University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
May 2018

Inducing Gene Expression of Major Urinary Proteins (MUPs) in the female mouse liver cell line Hepa1-6

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Abstract

Mice, *Mus musculus*, are a primarily nocturnal species that rely heavily on their olfactory system to detect changes in their environment. Specifically, mice rely on protein pheromones, the Major Urinary Proteins (MUPs), non-volatile molecules that are detected by the vomeronasal organ (VNO). MUPs are synthesized in the liver, excreted in the urine, and serve as genetically encoded pheromones which direct social behaviors such as countermarking, aggression, or mate preference. Mice can also use MUPs as a way to detect sex, status, and identity of the emitting individual. MUP expression is thought to be controlled by a set of hormonal axes consisting of testosterone, growth hormone, and thyroxine. The mouse genome encodes 21 MUPs, yet, each adult male mouse will express a unique set of 4-12 MUPs. The mechanism by which MUPs are chosen for expression is non-random, but not well understood. This study looks to understand how individual MUPs are chosen for expression by utilizing a cell culture model system. The female murine liver cell line Hepa1-6, is being used because it does not endogenously show expression of any MUPs, but previous studies have shown that female mice are capable of producing MUPs at male levels if they are exposed to testosterone. A combination of hormonal and drug treatments consisting of methylation inhibitors and deacetylation inhibitors are being used to induce MUP expression in these cultured cells. Following treatment of cells, they are harvested for RNA isolation, and the resulting cDNA library is examined for MUP expression. So far, the chosen treatment periods and concentrations of drugs and hormones have not been sufficient to induce MUP expression. As such, a working protocol for the induction of MUP expression is yet to be established. The creation of a working protocol will in the future contribute to the greater understanding of gene expression.

1. Introduction

How gene expression is regulated has been one of molecular biology's long standing questions. The major urinary protein (MUP) family in the house mouse *Mus musculus* serves as an interesting model system to study the mechanisms underlying gene expression control. The MUPs serve as genetically encoded pheromones, which are defined as a molecular signal that is excreted by an individual and elicits an innate behavioral response in a member of the same species[10]. These pheromones are detected using the vomeronasal organ (VNO) and trigger species specific responses such as aggression and countermarking in males, and mate preference in females[10,16]. The expression pattern found in males is much more expanded than that of females, and the males also have a greater number of behaviors linked to their MUP expression patterns[15].

Logan et al. characterized the *Mup* genes in the mouse, as well as identified orthologous loci in several other mammalian species providing phylogenetic and structural evidence that *Mup* gene families show significant lineage specificity, contributing to the idea of their role in species specific communication[10]. The *Mup* gene family is seen across terrestrial vertebrates, and its divergence has occurred multiple times in parallel in the mammalian lineage which is consistent to a species-specific function[9,14]. While there are many mammalian species that express MUPs,

the *Mup* gene family is expanded in the mouse genome which encodes 21 distinct MUPs localized on chromosome 4[10,12,15]. Each sexually mature male mouse will express a unique subset of 4-12 MUPs[10,11,14,15]. This expression is conserved throughout his lifetime, which has been shown using transcriptomic data[8,10,14,15]. The MUPs can be divided into two main categories based upon sequence similarity, the central and peripheral MUPs. Central MUPs are thought to have diverged due to an inverted duplication event of MUP2 and MUP1 which are part of the more ancestral peripheral MUPs[12]. Central MUPs can vary in as little as a single amino acid, while the peripheral MUPs have a greater degree of variability[12].

MUPs are synthesized in the liver, secreted by the kidneys, and excreted in the urine in milligram per milliliter quantities, a very metabolically costly action[8,10,17]. MUPs are highly homologous and part of the lipocalin family of proteins which are of a high molecular weight, and can bind specific small organic volatile molecules in the hydrophobic binding pocket created by the ß-barrel formed by its 8 anti-parallel ß-sheets[6,7,8,9,10,11,12,15,]. The ß-barrel structure of MUPs is extremely degradation resistant which allows both the MUP and its bound molecule to persist in the environment[8,12]. The subset of expressed MUPs varies among members of a population of wild mice, and has been shown to be highly heritable across generations of inbred lab strains, suggesting that the selection for certain MUPs over others is non-random[11,15]. MUPs can be used to differentiate between sex, status, and self or non-self[8,10,17]. The social and sexual selection towards a unique MUP signature confers the individual with the advantage of being recognized by females to prevent in breeding and allowing for selection of a mate with different genes[17]. The mechanism by which certain MUPs are chosen for expression over others is not well understood, but appears non-random as MUPs such as MUP20 are chosen more consistently for expression[2,12,13].

Due to the complexity of MUP expression, the MUP family serves as a promising system in which to study gene expression. The current study looks to induce MUP expression in the female Hepa1-6 liver cell line using a cell culture system, allowing for complete control over the cellular environment[,2,3,6,*10*]. Previous studies have shown that MUP expression can be induced through testosterone, growth hormone, and thyroxine *in vivo*[3,6]. A working *in vitro* model has not yet been established in studying the expression of MUPs. The current study looks to induce MUP expression through hormone treatments consisting of testosterone, growth hormone, and thyroxine over a 48 or 72-hour period. Following treatment, MUP expression is then examined at the mRNA level. The establishment of a working protocol using a cell culture will help us begin to understand the complexities of gene expression.

2. Methods

2.1 Cell culture

Female mouse liver cell line Hepa1-6 was obtained from American Type Culture Collection (ATCC, USA). The cells were allowed to grow to confluence in 1:1 F-12/DMEM media supplemented with dexamethasone, fetal bovine serum (FBS), and ITS. For each treatment, cells were split into a 12-well cell culture plate such that each well contained $5x10^5$ cells per milliliter. The cells were then incubated at 37ºC, 5% CO_2, and 95% humidity for about 24 hours in serum free (SF) media: 1:1 F-12/DMEM supplemented with dexamethasone, charcoal-striped FBS and ITS prior to treatment.

2.2 Hormone treatments

Cells were treated with a combination of hormones consisting of testosterone (Sigma-Aldrich, USA), dihydrotestosterone (DHT) (Sigma-Aldrich, USA), mouse growth hormone (mGH) (GenScript, USA), thyroxine (Fisher Scientific, USA), as well as with methylation inhibitor (Trichostatin A) (Promega, USA) and deacetylation inhibitor (5-aza-2'-deoxycytidine) (MP Biomedicals, USA) in varying concentrations (Table 1). Equivalent volumes of vehicles respective to their hormone/drug were used to serve as a control. Treatments were carried out over a 48 or 72-hour period and the hormones and/or drugs were replaced every 24 hours during this time.

2.3 Cell harvest

At the end of the treatment period, growth media was removed from each well and the cells were washed with phosphate buffered saline (PBS). The PBS was removed and replaced with trypsin and the cells were incubated at 37ºC. The cells were then resuspended in SF media. Identical treatment wells were combined.

2.4 RNA isolation

Cells were centrifuged for 5 minutes at 300xg. The supernatant was aspirated and RLT buffer combined with ß-mercaptoethanol (ß-ME) were added to the cell pellet to resuspend the cells. A QIAGEN RNeasy kit and QIAShredder column were used to isolate the RNA from the lysate. The purity of the RNA was then determined using a NanoDrop. Isolated RNA was stored at -80ºC.

2.5 cDNA synthesis

The isolated RNA was used to create cDNA using qScript cDNA Supermix (Quanta Biosciences) via reverse transcription. The Supermix, RNA template, and RNase/DNase free water were combined in a PCR tube for a 20 µL reaction and the following program was run in the thermocycler: 25ºC for 5 minutes, 42ºC for 30 minutes, 85ºC for 5 minutes. A NanoDrop was then used to determine the concentration of the cDNA and then diluted to 10 ng/µL in 10 mM tris-HCl (pH 8.0), 0.1 mM EDTA and stored at -20ºC.

2.6 Polymerase chain reaction (PCR) and Agarose gel visualization

PCR reactions were set up for a 25 µL reaction consisting of cDNA template (dilute), Promega PCR Mastermix, dH_2O, and a forward and reverse primer. The following program was run in the thermocycler (95ºC for 2 minutes, 95ºC for 1 minute, XºC (annealing temperature specific to primer) for 1 minute, 72ºC for 1 minute) 34x and then 72ºC for 10 minutes. Degenerate primers were used to target several central MUPs (labeled MUPB) and peripheral MUPs (labeled MUP25) due to their sequence similarity. Specific primers for ß-actin (BA), mouse androgen receptor (mAR), mGH receptors (mGHR), and thyroxine receptors (T4R) were also used to test viability of cells as well as for hormone receptors. A 2% agarose gel was used to visualize MUP expression with a 100 bp ladder and 6x loading dye used in each sample.

3. Results

3.1 MUP Expression

Degenerate primers were designed to target the central MUPs (MUPB) and peripheral MUPs (MUP25). MUP expression was examined in the control C57B1/6j liver tissue cDNA, AML-12 cDNA, and Hepa1-6 cDNA. The control C57B1/6j liver tissue cells expressed both central and peripheral MUPs around 500 bp (Fig 1). Central or peripheral MUP expression was not detected in the AML-12 or Hepa1-6 cells.(Fig 1). ß-actin (BA) served as a control for cell viability and was expressed around 250 bp (Fig 1).

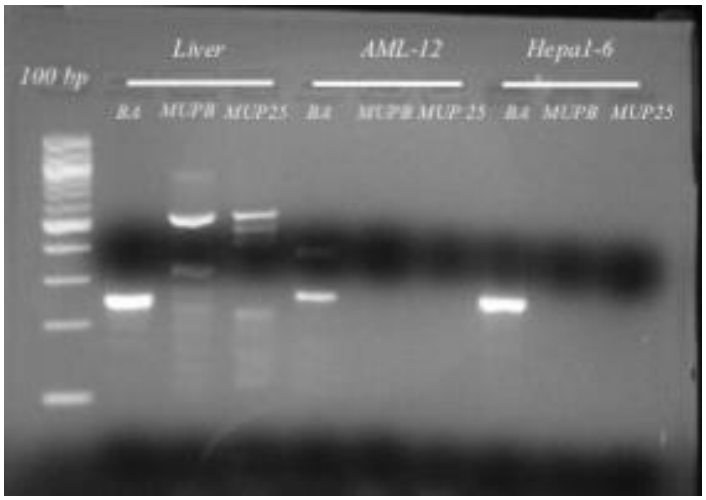


Figure 1. MUPs are endogenously expressed in male mouse liver tissue, but not in tested liver cell lines.

Figure 1. Degenerate primers were used to detect expression of central and peripheral MUP expression in cDNA libraries made from C57B1/6j adult male mouse liver tissue, as well as in the adult male liver cell line AML-12 and adult female liver cell line Hepa1-6. MUP primers successfully amplified MUP sequences in the liver. ß-actin (BA) served as a control for cell viability.

3.2 Hormone and Drug Treatments

Hormone and drug treatments as detailed in Table 1 were run and their corresponding treatments were examined at the mRNA level for the inducement of MUP expression. The chosen concentrations and treatment periods were not sufficient to induce the expression of MUPs.

Table 1. Hormone and Drug Treatment Schemes

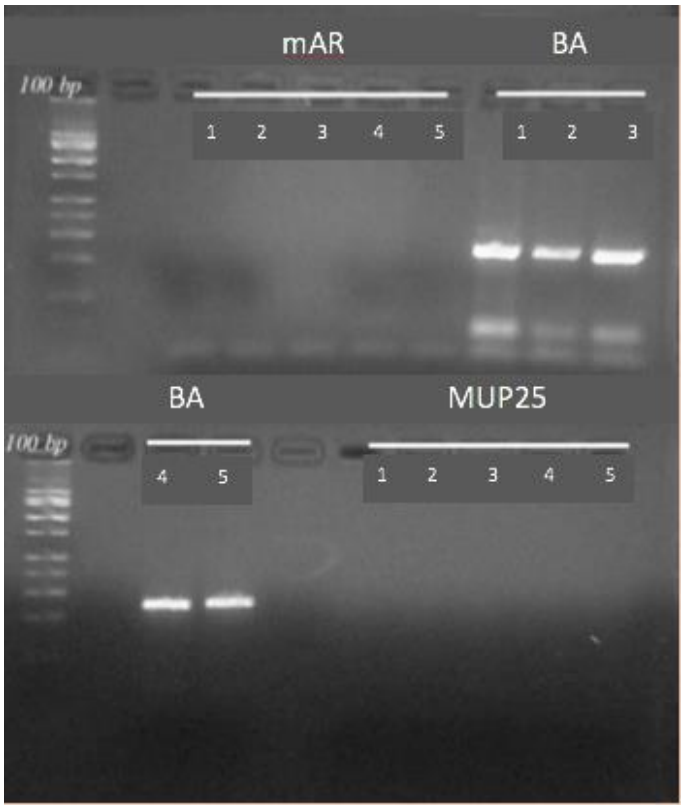
Treatment	Testosterone	DHT	Thyroxine	mGH	DAC	TCA
1		100 nM				
2		250 nM				
3		500 nM				
4		750 nM				
5	500 nM					25 nM
6	1000 nM					50 nM
7					2 uM	25 uM
8					2 uM	50 uM
9					5 uM	25 uM
10					5 uM	50 uM
11	10 nM		25 nM	100 nM		
12	25 nM		50 nM	100 nM		
13	10 nM		25 nM	100 nM	100 pM	
14	10 nM		25 nM	100 nM	250 pM	
15	20 nM			100 nM		200 nM
16	40 nM			100 nM		200 nM

Table 1 Treatment setup and concentrations are listed by row, with the corresponding hormone and drugs listed at the top. Treatments 1-4 used DHT, a strong agonist for the androgen receptor, to induce MUP expression. Treatments 5 and 6 used both a deacetylation inhibitor, as well as a methylation inhibitor to see if either deacetylation or methylation was inhibiting MUP expression. Treatments 7 and 8 used testosterone, thyroxine, and mGH at mammalian physiological levels to induce MUP expression. Treatments 9 and 10 used testosterone, thyroxine, mGH, and DAC to induce MUP expression by employing physiological levels of hormones and a deacetylation inhibitor. Treatments 11 and 12 used testosterone, mGH, and TCA to induce MUP expression, replicating a previously successful treatment.

3.2.1 Treatment with testosterone, thyroxine, and mouse growth hormone

A treatment employing conditions more similar to mammalian physiological levels did not yield MUP expression[6]. The treatment consisted of testosterone, thyroxine, and mGH at concentrations of 10 nM, 25 nM, 100 nM, and 25 nM, 50 nM, 100 nM respectively were carried out over a 72-hour period (Fig 2A-2B). ß-actin (BA) served as a control for cell viability and was expressed in this treatment around 250 bp (Fig 2A).

A.



B.

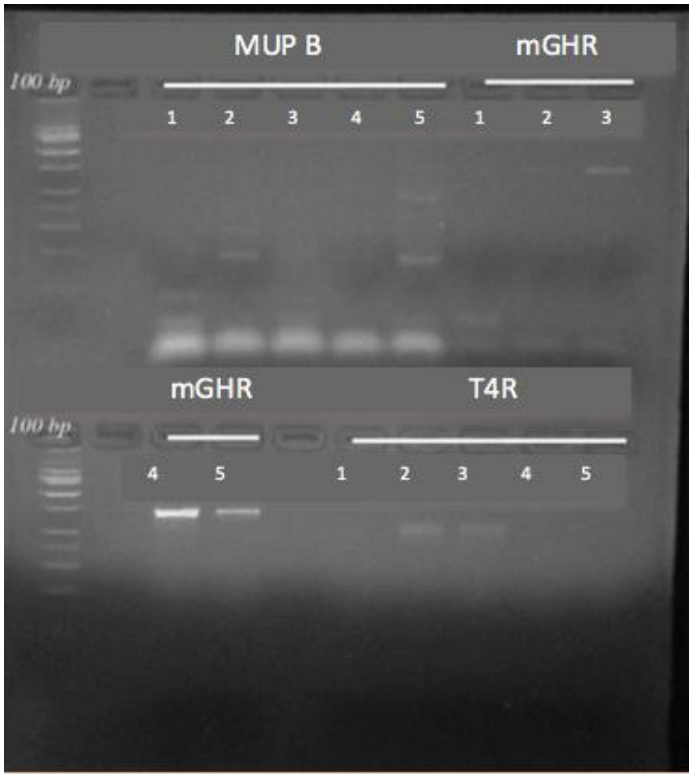


Figure 2. Hormone and MUP expression following treatment with testosterone, thyroxine, and growth hormone treatment of Hepa1-6 cells.

Figure 2. Combined treatment with testosterone, thyroxine and mouse growth hormone at tested concentrations did not induce MUP expression. Treatment setup: 1: control no vehicle, 2: control with low vehicle, 3: control with high vehicle, 4: 10 nM Testosterone, 25 nM thyroxine, and 100 nM mouse growth hormone, 5: 25 nM Testosterone, 50 nM thyroxine, and 100 nM mouse growth hormone. Neither the low concentrations (lanes labeled 4) nor the high concentrations (lanes labeled 5) tested resulted in induction of central (MUPB) or peripheral (MUP25) MUP expression (A, B). The cells did show expression of mouse growth hormone receptor (mGHR) and thyroxine receptor (T4R) (B). ß-actin (BA) served as a control for cell viability and was expressed in this treatment (A).

3.2.2 Treatment with testosterone, thyroxine, mouse growth hormone, and DAC

The addition of a methylation inhibitor with testosterone, mouse growth hormone, and thyroxine did not induce MUP expression. Treatments with testosterone, thyroxine, mGH, and DAC in concentrations of 10 nM, 25 nM, 100 nM, and 100/250 pM respectively over a 72-hour treatment period did not result in MUP expression (Fig 3). ß-actin (BA) served as a control for cell viability as was expressed in this treatment around 250 bp (Fig 3).

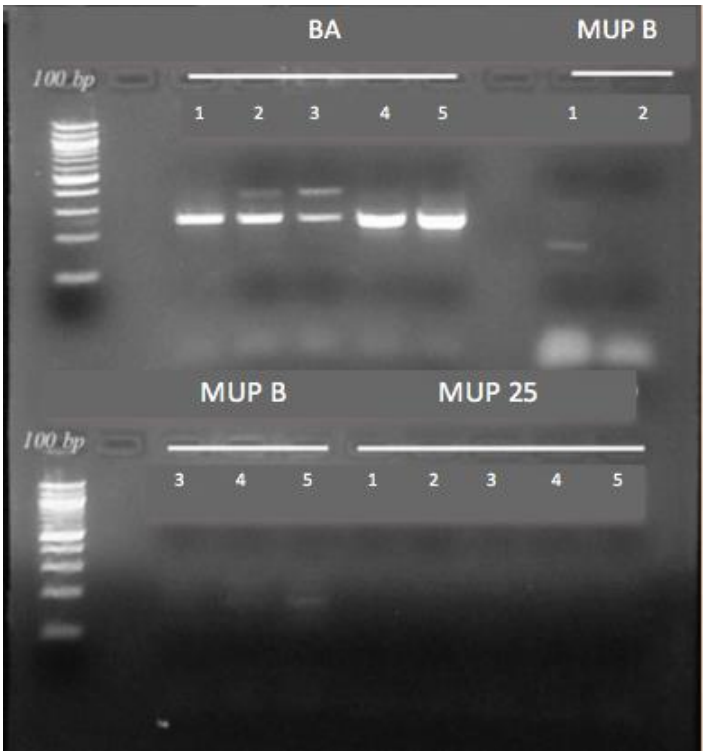


Figure 3. MUP amplification following treatment of Hepa1-6 cells with testosterone, thyroxine, growth hormone, and DAC

Figure 3. Combined treatment with testosterone, thyroxine, mouse growth hormone, and a methylation inhibitor (DAC) at tested concentrations did not induce MUP expression. Treatment setup: 1: control no vehicle, 2: low vehicle, 3: high vehicle, 4: 10 nM testosterone, 25 nM thyroxine, 100 nM mouse growth hormone, and 100 pM 5-aza-2'-deoxycytidine (DAC), 5: 10 nM testosterone, 25 nM thyroxine, 100 nM mouse growth hormone, and 250 pM 5-aza-2'-deoxycytidine (DAC). The addition of a methylation inhibitor did not induce expression of the central (MUPB) or peripheral (MUP25) MUPs. ß-actin (BA) served as a control for cell viability and was expressed in this treatment.

3.2.3 Treatment with testosterone, mouse growth hormone, and TCA

A deacetylation inhibitor combined with testosterone, and mouse growth hormone did not induce MUP expression. Treatments with testosterone, mGH, and TCA in 20/40 nM, 100 nM, and 200 nM respectively over a 72-hour period did not induce MUP expression. The TCA in combination with testosterone and mGH did not induce MUP expression (Fig 4). ß-actin (BA) served as a control for cell viability and was expressed in this treatment around 250 bp (Fig 4).

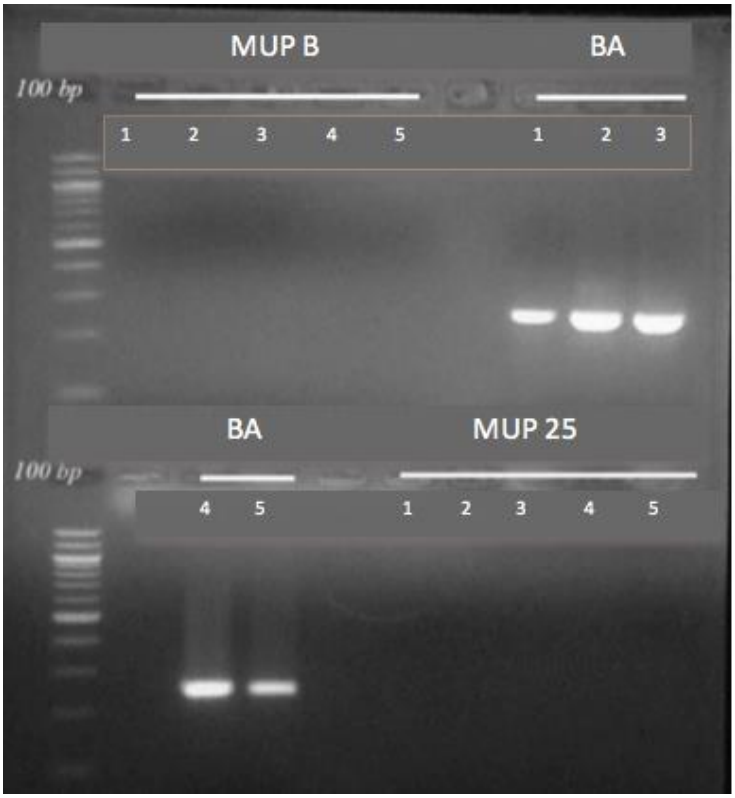


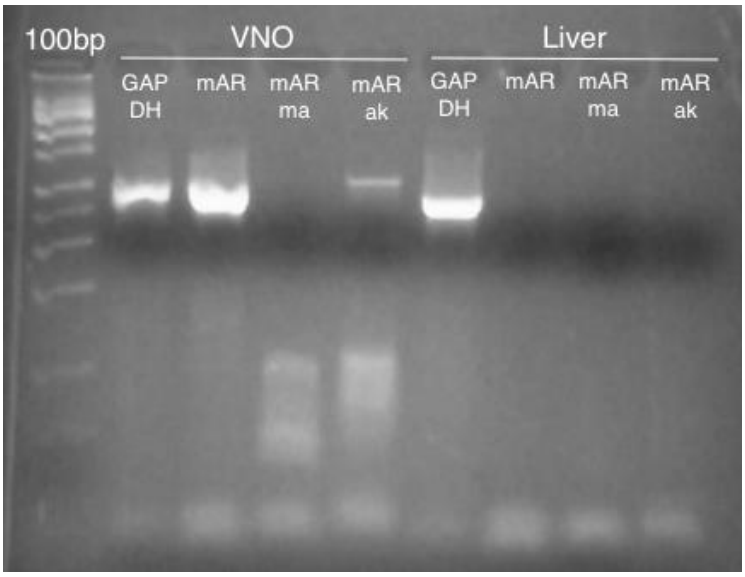
Figure 4. MUP expression following treatment with testosterone, growth hormone, and TCA treatments of Hepa1-6 cells.

Figure 4. Combined treatment with testosterone, mouse growth hormone, and a deacetylation inhibitor (TCA) at tested concentrations did not induce MUP expression. Treatment setup: 1: control no vehicle, 2: low vehicle, 3: high vehicle, 4: 20 nM testosterone, 100 nM mouse growth hormone, and 200 nM Trichostatin A (TCA), 5: 40 nM testosterone, 100 nM mouse growth hormone, and 200 nM Trichostatin A (TCA). The addition of a deacetylation inhibitor with testosterone and mouse growth hormone was insufficient to induce the expression of either the central (MUPB) or peripheral (MUP25) MUPs. ß-actin (BA) served as a control for cell viability and was expressed in this treatment.

3.3 Mouse Androgen Receptor Expression

The cell's expression of androgen receptors was examined in the adult male C57B1/6j VNO and liver (Fig 5A) as well as in the AML-12 male liver cell line (Fig 5B). The primer sets mAR, mARma, mARak, and mARcf were used to examine their potential for detecting androgen receptor expression. VNO cells actively expressed androgen receptors as determined using the primer sets mAR, mARak around 500 bp, and the primer set mARcf around 900 bp (Fig 5A-5B). Liver cells did not appear to express androgen receptors using the mAR, mAR, mARak, and mARcf primer sets (Fig 5A-5B). GAPDH served as a control for cell viability and was expressed around 500 bp (Fig 5A). ß-actin served as a control for cell viability and was expressed around 250 bp (Fig 5B).

A.



B.

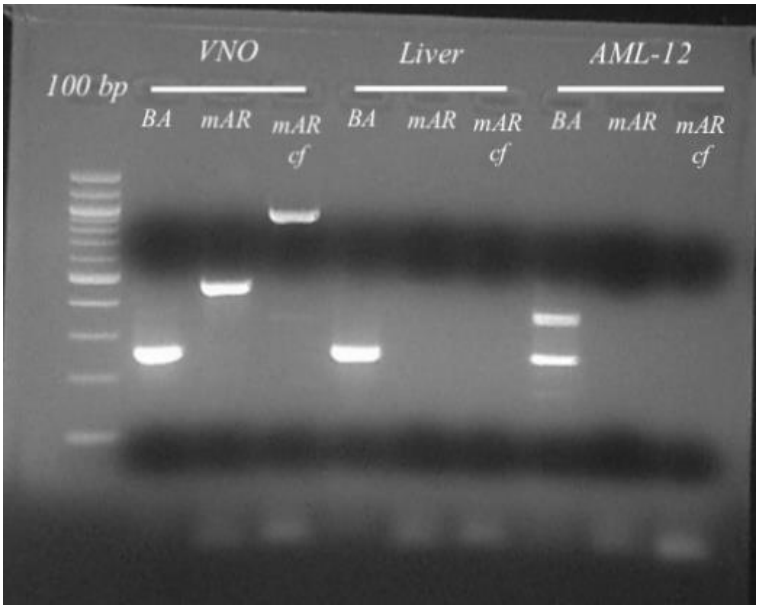


Figure 5. mAR primer test

Figure 5. Androgen receptors are endogenously expressed in VNO tissue, but not in the male liver or AML-12 cell line.

Figure 5. Specific androgen receptor primers were used to detect expression of androgen receptors in cDNA libraries made from C57B1/6j adult male mouse VNO and liver tissue, as well as in the adult male liver cell line AML-12. Androgen receptor primers successfully amplified androgen receptor sequences in the VNO (A,B). Three sets of androgen receptor specific primers were used and were amplified by PCR and visualized on a 2% agarose gel (A). A GAPDH primer served as a control (A). Two sets of androgen receptor specific primers were used and amplified by PCR and visualized on a 2% agarose gel (B). Androgen receptor specific primers could not be used to verify the expression of androgen receptors in the liver or AML-12 cells (A,B). A ß-actin (BA) primer served as a control for cell viability (B).

4. Discussion

The results of the hormone and drug treatments suggests that at the chosen combinations, concentrations and treatment periods, the use of DHT, testosterone, growth hormone, thyroxine, DAC, and TCA in cell culture are not sufficient to induce MUP expression. While the induction of MUP expression *in vivo* has been shown, a working protocol has not been established *in vitro*[1,3]. There is a great difference between the cellular environment of a mouse and cell culture, and any number of these factors may be the key to inducing MUP expression. Therefore, the establishment of a working protocol to induce MUP expression will help elucidate one of biology's long standing questions about gene expression.

Treatments employing the use of DHT were used following experiments with testosterone that did not induce MUP expression[1]. Testosterone concentrations and treatment periods were insufficient in inducing MUP expression, due to this DHT which has a higher affinity for the androgen receptor was used. The concentrations and treatment periods used, however, were insufficient in the induction of MUP expression. Because testosterone is thought to be a main driver of MUP expression, the presence of androgen receptors was examined. The VNO cell's expression of androgen receptors as detected by the use of the mAR primer set. However, mAR expression was not detected and in the control cDNA synthesized from adult male C57B1/6j liver cells. This may provide some insight into why mAR expression has not been successfully shown in Hepa1-6 cells, and why treatments with testosterone and DHT have so far failed to induce MUP expression. Conversely, this could indicate that testosterone does not have as great a role in driving MUP expression as determined by Clissold et al[3].

Treatments using a methylation inhibitor and/ or deacetylation inhibitor were used to induce MUP expression. TCA was obtained in order to determine if DNA methylation was inhibiting MUP expression. The DAC was obtained in order to determine if the *Mup* gene was being silenced. The TCA and DAC concentration and treatment period was not sufficient to induce the expression of MUPs. Treatments where DAC was used in conjunction with testosterone, thyroxine, and mGH at physiological levels did not induce MUP expression either, nor did treatments with testosterone, thyroxine, and mGH at physiological levels. A treatment carried out by a student colleague consisting of testosterone, mGH, and TCA was successful in inducing central MUP expression. Work is being done to replicate and confirm these findings. Further treatments are required to confirm exactly which combinations of hormones and drugs will be successful in inducing MUP expression. Perhaps MUP expression is determined by only testosterone and mGH, but not thyroxine. The success using TCA may elude to the need for acetylation to induce MUP expression in cultured cells.

An optimization of the PCR protocol as well as the current primers may also help towards the expression of MUPs. Varying amounts of DNA, annealing temperatures, and cycle numbers are being tried in the hope of visualizing expression of a low copy number of the MUPs. New androgen receptor primers are also being tested to try to target different sections of the androgen receptor.

The study, though unsuccessful in developing a working protocol for the induction of MUP expression, has reaffirmed the complexity of the MUPs as a model system in which to study gene expression. The establishment of a working protocol *in vitro* will contribute to the greater understanding of gene expression.

5. Acknowledgements

The author wishes to express her gratitude to the Undergraduate Research Program and Dr. Angel Kaur for the opportunity to engage in this research project. She would like to thank her lab members Morgan Ashley, Alice Kimbell, Anna Nazemi, and Rosanna Garris for their contributions to this project as well.

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