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

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**Introduction**

Diving mammals have the myoglobin in their muscles of any animal. 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 near the goal of exploring the activity of the dolphin myoglobin promoter in muscle cells growing in culture.

**Materials and Methods**

Polymerase chain reaction: PCR was done using Invitrogen Taq PCR enzyme on ~100 ng DNA, as recommended by the manufacturer. Primers used were:

- Dlp4R: 5' - TGTAAAACGACGGCCAGTACCCAAAGCTGRARTTCTA  -3'
- Dlp1.5F: 5' - CCACAGTACTATGTCCGTATT  -3'
- Dlp5R: 5' - TACTACATAATATGCATGCTCTTACATATTATATCCCCTTCAATTTCATCCCCACTGTATCCTATGGTC  -3'
- Dlp10F: 5' - CACAGTACTACGTCAGTATTAAAAGTAATTTGTTTTAAAAACATTTTACCGTACACATTACATACAC  -3'

The bands that appear in lanes 2, 4, 5, and 6 represent the pieces cut by the restriction enzymes. The bands that appear in lanes 4 and 5 were sent to Laragen for purification and sequencing.

**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.

We used the PCR to isolate sufficient quantities of mt DNA for sequence analysis. Four primers were used, 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 rightmost lanes were deemed adequate for sequence analysis.

The resulting DNA sequence was analyzed using a Neighbor-Joining algorithm as implemented by Witness For The Whales 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 functionality 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 plasmid DNA from e. coli.

The promoter fragment will direct the synthesis of the enzyme [beta]galactosidase under the control of the dolphin promoter. [beta]-galactosidase activity can then be assayed as a measure of the activity of the promoter (Figure 2, left, bottom).

However, because 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-T as an intermediate vector to solve this problem. In order to ensure that the resulting clones were in the correct orientation within the pGEM plasmid, 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 7.1x10^6 colonies per µg DNA. The second control tested how complete the digestion of the pGEM was, by transforming the enzyme-generated pGEM without any ligation: the "cut" pGEM gave a transformation frequency of 6.2x10^6 colonies 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 with the gel-purified promoter piece gave a transformation efficiency of 1.9x10^6 colonies per 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 demonstrate this by purifying several of the "positive" clones and restriction digesting them. The result from three positive clones is shown in Figure 2. All three show a restriction enzyme digestion pattern different from either the source vector, or the cut pGEM. This is the vector, and indicative of a successful subcloning, as schematically shown 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 swimming. 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 cetaceans, and not from 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 [beta]-galactosidase gene in response to transcriptional control by the dolphin myoglobin promoter region, in mammalian cells in culture. The [beta]-galactosidase is of no intrinsic interest, but will serve simply as a "reporter" for the activity of the promoter. The activity of the [beta]-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 cell culture in mammalian has been a valuable learning experience.

**References**


**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.