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A Study of a Proposed Molecular Mechanism for Idiopathic Cases of Hypertension:
Detection of Aldosterone-receptor Complex at Period Homolog 1 (*Per1*) Promoter Using
Chromatin Immunoprecipitation (ChIP)

By: Brent Colin Turner

Thesis

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

Fredericksburg, VA

May 1, 2009.

This thesis by Brent Colin Turner is accepted in its present form as satisfying the thesis requirements for Honors in Biology.

Date: Approved:

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ACKNOWLEDGEMENTS

ABSTRACT

Hypertension severely affects the quality and quantity of life of those afflicted with the disease. In most cases where the cause is known, hypertension is due to an abnormally high sodium condition; however, the mechanism by which this occurs is unknown. This phenomenon is particularly detrimental to patients considered to be non-dippers, experiencing no nocturnal dip in blood pressure. A proposed molecular mechanism for the relationship between dipping and sodium concentration involves the regulation of the circadian gene Period 1 homolog (*Per1*) by aldosterone and was studied by using chromatin immunoprecipitation (ChIP). This technique results in an enrichment of the chromatin fragments that are bound by the protein of interest, which after Polymerase Chain Reaction (PCR), it can be verified whether or not aldosterone directly increases the expression of *Per1*. Primers were successfully designed for two locations within the *Per1* gene. One set was for the -156 glucocorticoid regulatory element (GRE), a potential region where the aldosterone receptor binds and a second set to a 3' was designed as a control. Optimization of the enzymatic shearing conditions was determined to be ten minutes. There were no PCR products for the samples after the complete ChIP assay; however, the expected results were obtained for those samples without pre-clearing of chromatin and antibody treatment. For future study, samples should be tested systematically using PCR to determine in which step the DNA fragments are lost.

TABLE OF CONTENTS

INTRODUCTION

Hypertension & Its Associated Risks

Severe headache, altered mental status, fatigue, tinnitus (ringing in the ears), dizziness, vision problems, palpitations, hematuria (blood in the urine), confusion, shortness of breath and papilledema (swelling of the optic disc) are all signs of hypertension, a medical condition referring to chronically elevated blood pressure [1,2,3]. Unfortunately, a majority of those afflicted with high blood pressure show no preliminary signs and are unaware of their condition until it is too late to prevent the adverse side effects and associated risks.

Normal blood pressure values for a healthy human adult range from 120 to 139 systolic and between 80 and 89 diastolic [1]. A person with long-term hypertension, with values above 140/90mmHg, is at risk for a multitude of adverse side effects, many of which are life threatening [2, 3]. Cardiovascular problems include left ventricular hypertrophy, an increased chance of both atherosclerosis and arteriosclerosis, myocardial infarction, coronary artery disease (CAD), peripheral vascular disease, congestive heart failure (CHF) and aortic aneurysm [1]. Additionally, hypertension can result in renal failure, an increase of four to six times the chance of experiencing a cerebrovascular accident (CVA) and hypertensive retinopathy. Studies also show that chronic high blood pressure can reduce ones ability to think, ability to remember and learning capacity, all of are cognitive decline, which can ultimately lead to senile dementia [2].

In light of these associated risks it is evident that high blood pressure can dramatically shorten one's life expectancy as well as lessen the quality of living. Individuals over the age of fifty with hypertension suffer from seven years more of

cardiovascular problems and have a decrease in five years of life expectancy [3]. In 2004, it was estimated that approximately seventy-three million Americans, one in four, had hypertension (HTN) and close to 55,000 of these cases resulted in death [4]. Furthermore, 95% of causes of hypertension are still unknown, demonstrating the need for continuous research to discover the causes of this disease [1]. Furthermore, cardiovascular problems are more prevalent in those who have nocturnal hypertension demonstrating the necessity to find a specialized approach to treatment for these individuals [6].

Dipping and Non-Dipping

Extensive research shows that in healthy individuals, the rate of sodium reabsorption varies throughout the day, with less reabsorption at night causing a nocturnal dip in blood volume and, therefore; blood pressure [6]. This observation demonstrates that blood pressure exhibits circadian rhythm regulation patterns. Those who fail to dip at night have been classified as "non-dippers" and suffer from a greater risk of cardiovascular complications than those with hypertension who still experience a dip at night. The decrease in blood pressure is usually about 10-20% and typically the sodium level in the diet is irrelevant [7]. At night, there is naturally a decrease in the amount of natriuresis (excretion of sodium), due to lack of urination. A non-dipper is unable to compensate for this change and therefore continues to reabsorb sodium. Additionally, a non-dipper's elevated blood pressure is affected by a high sodium diet unlike that of a dipping person who would simply reabsorb less sodium at night. Interestingly, a renal transplantation can change one's status from non-dipper to dipper demonstrating the kidney's extensive control over this circadian rhythm [7].

Since blood pressure elevation during sleep has been linked with a greater detrimental effect on cardiovascular health it is important to better understand the mechanisms by which sodium reabsorption by circadian rhythm. Circadian rhythm refers to the twenty-four hour biological clock, which is reset by light cues from the environment. The sleep-wake cycle, heart beat, body temperature, hormone secretion and renal blood flow are all examples of other rhythmic fluctuations that are influenced by the circadian clock [5].

Hypertension typically refers to elevated arterial blood pressure; which in the majority of the known cases, it is a disease of elevated blood sodium concentration [1]. The amount of sodium in the blood is directly proportional to the amount of water, meaning high sodium concentration results in increased blood volume, which ultimately causes an elevation in blood pressure. Therefore, the ability of the kidney to regulate sodium levels is crucial in maintaining healthy blood pressure [4]. In order to adequately understand this regulation some relevant topics of basic renal physiology are addressed in the following section.

Basic Renal Physiology & Aldosterone's Role

In humans, there are normally two kidneys present, both lying on the outside of the peritoneal cavity, one on each side of the vertebral column. These organs have seven main functions: the regulation of water and electrolyte balance, the excretion of metabolic waste, excretion of bioactive substances, the regulation of arterial blood pressure, erythropoiesis (red blood cell formation), the regulation of Vitamin D production and gluconeogenesis (generation of glucose).

As shown in Figure 1, the outermost portion of the kidney is known as the renal cortex, which houses the renal tubules (except a portion of the Loop of Henle), blood vessels and cortical collecting ducts. The innermost portion of the kidney is referred to as the renal medulla and contains the remaining portions of the Loop of Henle. Urine formation occurs in the nephrons, the functional units that comprise the kidney. This process is divided into three stages; filtration, tubular reabsorption and tubular secretion. The process of urine formation is a crucial part of understanding the role of the kidneys in maintaining normal blood pressure values [4].

Filtration refers to the kidneys' ability to separate water and solutes from the blood that then leave the vascular system through the filtration barrier and enter Bowman's space, in the cortical region. The volume and solute contents in the filtrate and the urine ultimately represent two distinct values due to the processes of reabsorption and secretion. During these processes, additional substances are moved in and out of the lumen, both with and against their respective concentration gradients [1].

At the glomerulus, a capillary tuft surrounded by Bowman's capsule in the nephron, there is a high pressure gradient that causes the serum portion of the blood to be forced into the renal tubules. The direction of flow is through the proximal convoluted tubule (PCT), the Loop of Henle, the distal convoluted tubule (DCT) until finally the serum leaves the kidney by means of the collecting duct, where the adrenal cortical hormone aldosterone has its effect.

Aldosterone is a steroid hormone in the mineralocorticoid family produced by the adrenal cortex. This hormone reduces sodium excretion by stimulating its reabsorption in the renal tubules, with its target epithelium of action being the collecting duct [1]. The

signaling pathway of aldosterone is characteristic of a hormone-dependent gene

activation mechanism. In the absence of aldosterone the hormone receptor remains in the

cytoplasm; however, in the presence of aldosterone an activated hormone-receptor

complex is formed and enter the nucleus. The complex then binds to a promoter element

that causes the transcription of mRNA, which encodes for a number of proteins important

for sodium reabsorption [5].

When examining sodium levels specifically, it is apparent that the amount

excreted is only a very small fraction of what was originally filtered due to reabsorption.

This active process occurs in all tubular segments of the kidney except the descending

limb of the loop of Henle. The remaining segments of the nephron, shown in Figure 1,

reabsorb varying amounts of sodium as follows: 65% in the proximal tubule, 25% in the

ascending limb of Henle's loop, 5% in the distal convoluted tubule and 4-5% in the

collecting duct, with the latter being stimulated by aldosterone [1].
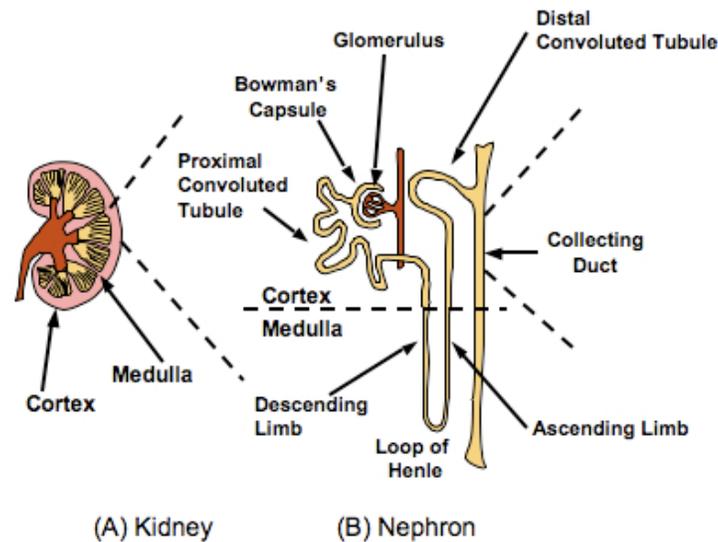

Figure 1- **The Kidney & The Nephron [10].** Part A of this figure shows a cross section
of the kidney with the cortex covering the internal medulla, while B depicts the nephron,
where the regulation of the concentration of water and soluble substances is performed.

While 98% of the filtered sodium is returned to the body by a variety of mechanisms, approximately 2% of sodium reabsorption is dependent on the concentration of the aldosterone. This last 2% is critical for the regulation of blood volume and blood pressure. Because of this, aldosterone is called the "all-purpose stimulator of sodium retention" [5]. Aldosterone stimulates renal reabsorption of salt which causes a transient positive fluid balance to maintain normal blood volume. Thus, if sodium intake is low, aldosterone cells increase so that the body can better maintain a normal sodium water balance by reabsorbing the additional 2% of filtered sodium. Conversely, after eating a meal high in sodium, aldosterone release is inhibited, causing a decrease in sodium reabsorption. In the absence of aldosterone a person would excrete the 2% filtered sodium, which is roughly an extra thirty grams of sodium chloride a day. While this seems to be a small percentage, the volume of the glomerular filtrate is so large that the fine regulation of this 2% within the minimal and maximal concentration of aldosterone can make drastic changes in ones blood pressure. Because of the direct relationship between sodium concentration, blood volume, and blood pressure under physiological conditions aldosterone plays an necessary role in both cardiovascular and renal health [5]. Already, studies have shown that aldosterone blockades in patients with high blood pressure can reduce both mortality rates and the amount of hospitalization. Additionally, these patients experienced a reduction of proteinuria, healing of vascular and glomerular lesions and the reduction of fibrosis [8].

Importance of Research Project

The recent discovery of early aldosterone-responsive transcripts through microarray analysis includes Period homolog 1 (*Per1*), a known circadian rhythm protein

[11]. This finding was the first step in identifying a link between these two important regulators of blood pressure. Studies performed by Gumz et al. demonstrated early upregulation of *Per1* expression after only one hour of aldosterone treatment in a mouse inner medullary collecting duct cell line, mIMCD-3 [11]. It is the goal of this research project to further investigate the link between aldosterone and *Per1* in order to determine whether the molecular mechanism responsible for this link is a direct effect of the mineralocorticoid aldosterone regulator binding to the promoter region.

The effects of hypertension can be drastic and greatly diminish both the quality and quantity of life. Moreover, the effects of hypertension are exacerbated by the non-dipping of blood pressure at night. Preliminary findings suggest a novel pathway for the regulation of dipping of blood pressure at night that involves aldosterone, a hormone associated with virtually all cases of inherited hypertension [11]. This mechanism involves the regulation of the circadian rhythm gene Period 1 (*Per1*), which could play a role in regulating the dipping of blood pressure during night. Since the upregulation of *Per1* has been shown to be regulated by aldosterone, this could be a new mechanism to explain the circadian rhythm associated with blood pressure. Understanding this newly proposed mechanism of regulation for *Per1* could reshape the current approach to treating the millions of cases of idiopathic hypertension, specifically for non-dippers.

This project has been carried out in collaboration with Dr. Michelle Gumz, a postdoctoral fellow in the laboratory of Dr. Charles Wingo. The Wingo Laboratory is located in the Department of Medicine, Division of Nephrology, Hypertension, and Renal Transplantation at the University of Florida's College of Medicine. The larger research project involves multiple objectives including: the characterization of the induction of

Per1 by aldosterone, defining the roles of the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) in aldosterone-mediated regulation of *Per1*, and the characterization of the role of *Per1* in the aldosterone-mediated regulation of Na^+ transport [12].   The specific aim of this proposed project is to determine the molecular mechanism by which aldosterone leads to the induction of *Per1* in cultured inner medullary collecting duct (mIMCD-3) cells. It is hypothesized that the aldosterone-receptor complex will bind to the promoter element of the *Per1*gene because early upregulation occurs after only one hour of aldosterone treatment.

To satisfy this objective, chromatin immunoprecipitation (ChIP) was performed, which results in an enrichment of the chromatin fragments that are bound by the protein of interest. Better understanding the role of aldosterone in the regulation of dipping and non-dipping could lead to a more effective treatment for many who suffer from the adverse effects of hypertension.

METHODS

This research project was performed at the University of Mary Washington (UMW) Jepson Science Center in Fredericksburg, Virginia. The study was carried out under the guidance of Faculty Advisor Dr. Deborah Zies.

Experimental Design

Discovering whether or not the aldosterone receptor binds to the *Per1* promoter is fundamental to the basic understanding of the molecular mechanism of aldosterone regulation of hypertension. Chromatin immunoprecipitation (ChIP) is one method by which these protein-DNA interactions can be assayed [9]. This technique monitors protein-nucleic acid interactions by using antibodies specific for the protein of interest. For this overall project the following antibodies would each provide necessary information because of their specialized function: MR1 (mineralocorticoid receptor), GR1 (glucocorticoid receptor), mK4H3 (dimethyl-lysine 4-histone 3), SRC1 (steroid receptor coactivator-1) and lastly, Pol II (RNA polymerase II) [12]. GR1 serves as the transcription factor that mediates the effect of glucocorticoids, while MR1 is the aldosterone receptor. In order to begin this study, anti-RNA polymerase II antibody was used for the antibody treatment. Pol II mediates transcription which is also expected to be present at any active promoter and was the chosen antibody treatment for the ChIP assay.

Primer Design

The three strongest putative hormone responsive elements (HREs) were previously determined to be at –996,-556 and -156 in the *Per1* promoter region. For the purposes of this project the putative -156 glucocorticoid response element (GRE) was

selected [11]. In order to discover whether or not aldosterone binds to the putative -156

(GRE) element in the *Per1* promoter in order to regulate expression at the transcriptional

level, two sets of uniquely designed primers were created using the FASTPCR program.

One set was designed to amplify the putative promoter element and one set was a

downstream set designed to amplify as a control. These primers were tested for

specificity and function on both pMLG107 (containing the promoter region) and pPer1

(containing the downstream region) plasmids. In order to have primers optimal for ChIP,

the following criteria was used: 20-24 nucleotides, melting temperature between 58-60ºC,

a guanine-cytosine ratio of 40-70%, and a final product of 150-250 base pairs [12]. Two

sets of primer oligonucleotides were synthesized by *SIGMA*-GENOSYS. The first set,

Set 3, amplifies a potential region where the aldosterone receptor binds, in pMLG107 and

is shown in Figure 2. Primer Set 6 was also created for the negative control using the

cDNA sequence for *Mus musculus Per1* (accession number: AF022992). In addition to

the aforementioned criteria, primers for set six were selected based on the furthest

possible distance from set three to best ensure that shearing would separate this region

from the putative binding element.
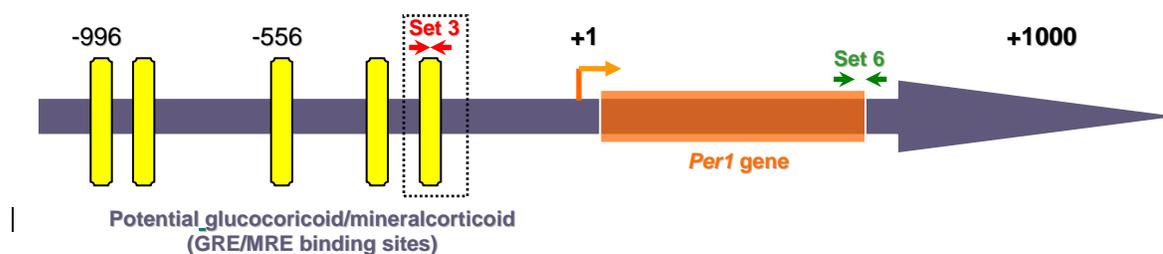
Figure 2 - Designing Primers for *Per1*. The arrow in purple represents the genomic
sequence surrounding the *Per1* gene, while the gene itself is highlighted in orange.
Primer sets 3 and 6 are denoted in red and green respectively. Putative hormone
responsive elements (HREs) are indicated by yellow rectangles. Numbering is relative to
the approximate transcription start site (+1). Set 3 primers were designed for the -156
putative binding element in order to see if aldosterone binds to the receptor. Set 6
primers were designed for a downstream region.

Testing ChIP Primers

Plasmid Constructs -Transformation. In order to amplify the amount of plasmids, pMLG107 and pPer1, available for polymerase chain reaction (PCR) and testing of the primer design, transformation was performed using the DH5α subcloning efficiency competent cells procedure (Invitrogen Inc.). Previously frozen cells were thawed on ice and then mixed with 5 μL of the respective plasmids. After thirty minutes of incubation on ice, a twenty second heat shock at 42ºC was performed. Tubes were then placed on ice for two minutes and 950 μL of LB-AMP media. Tubes were then incubated at 37ºC for one hour at 225 rpm. Approximately, 200 μL of each transformation was plated on prewarmed plates and finally incubated overnight at 37ºC.

Plasmid DNA Purification. Using the QIAPREP spin kit (Qiagen, Inc.), DNA purification was performed in order to extract the DNA from the cells in its pure form. After plates were allowed to incubate overnight, two colonies from each were transferred into their own respective vials with 3 mL of LB-AMP and placed in a shaker overnight at 37ºC. The bacteria were spun in a microcentifuge for one minute at 10,000 rpm. The supernatant was discarded and after repeating this last step the pellet was resuspended in P1 resuspensionbuffer. Next, 250 uL of P2 lysis buffer was added and mixed thoroughly through inversion. Then, 350 uL of N3 neutralization buffer was added and also mixed through inversion. After centrifugation for ten minutes at 13,000 rpm the supernatant was decanted into a QIAPREP spin column. The column was washed successively with PB and PE wash buffers and centrifuged with each wash for 30-60. Lastly, the QIAPREP column was placed in a 1.5 mL microcentifuge tube and spun for one minute with 50 μL of elution buffer to elute the plasmid DNA for collection.

Spectrophotomeric Analysis. In order to determine the DNA concentration and purity a spectrophotometric analysis was performed. The DNA samples were prepared in duplicate, 2 µl of each diluted with 50 µl of DI water. The absorbances for 260nm and 280nm wavelengths were obtained.

Restriction Enzyme Digest. The restriction enzyme digest was performed in order to confirm that the DNA isolated from the DH5α was in fact pMLG107 and pPer1. For the pPer1 plasmid, *Sal*I was used, which cuts the *Per1* insert at base pair 4 and pcmvsport6 at base pair one-hundred and twenty. *BamH*I and *Hind*III were used for pMLG107, which cut at base pairs 374, 2004 and 53, 595 respectively. Following the Promega procedure the following reagents were used in setting up the digest: 2 µl of 10X RE buffer, 0.2 µl of acetylated BSA, 1 µL of DNA, 16.3 µL of DI water, and 0.5 µL of RE enzyme in order to make a total volume of 20 µL. Restrictions were mixed and incubated at 37ºC for one and a half hours.

Agarose Gel Electrophoresis. Gel electrophoresis was performed to separate DNA fragments based on size in order determine if the predicted products were obtained from the restriction digest. Agarose powder (0.6 g) was mixed with 60 mL of 1XTAE buffer and 6 µL of Gel Red and then heated by microwave for one minute. After allowing to cool to approximately 50ºC, the gel was poured into a plate with a well comb and then left to solidify. A 5 µL aliquot of a 200bp ladder marker was used in addition to 24 µL of each of the plasmid samples, previously combined with 4 µl of gel loading dye.

PCR Reactions. Polymerase chain reaction was used to amplify the pMLG and pPer1 plasmids using their respective primers and then gel electrophoresis was performed in order to confirm that the predicted sizes were created. PCR reaction volumes were

optimized according to the directions specified by the Wingo Laboratory at the

University of Florida's College of Medicine [12]. Set up for the components of the

master mix for PCR analysis is shown in Table 1. For each reaction tube 1 µL of DNA

was added.

Table 1 - PCR Components

Reagent	1x reaction
10mM dNTP mix	0.2mM
10uM Forward Primer	0.2uM
10uM Reverse Primer	0.2uM
Go Taq	1.25 units
PCR 5x Buffer	1X
25mM $MgCl_2$	1mM
Total:	49 µl

PCR Cycle Conditions. Conventional PCR was performed using MJ Research

PTC-200 (Peltier thermocycler) DNA Engine Thermal Cycler PCR. Cycle temperatures

were used as outlined by Dr. Gumz [12]. Initial denaturation was at 95ºC for 15 minutes,

followed by 35 cycles of denaturing at 95ºC for 30 seconds, annealing at 55ºC for 30

seconds, and extension at 72ºC for 30 seconds. Final extension was at 72ºC for 10

minutes. To visualize PCR products reactions were electrophorized at 100V on 1%

agarose gels stained with Gel Red.

Chromatin Immunoprecipiation (ChIP)

The following procedures were previously outlined in the Active Motif

ChIP-IT^{TM} kit & Shearing Kit.

Tissue Culture and Conditions. In order to analyze the effect of aldosterone

treatment, chromatin was isolated from both treated and untreated inner medulary

collecting duct (IMCD-3) cells grown to 70-80% confluency. Dulbecco's Modified

Eagle Medium nutrient mixture with F-12 (DMEM/F$_{12}$) plus 10% fetal bovine serum was used.

IMCD-3 cells were grown on a 10 cm plate with 12 mL of the previously prepared DMEM F$_{12}$ media and 120 µL antibiotic/antimycotic at 37ºC. After cells were grown to 70-80% confluency they were split onto six plates using phosphate buffered saline (PBS) as a wash and 1 mL of trypsin, followed by five minute incubation at 37ºC.

ChIP Overview. During chromatin immunoprecipitation (ChIP), protein and DNA interactions are fixed using a formaldehyde treatment, then genomic DNA is isolated and sheared with an enzyme shearing cocktail solution. The sheared DNA is mixed with an antibody specific to proteins expected be bound to the promoter of interest. The antibody/protein/DNA complex is then precipitated using a G protein bead that recognizes the non-specific portion of the antibody. After reverse cross-linking and treatment with proteinase K, the DNA is purified and it can be analyzed using PCR with the primers previously described. The results can be visualized using gel electrophoresis.

Optimization of Chromatin Shearing - Enzymatic

Overview. DNA shearing was performed through enzymatic digestion. The following procedure was adapted from ChIP-IT chromatin immunoprecipiation kit and shearing kit (Active Motif, Inc.). In order to analyze the shearing efficiency and determine the proper amount of incubation time with the enzymatic shearing cocktail cell fixation, chromatin isolation and shearing was performed without adding phenylmethanesulphonylfluoride (PMSF) or protease inhibitors in order to maximize the number of shearing reactions that can be performed.

Cell Fixation. By treating cells with a formaldehyde fixation solution a cross-linking of DNA to any bound proteins occurs. After cells were allowed to grow to 70-80% confluency, on three separate 10 cm plates, the cells were harvested using a fixation solution, ice-cold 1x PBS, glycine stop-fix solution and cell scraping solutions.

After the medium was poured off of the three plates, 7.5 mL of the fixation solution (1.22 mL 37% formaldehyde and 30 mL Minimum Essential Medium) was added to each and then incubated on a shaking platform for 10 minutes. Next, the fixation solution was poured off and the plates were washed with 5 mL of ice-cold PBS (3.5 mL of 10x PBS and 47 mL of dH$_2$O) for 5 seconds. In order to stop the fixation reaction, 3.75 mL of glycine stop-fix solution (2.25 mL of 10x glycine buffer, 2.25 mL of 10x PBS and 18 mL of dH$_2$O) was added to each of the plates and rocked back and forth to cover the entire plate. Incubation and rocking was performed for 5 seconds at room temperature. The glycine stop-fix solution was poured off and the plates were again washed with 5 mL of ice-cold PBS for 5 seconds followed by the addition of 750 µL of cell scraping solution (450 µL PBS, and 4.0 mL of dH$_2$O) to each of the plates. The plates were held at an angle and scraped downwards using a rubber policeman so that they could be collected at the bottom of the plate. The cells from the three plates were collected in a 15 mL conical tube for centrifugation at 2500 rpm (720 RCF) at 4ºC. The supernatant was removed and the cell pellet was either be stored at -80ºC or sheared.

Chromatin Isolation and Shearing. The cells were lysed and the resulting DNA-protein complexes were sheared to cut the DNA into small enough fragments so that the potential putative binding elements would each be part of their own discrete units. This was performed by resuspending the pellet in 1mL of lysis buffer. Cells were transferred

into an ice-cold 2 mL dounce homogenizer, shown in Figure 3, and dounced gently on ice

with 10 strokes to aid in nuclei release. This apparatus is distinct from other

homogenizers because its tight-fitting glass pestle can be used manually to disrupt tissue

suspensions and obtain single cells or subcellular fragments without disrupting the nuclei.

Figure 3 - Dounce Homogenizer. This is a digital image of the dounce homogenizer
used for the ChIP assays performed.

Cells were transferred to a 15 mL conical tube and centrifuged at 5000 rpm (RCF

2400) for 10 minutes at 4°C to pellet the nuclei. After removing the supernatant, the

pellet was resuspended in 1.0 mL of digestion buffer. This solution was pre-warmed to

37°C for 5 minutes.

A working enzymatic cocktail solution was prepared by diluting the provided

enzymatic cocktail with 50% glycerol in dH_2O to a final concentration of 200 U/mL. For

optimization, 0.075 µL of stock enzymatic cocktail solution and 7.425 µL of 50%

glycerol were mixed to provide enough reagent for three reactions. Three reactions were

set up using 50 µL of chromatin and 2.5 µL of enzyme for 37°C incubation at 5, 10 and

15 minutes respectively. Additionally, one reaction was set up using 50 µL and 2.5 µL of

dH$_2$O, which was incubated at 37ºC for 10 minutes to serve as a control. After the incubations had completed, 1 µL of ice-cold EDTA was added to each tube and then chilled on ice for 10 minutes to stop the reactions. The sheared DNA samples were centrifuged at 14,500 rpm in a 4ºC micocentrifuge for 10 minutes so that the supernatant contained the chromatin could be collected. This chromatin could either be stored at -80ºC or the cross-links could be reversed immediately.

Reverse cross-links and remove RNA. Cross-links were reversed by adding 150 µL dH$_2$O, 8 µL 5M NaCl and 1 µL RNase to the DNA sheared samples. After vortexing to mix, the tubes were incubated overnight at 65ºC.

Treatment with proteinase K. Lastly, a Proteinase K treatment was performed by adding 2 µL to each tube and then incubating at 42ºC for 1.5 hours in order to digest protein and remove contamination. The DNA was then run on a 1% agarose gel to determine the optimal shearing conditions.

Optimizing Conditions. After the different reaction times conditions were categorized as under-digested, over-digested or optimized digestion through a comparison with a gel provided in the manufacturer's protocol. The ChIP assay was then performed using the procedures above with only the optimized digestion time.

ChIP assay

Chromatin preparation for the ChIP reactions had some notable deviations when compared to the optimization procedure. These are highlighted below in the corresponding sections.

Six plates were grown to 70-80% confluency, three treated with aldosterone and three untreated to serve as a control. Aldosterone treatment of the IMCD3 cells by

diluting 10 µL of 2.77 mM aldosterone into 15µL of 100 % ethanol. 12uL of this mixture was added to three plates, while three plates were treated with 12µL of 100% ethanol to serve as a control. After rocking the plates back and forth to mix, an incubation at 37ºC for an hour was performed.

During the cell scraping step, the cells from the three plates treated with aldosterone and those that were not were pipetted into two separate 15mL conical tube for centrifugation at 2500 rpm (720 RCF) at 4ºC. After removing the supernatant, the pellets were resuspended in 1.0 mL of digestion buffer as before, but additionally the buffer was supplemented with 5 µL of both PMSF and PIC in order to inhibit protease function. Additionally, after douncing the cells and pelleting the nuclei, the nuclei were resuspended in 1 mL of lysis buffer supplemented with 5 µL of both PIC and PMSF.

The enzymatic shearing cocktail working solution was prepared for two reactions for the chromatin to be used in ChIP reactions, one for the aldosterone treated cells and one for the control. This included 1 µL of stock enzyme and 99 µL of 50% glycerol. To each of the prewarmed nuclei, 50 µL of the working stock was added to each of the pre-warmed nuclei and then incubated at 37ºC for previously determined optimized time, 10 minutes. The reactions were stopped by adding 20 µL of EDTA followed by chilling on ice for 10 minutes. These samples were centrifuged at 14,500 rpm in a 4ºC microcentrifuge for 10 minutes so that the supernatant, which contains the sheared chromatin, could be collected.   The sheared chromatin was stored in 250 µL aliquots at -80ºC.

Pre-clearing of chromatin. Chromatin was pre-cleared with Protein G beads in order to reduce the non-specific background, which is commonly associated with ChIP.

The purpose of pre-clearing is to reduce the amount of non-specific binding to G protein beads.   Therefore, the DNA isolated is contaminated and not completely purified, but this step attempts to alleviate that intrinsic flaw. For the pre-clearing reactions the following reagents were combined into two microcentrifuged tubes, one for aldosterone treated and one for the untreated, to be rotated at 4ºC for 1-2 hours: 100 µL chromatin, 200 µL resuspended protein G beads, 118 µL ChIP IP buffer and 1 µL PIC. This provides a sufficient amount of reagents for two ChIP reactions for each tube. After rotation, the tubes were microcentrifuged for two minutes at 4,000 rpm and then placed on ice for two minutes to let the protein beads settle. The supernatant, chromatin, was then transferred to a fresh tube and the rotation and microcentrifugation steps were repeated to ensure complete removal of beads. From each of the pre-cleared chromatin samples, 10 µL was transferred to a microcentrifuge tube and stored at -20ºC to be used as input DNA for the PCR analysis. This DNA does not go through the steps specific to the ChIP assay and thus functions as a positive control.

Addition of antibodies to Pre-cleared Chromatin. The antibody treatment was performed using anti-RNA pol antibody (upstate 05-623) in order to precipitate the RNA polymerase present at promoters. PCR will determine whether or not this includes the *Per1* promoter, and therefore, if the aldosterone treatment induced binding of pol II to the promoter of the *Per1* gene.   Antibody incubations were performed in the kit's provided 0.65 mL siliconized tubes. From each sample, 170 µL of the pre-cleared chromatin was transferred to two tubes respectively. Then, 2.5 µL of anti-RNA pol antibody was added to one tube for each sample and nothing was added to the remaining two to serve as negative controls. These tubes were incubated overnight on a rotator at 4ºC.

Addition of Protein G to Antibody/Chromatin Mixture. Protein G beads, that bind the non-specific portion of the antibody used, were then added so that the antibody will attach, thereby making the complex heavier so centrifugation can precipitate out the desired DNA-protein-antibody-bead complex. After performing the incubation, 100 µL of resuspended protein G beads were added to each of the four antibody/chromatin mixtures and then incubated on a rotator for 1.5 hours at 4ºC.

Washing ChIP reactions. A series of washing of the ChIP reactions was performed to remove any protein-DNA complexes not specifically attached to the G protein bead. The following buffers were prepared: ChIP IP buffer, wash buffer 1, wash buffer 2 and wash buffer 3 (supplied and ready to use). The ChIP IP buffer was prepared by adding 1.6 mL to 8 µL PIC. For wash buffer 1, 6.4 µL PIC was added to 6.4 mL of wash buffer 1. Wash buffer 2 was prepared by adding 1.6 µL PIC to 1.6 mL of wash buffer 2. After prepared, each of the solutions were mixed and placed on ice.

After the incubation, the tubes were centrifuged for two minutes at 4,000 rpm and then allowed to settle for thirty seconds in order to pellet the beads. After removing the supernatant, 400 µL of ChIP IP buffer was added to each of the tubes, then vortexed and incubated on a rotator for one to three minutes. After pelleting the beads as stated above, beads were washed four times with 400 µL wash buffer 1; buffer was removed after each wash. Next, the samples were washed with 400 µL of wash buffer 2, beads were pelleted and then washed with wash buffer 3. After this final wash, as much buffer as possible was removed without disturbing the beads.

DNA elution from Protein G. DNA elution from protein G beads was performed in order to collect the immunoprecipitated DNA. This was performed using 50 µL of

ChIP elution buffer, which was prepared by adding 20 µL 1M NaHCO$_3$ to 400 µL of 1%SDS. After the addition of the elution buffer the tubes were vortexed and incubated for 15 minutes on a rotator at room temperature. Next, tubes were centrifuged at 4,000 rpm to pellet the beads and then the supernatant was transferred to microcentrifuge tubes.

Reverse cross-links and remove RNA. In addition to the ChIP elutions from above, the input DNA was also taken through the following steps of reversing the cross-links and removing RNA, which differ in the amount of reagents used from the same procedure for optimization. To each ChIP elution and the two input DNA samples 4 µL of 5 M NaCl and 1 µL RNase A was added and then mixed completely and centrifuged briefly. These tubes were incubated at 65ºC overnight.

Treatment with proteinase K. After overnight incubation, these samples were treated with proteinase K in order to digest protein and remove additional contaminants. This section is slightly different from the optimization portion of this procedure; the following three components were added to each tube: 2 µL 0.5 M EDTA, 2 µL 1M Tris-Cl pH 6.5, and 2 µL proteinase K solution. After vortexing to mix and a short centrifugation the tubes were incubated at 42ºC for two hours to be followed by DNA purification.

Purification of eluted DNA. Following incubation, 500 µL of DNA binding buffer was added to the proteinase K treated samples. Each of these samples were then transferred into respectively labeled DNA purification mini-columns and then centrifuged for 30 seconds at 14,500 rpm. After discarding the flow-through, 600 µL of DNA wash buffer was added to each mini-column and centrifugation for thirty seconds at 14,500 was performed. After removing this flow-through, 300 µL of DNA wash buffer was added

and centrifugation at 14,500 rpm was performed for two minutes. Then each of the mini-columns was placed in a microcentrifuge collecting tube and 50 µL of DEP-C treated H_2O was added directly to the resin of each mini-column. After incubation for three minutes at room temperature these samples were centrifuged at 14,500 rpm for one minute. This was repeated and then the eluted DNA was either stored at -20ºC or used for PCR analysis.

   PCR Analysis. PCR was performed as previously described in the testing primers section. By amplifying the DNA it will be possible to detect if *Per1* was precipitated using the RNA pol antibody that was immunoprecipitated by the ChIP procedure making it apparent whether or not aldosterone regulated *Per1* at the transcriptional level. If any of the proteins recognized by the specific antibody used were bound to the *Per1* promoter, then the *Per1* promoter should be amplified.

RESULTS

Designing Primers. Using the criteria discussed in the methods section both a forward and reverse primer for each set were successfully designed and are shown in Table 2.

Table 2 - Primers for Set 3 and Set 6

	Set 3	Set 6
Forward Primer	ATAGGAGGCGATCAGCTCACC (1504-1524)	GCCTCTAGCTTCTTAGCAGAGTGG (4265-4288)
Reverse Primer	GCTGTACTCATTCCACACTGGCAA (1635-1658)	TTGGGAGAGACAGCTCACTCT (4445-4465)

Spectrophotometric Analysis of DNA Samples. After transformation, isolations and DNA purification the *Per1* and *pMLG107* DNA samples were tested for concentration and purity using a spectrophotometer. The results for each plasmid are shown in Table 3.

Table 3 – Spectrophotometric Analysis of Primers

	Absorbance 260nm (A)	Absorbance 280nm (A)	[DNA] ng/µL	Purity
per1	0.249	0.131	311	1.90
pMLG107	0.248	0.132	310.	1.88

Restriction Enzyme Digest. The expected results for both the pMLG and pPer1 plasmids were not obtained after multiple restriction enzyme digests, but Figure 4C is representative of the results obtained. In the case of the pMLG plasmid, its digestion with *BAMH*I and *Hind*III is shown in Lanes 2 and 4 respectively. There are more products than expected in Lane 2, but the ~4,000, ~3,000, and ~400bp fragments are represented on the gel. In Lane 4, the expected bands of ~6,000 and ~500bp were visible, but an additional band of ~3,000bp was present as well. For the digestion of pPer1with *Sal*I, represented in Lane 5, bands of ~4,000 and ~5,000bp were visible as predicted.
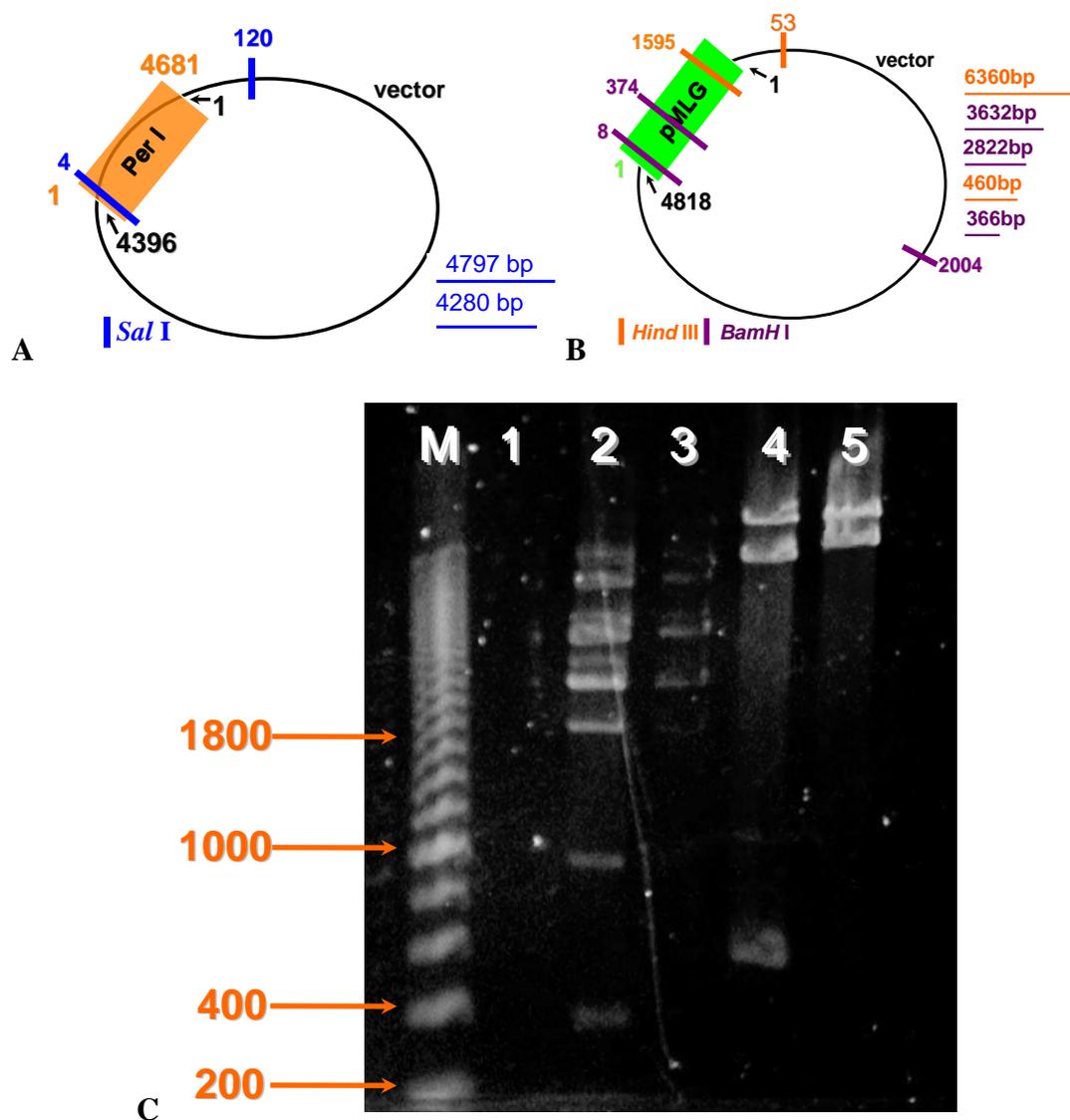
Figure 4 – Restriction Enzyme Digest for *Per1* and *pMLG107*. 4A shows the restriction enzyme digest for the pPer1 plasmid using *Sal*I. *Sal*I cuts the *Per1* insert at base pair 4 and at 120 in the vector. This results in fragment sizes of 4797bp and 4280bp. 4B shows the digest for the pMLG107 plasmid when using *Bamh*I and *Hind*III. *BamH*I cuts the insert at 8 and 374, while *Hind*III cuts at 1595. In the vector the enzymes cut at 595 and 2004 respectively. This results in fragment sizes of 3632, 2822 and 366bp. 4C shows the gel that was performed after the RE digest. Lane 1 and 3 were empty, but some spilling over occurred into Lane 3. Lane 2 represents the digestion of pMLG with *BAMH*I, while Lane 4 represents the digestion with *Hind*III. Lane 5 represents the digestion of pPer1 with *Sal*I. The 200bp molecular marker is indicated by "M," and the respective fragments sizes are shown on the left.

Amplification Using Set 3 and Set 6 Primers on pPer1 and pMLG107. For both

the Set 3 and Set 6 primers the expected products were visible on the gel shown in Figure

5. For pMLG with Set 3 primers the final product was expected to be 154bp, which did

appear on the gel in Lane 1 and 4. Set 3A and Set 3 B represent primers two sets of the

similar primers, synthesized at different times to avoid contamination, which was

previously a problem. The 200bp product was also shown in Lane 9 for the Set 6 primers

used with pPer1. Therefore, both Set 3 primers yielded the expected ~150bp product in

the pMLG lanes (1 & 4), while the Set 6 primers yielded the ~200bp in the pPer1 lane
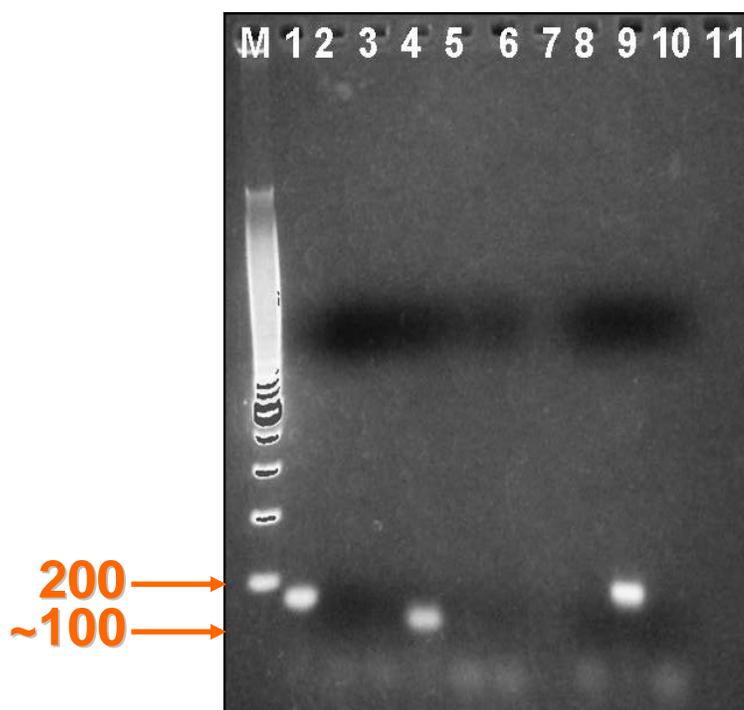
(9). There were no bands in the control lanes with dH_2O.

Figure 5 – PCR of pPer1 and pMLG107 Using Set 3 and Set 6 Primers. Lane 1,2 &
3 represent Set 3A primers with pMLG, pPer1, and dH_2O respectively. Lane 4,5 & 6
represent Set 3B primers with pMLG, pPer1, and dH_2O respectively. Lane 8, 9 & 10
represent Set 6 primers with pMLG, pPer1, and dH_2O respectively. Lanes 7 & 11 were
empty. The 100bp molecular marker is indicated by "M," and the respective fragments
sizes are shown on the left.

Chromatin Immunoprecipiation (ChIP)

Enzymatic Shearing Optimization. Enzymatic shearing was performed on three

samples of DNA for varying amounts of time and then the products were run on a gel as
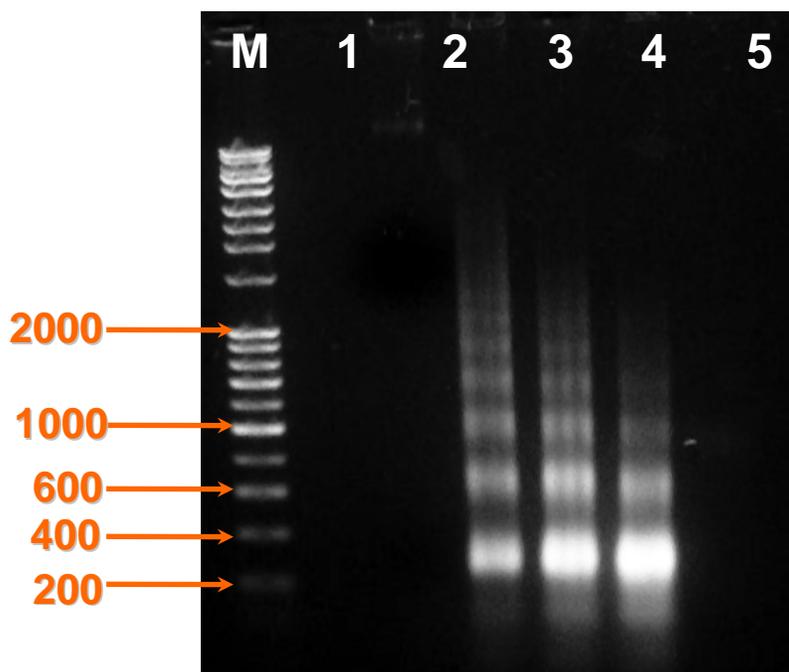
shown in Figure 6.

Figure 6 – Enzymatic Shearing Optimization. Lane 2 was empty. Lane 3 represents unsheared DNA. Lane 4 represents DNA treated for 5 minutes, which is under-digested. Lane 5 represents DNA treated for 10 minutes, which is optimized digestion. Lane 6 represents DNA treated for 15 minutes, which is over-digested. The 100bp molecular marker is indicated by "M," and the respective fragments sizes are shown on the left.

Amplification of ChIP Assay Products. After the entire ChIP protocol was

performed, PCR with primers sets three and six was performed and then the products

were run on a gel as shown in Figures 7A and B. Bands appeared as expected for the

plasmids; however, no bands were obtained in any of the ChIP products, including the
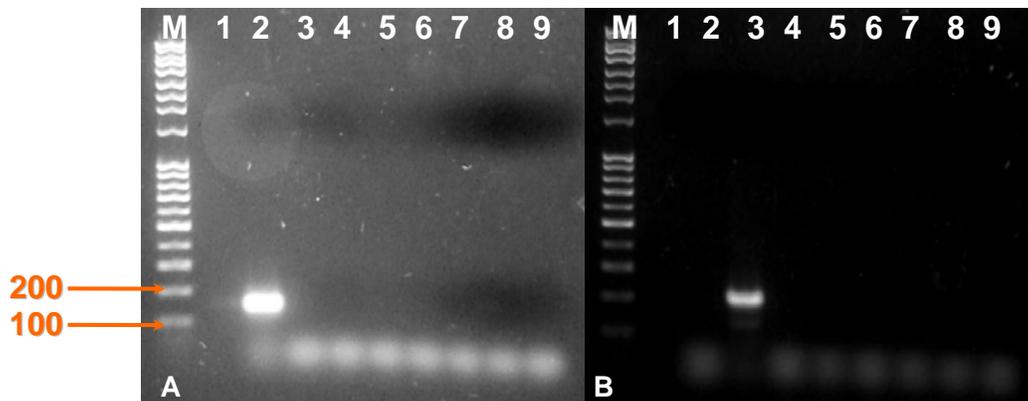
input DNA samples.

Figure 7– PCR with Set 3 & Set 6 After ChIP Assay. Gel A shows the results of using Set 3 primers, while Gel B shows the results of using Set 6 primers. The lanes for each respective gel are the same samples. Lane 1 was empty in both gels. Lanes 2 & 3 represent pMLG and pPer1 respectively. Gel A shows the expected ~150bp product for pMLG, while Gel B shows the expected ~200bp for pPer1. Lanes 4 & 5 represent the input DNA for the aldosterone-treated cells and the control cells respectively. Lanes 6 is the DNA for the aldosterone-treated cells, which received the antibody treatment, while Lane 7's cells did not. Lane 8 is the control cells, which received the antibody treatment, while Lane 9's cells did not. Lanes 4-9 did not show any bands, but have smudges at the bottom indicating unused primers. The 100bp molecular markers are indicated by "M," and the respective fragments sizes are shown on the left.

Amplification of ChIP Assay Products without Pre-clearing or Antibody

treatment, "Mock" ChIP. In an attempt to obtain results consistent with the expectations,

PCR was run on products that were sheared, had cross-links reversed, treated with

proteinase K, and purified. These products did not go through the pre-clearing or

antibody treatment steps. Figure 8 shows the results of the PCR on a gel, which includes

the expected product for the aldosterone treated cells for both Set 3 and Set 6 primers.

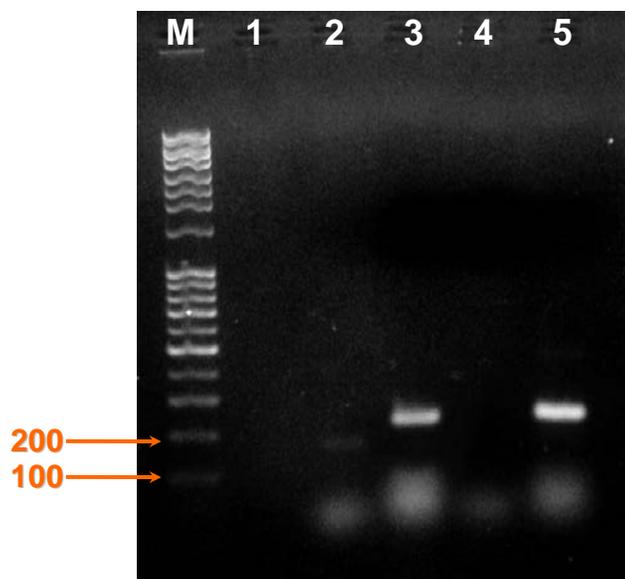Control cells only showed the expected 200bp product for Set 6 primers.

Figure 8 – PCR with Sets 3 & 6 "Mock" ChIP Assay; No Pre-clearing or Antibody treatment performed. Lane 1 was empty. Lane 2 & 3 represent input DNA for aldosterone treated cells using Set 3 & 6 respectively. Lane 4 & 5 represent control cells using Set 3 & 6 respectively. Each of these samples underwent a "Mock" ChIP, which includes reverse cross-linking, removal of RNA, proteinase K treatment and purification. The 100bp molecular markers are indicated by "M," and the respective fragments sizes are shown on the left.

DISCUSSION

Since there were no products visualized on the gel for the ChIP products that underwent PCR amplification results were inconclusive and it could not be determined whether or not the aldosterone-receptor complex was bound to the *Per1* promoter element. This is likely due to DNA loss that occurred in one of the ChIP assay steps, potentially the pre-clearing of chromatin step.

The primers created for the purposes of this study were consistent with the criteria ideal for ChIP samples. Set 3 represents the location where it is hypothesized that the aldosterone receptor binds and if this occurs in actuality, ChIP should confirm with a band of 154bp after PCR analysis of the products. Set 6 represents a control set that maximized the distance from the -156GRE region so that the aldosterone receptor would not bind in that region. This negative control should not result in precipitation of the fragments present at the promoter because the protein of interest, the aldosterone receptor, should not be bound to that region. After ChIP, these products should show a band of 200bp after PCR analysis. Before the designed primers were used on plasmids pMLG and pPer1, their respective concentrations and purities were determined using spectrophotometric analysis.

The DNA concentrations for pMLG and pPer1 were calculated to be 311 and 310 ng/μL respectively. Typically, a good quality DNA sample has a purity ratio of 1.7-2.0, which includes the determined values of 1.90 and 1.88 as shown in Table 2. Another method used to test the plasmids before the primers were used was the restriction enzyme digests.

Three separate restriction enzyme digests were performed in order to obtain results consistent with the restriction digest maps shown in Figures 4A and B. The gel depicted in Figure 4C is representative of these results and shows a successful digest for the pPer1 plasmid using *Sal*I, which includes the expected 4797bp and 4280bp fragments. For pMLG two separate enzymes were used to try and obtain predicted results, but for each digest additional bands were present in on the gels. The reason for the discrepancies of the restriction enzyme digest were not investigated since the PCR on the plasmids verified both the primer design and the presence of the appropriate fragment within each plasmid (Figure 5).

In order to start the ChIP protocol tissue culture conditions and enzymatic shearing conditions were optimized. The aldosterone treatment was performed for an hour before starting the cell fixation step. It is important to note that using the proper dounce homogenizer results in a drastic increase in the amount of DNA available for shearing. This is due to its gentle lysing nature, which aids in releasing intact nuclei.

Using the guidelines of the ChIP-It protocol, the results of Figure 6 were analyzed to best determine the optimal time for shearing for the ChIP assays. Upon comparison to a provided figure in the procedure, it was apparent that five minutes resulted in under-digestion of the DNA and that fifteen minutes resulted in the over-digestion. Ten minutes was determined to be the optimized time for digestion to use when completing the entire ChIP procedure.

After digesting the DNA sheared from the tissue cultured cells for the optimized time, these ChIP samples were taken through the entire protocol. The gel of the PCR products are shown in Figures 7A and B. The only bands that appeared were for the

respective plasmids and their primer sets (pMLG, Set 3 and pPer1, Set 6). These results were particularly unexpected because no bands appeared in the input DNA lanes, which represent genomic DNA prior to any precipitation. These samples traditionally serve as a positive control for PCR effectiveness and should have a band regardless of the treatment. These samples were not taken through the pre-clearing and antibody treatment steps. Since the positive control in this experiment was unsuccessful, the remainder of the results were uninterpretable. In order to discover a reason for this discrepancy, previously saved aliquots from DNA shearing were taken through a "mock ChIP" procedure that did not include the pre-clearing and antibody steps to determine the possibility of DNA loss in one of the steps.

As shown in Figure 8, the input DNA from the "mock ChIP" did in fact show the expected bands for both the primer sets regardless of aldosterone treatment, suggesting that an error occurred early in the actual ChIP experiment. The lack of a band in the lane representing the control cells with Set 3 primers in addition to the faint band in the lane for the aldosterone-treated cells with Set 3 indicate that perhaps the PCR conditions for Set 3 need to be adjusted to obtain better results. These results, combined with the lack of product in the ChIP PCR gel in Figures 7A and B suggest that DNA loss likely occurred in the pre-clearing step.

For future study, a systematic approach to determine if either the pre-clearing or antibody treatment steps result in DNA loss, should be performed. It is likely that DNA loss occurred during the pre-clearing, because input DNA from the same aliquot was used in the "mock" ChIP (without pre-clearing) and results were obtained as shown in Figure 8. By testing the samples at different stages of the ChIP protocol any possible error could

potentially be pinpointed to a specific step. If successful in that analysis, this part of the

procedure could be better examined to ensure that DNA is maintained in the samples.

Overall, this research project has been a success in that it has set the stage for

continued research concerning the proposed mechanism of aldosterone regulated *Per1*

circadian regulation of hypertension. Primers were successfully designed and tested for

both the pMLG and pPer1 plasmid confirming their identities. Tissue culture conditions

were successfully documented and the appropriate aldosterone treatment procedure was

determined. Additionally, the optimized digestion time for enzymatic shearing of the

chromatin was determined to be ten minutes. The initial steps of the ChIP protocol were

shown to be successful on the samples. The collected data and procedural notes provides

the backbone for the future success of this research project's original goal to verify the

binding of aldosterone and its receptor to the *Per1* promoter in order to potentially

regulate circadian blood pressure patterns.

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