# Determining the expression patterns of transcription factor MRF4 in developing *Xenopus laevis* embryos.

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**Abstract**

The development of the vertebrate embryo requires the coordinated differentiation of several different types of cells. This process is accomplished through the use of transcription factors, which bind to DNA and regulate the transcription of genes. By regulating the expression of specialized genes in response to cues, the embryonic cells will differentiate in the correct pattern to form a viable zygote. MRF4 is a transcription factor in the myogenic regulatory factor (MRF) family, which is known to trigger the differentiation of embryonic somites into myoctes. When using the African clawed frog (*Xenopus laevis*) as a model organism, MRF4 is found in the nucleus of the myocytes. However, MRF4 it is also found the cytoplasm of cells fated to become part of the eyes, brain, and nervous system. The expression patterns of MRF4 protein and mRNA throughout the entire *X. laevis* development cycle in muscle cells and nervous system cells are unknown.

**Project Goals**

The proposed project has two goals. The first goal is to determine the transcription pattern of MRF4 mRNA in the *X. laevis* embryo over the course of early embryo development. The second goal is to determine the expression pattern of MRF4 protein in the *X. laevis* embryo and see how it compares to the MRF4 mRNA transcription data. The expression of MRF4 protein will be studied though immunohistochemistry experiments, using labeled antibodies that can selectively reveal MRF4 protein either in the nucleus or cytoplasm. An in situ hybridization experiment will detect MRF4 mRNA transcription in the developing embryo. We hypothesize that MRF4 mRNA is transcribed in both muscle and nervous system tissue during development, but that the MRF4 protein will not be expressed in the same way for muscle and nervous system tissue.

**Introduction**

The African clawed frog, *Xenopus laevis*, is an important model organism for embryological research, owing to its large and easy-to manipulate embryo. The development process from zygote to tadpole is divided into 46 stages in Nieuwkoop and Faber's "Normal Table of *Xenopus laevis* (Daudin)", which is used as the standard reference for *X. laevis* development. The embryos respond well to molecular biology techniques, and there are established protocols for studying gene expression during embryo development.

A transcription factor is a protein that binds to a specific DNA sequence to influence the transcription of a gene into mRNA. They are made up of two components: a DNA-binding domain that recognizes a DNA sequence, and an active domain that interacts with other proteins. A given transcription factor will act as either an activator by up-regulating transcription, or a repressor by down-regulating transcription of an adjacent gene. There are several ways the active domain of a transcription factor can alter gene transcription: it can stabilize or inhibit the formation of the RNA polymerase complex which initiates transcription, manipulate the chromatin structure by acetylating or de-acetylating histones, or recruit additional co-activators

or co-repressors to the DNA-transcription factor complex. Through these mechanisms the cell can control the expression of genes.

The embryonic development of vertebrates is a complex process coordinated through altered patterns of gene expression and cell differentiation throughout the embryo. Transcription factors in the embryonic cells can respond to cues during the development process, expressing or silencing particular genes that will determine the fate of the embryonic cell. In particular, the development of muscle cells depends on a suite of transcription factors in the myogenic regulatory factors (MRF) family (Sabourin & Rudnicki 2000). There are four MRF proteins: MRF4, MyoD, Myf5, and myogenin (Hinterberger 2010). The presence of MRF4 triggers the differentiation of somites (Gaspera *et al.* 2006), which are undifferentiated regions of embryonic cells, into muscle cells (myocytes).

There has been some work done with *Xenopus laevis* embryos as a model organism for MRF expression. Two gene homologs for MRF4 have been identified in *Xenopus*, and their expression patterns during embryogenesis have been analyzed (Becker *et al.* 2003). MRF4 RNA is known to accumulate at a high level after birth and remain at high concentrations in the nuclei of adult cells (Rescan 2001).

The regulation of MRF gene transcription and its ability to bind to DNA as a transcription factor are known. The mRNA encoding the MRF4 protein itself leaves the nucleus and is translated in the cytoplasm. Translational modifications in the cytoplasm finish the MRF4 protein and allow it to re-enter the nucleus to act on DNA. What effect a transcription factor, which is designed to act when bound to DNA, can have in the cytoplasm is unknown.

Recent work in Dr. Hinterberger's lab at UAA has shown the presence of MRF4 mRNA in cells fated to become part of the nervous system, head mesenchyme, and eyes. This raises questions about the exclusivity of MRF4 as a transcription factor for developing muscles. The developing neural cells appear to not contain MRF4 protein in their nucleus; it is present only in the cytoplasm. The proposed study will test investigate the details of MRF4 mRNA and protein expression in the *X. laevis* nervous system throughout the embryonic development cycle. The expression of MRF4 mRNA outside of embryo somites needs to be confirmed, and the location of MRF4 protein during development will be examined. If MRF4 protein is found in the cytoplasm of non-somitic cells, then it might be part of an unknown mechanism in the differentiation of neural cells. Other work has revealed that over-expression of other MRFs lead to changes in brain morphology (Ludolph *et al.* 1994), lending support to this hypothesis.

**Project Design**

**Specific Aim #1: Determine the transcription patterns of MRF4 mRNA over the course of early *X. laevis* embryo development using in situ hybridization.**

The *X. laevis* embryos will be monitored and allowed to develop until they reach the development stage required for the project. The comparisons that can be made between MRF4

expression in the early developmental stages are a key part of the proposed project. Embryos can be observed and matched to the illustrations published in Nieuwkoop and Faber's "Normal Table of *Xenopus laevis* (Daudin)", providing a reliable way to terminate development and collect experimental data at the developmental stages of interest. Embryos at every second stage between stages 12 and 32 will be examined, giving an experimental set of 10 embryos.

The first part of the proposed project will be the in situ hybridization of MRF4 mRNA with a digoxigenin-labeled RNA probe. The goal of this experiment is to determine where and when the MRF4 gene is expressed during *Xenopus* embryo development. The embryos are prepared for this procedure by removing the vitelline membranes after proteinase K treatment, then being fixed with MEMFA. After rehydration, proteinase K is used again to increase the permeability of embryo cell membranes. Hybridization of the mRNA probe to MRF4 mRNA will require washes of ethanolamine, acetic anhydride, and hybridization buffers before the probe can be introduced and bound to mRNA in the embryonic cells. After hybridization, the digoxigenin in the probe can be visualized by incubating embryos with alkaline-phosphatase linked antibodies that are specific for digoxigenin. The antibodies will be stained, and the embryo embedded in agarose medium where it can be sectioned using a vibratome. The sections will then be imaged to determine the patterns of MRF4 gene expression throughout the development cycle.

**Specific Aim #2: Determine the expression of MRF4 protein in the cytoplasm and nucleus of somitic cells compared non-somitic cells using immunohistochemistry.**

*X. laevis* embryos at different stages of development will be washed and incubated with a fixative (a solution of MOPS, EGTA, $MgSO_4$, and Paraform, known as MEMFA) to fix the MRF4 antigen in place. Afterwards, embryos will be bleached with a bleaching solution that contains hydrogen peroxide to increase visibility of the stain. After the bleaching procedure, embryos must be rehydrated and then incubated in a solution of phosphate buffered saline with tritone (PBT). The prepared embryo will receive the first antibody, called the primary antibody. It attaches to antigen, in this case the MRF4 protein. After this, the secondary antibody is washed over the embryo. The secondary antibody is coupled to the horseradish peroxidase enzyme (HRP). Staining is done with a solution of 3,3'-diaminobenzidine (DAB) that reacts with the HRP protein, staining the complex brown. Thus, the complex made by the primary antibody, the secondary antibody, and the MRF4 protein will be visible. The staining pattern reveals the areas where MRF4 protein is expressed in the embryo. Stained embryos will be sectioned and imaged so the expression of cytoplasmic and nuclear MRF4 protein can be studied in somitic and non-somitic cells throughout the different embryonic development stages. The final result of the immunohistochemistry experiment will be a collection of images detailing the production of MRF4 protein during development.

**Anticipated Results**

The data from the two experiments outlined above will be in the form of images of sectioned embryos, stained for either MRF4 mRNA or protein, taken over the embryo's development. Comparisons between the two data sets will yield qualitative results showing where MRF4 is expressed on two levels: inside specific cells, and in the tissues of the embryo as a whole. The in situ hybridization experiments will show relative changes in mRNA transcription as the embryo develops, giving insight into the function of MRF4 during the differentiation of the nervous system. Fluctuations in the relative amount of mRNA expressed or changes in transcription in muscle and nervous tissues over the development cycle will address the hypothesis that MRF4 is used throughout the development of the nervous system. Examining the whole in situ hybridization data set will also reveal whether MRF4 is present throughout the entire process of nervous system differentiation, expressed at the beginning, or only expressed at the end of the process. The in situ hybridization data will also confirm that MRF4 mRNA is expressed in non-somitic cells.

Analysis of the immunohistochemistry dataset will likewise give qualitative results about the expression of MRF4 protein. The intracellular presence of MRF4 protein is stressed here, and the two subsets of data, one for nuclear proteins, the other for cytoplasmic proteins, will show whether MRF4 protein does have an effect on differentiation when in the cytoplasm. It is not known whether MRF4 protein is actually expressed outside of somites in the *X. laevis* embryo. If it is, the data will also reveal where the protein is expressed in non-somitic cells. The choice to use two different antibodies that target cytoplasmic and nuclear proteins will answer the question of whether MRF4 is acting as a transcription factor or if it has an unspecified function in the cytoplasm of nervous system cells. In the event of no detectable MRF4 protein expression outside of somites, a comparison between the two subsets of images (protein expression vs. mRNA transcription) could point to a mechanism where MRF4 mRNA, but not protein, has an unknown effect on the differentiation of the nervous system.

Ultimately, the results of the two experiments will further efforts to determine what role the MRF4 protein plays in each cell type. Additional work on this subject might use protocols such as real-time PCR to give quantifiable measurements of the levels of mRNA expression of the MRF4 gene. Other investigations could manipulate the levels of the MRF4 gene that are expressed through gene knockdown experiments. An attempt to find the molecular mechanism of action that the MRF4 protein uses in the cytoplasm could be in order if the proposed study shows that MRF4 is involved in that process.

**Budget**

The proposed project will be done in Dr. Tim Hinterberger's lab, under the supervision of Dr. Caroline Wilson, in the ConocoPhillips science building. The proposed experiments involve the use of living vertebrates, but Dr. Hinterberger's current IACUC permissions will cover the proposed project. There are some pieces of equipment there such as microscopes and dissection

tools that can be used. However, many items used in the proposed project may only be used once before being contaminated, or are consumed during the experimental protocols, and they are listed below.

**Antibodies for immunohistochemistry and in situ hybridization**
The use of these antibodies is critical to visualize the expression of MRF4 protein and mRNA in *X. laevis* embryos.

Two polyclonal antibodies: $500
Digoxigenin antibodies: $250

**Reagents for immunohistochemistry and in situ hybridization**
These reagents are required for the immunohistochemistry and in situ hybridization protocols.

Solutions: $500
PBS
PBT
PTw
MEMFA
Methanol
Hydrogen peroxide
Commercial hybridization buffer

Reagents: $250
DAB
BB/BA
Proteinase K
RNAse
Triethanolamine
Acetic anhydride

**Histology supplies**
These are disposable supplies needed to section and handle the *X. laevis* embryos.

Supplies: $250
Razor blades
Slides/coverslips
Slide holders

**Project References**

Becker, C., Gaspera, B., Guyot, M., Donsez, E., Armand, A., Charbonnier, F., & Launay, T. (2003). Expression of MRF4 Protein in Adult and in Regenerating Muscles in Xenopus. *Developmental Dynamics, 227*, 445-449.

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Hinterberger, T. J. (2010). A conserved MRF4 promoter drives transgenic expression in Xenopus embryonic somites and adult muscle. *International Journal of Developmental Biology, 54*, 617-625.

Rescan, P. Y. (2001). Regulation and functions of myogenic regulatory factors in lower vertebrates. *Comparative Biochemistry and Physiology Part B, 130*, 1-12.

Sabourin, L. A., & Rudnicki, M. A. (2000). The molecular regulation of myogenesis. *Clinical Genetics*, *57*, 16-25.

Ludolph, D. C., Neff, A. W., Mescher, A. L., Malacinski, G. M., Parker, M. A., & Smith, R. C. (1994). Overexpression of XMyoD or XMyf5 in Xenopus embryos induces the formation of enlarged myotomes through recruitment of cells of nonsomitic lineage. *Developmental Biology*, *166*(1), 18-33.

**Project Timeline**

- Early January, 2012: Learn sectioning techniques and how to handle embryos under Dr. Wilson, and order supplies for in situ hybridization and immunohistochemistry experiments.
- January 23, 2012: Begin in situ hybridization experiments.
- February 20, 2012: Begin immunohistochemistry experiments.
- March 19, 2012: All lab work to be finished. Review and begin analysis of data set.
- Mid-April 2012 – Presentation at the Undergraduate Research and Discovery Symposium
- May 15, 2012 – Expenditure deadline
- May 30, 2012 – Final written report deadline