CHARACTERIZATION OF A GENE ABUNDANTLY EXPRESSED IN STALLION TESTIS

A Thesis

by

JORDAN ELIZABETH SHIELDS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Animal Breeding

CHARACTERIZATION OF A GENE ABUNDANTLY EXPRESSED IN STALLION TESTIS

A Thesis

by

JORDAN ELIZABETH SHIELDS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee,	Penny K. Riggs
Committee Members,	Nancy Ing
	Thomas Welsh
	Terje Raudsepp
Head of Department,	Gary Acuff

December 2010

Major Subject: Animal Breeding

ABSTRACT

Characterization of a Gene Abundantly Expressed in Stallion Testis.

(December 2010)

Jordan Elizabeth Shields, B.S., Valdosta State University

Chair of Advisory Committee: Dr. Penny K. Riggs

NMES1 is a gene of unknown function first characterized in 2002. Reduction of the expression of this gene has been implicated in skin tumorigenesis in mice. Expression of *NMES1* is observed in epithelial tissue but expression in the testis is significantly higher than in epidermis. Because stallion fertility is an economically important trait, we decided to characterize the *NMES1* gene in stallions. We screened the CHORI241 library and obtained the full length equine *NMES1* genomic sequence by direct sequencing off of clone CH241-11J8. In order to experimentally determine the 5' and 3' untranslated regions (UTRs) we conducted RLM-RACE experiments using stallion testis RNA. The equine *NMES1* mRNA is 534 nt long and contains 5 exons.

Fluorescence *in situ* hybridization mapped *NMES1* to chromosome Eca1q23. *In situ* experiments to testis tissue sections were inconclusive and yielded no data confirming the physical expression pattern of *NMES1* in stallion testis tissue.

In order to determine the expression pattern of *NMES1* mRNA we conducted qRT-PCR assays on a panel of stallion testis samples from horses with normal and abnormal fertility. We found that expression was variable among both groups, with significantly less expression in some individuals. We also conducted the qRT-PCR assay on a panel of five equine tissues and found that the expression of *NMES1* was more than 100-fold greater in testis than in other tissues examined.

miR-147b is a miRNA of unknown target found within the 3’ UTR of *NMES1*. We conducted a miRNA qRT-PCR assay to determine the expression levels in stallion testis samples from fertile and sub-fertile stallions.   We observed similar expression among both groups and the ratio of mRNA to miRNA did not appear constant. We also investigated miR-147b expression in a panel of five equine tissues and found that equine spleen had more than 8-fold greater expression than testis.

DEDICATION

This thesis is dedicated to my family, both biological and spiritual. Thank you for your support throughout this process. Without y’all I never would have been able to accomplish any of this.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Riggs. Your guidance and mentorship has been constant. Thank you for taking a chance on me. I would also like to thank my committee members, Dr. Ing, Dr. Raudsepp, and Dr. Welsh, for their guidance and support throughout my project.

Thank you to my labmates, peers, friends, professors and staff for making my time at Texas A&M University a great experience.

Finally, thanks to my mom and dad, sisters and brother, for their unending support and encouragement.

NOMENCLATURE

FISH	Fluorescence *In Situ* Hybridization
ISH	*In Situ* Hybridization
PCR	Polymerase Chain Reaction
RLM-RACE	RNA Ligase Mediated Rapid Amplification of cDNA Ends
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction

TABLE OF CONTENTS

Page

ABSTRACT iii

DEDICATION v

ACKNOWLEDGEMENTS vi

NOMENCLATURE vii

TABLE OF CONTENTS viii

LIST OF FIGURES x

LIST OF TABLES xi

1. INTRODUCTION 1

2. LITERATURE REVIEW 3

    2.1 Industry Importance 3
    2.2 Testis Development 3
    2.3 Spermatogenesis 7
    2.4 *NMES1* 8

3. MATERIALS AND METHODS 11

    3.1 BAC Library Screening 11
    3.2 DNA Extraction 12
    3.3 Sequencing 12
    3.4 Primer Design 13
    3.5 RLM-RACE 15
    3.6 5’ and 3’ UTR Sequencing 17
    3.7 Labeling of BAC DNA with Digoxigenin 17
    3.8 Fluorescence *In Situ* Hybridization with Labeled BAC 18
    3.9 Testis Processing and Histology 19
    3.10 Probe Design 20
    3.11 Probe Labeling with Digoxigenin 23
    3.12 *In Situ* Hybridization with Poly d(T) Probe 24
    3.13 *In Situ* Hybridization with Digoxigenin-Labeled Probes 25

Page

3.14 RNA Extraction 27
3.15 Reverse Transcriptase Polymerase Chain Reaction 28
3.16 Quantitative Reverse Transcriptase Polymerase Chain Reaction 28
3.17 miRNA Reverse Transcriptase Polymerase Chain Reaction 29
3.18 miRNA Quantitative Reverse Transcriptase Polymerase Chain Reaction 30

4. RESULTS AND DISCUSSION 31

4.1 Genomic and UTR Sequences 31
4.2 Fluorescence *In Situ* Hybridization 37
4.3 *In Situ* Hybridization 39
4.4 Quantitative Reverse Transcriptase Polymerase Chain Reaction 41
4.5 miRNA Quantitative Reverse Transcriptase Polymerase Chain Reaction 43

5. SUMMARY AND CONCLUSIONS 47

REFERENCES 49

APPENDIX A 52

VITA 54

LIST OF FIGURES

Page

Figure 1 Superpools of the CHORI-241 BAC library were screened for the presence of the *NMES1* gene by PCR 32

Figure 2 Gel electrophoresis image of PCR products from extracted BAC DNA. 34

Figure 3 Screen capture of sequence analysis using Sequencher® 4.9 35

Figure 4 Nucleotide sequence for *Equus caballus NMES1* cDNA software 36

Figure 5 FISH results showing the chromosomal location of *NMES1* at Eca1q23 38

Figure 6 *NMES1* ISH results from equine testis tissue 40

Figure 7 Relative quantity of *NMES1* mRNA in stallion testis samples 42

Figure 8 Relative quantity of miR-147b in stallion testis samples 44

Figure 9 Relative quantity of miR-147b in an equine tissue panel 46

Figure 10 Relative quantity of *NMES1* mRNA in a panel of equine tissues 46

LIST OF TABLES

Page

Table 1 Complete list of primers used for sequencing CH241-11J8 14

1. INTRODUCTION

The *NMES1* gene was first identified in humans and is predicted to encode a relatively small protein (83 amino acids)of unknown function (Zhou *et al.* 2002). Although it was originally named *NMES1* after being discovered as a transcript identified as normal mucosa of the esophagus specific 1, current symbols for this gene are *C15orf48* (human) and *AA467197* (mouse). No consistent name currently exists for this gene. For simplicity, the gene will be referred to as *NMES1* in the remainder of the document. Previous experiments showed that *NMES1* was expressed abundantly in adult mouse testis, at a level 50 times greater than all other tissues examined (Riggs *et al.* unpublished). A microRNA (miRNA) has also been identified within the 3' UTR of the mouse *Nmes1* gene (Landgraf *et al.* 2007). The presence of the miRNA was computationally verified in *Equus caballus* as well (Zhou *et al.* 2009). This miRNA is identified as miR-147 in the mouse, but both the human and equine microRNA is labeled miR-147b.

Because *NMES1* transcripts are induced when terminal differentiation is induced in mouse epidermis (Riggs *et al.* 2005), and expression has been shown to affect cellular motility in vitro (Arai *et al.* 2008), we hypothesized that the abundance of *NMES1* in the testis might reflect a role for the gene during spermatogenesis. If *NMES1* is important during any stage of testis development or spermatogenesis, expression of this gene could also affect male fertility.

To date, the functions of proteins encoded by many genes involved in both testis development and spermatogenesis remain unknown or poorly characterized. Better understanding of genetic regulation of spermatogenesis is important because stallion fertility can

---

This thesis follows the style of *Animal Genetics*.

have significant economic consequences for the horse breeding industry. The economic impact due to stallion sub-fertility can be manifested in lost stud fees, increase in veterinary expenses, increased management costs, and ultimately a loss of genetic contribution from prized stud horses (Oristaglio Turner 2007). Stallion fertility traits are rarely selected for within the industry and thus have not been under strong selective pressure (Loomis 2006). This practice has led to an industry that economically benefits from stallion fertility, but has not selected towards increasing the success of this trait.

Because *NMES1* is abundantly expressed in the testis, but its function and role in spermatogenesis, if any, remain unknown, the work described in this thesis focused on genomic characterization of the *NMES1* gene. The objectives of this project were to characterize the *NMES1* gene in the equine genome and to begin analyses to determine the function of the NMES1 protein in stallion testis. If *NMES1* contributes to stallion fertility, these data could lend new knowledge toward understanding of autosomal gene contribution in male fertility.

2. LITERATURE REVIEW

2.1 INDUSTRY IMPORTANCE

The equine industry contributes approximately $39 billion dollars annually to the U.S. economy according to a 2005 study conducted by Deloitte Consulting, LLP for the American Horse Council Foundation. The industry is set-up in such a way that one stallion services many mares in the breeding pool. This structure allows for a large amount of potential money loss due to stallion infertility. Resources are lost when a stallion with a performance record is set up at stud and little to no progeny are produced. Breeding managers, and stallion owners, as well as mare owners, lose profits from costs associated with repeated breedings that produce no offspring. Throughout the domestic horse's history, many traits have been selectively bred for by humans; however, fertility traits have never been included on that list.

2.2 TESTIS DEVELOPMENT

The mammalian system for sex determination is unique in that mammals have a bi-potential gonadal system (Wilhelm *et al.* 2007). The bi-potential gonad is a mixed population of cells that contains both somatic and germ cell tissues (Kim *et al.* 2006). During early development, the presence of appropriate gene products tip the scale in one direction or another and starts the embryo on its path toward becoming male or female (Wilhelm *et al.* 2007). Before this fate has been determined, the urogenital ridge development is the same for males or females (Sim *et al.* 2008). In mammals, the presence or absence of the Y chromosome with an intact

SRY gene is the male determinant (Sekido *et al.* 2004). In the presence of *SRY* expression the urogenital ridge differentiates into a testis instead of an ovary. Expression of male hormones from the differentiated testis drives development of all other secondary sexual traits in males (Wilhelm *et al.* 2007).

The sex determining factor gene on the Y chromosome, *SRY,* was identified in 1990 (Sinclair *et al.* 1990). Sry is a member of the Sox family of transcription factors. This family of transcription factors contain a high mobility DNA binding domain, or HMG (Wilhelm *et al.* 2007).. Unlike other members of the Sox family, *SRY* has no classic domains for transcriptional activation and repression (Bernard *et al.* 2008). *SRY* is expressed specifically in the tissues that give rise to immature Sertoli cells, which are present in the developing testis (Wilhelm *et al.* 2007). In mice, expression of *Sry* starts around 10.5 days post coitum (dpc) and is characterized as being a wave of expression, starting from the center of the urogenital ridge and spreading outward towards the poles of the ridge (Wilhelm *et al.* 2007). This happens by 11.5 dpc. After expression has reached the poles, *Sry* is down-regulated starting in the center of the ridge and spreading out towards the poles again. *Sry* expression ceases at 12.5 dpc (Kidokoro *et al.* 2005). Soon after the expression of *Sry*, expression of Sox9 is up-regulated (Sekido *et al.* 2004). Because expression of *Sry* is no longer detectable by 12.5 dpc, *Sry* expression is not responsible for the continuation of male gonadal development, but rather only acts as a switch to start the process (Sim *et al.* 2008).

Sertoli cells derive from coelomic epithelium, part of the urogenital ridge that can give rise to interstitial cell types. Experiments were conducted to determine which cell types expressed *SRY* in embryonic mice. To answer this question Sekido and colleagues (Sekido *et al.* 2004) constructed two transgenes fused to the *SRY* promoter. They developed the first transgene using human placental alkaline phosphatase (hPLAP) inserted to replace the 5' end of the coding sequence to give a stable reporter, and developed the second transgene using a Myc-epitope tag inserted directly in front of the stop codon of *SRY*. They were then able to make transgenic mouse embryos and look at expression of the *SRY* transgenes by using an anti-Myc antibody to use for immunostaining during the developmental process and by looking at hPLAP enzyme activity. The results from the experiment showed that only the Sertoli precursor cells showed *SRY* expression. Only those cells which had *SRY* expression showed expression of *Sox9*. The results from this study concluded that only precursors of Sertoli cells express *SRY* and *Sox9* and continue on to become functional Sertoli cells without the recruitment of other cell types (Sekido *et al.* 2004).

Sox9 is a proposed target gene of *SRY*. *Sox9* is expressed at low levels in the urogenital ridge in both males and females at 10.5 dpc. At 11.5 dpc however, *Sox9* expression is strongly up-regulated in males and down-regulated in females (Sekido *et al.* 2004). It has been shown that *Sox9* expression is up-regulated in a similar wave pattern as that of *SRY*, starting in the center of the urogenital ridge and moving outward. This information suggests that *Sox9* is the first target of *SRY* (Bernard *et al.* 2008). It has been shown that if *SRY* is absent, supplementing with *Sox9* expression will induce testis formation. This evidence also points toward *Sox9* being a conserved target of *SRY* (Qin & Bishop 2005). *Sox9* affects the regulation of many genes, including anti-Mullerian hormone (*Amh*) and Vanin-1 (*Vnn1*) (Wilhelm *et al.* 2007).

Chaboissier *et al.* (2004) were interested in studying the in vivo effects of *Sox9* expression. They developed a *Sox9* conditional knock-out transgenic mouse line. Urogenital ridges were dissected from heterozygous and homozygous conditional knock-out mouse embryos and real-time quantitative PCR (qRT-PCR) was used to analyze expression levels of *Sox9*. In heterozygous male knock-outs, expression of *Sox9* was reduced to levels similar to that of females. The male embryos developed irregularly-formed sex cords. Expression of *Amh* behaved in linear fashion to the expression of *Sox9* suggesting again that *Amh* is a target for Sox9. Data for homozygous conditional knock-outs showed almost complete sex reversal from male to female. These male embryos lacked sex cord development and were histologically similar to the females. In fact, expression of *Bmp2* and *follistatin* was observed, both of which are markers for female sex determination (Chaboissier *et al.* 2004).

To date, the only known morphological event associated with the expression of *SRY* is an increase in cell proliferation. Throughout embryo development, a recurring theme is the proliferation and movement of progenitor cells. This is also an important event in testis development. Experiments designed to study cell proliferation in response to *SRY* expression were conducted by Schmahl and Capel (2003). It had been shown previously in other organ systems that if proliferation is blocked, differentiation is halted. In order to determine whether proliferation played any role in testis development, these researchers used cell proliferation inhibitors both in vivo and in culture. Pregnant female mice were injected with proliferation inhibitors at specific time points and then embryos were harvested for inspection and dissection. When proliferation inhibitors were injected between day 10 and 11 dpc, the male embryos failed to form testis cords. The gonads of both male and female embryos were smaller in size after injection when compared to controls (Schmahl & Capel 2003).

In the Schmahl and Capel study (2003), the relationship between cell proliferation and male-specific gene expression was examined. Under normal developmental conditions, *Amh* and *Sox9* expression can be detected at 12.5 dpc in Sertoli cells located within testis cords in male XY embryos. When embryos received injections in the critical window between day 10 and 11 dpc, expression of *Sox9* and *Amh* was abnormal in regards to localization, reduced expression, or loss of expression. These results demonstrated that Sertoli cells and testis cord formation are affected by inhibition of cell proliferation and that *Sox9* and *Amh* expression levels and patterns are also affected (Schmahl & Capel 2003). *SRY* has been shown to affect cell proliferation and it is proposed that this is one of the mechanisms by which *SRY* initiates male gonadal development (Kidokoro *et al.* 2005).

2.3 SPERMATOGENESIS

In stallions, a distinct pattern of maturation occurs within the testis. Lighter colored seminiferous tubules undergo spermatogenesis and are present in the testis core, while dark immature tissue surrounds the periphery. The testis matures slowly from the center outward as the stallion completes puberty and seminiferous tubules develop and start spermatogenesis (Clemmons *et al.* 1995).

As spermatogenesis begins during this process of inner light tissue spreading outwards during testis maturation, gonocytes are converted to spermatogonia. Spermatogenesis begins at this stage and occurs with a physiological wave of apoptosis (Rodriguez *et al.* 1997). As this first division of germ cells occurs, a surge of apoptosis spreads through the testis which aids in the development of seminiferous tubules by removing germ cells from the epithelium of the tubule. This process is necessary for maturation of the seminiferous tubules. In a study that measured apoptosis within the seminiferous tubules, a classification system was developed to rank the tubules from L1 to L7 according to the level of testis maturation (Staub *et al.* 2002). The classification system ranked the samples based on the lumen score of the seminiferous tubules. A score of L1 indicated that the lumen was completely closed and developmentally immature. Scores of L2-4 indicated that the lumens had increasing numbers of vacuoles within the tubules. A tubule with a score of L5 and L6 was given to tubules with large lumen and a complete lumen that was lacking complete generations of germ cells respectively. The L7 score was reserved for tubules with complete lumen formation that contained full germ cell populations. These experiments demonstrated a peak in apoptosis during the L6 type tubules that showed mature development of the tubule epithelium. The L6 tubules contained fewer germ cells than the next class of tubules (L7) that are marked by complete tubule development and spermatogenesis (Staub *et al.* 2002). This ordered and distinct pattern of tissue maturation and apoptotic wave leads to the final development of mature and complete seminiferous tubules where spermatogenesis occurs in the mature testis.

2.4 *NMES1*

NMES1 was first characterized by Zhou *et al.* (2002) during an investigation of gene expression in human esophageal squamous cell carcinoma. Experimental data from RT-PCR, northern blot, and immunohistochemistry showed a decreased expression of *NMES1* in esophageal carcinomas compared to the surrounding “normal” mucosal tissue. Some carcinomas completely lacked expression, and neither RNA nor protein could be detected (Zhou *et al.* 2002). A subsequent study (Riggs *et al.* 2005) identified differentially expressed genes in the epidermis of strains of mice either susceptible (DBA/2J) or resistant (C57BL/6J) to tumor formation in a two-stage carcinogenesis experiment. The phorbol ester, 12-O-tetradecanoyl-13-phorbol acetate

(TPA) can be used to promote tumor growth in mice following carcinogen exposure and are also known to induce terminal differentiation in mouse epidermis (DiGiovanni 1992). The two strains of mice were treated with TPA or vehicle (acetone), and RNA was extracted from dorsal epidermis tissue for microarray analysis. In the previous study (DiGiovanni 1992), tumor promotion sensitive mice (DBA/2J) demonstrated a marked increase in epidermal inflammation. The microarray data indicated that the promotion-resistant C57BL/6J mice strongly induced epidermal *NMES1* expression in response to TPA treatment. Expression of *Nmes1* in C57BL/6J epidermis was about 10-fold greater than expression in promotion-sensitive DBA/2J mice (Riggs *et al.* 2005).

Arai and colleagues (2008) investigated the methylation status of the promoter region of genes shown to exhibit higher expression after treatment with 5-aza-2'-deoxycytidine (DAC) in human esophageal squamous cell carcinoma (ESCC) cell lines. DAC is known to reactivate genes that have been silenced through methylation (Cameron *et al.* 1999). From microarray experiments conducted to identify genes induced by the DAC treatment. *NMES1* was identified as a candidate gene (Arai *et al.* 2008). Additionally, Boyden chamber assay experiments showed that when *NMES1* was over-expressed in two transfected ESCC lines, motility of the cells was significantly decreased compared to the parental cell lines (Arai *et al.* 2008). A similar microarray analysis was done on invasive cervical cancer cell lines by another lab and *NMES1* was found to be hypermethylated in cervical cancer cells (Sova *et al.* 2006). Based on these experiments, methylation and subsequent loss of *NMES1* gene expression may contribute to tumor development.

Most recently, Liu *et al.* (2009) demonstrated that *NMES1* encodes the microRNA, miR-147b, in its 3'-untranslated region (3'UTR), and showed that increased miR-147 expression dampens inflammatory response, particularly in macrophages. Interestingly, decreased miR-147 was implicated as a possible gene expression signature in coronary artery disease patients (Hoekstra *et al.*). The function of *NMES1* and its associated microRNA remain unclear, and it is not known if the *NMES1* gene can be expressed independently of the microRNA.

It has been noted that full lenth, capped, polyadenylated mRNAs can act as a primary miRNA transcript. These mRNAs are able to function normally as well, and can therefore be translated into proteins. It is also possible for the mRNAs to only function as pri-miRNAs (Cai *et al.* 2004). miRNAs have also been shown to act on genes involved in pathways that affect testis development and spermatogenesis. Expression of *Tnp2,*which is testis specific, has been shown to be reduced by miR-122a (Yu *et al.* 2005). miR-34c is a miRNA that is highly expressed in testicular germ cells and present in pachytene spermatocytes and round spermatids (Bouhallier *et al.* 2010). The miR-34 family of microRNAs have been shown to be regulated by p53 (He *et al.* 2007). miR-34c acts to reduce cell proliferation by down-regulating genes associated with cell proliferation within the testis and this is postulated to play a role in the spermatogenesis process (Bouhallier *et al.* 2010).

Based on unpublished data and previous studies (Riggs *et al.* 2005; Arai *et al.* 2008), we hypothesized that *NMES1* may play a role in cellular differentiation and growth. Because of the abundant expression in the testis, *NMES1* may play in integral role in the differentiation of spermatogonia within the seminiferous tubules. If the function of *NMES1* is important during spermatogenesis, its expression may affect stallion fertility.

3. MATERIALS AND METHODS

3.1 BAC LIBRARY SCREENING

A PCR assay designed to amplify the last exon of the equine *NMES1* gene was used to screen the CHORI-241 equine BAC library for a clone containing the *NMES1* gene. Super pools of CHORI-241 clones were first screened, and then positive single pools were identified. The single pools were then screened to determine column and row position of the positive clones within the BAC library.

The PCR assay consisted of 17.2 µl H_2O, 2.5 µl 10x PCR Buffer, 1.5 µl 25mM $MgCl_2$, 0.75 µl each forward (5’-sequence) and 0.75 µl reverse (5’-sequence) primers (IDT, location), 1.0 µl 2.5 mM dNTP mix (vendor, loc), 0.3 µl Amplitaq® (Applied Biosystems, Carlsbad, CA), and 1.0 µl template DNA (n ng) from the BAC library pools. The components were denatured at 94°C for 3 min, followed by 30 cycles of 94°C for 15 seconds, 60°C for 45 seconds, and a final extension step at 60°C for 6 min.

The PCR products were combined with 5µl 5x loading buffer (vendor) containing bromophenol blue dye and SYBR® green I (Invitrogen, Carlsbad, CA). For product visualization, 15 µl were electrophoresed through a 2% agarose gel in 1X tris-borate-edta buffer (TBE) at 65V for 2.5h. Bands in the gel were visualized under ultraviolet light. After screening the library, we identified a positive clone, CH241-11J8, and proceeded to generate BAC DNA.

3.2 DNA EXTRACTION

A large construct kit (Qiagen, Valencia, CA) was used for extraction of BAC DNA from the selected clone. Following manufacturer's instructions, 500 ml Luria broth (LB) cultures were inoculated with the selected BAC clone and DNA was extracted. BAC DNA quantity was measured on a NanoDrop ND-1000 spectrophotometer. The PCR assay used to screen the library was then performed on the extracted DNA to confirm the presence of our gene of interest, following the PCR assay protocol listed above in Section 3.1

3.3 SEQUENCING

Automated sequencing was performed with an ABI 3730xl sequencer. Reaction components included Big Dye® v.1.1 components (Applied Biosystems, Carlsbad, CA). Purified BAC DNA was used as the template material. Each 7µl reaction included 1 µg template DNA, 1.5 ul 5X buffer, 0.5 ul primer (10 uM). The reactions were denatured at 98°C for 2 min, then immediately quenched on ice for 2 min. 1 µl Big Dye v. 1.1 was added to each reaction and the sequences were loaded on an ABI 2720. The reactions were denatured at 95°C for 5 min then amplified for 99 cycles of: 96°C for 30 sec, 50°C for 10 sec, and 60°C for 4 min.

The sequences were purified through Dye-Ex spin columns (Qiagen, Valencia, CA) and the samples were dehydrated by vacuum centrifugation before being resuspended in 10 µl Hi-Di™ formamide (Applied Biosystems, Carlsbad, CA). The samples were transferred to a 96-

well plate, denatured at 98°C for 2 min and then quenched on ice for 2 min before being loaded onto an ABI 3730xl. The sequence data was analyzed with Sequencher® 4.8 software.

3.4 PRIMER DESIGN

Oligo 6® was used for primer design. The initial sequencing reaction was conducted with the primers assay already designed for screening the CH241 library while BAC DNA sequence derived from the initial sequencing reaction was used for further primer design to enable "primer walking" in the 5' and 3' directions. Primers were designed to amplify only the equine *NMES1* gene. The primers designed and used for the BAC sequencing experiment are listed in the Table 1.

Table 1 Complete list of primers used for sequencing CH241-11J8. All primers are written in 5’ to 3’ orientation.

GAATCCAAATGTCCACCAA	CTAAAAATGTAGCAGAAGCA
GTCGGGTACTGGGAACAA	GGAAGCCCATTGAAGAGT
CCTCCTTGACTGTTCTTGAA	AGTGTTCTTGCCCTGGTT
CATCCAGCGTTCTCCAA	AACCAGGGCAAGAACACT
TCGGACTCTGGATAAGGA	TTACTATGGGCGAGGCACT
ACGCTTGTGGCAGTGTTA	AGTGCCTCGCCCATAGTAA
ACGCCACCAAAGGAATAAGCTA	CTGGGGTGATAGAGAACTTGT
AGTGCCTCGCCCATAGTAA	GAGCACCATTTGTCAGCCTT
CATAAGGGAAAGGTAGAGA	

3.5 RLM-RACE

Total testis RNA from three different mature stallions was kindly provided by Dr. Nancy Ing, and was processed with the RNeasy cleanup kit (Qiagen, Valencia, CA) and DNAse treated (DNA-free™; Ambion, Austin, TX). Quality and integrity of RNA samples was assessed by capillary electrophoresis on an Agilent 2100 Bioanalyzer. RACE reactions were prepared with the First Choice® RLM-RACE kit (Ambion, Austin, TX). 1 µg starting RNA was used for each of the three samples. The kit protocol was followed precisely, adding 2 µl of glycogen during the RNA precipitation step. An oligo $d(t)_{20}$ primer was used for reverse transcription (RT). Four µl RT product was amplified by PCR. A set of primers was designed with Oligo 6® software. The forward primer was designed from the RACE adapter sequence. The reverse primer was gene-specific and based on *NMES1* sequence obtained from sequencing the BAC clone (5'-CGAAAAGAAATCCAGAACCT-3'). The reaction was run on an ABI 2720 at 94°C for 3 min, 35 cycles of 94°C for 30 sec, 58°C for 1 min, 72°C for one min, and then 72°C for 7 min.

The PCR products were evaluated by electrophoresis. 5 µl reaction plus 2 µl 5x loading buffer containing bromphenol blue and SYBR® green I was electrophoresed through a 2% TBE gel. The gel was run at 30 mA for 2.5 hrs. Bands were visualized under UV light.

Product was observed for only one of the three samples. Two products were amplified in the PCR reaction. For sequencing of each product, the bands were stabbed with a pipette tip and added to two new PCR reactions with the same components as above. The assay was repeated under the same parameters as above. After electrophoresis, each PCR reaction amplified a single discreet product.

The two PCR products were then ligated into the pCR®II vector (Invitrogen, Carlsbad, CA). 1 µl PCR reaction was combined with the vector in a ligation reaction according to manufacturer's recommendations. The ligation reaction was incubated at 14°C overnight and a portion of the reaction mix was transfected into chemically competent DH5α cells. 75 µl transformed cells were plated on agar plates containing 50 µg/ml ampicillin, 40 µl 100mM IPTG, and 40 mg/ml X-Gal. Plates were incubated at 37°C overnight. White colonies were selected and grown in 5 ml LB broth cultures with ampicillin (50 µg/ml) overnight.

To verify correct insertion of the PCR product into the vector, a PCR assay using standard M13 primers was performed on the broth cultures. 5 µl of culture was added to 100 µl of water and denatured for 10 min at 95°C. 5 µl of this sample was added to a PCR reaction containing and run at the on an ABI 2720 as follows: 95°C for 5 min, 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, and then 72°C for 7 min.

Once insertion of correct-sized product was verified, the PCR reaction was cleaned using the Psi-Clone kit (Princeton Separations, Freehold, NJ). The products were then quantified by NanoDrop spectrophotometry.

In order to obtain 3'UTR sequence of the NMES1 gene, 1 µg of starting RNA from the three stallion testis RNA samples prepared for the 5'RLM-RACE experiment were used. The 3' RACE protocol was followed, using the same First Choice® RLM-RACE kit (Ambion, Austin, TX). A gene specific forward primer (5'-GATCCTAGTGTACCTACAAA-3') was designed using the BAC sequence, while a reverse primer was designed from the 3' RACE adapter sequence.

The PCR products were analyzed by running 5 µl of the reaction plus 2 µl of 5x loading buffer with Bromphenol Blue and SYBR® green on a 2% TBE gel. The gel was run at 30 mAmps for 2.5 hours.. Bands were visualized over a UV light box. Discreet products were obtained so the reactions were cleaned using the Psi-Clone (Applied Biosystems, Carlsbad, CA) .

3.6 5’ AND 3’ UTR SEQUENCING

The sequencing reactions were carried out as described above except that, after addition of Big Dye®, PCR was conducted for 25 cycles of: 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 min. The sequence data was then analyzed using Sequencher® 4.9 software to build a contig and determine a consensus sequence.

3.7 LABELING OF BAC DNA WITH DIGOXIGENIN

To determine the chromosomal location of the equine *NMES1* gene, the CH241-11J8 clone DNA was labeled with digoxigenin by nick translation. 1 µg BAC DNA was added to 4 µl of dig nick translation mix and ddH_2O to a final volume of 20 µl. The reaction was incubated at 15°C for 90 min. After incubation, 25 µl double distilled H_2O was added to the reaction and the sample was purified on a spin-50 minicolumn (USA Scientific, Ocala, FL) following manufacturer’s protocol. 5 µl of the eluate was combined with 2 µl of loading buffer and electrophoresed through a 1% agarose gel for 30 min at 100 V to assess progress of nick translation reaction. The sample was mixed with 15 µl of 1µg/µl unlabeled equine genomic DNA and dehydrated. The probe was resuspended in 6 µl of H_2O and 14 µl of hybridization master mix (50% formamide, 10% dextran sulfate, and 2X SSC). The probe was stored at -20°C until used.

3.8 FLUORESCENCE *IN SITU* HYBRIDIZATION WITH LABELED BAC

Equine metaphase chromosome slides were obtained from Dr. Terje Raudsepp. The slides were examined with phase contrast microscopy and areas approximately 4x4mm^2 containing good quality metaphase spreads were marked with a diamond pencil. 500 µl RNase was added to the slides. A 24x60 mm cover slip was applied and the slides were incubated at 37° for 1 hour. The cover slip was removed and the slides were washed for 2 min in 2X SSC and then dried in an ascending ethanol series for 2 min each wash. The slides were denatured in 70% formamide with 2X SSC at 70°C for 10 min. The slides were then washed in ice-cold 70% EtOH and dehydrated in an ascending ethanol series and allowed to air dry.

The labeled probe from the above section was prepared for hybridization. 2.5 µl of probe was added to a microcentrifuge tube and was denatured at 95° for 11 min. The probe was then preannealed at 37°C for 20 min. The probe was then pipetted onto the marked areas on the slides and a cover slip was cut to fit the area. The cover slip was added and sealed with rubber cement. The slides were incubated at 37°C overnight in a humid chamber.

Following hybridization, the rubber cement was removed and slides were washed in 50 ml 2X SSC to remove the cover slips. The slides were washed in 3 changes of 50 ml 50% formamide with 2X SSC (5 min at 40°C); 3 changes of 50 ml 4X SSC with 0.05% Tween-20 (2 min with shaking); 50 ml 4X SSC for 2 min with shaking.

For signal detection, an antibody layering protocol was used. The antibodies were diluted in 200 µl per slide of 1X blocking solution. Layer 1 consisted of 0.8 µl anti-dig antibody in 200 µl 1X blocking solution and added to the slide. A cover slip was applied and the slide was incubated at for 30 min at 37°. The slides were washed in 3 changes of 50 ml 4X SSC with 0.05% Tween-20 (2 min with shaking), followed by 50 ml 4X SSC for 2 min with shaking. Layer 2 consisted of 0.4 µl of anti-mouse Ig-dig antibody added to 200 µl 1X blocking solution and added to the slide. A cover slip was applied and the slide was incubated at for 30 min at 37°. The slides were washed in 3 changes of 50 ml 4X SSC with 0.05% Tween-20 (2 min with shaking), followed by 50 ml 4X SSC for 2 min with shaking. Layer 3 consisted of 1 µl of antidig-rhodamine stock solution added to 200 µl 1X blocking solution and added to the slide. A cover slip was applied and the slide was incubated at for 30 min at 37°C. The slides were washed in 3 changes of 50 ml 4X SSC with 0.05% Tween-20 (2 min with shaking), followed by 50 ml 4X SSC for 2 min with shaking. The DAPI-antifade was applied to the slide and cover slips were mounted. Slides were stored at -20°C in the dark for approximately 20 min before being examined for signal. The slides were examined using fluorescence microscopy and analyzed using software..

3.9 TESTIS PROCESSING AND HISTOLOGY

Fresh testis tissue was collected from a 2.5 year old stallion. The tunica was removed and the testis was dissected into approximately 0.5 cm wide sections taken from the mature tissue containing seminiferous tubules. Tissue from the core of the testis that mostly contains collecting ducts was avoided.

The tissues were placed in individual 50 ml conical tubes and were incubated in 45 ml 4% paraformaldehyde rotating at room temperature for 24 hours. The paraformaldehyde was decanted and the tissues were washed and incubated in 45 ml 50% ethanol for 12 hours. The ethanol was changed and the tissues were incubated again for 12 hours. The 50 % ethanol was then decanted and replaced with 45 ml 70% ethanol. The tissues were incubated for 12 hours and the tissues were then placed in labeled cassettes and fresh 70% ethanol. Tissues were embedded in Paraplast® (Structure Probe, Inc., West Chester, PA) and sections cut at thickness of 5 µm for slides at the Veterinary Integrative Biosciences Histology Laboratory at Texas A&M University. The sections were affixed to plus coated slides and stored at 4°C. At least one slide per block was stained with hematoxylin and eosin to help determine tissue morphology.

3.10 PROBE DESIGN

An equine *NMES1* specific probe was designed to use for *in situ* hybridization experiments. The genomic NMES1 sequence determined from the BAC clone was aligned with mouse and human sequences to determine the probable coding sequence. Primers were then designed based on this sequence to make a 250 nt long product that was within the start and stop sites and complimentary to the *NMES1* mRNA transcript present in the testis. The primers were designed using Oligo 6 and specificity to the equine genome assembly was verified by BLAST.

cDNA was synthesized from total RNA that was extracted from the testis tissue that was collected for histologic analysis. This RNA processed through RNeasy clean-up columns (Qiagen, Valencia, CA) and was DNase treated with DNA-free™ (Ambion, Austin, TX). The RNA was quantified by NanoDrop spectrophotometry. Two µg of total RNA was used in the RT reaction and the protocol was followed as in Section 3.17.

The PCR assay was conducted using the above designed primers and was as follows: 17.2 µl H_2O, 2.5 µl 10x PCR Buffer, 1.5 µl 25mM $MgCl_2$, 0.75 µl gene specific forward primer, 0.75 µl gene specific reverse primer, 1.0 µl 2.5 mM dNTP mix, 0.3 µl Amplitaq®, and 1.0 µl template DNA from the BAC library pools. PCR was conducted under the following parameters: 94°C for 3 min followed by 30 cycles of 94°C for 15 sec, 60°C for 45 sec. The PCR products were electrophoresed through a 2% TBE gel for band visualization. 2µl 5x loading buffer with dye and Sybr® green were added to each 5 µl of the PCR reaction, then loaded onto the gel and run at 65 V for 2.5 hrs. The gel was then visualized over a UV light box. The PCR product was purified with the Psi-Clone kit and reagents to remove inhibitory reaction components.

This clean PCR product was ligated into a PCRII plasmid vector using the TA Cloning® kit with Dual Promoter from Invitrogen (Carlsbad, CA). 3µl of the product was used in the ligation reaction following the kit protocol. The reaction incubated overnight at 14°C. The plasmid was then transfected into Top10F' chemically competent cells, according to the manufacturer's recommendations. 20 µl of the transformed cells were plated on agar plates with 50 µg/ml ampicillin, 40 µl 100mM IPTG, and 40 mg/ml X-Gal. The plates were incubated overnight at 37°C. White colonies were selected and grown in 5 ml LB broth cultures with 50 µg/ml ampicillin. The cultures were incubated at 37°C in a shaking water bath overnight.

To further verify PCR product insertion, a PCR assay was conducted using standard M13 primers. The 50 µl assay contained the following: 31.4 µl H_2O, 5 µl 10x PCR Buffer, 5 µl 15mM $MgCl_2$, 1 µl 10mM M13 forward primer, 1 µl 10mM M13 reverse primer, 0.6 µl 5 mM dNTP mix, 1 µl Taq polymerase, and 5 µl template broth culture. PCR conditions consisted of 94°C for 3 min, followed by 30 cycles of 94°C for 15s, 60°C for 45s. The PCR products were visualized in a 2% agarose gel as described above. After successful ligation was confirmed, 200 µl of the 5ml broth culture was added to a 100 ml LB broth culture with ampicillin (50 µg/ml) and were incubated at 37°C overnight in a shaking water bath. Plasmid DNA was extracted from the culture with a large insert maxi-prep kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The plasmid DNA was then quantified by spectrophotometry.

The DNA was sequenced to determine the orientation of the PCR product insertion. Products were sequenced as previously described except that M13 forward or reverse primers were used.

The plasmid was then digested with two separate restriction enzymes in order to synthesize a sense and antisense probe. *SpeI* and *EcoRV* were used to digest the plasmid in two separate reactions. 10 µg of plasmid DNA in 170 µl water was added to 20 µl 10X restriction enzyme buffer and 10 µl of the appropriate restriction enzyme. The reaction was incubated at 37°C overnight. A sample of the digested plasmid was run on a 1% TBE gel for digest verification. 2µl 5x loading buffer with dye and SYBR® green were added to each 5 µl the PCR reaction, and then loaded onto the gel and electrophoresed at 50 V for 1 hr. The gel was then visualized over a UV light box.

The digested DNA was extracted from the digest reaction with an equal volume of phenol:chloroform:isoamyl alcohol, followed by chloroform extraction. The DNA was then precipitated with 3 volumes of 100% ethanol and 1/10th volume sodium acetate. The pelleted DNA was resuspended in 40 µl water and quantified.

3.11 PROBE LABELING WITH DIGOXIGENIN

The digested plasmid samples were labeled with digoxigenin by using the Dig RNA Labeling kit (SP6/T7) from Roche (Indianapolis, IN). The protocol given with the kit was used. 1 µg of the digested plasmid DNA from the above section was used. The sample digested with EcoRV was used with the SP6 promoter, while the sample digested with Spe1 was used with the T7 promoter. After the Dig incorporation reaction, the reactions were cleaned using Centri-Sep Spin Columns (Princeton Separations, Freehold, NJ). The transcripts were checked on a formaldehyde gel (68 ml DEPC treated ddH20, 8 ml 10X MOPS, 0.8 g Agarose, and 4 ml formaldehyde). The probe was prepared for loading (2.0 µl labeled probe, 0.5 µl formamide, 2.7 µl formaldehyde, 1.5 µl 10X MOPS, 1.6 µl Loading Buffer, 0.2 µl 10 ng/µl Ethidium Bromide, and 1.0 µl DEPC treated ddH_2O) then incubated at 70°C for 10 min and quenched on ice. The sample loaded on the gel and run at 100 Volts for 1 hr. The gel was then visualized on a UV light box to verify labeled transcripts.

To determine labeling efficiency, samples were tested on a dot blot. The dot blot was conducted using reagents from the DIG Nucleic acid Detection kit (Roche, Indianapolis, IN). The protocol from the kit was followed. The dot blot results indicated the efficiency and the probes were diluted to 10 ng/µl and stored at -80°.

3.12 *IN SITU* HYBRIDIZATION WITH POLY d(T) PROBE

To determine the RNA quality of the tissue, an *in situ* experiment was conducted using a poly d(T) probe that was obtained from Roche (Indianapolis, IN). Slides were incubated at 55°C for 10-15 min to melt the Paraplast. The slides were washed in 2 changes 50 ml Citrisolv (3 min), 2 changes 50 ml 100% EtOH (3 min), 50 ml 95% EtOH (3 min), and 50 ml DEPC-ddH_2O (3 min). The slides were incubated with 100 µl 5 µg/ml Proteinase K in 0.05M Tris/HCl pH 7.6 at 37°C for 30 min. The slides were washed in 2 changes 50 ml DEPC-ddH_2O (3 min), 50 ml 95% EtOH (3 min), and 50 ml 100% EtOH (3 min). The slides were allowed to air dry and 20 µl prepared probe hybridization solution was added to the slide. Cover slips were added and the slides were incubated in a humid chamber for 15 min at 65°C then at 37°C for 2 hrs.

Slides were washed in 3 changes 50 ml TBS with 0.1% Triton X-100 (3 min) and then incubated with 100 µl normal rabbit serum diluted 1:5 in TBS containing 3% BSA, 0.1% Triton X-100 for 10 min. The slides were incubated with 100 µl provided Antibody at a 1:200 dilution per slide for 30 min. The slides were washed for 2 changes in 50 ml TBS (3 min) and 50 ml alkaline phosphatase substrate buffer pH 9.0 (5 min). The slides were incubated with 100 µl color solution per slide, and cover slips were placed and sealed with rubber cement. The slides were incubated at room temperature overnight in the dark. The slides were washed with tap water (5 min) then mounted using Crystal Mount.

3.13 *IN SITU* HYBRIDIZATION WITH DIGOXIGENIN-LABELED PROBES

Once it was determined that the prepared tissue slides had sufficient quality RNA, the *in situ* experiment was repeated using the labeled probe that we synthesized. The slides were incubated at 55°C for 10-15 min to melt the Paraplast® (Structure Probe, Inc., West Chester, PA). The slides were washed in 3 changes 50 ml CitriSolv (5 min), 2 changes 50 ml of 100% EtOH (2.5 min), 2 changes 50 ml of 95% EtOH (2.5 min), 2 changes 50 ml 70% EtOH (2.5 min), 2 changes 50 ml of DEPC-ddH$_2$O (2 min), 2 changes 50 ml of 1X PBS (5 min), 50 ml 1X PBS with 0.3%Triton for 15 min, 2 changes 50 ml 1X PBS (5 min), 50 ml 5 µg/ml Proteinase K in 10mM Tris, 1mM EDTA, pH 8.0 for 10 min, 50 ml 1X PBS with 4% paraformaldehyde for 5 min at 4°C, 2 changes 50 ml 1X PBS (5 min), 2 changes 50 ml 1X PBS with 100mM glycine (3 min), 2 changes 50 ml 1X PBS (5 min), 2 changes 50 ml 0.1 M TEA with 125 µl of acetic anhydride (5 min), 2 changes 50 ml 1X PBS (5 min), 50 ml Pre-Hybe solution (4XSSC and 50% Formamide) for 1 hr at 37°C.

The probe was then prepared for hybridization to the section. For each slide, 2 µl dig-labeled probe (10 ng/µl) was added to 52 µl of hybridization buffer (40% de-ionized formamide, 10 % dextran sulfate, 1X Denhardt's solution, 4X SSC, 10 mM DTT, and 1 mg/ml yeast tRNA) and 6 µl denatured and sheared salmon sperm DNA (1 mg/ml). This probe mixture was denatured at 70°C for 10 min and then quenched on ice. 60 µl of the prepared probe was pipetted to the center of each slide, cover slipped, and sealed with rubber cement. The slides were incubated at 50°C in a humid chamber (chamber prepared with Whatman paper soaked in a solution 4X SSC and 50% Formamide) overnight.

Rubber cement was removed and the slides were washed in 50 ml 2X SSC for 5 min to remove cover slips, 2 changes 50 ml 2X SSC (15 min at 37°C with shaking), 50 ml NTE buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8) with 20 µg/ml RNAse A for 30 min at 37°C, 2 changes 50 ml 1X SSC (15 min at 37°C with shaking), and 50 ml Buffer 1 (100mM Tris-Hcl, 150 mM NaCl, pH 7.5) for 5 min. Slides were incubated with 100 µl Blocking solution (Buffer1 with 0.1% Triton and 2% normal sheep serum) per slide at room temperature for 22 min. The blocking solution was tipped off of each slide and 75 µl antibody solution (1:500 antibody dilution: anti-Dig antibody provided with the Roche Dig detection kit (Indianapolis, IN) and Buffer1 with 0.1% Triton and 1% normal sheep serum) was added to each slide and cover slipped. The slides were incubated at room temperature in a humid chamber for 2 hrs. The cover slips were removed and the slides were washed in 2 changes 50 ml Buffer 1 (10 min) and 50 ml Buffer 2 (0.1M Tris-HCl, 0.1 M NaCl, and 50 mM $MgCl_2$, pH 9.5) (10 min with shaking). The slides were incubated with 100 µl per slide Color Solution with Levamisole (2% NBT/BCIP stock solution from the Roche Dig detection kit, 97.9% Buffer 2, and 0.1% 1M Levamisole) and cover slips were added. The slides were sealed with rubber cement and kept in a humid chamber at room temperature for 24 hrs. The chamber was wrapped in aluminum foil to prevent light exposure.

The slides were washed in 50 ml Buffer 3 (10mM Tris-HCl pH 8.1 and 1 mM EDTA) for 5 min to remove cover slip then 50 ml water for 2 min. The slides were mounted with Crystal Mount™ (Sigma, St. Louis, MO) and stored at 4°C.