

The Expression of Glutamate Dehydrogenase
in CZB Cultured Mouse Embryos

A Biology Departmental Honors Thesis

by

Emily P. Martin

Thesis Advisor

Clare L. Chatot

Clare L. Chatot

Ball State University

Muncie, Indiana

May 1997

5/20/97
Theorie
LD
2489
.24
1997
.M31

ABSTRACT

CZB medium, which contains glutamine at a concentration of 1 mM, has been developed to culture mouse embryos from the 1-cell stage to the blastocyst stage (Chatot et al., 1990). Studies indicate that glutamine is essential to the development of some strains of mouse during preimplantation stages in vitro and it is used in vivo as an energy source (Chatot et al., 1989). Glutamate Dehydrogenase (GDH) is an enzyme that is necessary for the metabolism of glutamine. The purpose of our research is to determine the amount of GDH expressed, via the analysis of mRNA, in preimplantation mouse embryos that have developed to various stages both in vivo and in CZB culture. My specific goal is to analyze the GDH mRNA levels of those embryos that have been cultured in CZB medium using the technique of RT-PCR. All culture experiments had greater than 54% developing to the blastocyst stage, with the average percent developing into blastocysts being 78.5%. The overall mean of cells per embryo was 36.6 ± 1.85 (n=195), which is consistent with previous in vitro results by Chatot et al. (1990). Mutant GDH (mGDH) internal control plasmid has been isolated from DH5 α bacterial cells and subsequently linearized for RNA transcription via digestion with Hind III. In future experiments, mGDH internal control RNA will be transcribed from the pGDHmJL plasmid. RNA from CZB cultured embryos spiked with mGDH RNA will be subjected to quantitative RT-PCR to determine the developmental expression of endogenous GDH.

INTRODUCTION

CZB medium has been developed to culture mouse embryos from the 1-cell stage to the blastocyst stage. This in vitro system is unique because it contains glutamine at a concentration of 1 mM (Chatot et al., 1990). Studies indicate that glutamine is essential to the development of some strains of mouse during the preimplantation stages in vitro and it is used in vivo as an energy source (Chatot et al., 1989). Glutamate Dehydrogenase (GDH) is an enzyme that is necessary for the metabolism of glutamine.

The purpose of our research is to determine the amount of GDH expressed, via the analysis of mRNA, in preimplantation mouse embryos that have developed both in vivo and in vitro at various developmental stages. My specific goal is to analyze the GDH mRNA levels of those embryos that have been cultured in CZB medium using the technique of RT-PCR. The data collected will allow us to determine if our culture system permits GDH expression within the preimplantation embryos in an "in vivo like" manner.

LITERATURE REVIEW

Optimal CZB Culture System

"The ability of 1-cell mouse embryos to develop in vitro has been shown to be a function of mouse strain, medium components, and culture conditions" (Chatot, 1989). Certain mouse strains exhibit what is called a "2-cell block" in which the embryos become

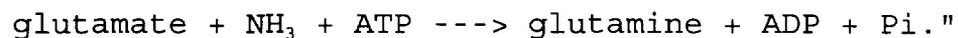
arrested at the 2-cell stage after one cleavage. In 1983, Goddard and Pratt showed, using a series of cytoplasmic-transfer experiments, that this 2-cell block is maternally regulated (Chatot, 1989).

Chatot et al. (1989) have developed a culture system in which mouse embryos will successfully develop from the 1-cell stage, past the 2-cell block, and into blastocysts. CZB medium, a further modification of modified BMOC-2, contains an amount of lactate to give an increased lactate/pyruvate ratio of 116, 1 mM-glutamine and 0.1 mM-EDTA but lacks glucose (Chatot et al., 1989). Chatot and colleagues showed in 1989 that the use of CZB medium for the culture of CF1 X B6SJLF1/J 1-cell embryos (which block at the 2-cell stage in vitro in most media) allowed for the development of 83% of these embryos beyond the 2-cell stage. They also showed in 1990 that 71% of CF1 X B6SJLF1/J embryos developed into blastocysts, as long as glucose was added to the CZB culture medium at 48 hours of culture. These results suggest that the optimal development of 1-cell embryos to the blastocyst stage does occur in this CZB culture system, with the addition of glucose to the medium at 48 hours. This suggestion was also supported by evidence showing that the blastocysts raised in the CZB culture system with the glucose addition at 48 hours had significantly more cells than did those embryos raised in CZB medium alone without the addition of glucose.

Glutamine Metabolism

Neerman et al. (1996) states that glutamine metabolism consists of a special metabolic network, glutaminolysis, which includes up to eight different pathways. "In addition to its function as an essential amino acid, glutamine acts as an important energy source when deaminated into the TCA intermediate α -ketoglutarate" (Neermann et al., 1996). It is known to function as both a primary energy source and a primary carbon source in a variety of cell types and embryos. Hankard et al. (1995) suggest that glutamine plays a significant role in adult humans by maintaining the protein homeostasis and acid-base balance and by being the preferred source of energy for the gut and for the immune system.

Erecinska et al. (1996) states that "glutamine synthetase catalyzes the omega-amidation of glutamate to form glutamine according to the equation:



These researchers suggest that this reaction occurs in two steps. In the first step, glutamate binds the enzyme via ATP- and Mg-dependent binding. ATP is cleaved, which forms the gamma-carboxyl-activated enzyme. In the second step, the enzyme-bound intermediate reacts with ammonia to form glutamine. Glutamine can be used to form glutamate and ammonia by the reverse reaction which is catalyzed by phosphate-activated glutaminase (Erecinska et al., 1996).

The results of experiments performed by Chatot et al. (1990)

suggest that "glutamine can be used as an energy source for the generation of CO₂ through the TCA cycle by all stages of preimplantation mouse development, whether raised in vivo or in vitro from the 1-cell stage." The primary influence of the glutamine is seen during the first 48 hours of culture in which it "promoted significant improvement in development from the 1-cell stage to the blastocyst stage" (Chatot et al., 1989). This suggests that the glutamine plays an important role in energy metabolism in the earlier embryonic stages in vitro. This correlates with in vivo evidence in which as much as 70% of the glutamine uptake by 2-cell embryos was converted into CO₂ (Chatot et al., 1990).

Glutamate (Glutamic Acid)

"Glutamic acid is an amino acid with multiple biological roles" (Erecinska et al., 1996). Banner et al. (1987) states that, within the central nervous system, glutamate is the most abundant free amino acid. It acts as a major excitatory neurotransmitter, and it's known to be a precursor in the biosynthesis of gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter. Glutamate links the metabolism of carbon and nitrogen because it can act as a source of energy or as a reservoir for ammonia (Erecinska et al., 1996).

Glutamate can be synthesized by three primary pathways. It can be formed by the hydrolytic cleavage of glutamine to glutamate, which is catalyzed by glutaminase (previously discussed). It can

be formed by transamination from other amino acids. Lastly, glutamate can be synthesized by the reductive amination exhibited in the following reaction, which is catalyzed by glutamate dehydrogenase (Erecinska et al., 1996):



Glutamate Dehydrogenase (GDH) - Molecular Properties and Roles

Glutamate Dehydrogenase is a mitochondrial enzyme that has an oligomeric structure. The smallest enzymatically active form of GDH is a hexamer with a molecular mass of approximately 320,000 daltons. The hexamer consists of only one type of subunit with each having a molecular mass of approximately 56,000 daltons. The subunits are "arranged in two layers of trimers in the form of a triangular antiprism" (Kvamme, 1988). Kvamme (1988) reports that at protein concentrations above 1.0 mg/ml ox liver glutamate dehydrogenase forms polymers that are larger than hexamers. Linear polymers (rods) may form, and the linear polymers may aggregate to form two-dimensional sheets or helical tubular structures. This type of polymerization is also exhibited by pig liver GDH, human liver GDH, and ox brain GDH. Rat liver GDH, on the other hand, does not exhibit this polymerization or will do so only slightly (Kvamme, 1988).

Glutamate dehydrogenase catalyzes the oxidative deamination of glutamate to synthesize α -ketoglutarate, and it catalyzes the reductive conversion of ammonium nitrogen into organic nitrogen in the production of glutamate (Das et al., 1996). This reversible

reaction occurs according to the following equation:



Plants and microorganisms will specifically use either NAD(H) or NADP(H) as a coenzyme, but vertebrates can use both of these coenzymes (Kvamme, 1988). Rat liver GDH, for example, uses NADPH for the synthesis of glutamate, but it uses NAD⁺ for the synthesis of α -ketoglutarate (Das et al., 1996).

GDH - Synthesis and Localization

Glutamate dehydrogenase is a ubiquitous enzyme, but its concentration varies among different tissues. The highest concentrations are found in the liver, brain, kidney, and pancreas, with the liver being the richest source (Das et al., 1996; Kvamme, 1988). Lower concentrations are also found in the intestinal and gastric mucosa, heart, spleen, skeletal muscle, and lungs (Kvamme, 1988). Geerts et al. (1996) suggest that gender may have an effect on the varying concentrations of GDH, at least in rat liver.

Kvamme (1988) reports that "within liver and brain cells glutamate dehydrogenase is localized in the mitochondrial matrix. There are reports, though, indicating that there are small amounts of GDH in the nucleus. Some researchers propose that the GDH is synthesized outside of the mitochondria and then transported into it. This view is supported by the evidence showing that GDH is able to bind to cardiolipin phospholipid, a primary component of the mitochondrial membrane (Kvamme, 1988).

GDH - Regulation of the Enzyme

GDH is an allosteric enzyme and each of its subunits has multiple binding sites for purine nucleotides (Wrzeszczynski et al., 1994). Allosteric inhibitors include guanosine triphosphate (GTP), adenosine triphosphate (ATP), and high concentrations of NADH. Allosteric activators include guanosine diphosphate (GDP) and adenosine diphosphate (ADP) (Wrzeszczynski et al., 1994; Stryer, 1988). Stryer (1988) states that this suggests that "a lowering of the energy charge accelerates the oxidation of amino acids." Research shows that ADP and NADH each bind at two sites per subunit. GTP also binds at two sites per subunit when NADH is present, but it binds only to one site in the absence of NADH (Wrzeszczynski et al., 1994).

There are a variety of other compounds that are able to help regulate GDH in vivo. Amino acids, such as alanine, leucine, and methionine, are known to be allosteric activators of GDH (Kvamme, 1988). Erecinska et al. (1996) states that it has even been reported recently that "mitochondria possess a factor, or factors, that enhance the sensitivity of GDH to leucine." Research shows that zinc ions, thyroxine, steroid derivatives, and phosphoenolpyruvate are all able to affect the activity of GDH. Erecinska et al. (1996) reports that steroid hormones act to allosterically inhibit GDH. Studies indicate that antipsychotic drugs including chlorpromazine, phenothiazine, and butyrophenone inhibit GDH in the ox brain and liver (Kvamme, 1988).

GDH - Enzyme Kinetics

The equilibrium of the reaction catalyzed by GDH is in favor of glutamate formation. The equilibrium constant (K_{eq}) can be determined by the following equation:

$$K_{eq} = [\alpha\text{-ketoglutarate}] [\text{NAD(P)H}] [\text{NH}_4^+] [\text{H}^+] / [\text{glutamate}] [\text{NAD(P)}^+] [\text{H}_2\text{O}].$$

Under the conditions thought to approximate those in the liver (38 °C and 0.25 ionic strength), the K_{eq} has been determined to be 6.97×10^{-15} M with NAD(H) and 4.48×10^{-15} M with NADP(H) (Kvamme, 1988). Erecinska et al. (1996) states that "the direction in which the enzyme operates in vivo depends not only on the value of K_{eq} but also on the prevailing intracellular concentrations of substrates and products of the reaction." The concentrations of the reactants in vivo, for example, can influence the activity of GDH in a couple of ways. First, they may determine the direction in which the enzyme operates by affecting the mass action ratio. Second, the reactant concentrations, when at levels below saturation, may determine the rate of the individual reaction by limiting the enzyme activity kinetically. Conditions in which glutamate is decreased and/or α -ketoglutarate, ammonia, and mitochondrial $[\text{NADH}]/[\text{NAD}]$ are increased will shift the reaction towards glutamate synthesis (Erecinska et al, 1996).

Brain Glutamate Dehydrogenase cDNA

The gene for GDH has been characterized in a variety of tissues. A cDNA for brain GDH is used in our lab. The cDNA has an open reading frame of 774 nucleotides. This reading frame

codes for 258 amino acids. This amino acid sequence corresponds to amino acids 244-501 of human liver GDH (Banner et al., 1987). Banner et al. (1987) reported that this human brain GDH cDNA is 95% homologous with the amino acid sequence of human liver GDH and 97% homologous with the bovine liver GDH sequence. This suggests that this particular gene has been conserved across species.

METHODS AND MATERIALS

Superovulation and Mating

NSA female mice from Colony 217 (Harlan Sprague Dawley, Indianapolis, IN) were superovulated via intraperitoneal injection of hormones. On Day 1 the females were injected with 10 IU Pregnant Mare Serum (PMS). Forty-eight hours later, 5 IU Human Chorionic Gonadotropin (hCG) were injected into the females. Following the hCG injection, the females were mated overnight with B6SJLF1/J males (Jackson Labs, Bar Harbor, ME).

CZB Culture Preparations

CZB medium was prepared in advance according to the procedures of Chatot et al. (1989). All holding dishes and culture dishes were prepared several hours prior to the collection of embryos. Sterile conditions were used at all times. When collecting embryos from 10 mice, 3 holding dishes were prepared, each with 9 50 μ l drops of CZB covered with CZB-rewashed oil. These holding dishes were placed into an incubator at 37 °C. Seven culture dishes were prepared, each with a wash drop and 2 final culture drops (50 μ l each); these were also covered with CZB-rewashed oil. The culture dishes were placed into a sealed chamber and gassed with 5% CO₂/5% O₂/90% N₂. The sealed chamber was placed into the incubator to equilibrate to 37 °C.

Embryo Collection and Culture Procedures

Female mice were sacrificed by rapid cervical dislocation immediately prior to the collection of 1-cell embryos at 25 hour post hCG. This procedure and all other animal protocols have been approved by ACUC #ACC 92-4E through 9/30/97. Following cervical dislocation, the oviducts were excised from the female mice and placed into 0.9% sterile saline solution. The oviducts were flushed, using a syringe and 30 gauge needle, with Hank's Balanced Salt Solution with Bovine Serum Albumin (HBSS+BSA). Embryos were washed through HBSS+BSA three times. It was then determined if 1-cell embryos were fertilized or unfertilized. Fertilized embryos were randomly segregated into groups of 25 embryos and each group was placed into a drop of CZB medium in a holding dish. Unfertilized embryos were discarded.

One-cell embryos were mouth-pipetted from the CZB holding drop, washed through the CZB wash drop, and placed into the final CZB culture drop in the culture dish. Culture dishes were placed back into the sealed chamber and regassed with the gas combination previously described. The chamber was placed into the 37 °C incubator. Cultures were removed from the incubator after 48 hours of incubation time, and 2.5 μ l of glucose (100 mg/ml) stock were added to each culture drop. The cultures were placed back into the sealed chamber, regassed, and returned to the incubator. Embryos were removed from culture in aliquots of 100 at the early and late 2-cell, 4-cell, 8- to 16-cell, and blastocyst stages. The embryos were washed with HBSS+BSA and

frozen at -70 °C until needed for RNA quantitational analysis.

Hoechst Stain

To confirm the developmental stage of the CZB cultured embryos, an aliquot of 25 embryos which had been cultured until Day 5 was removed from each culture. These embryos were fixed with 4% paraformaldehyde. The embryos were stained with Hoechst Stain (No. 33258; 10 μ g/ml) for 30 minutes. This stain is specific for DNA in the nucleus of the cell. The stained embryos were viewed and nuclei counted using a Zeiss Light Microscope with epifluorescence using UV filters. Phase-contrast and fluorescent photos were taken of the CZB-cultured blastocyst embryos using the Zeiss MC 80 Microscope Camera. Photos were also taken of in vivo developed blastocyst embryos for comparison purposes.

Mutant Internal Control GDH (mGDH) Plasmid Preparation and Digestion

The internal control plasmid construct, labeled as pGDHmJL, was previously prepared in our lab. It is a modified pYN751 that contains a 1796 bp GDH cDNA in the Promega pGEM-3Z plasmid vector that has been mutated to lose two AlwNI sites, one in the insert and one in the vector (Banner et al., 1987; Lawry, 1994). DH5 α Bacterial cells containing pGDHmJL construct were grown overnight in Luria Broth with ampicillin in a shaker incubator set at 37 °C. The construct contains an ampicillin-resistance region

which ensures that only those bacteria with the construct will survive and proliferate in the LB/Amp. The plasmid was isolated from the bacteria using a Wizard Miniprep DNA Purification Kit (Promega, Madison, WI). To produce a cleared lysate, it was first necessary to pellet the cells (10 ml) by centrifugation for 2 minutes at top speed. The pellet was resuspended in 200 μ l Cell Resuspension Solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100mg/ml RNase A). Cell Lysis Solution (200 μ l; 0.2 M NaOH, 1 mM EDTA) was added to the mixture and the tubes were inverted until the mixture was clear. This was followed by the addition of 200 μ l Neutralization Solution (1.32 M Potassium acetate, pH 4.8). The tubes were centrifuged for 5 minutes. The resulting supernatant was decanted to fresh tubes and the pellet was discarded. One ml of Wizard Miniprep DNA Purification Resin was added to the cleared lysate and the tubes were inverted. The Resin/DNA was vacuum dried over PCR Prep minicolumns to purify the plasmid DNA. The plasmid DNA was washed with 2 ml of Column Wash Solution (200 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA): 100% EtOH (50:50). The plasmid DNA was eluted from the columns using 50 μ l heated (65 °C) nuclease-free water. Spectrophotometric analysis was done to determine the DNA plasmid concentration. Plasmid DNA was stored at 4 °C until digest procedures.

It was necessary to linearize the plasmid DNA so it could act as the DNA template during the transcription reaction. Digestion of the plasmid was done using Hind III restriction

enzyme for 1 hour at 37 °C. Digested samples were extracted with an equal volume of phenol and chloroform:isoamyl alcohol (24:1) and ethanol precipitated to remove the enzyme and repurify the linearized plasmid. A portion of the digested sample and undigested sample, along with the Lambda Hind III standard, was electrophoresed on a 0.7% Agarose gel to confirm concentration and to determine if linearization was complete. Purified digested plasmid was stored at 4 °C until transcription procedures.

Mutant GDH Internal Control Plasmid Transcription Procedures

Purified linearized plasmid (1.0 $\mu\text{g}/\mu\text{l}$) was transcribed into RNA using a Riboprobe in vitro Transcription System from Promega. Gloves were worn at all times during this procedure. The linearized plasmid was heated to 68 °C for 5 minutes and cooled quickly on ice prior to the addition of the reaction mix. The reaction mix contained Transcription Optimized 5X Buffer, 100mM DTT, RNasin Ribonuclease Inhibitor (100 U), NTPs (ATP, GTP, UTP, GTP at 2.5 mM each), nuclease-free water, and T7 RNA Polymerase (30-40 U). All solutions were kept at room temperature except for the T7 RNA Polymerase which was kept at 4 °C. The reaction mix was added to the linearized plasmid template and this mixture was incubated at 38-39 °C for 1.5 hours to allow RNA synthesis to occur.

The DNA template was removed by the addition of RQ1-DNase (1 U/ μg) to the mixture, followed by the incubation at 37 °C for 30

minutes. After 30 minutes, 1 volume of phenol:chloroform (50:50) was added to the mixture. This solution was microcentrifuged and the upper, aqueous phase was transferred to a fresh tube. One volume of chloroform:isoamyl alcohol (24:1) was added to the aqueous solution. This was microcentrifuged for 2 minutes. The upper, aqueous phase was transferred to a fresh tube. This was followed by the addition of 100% ethanol (2.5 volume) and 7.5 M ammonium acetate (0.5 volume) to the aqueous solution. This mixture was precipitated for 30 minutes at -70 °C. After 30 minutes, the solution was centrifuged at 4 °C for 15 minutes.

To remove the unincorporated nucleotides, the supernatant was drained and the pellet was resuspended in 1 M ammonium acetate and 3 volume cold absolute ethanol. This was precipitated at -70 °C for 30 minutes. Microcentrifugation was done at 4 °C for 10 minutes. The supernatant was discarded and the pellet was rinsed with 70% ethanol. After the pellet had air-dried, it was resuspended in 20 μ l nuclease-free water. Spectrophotometric analysis was performed to determine the RNA concentration. Mutant GDH RNA was stored at -70 °C until the RT-PCR reactions.

RNA Isolation (Mouse Brain and Embryonic)

Sterile conditions were used; all glassware and pipette tips were autoclaved for 30 minutes and then dried for 10 minutes. Gloves were worn at all times. Mouse brain RNA was isolated to

be used as a control RNA. Two mice were sacrificed by rapid cervical dislocation and the brain tissue was dissected out of the skull. The RNA isolation was completed using the acid guanidine thiocyanate phenol chloroform (AGPC) method of Chomczynski and Sacchi (1987). Brain tissue was homogenized with 5 volumes of Solution D (200 μ l per 100 embryo aliquot) (Solution D contains 4M Guanidine Thiocyanate, 25mM Sodium Citrate-pH 7, 0.5% Sodium Lauryl Sacrcosine, and 0.1 M 2-mercaptoethanol) using a tissue grinder (embryos are vortexed for 1-2 minutes). After homogenization, 0.1 volume 2 M NaOAc, 1 volume water-saturated phenol, and 0.2 volume chloroform:isoamyl alcohol (49:1) were added to the brain or embryo extracts. This solution was placed on ice for 15 minutes and centrifuged for 20 minutes at 8 °C at 10,000X g. The aqueous phase was removed and an equal volume of isopropanol was added. This mixture was placed into the -20 °C freezer for 1 hour. The mixture was centrifuged for 20 minutes at 4 °C at 10,000X g, and the resulting pellet was resuspended in 2 ml Solution D (200 μ l for embryos). This was followed by the addition of 2 ml isopropanol (200 μ l for embryos) and a subsequent precipitation for 1 hour at -20 °C. Centrifugation was done for 15 minutes at 4 °C at 10,000X g, and the resulting pellet was washed with 70% cold EtOH. The air-dried pellet was resuspended in 500 μ l nuclease-free water. The total RNA concentration of brain tissue was determined using a Beckman Spectrophotometer. The RNA was aliquoted into 20 μ l/tube and stored at -70 °C until needed for RT-PCR. Prior to embryo RNA

isolation, the mutant control RNA was added to the embryo tubes in concentrations ranging from 1 fg to 1000 fg per embryo.

Embryonic RNA was then isolated according to the AGPC method from 1-, 2-, 4-, 8-16-cell, and blastocyst stages of CZB cultured embryos. This RNA was also stored at -70 °C until the RT-PCR reactions.

Embryo and Control RT-PCR, and Product Purification

RNA from 50 embryo equivalents containing a predetermined amount of mGDH internal control RNA per embryo will be reverse transcribed using a 3' antisense GDH gene specific primer to produce a GDH specific cDNA. The cDNA will be amplified using the polymerase chain reaction overnight in an ERICOMP Thermocycler. This will be done using the same 3' antisense primer and a 5' sense primer. The 3' primer is a 24mer with the sequence of 5' CCT TTC GTA CCA CCT TGA TAA GGG 3'. The 5' primer is a 25mer with the sequence of 5' G GCA AAG CCT TAT GAA GGA AGC ATC 3'. A human brain GDH cDNA sequence was used to design the primer set (Banner et al., 1987). Cycle 1 involves the denaturation of the samples at 94 °C for 5 minutes. Cycle 2 consists of 3 steps: denaturation at 94 °C for 1 minute, reannealing at 42 °C for 1 minute, and polymerization at 72 °C for 1 minute. This PCR cycle is repeated 60 times. Cycle 3 is an extension step at 72 °C for 10 minutes. The samples are held at 4 °C until they are removed from the PCR machine.

The cDNAs are purified using a Wizard PCR Preps DNA

Purification System with PCR Preps minicolumns. The PCR products were transferred into 100 μ l of Direct Purification Buffer (50 mM KCL, 10 mM Tris-HCl pH 8.8, 1.5 mM MgCl₂, 0.1% Triton X-100). One ml of PCR Preps DNA Purification Resin was added to the PCR Product/Buffer solution and this mixture was vortexed. This mixture was vacuum dried over the PCR Prep minicolumns to purify the cDNA. The cDNAs were washed with 2 ml 80% isopropanol. The cDNA was eluted from the columns using 50 μ l heated (65 °C) nuclease-free water. Purified products are stored at 4 °C until they are digested. Positive controls will include the RT-PCR of mouse brain RNA and mutant control RNA alone, and PCR of the intact control plasmid. Negative controls will include RT-PCR of a no RNA sample.

Product Digestion, Southern Blotting, and Quantitative Analysis

Digestion of RT-PCR products will be done using AlwN1 restriction enzyme for 1 hour at 37 °C. Digestion with this restriction enzyme will allow us to distinguish between the product of the mutant GDH internal control RNA of known concentration and the embryo specific cDNA for GDH of unknown concentration. A difference will be seen in the number of bands that result and in their locations. The mGDH internal control was specifically constructed such that it lacks the AlwN1 restriction site (Lawry, 1994). This restriction site is present normally in endogenous GDH. The products will be electrophoresed on two-percent agarose gels, along with pBR322 MspI as a

standard. Southern Blotting procedures may also be used to confirm the concentration of cDNA present. This would involve the use of a digoxigenin-labeled GDH specific probe to visualize the RT-PCR product bands. The digestion of the GDH endogenous cDNA will yield two fragments that are 220 bp and 149 bp in length. The mGDH will yield one band that will be 365 bp in length. The intensity of the embryonic bands will be compared to the intensity of the mutant GDH internal control band. Quantitative analysis will be done using scanning densitometry and peak height analysis via Gaussian Distribution. Each experiment will be repeated three times.

RESULTS AND DISCUSSION

Embryo Culture

Embryos were cultured in CZB media from the 1-cell stage to the early and late 2-cell, 4-cell, 8- to 16-cell, and blastocyst stages. Embryos, in aliquots of 100, were frozen at these developmental stages for the subsequent RT-PCR reactions. From each culture, a set of embryos (usually 25) was permitted to develop to the blastocyst stage. Results (Table 1) indicate that the embryos successfully developed into blastocysts. All culture systems had greater than 54% developing to the blastocyst stage. Several cultures had 100% of the embryos developing into blastocysts. The average percent developing into blastocysts was 78.5%. These results are consistent with those of Chatot et al. (1990) who have previously shown that "one-cell embryos cultured in CZB medium with 1 mM-glutamine and a Day-3 glucose injection developed better than any other variation, with 71% reaching the blastocyst stage."

These blastocysts were stained with Hoechst Stain to visualize the nuclei, which acts as an indicator of the number of cells per embryo. The results (Table 1) showed a range of 22.5 to 67.3 average cells per embryo, with the overall mean being 36.6 ± 1.85 (n=195) cells per embryo. This is consistent with the results of Chatot et al. (1990) who found that there were 34.33 ± 1.16 (n=69) cells per embryo in blastocysts developing in the CZB medium with the glucose addition on Day 3 of culture.

Table 1: Culture results from 1-cell embryos in CZB medium.

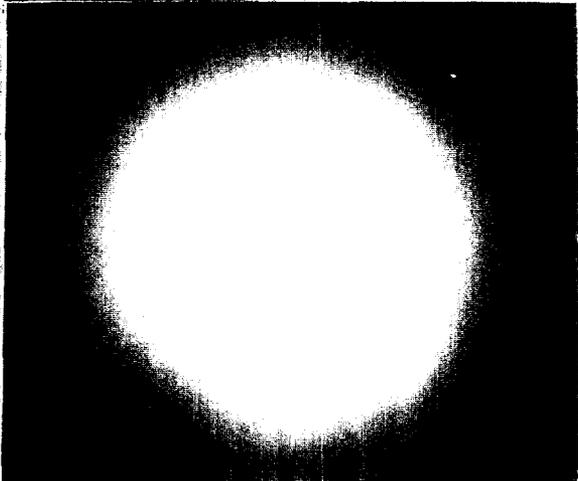
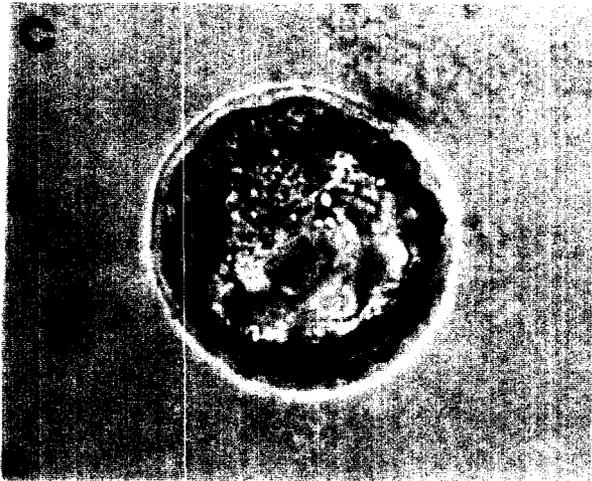
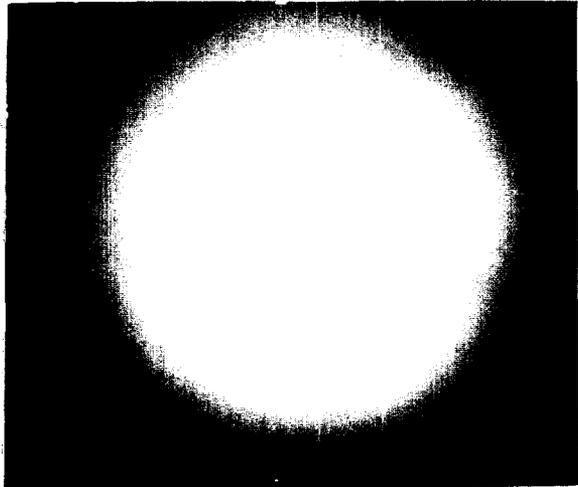
Culture #	# emb. cult.	stage frozen	# frozen	# Day 5 M+B1	% Day 5 M+B1	ave. # cells/embryo
1	53	Bl	-	29	55	67.3
2	223	Bl	144	162	73	24.5
3	261	e2-C	224	25	100	34.2
4	271	e2-C	248	16	70	28.6
5	232	8-C	198	25	100	28.4
6	290	12-C	249	25	100	22.5
7	271	12-C	199	16	64	35.2
8	229	D4M	188	14	56	24.4
9	238	3-8-C	200	38	100	44.6
10	103	3-8-C	97	2	67	38.0

Abbreviations: emb., embryos; e2-C, early 2-cells; 12-C, late 2-cells; 3-8-C, 3-8-cells; 8-C, 8-cells; D4M or M, morula taken on Day 4 of culture; Bl, blastocysts; ave., average.

Figure 1

Photos of blastocysts were taken for comparison purposes. Figures A and B are Phase-Contrast and Hoechst stained Fluorescence photographs respectively of CZB cultured blastocysts. Figures C and D are Phase-Contrast and Hoechst stained Fluorescence photographs respectively of in vivo developed blastocysts.

A



This in vitro data correlates with in vivo data which yielded an average of 38.0 ± 4.96 (n=10) cells per embryo.

Photos of in vitro (Fig. 1A, B) and in vivo (Fig. 1C, D) developed embryos are shown for comparison purposes. Figures 1A and 1C are Phase-Contrast photos of the blastocysts and Figures 1B and 1D are Fluorescent photos showing the Hoechst-stained nuclei. Visual observation suggests that the in vivo and cultured embryos have developed similarly.

pGDHmJL Plasmid Isolation and Digestion

pGDHmJL plasmid DNA was isolated from the DH5 α bacterial cells and digested with Hind III according to the procedures. The resulting DNA (digested and undigested) was electrophoresed on 0.7% agarose gels with Lambda Hind III standards (Figure 2). Figure 3 shows the Hind III digestion of 5 samples of mGDH that were subsequently used for RNA transcription. All digestions (Figure 2 and 3) were successful. The linearized DNA runs as a single band at approximately 4.535 kilobases (Lawry, 1994). These single bands differ from the double bands exhibited by the undigested samples in Figure 2. The undigested DNA runs faster on the agarose gel due to supercoiling of the DNA as compared to the Hind III linearized DNA, which runs true to size (Figure 2). The difference in size (4 bp) between the GDH plasmid and the mGDH plasmid can not be detected on this gel. They appear to run at the same rate.

Figure 2

Photograph of a 0.7% agarose gel of GDH and mGDH plasmids undigested and digested with Hind III. The Lambda Hind III standards, in lane 1, yield bands at 23.13, 9.42, 6.56, 4.36, 2.32, and 2.03 kilobases from the top to the bottom. The GDH (lanes 4, 5) and mGDH (lanes 6,7) plasmid samples, undigested and digested respectively, are shown. * indicates digested GDH plasmid; ** indicates digested mGDH plasmid.

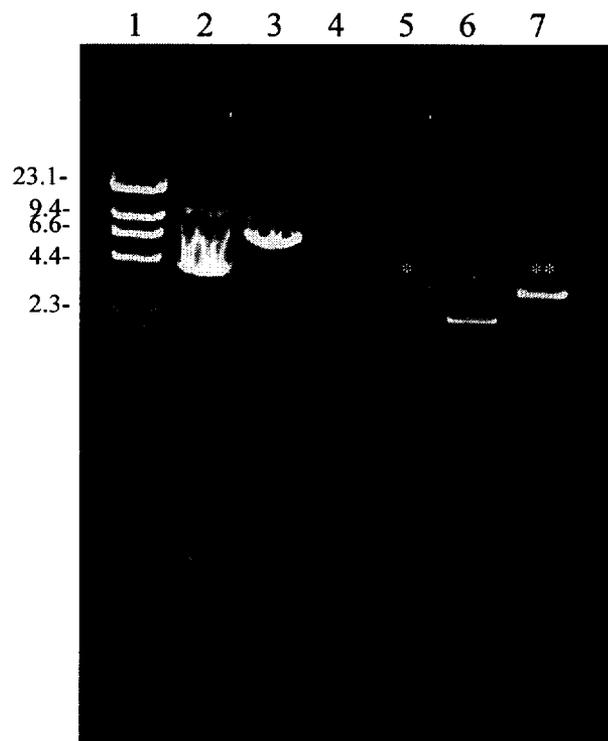
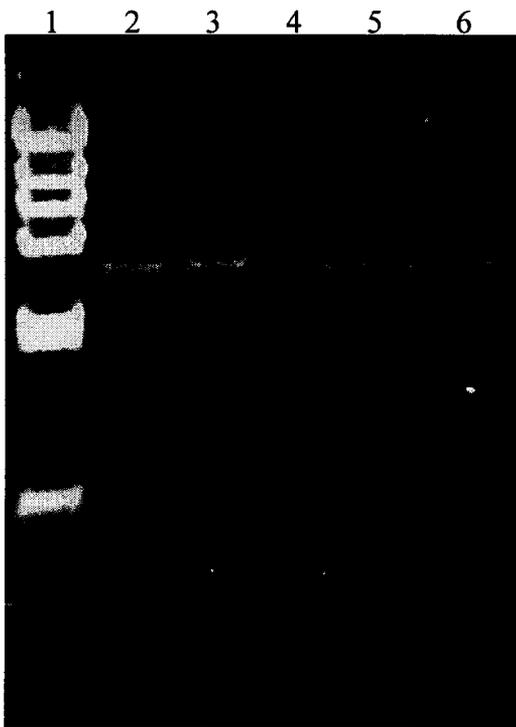


Figure 3

Photograph of 0.7% agarose gel showing 5 samples of mGDH plasmid digested with Hind III (lanes 2-6). Lambda Hind III standards (lane 1) are used for this gel (see legend for Figure 2 for sizes).



RNA Transcription

RNA was transcribed from purified linearized pGDHmJL plasmid DNA. Following the spectrophotometric analysis of the RNA, it was subjected to RT-PCR to assess RNA quality. The reverse transcription reaction was performed to yield cDNA. This cDNA was amplified using the Polymerase Chain Reaction. Figure 4 shows a photo of a 2.0% gel containing the RT-PCR products. The RT-PCR reaction should yield a band of 369 bp for endogenous GDH and 365 bp for mGDH. The products from the PCR alone of mGDH plasmid (lane 6) yielded a band of 365 bp indicating that the PCR reaction was successful. RT-PCR of mouse brain RNA (lane 7) yielded an appropriate 369 bp band. RT-PCR of mGDH RNA (lane 8) did not yield a band of the appropriate 365 bp size suggesting that the RNA transcription was not optimum. In addition, the background in the PCR reaction was high. The no RNA control (lane 2) demonstrates lack of reagent contamination in the reaction.

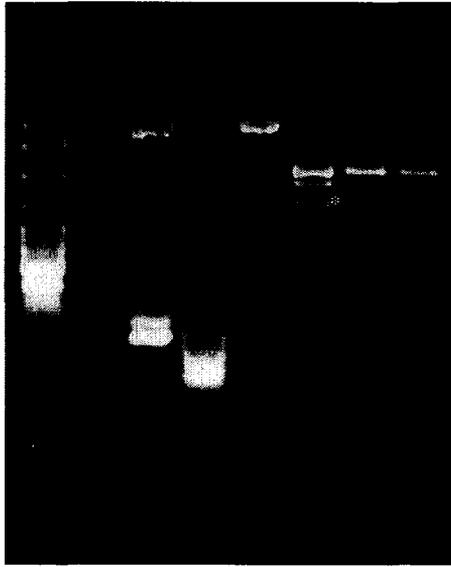
There are a variety of factors that may be hindering the success of the RNA Transcription and RT-PCR reactions. The most common problem that occurs when working with RNA is the degradation of the RNA by RNase contamination. This type of contamination can degrade the newly transcribed RNA during the transcription reaction causing it to be unsuccessful, which will subsequently cause the RT-PCR to be unsuccessful. It is highly unlikely that this is the source of our problems because we use RNasin Ribonuclease Inhibitor from Promega in our experiments.

Figure 4

Photograph of 2.0% agarose gel of positive control RT-PCR products. The pBR322 MspI Standard, shown in lane 1, has its first five bands from the top at 622, 527, 404, 307, and 238/242 base pairs. Lane 2 is the no RNA control. Lane 5 shows the PCR of the mGDH plasmid. Lane 6 shows the RT-PCR of GDH mouse brain RNA. Lane 7 shows the RT-PCR of mGDH RNA. * indicates 365-369 bp RT-PCR products.

1 2 3 4 5 6 7 8

.622-
.404-
.397-
.242-



It is possible that, during the transcription reaction, the DNA template is being precipitated by the spermidine in the Transcription Optimized 5X Buffer. This can be prevented by adding the components of the reaction in the stated order and at room temperature. Another problem could be that incomplete transcripts are being synthesized due to premature termination of the RNA transcription reaction. In some cases, changing the incubation temperature from 37 °C to 30 °C may increase the proportion of full-length transcripts. It also known that the carryover of reagents (SDS, heparin, and guanidine thiocyanate, NaCl) from some RNA purification methods can interfere with the RT-PCR reaction. Doyle (1996) suggests solving this problem with additional purification steps or changes in the purification protocol, along with a reduction in the volume of target RNA. Studies show that any residual NaCl (used to precipitate the template DNA) may inhibit the RNA Polymerase by as much as 50%. Doyle (1996) suggests that desalting of the template DNA may be accomplished by column chromatography, reprecipitating the template in the presence of another salt, and washing the pellet 1-2 times with 70% ethanol. The RT-PCR reaction may not be optimal due to inhibitors present in the RNA sample. To improve this reaction, it may be necessary to ethanol precipitate the RNA reaction mixture prior to the RT reaction to remove possible inhibitors. Doyle (1996) also suggests reducing the volume of the sample in the reaction. It also possible that the Mg^{2+} concentration is not optimal for the transcribed RNA, even though

we have shown it to be effective for the mouse brain RNA. Incorporation of some or all of these changes may help to optimize the transcription and RT-PCR reactions.

Future Experiments

The transcription reaction will be repeated to synthesize new mutant GDH (mGDH) internal control RNA from the pGDHmJL plasmid. This RNA will be added to the RNA from 50 embryo equivalents, and the mixture will be reverse transcribed to synthesize cDNA. The cDNA will be amplified using the polymerase chain reaction, and the amplified cDNAs will be digested with AlwN1 to distinguish between the endogenous RNA and the internal control RNA. To complete this project, quantitative analysis will be done to determine the amount of GDH RNA expressed at various developmental stages in CZB cultured mouse embryos.

LITERATURE CITED

- Banner, C., Silverman, S., Thomas, J.W., Lampel, K.A., Vitkovic, L., Huie, D. and Wenthold, R.J. (1987). Isolation of human brain cDNA for glutamate dehydrogenase. Journal of Neurochemistry. 49: 247-251.
- Chatot, C.L., Ziomek, C.A., Bavister, B.D., Lewis, J.L. and Torres, I. (1989). An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J. Reprod. Fert. 86: 679-688.
- Chatot, C.L., Tasca, R.J. and Ziomek, C.A. (1990). Glutamine uptake utilization by preimplantation mouse embryos in CZB medium. J. Reprod. Fert. 89: 335-346.
- Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.
- Das, A.T., Salvado, J., Boon, L., Biharie, G., Moorman, A.F. and Lamers, W.H. (1996). Regulation of glutamate dehydrogenase expression in the developing rat liver. Eur. J. Biochem. 235: 677-682.
- Doyle, K. (1996). Promega: Protocols and Applications Guide., Third edition, Promega Corporation, Madison, Wisconsin: 114-122, 192-206.

- Erecinska, M. and Silver, I.A. (1990). Metabolism and role of glutamate in mammalian brain. Progress in Neurobiology. 35: 245-296.
- Geerts, W.J., Verburg, M., Jonker, A., Das, A.T., Boon, L., Charles, R., Lamers, W.H. and Van Noorden, C.J. (1996). Gender-dependent regulation of glutamate dehydrogenase expression in periportal and pericentral zones of rat liver lobules. J. Histochem. Cytochem. 44: 1153-1159.
- Hankard, R.G., Darmaun, D., Sager, B.K., D'Amore, D., Parsons, W.R., and Haymond, M. (1995). Response of glutamine metabolism to exogenous glutamine in humans. Am. J. Physiol. 269: E663-E670.
- Kvamme, E. (1988). Glutamine and Glutamate in Mammals. CRC Press, Inc., Florida: 82-93.
- Lawry, J.R. (1994). Characterization of the expression of glutamate dehydrogenase in preimplantation mouse embryos using competitive reverse transcription-polymerase chain reactions. Graduate School Thesis, Ball State University, Muncie, IN.
- Neerman, J. and Wagner, R. (1996). Comparative analysis of glucose and glutamine metabolism in transformed mammalian cell lines, insect and primary liver cells. J. Cell. Physiol. 166: 152-169.
- Stryer, L. (1988). Biochemistry. W.H. Freeman and Company, New York: 495-496, 575-579.

Wrzeszczynski, K.O. and Colman, R.F. (1994). Activation of bovine liver glutamate dehydrogenase by covalent reaction of adenosine 5'-O-[S-(4-Bromo-2,3-dioxobutyl)thiophosphate] with arginine-459 at an ADP regulatory site. Biochemistry. 33: 11544-11553.