IDENTIFICATION OF SENSITIVITY GENES INVOLVED IN TERATOGEN-INDUCED NEURAL TUBE DEFECTS

by

Blaine G. Hayes

Submitted to the Office of Honors Programs and Academic Scholorships Texas A&M University in partial fulfillment of the requirements for the 1998-99 UNIVERSITY HONORS UNDERGRADUATE RESEARCH PROGRAM

April 15, 1999

Approved as to style and content by:

Richard Finnell (Faculty Advisor)
Department of Veterinary Anatomy and Public Health

Susanna Finnell, Executive Director
Honors Program and Academic Scholorships

Fellows Group: Biology

Abstract

Identification of Sensitivity Genes Involved in Teratogen-Induced Neural Tube Defects

Blaine Gabrielle Hayes, (Dr. Richard Finnell)

University Undergraduate Fellow, 1998-99, Texas A&M University,

Department of Veterinary Anatomy and Public Health

Neural tube defects (NTDs) are common human congenital anomalies affecting 1 per 1000 liveborn infants in the United States. NTDs are medical conditions that result from improper formation of the brain and spinal cord. There is evidence to suggest that NTDs have a strong genetic component to their development. My project is designed to examine differential expression patterns of genes within the neural tube cells of inbred LM/Bc mouse fetuses following exposure to the known teratogenic agent, Valproic acid (VPA). In this project I am comparing gene expression patterns in both control and treated mice and attempting to identify those genes whose expression patterns have been significantly altered. My experimental methods include isolating total RNA from LM/Bc mouse neural tissue, reverse transcribing the RNA into cDNA and PCR amplifying the cDNA using a radioactively end labeled primer in order to visualize the amplified fragment. The labeled cDNA fragment is then loaded onto a denaturing gel and separated by electrophoresis. The gel is then exposed to film and examined. Differentially expressed cDNAs are then isolated from the gel and sequenced. The results of this project show the identification of differentially expressed genes that may regulate the occurrence of NTDs.

ACKNOWLEDGMENTS

I would like to extend my appreciation to Dr. Richard H. Finnell for the opportunity to commence my journey into the scientific community. As a result of his guidance, I have been able to develop new approaches to research and intellectual endeavors. Next, I wish to thank my co-workers and mentors in Dr. Finnell' s lab. I am particularly grateful for the help of Dr. Robert Barber for sharing his knowledge. With his positive attitude and support, I was able to be continually challenged by the research process and invigorated by its rewards. Finally, I wish to thank my parents Jim Hayes and Cathy Dickerson for giving me the understanding of perseverance and love.

TABLE OF CONTENTS

Page

LIST OF TABLES

Number *Page*

LIST OF FIGURES

Number *Page*

Chapter 1

I. INTRODUCTION

I.A Neural tube defects overview

Neural tube defects are common human congenital anomalies affecting 1-per1000 liveborn infants in the United States (Campbell et, al., 1986) making them amongst the most common of all human birth defects. NTDs occur when the neural tube, responsible for brain and spinal cord formation, fails to close properly. As a result, central nervous system cababilities are impeded and serious problems persist.

Unfortunately, the mechanism behind NTD formation is still not fully understood (Martin et al., 1983). There is evidence in support of a genetic component to NTD etiology, including the reoccuring incidence of NTDs within geographic or ethnic populations and amongst infants of parents with NTDs (Vacha, 1997). Both patterns suggest a clustering of NTD liability genes within families and populations and it turn the involvment of a genetic factor in the susceptibility and/or occurrence of NTDs.

In addition to a genetic component, a number of associations have been made correlating NTDs with various environmental conditions (Vacha, 1997). For an example, there have been reported increased rates of NTDs within populations exposed to chemical agents in the environment such as those in soft water (Elwood and Coldman, 1981). Additional environmental factors include maternal age, parity, social class, metabolic disease, occupational exposure and teratogenic drugs. All factors should be regarded as potentially

hazardous to the developing brain and have been shown to have adverse affects on offspring (Sever, 1995).

These NTD rate increases suggest an interaction between environmental factors and genes within affected individuals. The combined NTD etiology including both genetic and environmental components is known as the multifactorial model of inheritance (Carter and Evans 1973). Currently, it is the basis for most NTD research in the scientific community as well as the basis for my project.

I.A 1. Classification of neural tube defects

The term " neural tube defect" is often used to refer to congenital defects of the central nervous system (Campbell et al., 1986). Neural tube defects commonly occur when the embryonic neural tube, which ultimately forms the brain and spinal cord, fails to properly close during the first few weeks of development (Norman et al., 1995). As a result of this incomplete closure, the nerval tissue is exposed to the intra uterine environment. Neural tube malformations are usually divided into 3 types. The most common NTDs are anencephaly, spina bifida and encephaloceles (Copp et al., 1990).

1. **Anencephaly** is a NTD that has been subcatagorized into two different classifications.

   First, holo-anecephaly, is a severe defect as a result of the complete protrusion of neural tissue into the CSF. Individuals with this defect have a complete absence of brain tissue and are normally stillborn. The second, mero-anencephaly, is due to the partial absence of the brain tissue (Hunter, 1993).

2. **Spina bifida** may also be characterized based upon its association with overlying skin tissue or with an associated sac (spina bifida occulta or spina

bifida cystica) (Hunter, 1993). It the sac is present, the defect can then be further classified as to its contents. If it contains meninges and cerebral spinal fluid (CSF), it is referred to as a meningocele. By contrast, if the sac contains portions of the spinal cord or nerve fibers, the defect is classified as meningomyeloceles (Hunter, 1993).

3. The last type of defect with common clinical manifestations is known as **encephaloceles**. Cephaloceleous defects are characterized by the presence of an epithelial-covered sac, which normally forms the inner lining of the ventricles, protruding from the skull. If the sac contains brain tissue, it is classified as encephocele. If it does not contain brain tissue, it is referred to as a cranial meningocele (Hunter, 1993).

I.A.2. Prevalence of neural tube defects

Neural tube defects are major congenital anomalies of the Central Nervous System. Among malformations, they are the most common cause of prenatal death and are second only to congenital heart defects as a cause of infant mortality and account for approximately 15% of perinatal deaths in the United Kingdom (Copp et al., 1990). Studies have found that NTD prevalence rates vary between geographical sampling regions as well as ethnicity of the individuals residing within the region. For example, Japan has an average prevelance rate of (0.6/1000), while Hispanics in the United States have a prevenance rate of (1.00/1000) (Vacha, 1997). Prevalence of NTDs also varies across regions. In Great Britain there is a downward trend in rates from the Northwest to the Southeast (Vacha, 1997). In addition, family studies have shown clustering of NTDs within specific gene pools. The rates for

Sikhs, Ashkenazi Jews, and United States African Americans are consistently low regardless of geographical location (Vacha, 1997).

In a Los Angeles County hospital during a 3-month period in 1976 to 1977, 7 out of every 923 babies were born with NTDs, far exceeding the normal rate of one per 1000 (Vacha, 1997). A more recent cluster of NTDs occurred in Cameron County, Texas. During the month of April in 1991, three babies in Brownsville, Texas were born with anecephaly in a 36-hr period. This unusual high occurrence of anencephaly suggests a genetic/environmental component was affecting the Hispanic population (Sever, 1995).

Although neural tube defects affect a major portion of the world's population, they still remain poorly understood (Sever, 1995). Consequently, babies are still being affected, some even dying. This supports the need for continued research in attempt to understand the etiology of NTDs.

I.A.3. Multifactorial model

There is overwhelming evidence supporting a multifactorial etiology for NTDs. As previously discussed, NTDs are phenotypically heterogenic., involving both the combined action of multiple genes and environmental factors in establishing a range of susceptibility within individuals (Campbell et al., 1986). The multifactorial model can be conceptualized as a liability distribution curve within a population. *See Figure 1.1.* One tail of the curve represents the population's background prevalence of NTDs implying a genetic

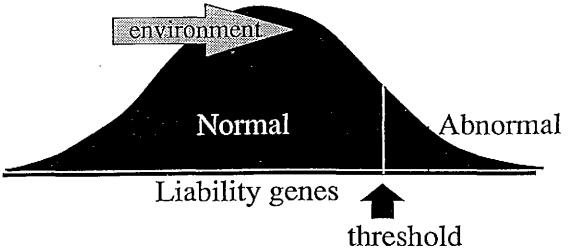


Figure 1. Multifactorial threshold model for NTDs. This figure represents a normal distribution of "liability" genes within a population. Individuals to the right of a threshold would be expected to possess many liability genes and would express a NTD phenotype

predisposition for the NTD phenotype. At a given point in the distribution within the normal curve, there is a vertical line known as the " threshold" . The threshold defines the boundary of the distribution of liability genes and the phenotypic expression of a NTD. Individuals to the right of the threshold line who possessed liability genes within their genotype would be expected to show the NTD phenotype, individuals to the left would not. Liabilities are illustrated as being a combined possession of specific liability genes and the influence of mysterious environmental factors (Vacha, 1997).

I.B. Normal neurulation

Primary neurulation commences with the formation of the neural primordium, the neural plate. This occurs when the stem cell epiblast transforms into a columnar epithelium situated along the dorsal midline of the embryo. The onset of this cell differentiation within the epiblast results from an interaction between the embryonic epiblast layer and the underlying notochord and paraxial mesoderm. This process is referred to as neural induction (Copp et al., 1990). Neural induction is a signaling process between two sets of

tissues. One set (the embryonic epiblast) is the signaling tissue, the other (the underlying notochord) is the responding or target tissue (Vacha, 1997).

The second step in primary neurulation is the elevation and apposition of the neural folds. Directly after formation, the edges of the neural plate become elevated relative to its midline, converting the flat plate into a groove. Elevation is initiated at the junction between the neural plate and surface ectoderm and proceeds by a smooth continuation of neural groove deepening. This deepening occurs until both sides of the neural folds are opposed (Copp et al.,1990).

The third step in primary neurulation is the fusion of the neural folds at the dorsal midline. Consequently, continuity between the neural plate and surface ectoderm is lost and the surface ectoderm n the right and left sides of the embryo become continuous, forming a complete epidermal layer externally. At the same time, the two sides of the neural fold come together to form an internal roof plate. It is at this point in neurulation when NTDs may arise from either defective neural fold elevation or failure on neural fold fusion. The final event in primary neurulation is closure of the posterior neuropore (Copp et al., 1990).

The last phase of brain and spinal cord development is known as secondary neurulation. Secondary neurulation begins by the segregation of cells from adjacent regions to form the medullary cord. Multiple cavities then form in the medullary cord followed by coalescence of all the cavities into a single lumen which becomes continguous with the central canal of the primary neural tube (Campbell et al., 1986).

Neural tube defects occur when certain steps of the neurulation process are altered such that neural tube closure does not occur. There is evidence that indicates a mult-site closure for human beings controlled by individually expressed genes. When expression is altered by a teratogenic agent such as Valproic acid, the neural tube fails to form. Consequently, NTDs are soon to follow (Allen, 1993).

I.C. Valproic Acid as a teratogen

Valproic acid (VPA) is a simple, eight-carbon, branched-chain fatty acid that is used for the treatment of seizures for patients diagnosed with epilepsy. VPA's effectiveness in treating seizures was accidentally discovered while it was being used as a control vehicle for anticonvulsant drug transport. Additional experimental use showed that VPA could not only be used as a transport vehicle, but also as an anticonvulsant drug. In 1978, the United States' Food and Drug Administration approved the use of VPA for the treatment of absence seizures and it is still widely prescribed today (Lammer, 1987).

Human studies have determined that VPA has the ability to cross the placenta and umbilical cord during pregnancy. The usual chronic adult dosage, 1,000-1,600 mg per day have been exceeded in umbilical sampling upon delivery (Lammer, 1987). This indicates the fetus is being exposed to concentrations of VPA possibly even higher than that of the mother.

VPA use during pregnancy results in an absolute increase in the susceptibility of NTDs. Administration of VPA during pregnancy may result in a 5-to 20 fold increase of NTD in the offspring above the background incidence for the general population in most countries (Wegner, 1992). The rate of occurrence of spina bifida has been known to increase to 1-2% for the VPA exposed fetus. Additional defects of flat nasal bridge, fingerlike thumbs, prominent forehead, eyebrow depigmentation, low-set malformed ears, micrognathia, microcephaly, cleft lip, and cleft palate were also found to occur in humans beings as well as in mice following VPA exposure (Lammer, 1987).

I.D. Mouse models for NTDs

The precise molecular mechanisms of the action of VPA, as well as most human teratogens, still remain unknown. Animal studies have been proven to be an effective way to extrapolate

data while avoiding ethical and time constraints (Wegner, 1992). One particularly useful species used for NTD studies is the mouse. Similarity has been found between the NTD phenotypes of mice and humans (Embury, 1978). From that study, we can assume that genes responsible for causing NTDs in humans are also present and responsible in mice. This makes them great models for NTD research.

There are many advantages in using mice as experimental models. One major benefit is the shorter length of murine gestation. Human neural tube closure occurs at approximately Gestational Day (GD) 18 compared to GD 8.5 in mice. This enables mice to be used as a faster, more easily controlled model.

When using mice as models, we have the ability to produce inbred strains of mice. Inbred strains are produced by numerous generations of inbreeding between a specific mice colony. At the end of the process, the inbred strain is 99% identical in genotype. This enables us to treat numerous mice as if they were the same individual mouse. The experimental results are then used to form conclusions regarding mechanisms for NTDs in humans. An additional benefit of using mice is the abundance of known information about gene expression, cloning and sequencing in mice. Consequently, altered genes are easily identified and studied.

There are approximately 250 existing mouse strains. We used the LM/Bc/Fnn strain for this study due to its ready availability and its reputation as being susceptible to VPA induced NTDs. Approximately 100% of treated LM/Bc/Fnn dams produced offspring with NTDs when exposed to 600mg/Kg of of VPA during neural tube closure on GD 8.5.

I.E. Hypothesis

Embryonic neural tube development is a heterogeneous process involving several coordinated cellular and morphogenic mechanisms. My project has been based upon the hypothesis that alterations in gene expresion patterns of LM/Bc/Fnn embryos will persist

upon exposure to VPA during neural tube closure. I believe that this may also alter the individual's susceptibility to NTDs. The method by which the expression patterns have been examined is by the use of Differential Display/PCR.

CHAPTER 2

II. EXPERIMENTAL METHODS

II.A. Maintenance and VPA treatment of mice

Highly inbred LM/Bc/Fnn mice were maintained on a 12 hour light cycle at the Laboratory Animal Research and Resources Building, Texas A&M University. Virgin, pathogen-free females, 40-60 days of age, were bred with experienced males and checked the following morning for the presence of a vaginal plug. Upon detecting a plug, gestation day 0 was defined as 10 PM of the previous night, the midpoint of the dark cycle (Snell et al., 1948). Collected dams were then exposed to the teratogenic insult at the period of peak sensitivity to the induction of NTDs, at GD 8.5 (Wlodarczyk et al., 1996). Dams were sacrificed by cervical dislocation on GD 9. The uterus was exteriorized and the embryos were removed from the fetal membranes with Watchmaker forceps. Once visualized, the anterior portion of the neural tubes were removed by the use of tungsten needles under a dissecting microscope (Wild M8, Heerbrugg, Switzerland). Finally, these anterior neural tubes were individually placed in physiological saline, and stored at -80°C for later molecular analysis by differential display.

II.B. Differential display/PCR

II.B.1. Background

In 1991, Drs. Peng Liang and Arthur Pardee coupled the use of PCR with differential gene expression patterns to develop the procedure known as 'differential display'. Since then, examining genetic expression patterns by means of differential display has become an important tool in the study of normal embryogenesis (Vacha, 1997). It is estimated that only 15% of the entire mammalian genome is expressed at any given time (Maniatis et al., 1987). While a seemingly small percentage of the total, this amounts to approximately 10,000 to 20,000 different mRNAs in a given mammalian cell (Alberts et al., 1983). Differential display (DD) was designed to provide a visual representation of these mRNAs, just as 2D gel electrophoresis has been used to visualize protein expression patterns. Differentially expressed genes can be detected and cloned for further characterization by comparing the resulting autoradiographic patterns of mRNAs across treatment or other comparison groups. The differential display approach allows for the screening of literally thousands of genes, with the potential to identify genes that may be transcriptionally regulated in any experimental system. There are several advantages to the use of differential display (Sambrook et al.,). First, the DD reactions may be performed with very little mRNA. This is accomplished through the power of PCR amplification. Second, numerous subtractions are not necessary because DD simultaneously reveals the gene expression patterns of multiple comparison groups. Third, DD is more amenable to troubleshooting than alternate techniques of subtractive and differential hybridization. Finally, DD is a relatively simple and fast procedure (Vacha, 1997).

The procedure begins with approximately 250 ng of total RNA being isolated from the tissue of interest by use of guanidinium thiocyanate. Next, it is divided into three centrifuge tubes for reverse transcription into cDNA, using an MMLV reverse transcriptase (Gene Hunter, Brookline, MA) and a specific T11V primer. The T11V primer contains a 12 bp poly-T tract with a specific 3' sequence, where V refers to either adenine, cytosine, or guanine. The poly d(T)11 portion of the primer is complementary to the poly A tail of mRNAs, while the 3' base of the oligomer serves to anchor it specifically to a subset of mRNAs containing the complementary 3' sequence. Consequently, by the use of three separate primers (T11A, T11C, T11G), the entire mRNA population is theoretically transcribed as three distinct subpopulations of cDNA (Figure 2.1). The cDNA is subsequently amplified by PCR using the same T11V primer used in reverse transcription. In addition, a select 20 mer is used at the opposing primer (Figure 2.2). By amplifying with several different primer combinations (Figure 2.3) a known percentage of the mRNA population can be profiled. The resulting PCR products are then run on a 6% denaturing acrylamide gel, such that samples of identical primer combinations, loaded adjacently may be easily compared. Following electophoresis, the differential display gel is exposed to X-ray film to resolve the pattern of message expression (Vacha, 1997). *See Figure 2.4 for DD overview.*

Figure 2.1 DD/PCR Primers

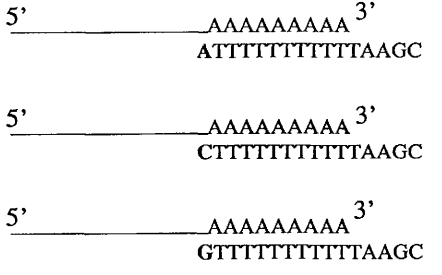


Figure 2.2

ARBITRARY DD-PCR PRIMERS

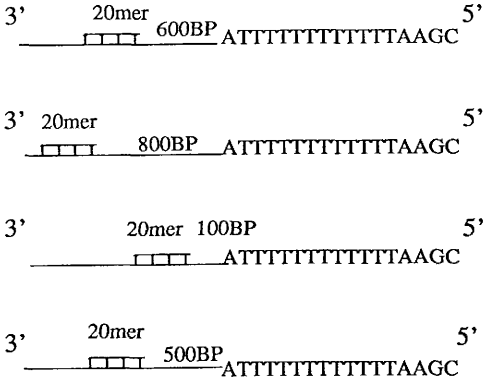


Figure 2.3

DD-PCR PRIMER COMBINATIONS

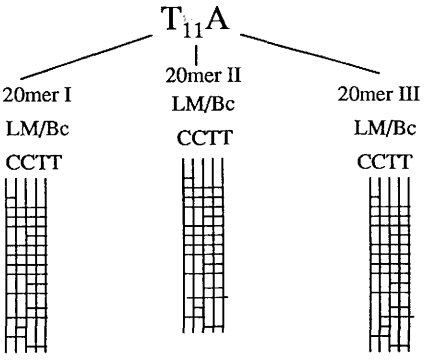
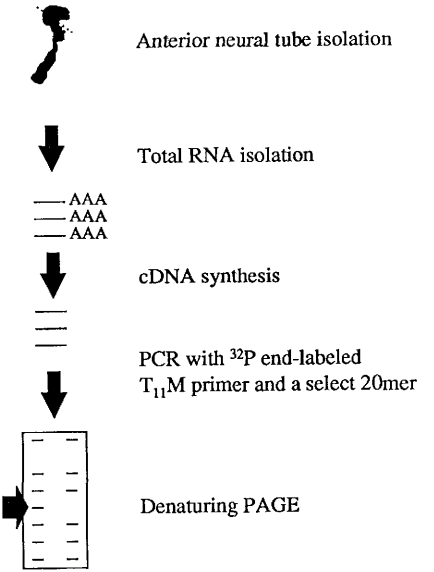


Figure 2.4 DD/PCR Overview



Once a gene has been identified as being differentially expressed, it was isolated from the acrylamide gel for further characterization (Figure 2.5). This was accomplished by overlaying the developed film onto the acrylamide gel, and removing the associated band with a razor blade. It was imperative that the gel and film be properly aligned to avoid removing adjacent genes that would bring false results. This was overcome by punching holes in to gel and film prior to exposure (Callard et al., 1994).

II.B.2. Procedure

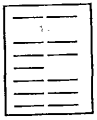
II.B.2.a. RNA isolation

Ten embryonic neural tubes from each treatment group were pooled together, and the surrounding physiological saline removed by pipetting to result in a pellet of neural tissue. While single embryos have been successfully used for DD/PCR (unpublished results), this study utilized pooled embryos in order to more accurately quantify the extracted RNA. DNA-free RNA was then isolated from the neural tissue with the aid of the TriPure isolation reagent (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocols.

II.B.2.b. Reverse Transcription

Total RNA from each of the two experimental groups was divided into 3 tubes (300 ng aliquots) for reverse transcription to cDNA. Each tube received a single anchored primer (5'-TAGGGAGGACCATTTTTTTTTTV-3') to a final concentration of 0.2 μM,

Figure 2.5



Gel isolation

Reamplification/ Cloning

Ribonuclease Protection Assay

reaction buffer [125 mM, Tris (pH 8.3), 188mM KCL, 7.5 mM MgCl, 25 mM DTT] and deoxyribonucleotides to a final concentration of 20 μM. The RNA was then denatured to foster proper annealing. One μl of MMLV reverse transcriptase (GeneHunter, Brookline, MA) was added to initiate reverse transcription. This reaction was allowed to incubate for 1 hour at 37°C, followed by a 5-minute incubation at 75°C to inactivate the reverse transcriptase prior to the PCR. The resulting cDNA was then stored at -20°C for later PCR amplification.

II.B.2.c. Primer sequences

The sequences of anchored primers were designed to take advantage of numerous modifications introduced in the DD literature (*see Table 2.1*). Reverse transcription 'anchored' primers included 5'-TAGGGAGGACCATTTTTTTTTTV-3', where V refers to adenine, cytosine, or guanine. This sequence contains a poly d(T)11 tract for complementary base pairing to poly A mRNAs as well as an *in vitro* transcription efficiency sequence (5'-GGAGGA-3') for later manipulations (Milligan et al., 19873'). All primers were designed to have a denaturation temperature near 60°C for increased PCR stringency. Primers will be end-labeled with [γ32P]-CTP and T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocols. The sequences of DD primers have been summarized in *Table 2.2*.

Table 2.1. *Criteria for the design of DD anchored primer sequences*

Must contain at least 20 nucleotides to increase PCR stringency
Melting temperature must be at least 60°C for PCR stringency
Sequence must not be capable of forming loop structures
Sequence must not be capable of forming stable duplex structures
The 3' nucleotide must be adenine, cytosine, or guanine for specificity
Oligonucleotide must have an internally stable 5' sequence
Oligonucleotide must have an internally unstable 3' sequence

Table 2.2. Sequences of DD/PCR primers

Primer designation	Primer sequence (listed 5'-3' according to IUPAC symbols)
60°T11A	TAGGGAGGACCATTTTTTTTTTA
60°T11C	TAGGGAGGACCATTTTTTTTTTC
60°T11G	TAGGGAGGACCATTTTTTTTTTG
SV1	TGTGGCAACCCTCTTGTC
SV2	TGTGGCAACCCGGAACCAATC
SV3	TGTGGCAACCTCGGTCATAG
SV4	TGTGGCAACCCTGCTTGATG
SV5	TGTGGCAACCGAGGATGAC

PCR was performed using the following mixture per reaction: 2.5 µl 10X TAQ buffer [500 mM Tris (pH 8.3), 2.5 mg/ml BSA, 20% (w/v) sucrose, 1 mM cresol red], .25 µl arbitrary primer, 1.5 µl end-labeled anchored primer, 1.5 µl MgCl2, 2 µl cDNA from reverse transcription and .25 µl Sigma Taq polymerase (Promega, Madison, WI) and approximately 10 µl mineral oil. The first cycle of PCR was performed according to the following conditions: 94°C denaturing for 1 second, 42°C annealing for 1 second, and 73°C elongation for 15 seconds. The conditions of cycles 2-30 were the same as the first cycle, save for the annealing temperature, which was increased, to 60°C. After 30 cycles of PCR, a 73°C hold cycle was performed for 15 seconds to allow for complete elongation of the resulting amplicons. These reaction times are typical for this type of thermocycler, as recommended by the manufacturer (Idaho Technology).

II.B.2.d. Electrophoresis

The resulting PCR products were mixed with one volume gel loading buffer [95% formide, 10mM EDTA (pH 8.0), 0.09% xylene cyanole FF, and 0.09% bromphenol blue], heat denatured by incubating for 4 minutes at 80°C, and loaded on a 4.5% denaturing polyacrylamide gel such that treatment groups of identical primer combinations are loaded adjacently. Electrophoresis was then performed on a Genomyx LR DNA sequencingsystem (Foster City, CA) according to the manufacturer's protocols. Following electrophoresis, the DD/PCR gel was then exposed to BIOMAX film (Kodak, Rochester, NY) to resolve the pattern of message expression.

II.B.2.e. Isolation/Reamplification of differentially expressed cDNAs

Differentially expressed cDNAs were directly isolated from the polyacrylamide gel by overlaying the resulting autoradiograph and isolating the corresponding polyacrylamide slice with a sterile scalpel blade. Each differentially expressed band was then placed into a separate microcentrifuge tube for reamplification and spun down on a centrifuge. Remaining liquid was then removed by pipette. To each tube 250 µl Solution D [12.5 ml GCT solution (62.5g guanidium thiocyanate, 4.4 ml 750mM sodium citrate (pH 7.0), 6.6 ml 10% sarcosyl), 90 µl b-meracaptoethanol] was added. Each mixture was sonicated followed by the addition of 25 µl NaOAc (pH 4.0), 250 µl acidic phenol and 100 µl chloroform. The mixtures were then chilled on ice for 20 minutes and spun on the centrifuge at 8,000 rpm for 10 minutes. The aqeous phase from each tube was transferred into new tubes followed by the addition of .75 µl mg Glycogen (20 mg/ml). 375 µl EtOH was added and each tube was vortex and precipitated at -20°C for 90 minutes. The tubes were then spun down at 14,000 rpm for 30 minutes at 4°C and supernatant was removed. Each pellet was then air dried and resuspended in 50µl DEPC-txt water. These products were then purified with the aid of the QIAquick PCR purification kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions, and stored at -20°C for further identification procedures.

II.C. Cloning

II.C.1. Background

Cloning of genes by TA Cloning Kit (INVITROGEN; La Jolla, CA) involved four separate reactions of PCI extraction, transformation and the growing of liquid bacterial cultures to form mini preps. Genes of interest were cloned into a plasmid for the ease of future

amplification and storage. Future characterizations of the identified genes were performed with the use of plasmid miniprep stocks. *See Figure 2.6 for an overview.*

II.C.2. Procedure

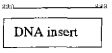
PCI extraction was performed on each purified PCR product by the addition of 100 µl PCI extract to 100 µl PCR product. The aqueous phase was then removed and to it were added 10 µl 3M NaOAc and 200 µl EtOH. This reaction was incubated overnight at -20°C and was verified on a 0.8% agarose gel. *See Figure 2.7.*

Ligation began with the spinning of extraction products at 14,000 rpm for 30 minutes. To each reaction, 1µl 10X T4 DNA Ligase buffer (INVITROGEN; La Jolla CA) 2 µl (25 ηg/ml) PCR 2-1 vector, 5 µl water and 1µl (40/ml) T4 DNA Ligase (INVITROGEN; La Jolla, CA) were added. Each mixture was then stored at 14°C overnight.

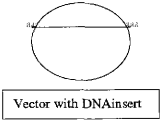
Transformation was performed on the ligated plasmids into DH5α, competent E. coli cells (INVITROGEN; La Jolla, CA) according to the manufacturer's instructions and

Figure 2.6. Cloning Overview

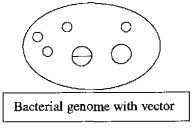
1. **PCI Extraction:** Extracts Linear DNA



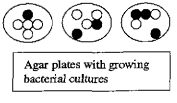
2. **Ligation:** Glues insert into vector



3. **Transformation:** Transfers vector into bacterial genome



4. ***Growing of Liquid Bacterial Cultures:*** Amplifies vector



5. **Mini-Preps:** Screening of Blue/White Colonies for bacteria with vector: White Colonies, those that contain vector, are selected for sequencing

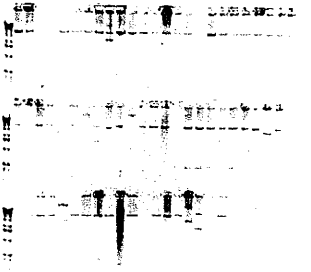


Figure 2.8 EcoR 1 Digestion on Agarose Gel

Table 2.3 Criterion for the interpretatin of BLAST DNA sequence alignment results

•Only alignments to 3' untranslated sequences were observed due to the use of a poly (T) anchored primer during DD
•Intron sequences were not considered since DD ampicons were intron-less cDNAs
•Genomic DNA sequences were not considered since RNA was collected for DD
•Transposon sequences were fully considered
•Pseudogene sequences were not considered since they are not transcriptionally active
•Non-murine species sequences were considered only when the corresponding murine gene was absent from the database

plated onto LB agar plates containing 100 µg/ml ampicilin and incubated at 37°C overnight. This procedure resulted in the formation of liquid cultures each screened by blue medium. Positive white colonies were then chosen and shook at 37°C overnight.

Liquid cultures were grown to provide sufficient plasmid DNA for recovery by mini prep analysis according to the manufacturer's instructions, followed by EcoR 1 digestion. *See Figure 2.8.* Endonuclease diigestion involved the addition of 33 µl water, 3 µl plasmid DNA, .25 µl MEB EcoR 1 and 10X MEB buffer to each reaction and subsequent overnight incubation at 37°C. See figure 2.8 for 0.8% Agarose gel of Digested inserts.

II.D. Sequencing

II.D.1. Background

In order to determine the identity of the genes whose expression patterns were altered upon VPA insult, the isolated clones were subjected to DNA sequence analysis. We utilized the Thermo Sequenase kit (Amersham Pharmacia Biotech; Cleveland, OH) to sequence fragments that had been cloned as described in the previous section. The identified sequences were then aligned to the GeneBank database. *Table 2.3* lists the criterion used for the interpretation of BLAST DNA sequence alignment results.

II.D.2. Procedure

A reaction mixture was prepared containing each of the following /template: 14 µl water, 2 µl reaction buffer, 1µl template DNA (DD/PCR product), 1 µl primer, 2 µl (4U/µl) Thermo Sequenase DNA polymerase. 4.5µl of each reaction mixture was then added to one of four cycling termination reaction tubes and put on ice. Four termination mixes were then prepared on ice containing 2µl dGTP and 0.5µl of [α-33P]ddNTP (G,A,T, or C-one per sequence) and added to their appropriate reaction tube. Each tube was then covered with 10-20µl of mineral oil and placed s the Robocycler (Stratagene, La Jolla, CA) at the following conditions: 95°C for I minute, 30 cycles of 95°C for 1 minute, 56°for 1 minute, and 72°C for I minute, and a 4 °C soak. 4 µl stop solution was added to each of the termination reactions that were stored at -20°C until further use.

Prior to gel loading, each sample was denatured at 95°C for 10 minutes and 3-5 µl of each sample was electrophoresed onto a 6% Genomyx denaturing acrylamide gel. Sequences were then identified as described in the previous section (II.D.1) with the aid of Microsoft Digital science SQ software (Kodak; New Haven, CT.).

CHAPTER 3

III. RESULTS

The following section lists and interprets sequencing results from those genes with altered gene expression patterns as show by DD/PCR.

Gene sequencing, as desrcibed in the previous chapter, enables us to visualize the separation of cDNA nucleotides and determine the identiy of altemately expressed genes. *Figure 3.1* shows a picture of a sequencing gel that has separated the four cDNA nucleotides, Adenine, Guanine, Cyctosine, and Thymine into bands. After separation, the derived sequences are then matched to a BLAST sequence. *Figure 3.2* lists the sequences of three novel genes which aligned to the database. Although these genes may play an important role in embryonic neural tube development, their exact functions are currently unknown. Another possibility due to the limitations of the DD/PCR technique, is that the genes being expressed may be false positive readings. In addition, because it is possible for multiple amplicons to co-migrate with the DD band of interest, the sequences detected may actually represent these co-migrating fragments.

Additionally, two promising genes also matched sequences in the BLAST database. *Figure 3.3* shows the identified sequence of Inorganic Pyrophosphatase (IPPase) and *Figure 3.4* shows the sequence that aligned to the mitochondrial genome.

Figure 3.1 Sequencing Gel

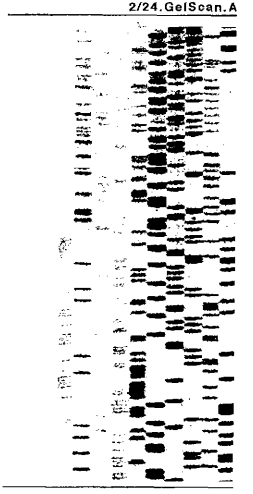


Figure 3.2. Novel Gene Sequences

1: CTG GTC ATA GTT TTT AAT GTT AAA CAG TAA ATG GCA GTA GTG ACC AAG CAC AGT GCT TAT ACA CAC TAT AAT CAT TTT TAA TTC ATC TTT

2: ATA GCT GGT ATT ACT TTG TTT TTA AGG ATA TAT ATT TCA RRR GAA ATT ATA AAA GTT CTT TGC TAT GCT GCA AGT TGT GTA TTA AAA CAT TAC TTT AAA ATT AAA TCG GTT ACG TTA TGA TTC TAA AAA AAA ATG GTC TCC TAT GAC GAG TGC ACA AAG CGA

3: CTA GAT GCA TGC TGC AGC GGC CGC CAG TGT GAT GGA TAT CTG CAG AAT TCG GCT TTG TGG CAA TCA TAG AAG TGT TAG GCT GGT TAA ACT AAT TAG AAA ACT ATA TGA GGT TAC GTT TGT TCA GGT TTT TTA GGG GCT

Figure 3.3 Inorganic Pyrophosphatase Sequence

TGT AGC AAT AGC GGC GGC GGC

GGC TGT TTC CCC GGC ACG ATG

AGC AGC CTT CAG CAG CGA GGA

GCG CGC GGC GCC CTT CAC CCT

CGA GTA CCG AGT CTT CCT CAA

AAA TGA AAA AAG GAC AAT ATA TCT

TCT CCA TTT CAT GAT ATT CCA A

Figure 3.4 Mitochondrial Genome Sequence

ATG CTA AAA ATT ATT CTT CCC TCA

CTA ATG CTA CTA CCA CTA ACC

TGA CTA TCA AGC CCT AAA AAA ACC

TGA ACA AAC GTA ACC TCA TAT

AGT TTT CTA ATT AGT TTA TTA

ACC AGC CTA ACA CTT CTA TGA CAA

ACC GAC G

CHAPTER 4

IV. DISCUSSION

IV.A. Inorganic Pyrophosphatase Sequence

The gene that codes for the production of inorganic pyrophosphatase (IPPase) was identified as being overly expressed in neural tissue form embryos treated with VPA. The IPPase gene, EC 3.6.1.1, is most commonly expressed in E.coli and yeast cells (Shintani et al., 1998). In addition, a cDNA sequence homology has also been isolated in mammalian liver cells and from a library of developing barley grains. IPPase has been found to be expressed in nondormant barly grains with metabolically active embryonic tissue. Addition of recombinant IPPase enhanced germination rate in isolated dormant embryos (Vissor et al., 1998). This supports the hypothesis that IPPase activity may influence energy production in developing embryos. The greater the activity, the more energy that can be produced.

When applied to the concept of neurulation, it can be hypothesized that this increase in energy may cause increased rates of cell differentiation. Section I.B describes the complex process of neurulation as beginning with the formation of the neural plate followed by neural induction. In review, neural induction begins with cell differentiation within the epiblast resulting from tissue interaction between the embryonic epiblast layer and the underlying notochord and paraxial mesoderm. If the energy levels alterered by increased IPPase activity exceed the necessary levels for proper neural induction, it is possible that cell

differentiation may occur prematurely. Consequently, the rate of primary neurulation would be altered, and the resulting product would be a malformed neural tube that failed to close. There has been a reported incidence of a reduced rate of cell proliferation in the notochord and hindgut a developing brain associated with growth imbalance and mechanical distortion of the caudal embryonic region in the form of ventral curvature. This process is known to oppose neural tube closure and lead to spina bifida (Copp et al., 1990). I believe decreasing rates of cell proliferation may also cause a growth imbalance and consequently NTDs would result. This is what I believe may have contributed to the occurrence of NTDs in the VPA treated mice of my project.

IV.B Mitochondrial Genome Sequence (ORF 4)

The sequencing results show a portion of the mitochondrial genome, open reading frame 4 (ORF 4), as being alternately expressed in the treated mouse embryonic neural tissue. Little is known regarding ORF 4, however, due to the tendency of the majority of known mtDNA sequences to code for proteins associated with mitochondrial energy production, it is logical to hypothesize that this ORF also may code for similar proteins. I would like to discuss the mechanisms of energy production that take place within this cellular organelle. Mitochondrion are small, membrane-bound cellular organelles responsible for converting nutrients into the energy required for cellular activity by aerobic respiration. This process results from the breakdown of pyruvic acid molecules that are imported into the mitochondria from the cell's cytoplasm. Upon entering the mitochondria, pyruvic acid molecules react with water to produce carbon dioxide and ten hydrogen atoms. This process is known as the Kreb's cycle. After this reaction occurs, the hydrogen atoms are transported via special carrier molecules known as coenzymes to the cristae of the inner

membrane. Here, the coenzymes donate the hydrogens to a series of membrane-bound proteins called the electron transport chain (Sherwood, 1997).

The electron transport chain separates the electron and the proton in each of the ten hydrogen atoms. The ten electrons are then sent through the chain and eventually combine with oxygen and the protons to form water. Energy is released as the electrons flow coenzymes to oxygen atoms and are trapped by components of the electron transport chain. As electrons flow from one component to another, random protons are pumped from the mitochondrial matrix to the space between the inner and the outer membrane. A membrane-protein complex allows the one way transport of protons back into the matrix only if a phosphate group is added to the chemical ADP to form ATP in the process of oxidative phosphorylation. The overall result of this series of mitochondrial reactions is approximately 38 ATP molecules (per molecule of Glucose) (Sherwood, 1997) that is used by the cell for virtually every energy-requiring reaction used to sustain its life as well as that which is used during cell division and reproduction.

Assuming the sequnece we determined to match ORF 4 codes for proteins that cause an increase in the production of cellular energy, there is a possibility that ORF 4's expression in treated mice also contributes to altered rates of cell proliferation. Subsequently, the change in the rate of cell proliferation may produce a mechanical distortion in specific regions of the neural tube and inherently cause NTDs.

CHAPTER 5

V. CONCLUSIONS AND FURTHER RESEARCH

Neural tube defects are important causes of infant mortality and morbidity (Sever, 1995). Children with NTDs who survive beyond the neonatal period are often multiply handicapped (Copp et al., 1990). As NTD affected children develop into their adult years, they are being faced with a health care system that has a widespread absence of coordinated multidisciplinary care for NTDs such as spina bifida (Sawyer et al., 1998). If by chance they do get quality health care, then they are still faced with astronomical medical bills. Lifetime cost for one child can exceed $540,000 and combined lifetime costs of just 18 conditions is $8 billion annually (Finnell et al., 1995). The non-monetary costs are incalculable.

For an example, one specific non-monetary cost is the frustration associated with hopelessness. Because our understanding of the mechanisms of NTDs is fairly low, people with loved ones experiencing neural disorders are unable to obtain efficient methods of treatment and prevention. They are incapable of ending the intense pain their loved ones are experiencing. This illustrates the need for better understanding of neural tube malformations accompanied by joint scientific thought leading to large-scale research projects that aim to solve the mystery of NTDs. There is an ever-increasing need for a molecular, genetic based approach to enhancing our understanding of abnormal neural tube morphogenesis (Vacha, 1997). It will continue to increase until comprehension is achieved and NTDs are no longer a threat to human society.

My project used the multifactorial approach to go one step further in the identification of this mechanism. By the use of DD/PCR technology, we were able to show differential expression patterns in neural tissue that may prove to affect individuals' susceptibility to NTDs. In doing so, we chose to accept our original hypothesis that altered gene expression would follow VPA teratogenic insult. Altered genes that were identified by means of sequencing and alignment to the BLAST database, are speculated to play important roles in energy production and embryonic cell development as listed in the previous section in *Table 3.1.* Clearly, there is still need for further research and verification as to the role these genes play in the formation of NTDs.

Further research

The follow-up experiment to this research project might involve further characterizing of the functions of the differentially expressed genes. Further identification could be accomplished by means of ribonuclease protection assay (RPA) which has the ability to screen past possible " false positives" produced byDD/PCR.

The procedure involves the generation of in vitro-transcribed riboprobes from differentially expressed amplicons following their isolation and subsequent reamplification. The T7 RNA Polymerase binding site is located within the vector (PCR 2.1). If the gene is truly expressed, its mRNA would be available for hybridization to the complimentary antisense riboprobe.

Single-stranded, unbound RNAs are degraded by a treatment of RNAse A and the resulting hybridized mRNA/riboprobe fragments are visualized by polyacrylamide gel electrophoresis. The banding patterns from both gels resulting from differential display and

RPA techniques could then be compared. If they contain the same banding pattern, they are most likely the same gene (Vacha, 1997).

In addition to RPA varification, it would also be necessary to repeat this experiment in additional animal models at different periods of embryonic development. The excision of neural tubes was chosen to be approximately GD 8.5 due to the widely accepted concept that neurulation occurs during this time period. This experiment could also be done at different times in embryonic development to determine if gene expression is solely being altered at this time point, or if it is being altered through-out the entire growth period upon VPA exposure. Examining different time points within the area of neural tube closure might provide a more complete explanation as to the true effect that altered expression is having on the developing organism.

REFERENCES

Alberts, B, D Bray, J Lewis, M Raff, K Roberts and JD Watson.1983. *Molecular biology of the cell.* 3rd Ed., p409. Garland Publishing, Inc, New York.

Callard D, B Lescure and L Mazzolini. 1994. A method for the elimination of false positives generated by the mRNA differential display technique. *Biotechniques* **16**: 1096.

Campbell, LR, DH Dayton and GS Sohal. 1986. Neural tube defects: a review of human and animal studies on the etiology of neural tube defects. *Teratology* **34**: 171-187.

Carter, CO.and K Evans. 1973. Spina bifida and anencephalus in Greater London. *J. Med. Genet.* **10**: 209-234.

Copp, AJ, FA.Brook, JP Estibeiro, A Shum and DL Cockroft. 1990. The embryonic development of mammalian neural tube defects. *Prog. Neurobiol.* **35**: 363-403.

Elwood, JM and AJ Coldman. 1981. Water composition in the etiology of anencephalus. *Amer. J. Epidemiol.* **113**(6): 681-690.

Embury, S, MJ. Seller, M Adinolfi and PE Polani. 1978. Neural tube defects in curly-tail mice. I. Incidence, expression and similarity to the human condition. *Proc. R. Soc. Lond. B.* **206**: 85-94.

Finnell, RH, MS Yerby, H Nau. 1995. General Principles: Teratogenicity of antiepileptic drugs. (eds. Levy, RH, RH Mattison, BS Meldrum), *Antiepileptic Drugs, 4th ed.* p 209-230. Raven Press, New York.

Hunter, AG. 1993. Brain and spinal cord In: *Human malformations and related anomalies.* (ed. Stevenson, RE), vol II, Oxford University Press, Oxford.

Lammer, EJ, LE Sever and GP Oakley Jr. 1987. Teratogen update: valproic acid. *Teratology* **35**: 465-473.

Martin, RA, RM Fineman and LB Jorde. 1983. Phenotypic heterogeneity in neural tube defects: a clue to causal heterogeneity. *Am. J. Med. Gen.* **16**: 519-525.

Maniatis, T, S Goodbourn and JA Fischer. 1987. Regulation of inducible and tissue specific gene-expression. *Science* **236**(4806): 1237-1245.

Milligan, JF, DR Groebe, GW Witherell and OC Uhlenbeck. 1987. Oligoribonucleotide synthesis using T7 RNA Polymerase and synthetic DNA template. *Nucleic Acids Res.* **15**: 8783-8798.

Norman, MG, BC McGillivray, DK Kalousek, A Hill and KJ Poskitt. 1995. *Congenital malformations in the brain: pathogenic, embryologic, clinical, radiologic and genetic aspects,* Oxford University Press, Oxford.

Sambrook, J, EF Fritsch and T Maniatis. 1989. *Molecular cloning: a laboratory manual,* 2nd Edition, p 10.38, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sawyer, SM, N Collins, D Bryan, D Brown, MA Hope and G Bowes. 1998. Young people with spina bifida: transfer from paediatric to adult health care. *J. Paediatrics & Child Health.* **34**(5): 414-7.

Sever, LE. 1995. Looking for causes of neural tube defects: Where does the environment fit in? *Envion Health Perspect* **103**(Suppl 6): 165-71.

Shinitani, T, T Uchiumi, T Yonezawa, A Saminen, AA Baykov, R Lahti and A Hachimori. 1998. Cloning and expression of a unique inorganic pyrophosphatase from Bacillus subtilis: evidence for a new family of enzymes. *FEBS Letters* **439**(3): 263-6.

Sherwood, L. 1997. *Human Physiology: from cells to systems 3rd* Edition, p31-33,Wadsworth Publishing Company, Belmont, CA.

Snell, GD, E Fekete and KP Hummel. 1948. The relation of mating, ovulation and the estus smear in the house mouse to the time of day. *Anat. Rec.* **76**: 39-54.

Texas Department of Public Health. 1992. *An investigation of a cluster of neural tube defects in Cameron County, Texas,* Austin, Texas.

Vacha, SJ. 1997. The use of differential display to identify candidate genes for susceptibility to heat-induced exencephaly in a mouse model. Texas A&M University Dissertation.

Van Allen, M, DK Kalousek, GF Chernoff, D Juiloff, M Harris, BC McGillivray, S Yong, S Langlois, PM MacLeod, D Chitayat, JM Friedman, RD Wilson, D McFadden, J Pantzar, S Ritchie and JG Hall. 1993. Evidence for multisite closure of the neural tube in humans. *Am. J. Med. Gen.* **47**: 723-743.

Vissor, K, S Heimovaara-Dijkstra, JW Kijne and M Wang. 1998. Molecular cloning and characterization of an inorganic pyrophosphatase from barley. *Plant Molec. Biol.* **37**(1): 131-40.

Wegner, C. 1992. Alteration of embryonic folate metabolism by valproic acid during organogenesis: Implications for mechanism of terotogenesis. *Neurology* **42**(suppl 5): 17-24.

Wlodarczyk, B, GD Bennett, J Calvin and RH Finnell. 1996b. Arsenic induced neural tube defects in mice: alterations in cell cycle gene expression. *Repro. Tox.* **10**: 447-454.

VITA

Blaine Gabrielle Hayes
4120 NW 75th St.
Kansas City, Missouri 64151

Education: Texas A&M University, College Station, Texas
Bachelor of Science in Biomedical Science
Graduation Date May 2000

Experience: Texas A&M University Undergraduate Fellows Research Program, College Station, Texas
Department of Veterinary Anatomy and Public Health; Conducted research project on Teratogen-Induced Neural Tube Birth Defects, June 1998-present

Texas A&M University Study Abroad Program, Costa Costa Rica, Department of Nutrition; Conducted Group Maternal/Child Health Study, June 1998

Activities: Texas A&M Honors Program
Elementary Student Tutor
Order of Omega Honor Society
Greek Week Co-Chair
Kappa Kappa Gamma Sorority Member
HOSTS Member (Helping One Student To Succeed)