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Expression of *MRF4* During Early *Xenopus* Development

Abstract:

MRF4, Myf5, MyoD, and myogenin, the myogenic regulatory factors (MRFs), are a family of proteins expressed during skeletal muscle development, growth, and regeneration. Although MRF4 is known to function in the maintenance of the differentiated muscle cell type and is expressed during embryonic myogenesis, its role is incompletely understood. Current findings of *MRF4* expression in Dr. Hinterberger's lab using *Xenopus laevis*, has led us to believe that the gene expression is not restricted to the myogenic cells. To further explore the expression of *Xenopus MRF4*, I will perform additional in situ hybridization on whole embryos and sectioned tissues over a range of stages to determine cell specificity within the brain, eyes, brachial arches, otic vesicles, and head mesoderm. I will utilize different probe sequences coding for *MRF4* as a control for non-specific probe binding. I will determine any artifacts within the whole-mount in situ hybridization specimens by comparing them to specimens sectioned prior to in situ hybridization. The findings will open doors to future studies of the transcriptional regulation of the *MRF4* gene during differentiation of muscle and possibly other cell types. This will lead to better understanding of the regulatory function of MRFs and how genes are controlled.

Specific Aim #1: Determine tissue localization of specific cells of X. *laevis* that express *MRF4* in the head and neck at early neural through metamorphic stages by sectioning specimens of whole mount in situ hybridization.

Our recent findings of in situ hybridization of whole mount X. *laevis* embryos contradict what previous scientists have found. We plan to test and pin point the exact cells of X. *laevis* which express the *MRF4* gene. Furthermore, we will determine whether our results are consistent when probes that correspond to different regions of the *MRF4* gene sequence are used.

Specific Aim #2: Determine cell specificity in X. *laevis* expressing *MRF4* in the head and neck at neural through metamorphic stages by section in situ hybridization.

To assure that our probe is reaching and binding selectively to the target mRNA sequence of the specimen, we will perform section in situ hybridization. The intensity of staining will reveal whether any of the whole-mount staining is an artifact.

Projects Goals: The goal of this research project is to further understand the expression of *MRF4* during early *Xenopus* development. This genetic study will present pioneering information about the transcriptional and signaling mechanisms controlling the expression of the *MRF4* gene during specification and differentiation of muscle growth. By determining the specific cells expressing the *MRF4* gene, we will gain a better understanding of the regulatory functions of the MRF's. This information will allow for further studies on how all genes are controlled and regulated.

Introduction:

Identifying genes and the proteins they encode is a focal point of research in molecular biology. Recently, there are increasing studies of a family of muscle-specific basic helix-loop-helix (bHLH) transcription factors referred to as the myogenic regulatory factors (MRFs). The MRF family consists of four members–*Myf-5* (Braun et al., 1989), *MyoD* (Davis et al., 1987), *myogenin* (Edmondson and Olson, 1989; Wright et al., 1989), and *MRF4* (Rhodes and Konieczny, 1989; Braun et al., 1990; Miner and Wold, 1990). In vitro and in vivo studies document MRFs controlling gene expression during myoblast specification and throughout myofiber differentiation, maintenance, repair, regeneration, and hypertrophy; however, the individual role of each MRF during these specific cellular events is poorly understood. Studies by Rudnicki et al. (1992) and Wang et al. (1996) suggest the MRFs perform identical functions. However, differences in their spatial and temporal expression patterns (Smith et al., 1993) as well as differences in MRF null mutant (knockout) phenotypes (Braun et al., 1992; Flobe et al., 1996) indicate that the MRFs also exhibit slightly different roles during muscle differentiation (Pit et al. 1997). Some MRFs exhibit the ability to activate one another's expression as well as autoregulate their own function, suggesting a feedback regulatory network (Weintraub, H. 1993). One of the members of the myogenic regulatory factor family, MRF4, is usually thought of as being expressed at a time when the embryonic muscle cells already show many differentiated features, implying that MRF4 is not involved in the commitment of muscle cells. For many years MyoD and Myf5 classified as the main factors for myoblast determination. More recent studies with the absence of both *MyoD* and *Myf5* show that *MRF4* can function as a determination gene rather than simply a myofiber differentiation factor (Kassar-Duchossoy et al. 2004).

Lately, the myogenic regulatory networks of non-mammalian vertebrate species are receiving more attention. Studies show significant differences between rodents and *Xenopus* indicating that the *MRF* gene regulatory network must function differently in *Xenopus* than it does in mammals. Analysis of gene expression patterns in *Xenopus laevis* has revealed that the MRFs are expressed exclusively in myogenic tissues (Hinterberger et al., 1991; Ott et al., 1991; Smith et al., 1993). In addition to the unique timing of *MRF* gene expression during development, immunohistochemical staining has shown that the *MRF* genes also exhibit unique differences in spatial expression patterns (Smith et al., 1994). Specifically, the embryonic expression of *MRF4* in X. *laevis* has been described as restricted to the myotome (Gaspera, D.B. et al. 2006). Recently however, Dr. Hinterberger and I have seen expression of *MRF4* localized in the brain, eyes, branchial arches, otic vesicles, and head mesoderm through our own sectioning of whole mount in situ hybridized specimens. This new finding sheds light on how the *MRF4* gene may play a role in neural development in X. *laevis*. Under the guidance of Dr. Hinterberger, I will further investigate this discovery by determining cell specificity in X. *laevis* expressing *MRF4* in the head and neck at neural through metamorphic stages by sectioning of whole mount in situ hybridization as well as section in situ hybridization. For many purposes, whole mount in situ hybridization is sufficient, but the process of sectioning embryos afterwards can result in a loss of signal intensity and often times there is the possibility of probe binding

non-specifically to the embryo, trapping of the probe, and external surface penetration. To avoid these artifacts, it is possible to perform in situ hybridization on embryo sections.

An extensive understanding of the expression of *MRF4* gene will provide new insight into the regulatory functions of the MRFs and the transcriptional and signaling mechanism that control their expression during the specific and differentiation of muscle progenitors.

Research Design:

Specific Aim #1: Determine tissue localization of specific cells of X. *laevis* that express *MRF4* in the head and neck at early neural through metamorphic stages by sectioning specimens of whole mount in situ hybridization.

The whole mount in situ hybridization steps will be performed according to the procedures in use in Dr. Hinterberger's lab. We will first execute artificial fertilization with eggs retrieved from a female *Xenopus* and sperm extracted from a male *Xenopus*. After fertilization, the eggs will be allowed to develop to different stages. We will fix the embryos with formaldehyde, and divide them into batches each of which will receive a different probe. Three probes are currently available in the lab: one is for the full-length MRF4 sequence, one is for nucleotides 1-230, and one is for nucleotides 735-963. After the proper washes, treatments, pre-hybridization, hybridization, pre-incubation, antibody incubation, and BM Purple color reaction have been performed, we will fix the embryos in Bouin's fixative. The embryos will then be bleached to remove any melanin pigment. A cryostat will be used to section the whole embryos. Embryos will first be prepared for sectioning and then embedded in Tissue-Tek medium and frozen at -25 degrees Celsius. Sections will be cut between 30-50 mm and mounted on polylysine coated slides. Sections will be examined under a microscope for expression of *MRF4* in individual cells.

Specific Aim #2: Determine cell specificity in X. *laevis* expressing *MRF4* in the head and neck at neural through metamorphic stages by section in situ hybridization.

Separate in situ hybridization on tissue sections will be performed. Embryos will be embedded in the Tissue-Tek medium, frozen at -25 degrees Celsius, cut with a cryostat between 30-50 mm, and mounted on polylysine coated slides. Section will air dry for no longer than 3 hours to prevent degradation of mRNA. Sections will be fixed in 4% paraformaldehyde and washed accordingly with acetic anhydride and PBS. Sections will be pre-hybridized with hybridization buffer overnight in a chamber humidified with 5x SSC. The pre-hybridization solution is then sucked off and replaced by hybridization mixture. The sections will be covered with siliconized coverslips and sealed with rubber cement, which helps to maintain the concentration of salt and formamide in the hybridization buffer. The hybridization is done overnight at 72 degrees Celsius and the cover slips are allowed to slide off. Next the section will be washed with .2x SSC, rinsed with buffer, blocked in buffer and equilibrated in buffer. To perform the color reaction, slides will be place upside-down in a tray with BM Purple reagent.

The slides will sit in the dark until they reach the desired intensity then rinsed with buffer. Slides will be examined under a microscope.

After we determine *MRF4* expression in the sectioned whole mount in situ hybridization specimens and section in situ hybridization specimens, we will identify any variation among the two samples. The slides will be placed under a microscope for further analysis. We will locate the specific tissue that expresses the *MRF4* gene and distinguish where the whole mount in situ hybridization may have faulted.

In the case of section in situ hybridization, probe penetration through the embryo and probe trapping in internal spaces are not an issue, and any surface-binding artifacts would be located differently. Through these steps, I can describe the true patterns of gene expression in the section in situ hybridization and sections of whole mount in situ hybridization of the X. *laevis* and validate any artifacts. Also, by utilizing probes corresponding to regions of the cDNA, I can confirm that the probe is binding genuinely.

Anticipated Results:

Previous research (Della Gaspera et al., 2006) showed that *MRF4* expression is found in the myotome and somites of *Xenopus* embryos. However, in our lab we have findings of *MRF4* expression in the brain, eyes, branchial arches, otic vesicles, and head mesoderm. This leads me to believe that further experiments of in situ hybridization on *Xenopus* embryos will result with consistent findings of *MRF4* expression in not only the somites and myotome, but in the tissues of the brain, eyes, branchial arches, otic vesicles, and head mesoderm. This will allow me to track *MRF4* expression in great detail in the myotomes as well. I hypothesize that *MRF4* plays the role of a determination gene, like the other members *MyoD, Myf5*, and *myogenin*, and is involved in the sequence of cellular events leading to the formation of adult muscle that occurs in animals. I expect to see intensified expression of *MRF4* in the muscle cells as the embryos develop.

It is important that the probe reaches the mRNA of the target gene located in our sample and binds selectively. With section in situ hybridization, the obstacle of the probe infiltrating the membrane is greatly reduced. Furthermore, the issue of probe trapping as well as binding of the probe to non-specific cell components or other closely related mRNA sequences leading to coloration within tissues that normally would not express *MRF4* is eliminated. We can confidently say that the hybridization reaction is specific and that the probe is in fact binding selectively to the *MRF4* sequence.

To gain a better understanding of the expression of *MRF4* during cellular maturation is exciting. There is little knowledge about this gene and distinguishing its individual role during cellular events such as myofiber specification, maintenance, repair, hypertrophy and regeneration

would be beneficial. I believe this research project is a stepping stone to deciphering how genes are controlled and regulated and will open doors to future studies.

Proposed Budget:

-*Xenopus laevis*, food, etc.: $500

-Sectioning supplies (embedding medium, disposable blades, etc.): $750

-Hybridization chamber: $500

-In situ hybridization probe synthesis buffers (2 kits): $700

-In situ hybridization reagents: $1000

-Sterile plastic/glass ware, filters, vials, slides, cover slips: $1000

Total=$4,450

Budget Justification:

All of the above materials are essential to the experiments. This should be sufficient funding for our project for at least a year. Some reagents and supplies are already on hand in Dr. Hinterberger's lab.

References:

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Timeline:

November 2008: Enter lab and practice in situ hybridization of whole mount embryos and tissue section in situ hybridization

December-January 2009: Carry out Specific Aim #1 of the project. This time frame will vary depending on the results obtained and further trials that may be needed

February 2009: Carry out Specific Aim #2 of the project. This time frame will vary depending on the results obtained and further trials that may be needed.

March 2009: Carry out Specific Aims #3 and #4 of the project. Analyze data and work on completing my final report of the project. Prepare presentation for Undergraduate Research Symposium.

April 2009: Present research at Undergraduate Research Symposium and also AHI members.

Academic Achievement:

-Recipient of UA Scholarship

-Recipient of Key Club International Scholarship

-Dean's list at UAA

-Member of the University Honors Program

-Member of National Society of Collegiate Scholars

-Overall 3.52 GPA

-Experienced background in chemistry and biology

-Experienced background in individual research