC) promoter (~5000 bp) and the polyadenylation (poly-A) signal of the Human Growth Hormone (HGH) (~ 600 bp) into the pBluescript II SK+ plasmid (~ 4000 bp).A working stock of the *α-MHCpBluescript II SK+* plasmid was made. The antibiotic used for colony selection for this plasmid was *Ampicillin*.

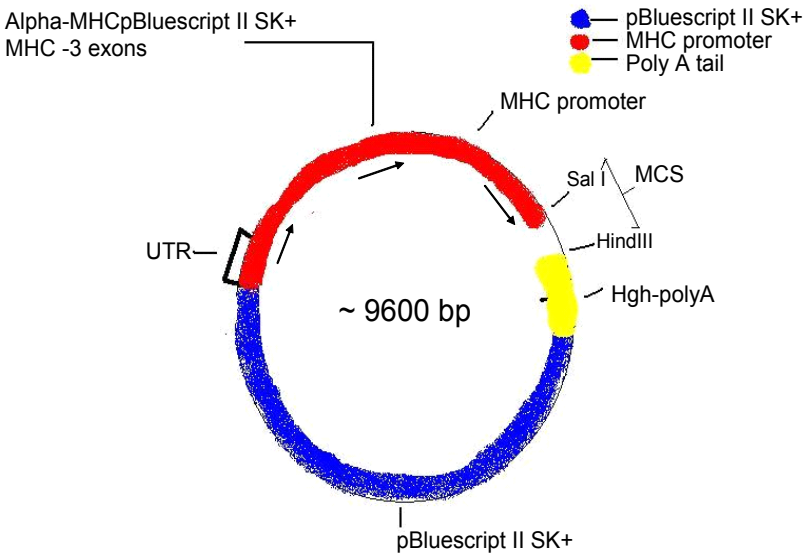


Figure 4 α- MHCpBluescript II SK+ plasmid.

3.2 PREPARATION OF BACTERIAL CULTURE MEDIA AND PLATES

Materials

1 µl *plasmid*

40 µl chemically competent *XL* Blue cells (Stratagene cat# 200249)

500 µl SOC Medium (Invitrogen cat# 15544-034)

LB/*Antibiotic* Broth

Amplicillin (50 ug/ml) (Fisher cat# BP1760-25).

Kanamycin (50 ug/ml) (MPBiomedical cat# 194531).

10g Tryptone (Becton-Dickerson cat# 211705)

10g NaCl (VWR cat# VW6430-5)

5g Dried yeast extract (Becton-Dickerson cat# 212750)

1L dH_20

Autoclave for 20 minutes to sterilize.

Add *Antibiotic* when broth cools to 50-55^oC.

Store at 4^oC.

LB/*Antibiotic* Plates

10g Tryptone

10g NaCl

5g Dried yeast

15g Agar (Becton-Dickerson cat# 214010)

1L dH_20

Autoclave for 20 minutes to sterilize.

Set out plates. Add *Antibiotic* when broth cools to 50-55^oC.

Pipette 25 ml of LB/*Antibiotic* to each plate.

Cool at RT until solidified. Store at 4^oC.

3.3 TRANSFORMATION OF CHEMICALLY-COMPETENT CELLS AND ISOLATION OF PLASMID DNA

The chemically-competent XL Blue cells were purchased from stratagene and stored at -80oc. At the time of transformation, a vial of the competent cells was removed from the -80oc freezer and thawed on ice for 5 minutes. The Plasmid DNA (with or without insert) was removed from

the -20oc freezer and also placed on ice to thaw. A 40 µl aliquot of the chemically-competent XL Blue cells was transferred to a sterile, labeled 1.5 ml tube for transformation. One micro liter of the Plasmid DNA was transferred to the tube containing the 40 µl of competent cells and gently mixed. The transformation mixture was incubated on the ice on the ice for 10 minutes followed by 50 seconds incubation in a 50 oC dry heat block. During this period the competent cells were heat shocked and their pores opened to accept the plasmid DNA. After the 50 seconds were up the tube was immediately placed on ice for 2 minutes. During this phase of the transformation the cells were cold shocked and their pores were forced close trapping the clone within the cell.

Then 500 µl of SOC medium (Invitrogen cat# 15544-034) was added to the mixture and the tube was placed in a shaker set at 37^{o}C and 250 rpm to incubate for one hour. During this time the traumatized cells can revive in a rich nutrient media and be ready for plating after one hour. During this time two LB/ *Antibiotic* plates were placed in a 37^{o}C incubator to warm before plating. After the incubation the cells were removed from the shaker and the two LB/*Antibiotic* plates retrieved from the incubator. A 1:10 dilution of the cells using sterile dH2O was performed and 1 µl of the dilution was transferred and spread to one plate and 1 µl of the undiluted cells was transferred and spread on the other plate. The plates were covered and inverted for placement back into the incubator for at least 18 hours.

The plates were removed from the incubator. Then 250 ml of room temperature LB/*Antibiotic* broth was added to a sterile 500 ml flask. One colony from the plate was used to inoculate the LB media. The flask was covered with parafilm to avoid splash out. The flask was then placed in a 37^{o}C shaker at 250 rpm for 18 hours. The flask was removed the next day and the cultured media was transferred to a centrifuge bottle. The broth was centrifuged at 6000 rpm at 4^{o}C for 30 minutes. The bottle was removed from the centrifuge and the supernatant was

discarded. Then the pellet was processed for plasmid purification using the Qiagen Maxi-plasmid prep kit (cat# 12162**)** and following the Maxi prep protocol in the Qiagen Plasmid Purification Handbook. The purified plasmid was resuspended in 1 ml of 1x TE. A small aliquot of the resuspension was placed in a spectrophotometer to measure concentration as well as purity of the DNA stock.

3.4 AUTOMATED DNA SEQUENCING

BigDye Terminator Sequencing was used to verify that the insert was intact. Sequencing reactions were performed using the primers in Table 1 below. These primers sets were designed to overlap each other and span the entire cDNA region of the GPD1L gene.

Table 1 GPD1L Primers used for DNA sequencing.

Name	Primer Sequence 5' to 3'
1F	CCGGCCAGGGAAGCACG
1R	ATGAGTCCCAGGCGGATGACG
2F	CTTAAGAACATCGTAGCTG
2R	GTAAAAATTCATACATATCCG
3F	TGATCAATCTTTTGGGTTCAC
3R	CCTCTGAGCCTTGACGAA
4F	TCCCCCTCACTGCAGTTGTC
4R	TTGACCTTTCCAGGGAACACC
5F	GTCCCAAGAGCCAGTGATTAT
5R	TGAGACAAGCAAAGTGGGTTA
6F	CACAGAAGTATACGAAAGCAC
6R	TGAGCTGCTAATCTAGGTGT
7F	CTGTGGTCCATTGTTCAT
7R	ATGTATGTAAAAGGCTAGCAG

Set up the Bigdye reaction.

50-100 ng Plasmid DNA

1 µl 1µm primer (forward or reverse)

3 µl Bigdye reaction mix v.1.1 (Applied Biosystems cat# 4337455)

X-10 µl dH_20

10 µl

Amplify in the ABI Thermocycler 9700

25 cycles

95°C 5 seconds

50 °C 10 seconds

60 °C 4 minutes

4 °C forever

Remove the tubes from the 9700 and place them in a 96 well PCR tube plate. The following protocol was used to remove residual bigdye terminator prior to the sequence analysis. Transfer 10µl of dH_20, 2 µl 2M $NaAO_c$, and 50 µl of 100% ethanol to the each reaction tube. Recap the tubes and invert the plate three times. Place the plate in a centrifuge and spin at 2000 rpm at RT

for 30 minutes. Remove the tubes from the centrifuge and uncap them. Invert the tube plate on a folded paper towel to remove almost all of the supernatant by gently shaking once. Then transfer 150 µl of 70% ethanol to the tubes. Recap the tubes and place the plate back in the centrifuge for 10 minutes. Remove the plate and uncap the tubes. Invert the plate onto a folded paper towel and placed back in the centrifuge in the inverted position with the paper towel. Centrifuge at 700 rpm for 1 minute. Remove the plate and discard the paper towel. To the dry tubes add 30 µl of Deionized Di-formamide.

Transfer the 30 µl of Deionized Di-formamide (Applied Biosystems cat# 4311320) in each tube to an ABI 310 sample vial (Applied Biosystems cat# 401957). Place a rubber septa (Applied Biosystems cat# 401956) on the vial. Denature the sample at 95^{o}C for 2 minutes. Place on the ABI 310 for sequencing and analyzed using sequencing analysis and blast (http//www.ncbi.nim.nih).

3.5 MUTAGENESIS

The A280V form of the GPD1L was produced with mutagenesis by the incorporation of the C/T base change into the WT clone. Primers were designed for priming the polymerase chain reaction on the sense and anti-sense strands of the plasmid. These primers are sense; 5'GGTGGCCGAGGCCTTCG***T***CAGAACTGGGAAGACC-3' and anti-sense; 5'-GGTCTTCCCAGTTCTG***A***CGAAGGCCTCGGCCACC-3'. Mutagenesis was performed using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene cat# 200514).

Set up the PCR

100 ng Plasmid DNA

1 µl 20 µm sense primer (IDT Technologies)

1 µl 20 µm antisense primer (IDT Technologies)

5 µl 10X Reaction Buffer

1 µl 10 mM dNTPs

1 µl *QuikChange* Multi enzyme blend

x to50µl dH_20

50 µl

Amplified in the ABI 9700 using the following PCR parameters:

1 cycle

95°C 1 minutes

30 cycles

95°C 1 minute

55°C 1 minute

65°C 8-12 minutes (2 minutes for every 1 kb of plasmid length)

1 cycle

4°C forever

The PCR products were digested with Dpn I by transfer of 1µl *Dpn I* RE (NEB cat# R0176S) to the PCR products at 37°C for 1 hour. The Dpn I will selectively degrade the methylated original WT PCR template and leave only PCR products containing the site-specific mutations.

A two to four microliter aliquot of the digestion mixture was directly used to transform the *XL* Blue cells as described above for the wild type plasmid except for the following changes. Three plates were used to plate 25µl, 50µl and 100µl volumes. The plates were covered and inverted for placement back into the incubator for a minimum of 18 hours. Then 20 colonies from mutant plates were picked and placed in 20 sterile labeled 15 ml tubes containing 10 ml of room temperature LB/Antibiotic broth. These tubes were placed in a 37°C shaker at 250 rpm overnight. The next day the tubes were processed using the Qiagen Mini-plasmid prep kit (cat# 27106) and following the mini prep protocol in the Qiaprep Miniprep Handbook. The 20 clones were then analyzed by direct sequencing of the insert to make sure inserted mutation is present and intact using Bigdye terminator as follows. A stock concentration is carried out as above and the mutant clone was ready for use. The mutant clone was referred to as A280V Mutant (MUT).

3.6 RESTRICTION ENZYME DIGESTS

The restriction enzymes and reaction conditions used in this study are summarized in Table 2 below.

Table 2 Conditions for Restriction Enzyme digests.

RE	NEB Cat#	Buffer #	BSA	Temp ^{o}C
EcoRI	R0101S	EcoRI	Yes	37
BamHI	R0136S	used EcoRI	Yes	37
Bsm I	R0134S	2	No	65
HindIII	R0104S	2	No	37
Sal I	R0138S	3	Yes	37
Xho I	R0146S	2	No	37
Bst UI	R0518S	2	No	60
Not I	R0189S	2	Yes	37
Kpn I	R0142S	1	Yes	37
Sac I	R0156S	1	Yes	37
Cla I	R0197S	4	Yes	37

Bovine Serum Albumin (BSA) - 10 mg/ml. (NEB cat# B9001S)

The following reactions were used for restriction digestion. Digestions with both a single enzyme and two enzymes simultaneously (double digest) are shown.

Reaction setup

Single Digests

Plasmid DNA	10 µl
Restriction Enzyme	2 µl
10x BUFFER	5 µl
BSA (if needed)	0.5 µl
dH2O	32.5 µl
	50.0 µl

Double Digests

Plasmid DNA	10 µl
Restriction Enzyme	1.5 µl

Restriction Enzyme	1.5 µl
10x BUFFER	5 µl
BSA (if needed)	0.5 µl
dH2O	31.5 µl
	50.0 µl

The reactions were set up in a sterile labeled 0.5 ml tube. The tubes were capped and covered with parafilm to reduce evaporation. The reactions were then incubated overnight in a (enzyme specific temperature) heat block. The next day the restriction products were examined by electrophoreses on a 1% agarose gel (Low EOE/*BioExpress* E-3120-500) Agarose containing Ethidium Bromide(ETBR) (Fisher cat# BP102-1)) staining. A *Lambda* BstE-digest ladder (New England Biolabs cat# N3014S)) was used for molecular weight standard. The products were gel extracted using the Qiagen Gel extraction kit (cat# 28704) and following the *Qiaquick* protocol in the *Qiaquick* Spin Handbook pp.23-24. The products were then used for the ligation reaction below.

3.7 LIGATION AND TRANSFORMATION OF GPD1L INSERT AND PLASMID DIGESTED PRODUCTS:

A typical ligation reaction was set up as the following:

5 µl	*GPD1L* (WT) insert
1 µl	*plasmid*
1 µl	10x *T4* Ligation Buffer
2 µl	dH2O
1 µl	*T4* Ligase (NEB cat# M0202S)
10 µl	

The ligation reaction was set up in a sterile labeled 0.5 ml tube. The tube was capped and covered with parafilm to reduce evaporation. Then placed in a 16^{o}C water bath and incubated overnight. The next day the ligation was transformed with chemically competent *XL* Blue cells as before. During the incubation of the cells in 500 µl of SOC media for 1 hour; three LB/*Antibiotic* plates for both WT and MUT transformants were placed in the incubator for pre-warming. The cells were plated as follows:

Plate 1_25 µl of transformation mixture

Plate 2_50 µl of transformation mixture

Plate 3_100 µl of transformation mixture

The plates were covered and inverted for placement back into the incubator for 18 hours. Then 20 colonies from the WT plates and the MUT plates were picked and placed in labeled sterile 15 ml tubes containing 10 ml of room temperature LB/*Antibiotic* broth. These tubes were incubated in a 37^{o}C shaker at 250 rpm overnight. The next day the tubes were processed using the Qiagen plasma purification kit as above. The 20 colonies from both *wild type* and *Mutant* were verified by direct sequencing of the insert to make sure *GPD1L* transcript was intact using bigdye terminator primers from Table 1.

3.8 PHENOL/CHLOROFORM EXTRACTION

To 1.5 ml tubes containing the plasmid DNA add 50/50 phenol chloroform solution to two times the DNA suspension volume. Then in a microcentrifuge spin the mixture 14,000 rpm for 2 minutes. Transfer only the top layer of the mixture to a new 1.5 ml tube and add 100% chloroform to two times the extracted volume in the tube. Discard the bottom layer of phenol remaining in the tube to the proper waste jar. Centrifuge for this time for 15 minutes at 14000 rpm. Remove the tube from the centrifuge and discard any phenol left at the bottom of the tube. Next proceed to Ethanol/Acetate precipitation of DNA.

3.9 ETHANOL PRECIPITATION

To the DNA suspension add 1/10 the volume of 7.5 M NH_4AO_c and 3 µl 100 mg/ml Glycogen (Invitrogen cat# 10814-010). Then add 900 µl cold 100 % ethanol and place the mixture in a -80^oC freezer for 15-30 minutes. Remove the mixture tube from the freezer and place in a microcentrifuge to spin at 14,000 rpm at a temperature of 4^oC for 30 minutes. Remove the tube from the centrifuge and discard the supernatant carefully to avoid disturbing the DNA pellet. Next add 500µl of 70% ethanol and place the tube back in a microcentrifuge at 14,000 rpm at 4^oC for 10 minutes. Remove the tube and again discard supernatant carefully. Invert the tube on a paper towel to dry the pellet for five to ten minutes. Then store the tube containing the dry pellet in a -20^oC freezer or resuspend it in 1x TE buffer. After the clean-up the linearized α-*MHCpBluescript II SK+* plasmid and the GPD1L fragments were blunt ended with the Klenow fragment.

3.10 KLENOW REACTION

Klenow reaction of DNA fragments was conducted according to the protocol described by the manufacturer (New England Biolabs, NEB cat# M0212S). Briefly, add 5 µl of reaction buffer, 1µl of 10 mM dNTPs and 1µl of Klenow fragment to a tube containing the plasmid DNA. Bring the total reaction volume to 50 µl with sterile dH20 and incubate for 30 minutes in a 37^oC heat block. Remove the tube and cleanup the DNA using the phenol/chloroform and ethanol precipitation protocols mentioned above.

3.11 CIP (CALF INTESTINAL/ ALKALINE PHOSPHATASE) REACTION

The CIP reaction was conducted according to the protocol described by the manufacturer (New England Biolabs, NEB cat# M0290S). Briefly, add 5 µl of reaction buffer, 0.5µl of 10 mg/ml BSA and 1µl of Calf Intestinal/ Alkaline Phosphatase to a tube containing the plasmid DNA. Bring the total reaction volume to 50 µl with sterile dH20 and incubate for 60 minutes in a 37^{o}C heat block. Remove the tube and cleanup the DNA using the phenol/chloroform and ethanol precipitation protocols mentioned above.

4.0 RESULTS

4.1 CONSTRUCTION OF THE pBLUESCRIPTR/GPD1L CLONE

The initial vector containing the entire cDNA of human GPD1L was purchased pre-constructed from Invitrogen and was constructed as follows in figure 5. This plasmid was used as the initial wild type clone of the GPD1L gene.

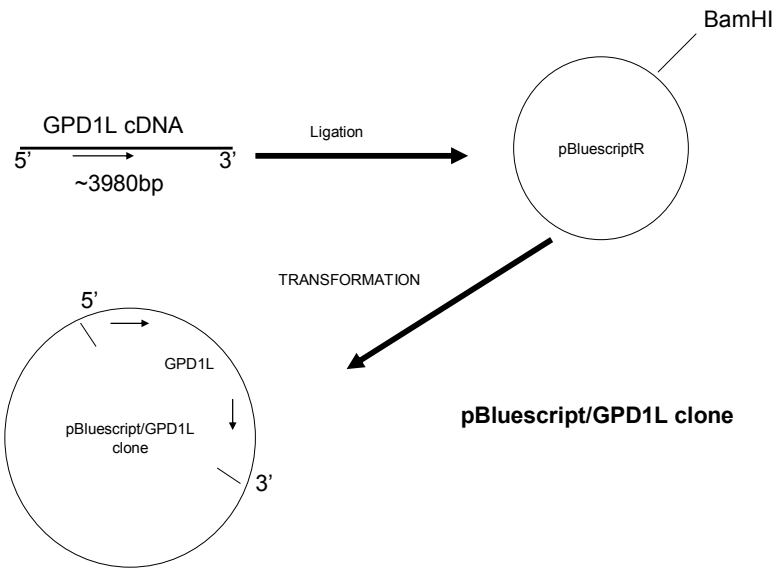


Figure 5 Construction of pBluescriptR/GPD1L clone.

The GPD1L cDNA transcript (~3980 bp) was inserted into the pBluescriptR plasmid (~2900 bp) utilizing the BamHI cut site of the plasmid multiple cloning sites. The produced clone is ~ 6900 bp in length.

4.2 MUTAGENESIS OF pBLUESCRIPT/GPD1L WILD TYPE CLONE

The *pBluescript/GPD1L* wild type clone was used to create the A280V mutant with a technique known as site directed mutatgenesis. Site directed mutagenesis of the pBluescriptR/GPD1L wild type clone (6900 bp) was performed by using both a sense strand and an anti-sense strand oligo to incorporate the A280V mutation into the GPD1L transcript during a polymerase chain reaction (PCR). The two specific oligos ensured that the PCR would produce two complementary strands, each containing the A280V mutation, and eventually a double-stranded mutant homozygote as the final result. The wild type DNA was then removed from the reaction tube with the Dpn I enzyme. The Dpn I endonuclease recognizes *dam* methylated DNA and digests the DNA sequences at these sites. The wild type clone contained the *dam* methylated sites when it was originally isolated using a bacteria host. A bacterial cell uses *dam* methylation as a way to protect its genomic DNA from degradation by restriction enzymes. Methylation occurs on the adenine of every GATC string in the DNA transcript of the clone and the Dpn I cut at these sites. The remaining mutant products were transformed and the mutant clone was produced. Below in figure 6 is an illustration of site-directed mutagenesis process and in figure 7 are the DNA sequences of the wild type and mutant clones showing the homozygous C to T base change.

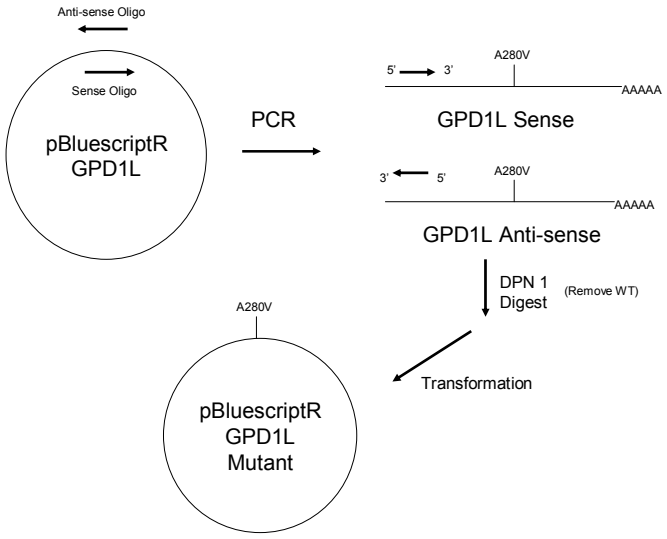


Figure 6 Site-directed mutagenesis of the pBluescriptR/GPD1L wild type clone.

The selected clone was verified by DNA sequencing. Below is a comparison of the wild type and mutant clone.

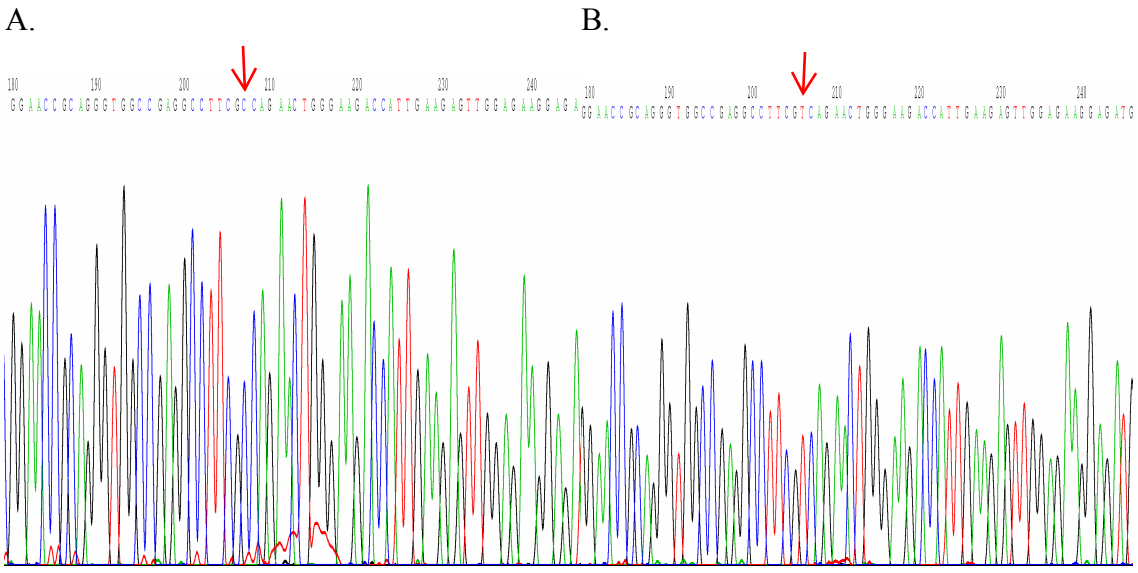


Figure 7 Sequence comparison of wild type and mutant GPD1L.

A. The red arrow shows the wild type Alanine amino acid (GCC). B. The red arrow shows the mutant valine residue (GTC) after mutagenesis.

4.3 CREATION OF pBK-CMV/GPD1L INTERMEDIATE CLONE

The wild type and mutant GPD1L clones were then inserted into the pBK-CMV vector to create the intermediate construct. The *pBluescript/GPD1L* clones and the *pBK-CMV* vector were digested with restriction enzymes *EcoR1* and *BamHI*. Both enzymes produce four base *single strand* overhangs also called sticky ends. The complementary sticky ends of the *GPD1L* clones and the *pBK-CMV* vector were ligated to form the new recombinant. The restriction products were examined by electrophoreses on a 1% agarose gel (Low EOE/*BioExpress* E-3120-500) Agarose containing ETBR (Fisher cat# BP102-1)) staining. A *Lambda* BstE-digest ladder (New England Biolabs cat# N3014S)) was used for molecular weight standard. The *pBluescript/GPD1L* digests produced two bands. The *GPD1L* cDNA band was approximately 3627 base pairs (bp) and the linearized *pBluescript* plasmid band was around 3273 bp. The 3627 bp *GPD1L* band was excised and the *pBluescript* band was discarded. Below in figure 8 is an illustration of the cloning process and in figure 9 is a gel picture of the pBluescriptR (pBsR) plasmid and GPD1L clone with the digestion.

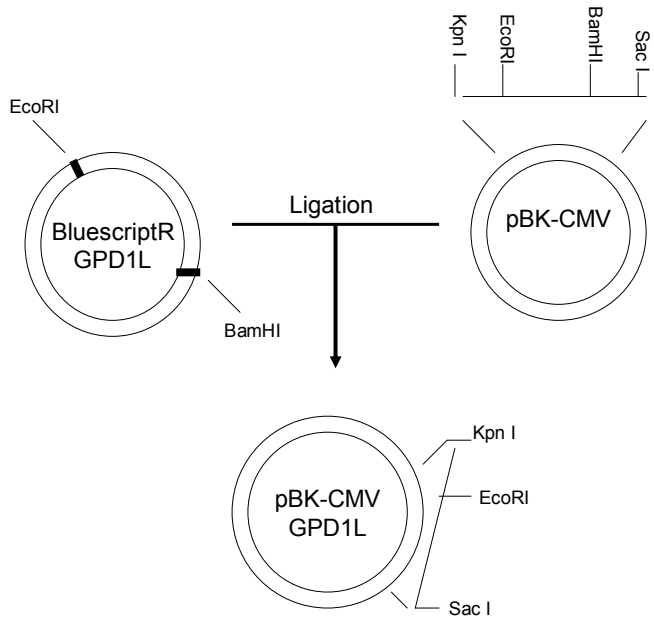


Figure 8 Construction of pBK-CMV/GPD1L clone (~8127 bp).

EcoRI and BamHI digestion excised the GPD1L transcript (~3627 bp) from the *pBluescript/GPD1L* clones and allowed insertion into the *pBK-CMV* vector (4500 bp). The illustration also shows the restriction enzyme cut sites used to verify the final clone.

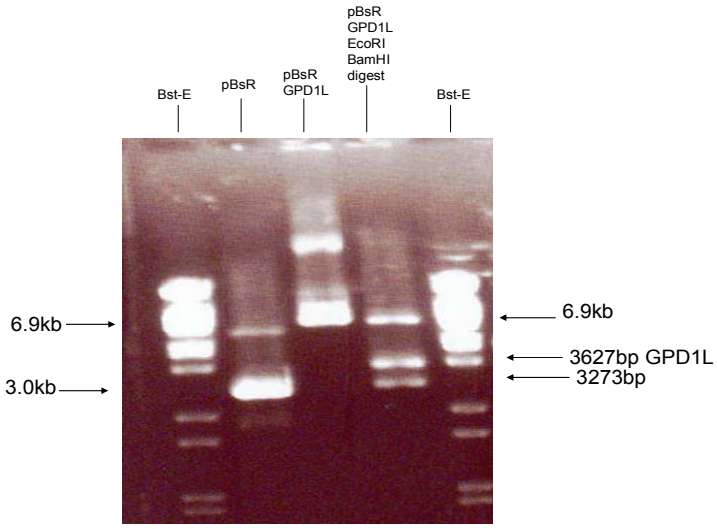


Figure 9 Gel picture of pBluescriptR/GPD1L clone and EcoRI/BamHI digest.

The Lanes left to right show Bst-E ladder, linearized pBluescriptR plasmid, Linearized pBluescriptR/GPD1L clone, EcoRI/BamHI digest of pBluescriptR/GPD1L clone and Bst-E ladder, respectively.

The linearized *pBK-CMV* plasmid and the 3627 bp GPD1L fragment were ligated to form the finished GPD1L intermediate construct (8127 bp). To characterize the clones obtained with cloning, partial clones were analyzed using restriction enzyme digestion. Below in figure 10 is a gel picture showing the pBK-CMV plasmid (4500 bp) and the pBK-CMV/GPD1L clone (8127 bp) linearized with Sac I digestion. Also pictured is the pBK-CMV/GPD1L linearized with a double digest using Kpn I and Sac I restriction enzymes. As expected the excised GPD1L fragment (3725 bp) and linearized pBK-CMV plasmid (4402 bp) were visible after the Kpn I/ Sac I digests. The fragments were sized with the Bst-E molecular size marker shown.

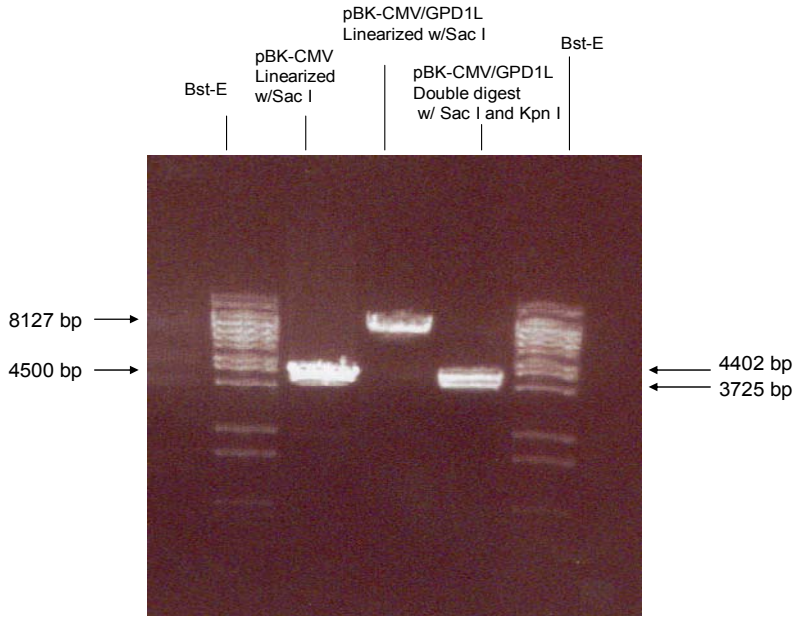


Figure 10 Restriction analysis of pBK-CMV clone.

Lanes left to right show Bst-E ladder, Sac I linearized pBK-CMV (4500 bp), Sac I linearized pBK-CMV/GPD1L (8127 bp), double digest Kpn I/Sac I of pBK-CMV/GPD1L (4402 bp plasmid and 3125 bp GPD1L insert and Bst-E ladder, respectively.

4.4 A-*MHC*p*BLUESCRIPT II SK*+ /GPD1L CLONE CONSTRUCT

The α-*MHCpBluescript II SK*+ plasmid was a vector used to build transgenic constructs for generating transgenic mice. The GPD1L intermediate clone, pBK-CMV/GPD1L, was used to clone the GPD1L gene into this vector. The cloning process to create the constructs required more steps than making the first recombinant. There were three rounds of digests in this process. The strategy for doing the cloning this way was to first of all make use of available complementary cut sites within the *GPD1L* and *MHC* vector, and second was to make sure the piece would be ligated into the vector in the right orientation for expression. The MHC promoter will initiate transcription of the 5' end containing the start codon (ATG or Methionine) of the *GPD1L* transcript and therefore needs to precede the 5' end. The 3' end containing the *GPD1L* stop codon (TAA) must precede the polyadenylation sequence in order for the addition of the poly-A tail on the 3' end of the transcribed message. To achieve the desired orientation with the limited cut sites available the process took three rounds of restriction digests.

4.4.1 First round:

The first round of digests was as follows. The *Bsm1* RE creates a blunt end cut. This blunt end was ligated to the *HindIII* RE end of the vector. However the *HindIII* RE left a four base overhang and was not compatible with the *BsmI* blunt end. Therefore an extra step was added to blunt the *HindIII* RE end. This technique was accomplished with the help of an enzyme called Klenow. The Klenow fragment is actually a DNA polymerase that can be used to fill in the overhang and blunt it. After the Klenow reaction the second digest was performed.

4.4.2 Second round:

The second digest created a compatible sticky end (overhang) on the *GPD1L* cDNA fragment and the linearized *α-MHCpBluescript II SK+* plasmid. The products were digested to produce the sticky ends needed for ligation and orientation into the vector. It was most important to produce the sticky ends to ensure that the DNA fragments were ligated in the right orientation. A double blunt-ended ligation can orient either way and the resulting clone would be useless if the promoter was not preceding the 5' end of the GPD1L transcript. The restriction sites available for the compatible sticky ends were very limited so a special technique was used. Some restriction enzymes are unique in that the overhang of one enzyme may be similar enough to the overhang of another that they will ligate together. However this kind of ligation usually destroys the specificity of the recognition site and it cannot be cut with one or both of the enzymes ever again. However, because of the limited sites to use this was the best option available. The two enzymes that are most commonly used in this fashion are *Sal 1* RE and *Xho 1* RE. They both create a four base 5' overhang of AGCT (sticky end) and therefore are compatible in a ligation reaction (figure 11).

***Sal 1* recognition site**

***Xho 1* recognition site**

Figure 11 Compatible overhangs for Sal I and Xho I

Notice that the two recognition sites are very similar. The overhangs produced can be ligated together but the site will be altered so that the original recognition site is changed and unrecognizable by one or both of the enzymes.

After the digest the restriction products were examined by electrophoreses on a 1% agarose gel containing ETBR staining. Only a small aliquot of the *GPD1L* products was examined. A Lambda BstE-digest ladder was used for molecular weight standard. The *pBK-CMV/GPD1L* digests produced several bands. The *GPD1L* band (3423 bp) was too close in size to the plasmid band (4260 bp) and did not separate enough on a 1% agarose gel to excise the band with certainty. For this reason a third round of digest was performed on the *GPD1L* products to ensure the right product was excised.

4.4.3 Third round:

The third round was to digest the plasmid fragments so that the *GPD1L* fragment was distinguishable and could be excised from the gel. The enzyme *BstU1* RE was chosen because there were many recognition sites in the *pBK-CMV* plasmid but none in the *GPD1L* piece. The restriction products were examined by electrophoresis on a 1% agarose gel Agarose containing ETBR staining. A *Lambda* BstE-digest ladder was used for molecular weight standard. The digest can be seen in the cartoon below in figure 12. After the fragment became distinguishable from the plasmid fragment; the entire digested product was run on another agarose gel and the *GPD1L* band (3423 bp) was excised. The product was gel extracted and purified for the ligation reaction.

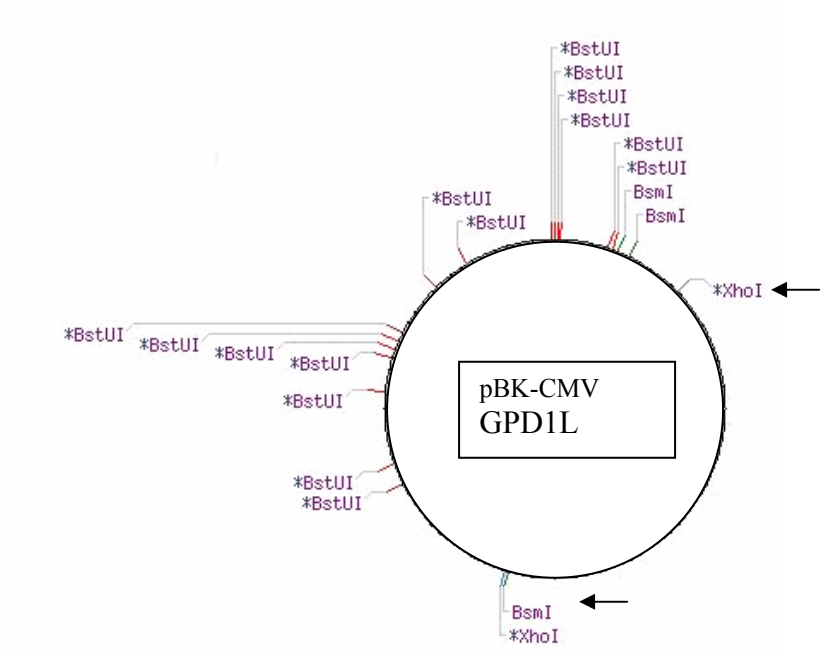


Figure 12 Illustration of BSTUI digest.

The GPD1L transcript (3423 bp) is located between the upper *Xho I* and lower *Bsm I* sites as shown by the arrows. There are many *BstUI* recognition sites in the *pBK-CMV* fragment that help to distinguish the GPD1L fragment on a 1% agarose gel.

Below in figure 13 is an illustration of the cloning process and in figure 14 an illustration of the final clone.

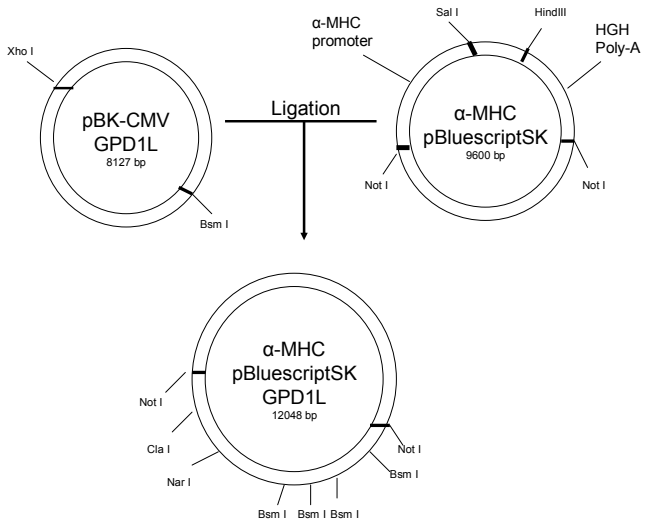


Figure 13 Creation of final construct.

The *pBK-CMV/GPD1L* clones were digested with the *Bsm I* and *Xho I* restriction enzymes to remove the GPD1L transcript. The *α-MHCpBluescript II SK* plasmid was digested with the *HindIII* and *Sal I* restriction enzymes to allow the insertion of the GPD1L transcript. The illustration also shows the other restriction enzyme cut sites used to verify the final clone.

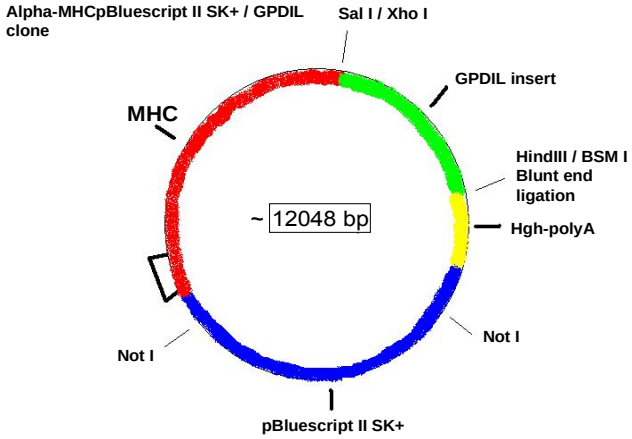


Figure 14 α-MHCpBluescript II SK+GPD1L clone.

The figure shows the final vector with the GPD1L insert. The GPD1L insert is oriented with the 5' end where the Sal 1/Xho I ligated site and the 3'end at the HindIII/BsmI site. The GPD1L construct is now complete with a promoter, coding region and poly-A signal.

The digested products for the molecular cloning process for making the constructs is shown on a 1% agarose gel in figure 15 and 16. Figures 17, 18 and 19 show the verification digests for the clone. Figure 17 shows the α-*MHCpBluescript II SK+* plasmid and α-*MHCpBluescript II SK+* /GPD1L clone linearized with a single cut of the *Cla I* restriction enzyme to verify the presence of the GPD1L transcript in the clone. Figure 18 shows the *Not I* digestion of the α-*MHCpBluescript II SK+* /GPD1L clone and in figure 19 an illustration of the final transcript. This was the final product sent to the University of Pittsburgh Transgenic Facility.

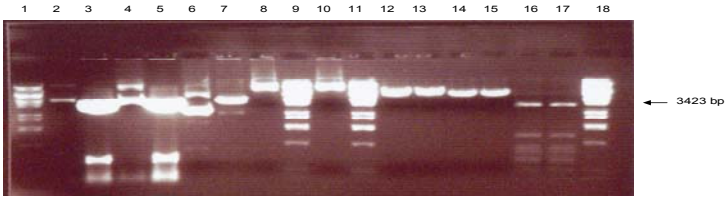


Figure 15 Gel showing the creation of WT1 and MUT1.

Shown also is the extraction from the *pBK-CMV* plasmid for the final cloning. Lanes 1, 9, 11 and 18 are the BSTE ladder for fragment sizes. Lanes 2 and 4 show the WT and MUT clones. Lanes 3 and 5 show the EcoRI and BamHI double digest of the two clones. Lane 6 is the *pBK-CMV* plasmid. Lane 7 is the EcoRI and BamHI double digest of the plasmid. Lanes 8 and 10 show the WT1 and MUT1 clones. Lanes 12 and 13 is the *Bsm I* digest of WT1 and MUT1. Lanes 14 and 15 show the *Xho I* digest. Finally, Lanes 16 and 17 are the third round *BstUI* digested products of WT1 and MUT1. Notice the 3423 bp piece.

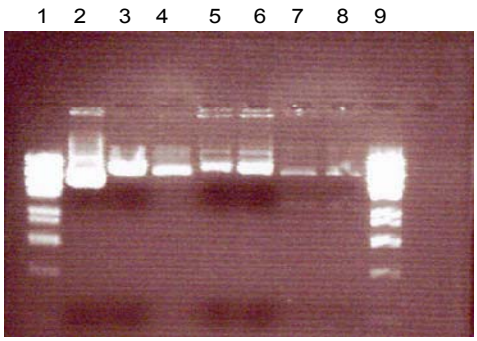


Figure 16 Gel showing the creation of WT2 and MUT2.

Lanes 1 and 9 are the BSTE ladder. Lane 2 is the uncut α-*MHCpBluescript II SK*+ plasmid. Lanes 3 and 4 show the *HindIII* and *Sal I* digested α-*MHCpBluescript II SK*+ plasmid, respectively. Lanes 5 and 6 are the WT2 and MUT2 clones. Lanes7 and 8 show the final *Not I* digest of WT2 and MUT2.

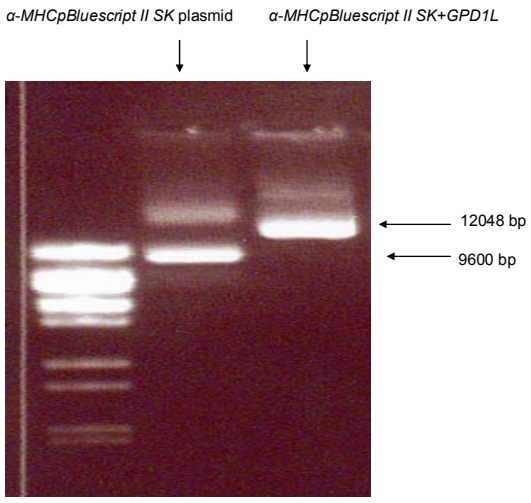


Figure 17 Gel picture showing Cla I linearized α-MHCpBluescript II SK+ plasmid

and α-*MHCpBluescript II SK*+ /GPD1L to verify presence of GPD1L transcript in clone.

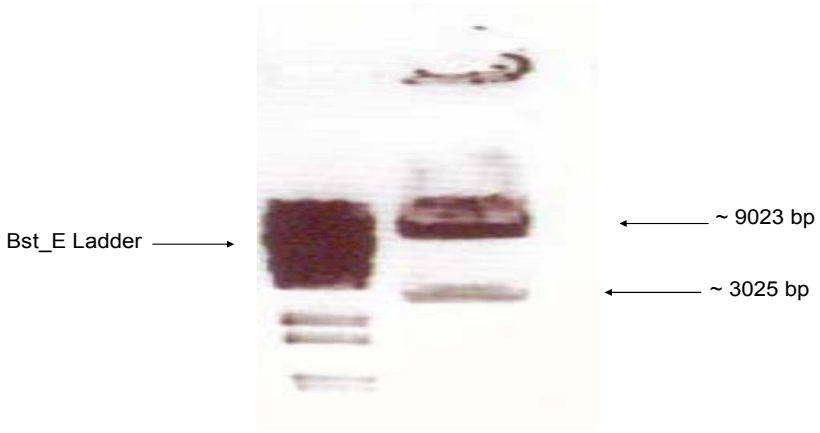


Figure 18 Not I digestion

producing final transcript sent to mouse facility. The 9023 bp fragment is the final transcript containing the MHC promoter, GPD1L transcript and the HGH poly-A signal sequence.

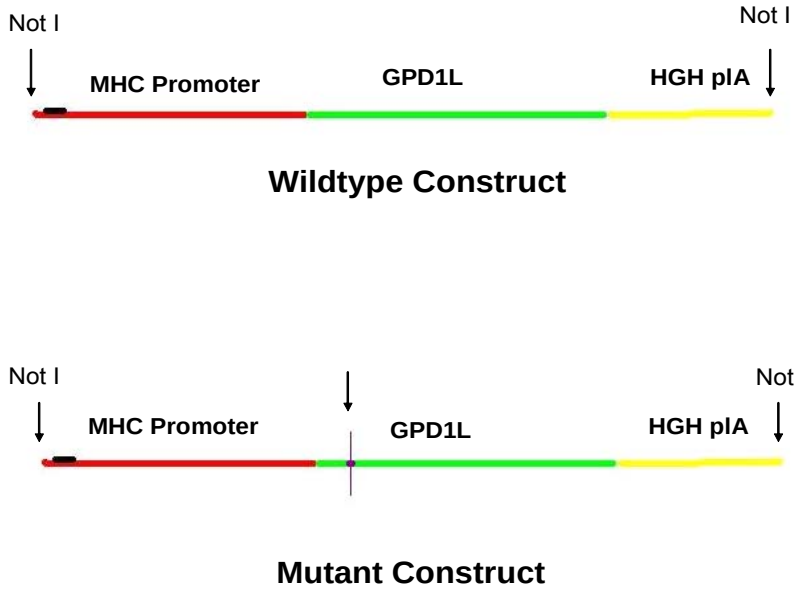


Figure 19 Final construct after Not 1 RE digest.

The figure shows the final constructs contain a promoter, the GPD1L transcript and the HGH polyadenylation signal sequence with the two *Not I* restriction site locations. The mutant is also labeled with an arrow showing that the A280V mutation is present.

The final transformation produced a wild type clone but not a mutant. There is the possibility that the mutant GPD1L fragment was altered during the three rounds of digests and the resulting 5' and 3' ends were made incompatible with the corresponding plasmid ends. During a restriction digests the sequence of the 5' or 3'end of a DNA fragment can be altered slightly leaving it incompatible for ligation. The three digests were performed on the intermediate mutant clone again. The ligation reaction was set up and no colonies were produced after transformation a second time. Finally after one more attempt to produce the mutant, mutagenesis was performed on the wild type clone to produce a valid mutant clone. The same protocol was used as in the production of the pBluescriptR/GPD1L mutant shown above in B and shown in figure 20 below.

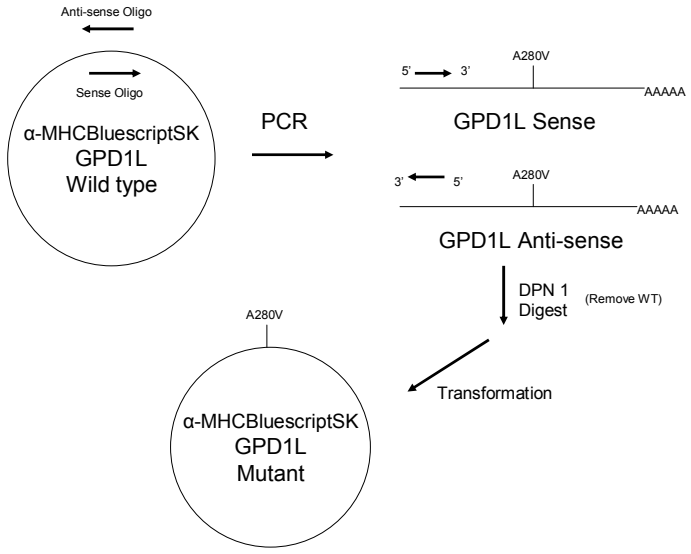


Figure 20 Mutagenesis of α-MHCpBluescript II SK+ /GPD1L wild type clone.

4.5 PRONUCLEAR INJECTION

The wild type and mutant clones of GPD1L were used for pronuclear injection to generate transgenic mice. The first two litters were obtained two months after injection. Mouse tails were obtained from these litters and tail DNA was extracted from 24 offspring produced from the wild type GPD1L transgene injection and 14 tails from the mutant GPD1L injection. Kenny Rahl, a molecular technician in the lab, performed a southern blot on the DNA. Southern blotting is a method using radio-isotope labeled probes to detect the presence of a specific DNA sequences or a transgene. Two probes were designed to test for the presence of the transgene. One probe was homologous to the MHC promoter and the other probe was homologous to the human *GPD1L* transcript. The probe for the MHC promoter should hybridize to the native mouse MHC transcript as well as the transgenic copy producing two bands on the blot. The *GPD1L* probe was homologous to the human *GPD1L* and one band should be seen only if the transgene is present in the mouse genome. Unfortunately, the southern blot showed that the transgenic MHC and the *GPD1L* transgene were not present. Further investigation of the mouse tail DNA was performed using PCR with *GPD1L* cDNA primers specific for the transgene. The PCR results confirmed the negative results of the southern blot. The negative PCR results can be seen below in figure 21.

Table 3 PCR primers for transgene conformation: F & #1: 500 bp; F & #2: 800 bp

Forward for set 1&2	5’-CCCAGCTGCCCGGCACTCTTA-3’
Reverse #1	5’-CTTGTGTCCAGGAAGATATTT-3’
Reverse #2	5’-ATGAGTCACAGGCGGATGACG-3’

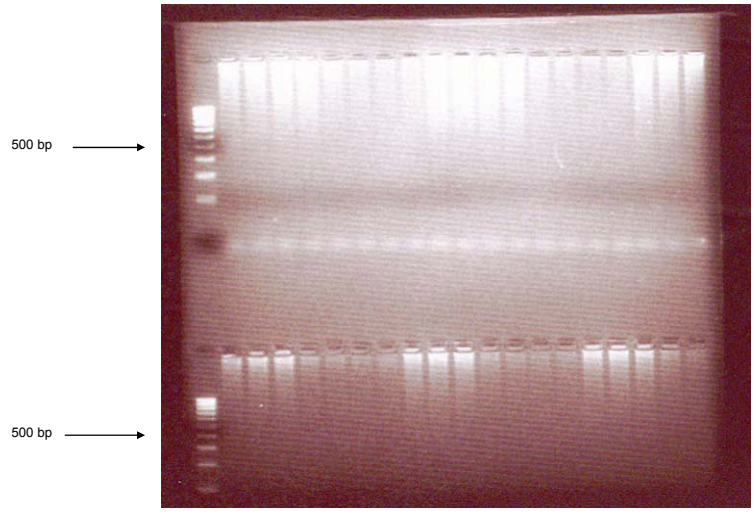
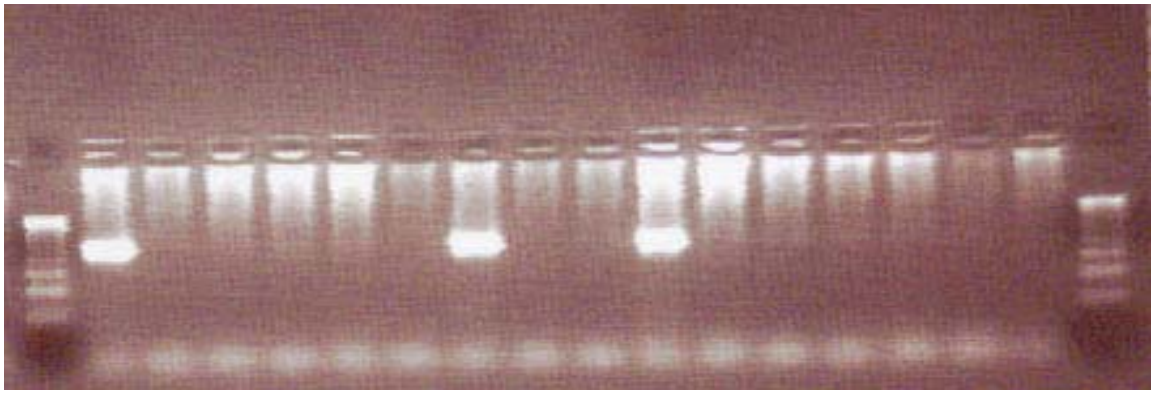


Figure 21 PCR of mouse tail DNA using the forward and reverse #1

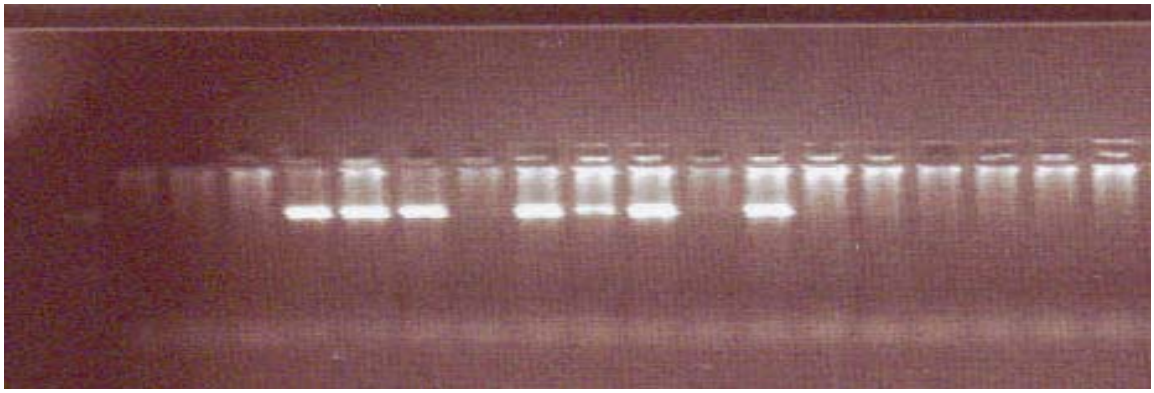
Well # 2-20 and 22-26 are the 24 wild type and well 27-40 are the14 mutants. All are negative for the presence of the GPD1L transgene.

The pronuclear injection was performed again. At six weeks after injection, we received the first pups to test. Thirty-three offspring were produced from two litters for the wild type construct. Again, Kenneth Rahl extracted the tail DNA and performed another southern blot. Of the 33 pups in the litter there were ten that were positive for the presence of the transgene on the blot. As before all 33 DNA samples were tested by PCR using the primers specific for the Human *GPD1L* transcript above. The same ten positive tail samples showed a visible PCR product for the gene. Below is figure 22 are the PCR results for the wild type offspring.

A.



B.



C.

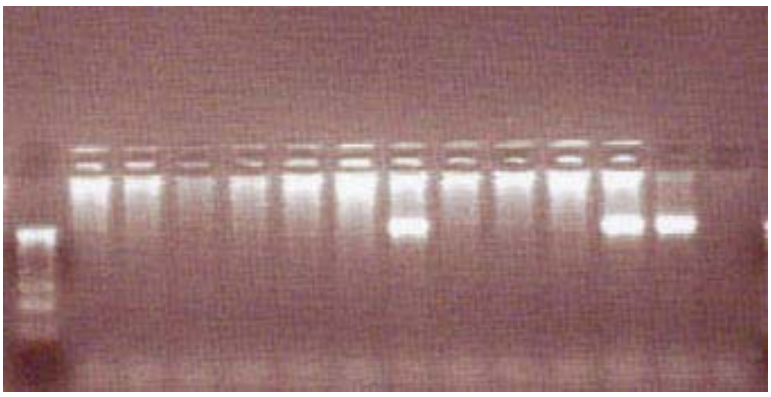


Figure 22 Second pronuclear injection test PCR of wild type.

Gels A and B show the results of the forward primer and reverse primer #1.Gels C and D show the results of the forward primer and reverse primer #2.

The offspring for the mutant *GPD1L* transgene was obtained one week after the wild type. There were 6 possible lines in this litter. The DNA was extracted and analyzed by southern blot analysis. The blot showed one positive mouse line for the mutant construct. PCR analysis was performed using the Human *GPD1L* primers to validate the southern blot results. One of the six mouse tail DNA samples produced a visible PCR band suggesting that the transgene was present. The transgenics was successful with the production of ten wild type *GPD1L* lines and one mutant *GPD1L* line. In figure 23 the PCR analysis shows the presence of the transgene in ten wild type offspring and one mutant offspring using the forward primer and reverse primer #1.

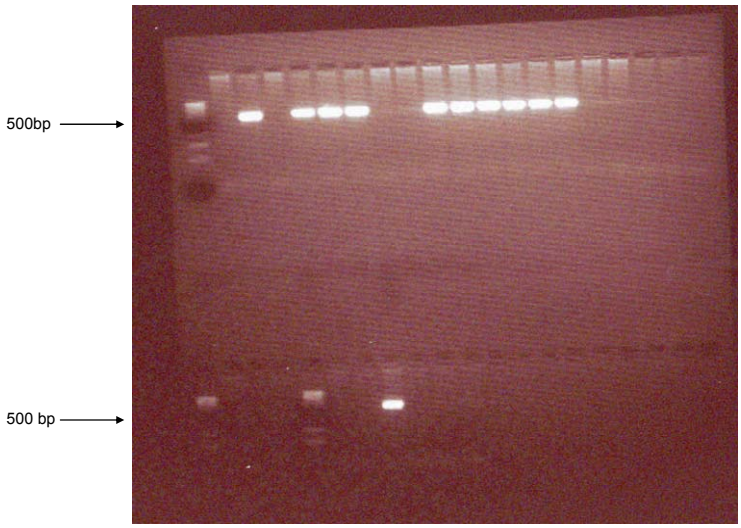


Figure 23 Mutant PCR

This gel shows ten positive wild type lines on the top row and ten negative wild type lines. There is one positive mutant line on the bottom row out of six total lines. PCR was produced using the forward primer and the reverse primer #1.

In summary, we were able to generate both wild type and mutant GPD1L transgenic mouse models and these mice can be used for future functional studies.

5.0 DISCUSSION

The construction of the GPD1L over expression clones was a little more difficult then anticipated. The main problem was the *α-MHCpBluescript II SK+* plasmid (MHC vector) used for the final vector of the construct. The MHC vector was the only one available to complete the project. This vector was important to the project because it had the MHC promoter (a common promoter found in heart tissue for tissue specific expression) and a poly-A signal within its transcript. Unfortunately, the available restriction sites were very limited and made the design difficult. The problem was that the MHC vector was designed for use in a project other than that of the GPD1L gene study. Cloning into the *α-MHCpBluescript II SK+* plasmid also required the use of another vector, namely the pBK-CMV plasmid, which was one of the many extra steps that were involved in the cloning process. The other molecular techniques like blunt ending with the Klenow fragment and removing the active phosphates from the linear plasmid were very important in assuring that the cloning process ended in such a great success. For the future projects, including the design and engineering of transgenic constructs, a different final plasmid vector would be ideal. Possibly one that contains multiple restriction sites allowing a greater ease for the design and creation of a transgenic construct. The same MHC plasmid could be used again but only by first inserting a ready made DNA linker to introduce more restriction sites first. Another factor that was important in this project and probably in all future projects involving transgenics is the mutagenesis protocol. This technique was especially important when the

several attempts to produce the mutant final construct failed. This technique saved both time and money during this study.

There are a few reasons why the first pronuclear injection may not have worked. One reason is that the transgenes expression could be potentially lethal to the developing embryo. However, in most successful pronuclear injections only about 10% of the offspring actually express the inserted transgene (11). This would suggest that at least one pup should have been produced with the transgene inserted in its genome without expression of the inserted gene. Lethality could still be a reason for no transgenic offspring if the random insertion of the transgene is close to or within another gene in the mouse genome. If this was the case the insertion could have altered gene expression of one or more genes and become potentially lethal to the developing embryo. Although there is a chance of this happening the opposite is of equal chance and a non-lethal insertion occurring. Another problem that is common to the pronuclear injection process is that a great many of the developing zygotes can be lysed during the injection and are subsequently lost during the process. One other possibility is that the integrity of the DNA was poor. To check the integrity of the DNA, the GPD1L clones were sequenced with BigDye terminator sequencing again. The results of the sequencing showed that the DNA sequences were unaltered and intact. The integrity of the DNA could also have been affected by the final cleanup process used before the injection. The Gel extraction method used on the *Not I* digested products may have greatly affected the integrity of the GPD1L DNA. The final *Not 1* RE digest was performed again but the products were not gel extracted with the Qiagen kit as before. Instead they were sent to the transgenic facility in the reaction tubes. The facility used a different technique that incorporated a specific column that had time and time again, proven to give them better success. The pronuclear injection was then performed again. The injection was a

success. The use of a special column proved to be a much better method than the gel extraction method.

The study was overall a great success. The creation of the two over expression constructs will not only aid in the characterization of the novel GPD1L gene but they will provide the first Brugada Syndrome murine model. The importance of these mouse models to public health is how they will contribute to the study of cardiovascular disease (CVD). CVD is an important public health issue that claims the lives of millions of people each year. The Brugada syndrome is a form of CVD and a well known cause of sudden cardiac death. In the past decade many important advances have been made for the identification and characterization of this disease but successful therapeutic solutions are not available. The absence of successful medical intervention is partly due to the lack in identifying novel genes and mutations that lead to cardiac arrhythmia and sudden cardiac death. The GPD1L transgenic mice produced in this study will become a valuable tool to understand the electrophysiology and molecular mechanisms of this novel gene. The models will aid in improving diagnosis, assessment of prognosis and the possibility of therapeutic alternatives for this rare but deadly disease.

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