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Gruber, Margaret, "The expression of the CRF1 gene in mouse embryonic development" (1999). *Presidential Scholars Theses (1990 – 2006)*. 75.
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The Expression of the CRF1 Gene in Mouse Embryonic Development

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Presidential Scholar Thesis

Class of 1999

Abstract

A mouse gene cloned in the laboratory of James and Olivia Smith at Baylor College of Medicine was found to be involved in motor function. This gene was named CRF1 for *C*1q-*R*elated *F*actor 1, and using in situ hybridization, it was found to be expressed mainly in the brainstem of adult mice. There is a gene similar to the CRF1 gene in humans. By determining this gene's role in mouse development, more will be known about the development of human motor function.

To determine the stage of embryonic development at which the expression of this gene begins, a series of in situ hybridization experiments were performed on saggital sections of embryos at 11.5, 12.5, 13.5, 14.5, and 16.5 days of development using a dig-labeled riboprobe made from CRF template DNA. Two types of substrate detection, NBT/BCIP and fluorescence, were used to visualize the probe. In several experiments, no specific hybridization occurred. In the remainder of the experiments, the hybridization that occurred in specific tissues was not repeatable and therefore unreliable.

Introduction

In recent years, we have been able to discover many new genes and their functions. Many times genes are discovered in animals that are related to genes in humans. We are very interested in finding out what these new genes do in humans and what abnormalities and diseases they may cause. The first step in discovering these genes' roles in human life is finding out what the related gene does in animals' lives.

Many genes have been found that play a role in development. These genes determine an organism's body type, musculature, physical characteristics, and so forth. If

these genes have a role in human development, we will begin to understand how we are formed into such complex beings.

The gene that the Smith lab discovered is one such gene. The CRF1 gene was found in mice, and humans have a gene that is related to it. This gene was determined to be important in motor function of adult mice. Without it, mice would not be physically capable animals. The implications on human life are enormous. By discovering when and where this gene is expressed in humans, we may be able to learn more about our own motor abilities and how our muscles and nerves work together to enable us to perform complex motor tasks.

By first determining when and where this gene is expressed in mice, we can begin to answer some of these questions about humans. A laboratory technique called in situ hybridization allows us to determine this. In situ hybridization, from Latin for "in site", allows us to determine where in the body a particular gene is expressed. A probe is made of genetic material that is identical or similar to the gene in question. It is usually labeled with an enzyme or radioactivity that is easily observed. By then applying the probe to a tissue, it is possible to see if the gene is expressed at that site. If a series of tissues are used that are at different stages of development, it would also be possible to determine when the gene is expressed--whether during embryonic development or after birth.

Materials and Methods

Embryo Preparation

The embryos must be prepared so they stay fresh and can be used in many experiments. The tissue is embedded in paraffin wax so that it remains intact when

sliced. Thin slices are made through each embryo so that the solutions applied in the experiments have contact with cells and have access to the content of the nuclei.

Mouse embryos at various stages of development were obtained. These embryos were 11.5, 12.5, 13.5, 14.5, 16.5 and 18.5 days old and had been dissected in cold, sterile phosphate buffered saline (PBS) and placed in 4% paraformaldehyde in PBS. The embryos remained in this solution at 4°C until ready for fixation. The embryos were fixed at 4°C by first replacing the paraformaldehyde with enough 0.9% sodium chloride (NaCl) to cover the entire embryo. The length of time the embryos remained in this solution was determined by its stage of development and therefore size. This information is outlined in Table 1 below for each embryo stage and each solution. The embryos were then transferred to a solution of 30% ethanol (EtOH) and 0.9% NaCl. This was replaced with a solution of 50% EtOH and 0.9% NaCl. Finally, the embryos were placed in 70%, 90% and 100% EtOH.

The embryos were then prepared for embedding in paraffin. Each embryo was covered with a 1:1 solution of 100% EtOH and xylene for 45 minutes at room temperature. The solution was then replaced with xylene for 1 hour, also at room temperature. A 1:1 solution of xylene and paraffin was prepared. The embryos were covered with this solution and allowed to sit for 45 minutes at room temperature. The embryos were then placed in a Tissue Tek cassette and covered with paraffin. The cassette was placed in a 60°C oven for 1 hour. This was repeated with fresh paraffin 2 more times for 1 hour each time. The paraffin was removed and the embryo was covered with fresh paraffin. The cassette was allowed to sit at room temperature overnight. At

that point, the paraffin had hardened and the embryos could be sectioned or stored indefinitely. Five-micron thick sections of embryos were used in the following experiments. These sections were prepared using a microtome and were placed on Probe-On Plus slides. These slides were then stored at 4°C until use.

Probe Preparation

Probes contain a nucleic acid sequence identical or similar to the gene in question. They are labeled radioactively or enzymatically for easy identification later. They are applied to a tissue and will hybridize with, or bind to, areas expressing a particular gene.

A master mix for sense and antisense probes was prepared using the materials and quantities outlined in Table 2. The master mixes were then separated into 4 Eppendorf tubes for both sense and antisense. These 8 tubes were then loaded into the PCR machine and run using the program outlines in Table 3.

An agarose gel was prepared with 50ml TAE, 0.5 g agarose and 0.5µl ethidium bromide. The PCR products were loaded on the gel after adding a loading buffer (1µl buffer: 10µl sample). A 1Kb ladder was also loaded on the gel. The gel was then run for 40 minutes at 100 volts.

The QIAquick Gel Extraction Kit and protocol was used to extract the DNA from the gel. The DNA fragments were cut out of the gel with a scalpel and weighed. Each section weighed approximately 700mg. The sections were split up into several colorless Eppendorf tubes. Three volumes (2.1ml total) of Buffer QX1 from the kit was added to

each colorless tube. The tubes were then placed in a 50°C water bath and vortexed occasionally until the gel had dissolved.

One volume of isopropanol was added to each sample and mixed. A spin column was then placed in a 2-ml collection tube and 800μl of the sample was added to the column. The column was then centrifuged for 1 minute. Repeated centrifugations were done until the entire sample had been applied to the column. After each, the flow-through was discarded. To the column, 750μl of Buffer PE from the kit was added and centrifuged for 1 minute. The flow-through was discarded and the column was spun again for another minute to ensure all liquid was removed. The column was then transferred to a 1.5ml microfuge tube and 50μl of DEPC water was added to the center of the column. The column was then centrifuged for 1 minute to elute the DNA. The concentration of the DNA in each sample was determined using a fluorometer. The antisense sample was determined to contain 3.4μg of DNA while the sense sample contained 2.4μg of DNA.

To reprecipitate the DNA, 1/10 volume of 3M NaOAc and 2.5 volume of EtOH was added to each sample. The tubes were then stored at -70°C for 30 minutes and centrifuged for 15 minutes at 4°C. The supernatant was poured off and the pellet was washed with 1ml of 70% EtOH and centrifuged for 10 minutes at 4°C. The pellet was vacuum-dried and resuspended in DEPC water to get a final concentration of 500ng/μl (4.8μl water for sense and 6.8μl water for antisense).

The DNA sample was then transcribed into a dig-labeled riboprobe using the components outlined in Table 4. The tubes were incubated for 3 hours at 37°C and to

each tube 1μl glycogen, 2μl 4M lithium chloride, and 60μl 100% EtOH were added. The tubes were then stored at -70°C for 30 minutes. The samples were centrifuged for 15 minutes at 4°C and the supernatant was poured off. The pellet was washed by adding 200μl EtOH and centrifuging for 10 minutes at 4°C. The supernatant was poured off and the pellet was vacuum-dried for 5 minutes. The pellet was resuspended in 50μl of DEPC water. A 1:100 dilution of each sample was prepared and the RNA concentration was calculated using a spectrophotometer. The sense probe had a concentration of 364ng/μl and the antisense probe had a concentration of 309ng/μl. The probes were then stored in small aliquots at -80°C.

In situ Hybridization with NBT Detection

In this technique, a probe is applied to a tissue and is visualized using a chemical that turns it brown or purple. The areas that bind the probe are therefore easy to see under a light microscope and show where the gene is being expressed.

The slides prepared earlier were removed from 4°C and allowed to come to room temperature. Two slides were selected from each of the 11.5, 12.5, and 13.5 day embryos and labeled. One slide from each age was used with the sense probe and one slide was used with the antisense probe. One extra slide was randomly selected to serve as the control and was labeled for no probe. These slides were soaked in xylene for 10 minutes, and fresh xylene was added for a second 10-minute soak. This removed the paraffin from the section.

The tissue was then rehydrated by soaking the slides in graded alcohol. A quick was performed with 100% EtOH. Fresh 100% EtOH was added and the slides were

soaked for 2 minutes. The excess EtOH was wiped from the slides and a Pap Pen was used to create a well around the tissue on each slide. The graded alcohol soaking was continued with 90% and 70% for 1 minute each. The slides were then washed in PBS. The slides were held here while the proteinase treatment and acetylation solutions were prepared.

A 25µg/ml solution of proteinase K was prepared by combining 1µl of the 25mg/ml stock proteinase K and 1ml of water. The acetylation solution (0.25% acetic anhydride and 0.1M triethanolamine pH 8.0) was prepared by combining 938µl triethanolamine, 70ml PBS, and 175µl acetic anhydride. The slides were soaked in 0.2M hydrochloric acid (HCl) for 10 minutes at room temperature and then 200µl of the proteinase K solution was added to each slide. This was allowed to sit for 10 minutes at room temperature. The slides were washed with PBS for 5 minutes and 0.9% NaCl for 5 minutes.

The tissue was then dehydrated by placing the slides in 30%, 50%, 70%, 80%, 95%, and 100% EtOH for 1 minute each. The slides were then allowed to air dry. The slides were incubated with 200µl prehybridization solution (50% formamide, 600mM NaCl, 10mM EDTA, 10mM Tris-HCl pH 7.5, 50µg/ml heparin, 10mM DTT, 10% PEG 8000, and 10X denhardt solution) for 2 hours in a 60°C moat. The excess was wiped off and 1600µl hybridization solution was prepared (200µl for each slide). The hybridization solution was the same as the prehybridization solution except it also contained 0.5mg/ml carrier DNA and 0.5mg/ml tRNA as well as the probe. For this experiment, a probe concentration of 3ng/µl was desired. To the volume of hybridization solution for the

sense slides (800µl total), 6.72µl of a sense probe prepared earlier was added. To the volume used for the antisense slides, 12.24µl of an antisense probe was added. These solutions were mixed and 200µl were added to each slide. The slides were incubated overnight in a 60°C slide moat.

The following day, the slides were washed in 5X SSC at 65°C for 5 minutes. The slides were then transferred to 2X SSC diluted with 50% formamide at 65°C for 30 minutes. A 10U RNAse T solution was made by combining 1ml 2X SSC and 0.1µl 100,000U RNAse T. Each slide was treated with 200µl of this solution and incubated for 1 hour at a 37°C slide moat.

The slides were washed with 2X SSC for 30 minutes at 65°C and then twice for 15 minutes each time with 0.2X SSC. A blocking solution was made with 20µl normal sheep serum, 3µl Triton X, and 977µl Buffer #1 (100mM Tris-HCl, 150mM NaCl, pH 7.5). Each slide was treated with 200µl of the blocking solution for 30 minutes at room temperature. The anti-digoxigenin solution was prepared by combining 977µl Buffer #1, 10µl normal sheep serum, 10µl sheep anti-dig-Alk-Phos, and 3µl Triton X. Each slide was treated with 200µl of this solution and incubated for 1 hour at room temperature. The slides were then rinsed with Buffer #1 twice and three times with Buffer #2 (100mM Tris-HCl, 100mM NaCl, 50mM MgCl, pH 9.5).

The probe was detected using a solution of NBT/BCIP. This was made by combining 2ml Buffer #2, 20µl levamisol, and 40µl NBT/BCIP. Each slide was treated with 200µl. The slides were placed in a sealed container covered in foil. This container

was placed in 4°C overnight for development. The following day, the slides were rinsed in distilled water and observed under a microscope to visualize the probe.

An additional experiment was performed using NBT detection using 13.5 day embryos to test new probes. This experiment followed the protocol outlines above with hybridization at 60°C, probe concentration of 3 and 5ng/µl, and antibody incubation at 4°C overnight. The slides were counterstained with hematoxylin (3ml in 50ml water) to provide a better contrast for visualization of the probe.

In situ Hybridization with Fluorescent Detection

In this technique, a probe is applied to a tissue and is visualized using a chemical that turns it green or yellow against a blue background. The areas that bind the probe are therefore easy to see under a fluorescent microscope and show where the gene is being expressed.

An experiment was performed using 11.5, 12.5, and 13.5 day embryos following the same protocol outlined above with the following changes. The probe concentration used was 3ng/µl for both sense and antisense slides. Incubation with anti-dig was done overnight at 4°C instead of 1 hour at room temperature. Also, fluorescent detection was used instead of NBT/BCIP.

After incubating with anti-dig, the slides were washed twice for 10 minutes each time in a solution of 100mM Tris-HCl and 100mM NaCl. The fluorescent detection solutions were obtained from the eLF-97 Detection Kit. This kit included a washing buffer, developing buffer, a substrate reagent, Additive #1 and Additive #2. A 1X

washing buffer was made from the 10X stock solution and used to wash the slides twice for 10 minutes each time.

A substrate solution was made by combining 2ml developing buffer, 200µl substrate reagent, 4µl Additive #1 and 4µl Additive #2. Each slide was treated with 200µl of this solution. The slides were placed in a sealed, darkened container and monitored throughout the day for development. When proper development had occurred, the slides were washed with 1X washing buffer twice and counterstained with Hoechst dye (30µl Hoechst in 30ml water) for 30 seconds.

A variety of additional experiments were carried out with minor protocol changes. An experiment was performed using 13.5 day embryos with probe concentrations of 1, 3, and 5ng/µl for both sense and antisense. Hybridization occurred at 60°C overnight, and antibody incubation occurred at 4°C overnight. The same protocol for fluorescent detection was used.

Another fluorescent experiment was performed to test new probes using 14.5 day embryos. The protocol outlined above was followed with hybridization at 60°C overnight, probe concentrations of 3 and 5ng/µl and antibody incubation at 4°C overnight.

A final fluorescent experiment was performed to test another set of new probes using 11.5, 12.5, 13.5, 14.5, and 16.5 day embryos. The same protocol was used with hybridization at 60°C overnight, probe concentration of 3.5ng/µl and antibody incubation for 1 hour at room temperature. The fluorescent detection substrate reagent was diluted (100µl used instead of 200µl) and incubated at room temperature overnight.

Results

In situ Hybridization with NBT Detection

Experiment 1: The hybridization appeared more specific on the 11.5 day embryo than on the 12.5 and 13.5 day embryos. The hybridization appeared to occur on the liver and the brain of the 11.5 day embryo on the antisense slides. A precipitate formed on top of the tissue of the 12.5 and 13.5 day embryos and no specific hybridization was noted.

Experiment 2: Hybridization appeared in two areas of the 13.5 day embryos for both probe concentrations (3ng/µl and 5ng/µl) on the antisense slides. Prior to counterstaining, these areas appeared to be in the brain and the intestines. After counterstaining, these areas of hybridization were much less apparent.

In situ Hybridization with Fluorescent Detection

Experiment 1: The hybridization that occurred with all embryos (11.5, 12.5, and 13.5 day) was nonspecific on the antisense slides. The substrate was visible in all areas of the tissue with uniform density.

Experiment 2: The probes appeared to be switched in this experiment. The sense slides, which should show no hybridization, showed nonspecific hybridization throughout the tissue. The antisense slide, which should show hybridization, showed none.

Experiment 3: Hybridization was noted along the vertebrae of the 14.5 day embryo on the antisense slides.

Experiment 4: Prior to counterstaining the slides with Hoechst dye, hybridization was apparent on the 11.5 and 12.5 day embryos on the antisense slides. After

counterstaining, this hybridization was undetectable. Autofluorescence was very apparent in the liver, however.

<u>Discussion</u>

The expression experiments were in the early stages at the time these were performed. These experiments required many protocol adjustments. Most of the hybridization that occurred was nonspecific. This may have been caused by a relatively low hybridization temperature of 60°C. If the hybridization temperature was raised, perhaps a more stringent hybridization would have occurred. The probes also did not appear to be functioning properly. The antisense probes should have given a positive signal and the sense probes should not have hybridized. On several occasions, the hybridization on the sense slides occurred in the same type of tissue as the antisense slides but with lesser intensity.

To try to determine where the problem was occurring, the probes were run on an agarose gel prior to use. When this was done, it was apparent that the probes were contaminated. This discovery led to the production of new probes, but each attempt was unsuccessful. After each new set of probes was made, the gel again showed that the probes were contaminated. Improper handling of the samples, a contaminated sample of template DNA, or improper primers for the PCR reaction could have caused this contamination.

The fluorescent technique for probe detection is not a good way to pinpoint the expression of a gene if no tissue has been ruled out. The technique is very sensitive and requires precise temperatures and concentrations. There were not any repeatable results

attained by this series of experiments. The organs that appeared to be expressing the gene in one experiment did not show expression in other experiments. No pattern was apparent regarding the role of this gene in development.

Another technique would be a better choice to narrow down the location of the gene to a region of the embryo. The use of radioactive probes would be a better technique to begin with. By performing autoradiography on the slides, it would be easier to narrow down the location of the gene to a region of the embryo, and perhaps it would be easier to determine the stage of development at which this gene is expressed.

Embryo Stage (Days)	11.5 to 15.5	16.0 to 18.5
0.9% NaCl	30 minutes	2 hours
30% EtOH/0.9% NaCl	90 minutes	2 hours
50% EtOH/0.9% NaCl	90 minutes	2 hours
70% EtOH	2 hours	≥12 hours
90% EtOH	2 hours	2 hours
100% EtOH	2 hours	5 hours

Table 1: The time required for each step of the fixation of mouse embryos. The embryos were covered with each solution and kept at 4°C for each step.

Material	Quantity (µl)
DEPC Water	162
Template DNA (1ng/µl)	4
10X PCR Buffer	20
MgCl2 (50mM)	6
10X dNTPs (10mM)	4
Primer 1 (50µM)	2
Primer 2 (50µM)	2
Taq Polymerase (5U/µl)	1.2

Table 2: The contents of a PCR master mix for sense and antisense probes. Primer 1 was 5' T7B and Primer 2 was 3' 47601 for the sense probe. Primer 1 was Ex1-F and Primer 2 was 4760-T7 for the antisense probe. All of these primers and the template DNA (of the CRF1 gene) were obtained from the laboratory stock.

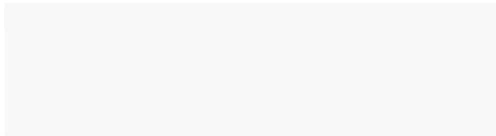
Step	Time
1. 95°C	3 minutes
2. 95°C	30 seconds
3. 60°C	30 seconds
4. 72°C	1 minute
5. Goto Step 2	29 more times
6. 72°C	10 minutes
7. 4°C	Hold up to 24 hours

Table 3: The PCR program used to amplify each sample of template DNA for the CRF1 gene.

Material	Quantity (μl)
5X Buffer	4
DTT (100mM)	2
5X dNTPs (25mM)	4
UTP (2.5mM)	2.6
Dig-UTP (5mM)	0.7
DNA sample (500ng/μl)	2
T7 Polymerase	1
RNAsin	1
DEPC Water	1.7

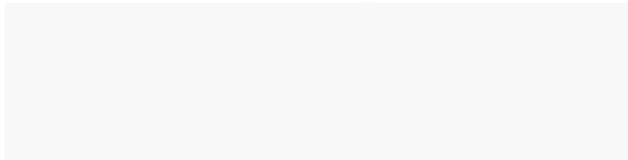
Table 4: The materials used to transcribe the DNA samples into dig-labeled riboprobes. These components were incubated at 37°C for 3 hours to facilitate the transcription process.

Approval



- excellent job!

Robert Seager
Professor, Biology Department
Faculty Advisor



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