Characterization of the Leptin Receptor (Lepr) Isoforms in Bowhead Whales using RACE and cDNA Sequencing

Rachel Sarah Bright

Department of Biology

Honors Research Project

Submitted to

The Honors College
ABSTRACT

Leptin and its receptor play a key role in adipose tissue regulation. The whale, *Balaena mysticetus* (Bowhead whale) has vast amounts of adipose tissue (blubber) needed to survive the arctic waters and thus represents an extreme organism with respect to how fat must be regulated. To better understand how this whale may regulate this large blubber mass further characterization of the leptin receptor gene (Lepr) and its different isoforms was undertaken. Sequences from GenBank of ungulates were aligned and used to predict what the sequences of the bowhead whale could be, given that whales are related to ungulates. To demonstrate the actual presence of these isoforms in whales, rapid amplification of cDNA ends (RACE) was performed on RNA extracted from bowhead kidney tissue. Resulting products were cloned, and the nucleotide sequences determined. Multiple attempts to derive these isoforms from RNA samples proved problematic. However, a PCR on extracted genomic DNA was successful and produced a 230 base pair sequence. This sequence was aligned and identified to be most similar to a portion of the long isoform (ObRb) of ungulates. This sequence confirms the presence of a receptor gene in bowhead whale similar to that of ungulates as predicted.
ACKNOWLEDGMENTS

I would like to thank Dr. Joel Duff and Hope Ball for giving me the opportunity to participate in this project. Thank you to Dr. Stephen Weeks for organizing the Tiered Mentoring Program, which sparked my interest in this project and helped me to better my understanding of genetics and molecular techniques used in a research setting. I thank Dr. Duff for being supportive on my path to dental school and being both a role model and mentor. I thank Hope Ball for being so patient and resourceful. Your help with learning in the lab and making sure everything was going smoothly cannot be thanked enough. I thank Dr. Hans Thweissen for collecting the Bowhead samples from the Inuit hunts. I want to thank my project readers: Dr. Rich Londraville and Dr. Francisco Moore. Also, thank you to the Department of Biology chair, Dr. Monte Turner and my department Honors Faculty Advisor, Dr. Jim Holda. Thank you to the Honors College for overseeing this process and encouraging student faculty relations. Lastly, I thank my parents Ellen and James K. Bright for their support through my undergraduate career and with this project. I would not be nearly as successful as a student without your support from an early age and I love you very much. As I continue on to the Ohio State College of Dentistry this experience will remain as my most valuable and difficult one that I encountered as an undergraduate.
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... 5
LIST OF TABLES ............................................................................................................. 6

CHAPTER

I. INTRODUCTION ........................................................................................................... 7
II. MATERIALS AND METHODS ..................................................................................... 15
III. RESULTS ..................................................................................................................... 29
IV. DISCUSSION AND CONCLUSIONS ......................................................................... 33

REFERENCES CITED ..................................................................................................... 35
LIST OF FIGURES

1. Graph of the structure of the alternately spliced leptin isoforms (Ahima and Osei, 2004).

2. Flow chart of biological responses to varying adipose tissue and leptin levels (Friedman, 2002).


4. Map showing location of bowhead whale populations (Braham *et al.* 1984)

5. Sequencher® alignment of cow mouse sheep and human to make primers.

6. 5’ RACE scan

7. 3’ RACE scan

8. 3’ RACE clone check scan

9. Alignment of *Bos taurus* Lepr and PCR Clone check of kidney tissue from Bowhead whale.
LIST OF TABLES

1. 5’ RACE primers
2. 3’ RACE primers
3. 5’ RACE sample trial values
4. 5’ RACE PCR reaction specifics
5. 3’ RACE sample trial values
6. Vector primer sequences
7. PCR reaction specifics for clone checks
8. Sequencing reaction specifics
CHAPTER I
INTRODUCTION

LEPTIN (GENERAL)

Leptin, a peptide hormone most closely associated with adipose tissue, was first identified in 1994 (Zhang et al., 1994). With a molecular weight of 16 kd, the amino acid sequence is 84% similar between human and mouse (Zhang et al., 1994). Produced by adipose cells, leptin plays a role in appetite control and energy balance through interactions with the leptin receptor (Lepr) in the hypothalamus (Friedman 2009, Trayhurn et al., 2006, & Zhang et al., 1994). Individuals with more adipose tissue show larger amounts of leptin (Villafuerte, 2000). Individuals with lower fat concentrations exhibit lower levels of leptin, leading to stimulated appetite and decreased energy expenditure until fat levels increase (Friedman, 2009). Mutations in the genes encoding the hormone itself and receptor have been linked to profound obesity in both rodents and humans (Trayhurn et al., 2006, & Zhang et al., 1994). Some leptin deficiency problems have been found to be treatable with leptin replacement therapies, though obese subjects tend to be leptin resistant (Freidman 2009). Though highly conserved in mammals, the leptin gene is poorly conserved in other classes of vertebrates as shown by a study of leptin in puffer fish (Kurokawa, 2004) and the South African clawed frog (Crespi et al., 2006).

LEPTIN RECEPTOR

For a peptide hormone to cause a reaction within a cell, a specific receptor for that hormone must be present on the cell surface. The leptin receptor (Lepr) is a single
membrane spanning cytokine-1 family receptor and was identified in 1995 by the Tartaglia lab group (Friedman 2009, Tartaglia 1995). Lepr, in mammals, is known to have six isoforms, ObRa-f (Chen, et al., 1996, Lee et al., 1996 and Tartaglia, et al., 1995). These isoforms are formed from alternate splicing of the same Lepr gene (Lee, et al. 1996, Figure 1 Ahima 2004, Chen, et al. 1996, & Friedman 2009). The amino acid sequences of Lepr are highly conserved and are shown to be over 70% similar in comparing mouse and human isoforms (Chen, et al. 1996, & Kawachi, et al., 2007).

Of the six isoforms three have been studied in detail (ObRb, ObRa and ObRe). ObRb is the longest of the known isoforms and is expressed predominantly in the hypothalamus and cerebellum, compared to other tissues. This is thought to help regulate the weight reducing effects of leptin (Bjørbæk, et al. 1998, Lee, et al. 1996 and Uotani, et al 1999). ObRa, a short isoform, is thought to help transport leptin across the blood-brain barrier (Uotani, et al.1999) (Bjørbæk, et al. 1998) and is expressed on the cell surface much more often then ObRb possibly due to ObRa being recycled and ObRb being degraded (Uotani, 1999). Lepr isoforms ObRa and ObRb are also thought to contribute to leptin degradation and transport throughout the body as well (Uotani, 1999). ObRe is soluble, non- membrane spanning and the shortest of the isoforms and is the major determinant of plasma leptin levels (Yang, et al. 2004). It is expressed heavily in the hypothalamus, adipose tissue, heart, testes and produced by the placenta during development (Gavrilova, et al.1997, Lee, et al.1996), though the effect on circulating ObRe is still unknown (Yang, et al. 2004).
LEPTIN SIGNALING

Leptin signaling functions as a negative feedback loop, maintaining homeostasis of adipose tissue by regulating energy output and food intake through neural control (Friedman, 2009). Effects of changes of adipose tissue and leptin levels can be seen below in Figure 2 (Friedman, 2002). To have an effect on a target cell, leptin must bind to ObRb (the long isoform) that sits on the cells surface causing the activation of a pathway known as JAK2 (Yang, et al. 2004). When leptin is bound to ObRe, the leptin-receptor complex is not capable of activating ObRb, and therefore having an effect on a target cell (Yang, et al. 2004).
Figure 2: Flow chart of biological responses to varying adipose tissue and leptin levels (Friedman, 2002).

OTHER STUDIES

Current studies in ungulates have shown differences in the amounts of each Lepr isoform found in differing tissues (Kawachi, et al., 2007). Other research groups have had success at identifying and sequencing the Lepr and its isoforms in bovine (Kawachi, et al., 2007). They identified and sequenced isoforms Ob-Ra, b and c. In this study, Ob-Ra was found in all tissues examined, Ob-Rb was found in low levels in the lungs and testes, and Ob-Rc was found in low levels in the lung and spleen. The Kawachi lab group
did RACE to determine the cDNA sequence for cows and this approach is similar to what has been attempted here for the Bowhead whale (2007). Below, figure 3 (Kawachi, et al., 2007) shows the presence of three Lepr isoforms in tissues throughout the body in cow.

**Figure 3: Presence of Lepr isoforms in *Bos Taurus* tissues Kawachi et al. 2007.**

**BLUBBER:**

Cetaceans and other marine mammals have a thick layer of fat, called blubber, which is useful for insulation in the cool arctic waters and acts as an energy reservoir (Iverson, 2002). Firm and fibrous in nature, blubber can also affect animal buoyancy and can help with streamlining (Iverson, 2002). A layer of blubber is almost continuous.
across the body and can account for over 50% of total body mass (Iverson, 2002).

Blubber is cooler near the skin then it is near the interior (muscles or body core) of the animal and changes biochemically with depth as well (Iverson, 2002). Bowhead blubber is thought to have two distinct layers (Budge, 2008). In a study of 64 bowhead whale samples, blubber fatty acid composition was found to be significantly different for whale age, season hunted and year hunted, but gender was not noticed to be a factor (Budge, 2008).

WHALE ECOLOGY:

Bowhead whales (Balaena mysticicetus) (Linnaeus 1758) are large, stocky baleen whales which feed exclusively on one of the smallest organisms in the oceans, phytoplankton (Mackas et. al. 1985). They are also known as the Greenland whale, Greenland Right whale or Polar whale (COSEWIC 2005). Their head, ideally shaped for pushing up through ice to breath, is large in proportion to the rest of their body, composing around 30% of their total body length (George et al. 1989). They have small paddle- shaped flippers and no dorsal fin or dorsal bump (Haldiman and Tarpley, 1993). The lifespan of bowheads may exceed 100 years, with sexual maturity occurring in the 25th year (George et al. 1999). Generally, females give birth to a single calf once every three years (Miller et. al 1992, & Rugh et al. 1992). Bowheads, along with other arctic mammals, are equipped with numerous adaptations to survive their cold habitat. They have their massive energy storage in the form of blubber that also helps to insulate internal body temperature, a sophisticated acoustic sense for ice navigation and long range communication (Ellison et al. 1987, & George et al. 1989).
Worldwide, all five recognized populations of Bowhead whale (see Figure 4) are located in the northern hemisphere (Braham et al. 1984). As of May 2005, three populations of bowhead whales can be found off the coast of Canada, with two populations considered threatened and one population under special concern (Cosewic 2005). All populations in Canadian waters are designated endangered since 1980, and are currently listed as endangered in the U.S. under the Endangered Species Act of 1973 and as depleted under the U.S. Marine Mammal Protection Act of 1972 (Cosewic 2005). Reasons for population threats include hurt from previous commercial whaling from 1860-1915 (Ross 1974). This whaling left the populations vulnerable and today they are still threatened by illegal hunting and predation due to reduced ice coverage caused by human effects (Cosewic 2005). Other threats to population growth include low fecundity, the small ecological niche where bowheads live and the recent climate change which is affecting ice coverage (Cosewic 2005). The killer whale, *Orcinus orca* (Linnaeus, 1758), is the only known natural predator of the bowhead whale, besides man (George et al. 1994). Today, bowheads are legally hunted by the Inuit natives in small quantities bi-annually in the Canadian arctic (NWMB 2000). Samples used in this study were obtained from whales of the Bering-Chukchi-Beaufort Sea population in hunts of this nature. (Gavrilova, 1997)
WHY LEPTIN RECEPTOR IN WHALES

The goal of this project is to provide sequence evidence for bowhead whale Lepr. By examining sequences of Lepr and its isoforms, we hope to identify known isoforms and make note of any variation found in the bowhead whale sequence due to alternate splicing at the 3’ end. Variation at this site is seen when comparing other mammal sequences. Knowledge of the alternate splicing and sequence at the 3’ end will be useful to future research on leptin signaling and fat storage of cetaceans. Given the origin of whales from ungulates it is hypothesized that whales will exhibit similar leptin isoforms as those that have been identified in other ungulates, specifically Bos taurus.
**CHAPTER II**

**MATERIALS AND METHODS**

**BIOINFORMATICS:**

First, using the national data base NCBI, a search was conducted for Lepr sequences from mammals. Sequences obtained were of *Bos taurus, Mus musculus, Homo sapien, and Ovis aries* (bull, mouse, human and sheep). Once obtained, these sequences were aligned using the program Sequencher® (4.10.1 Gene Codes Corporation). This alignment was useful when determining location of useful primer sites by finding portions of the sequence that were highly conserved among all the samples and thus could be expected to be conserved in the target whale species.

**Figure 5: Sequencher® alignment of cow mouse sheep and human to make primers.**

Blue=2502, Green= overlap, Yellow=2520, Pink=1469, 410 not shown.
SAMPLE COLLECTION:

Bowhead whale tissue samples were all obtained by Dr. Hans Thweissen (NEOUCOMP) with permission from the National Marine Fisheries Services (permit #814-1899-01) and with cooperation from native Alaskan Inuit hunters. These hunts happened in Barrow, Alaska twice yearly, during spring and fall hunts. The bowhead whales most likely came from Beaufort-Bering Sea population. The number of whales caught during each hunt change from hunt to hunt and year to year. Race was performed of sample #35, a kidney sample from 09B9, an adult, male bowhead whale. The tissue was stored in RNA later® solution (Ambion®) for molecular data.
RNA EXTRCTIONS Ambion TRI Reagent®

RNA extractions were completed on tissue stored in RNA-later from the bowhead hunts. All RNA extractions were completed using TRI Reagent® (Ambion®). Methodology followed manufacturer’s suggested protocols with recommended additional steps for extraction of RNA from fatty tissues. In brief, tissue placed in an RNase free tube was homogenized using a sonicator (Fisher Scientific 60 Sonic Disembrator) machine XuL of TRI Reagent® solution. Next, the homogenate was incubated for 5 minutes at room temperature then centrifuged at 12,000 xg for 10 minutes at 4°C. The supernatant was transferred to a fresh RNase free tube and 100 µl of BCP was added per 1 mL of TRI Reagent solution. The tubes were then capped tightly, vortexed and incubated at room temperature for 10 minutes. Next the tubes were centrifuged at 12,000xg for 15 minutes at 4°C. The colorless top aqueous layer was then transferred again to a new RNase free tube. In the tube, 500 µl of isopropanol was added per mL of TRI Reagent solution. The mixture was vortexed and then incubated at room temperature for 10 minutes. Again, the tube was centrifuged at 12,000xg for 8 minutes at 4°C. The supernatant liquid was discarded leaving a gel like white pellet on the side and bottom of the tube. Then 1mL of 75% ethanol was added to each extraction (the previous wording was too close to the exact protocol directions). Tubes were then centrifuged at 7,500 xg for 5 minutes at 4°C to wash the pellet. The ethanol wash was next discarded without disturbing the pellet and RNA was left to air dry for 5 minutes. The samples were eluted in 25 µl of molecular water, nano-dropped to
determine RNA concentration and samples were stored at -80°C in the department Ultra-Low freezer (VWR Scientific Products).

PRIMERS

Primers were made based on *Bos taurus* sequences for leptin receptor in highly conserved areas near the 3’ end. These primers contained not more than 50% cytosine or guanine due to the possibility of the primers folding on top of themselves. An alignment was made from NCBI on the computer program Sequencher® 4.10.1 (Gene Codes Corporation). Other primers used in the charts below that are not gene specific came as part of an Applied Biosystems Fist Choice RLM-RACE Kit.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Base Pair Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ RACE Adapter</td>
<td>5’-GCUGAUAGCGAUAGGAAUUGAAGAATACUGGCUUUCUCUGCUUGAAG-3’</td>
<td>44</td>
</tr>
<tr>
<td>5’ RACE Outer Primer</td>
<td>5’-GCTGATGGCGATGAAATGAACTG-3’</td>
<td>24</td>
</tr>
<tr>
<td>5’ RACE Inner Primer</td>
<td>5’-CGCGGATCCGAACTGCGTTTGCGTCTTGATG-3’</td>
<td>35</td>
</tr>
<tr>
<td>410 OBR Reverse</td>
<td>5’-AGTCCTCTTTTCATCCAGCACTGTATGT-3’</td>
<td>29</td>
</tr>
<tr>
<td>1469 OBR Reverse</td>
<td>5’-CATCAGAAACGACAGGGGTGCCTCCT-3’</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 1: 5’ RACE Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Base Pair Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’ RACE Adapter</td>
<td>5’-GCCAGCAGAGAATATTACGACTCAGTATAGGT12VN-3’*</td>
<td>57</td>
</tr>
<tr>
<td>3’ RACE Outer Primer</td>
<td>5’-GCCAGCAGAGAATTATTACGACT-3’</td>
<td>23</td>
</tr>
<tr>
<td>3’ RACE Inner Primer</td>
<td>5’-CGCGGATCCGAAATACGACTATAGG-3’</td>
<td>32</td>
</tr>
<tr>
<td>2502 OBR Forward</td>
<td>5’-CACCCAGCATGATGCAGATCT-3’</td>
<td>20</td>
</tr>
<tr>
<td>2520 OBR Forward</td>
<td>5’-CCCCATTGAAAATACGATTCAGTC-3’</td>
<td>26</td>
</tr>
</tbody>
</table>

Table 2: 3’ RACE Primers

* (V=G+A+C, N=A+C+G+T)
RACE 5’

Rapid amplification of cDNA ends, or RACE, was performed on the RNA extractions from the bowhead blubber tissue. A kit from Applied Biosystems was used, entitled First Choice RLM-RACE Kit. RACE is a variation of a polymerase chain reaction (PCR) designed to help clone cDNA segments. Methodology followed manufacturer’s protocols. In brief, RNA was extracted, and 5’ RACE reaction was attempted.

A. RNA Processing

In an RNase-free micro-centrifuge tube the following was assembled: 10 µg of RNA, 2 µl of 10X CIP buffer, 2 µl Calf Intestine Alkaline Phosphatase (CIP), and 6 µl of Nuclease-free water. Multiple attempts were completed with varying amounts of starting RNA (see chart below). The tube was vortexed, spun briefly and incubated at 37°C for one hour. Next 15 µl of ammonium acetate, 115 µl of nuclease free water and 150 µl of acid phenol chloroform was added to the tube. Reaction mixture was vortexed and centrifuged at room temperature for 5 minutes at 10,000 xg. The top aqueous layer was then transferred to a new RNase free tube. 150 µl of chloroform was then added, the tube was vortexed and centrifuged again at room temperature and 10,000 xg. The top aqueous layer again was transferred to a new RNase free tube and 150 µl of isopropanol was added. The reaction was then vortexed and chilled on ice for 10 minutes. After chilling on ice the reaction was centrifuged for 20 minutes. A pellet at the bottom of the tube was rinsed with 0.5 mL cold 70% ethanol and centrifuged for 5 minutes. Ethanol was removed carefully, not disturbing the washed pellet and the
pellet was allowed to air dry. Pellet was resuspended in 11 µl of nuclease free water and the sample was placed on ice. The following was then assembled in an RNase free tube: 5µL CIP’d RNA from previous reaction, 1 µl of 10X TAP buffer, 2 µl of Tobacco Acid Pyrophosphatase, and 2 µl of nuclease free water. Reaction was vortexed, spun briefly and incubated at 37°C over a heat block for 1 hour. After incubation the following was assembled in an RNase free tube: 2 µl of CIP/TAP-treated RNA from previous reaction, 1 µl 5’Race adapter, 1 µl of warm 10X RNA Ligase buffer, 2 µl of T4 RNA ligase (2.5U/µL), and 4 µl of Nuclease free water. The reaction mixture was mixed and spun briefly, then incubated at 37°C. The reaction was stored at -20°C.

<table>
<thead>
<tr>
<th>Trial #</th>
<th>Sample</th>
<th>Tissue Type</th>
<th>Nano-drop value</th>
<th>1 µg RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>#35</td>
<td>Kidney</td>
<td>345.6 µg/µL</td>
<td>~10 µl</td>
</tr>
<tr>
<td>2</td>
<td>#35</td>
<td>Kidney</td>
<td>345.6 µg/µL</td>
<td>~16 µl</td>
</tr>
</tbody>
</table>

**Table 3: 5’ RACE Sample trial values**

B. Reverse Transcription

For the reverse transcription reaction, the following was assembled in an RNase free microfuge tube on ice: 2 µl of Ligated RNA, 4 µl of dNTPMix, 2 µl of Random Decamers, 2 µl of 10X RT Buffer, 1 µl of RNase Inhibitor, 1 µl of M-MLV Reverse Transcriptase and 8 µl of Nuclease free water. The reaction mixture was mixed gently, spun briefly and incubated at 42°C for one hour.

C. Nested PCR for 5’ RLM-RACE
First the following components were combined in a PCR tube on ice: 1 µl of RT reaction, 5 µl of 10XPCR Buffer, 4 µl of dNTP Mix, 2 µl of 5’ Race gene-specific outer primer (10 µM), 2 µl of 5’ Race outer primer, 36 µl of Nuclease-free water, and 1.25 U of thermostable DNA polymerase (0.25 µl of 5 U/µl) (Takara LA PCR kit version 2.1) Next, the tube was mixed gently, spun briefly and following the conditions shown below was placed in a thermocycler (Eppendorf Mastercycler Personal, model #22331 Hamburg).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Reps</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>1</td>
<td>94°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Amplification</td>
<td>2</td>
<td>94°C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60°C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>2 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>3</td>
<td>72°C</td>
<td>7 min</td>
</tr>
</tbody>
</table>

Table 4: 5’ RACE PCR reaction specifics.

Once the previously mentioned cycle was complete, the following was combined in a PCR tube on ice: 2 µl of Outer PCR product, 5 µl of 10X PCR Buffer, 4 µl of dNTP Mix, 2 µl of 5’ RACE gene specific inner primer (10µM), 2 µl of 5’ RACE Inner Primer, 35 µl of Nuclease-free water and 1.25 U of thermostable DNA polymerase (0.25 µl of 5 U/µl) (Takara). The same cycle parameters were used as in Table X in the thermocycler.

D. Gel Analysis of Products

After the second PCR was complete, gel electrophoresis was performed. A 1% agarose gel was poured in to a horizontal electrophoresis system (OWL model
B1A). The gel was allowed to set with a plastic comb inserted to make wells for the DNA ladder and samples. After the gel was set, it was submersed in PCR buffer (TAE 1x) and the ladder (Axygen Biosciences 100bp Ladder DNA market) and samples with 3 µl of load dye (Promega Blue/Orange 6x Loading Dye) were loaded. Running conditions for each gel followed 70 mA for approximately 30 minutes. Once ran the gel was submerged for three minutes in ethidium bromide in darkness to develop. Next the gel was rinsed in water and visualized using Gel Snap® computer program and G: Box gel imager (both by Syngene).

RACE 3’

A. Reverse Transcription

The following was combined in an RNase-free tube: 2 µl RNA, 4 µl of dNTP Mix, 2 µl 3’ RACE Adapter, 2 µl 10X RT Buffer, 1 µl RNase Inhibitor, 1 µl M-MLV Reverse Transcriptase, 8 µl Nuclease-free water. The reaction mixture was mixed gently spun briefly and incubated at 42°C for one hour.

B. PCR for 3’ RLM-RACE

First, the following was added to a PCR tube on ice: 1 µl of the RT reaction, 5 µl 10X PCR buffer, 4 µl dNTP mix, 2 µl of 3’ RACE gene specific outer primer (to uM), 2 µl of 3’ RACE outer primer, 36 µl Nuclease-free water, and 1.25 U of thermostable DNA polymerase (0.25 µl of 5 U/µl) (Takara). Multiple attempts were completed with varying amounts of starting RNA (see chart below). The
tube was flicked gently and spun briefly. The same cycle parameters were used as in Table X in a thermocycler. Second, First, the following was added to a PCR tube on ice: 1 µl of the RT reaction, 5 µl 10X PCR buffer, 4 µl of dNTP mix, 2 µl of 3’ RACE gene specific inner primer (to µM), 2 µl of 3’ RACE inner primer, 36 µl Nuclease-free water, and 1.25 U of thermostable DNA polymerase (0.25 µl of 5 U/µl) (Takara). The tube was flicked gently and spun briefly. The same cycle parameters were used as in Table X in a thermocycler. Once PCR was complete results were visualized using gel electrophoresis.

<table>
<thead>
<tr>
<th>Trial #</th>
<th>Sample</th>
<th>Tissue Type</th>
<th>Nano-drop value</th>
<th>1 µg RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>#35</td>
<td>Kidney</td>
<td>345.6 µg/µL</td>
<td>~3.5 µL</td>
</tr>
<tr>
<td>2</td>
<td>#35</td>
<td>Kidney</td>
<td>345.6 µg/µL</td>
<td>~7 µL</td>
</tr>
<tr>
<td>3</td>
<td>#35</td>
<td>Kidney</td>
<td>345.6 µg/µL</td>
<td>~3 µL</td>
</tr>
</tbody>
</table>

Table 5: 3’ RACE sample trial values

Gel Purification

Purification of RACE products were done using spin tubes from a Millipore Ultrafree®-DA kit. Product bands that were cut out of the 1% agarose gels using a sterile razor blade in the Kodak Gel Logic 2200 Imaging System. Once cut out of the gel, the small fragments were placed into spin columns included in the kit that have a mesh to let the cDNA products slip to the bottom of the tube and catch the agarose medium. Tubes were placed into the centrifuge for 10 minutes so that the product would collect at the bottom for cloning.
CLONING TA TOPO Kit

In an RNase free tube the following was assembled: 2 µl of RACE PCR products from gel cleanups, 0.5 µl of TOPO Kit salt solution, and 0.5 µl of TOPO® vector. This mixture was incubated at room temperature for 12 minutes and then placed on ice. While on ice, X-gal (Shelton Scientific, Inc.) was applied to pre-poured Petri dishes and set in the incubator (Fisher Scientific Isotemp Incubator) at 37°C to dry. Bacteria (One Shot® Chemically Competent E.coli) were also removed from the ultra-low freezer and placed on ice to thaw at this time. Once on ice, 25 µl of bacteria were added to each reaction and the reactions were mixed gently (not by pipetting up and down so as not to kill the bacteria due to friction). The reactions were then heat shocked for 30 seconds at 42°C then immediately placed on ice. Next the 125 µl of S.O.C. medium was added to the reactions and they were placed on the shaker at 37° (Orbit Lab-Line Environ Shaker) for one hour. After being incubated in the shaker, between 40 µl and 80 µl of each reaction was spread onto the pre-warmed petri dishes with the X-gal coating. Bacteria were left in the incubator over night to grow colonies.

Colonies formed overnight, and ones which did not turn blue were selected using a pipette tip. A culture was formed using 1 mL of LB/amp broth and selected colonies. The cultures were placed in the shaker overnight.

Clones were checked (Qiagen PCR Kit) to insure an insert was present in the vector inserted in the bacteria. The following was assembled in a PCR tube: 3.5 µl of nuclease-free water, 6.0 µl Q-Master Mix, 0.25 µl of T7 primer, 0.25 µl of T3 primer and 0.1 µl of the bacterial culture. Reaction mixtures were placed into a thermocycle Results were visualized using gel electrophoresis to identify insert size.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Base Pair Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7</td>
<td>5’-TAATACGACTCACTATAGGG-3’</td>
<td>20</td>
</tr>
<tr>
<td>T3</td>
<td>5’-ATTAACCCTCACTAAAGGGA-3’</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table 6: Vector Primer Sequences**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Stage</th>
<th>Reps</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>1.5 min</td>
</tr>
<tr>
<td>Amplification</td>
<td>2</td>
<td>25</td>
<td>95°C</td>
<td>45 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>49°C</td>
<td>45 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C</td>
<td>1.5 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>3</td>
<td>1</td>
<td>72°C</td>
<td>4 min</td>
</tr>
</tbody>
</table>

**Table 7: PCR reaction specifics for clone check**

PLASMID PREP (Qiagen MiniPrep Kit)

Once clones were found to have proper insert size for the Lepr product, in an RNase free tube, 1.5 mL of bacterial culture was added and tube was placed in the centrifuge for 8 minutes at 8000 rpm. After spinning, a bacterial pellet remained at the bottom of the tube and the liquid broth was discarded. Next 250 µl of P1 solution was added and was pipetted up and down to resuspend the pellet. Then 250 µl of P2 solution was added and the tube was closed and inverted several times. Following, 350 µl of N3 solution was added and again the reaction mixture was inverted to mix, this time turning cloudy. The reaction mixture was next centrifuged on high for ten minutes. All of the reaction liquid was then transferred to a pre-labeled QIAprep spin column (membrane and clear catch tube), being careful not to transfer any precipitate at the bottom of the reaction tube. The spin column was placed in the centrifuge on high for 60 seconds. Next,
the liquid was discarded and 725 µl of PE buffer was added and again the reaction mixture was centrifuges for 60 seconds. The liquid was discarded and the spin column was centrifuged for 60 seconds on high to dry. The clear catch tube was discarded and the spin column membrane was inserted into a new RNase free 1.5 mL tube. 40 mL of nuclease-free water was added on top of the spin column membrane and the reaction mixture was centrifuged on high for 60 seconds. The spin column was finally discarded and the purified DNA in water was then used for sequencing.

SEQUENCING

A sequencing reaction was assembled in an RNase free tube containing the following: 2.3 µl of Big Dye Reaction Mix (specifics), 0.5 µl of primer (~3.2 pmol) and 5.0 µl of DNA. The reaction was then placed in the thermocycler and ran under the following condition.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Reps</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient Flux</td>
<td>1</td>
<td>29°C</td>
<td>20 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>44°C</td>
<td>10 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60°C</td>
<td>4 min</td>
</tr>
</tbody>
</table>

Table 8: Sequencing reaction specifics

ETHANOL SEQUENCE REACTION CLEAN-UPS

In a clean 1.5 mL tube 1.7 µl of 125 mm EDTA was added. In the sequencing reaction tubes from the following reaction, 19 µl of 100% ethanol was added to the sequencing mixture and the entire contents were moved to the clean 1.5 mL tube containing the EDTA. The reaction tube was allowed to sit at room temperature for 15
minutes then centrifuged on high for 25 minutes with the hinges facing outward. Facing the hinges in the same direction allowed for the pellet to form in a predicted area. Once centrifuged, the liquid in the reaction tube was removed by pipetting up opposite of where the pellet should have formed. This liquid was discarded. Next 30 µl of 70% ethanol was added to the reaction tube containing only the pellet and this was allowed to sit for one minute before returning to the centrifuge to spin on high for another 15 minutes, with hinges facing out. Once spun, again, the liquid was carefully removed trying not to disturb the pellet, and the reaction was set in the preheated DNA Speed Vac® (Savant) at medium setting for 10 minutes to dry the sample. Once reaction mixture was dried in the Speed Vac®, 28 µl of HiDi formamide™ (Applied Biosystems) was added and the reaction was placed in a preheated heat-block (Fisher Scientific, Dry Bath Incubator®) at 95°C for 2 minutes. Next the tube was cooled over ice for 2 minutes and the reaction mixture was transferred to a 96 well plate for Senger sequencing analysis in the 3130x Genetic Analyzer (Applied Biosystems).

SEQUENCE ANALYSIS

Once sequences were obtained, results were BLAST searched on the national database, NCBI. BLAST searching told if the fragments we obtained were ungulate Lepr (most closely associated reported sequence). Sequences which when BLAST searched were identified as ungulate sequence were place in the Sequencher® 4.10.1 (Gene Codes Corporation) program and aligned with previously looked at Lepr sequences to determine where our results were in relation to known exons.
CHAPTER III

RESULTS

RNA was extracted following a TRIReagent® protocol from RNAlater® preserved tissue of a male bowhead (B9) kidney. The extracted RNA was then quantified using a nano-drop. This analysis yielded 345.6 ng/µl of RNA. First strand cDNA synthesis was performed using recommended protocols for 5’ RACE (Applied Biosystems) and 3’ RACE and results were evaluated on agarose gels (see below). Gene specific primers for the RACE PCR reactions were designed based upon a Lepr alignment from sequence found on Genbank (see figure 5 in Materials and Methods).

5’ RACE:

First results of attempts at 5’ RACE seemed promising. Smears were seen indicating possible capture of differently spliced isoforms. The gel image below (Figure 6) shows smears indicating possible isoforms of OBR in whale kidney using designed gene-specific and RACE supplied primers. Attempts were made to clone the products into bacterial vectors, but the resultant sequences were not shown to be similar to any leptin receptor sequences in Genbank.
3’ RACE:

The RNA extracted from whale kidney was also used to attempt 3’ RACE. Gene-specific and RACE supplied primers were used in first strand cDNA synthesis. Results were visualized on an agarose gel (see Figure 7). Smears again were seen indicating possible different Lepr isoforms. These were cut out of the gel and gel purified (Millipore Ultrafree®-DA kit). Inner primer 3’RACE was performed (see Materials and Methods for details) using supplied inner primers and the same gene-specific primers. Bands were seen, gel purified (Millipore Ultrafree®-DA kit) and cloned (TOPO® vector kit) into a bacterial vector. Clone checks were performed (Figure 8) and bands were seen indicating that different isoforms were cloned into bacterial vectors. Attempts at
sequencing these clones were unsuccessful. A small portion of the sequence was acquired from a cloned PCR product (see alignment Figure 9) and results from this demonstrate that expected Lepr sequence in whales shares a high degree of similarity with ungulate taxa.

Figure 7: 3’ RACE PCR #1 smears show possible isoforms
Figure 8: 3’ Agarose gel showing PCR products from clones of products of 3’ RACE PCR in figure 7. The left lane is molecular ladder and bands in other lanes indicate inserts are present in individual plasmids from those colonies.

Figure 9: Alignment of *Bos taurus* Lepr and PCR Clone check of kidney tissue from Bowhead whale. Base pairs that differ are highlighted in yellow.
CHAPTER IV
DISCUSSION AND CONCLUSION

This project employed a number of bioinformatic and molecular DNA methods to attempt to sequence and identify known and possible unknown isoforms of Lepr in bowhead whale tissue samples. Previous studies suggested that RACE is an efficient method of obtaining large sections of sequence such as seen in the Kawachi lab study on *Bos taurus*.

The 5’ RACE protocol focused on making elongated cDNA copies of the present RNAs in the bowhead whale kidney tissue sample used. In the first attempt 5’ RACE products appeared as smears on the gels (figure 6) suggesting possible isoforms of Lepr with the gene specific primers made using the alignment shown above had worked. However, subsequent cloning of these products and then sequencing of those products did not confirm the presence of Lepr sequences. The lack of success could be due to possible low Lepr RNA copy number in the kidney sample used, RNA degradation so that the 5’ “cap” could not attach properly, or human error when following the protocol.

Lacking success for 5’ RACE, 3’ RACE was performed. For the 3’ RACE, RNA is copied from the poly-A 3’ end found on mRNAs. Results here were more hopeful as smears (figure 7) again were seen once PCR took place, suggesting capture of multiple isoforms. Though some 20 base pair fragments were identified from the sequencing reactions, BLAST search and comparisons to our primers revealed that only our primer sequence was obtained in return with the larger sequences. Again, some possible issues
could have caused by the bacteria only up taking a portion of the cDNA into vector, possible to much or too little starting RNA, or human error in following protocol.

Though problems still persist, using RACE to obtain sequence of the Lepr isoforms is still a possibility. More trouble shooting with primers and RACE protocol still could provide answers. In lieu of finding full length Lepr sequences an attempt to directly amplify a portion of the Lepr sequence from genomic DNA was attempted. This resulted in a positive PCR product which was cloned and sequenced resulting in a ~230 base pair sequence of bow head Lepr was obtained. This short sequence was aligned and found to be most similar to *Bos taurus* Ob-Rb, confirming that Bowhead whales do have Lepr. If whales make leptin, but the leptin is signaling in these animals the way it does in other mammals, then how is it possible that they can sustain such large fat deposits in the form of blubber. Is it possible something with their receptor? Comparisons of this 230 base pair sequence reveal only a single nucleotide sequence difference with ungulates and so it does not appear that there is a significant difference in their primary sequence but this sequence only represents a small portion of the total Lepr gene sequence.
REFERENCES CITED