Developmental Biology of the Mouse Embryo Glutamate Dehydrogenase

An Honors Thesis (HONRS 499)

by

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Abstract

The purpose of this thesis was to obtain new data about the development of the mouse embryo during preimplantation stages. The research was based on Dr. Clare Chatot's earlier experiments and data which indicated that glutamine is used as an energy substrate in preimplantation mouse embryos and that the enzyme glutaminase was present in all preimplantation stages. We hypothesized that the next step in the embryo's metabolism would be the utilization of the enzyme glutamate dehydrogenase to convert glutamate to α-ketoglutarate. The experimental purpose was to detect this enzyme at the 1-cell, 2-cell, 8-cell and blastocyst stages of the embryo's development. The paper provides background information obtained from previous experiments which helped develop our hypothesis. Experiments were completed to test the hypothesis. Results provided control data suggesting that the PCR reaction and RNA preparation were working efficiently. Finally, the paper discusses the results and the conclusions drawn from them.

Introduction

Preimplantation Embryo Development

The embryonic development of the mouse is a complex series of events triggered after fertilization of the egg by the sperm when the pronuclei of both migrate to the center of the egg and unite. In the case of the mouse, preimplantation development of the embryo is slow allowing time for the uterine tissue to prepare for receiving the embryo. After 24 hours, the embryo is still at the two-cell stage. The four-cell stage is achieved after 36 hours, eight-cell stage is achieved after two days, and blastocyst is complete after four days. These slow divisions, without any increase in mass, continue as the embryo moves along the oviduct into the uterus until implantation 4.5 days after fertilization (Hogan, 1986).

During preimplantation development many changes in the pattern of RNA and protein synthesis occur. These changes in patterns of protein synthesis are the result of several processes after fertilization. During the one to two-cell stage there is an increased turnover rate of some proteins made on stable maternal mRNAs (Howlett and Bolton 1985). Also there is substantial evidence of posttranslational modification of proteins synthesized on either maternal or embryonic RNA. These modifications include phosphorylation, glycosylation, or proteolytic cleavage (Banner et al. 1987). Studies have also shown that some maternal mRNAs are utilized or suppressed selectively. At the mid-two-cell stage degradation of specific maternal mRNAs carried over from the oocyte occur and synthesis of proteins from embryonic mRNAs begin.

However, some maternally encoded proteins can persist beyond this stage (Hogan, 1986).

At the eight-cell stage compaction begins. This is a calcium sensitive process where the blastomeres flatten and increase their contact with each other forming tight junctions. Blastomeres also develop distinct apical and basal membrane and cytoplasmic domains. During compaction, cellular changes occur that alter both the cells surface properties and the cytoskeleton. One surface change involves the cells increase in Ca^{++} dependent adhesiveness, which allows the cells to adhere to one another and to lectin-coated beads. Also lamellipodia-like cell processes spread over the cells to increase their adhesive surfaces. Regionalization of microvilli, lectin binding sites, and intracellular organelles allow the cells to express contact-induced cell polarization necessary for compaction. The cells during this time also establish gap junction-mediated intercellular communication between all other cells of the morula unit. Lastly the cells gradually develop apical, zonular tight junctions between outside cells to generate an impermeable outer epithelial layer (Hogan, 1986).

After compaction is complete the embryo has entered the blastocyst stage of development. During this stage, fluid is transported from the embryonic cells to a central blastocoel. This occurs via a basally localized sodium-potassium ATPase pump.

Energy Metabolism in Preimplantation Embryos

For it to be possible for the 1-cell embryo to develop into a blastocyst energy metabolism must be utilized. One metabolic pathway that provides the energy necessary for growth and

development is the Tricarboxylic acid (TCA) cycle. During early development of the embryo glutamine, glucose, pyruvate, and lactate are the energy substrates used by the TCA cycle to generate CO_2. Studies done in vitro and in vivo have shown that glutamine can be utilized as an energy substrate by all preimplantation stage embryos by oxidation to CO_2 through the TCA cycle (Chatot et al. 1990). The percentage of total [^{14}C]glutamine utilized to generate CO_2 in embryos grown in vivo ranged from 70% at the 2-cell stage to 20% at the 8-cell and blastocyst stages. Studies of preimplantation mouse embryos in culture suggest that at the 8-cell stage the embryo increases its utilization of glucose as its energy substrate to free glutamine for other cellular functions. This is required due to the increase in cell numbers and the embryos' need for increased synthesis of nucleic acids and proteins. The presence of glutamine throughout the preimplantation culture period and the requirement for glucose after Day 3 of culture facilitated increased development of embryos to the blastocyst stage. These findings suggest that in vivo glutamine is utilized with lactate and pyruvate as an energy source up to the 8-cell stage when glucose becomes the primary energy source (Chatot et al. 1989-1990).

Glutamine Regulation

Glutamine requires much regulation during this time. Regulation of the amino acid glutamine is dependent upon glutamine synthetase and glutaminase in brain and liver (Nicklas, 1988). Glutamine synthetase replenishes glutamine as it is utilized in the "glutamine cycle" and glutaminase converts glutamine to glutamate

depending on the balance required in the cell. In this process, glutamate and gamma-aminobutyric acid (GABA) levels are maintained by glutamine derived from the extracellular space which in turn can be replenished by synthesis in the glia by glutamine synthetase. Therefore glutamine is important in producing glutamate which is then utilized in the TCA cycle and other reactions.

Glutamate

Glutamate is a free amino acid that is involved in many regulatory functions. Physiological, pharmacological, and biochemical studies provide compelling evidence that glutamate is a major excitatory neurotransmitter. The amino acid can also be

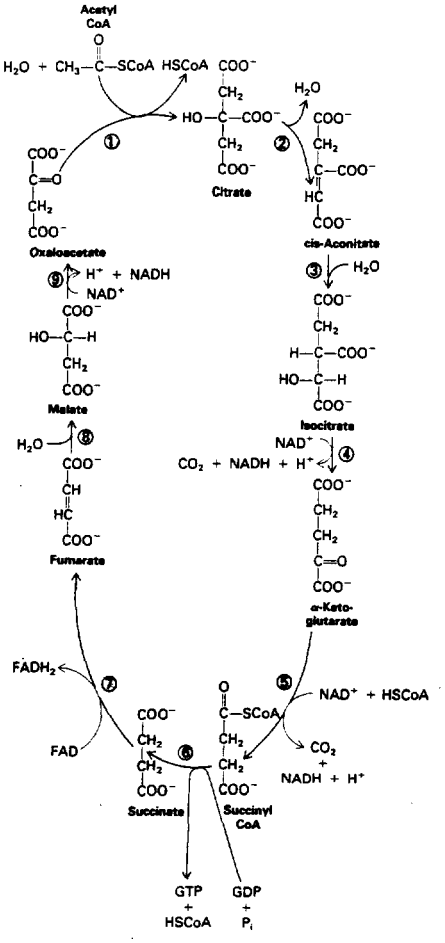
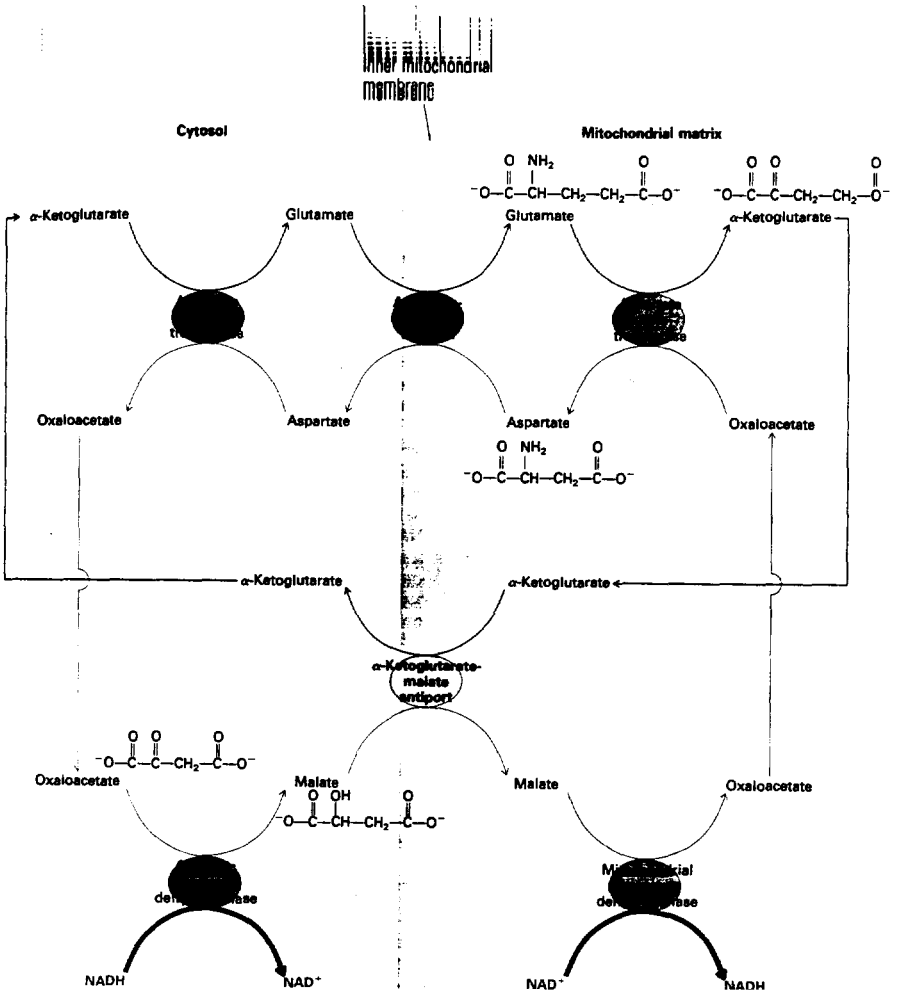


Figure 1. Tricarboxylic Acid Cycle.

further metabolized to gamma-aminobutyric acid (GABA), which works as an inhibitory neurotransmitter necessary for cellular regulation in the brain (Banner et al., 1987). Glutamate also serves as an intricate component to the TCA cycle (Figure 1). This nine step cycle begins with the condensation of the two-carbon acetyl group from acetyl CoA with the four-carbon molecule oxaloacetate to form the six-carbon molecule citrate. The enzymatically catalyzed reaction of steps 2 through 9 convert each molecule of citrate back to oxaloacetate for reuse. In this process each cycle loses two CO_2 molecules. Also in this process 4 pairs of electrons are removed from the carbon atom: three pairs are transferred to 3 molecules



of NAD^+ to form 3NADH + $3H^+$; the remaining pair is transferred to the acceptor FAD to form $FADH_2$. The regeneration of the molecule oxaloacetate is propelled by the malate shuttle in the last three reactions of the cycle (Darnell et al. 1990) (figure 2). Here the cytosolic $NADH^+$ reduces the four-carbon oxaloacetate to malate. Malate crosses the inner membrane in exchange for α-ketoglutarate and reduces NAD^+, forming $NADH^+$ as well as oxaloacetate in the matrix. But matrix oxaloacetate is impermeable to the inner mitochondrial membrane and can not pass directly back to the cytosol. So matrix oxaloacetate is converted into the amino acid asparate, which crosses the inner mitochondrial membrane to the cytosol in exchange for glutamate. In the cytosol the asparate is reconverted to oxaloacetate, completing the cycle. To allow the cycle to proceed, α-ketoglutarate is converted to the amino acid glutamate in the cytosol and crosses to the matrix, where it is reconverted to α-ketoglutarate. This complex electron shuttle involving glutamate allows oxaloacetate to be regenerated for use in the TCA cycle. The shuttle also oxidizes the cytosolic $NADH^+$ to NAD^+, and reduces the matrix NAD^+ to $NADH^+$ simultaneously. This process allows NAD^+ to be available for glycolysis, conversion of glucose to pyruvate in the cytosol. The $NADH^+$ and H^+ will also allow ATP to be synthesized (Darnell et al., 1990). Because of its varied and critical roles in cellular functions, it is essential that glutamate production and degradation be finely regulated.

Glutamate can also be converted directly to α-ketoglutarate. One enzyme central to the metabolism of glutamate to α-ketoglutarate is glutamate dehydrogenase [GDH; L-glutamate:$NAD(P)^+$

oxidoreductase (deaminating); EC 1.4.1.3]. The hexameric structured enzyme is found in the mitochondrial matrix and is thought to be localized predominantly in astroglial cells in brain. It catalyzes the following reversible reaction: α-ketoglutarate + NH_4 + NAD(P)H <-> Glu + H_2O + $NAD(P)^+$. Thus glutamate dehydrogenase along with certain ligands control the production and degradation of the amino acid glutamate. It is hypothesized that the ligands adhere to distinct but overlapping binding sites on the enzyme. The overlapping nature of these sites allows for the binding of some ligands and excludes the binding of others. This would explain the allosteric behavior of the enzyme in which the ligands either inhibit or activate its activity (Fisher et al. 1973). The coenzyme $NADH^+$ at higher concentrations inhibits the activity of the enzyme by binding to a second noncatalytic binding site which has a low affinity for the coenzyme. Studies have also revealed that the amino acid glutamate enhances the binding of the coenzyme $NADH^+$, but this high substrate inhibition by $NADH^+$ can be abolished at high enzyme concentrations. The coenzyme GTP also operates as an inhibitor by promoting the binding of $NADH^+$ to its inhibitory site. The extent of GTP inhibition depends on the concentrations of inorganic phosphate and magnesium ions, both of which decrease the binding of GTP to glutamate dehydrogenase. Other inhibitors to the enzyme include GDP, inositol triphosphate, and inositol diphosphate. In contrast ADP acts as an activator. It decreases the affinity of the catalytic site for NAD(P)(H) thus activating the oxidation of glutamate. This coenzyme also has the ability to displace $NADH^+$ from its inhibitory binding site in an apparently

competitive manner. Other activators of glutamate dehydrogenase include a number of monocarboxylic L-amino acids. Of these, leucine has been most extensively studied. It appears to behave in a similar enzyme-coenzyme interaction as ADP although the site to which it binds is distinct from that occupied by ADP (Tipton & Couee, 1988).

A complete functional model on this mitochondrial enzyme which accounts for its complex allosteric regulation by the various ligands is still unclear. But its importance in regulation of embryo growth and development at the cellular level is recognized. For our experimental purposes, we are studying GDH expression at different stages of preimplantation mouse development. This will provide information about utilization of glutamate and glutamine at the different stages, and their role in embryonic metabolism.

Materials and Methods

RNA Isolation

Total RNA from adult mouse brain tissue or mouse embryos at the 1-cell and blastocyst stage was isolated by the Acid-Guanidine-Phenol-Chloroform method of Chomczynski and Sacchi (1987). Brain tissue (or embryo) was homogenized and thoroughly dissolved in 2ml (200μl for embryos) of denaturing solution [4M guanidinium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1M 2-mercaptoethanol]. In a corex tube, 0.2ml (20μl for embryos) of 2M sodium acetate pH 4.0, 2.0ml (200μl for embryos) of water-saturated phenol, and 0.4ml (40μl for embryos) of chloroform:isoamylalcohol (49:1) were added sequentially to the tissue guanidine solution, inverting the corex tube after each addition. The tubes were held on ice for 15 minutes. This was centrifuged at 10,000g for 20 minutes at 4°C to separate the RNA from the DNA and proteins. The isolated RNA in the aqueous phase was precipitated with 2ml (200μl for embryos) of isopropanol for 1 hour at -20°C and again centrifuged at 10,000g for 20 minutes at 4°C. The liquid was removed and the pellet was resuspended in 0.6ml of denaturing solution and transferred to a 1.5ml eppendorf tube. This was reprecipitated with 1 volume of isopropanol for 1 hour at -20°C and microfuged for 10 minutes at 4°C. The RNA pellet was washed with 1ml of 75% EtOH. The RNA was dissolved in sterile tissue culture water (50-100μl) and 10μl of the RNA was used to determine its concentration. This was calculated from optical density measurements made in triplicate at 260nm. Aliquots (1-2μl) of brain RNA were electrophoresed in 0.7% agarose gels in TBE and

ethidium bromide to assess RNA integrity and purity (i.e. absence of DNA contamination).

RT-PCR

The procedure for reverse transcription and PCR amplification was similar to published methods (Sambrook et al. 1989). Two micrograms of RNA from mouse brain or RNA from 25 embryo equivalents were transferred to a 0.5ml eppendorf tube and stabilized with 1μl of calf thymus-tRNA (5mg/ml). The tube was heated in a 70°C water bath for 5 minutes and cooled on ice. The following were added: reverse transcriptase buffer (6μl of 5x stock: 250mM Tris-HCL,pH 8.3, 200mM KCL, 30mM $MgCl_2$. BRL cat #8025SA), 20mM dNTPs [3μl of stock prepared as in Maniatis cloning manual, (Sambrook et al. 1989), dissolved in 100-125μl water, neutralized with 1N NaOH to 6.5-7.0], 3'antisense primer to a portion of the nucleotide sequence of the coding region of the human GDH cDNA clone reading 5' GGA AAG CAT GGT GGA ACT ATT CCC 3' (Banner et. al. 1987) (1μl), RNasin (1μl, Promega), H_2O (1μl, sterile tissue culture water, Sigma), DTT (3μl of 0.1mM stock, BRL), and Moloney murine leukemia virus reverse transcriptase (1.5μl, 200 units/μl, BRL cat #8025SA). Reactions were incubated for 1 hour at 37°C and then heated to 95°C for 5 minutes. After reverse transcription, BSA (0.5μl of 100x stock, 10mg/ml, BioLabs), PCR buffer (5μl of 10x stock, 100mM KCL, 200mM Tris-HCL pH 8.8, 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1.0% Triton X-100), sterile tissue culture water (42μl), 5'sense primer to a portion of the nucleotide sequence of the coding region of the human GDH cDNA clone reading 5' G GCA AAG CCT TAT GAA GGA AGC ATC 3' (Banner et. al. 1987)

(1μl), and Vent polymerase (1μl, 1,000 U/ml, #254S BioLabs) were added. The mixture was overlayed with 50μl of mineral oil and transferred to a Precision Scientific Genetics thermocycler for 60 cycles of PCR. A negative control tube for both RT and PCR included all components except RNA or DNA. Each PCR cycle consisted of a denaturing step (95°C, 1 minute), an annealing step (42°C, 1 minute) and an elongation step (72°C, 1 minute). For the first cycle only, the duration of the denaturing step was 6 minutes and for the final cycle only, the length of the elongation step was 9 minutes. After the 60 cycles were completed the tubes were cooled to 22°C for 4 minutes. Reactions were stored at 4°C until analysis by 2% agarose gel electrophoresis in TBE and ethidium bromide.

Control PCR Reaction

Linearizing GDH Plasmid

GDH plasmid was cut with EcoRI into linear DNA for use as a control template for PCR reactions. The following were added together in a 0.5ml eppendorf tube: DNA plasmid (4μl, 1μg GDH), TE pH 7.6 (9.5μl of 1x stock), EcoRI buffer (1.6μl of 10x stock: 500mM NaCl, 1000mM Tris-HCL, 50mM $MgCl_2$, .25% Triton x-100 pH 7.9), and EcoRI (1.0μl). The tube was put in a 37°C water bath for 1 hour to digest. After digestion was completed, 2M sodium acetate pH 4.0 (1.6μl) and cold absolute EtOH (2 vol.) were added. The tube was placed in the -70°C freezer for 30 minutes. Digested DNA was pelleted in the microfuge for 5 minutes. The pellet is saved and dissolved in 75μl of 1 x TE pH 7.6 to give a concentration of about 20-30ng/μl. The cut GDH plasmid is stored at 4°C. To visually

check the results of the digestion, electrophoresis in a 0.7% nondenaturing agarose gel with Tris-Borate-EDTA running buffer containing ethidium bromide was used.

PCR

The procedure for PCR amplification was performed as a control for the RT-PCR experiments. The following were added to 1μl of cut GDH plasmid: 0.5μl of BSA (100x stock, 10mg/ml, BioLabs), 5.0μl of PCR buffer (10x stock, 100mM KCL, 200mM Tris-HCL pH 8.8, 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1.0% Triton X-100), 1.5μl of 20mM dNTPs, 1.0μl of 5'sense GDH primer, 1.0μl of 3'antisense GDH primer, 42μl of sterile tissue culture water, 1.0μl of Vent polymerase. The mixture was then overlayed with 50μl of mineral oil and placed in the Precision Scientific Genetic thermocycler for 60 cycles as described for the reverse transcribed RNA samples.

Results

In this investigation we examined the GDH expression in mouse brain which is encoded on RNA. Therefore, RNA needed to be extracted and isolated from mouse brain. This was accomplished by the Acid-Guanidine-Phenol-Chloroform method of Chomczynski and Sacchi (1987) described in methods. To assess the integrity and purity of RNA, aliquots (1-2μg) of brain RNA were electrophoresed in a 0.7% agarose gel in TBE and ethidium bromide. If DNA was

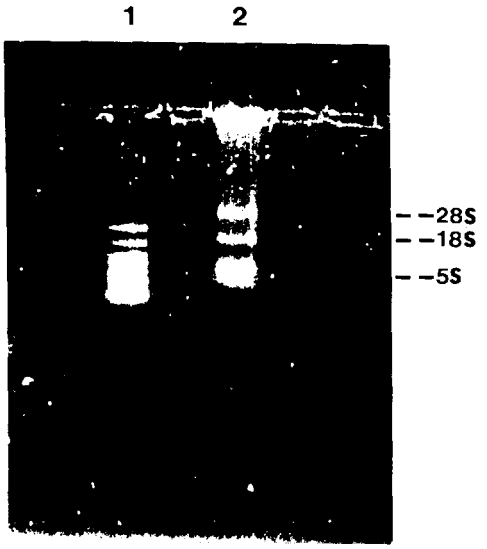


Figure 3. Agarose electrophoresis gel of mouse brain RNA isolated by the Acid-Guanidine-Phenol-Chloroform method. Lane 1 shows the bands of the standard used for markers. Lane 2 is isolated mouse brain RNA, 18S, 28S and 5S rRNA bands are indicated.

present, it could be detected as a orange fluorescent band at the

well of the gel. The RNA appeared as 18S and 28S rRNA bands, a combined 5S rRNA and 4S tRNA band, and an mRNA smear between 18S and at least 28S or larger (Figure 3, lane 2).

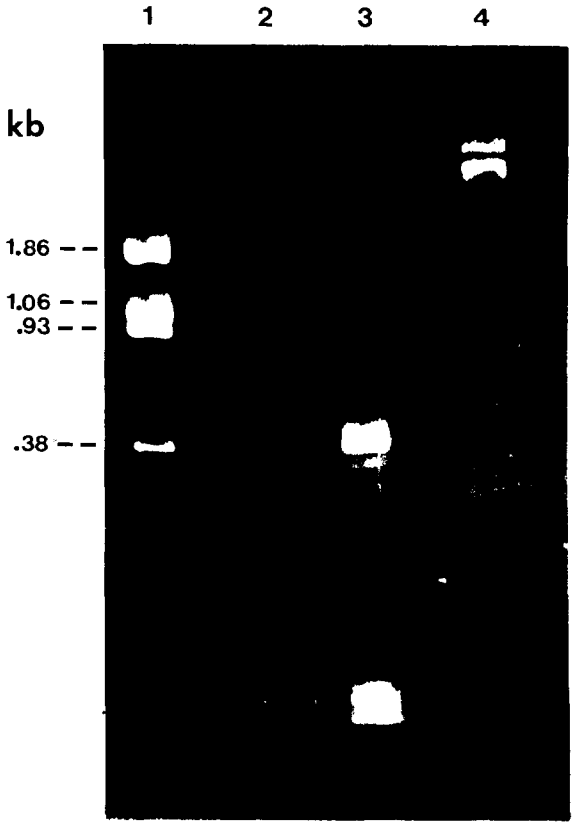


Figure 4. Agarose electrophoresis gel of linearized GDH plasmid and the amplified portion of the linearized GDH plasmid by PCR. Lane 1 contains Bst NI digested pBR322 standards which are used for markers. Lane 2 contains PCR reaction products to which no DNA was added (negative control). Lane 3 contains the PCR amplified fragment of the linearized GDH plasmid, approximately 369 base pairs in length. Lane 4 contains the whole linearized GDH plasmid, approximately 4500 base pairs in length.

In order to provide a positive control template for the PCR the GDH (pYN751) plasmid was cut with EcoRI into linear DNA. To

assess the success of the digestion the cut GDH plasmid was electrophoresed in a 0.7% agarose gel in TBE and ethidium bromide. The cut GDH DNA was detected as a orange fluorescent band of approximately 4500 base pairs (Figure 4, lane 4).

This cut GDH plasmid was first used to test the PCR reaction to show that the solutions, dNTPs, primers, and Vent polymerase were working and not contaminated with GDH sequences. For this experiment two samples were prepared, one without cut GDH and the other with cut GDH (1-2μg). These samples were both amplified with a 3′ antisense and a 5′ sense primer for a portion of the coding region of the human GDH cDNA clone. The product is expected to contain a portion of the DNA sequence for GDH approximately 369 base pairs long. The results are shown in figure 4 lanes 2 and 3. The control (lane 2) shows no GDH specific 369bp band indicating no contamination of solutions, while the plasmid (lane 3) produced the 369bp band as expected. Figure 4 also shows the primers. They appear as low molecular weight bands at the bottom of the gel indicating that they are not forming primer dimers.

To detect the GDH message in isolated mouse brain RNA we used RT-PCR. This technique involves reverse-transcribing RNA and then amplifying RNA-DNA hybrids by PCR. We expected that if we were to reverse-transcribe total RNA with a 3′antisense primer for a portion of the coding region of the human GDH cDNA clone, and then amplified with a 5′sense primer for the same, the product should contain a portion of the RNA sequence for GDH approximately 369 base pairs long. Unfortunately, analysis by 2.0% agarose gel electrophoresis in TBE and ethidium bromide showed no trace of the

appropriate sequence (Data not shown).

To see whether the problem occurred in the RT or PCR portion of the procedure, two samples of cut GDH DNA were amplified by PCR. Both samples contained the same 3'antisense primer and 5'sense primer, dNTPs and Vent polymerase, but had different PCR buffers (one from BRL and one prepared in the laboratory). Analysis of

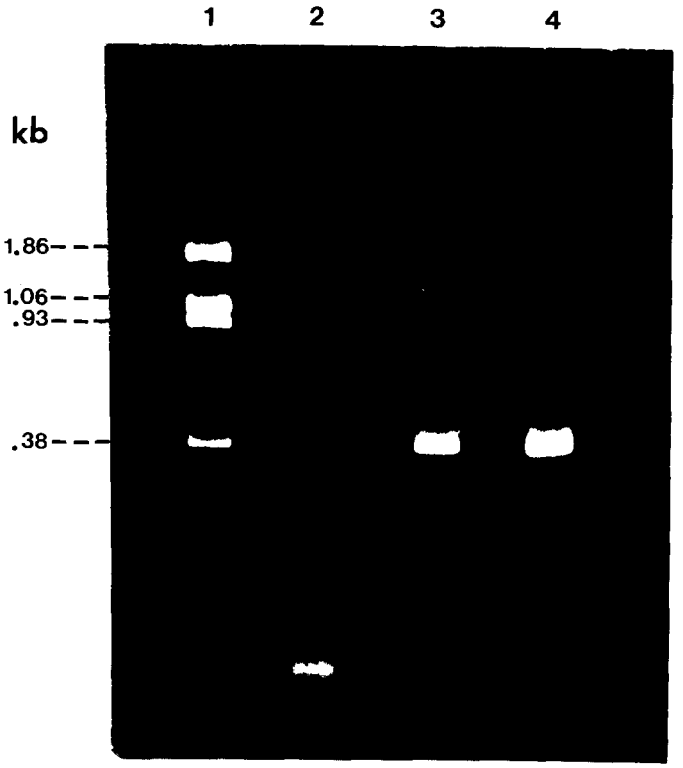


Figure 5. Agarose electrophoresis gel of the amplification of linear GDH plasmid DNA by PCR. Lane 1 contains the pBR322 Bst NI standards. Lane 2 contains the control to detect contamination of PCR solutions. Lane 3 contains the amplified portion of the linear GDH DNA (PCR buffer prepared in the laboratory). Lane 4 contains the amplified portion of the linear GDH DNA (PCR buffer from BRL).

cDNA products by 2.0% agarose gel electrophoresis showed the PCR amplification procedure to be running successfully, resulting in an orange fluorescent band of approximately 369 base pairs under both buffer conditions (Figure 5, lanes 3 and 4). GDH plamid (-) control again showed no 369bp band suggesting no contamination of solutions (Figure 5, lane 2).

Since the PCR portion of the reaction has been consistently successful, the problem must lie with the RT portion of the procedure. To remedy the problem a number of ideas were tried. New reverse-transcriptase and 5x buffer were ordered, water and solutions were re-autoclaved, and newer RNasin (to prevent RNA breakdown prior to RT) and dNTPs were used. RT-PCR following each of these changes was still unsuccessful (data not shown).

Discussion

From these experiments we have drawn several conclusions. The PCR method used on the linearized GDH plasmid yielded the expected 369 base pair fragment. But the RT-PCR was unsuccessful. This was most likely due to the sensitivity of the reverse transcriptase to heavy metals and contaminants still present in our water supply even following deionization and Milli Q purification. Other laboratories have required additional charcoal filtration for some molecular reactions to work properly. This will be implemented in future experiments.

If these experiments, RT-PCR using fragment GDH coding primers, were used on embryo RNA at the 1-cell, 2-cell, 8-cell, and blastocyst stages we would expect to find GDH expression at all stages. The original proposal involved studying GDH RNA expression in mouse embryos at the 1-cell, 2-cell, 8-cell, and blastocyst stages. We hypothesized that RT-PCR specific for GDH using preimplantation embryos should show expression at all stages. This is based on studies of glutamine which shows that it is used as an energy substrate at all stages (Chatot et al. 1990). Glutaminase, the enzyme which converts glutamine to glutamate is also present to varying degrees at all stages (Chatot et al., manuscript in preparation). Most probably the next step in glutamine utilization in the TCA cycle involves GDH conversion of glutamate to α-ketoglutarate. Since GDH indirectly regulates the activity of glutaminase by the degradation and synthesis of glutamate, it would be expected to also be found at each stage, perhaps at varying amounts.

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