

Regulation of the myoglobin gene from the common dolphin, *Delphinus capensis*

Brandie Garman, Diana Lopez, and Stephanie Spencer: CI HSI-STEM Institute, Summer 2010
Advisor: Charles Sackerson



Introduction

Diving mammals have the most myoglobin in their muscles of any animals. This high level of myoglobin is important for carrying oxygen and handling the by-products of the vigorous exercise they do while swimming, diving, feeding and escaping predators. We would like to study the regulation of the gene for myoglobin, to understand the mechanisms by which it is turned on to such high levels. This project will use a cloned promoter region from a dolphin myoglobin gene to drive a reporter gene in cell culture. In these experiments, we have taken two important steps toward that goal.

First, we have used the polymerase chain reaction (PCR) to confirm the species of origin of the myoglobin gene promoter to be used in the experiments. The tissue sample had originally been assigned to the species *Delphinus capensis*, the long-beaked common dolphin, and we have confirmed that assignment (Figure 1).

Second, we have moved the promoter fragment, which was previously cloned and sequenced, into an intermediate vector, to facilitate the final cloning into the expression vector (Figure 2).

We will describe here these two sets of experiments. Both sets of experiments were successful, and the project is now nearer the goal of exploring the activity of the dolphin myoglobin promoter in muscle cells growing in culture.

Results

Species identification by mitochondrial control region DNA sequence analysis:

The mitochondrial (mt) control region is one of the most rapidly changing regions of DNA in eukaryotic cells. Even closely related species will have differences that make them unique for this stretch of DNA, making it useful for an unambiguous identification of species, populations, and even individuals¹.

DNA had been previously isolated from a muscle sample (provided by Rachel Cartwright) identified as being from *Delphinus capensis*. This DNA has been used to clone the myoglobin promoter. We used the PCR to isolate sufficient quantities of mt DNA for sequence analysis. Four primers were used², two "pointing to the right" and two "pointing to the left", in all combinations to give four different reactions. The results are shown in Figure 1, left panel; the efficiency of the reaction differed for each of the combinations. The two samples in the right-most lanes were deemed adequate for sequence analysis.

The resulting DNA sequence was analyzed using a Neighbor-Joining algorithm⁴ as implemented by Witness For The Whales⁵ against their database of reference nucleotide sequences. The result is shown in Figure 1, right panel. The closest reference sequence belongs to the "long-beaked common dolphin", *Delphinus capensis*. This confirms the identity of the tissue sample used for the myoglobin promoter cloning.

Subcloning of the dolphin myoglobin promoter:

In order to test the properties of the myoglobin promoter, it will need to be incorporated into an "expression vector" suitable for use in muscle cells growing in culture. Making the expression vector will involve using restriction enzymes and DNA ligase to recombine the promoter into the *lacZ* gene from *E. coli*; the *lacZ* gene will direct the synthesis of the enzyme β -galactosidase under the control of the dolphin promoter. β -galactosidase activity can then be assayed as a measure of the activity of the promoter (Figure 2, left, bottom).

However, the clone that had been previously produced could not be used for production of the expression vector, because the available restriction enzyme sites will not work. We chose to use pGEM-11 as an intermediate vector to solve this problem. In order to ensure that the resulting clones were in the "target" vector, pGEM, and not in the original "source" vector, we "gel-purified" the promoter fragment away from the source vector before doing the ligation into pGEM. This process is shown in Figure 2.

After ligation of the promoter into pGEM, the product was transformed into *E. coli*. We included two important controls in the transformation: The first control tested the transformation efficiency of the *E. coli* cells, to make sure that the cells were competent for DNA transformation. We found a transformation frequency of 7.1×10^5 colonies per μg DNA. The second control tested how complete the digestion of the pGEM was, by transforming the enzyme-digested pGEM without any ligation: the "cut" pGEM gave a transformation frequency of 6.2×10^1 per μg DNA, and all these colonies were blue, indicating that these colonies resulted from undigested pGEM, and that the enzyme digestion was approximately 99.99% complete.

The ligation reaction of the cut pGEM + the gel-purified promoter piece gave a transformation efficiency of 1.9×10^2 , about 3X more than the cut pGEM alone. In addition, half of the colonies were white, implying that the colonies resulted from pGEM molecules that had the desired promoter fragment inserted.

We checked this by purifying several of the "putative" clones and restriction digesting them. The result from three putative clones is shown in Figure 2. All three show a restriction enzyme digestion pattern different from either the source vector, or from the original pGEM target vector, and are indicative of a successful subcloning, as schematized in Figure 2.

Discussion

Myoglobin levels in the muscles of diving mammals is among the highest in any species. The myoglobin in cetaceans is essential to their ability to feed and escape predators after weaning. Therefore it is in our interest to understand the molecular mechanisms that trigger the expression of high levels of myoglobin.

Toward this end, we are working on a DNA clone of the 5' regulatory region of the myoglobin gene from *Delphinus capensis*. We have demonstrated that the clone being used is, indeed, from *D. capensis* using the hypervariable control region from the mitochondrial genome. This is an important step, in that the muscle samples used in this research are from "forensic" samples derived from beached, dead animals. Therefore some of the markers important to species identification may be missing or damaged in some samples. Our use of mtDNA for identification eliminates any question as to the identity of the samples.

We have also been successful in moving the existing clone into an intermediate plasmid vector from which it can readily be used to construct an expression vector. An expression vector is a plasmid designed to direct the synthesis of a protein product, in response to appropriate regulatory regions. This expression vector will express the *E. coli* β -galactosidase gene in response to transcriptional control by the dolphin myoglobin promoter region, in mammalian cells in culture. The β -galactosidase is of no intrinsic interest, but will serve simply as a "reporter" for the activity of the promoter. The activity of the β -galactosidase enzyme is readily assayed, and this will allow studies of the conditions that regulate the myoglobin gene at such high levels.

We have also, in work not shown, learned to culture mammalian cells to use for the experiments. The cells we have been using, HeLa, are not the muscle cells which will be used in the final experiments, but our ability to maintain mammalian cells in culture has been a valuable learning experience.

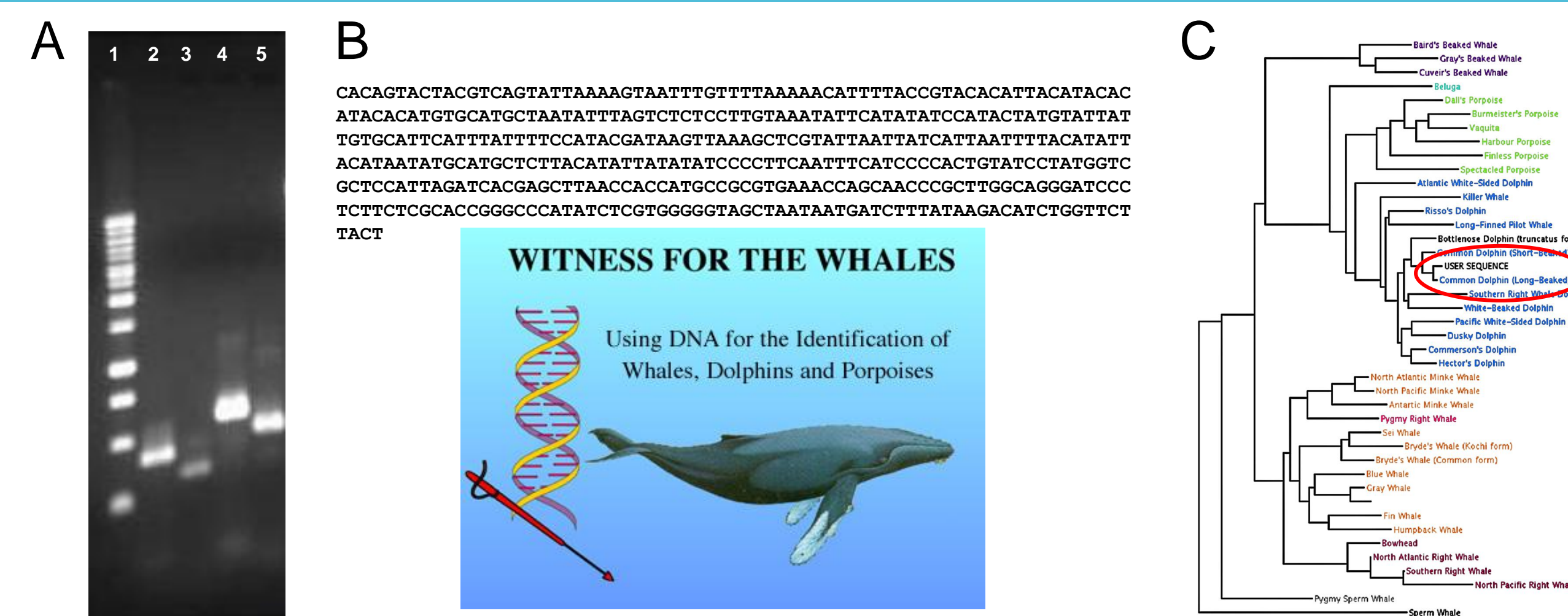


Figure 1: Mitochondrial sequence isolation and analysis.

A: Gel electrophoresis of the products of four PCRs: lane 1: size standards; lane 2: reaction using primers Dlp4R and Dlp1.5F; lane 3: reaction using primers Dlp4R and Dlp10F; lane 4: reaction using primers Dlp5R and Dlp1.5F; lane 5: reaction using primers Dlp5R and Dlp10F. The remainder (20 μl) of the reactions from lanes 4 and 5 were sent to Laragen for purification and sequencing.

B: Top: Compiled sequence from the mtDNA control region. Bottom: This sequence was submitted to the Witness For The Whales site at the University of Auckland, New Zealand for comparison to their curated database of mtDNA control region sequences from Cetacean species.

C: The output from the analysis indicates that the closest match (circled in red) to our submitted sequence is that from the "Common Dolphin (Long-Beaked)", latin name *Delphinus capensis*.

Materials and Methods

Polymerase chain reaction: PCR was done using Illustra puReTaq PCR beads on ~100 ng DNA, as recommended by the manufacturer. Primers used were:

Dlp1.5F: 5'-TGTAACACGACGCGCCAGTTCACCCAAAGCTGRARTTCTA-3'

Dlp10F: 5'-CCACAGTACTATGTCGGTATT-3'

Dlp4R: 5'-GCGGGWTRYTGRTTTCACG-3'

Dlp5R: 5'-CCATCGWGATGCTCTATTAAAGRGAA-3'

Sequence analysis: Unpurified PCR products were sent to Laragen, Inc., Los Angeles for purification and sequencing. Two independent products were sequenced in both directions, and the final sequence compiled from the region that gave consensus from three of the four sequences.

The sequence was submitted to Witness For The Whales (<http://www.cebl.auckland.ac.nz:9000/page/whales/title>) for comparison to their database of mtDNA sequences.

Subcloning: A previously described clone of the *D. capensis* myoglobin promoter region (757 bp) was in the vector pBluescriptII (Stratagene); it was moved into pGEM-11 (Promega) using standard methods⁶.

Acknowledgements

We are grateful for the opportunity given to us by the HSI-STEM Summer Institute 2010, CSUCI, and Dr. Charles Sackerson to participate in this research.

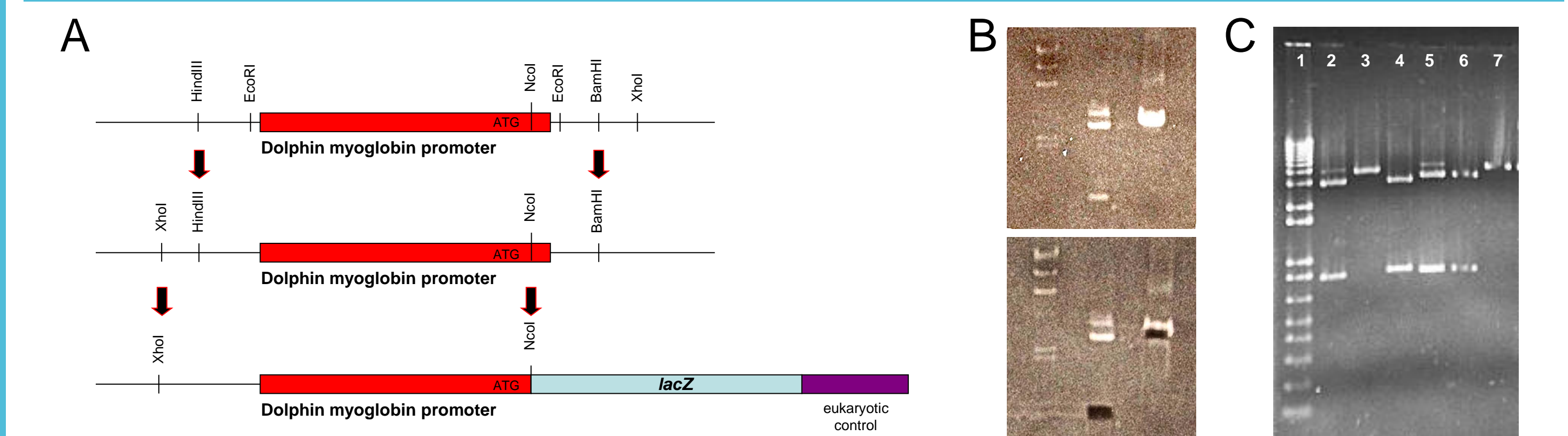


Figure 2: Subcloning of the dolphin promoter into pGEM.

A: Top: The source clone has the promoter DNA flanked by EcoRI sites; both XhoI and HindIII are 3' of the insert, whereas the cloning into the expression vector, p501blue requires that either HindIII or XhoI be 5' of the promoter. Center: Cutting the source vector with HindIII and BamHI allows the promoter to be moved into pGEM-11. Bottom: The pGEM clone can be used to move the promoter into p501blue using XhoI and NcoI.

B: Gel purification of the dolphin promoter fragment and the cut pGEM vector.

C: Restriction digestion of the pGEM subclones. Depending on where the restriction enzymes, HindIII, BamHI and XhoI are positioned on the plasmid determines the presence of the band on the electrophoretic gel. The top row of bands represent the entire vector. The lower bands represent the pieces cut by the restriction enzymes. The bands that appear in lanes 2, 4, 5, and 6 appear because the distance between the restriction enzymes are large enough to produce a band that can be seen. In lanes 3 and 7 the bands are present but not large enough to be seen.

References

- (1) Baker, C.S. and S.R. Palumbi (1994). Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. *Mol Biol Evol.* 11, 426-435.
- (2) Dalebout, M. L., C. S. Baker, V. G. Cockcroft, J. G. Mead, and T. K. Yamada (2004). A comprehensive molecular taxonomy of beaked whales (Cetacea: Ziphiidae) using a validated mitochondrial and nuclear DNA database. *Journal of Heredity*, 95, 459-473.
- (3) Iwanami, K., Mita, H., Yamamoto, Y., Fujise, Y., Yamada, T., & Suzuki, T. (2006). cDNA-derived amino acid sequences of myoglobins from nine species of whales and dolphins. *Comparative Biochemistry and Physiology Part B Biochemistry & Molecular Biology*, 145(2), 249-256.
- (4) Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.
- (5) <http://www.cebl.auckland.ac.nz:9000/page/whales/title>
- (6) Sambrook, J and D. Russel (2001). *Molecular Cloning: A Laboratory Manual*. CSHL Press.