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**DIFFERENTIAL GENE EXPRESSION IN RESPONSE TO SUB-ZERO
TEMPERATURES IN THE FREEZE TOLERANT WOOD FROG,
*RANA SYLVATICA***

By

Dawn Heather Lobsinger

B.Sc. (Hons). University of Waterloo. 1993

A thesis submitted to
the faculty of Graduate Studies and Research
in partial fulfillment of
the requirements of the degree of

Master of Science

Department of Biology

Ottawa-Carleton Institute of Biology
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"DIFFERENTIAL GENE EXPRESSION IN RESPONSE TO SUB-ZERO
TEMPERATURES IN THE FREEZE TOLERANT WOOD FROG, *RANA SYLVATICA*"

Submitted by Dawn Heather Lobsinger, B.Sc. (Hons)

in partial fulfillment of the requirements for
the degree of Master of Science

Thesis Supervisor

Chair, Department of Biology

ABSTRACT

Exposure to subzero freezing temperatures causes a variety of biochemical changes in the freeze-tolerant wood frog, *Rana sylvatica*. Cellular responses to freezing commence a mere five minutes after the initial formation of epidermal ice. To obtain a general overview of differential protein expression in response to freezing, *in vitro* translation of mRNA isolated from tissues (liver, brain, heart, muscle, kidney and gut) was conducted and compared for control (5°C), frozen (24 hours at -2.5°C) and recovered (24 hour thaw at 5°C) wood frogs. ³⁵S-labeled proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and located by fluorography. Results showed changes in the translatable mRNA population of freezing exposed wood frogs. Differential protein expression was greatest in the comparison of liver from control versus frozen frogs but other tissues each showed one or two differentially expressed proteins in either the frozen or thawed states. Subsequently, isolation of a specific cDNA clone, the *R. sylvatica* liver glucose transporter (glut2), was attempted via heterologous probing of the liver cDNA library from frozen frogs with a human glut2 cDNA fragment. Potential clones were chosen from a primary screening and further purification was achieved with secondary and tertiary screens. Analysis of the partial nucleic acid sequence of fifteen potential positive clones using the GenBank database did not reveal the presence of the wood frog glut2 cDNA. In another effort to find a wood frog glut2, TA cloning, a PCR based method, was attempted. A PCR product was amplified using a variety of degenerate primers and one product was cloned into a T-vector. The insert of the resulting recombinant plasmid was sequenced and comparison of this with the GenBank database did not reveal its identity as the liver glucose transporter.

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INTRODUCTION

The occurrence of seasonal freezing temperatures throughout vast areas of our planet has necessitated the evolution of freeze tolerance amongst a diverse group of organisms. Freeze tolerance has been documented in bacteria (Gounot, 1991), plants (Lipp *et al.*, 1994), reptiles (Storey *et al.*, 1988; Costanzo and Claussen, 1990; Churchill and Storey, 1992), insects (Duman *et al.*, 1991; Zachariassen, 1985), amphibians (Storey and Storey, 1992) and various intertidal invertebrates (Aarser, 1982). Vertebrate freeze tolerance is of particular interest to medical science as these animals represent a model system for the development of techniques for human organ cryopreservation. While freezing protocols for simple tissues such as gametes, skin and bone marrow have been perfected, large organs (liver, heart, kidney, etc.) suffer irreparable damage when frozen (Costanzo *et al.*, 1995). Tissue injury occurs due to the inability of cells to maintain integrity, regulate volume and avoid metabolic decline (Storey, 1990).

Freeze tolerant organisms have developed several physiological and molecular adaptations to survive subzero temperatures. One is the ability to control extracellular ice formation. This is achieved by the production of ice-nucleating agents (INA), unique to freeze tolerant animals. These hydrophilic proteins function to control extracellular ice formation thereby ensuring low rates of ice propagation. This controlled rate ensures minimal osmotic stress on cells, minimal membrane shrinkage/deformation and a gradually applied ischemic state (Storey and Storey, 1988). Spontaneous ice formation would cause potentially lethal cellular damage in the form of punctured membranes, disrupted compartmentation and the destruction of subcellular structure (Storey, 1987; McGann *et al.*, 1988). The mechanism of action of INAs is unknown. *In vitro* studies of wood frog INAs suggest that they may not initiate ice formation as temperatures of -6°C are required before nucleation is observed in plasma or solution (Storey and Storey, 1992). It is believed that their "water-loving" nature creates an ordering effect on water

molecules hence reducing the energy barrier to nucleation (Duman *et al.*, 1982) and controlling the subsequent growth of ice crystals (Storey and Storey, 1992).

As well as the potential physical damage, extracellular ice creates a hypertonic extracellular environment which threatens cellular water loss to the point of irreversible dehydration. This pull to withdraw water from cells to correct the ionic and osmotic imbalance is countered by the production of high concentrations of low molecular weight cryoprotectants which are taken up by the cells. Most cryoprotectants are three to six carbon sugars or their corresponding alcohols such as glycerol and sorbitol, commonly synthesized by freeze tolerant insects, and glucose, which is used by most of the freeze tolerant frogs (Duman, 1982; Storey, 1990). These molecules have a colligative effect (and can also bind water to render it unfreezable) which helps to limit the total amount of water that can be converted to ice and to prevent cell volume from dropping below its critical minimum volume (Storey, 1990; Mommsen and Storey, 1992).

Freezing of blood plasma also causes ischemia, the interruption of oxygen, nutrient and waste transport between the blood and cells. Therefore, tissues must be suited to endure anoxia throughout the freeze. Organs must survive autonomously with energy derived from glycogen reserves by fermentative glycolysis producing lactate and alanine as end products and with depletion of phosphagen and adenylate pools (Storey and Storey, 1985c). Specific mechanisms of metabolic rate depression may also be employed to reduce overall energy needs in the frozen state and thereby prolong the potential survival time. Unneeded metabolic pathways are shut down, and the rates of energy use and production as well as those of passive metabolism are rebalanced in an efficient manner (Storey and Storey, 1989).

Safeguards must also be in place to maintain protein structure and membrane integrity during the freeze. Protein stability against the stresses of low temperature, high ionic strength and low water content, is ensured by the cryoprotectants already mentioned (Mommsen and Storey, 1992; Costanzo *et al.*, 1993a). Lipid bilayer integrity is

threatened by physical stress, direct damage by ice, dehydration and high salt concentrations. Membrane integrity is maintained by the insertion of low molecular weight molecules (i.e., proline and trehalose) into the membrane. These function to stabilize the bilayer structure which can otherwise break down to the gel state as a result of compressive forces on the membrane as cell volume shrinks (Storey, 1990). Trehalose operates by binding with polar head groups on phospholipids thereby spreading out the bilayer (Rudolph and Crowe, 1985). The mechanism of action of proline, while having a similar effect on the bilayer, is unknown.

The wood frog, *Rana sylvatica*, is the best studied of the five known freeze-tolerant amphibians (Storey and Storey, 1988; Costanzo *et al.*, 1993b). The initial response of the frog to subzero temperatures is to let body fluids supercool by a few degrees below the equilibrium freezing point. However, the supercooled state typically persists for only a short time until internal freezing is triggered by contact with external ice that propagates across the skin (Storey and Storey, 1985a). The nucleation of body fluids is marked by a sharp jump in body temperature due to the release of heat (latent heat of fusion) during crystal formation. This exotherm may persist for 3-20 hours (depending on the mass of the frog, here 2 to 14 g) during which time the body temperature of the frog slowly falls until it equilibrates with the ambient temperature when ice formation reaches its maximum (Storey and Storey, 1985a).

R. sylvatica produces glucose as its cryoprotectant, this having several advantages: 1. it is liberated quickly from glycogen via a simple three step pathway; 2. it can serve as a fermentative fuel for cellular metabolism under the ischemic frozen state; 3. it can be reconverted easily to a storage form (glycogen) when the stress is removed (Storey, 1987b; Storey, 1990). Hepatic glycogenolysis commences a mere five minutes after the appearance of the exotherm producing cryoprotectant at a rate of 20-35 $\mu\text{mol glucose/g/h}$ which is then exported for transfer to all tissues via the blood (Storey, 1987a;

Costanzo *et al.*, 1995). Cryoprotectant distribution is aided by exotherm-induced cardioacceleration (Layne *et al.*, 1989; Costanzo *et al.*, 1993b). Glucose concentrations which normally range from 1-5 $\mu\text{mol/g}$ wet weight (Storey, 1990) increase to 50 - 350 $\mu\text{mol/g}$ wet weight (Storey and Storey, 1984). Generally, the muscle tissue has the lowest concentration of glucose and the liver, blood and heart have the highest. This distribution of cryoprotectant reflects the pattern of ice formation: peripheral tissues freeze first with the core organs being spared until the last minutes. As freezing progresses, blood is constricted from the vasculature and forms a pool in the region just above the heart (Storey, 1987a).

Extracellular ice formation is initiated and regulated by an ice-nucleating protein. This seasonally expressed plasma protein, determined to be >300 kD, prevents extensive supercooling of blood plasma and therefore ensures that freezing occurs at a relatively high subzero temperature (-2 to -3°C) where the subsequent rate of ice formation will be slow (Wolanczyk *et al.*, 1990). This minimizes the extent of cellular osmotic shock, provides the time necessary for cryoprotectant synthesis and distribution and for other cellular responses to freezing (Storey and Storey, 1993). Up to 65% of the total body water of *R. sylvatica* is frozen, this is generally typical of the maximal amount of water that can be lost by the cell(s) without surpassing their minimal critical volume (Storey and Storey, 1993). Most of the ice formation occurs within the first 12 hours of freezing (Lee *et al.*, 1992) and large amounts of ice accumulates in extra-organ sites such as the coelomic cavity or between the skin and skeletal muscle (Storey 1990; Costanzo *et al.*, 1993a). Not only do cells lose water and shrink during freezing but whole organs also experience a net water loss as water exits to freeze in these extra-organ ice masses (Lee *et al.*, 1992).

As opposed to the behaviour of freeze tolerant insects which build up their cryoprotective capacity during weeks of autumn cold hardening (Duman *et al.*, 1991), the synthesis of cryoprotectants (and also perhaps other cryoprotective measures) by frogs is

not anticipatory and occurs only as a direct response to freezing in peripheral tissues. Attempts to induce cryoprotectant synthesis by prolonged exposure to 3°C (up to eleven weeks) or by acclimation (decreasing the temperature 1°C/day from 23°C to 0°C) have been unsuccessful. Similarly, the activities of some enzymes known to be involved in glycogenolysis (i.e., glycogen phosphorylase) increase only upon freezing exposure (Storey and Storey, 1984). The trigger for altered biochemical activity is believed to be hormonal involving epinephrine, the predominant catecholamine among lower vertebrates (Hemmings and Storey, 1994). The β_2 -adrenergic receptor, through which epinephrine can act, is the dominant receptor in amphibian liver (Hemmings and Storey, 1994). Blockage of hormone binding at this receptor by propranolol strongly reduces the frog's ability to produce cryoprotectant in response to freezing (Storey, 1990). This information lends support to the hypothesis that the cryoprotectant output response to freezing is an enhancement of the "fight or flight" catecholamine-mediated glycogenolysis that is common to all vertebrates (Storey 1990; Costanzo *et al.*, 1993b).

Frogs recover fully from freezing within about 24 hours after ambient temperatures rise above 0°C. Thawing begins from the core organs (where high glucose concentrations give these tissues the lowest melting point) and progresses to the periphery, the opposite of freezing. The first sign of life is the resumption of heartbeat, followed by cutaneous blood flow, and then breathing (Layne and First, 1991; Costanzo *et al.*, 1995). Tissue glucose concentrations begin to fall rapidly, with decreases seen initially in the liver and blood (Storey, 1987a). Complete restoration of normal glucose levels can take several days, from 2 days for liver to 8 days for skeletal muscle (values for 2 g frogs) (Storey and Storey, 1985b). The glucose is reconverted to glycogen and replenishes the hepatic reserves (Storey, 1987b). Organ water content is also quickly restored; for example, hydration reached pre-freeze levels within 3 hours in the gracilis major muscle and within 6 hours in liver whereas complete rehydration of intestine took

>48 hours (Lee *et al.*, 1992). Resumption of normal motor activities in wood frogs has been observed with 14-24 hours of thawing (Layne and First, 1991).

A variety of molecular changes that support freeze tolerance may be regulated at the level of the gene. Gene expression is controlled at the levels of transcription and translation. Both processes can be divided into three events: initiation, elongation and termination (Latchman, 1990). Transcription, which occurs in the nucleus, is the production of a RNA copy of the DNA template (gene) by a DNA-dependent RNA polymerase (RNAP, specifically RNAP II). The RNAP and its associated proteins (transcription factors) assemble at the promoter region located upstream of the transcriptional start site of the gene (initiation). Here the RNAP or protein factors cause local unwinding of the double stranded DNA template which then allows the polymerase to move along the template (elongation) producing heterologous nuclear RNA (hnRNA). The hnRNA is "capped" with a modified guanosine residue (7-methylguanosine) after transcription of approximately 30 nucleotides. Termination is signaled by the sequence AAUAAA which is located upstream of the eventual polyadenylation (cleavage) site and by a GU-rich region 3' of the cleavage site. A poly(A) polymerase then polyadenylates the 3' end of the transcript; the average tail length is 150-200 residues. Both the 5' cap and 3' poly(A) tail serve to stabilize the transcript (i.e., prevent its degradation). The resulting hnRNA, which contains exons (coding sequence) and introns (non-coding sequence) is then processed by small nuclear ribonucleoproteins (snRNPs) to remove the introns and produce translatable message RNA (mRNA) (Wingender, 1993).

The mRNA is then transported to the cytoplasm to be translated. Ribosomes (rRNA) are the translation machinery of the cell and, in eukaryotes, are composed of 60S and 40S subunits and associated proteins (initiation, elongation and release factors). The ribosomal subunits come together to form a functional 80S unit which facilitates translation of the mRNA to protein (initiation). The ribonucleic acids of the message are read in groups of three nucleotides called codons. Of the 64 possible codons, 61 code for

amino acids and three are nonsense or stop codons. Translation always begins at the first AUG codon (which codes for methionine). As each codon is read a transfer RNA (tRNA) molecule displaying the complementary anticodon and carrying the corresponding amino acid (charged tRNA) is complexed with the ribosome. The tRNA molecules enter the ribosome at a specific position known as the A site. Adjacent to this is the P site which contains a tRNA with the nascent polypeptide. The growing protein is transferred to the amino acid carried by the tRNA in the A site in a process known as transpeptidation. The uncharged tRNA is then released and the tRNA + peptide is translocated to the P site and so the cycle continues (elongation). Translation continues until a stop codon is encountered at which point a release factor liberates the ribosome from the mRNA (termination) (Voet and Voet, 1990). Many ribosomes may bind the mRNA to produce multiple copies of the protein before the template is degraded. The half life of an eukaryotic mRNA can range from several minutes (i.e., regulatory proteins) to hours (i.e., constitutively expressed proteins) (Wingender, 1993).

The primary point of gene regulation is transcription (Lowenhaupt *et al.*, 1978; Darnell 1982), specifically at initiation (Bentley and Groudine, 1986). Transcription is mediated by many proteins which bind sequences known as promoters, enhancers, silencers and response elements. The promoter region may consist of several elements (highly conserved sequences) including the well-known TATAA and CCAAT boxes. The TATAA box, located 25 bases upstream of the gene, directs the RNAP to the correct transcriptional starting position. The CCAAT box, located 50 bases upstream from the TATTA box, ensures optimal promoter activity (Wingender, 1993). Enhancers stimulate transcription 10-100 fold (Boulet *et al.*, 1986); silencers negatively regulate expression (Brand *et al.*, 1985). These elements can be found upstream or downstream of the gene or even intragenically (Wingender, 1993). There are also a wide variety of response elements and their associated binding proteins which up- or down-regulate specific genes in response to certain physiological conditions. A few examples include the heat shock

consensus element (HSCE), cAMP response element (CRE), glucocorticoid response element (GRE) and thyroid hormone response element (THRE) (Latchman, 1990).

Regulatory element binding proteins can effect transcriptional initiation in two ways.

The first mode of action is demonstrated by the GRE binding protein (GREP). Binding of the GREP to the GRE causes a conformational change in the DNA which exposes the transcriptional start site. This facilitates binding by transcriptional factors and thereby promotes the action of RNAP (Latchman, 1990). The second regulatory mechanism is exemplified by the HSCE binding protein (HSCEP). The HSCEP binds the HSCE in close proximity to the TATAA box binding protein. Interaction between the protein factors either stabilizes the transcription complex (RNAP+ factors) or alters its structure to enhance transcriptional activity (Latchman, 1990).

RNA splicing and stability constitute post-transcriptional levels of gene regulation. RNA splicing, which removes exons, occurs on a complex of small nuclear ribonucleoproteins (snRNPs) known as a spliceosome. Failure to remove the exons inhibits transport of the transcript to the cytoplasm or causes the production of a dysfunctional protein should it be translated (Sharp, 1987). Stability of the mRNA determines the number of proteins produced from the transcript: the less stable the mRNA, the fewer proteins that are produced. Increased stability of the mRNA is paralleled with an increased rate of transcription. This level of regulation occurs primarily in transient states when protein production must be rapid (Guyette, 1989; Marzluff and Pandey, 1988).

Translational control of gene expression is rare and usually coupled with transcriptional controls (Latchman, 1990). This level of regulation generally occurs under conditions requiring rapid changes, i.e., during heat shock. Mechanisms of regulation include modification of ribosomally-associated proteins (i.e., by phosphorylation) (Casey *et al.*, 1988) or alteration of the mRNA structure itself (i.e., stem loop structures) (Latchman 1990).

As already discussed, glucose is a key molecule in *R. sylvatica* freeze-tolerance. The diffusion rate of sugars across cell membranes is very low due to their poor permeability and hence, the primary mode of glucose movement across membranes is via facilitated transport. Bidirectional glucose transport is mediated by a family of transmembrane carriers designated GLUT1-7 (note: GLUT will refer to the protein, glut will refer to the gene) (Mueckler, 1994). These proteins span the membrane twelve times, have cytoplasmically located N- and C- termini and one intra- and extra-cellular loop. The amino acid sequences of the transmembrane regions are highly conserved between species while that of the exo- and endo-plasmic loops are more variable (Bell *et al.*, 1993). Generally, each transporter is specific to certain cell or tissue types (Gould and Holman, 1993). The liver specific isoform, GLUT2, is located on the sinusoidal face of the hepatocyte membrane (Thorens *et al.*, 1993) and is responsible for the release and uptake of glucose to and from the blood (Postic *et al.*, 1993). Mammalian glut2 expression is regulated by glucose at the transcriptional level as determined by nuclear run-on assays (Waeber *et al.*, 1994). Several other studies have reported the positive regulation of glut2 expression under increased glucose concentrations both *in vivo* and *in vitro* (Postic *et al.*, 1993; Waeber *et al.*, 1994). Investigation of the murine and human 5' upstream region of glut2 has identified the presence of an undefined carbohydrate response element (Waeber *et al.*, 1994). Studies on *R. sylvatica* glut2 have just begun. Initial comparative studies between the wood frog and the non-freeze tolerant leopard frog, *R. pipiens*, have revealed differences in glucose transport capacity. The difference is believed to be due to a greater number of constitutive carriers found in the hepatocyte membranes of the freeze-tolerant frog (King *et al.*, 1993). This may represent an adaptation for freeze tolerance. Indeed, a more recent study has shown that the concentration of GLUT2 in the membrane increases during the autumn in the wood frog

(8.5 fold greater than in summer frogs) suggesting an anticipatory mechanism of freeze tolerance (King *et al.*, 1995).

This present investigation had two aims. The first goal was to determine whether freezing elicits differential protein expression as seen in response to other stresses in animals (i.e., heat shock, acute phase response). It is thought that even though metabolism and life functions are decreasing and eventually halting, there must be an increase in specific proteins which are necessary for a safe decline into the frozen state. This was examined by *in vitro* translation. *In vitro* translation is a useful technique to detect changes in the translatable mRNA population of specific cells. This method has been used to observe such changes in renal cell carcinoma cells (Paradis *et al.*, 1991) and anoxia tolerant turtle tissues (Douglas *et al.*, 1994). Simply, isolated total RNA or mRNA is added to a cell free extract containing the required translation machinery (ribosomes, tRNA, amino acids, etc.). Here, RNA isolated from tissues of control (5°C), frozen (24 hours at -2.5°C) and recovered (24 hour thaw at 5°C) wood frogs was used for the procedure. Translation occur in the presence of a radiolabeled amino acid, usually ³⁵S-methionine or ³H-leucine (this study utilized ³⁵S-methionine). The translation products are separated by SDS-PAGE and analyzed by autoradiography. The detection of new proteins in the resulting banding pattern between comparable samples indicate the alteration of the mRNA population. Elucidation of the presence, absence or enhancement of proteins during freezing was the primary goal of this experiment. Synthesis of new mRNAs and degradation of other messages were detected by the presence of new, radioactive bands and the absence of proteins present in control samples.

The second goal of the study was to isolate and characterize a wood frog glut2. Since copious amounts of glucose are transported via the blood to all tissues to serve as a cryoprotectant, its export from the producing organ, the liver, may be enhanced to meet the extraordinary demand. Enhancement may be due to up-regulation of the gene (which

can be determined by northern analysis) or possibly the structure of wood frog GLUT2 is modified to suit its role in freeze tolerance (which may be determined by analysis of the deduced amino acid sequence of the cDNA). A cDNA library made from liver of frozen frogs was heterologously probed with a human glut2 cDNA fragment (1-1762 bp, full coding sequence) in an attempt to isolate the frog glut2 cDNA with the goal of sequencing and subsequently comparing the frog protein with known mammalian GLUT2 proteins.

MATERIALS AND METHODS

Animal care and Tissue Sampling

Live *Rana sylvatica* (males, 5-7 g) were obtained from breeding ponds in wooded areas around Ottawa in April and then maintained at 5°C in beds of damp moss for approximately 4 weeks without feeding. Control animals were taken directly from the 5°C acclimated group. The remaining frogs were transferred to an incubator set at -2.5°C and allowed to freeze. Body temperatures of selected individuals were monitored by a thermistor secured to the abdomen; in general, freezing began within 45-60 min after placing in the incubator. A portion of the population was sacrificed after exposure to freezing conditions for 24 hours. Remaining frozen frogs were transferred back to 5°C and allowed to thaw and recover for 24 hours. All animals were sacrificed by spinal pithing; tissues were quickly excised within 90-120 seconds, frozen in liquid nitrogen, and then transferred for long term storage to -70°C. Tissues sampled included liver, muscle, brain, heart, kidney and gut (stomach and intestine combined).

Chemicals

All chemicals used were molecular biology grade or their equivalent and of the highest purity.

PART I - *IN VITRO* TRANSLATIONS

Overview

In vitro translations were performed using total RNA isolated from various tissues of the wood frog to obtain a general view of differential protein expression during whole body freezing. Tissues analyzed included liver, muscle, brain, heart, kidney and gut. Briefly, total RNA was extracted from the tissues using a one step isolation method and analyzed electrophoretically. The mRNA component of the total RNA was the template for protein synthesis in a cell free lysate containing a radiolabeled amino acid (³⁵S-methionine). Proteins were then separated by SDS-PAGE and analyzed by fluorography and autoradiography. Laser densitometry/desktop scanning was then used to produce graphic representations of the autoradiographs.

Preparation of materials used for RNA isolation

Gloves were worn at all times while manipulating the RNA and reagents. All solutions used in the isolation and subsequent handling of RNA were prepared in double distilled water treated with 0.1% v/v diethylpyrocarbonate (DEPC), incubated for two hours at 37°C and autoclaved. Plastic 1.5 ml Eppendorf tubes, 50 ml oak ridge tubes and Pipetteman tips were handled with gloved hands only and autoclaved.

Total RNA isolation

Total RNA was isolated from the tissues using Trizol™ reagent (Gibco-BRL, Bethesda, MD) according to the manufacturer's instructions. Tissues were homogenized

directly in a specific volume of the Trizol™ reagent (1 ml/ 50-100 mg of tissue) in 50 ml oak ridge tubes and allowed to incubate at room temperature for five minutes. Chloroform was added (0.2 ml/ ml of reagent), the sample was shaken vigorously for 15 seconds and then allowed to rest at room temperature for two minutes. The sample was then centrifuged at 12 000 xg for 15 minutes at 4°C to separate the organic and aqueous phases. The upper aqueous phase containing the RNA was transferred to 1.5 ml Eppendorf tubes in 0.6 ml aliquots and 0.5 ml of isopropanol (at room temperature) was added to precipitate the nucleic acids. If precipitation did not occur immediately upon the addition of the isopropanol, the tubes were placed at 4°C for 2-4 hours to facilitate the process. The RNA was pelleted by centrifugation at 12 000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed with 1 ml of ice cold 70% ethanol, re-pelleted (7 500 x g, 5 minutes, 4°C) and then allowed to dry (37°C). RNA pellets were dissolved in a small volume of DEPC treated water (20-100 µl) in a 55°C waterbath (10-20 minutes) and pooled, if required. Absorbency readings were taken at 260 nm (concentration) and 280 nm (purity) using 10 µl of sample diluted in 990 µl of water (Gilford spectrophotometer 240 or 260). If necessary, the sample was diluted to a working concentration (2 µg/µl) and stored at -20°C in 20 µl aliquots.

Electrophoretic analysis of total RNA

The denaturing agarose gel running buffer was 1x MOPS diluted from a 10x stock which contained 0.2 M MOPS (3-[N-Morpholino] propanesulfonic acid), 1 mM EDTA (ethylenediamine tetraacetate), pH 8, and 50 mM sodium acetate. Two to four µg of total RNA was denatured at 55°C for 15 minutes in a solution containing 1x MOPS, 1.8 M formaldehyde, 3.8% deionized formamide and 200 ng of ethidium bromide (400 µg/ml stock in water). Samples were then incubated on ice for 2 minutes. RNA loading buffer was added to a final concentration of 1x from a 6x stock containing 50% deionized

formamide, 1x MOPS, 40% glycerol and 0.5% bromphenol blue. Samples were then loaded onto a denaturing, 1.5% agarose gel prepared as follows: solid agarose was dissolved by boiling in a solution containing 1x MOPS and 2.2 M formaldehyde. Once cooled to 55°C, the agarose solution was poured into a horizontal gel slab and allowed to solidify. The gel was covered with 1x MOPS running buffer (Maniatis *et al.*, 1989).

Samples were electrophoresed at a constant 70 volts until the bromphenol blue dye had migrated 2/3 the length of the gel. The gel was then viewed on a UV transilluminator (310 nm) and a photograph was taken using the Polaroid DS-34 instant camera.

***In vitro* translations of total RNA**

In vitro translations were performed using the Wheat Germ Extract kit from Promega (Madison, WI) according to the instructions. Reaction tubes contained the following: 80 µM amino acid mixture (minus methionine), total RNA (in water) ranging from 100 µg/ml to 200 µg/ml, potassium acetate ranging from 53 mM to 200 mM, 20 U of RNasin ribonuclease inhibitor, 12.5 mCi of ³⁵S- L-methionine, wheat germ extract (50% of total volume) and nuclease-free water to a final volume of 25 µl. The reaction proceeded for one hour at 25°C. To determine the efficiency of the reaction by percent incorporation of radiolabel, two µl of the reaction sample was transferred to a 1.5 ml Eppendorf tube containing 98 µl of 1 M NaOH and these tubes were incubated at 37°C for 10 minutes. Subsequently, 900 µl of 25% TCA (trichloroacetic acid)/2% casein hydrosylate was added to the samples which were then placed on ice for 30 minutes. One-quarter of the sample was vacuum filtered onto glass fiber discs (GF/A) and washed three times with ice cold 5% TCA and once with ice cold 70% ethanol. These filters represented incorporated counts. Five µl of the same samples were spotted directly onto glass fiber discs without washing and represented total counts. Glass fiber discs were

placed into scintillation vials containing 5 ml of Econofluor liquid scintillation fluid (NEN-Dupont, Boston, MA) and radioactive counts were determined using a Packard Tri-carb Liquid Scintillation analyzer (Model 1900CA). Percent incorporation was determined from the resulting cpm (counts per minute) values according to the following equation:

$$(\text{incorporated counts} / [\text{unincorporated counts} \times 50]) \times 100.$$

The original reaction samples were stored at -20°C until analyzed by SDS-PAGE.

SDS-Polyacrylamide gel electrophoresis analysis of translation products

SDS-PAGE was performed using the discontinuous buffer system as described by Laemmli (1970) and the Mini-Protein II electrophoresis apparatus (Bio-Rad, Hercules, CA). The resolving gel consisted of an 8 - 15% linear gradient of polyacrylamide; the stacking gel was 5% polyacrylamide (30:0.8 acrylamide: bis-acrylamide). Aliquots of translation samples containing 20,000 - 100,000 acid-precipitable counts (cpm) were mixed with an equal volume of loading buffer (50 mM DTT (dithiothreitol), 20 % v/v glycerol, 1% w/v SDS (sodium dodecylsulfate), 50 mM Tris-HCl, pH 8.8, 0.04 % w/v bromophenol blue), boiled for 5 minutes, loaded onto the gel and electrophoresed at 40 mA until the dye front ran off the gel. Broad range molecular weight standards (Bio-Rad, Hercules, CA) were included. The gel was then fixed for 30 minutes (25% v/v methanol, 10% v/v acetic acid), stained for 1-2 hours (0.25% w/v Coomassie Brilliant blue, 50% v/v methanol, 7.5% v/v acetic acid) and destained for 2 -16 hours (25% v/v methanol, 10% v/v acetic acid). The gel was treated with the fluorography reagent EnHance™ or Entensify™ (NEN-Dupont, Boston, MA) as directed by the manufacturer. The gel was dried onto Whatman 3M filter paper using the Bio-Rad Model 238 gel drier programmed for two hours at 80°C. Once dried, the gel was exposed to x-ray film (Kodak XAR5) for an appropriate amount of time (ranging from 1 to 10 days) at room temperature with no

intensifying screens. Autoradiographs were developed manually using Kodak chemicals (Rochester, NY).

Analysis of *in vitro* translation data

Translation autoradiographs were analyzed using a Molecular Dynamics Laser Densitometer or Hewlett Packard ScanJet 3c. Densitometry traces representing the autoradiographs were produced using Imagequant v. 3.1 and Sigma Plot and pairwise comparisons were made as follows: control vs frozen, control vs recovered and frozen vs recovered for each tissue. The molecular weights of differentially expressed proteins were interpolated/extrapolated from a plot of log molecular weight vs distance migrated of the standard proteins.

PART II - *Rana sylvatica* LIVER GLUCOSE TRANSPORTER cDNA

Overview

The isolation of the liver-specific glucose transporter of *Rana sylvatica* was attempted via three approaches. The initial approach involved an attempt to isolate a human *glut2* DNA fragment via the polymerase chain reaction (PCR) from a human liver cDNA library with primers specific to the sequence. The amplified product would have served as a probe for heterologous screening of a cDNA library constructed from liver of 24 hour frozen frogs. The second approach consisted of heterologous probing of the frozen frog liver cDNA library with a human *glut2* cDNA clone (1-1762 bp fragment). Potential clones were chosen after a primary screening of the library. Secondary and tertiary screens of these potentials followed to further identify and purify positive clones.

Final clones were characterized and the translated sequences were compared to database sequences using the Genobase BLAST server. Isolation of wood frog glut2 was finally attempted via PCR with degenerate primers made to conserved regions of known mammalian GLUT2 sequences and subsequent TA cloning and sequencing.

Initial approach to finding wood frog glut2

An initial approach to the identification of the wood frog liver glucose transporter (glut2) involved isolation of a human glut2 fragment from a human liver cDNA library (in lambda ZAPII, kindly provided by Dr. Robert Korneluk, Children's Hospital Research Institute, Ottawa) to use as a probe to screen a cDNA library made from liver of freezing-exposed frogs. Primers complementary to sequence at 387-401 bp, 816-830 bp and 1598-1613 bp of human glut2 were synthesized (kind gift from Brian Martin, National Institute of Mental Health, Bethesda, MD) for use in polymerase chain reactions (PCR). PCR was performed using a variety of conditions including 1 - 5 mM MgCl₂, 0.05 - 0.5 μ M primer, 1% and 3% v/v DMSO, 1% and 5% v/v formamide, 1% and 5% v/v formamide with 10% v/v glycerol, 1/100, 1/10 and undiluted template (human liver cDNA library phage) and four different cycling protocols: 1. one cycle of 3' at 94°; 35 cycles of 45" at 94°, 30" at 55°, 1'30" at 72°; 1 cycle of 10' at 72°, 4°C indefinite: 2. one cycle of 5' at 94°; 24 cycles of 2' at 60°, 2' at 72°, 1'30" at 94°; one cycle of 2' at 60°, 7' at 72°; 4°C indefinite: 3. twenty-seven cycles of 10" at 95°, 10" at 68°, 1' at 70°; 4°C indefinite: 4. one cycle of 5' at 95°, 45" at 55°, 2' at 72°; 30 cycles at 45" at 95°, 45" at 55°, 2' at 72°; one cycle of 45" at 95°, 45" at 55°, 5' at 72°; 4°C indefinite. Concurrently, one of the primers (complementary to a middle portion of the transporter sequence) was employed as a probe to screen the human liver cDNA library for a human glut2 cDNA (see the next section for detailed explanation of cDNA library screening). Primary and secondary screens were performed under conditions outlined for oligo probes by Amersham

(Cleveland, OH): prehybridization solution contained 6x SSC (from 20x SSC stock: 3.0 M NaCl, 0.3 M sodium citrate, pH 7.0), 20 mM sodium dihydrogen phosphate, 0.4% SDS, 5x Denhardt's solution (5% w/v bovine serum albumin, fraction V, 5% w/v PVP (polyvinylpropylene) 40,000, 5% w/v Ficoll 400) and 0.5 mg/ml denatured salmon sperm DNA; hybridization solution was the same but omitting the Denhardt's solution.

Hybridization was performed at 65°C overnight. Washes were in a solution of 6x SSC and 0.1% SDS at room temperature for 30 minutes, then at 65°C for two times 30 minutes. Plaque lifts were exposed to x-ray film at -70°C overnight. The primary screen revealed 25 potential clones which were then isolated and subjected to a second round of screening that reduced the number of clones to fifteen. These potential clones were then tested by the PCR using the middle and C-terminal primers described above under the following conditions: 1x reaction buffer (Promega), 200 µM dNTP, 0.5 µM of each primer, 2 mM MgCl₂, 5 U Taq polymerase (Promega), and 10 µl template. The following cycling conditions were employed: one cycle of 5' at 95°, 45" at 55°, 2' at 72°; 30 cycles of 45" at 95°, 45" of 55°, 2' of 72°; one cycle of 45" at 95°, 45" at 55°, 5' at 72°; 4°C indefinite. Four of the fifteen clones displayed amplification products of the expected size (800 bp). These potential human glut2 clones were converted to phagemid via *in vivo* excision (Stratagene, La Jolla, CA) and one was prepared for sequencing. Initial sequence analysis did not demonstrate that this one clone was human glut2. Sequence determination of the remaining clones was not performed as a human glut2 cDNA clone was acquired.

Second approach to finding wood frog glut2

Primary screening of frog liver cDNA library

A unidirectional cDNA library was constructed in the lambda ZAP II vector by Stratagene (La Jolla, CA) from poly A⁺ mRNA isolated from liver of 24 hour frozen *R. sylvatica* and consisted of greater than 10^6 primary clones. The amplified library was screened with radiolabeled human glut2 (liver-specific glucose transport protein, 1-1762 bp) kindly provided by Dr. Graeme Bell, University of Chicago Medical Center. The probe was prepared by initially releasing the insert from the vector by restriction digestion with *Bgl*II and then purifying that fragment from the vector via agarose gel electrophoresis. Recovery of the fragment from the gel matrix was facilitated by GeneClean III (Bio101, Vista, CA). This human glut2 fragment was then radiolabeled with α -³²P-dCTP using random primers and the Klenow fragment of DNA polymerase I (NEBlot kit, NEB, Mississauga, Ont.). The primary screen consisted of four, 24 x 24 cm NZY plates (5g NaCl, 2g MgSO₄·7H₂O, 5g yeast extract, 10 g peptone/L, pH 7.5) each containing approximately 30,000 plaque forming units (pfu). Plaques were lifted onto Hybond-N 0.45 μ m nylon membranes (Amersham, Cleveland, OH) with an adsorption time of two minutes. The membranes were then treated as follows: denaturation (1.5 M NaCl, 0.5 M NaOH) for 2 minutes, neutralization (1.5 M NaCl, 0.5 M Tris-HCl, pH 8) for 5 minutes, and rinse (0.2 M Tris-HCl, pH 8, 2 x SSC for 30 seconds. Plaque lifts were placed on a piece of Whatman 3M filter paper and excess rinse solution was allowed to drain from the membrane. Phage DNA was then UV cross-linked to the membrane using a UVP Ultraviolet Crosslinker Model CL-1000 (VWRCanlab, London, Ont.) set to deliver 120,000 microjoules/cm² (254 nm). The plaque lifts were pre-hybridized in 50 ml of solution containing 30% v/v formamide, 5 x Denhardt's solution, 0.2 mg/ml salmon sperm DNA, 0.1% w/v SDS and 5 x SSPE (0.75 M NaCl, 0.04 M NaH₂PO₄-H₂O, 0.005 M EDTA, pH 7.4) for one hour at 42°C in a Labline Model 308 hybridization oven (VWRCanlab, London, Ont.). Radiolabeled human glut2 probe was denatured by boiling for 5 minutes and then incubated on ice for two minutes before addition to 50 ml of hybridization solution (same as above) at a concentration of 1×10^6 cpm/ml. The pre-

hybridization solution was discarded and replaced by the hybridization solution containing the human glut2 probe. Hybridization proceeded overnight (18-20 hours). The membranes were washed in 250 ml of 6 x SSC, 0.2% w/v SDS for 30 minutes at room temperature. They were then exposed to Kodak XAR5 film at -70°C overnight. Autoradiographs revealed 19 potential positive cDNA clones. Each plaque corresponding to a positive signal on the autoradiograph was removed using a sterile Pasteur pipette to a 1.5 ml Eppendorf tube containing 500 µl of SM buffer (50 mM TRIS-HCl, pH 7.5, 100 mM NaCl, 8 mM MgSO₄, 0.01% w/v gelatin). Chloroform was added to 5% v/v, the contents were mixed by brief vortexing and were then allowed to sit at room temperature for 30 minutes. The tubes were centrifuged at 4°C for 15 minutes at 5,000 xg. The supernatants containing isolated phage were transferred to a new Eppendorf tubes and stored at 4°C. A spot titer of each phage preparation was performed to determine the approximate number of plaque forming units/ml (pfu/ml). To obtain the titer, XL1BLUE cells were cultured at 37°C overnight in five ml of LB broth (10 g tryptone, 5 g yeast extract, 5 g NaCl/L, pH 7.5). Ten µl of the cells were mixed with pre-warmed (55°C), five ml TB top agar aliquots (10 g tryptone, 5 g NaCl, 8 g agar, 0.01 M MgSO₄ /L) and poured onto LB plates (LB broth + 15 g agar/L). Once the top agar had solidified, the plates were inverted and incubated at 37°C for one hour. During that time, serial dilutions of the phage solutions were prepared (1/100-1/10000, in SM buffer). Ten µl of each dilution was spotted onto a LB plate and then replaced at 37°C overnight. The titer of each phage preparation was then determined according to the following formula: pfu/ml = number of plaques counted x dilution / volume plated. These nineteen potential positive clones were then subjected to a second round of screening.

Secondary screening of potential positive cDNA clones

The potential positive clones from the primary screening were plated at low density (50-100 pfu/plate) on 100 x 85mm NZY plates. Plaque lifts and subsequent processing were performed as described above. Hybridization of the plaque lifts also proceeded as outlined above. Washes were in a solution of 30% v/v formamide, 5 x SSC and 1% w/v SDS, four times for 30 minutes each at 42°C. The plaque lifts were exposed to x-ray film at -70°C for two days. From the autoradiographs, fifteen positive cDNA clones were chosen. These phage were then converted to phagemid (pBLUESCRIPT) using the *in vivo* excision protocol provided by Stratagene. Briefly, mid-log phase XL1BLUE cells ($OD_{600}=0.2-0.5$) were combined with the phage of interest along with helper phage, Ex-Assist ($\sim 10^7$ pfu), and incubated at 37°C for 15 minutes. To this was added 3 ml of LB broth and incubation was extended for 2.5 - 3 hours. The XL1BLUE cells were then pelleted at 2,000 xg for 15 minutes and the supernatant containing the intermediate filamentous phage was transferred to a new tube and heated at 70°C for 15 minutes. Lingering cells were pelleted at 4,000 x g and the supernatant was again transferred to a new tube. These stocks of filamentous phage (containing the clone of interest) were stored at 4°C. Two hundred μ l of log phase *E. coli* SOLR cells ($OD_{600}=1.0$) were separately infected with 100 μ l of each of the filamentous phage stocks and incubated at 37°C for 15 minutes. One third of this volume was used to create a bacterial lawn on LB-ampicillin (25 μ g/ml) plates incubated at 37°C overnight. A single colony from the bacterial lawns was streaked onto a new LB-ampicillin plate and grown overnight at 37°C to produce master plates of SOLR colonies harboring pBLUESCRIPT which contained the clones of interest. Single colonies from the master plates of each of the fifteen potentials were used to separately inoculate 5 ml of LB broth and were grown overnight at 37°C with shaking. A mini plasmid preparation (RPM, Bio 101, Vista, CA) was used to isolate pBLUESCRIPT from each of the overnight liquid cultures. Five μ l of the final phagemid preparation was linearized with *Xba*I (Pharmacia, Baie d'Urfe, Que.) and analyzed for size, homogeneity and concentration by agarose gel

electrophoresis (1% TAE gel (0.5x TAE: 20 mM TRIS-acetate, 0.5 mM EDTA, pH 8.3)). This phagemid DNA was then used in the tertiary screen.

Tertiary screening of potential positive cDNA clones

A dot blot was used for the tertiary screen. The phagemid DNA isolated from the mini preparations of each potential clone was denatured prior to application to a nylon membrane. Denaturation of 150 ng of each potential phagemid was accomplished by the addition of 0.4 M NaOH and 10 mM EDTA in a final volume of 100 μ l followed by boiling for 10 minutes. The DNA solution was neutralized with the addition of an equal volume of 2 M ammonium acetate (pH 7). The phagemid DNA was then applied to a 0.2 μ m nylon membrane (Schleicher & Schuell, Keene, NH) following the instructions provided with the Bio-Dot Microfiltration Apparatus (Biorad, Hercules, CA). The DNA was UV cross-linked to the membrane as previously described. Pre-hybridization and hybridization was performed as described above except the solutions contained 50% v/v formamide. The dot blot was then washed at room temperature for 30 minutes each in 100 ml of the following solutions in order: 6x SSC, 0.2% SDS; 4x SSC, 0.2% SDS; 2x SSC, 0.2% SDS; 1x SSC, 0.2% SDS; 0.5x SSC, 0.2% SDS; and 0.2x SSC, 0.2% SDS. The dot blot was then exposed to x-ray film (Kodak XAR5) at -70°C overnight. A negative control (a 1700 bp clone of *Xenopus* elongation factor 1) was included in the procedure.

Sequencing of potential cDNA clones

All fifteen potential positive clones were subjected to sequence analysis in both the T3 and T7 (forward and reverse, respectively) direction. Phagemid DNA was isolated using a large scale alkaline lysis protocol (Maniatis, 1989). Ten μ g aliquots of the DNA

were then denatured by addition of NaOH to 0.2 M and incubation at 37°C for 30 minutes. The DNA solution was neutralized by addition of sodium acetate to 0.3 M. Glycogen was added as a carrier to a final concentration of 50 ng/μl. The phagemid DNA was then precipitated with the addition of 3 volumes of ethanol/0.16 M sodium acetate and overnight incubation at -20°C. The phagemid DNA was recovered by centrifugation at 4°C for 20 minutes at 12,000 xg. The nucleic acid pellet was washed once with ice cold 70% ethanol, the ethanol was aspirated and the pellet dissolved in a small volume of water (14 μl) in preparation for sequencing. Sequencing reactions were performed using the Sequenase v. 2.0 sequencing kit (USB, Arlington Heights, IL) according to the manufacturer's instructions with incorporation of 35S-dATP. Samples were heated to 75°C for two minutes prior to loading onto a 35 x 43 cm, 0.4 mm thick, 6% sequencing gel (6% acrylamide, 7M urea, 0.5x TBE). Gels were electrophoresed at a constant power of 60 watts for two to six hours, fixed in a solution of 10% v/v methanol, 5% v/v glacial acetic acid and then dried onto Whatman 3M filter paper at 80°C for one hour. The dried gel was exposed to x-ray film overnight at room temperature. Initial sequence (approximately 400 bp) was obtained using T3 and T7 primers (USB, Arlington Heights, IL). Subsequent sequence was obtained using custom primers designed using Oligo 4.0 and synthesized at Queen's University's DNA synthesis facility.

Sequence Analysis

The sequence obtained from the potential clones was compared with known sequences contained in databases using the Genobase BLAST server available on the World Wide Web. Sequence analysis was performed using Lasergene software from DNASTAR (Madison, WI)

Re-screening of the fifteen potential clones via Southern blot analysis

The fifteen potential frog glucose transporter clones were re-screened with radiolabeled human glut2 cDNA fragment. All clones were digested with *EcoRI/XhoI* to release the insert from the vector and the fragments were separated on a 1% TAE agarose gel. After the gel was stained and photographed, a Southern blot was performed using the downward alkali method described by Chomczynski (1992). Prior to transfer, the DNA contained in the agarose gel was denatured by gently shaking in a solution of 0.5 M NaOH and 1.5 M NaCl (500 ml, two times for thirty minutes). The stacking order of transfer components from bottom to top were as follows: a five cm stack of paper towels, cut slightly larger than the gel; five gel-sized pieces of Whatman 3M filter paper; one gel-sized piece of filter paper pre-wet in transfer buffer (20x SSC); one gel-sized piece of 0.2 μ m nylon membrane (Schleicher & Schuell, Keene, NH); the agarose gel; three pieces of pre-wet, gel-sized filter paper; wick. The ends of the wick were immersed in transfer buffer. The transfer proceeded for two hours. The DNA on the blot was then neutralized in a solution of 0.25 M TRIS-HCl, pH 7.0, 1.5 M NaCl for five minutes and subsequently cross-linked to the membrane. Pre-hybridization of the membrane was carried out at 42°C in 10 ml of a solution of 50% v/v formamide, 5x Denhardt's solution, 0.2 mg/ml salmon sperm DNA, 0.1% SDS and 5x SSPE for one hour. Hybridization with denatured, radiolabeled human glut2 cDNA continued overnight with a fresh aliquot of solution. Excess radioactive counts were then washed from the Southern blot in a solution of 2x SSC, 0.1% SDS (250 ml, two times at thirty minutes intervals). The blot was exposed to x-ray film at -70°C for 3 days.

Northern Analysis of two identified clones

Northern analysis was employed to determine if two of the identified clones were regulated at the level of transcription during freezing. Total RNA isolated from liver of

control, four hour frozen, 24 hour frozen and 24 hour recovered frogs was separated on a denaturing 1.5% agarose gel and then transferred onto 0.2 μ m nylon membrane and cross-linked. Northern transfer occurred as described for the Southern transfer with the denaturation step replaced by two, thirty minute washes of the formaldehyde gel in double distilled water. Phagemids containing the cDNA clones identified as ATPase and α chain fibrinogen were isolated using a small scale alkaline lysis plasmid preparation modified from Maniatis *et al.* (1989). The DNA was digested with *EcoRI/XhoI* to release the cDNA inserts of interest (ATPase and α chain fibrinogen). The inserts were purified from the vector by agarose gel electrophoresis and then removed from the matrix using GeneClean III (Bio 101, Vista, CA). The inserts were labelled with α -³²P-dCTP using random primers and the Klenow fragment of DNA polymerase I (NEB, Mississauga, Ont.) and used to probe duplicate northern blots separately. The northern blots were pre-hybridized in 10 ml of hybridization (as described for the Southern blot) at 42°C for one hour. At this point the solution was replaced and denatured probe was added (one labelled probe/blot in separate tubes). Hybridization proceeded overnight at 42°C. Excess probe was removed from the northern blots by washing in a solution of 2x SSC, 0.1% SDS for thirty minutes at room temperature. The blots were then exposed to x-ray film at -70°C for six days. The approximate size of resulting bands on the autoradiograph were interpolated from a plot of log molecular weight vs distance migrated for a RNA molecular weight ladder (Gibco-BRL, Bethesda, MD).

Final approach to finding wood frog glut2

A final approach to the isolation of the liver glucose transporter of *Rana sylvatica* employed new degenerate primers specific to conserved regions (transmembrane segments, designated TM1-12) of known mammalian GLUT2 sequences, PCR and TA cloning. The primers described in Table 1. The sixteen possible primer pairs were

employed in PCR protocols using the frozen frog liver cDNA library as template. PCR conditions were as follows: 1x reaction buffer (Promega), 200 μ M dNTPs, 2 mM $MgCl_2$, 0.5 μ M each primer, 1 U Taq polymerase (Promega) and 5 μ l template. Cycling parameters were as follows: one cycle of 5' at 95°; 30 cycles of 45" at 95°, 45" at 60°, 1' 30" at 72°; one cycle of 45" at 95°, 45" at 60°, 5' at 72°; 4°C indefinite. Amplification products of approximate expected sizes (~1000 - 1350 bp) were obtained with four primer pairs. One of the primer pairs was utilized to amplify enough PCR product to ligate into the pGEM-T vector (Promega, Madison, WI). Successful TA cloning was indicated by blue/white screening of competent *E. coli* HB101 cells transformed with the recombinant plasmid grown on X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside)/ IPTG (isopropylthio- β -galactoside) containing media. Thirteen white colonies were chosen for plasmid isolation (mini alkaline lysis protocol from Maniatis *et al.* (1989)) and subsequent analysis of that DNA by restriction digestion (NdeI and SacII) and agarose gel electrophoresis (0.5x TAE, 0.8%, 80V constant). Of those, only one suggested the presence of an insert. This plasmid was then isolated using a mini alkaline lysis procedure (as above) and prepared for sequencing by alkali denaturation (described above). Sequence obtained from this clone was compared with known sequences in GenBank via the BLAST server.

RESULTS

PART ONE - RNA *IN VITRO* TRANSLATION

Total RNA was isolated from selected tissues of wood frogs using Trizol™ reagent and then separation on denaturing agarose gels was used to determine purity and intactness. Figure 1 shows photographs taken of these gels after staining with ethidium bromide and viewed under UV light (310 nm). Tissues are liver (a), brain (b) and heart (c). Figure 2 shows the equivalent gels of total RNA from skeletal muscle (a), kidney (b) and gut (c). The top band represents the 28S rRNA subunit; the bottom band is the 18S rRNA subunit. The background smearing is mRNA. No signs of degradation were observed (i.e., no smears of nucleic acid down the entire length of the gel) for extracts from any of the tissues. Separate spectrophotometric analysis of the total RNA from each tissue showed OD_{260/280} ratios that ranged from 1.6-2.0, expected values as indicated by the manufacturer of the reagent (Gibco-BRL, Bethesda, MD). Therefore, samples were pure and generally free of contamination (i.e., excess protein or phenol would lower the ratio beyond the acceptable range). Contamination by these agents may have rendered the RNA highly or completely untranslatable.

Extracts of total RNA were then used for *in vitro* translation. Translation products were analyzed for all six tissues of wood frogs and from the following experimental states for each: control (5°C), 24 hour frozen (at -2.5°C) and 24 hour recovered (thawed at 5°C). Translation was allowed to occur for one hour; previous study in the lab demonstrated linear translation rates over a four hour period with one hour as the optimal length of incubation for the reaction. Radiolabeled translation products were separated on 1-dimensional SDS-PAGE gels, the gels were stained and then subjected to fluorography. All autoradiograms are oriented such that these experimental states are presented from left to right. Developed autoradiograms were then scanned to facilitate the identification

of new translation products or changes in the content of products between the experimental states. The predicted molecular weights of translation products were estimates and accurate to $\pm 10\%$, a value previously determined in the lab.

Figure 3 is a photograph of the autoradiogram showing the radiolabeled *in vitro* translation products made from total RNA extracted from frog liver. Each lane was loaded with 20,000 acid precipitable counts (cpm); exposure time to x-ray film at room temperature was ten days. Translation of this sample was attempted seven times, with two autoradiograms displaying the same banding pattern, the remaining not being discernable due to over- or under-exposure of the film (similarly for subsequent translation results descriptions). Visual comparison of the banding patterns revealed many differences between the three experimental states. These differences were even more apparent in the densitometry scans of the photograph (Figure 4a,b,c). Most noticeable was the greatly increased abundance of a >200 kD protein in the frozen and recovered states, as compared with the control (Figure 4a and c). A ~ 45 kD protein was also seen to be enhanced in the frozen state (Figure 4a). Several new translation products ($\sim 41 - 15$ kD) were identified in the frozen state as compared with the control state (Figure 4a). However, by contrast with the multiple new proteins identified as a response to freezing, the comparison translation products in frozen versus thawed liver revealed three proteins that were present in the frozen state and had disappeared after thawing ($\sim 41, 22$ and 16 kD) (Figure 4c). The comparison of recovered with control states revealed that proteins of $\sim >200, 34, 28, 23, 22, 16$, and 15 kD that first appeared during freezing, remained elevated after thawing (Figure 4c).

The photograph of the autoradiogram of the radiolabeled *in vitro* translation products resulting from brain mRNA is shown in Figure 5. Each lane was loaded with 100,000 acid precipitable counts (cpm); exposure time to x-ray film at room temperature was one day. Translation of this sample was attempted three times, with two autoradiograms displaying the same banding pattern. Figure 6a, b and c show the

densitometry scans from this photograph. Figure 6a compares the results for the control and frozen states; these revealed the addition of one new protein (~59 kD) produced upon freezing exposure and one protein (~6 kD) that was present in the control state but disappeared in the frozen state. Scans of the lanes from frozen versus recovered states were essentially identical (Figure 6b) suggesting no changes in the mRNA populations between the two states. A comparison of control and recovered states revealed the persistence of the ~59 kD protein (identified in translation extracts from frozen frogs) into recovery and continuing absence of the ~6 kD protein in thawed animals (Figure 6c).

The photograph of the autoradiogram of radiolabeled *in vitro* translation products produced from heart mRNA is shown in Figure 7 and the gel scans in Figure 8. Each lane was loaded with 100,000 acid precipitable counts (cpm); exposure time to x-ray film at room temperature was one day. Translation of this sample was attempted four times, with three autoradiograms displaying the same banding pattern. Figure 8a compares the densitometry scans of the gels produced from control and frozen heart samples and shows the presence of a new ~6 kD protein in the latter. This protein product disappeared, however, during thawing (Figure 8b). A ~40 kD protein was observed in the samples from control and recovered states but was not present during freezing exposure (Figures 8a and 8c).

Figure 9 shows a photograph of the autoradiogram of the radiolabeled *in vitro* translation products produced from hind leg skeletal muscle mRNA. Each lane was loaded with 50,000 acid precipitable counts (cpm); exposure time to x-ray film at room temperature was ten days. Translation of this sample was attempted six times, with four autoradiograms displaying the same banding pattern. Differences between the control and frozen states were observed from the densitometry scans presented in Figure 10a. Two proteins, one unique (~116 kD) and one enhanced (~53 kD), were clearly discernable in the frozen state, as compared with the control. Note from Figure 10b that these two proteins were expressed due to freezing conditions since they were lost or

reduced, respectively, upon thawing. No differences were observed in the comparison of control and recovered states (Figure 10c).

Figure 11 is a photograph of the autoradiogram of the radiolabeled *in vitro* translation proteins produced from kidney mRNA. Each lane was loaded with 50,000 acid precipitable counts (cpm); exposure time to x-ray film at room temperature was ten days. Translation of this sample was attempted twice times, with both autoradiograms displaying the same banding pattern. Only one difference, the enhanced production of a >200 kD protein during freezing, was noted when contrasting the densitometry scans of control and frozen states (Figure 12a) and frozen and recovered states (Figure 12b). The comparison of control versus recovered states showed no differences (Figure 12c).

The autoradiogram of *in vitro* translation products produced from gut extracts is shown in Figure 13. Each lane was loaded with 50,000 acid precipitable counts (cpm); exposure time to x-ray film at room temperature was ten days. Translation of this sample was attempted twice; one autoradiogram displayed a clear banding pattern. Pairwise comparisons of the three states revealed no differences in the proteins produced upon translation in any of the 3 states (Figures 14a, b and c) indicating a lack of differential gene expression in this tissue in response to freezing.

PART II - *RANA SYLVATICA* LIVER GLUCOSE TRANSPORTER cDNA

Initial approach to finding wood frog glut2

PCR amplification of a human glut2 fragment from a human liver cDNA library was unsuccessful as demonstrated by agarose gel electrophoresis despite varied alteration of all PCR components and addition of several non-conventional components (DMSO, formamide, glycerol). As well, probing the human liver cDNA library with an oligo (one

of the PCR primers) was fruitless. Of the fifteen clones isolated by the screening procedure, only four would amplify by the PCR. Sequence of one of these clones and subsequent GenBank analysis revealed that the clone was not a cDNA fragment of the human glut2.

Second approach to finding wood frog glut2

The DNA fragment used to screen the frozen frog liver cDNA library, a human glut2 cDNA (1-1762 bp), was released from its vector by restriction digestion and subsequently purified by agarose gel electrophoresis. The ethidium bromide stained gel showing the free cDNA fragment (arrow) is shown in Figure 15.

Primary and secondary screening of the frog liver library was carried out at lower stringency (only 30% v/v formamide in the hybridization solution) as described by Thorens *et al.* (1988) due to the heterologous nature of the probe. Since the probe was mammalian in origin and the target cDNA was amphibian, stringency was lowered to allow for mismatches between the base pairing of probe and target. The tertiary screen was performed at normal stringency (50% v/v formamide) to better ensure the selection of positive clones.

The primary screen of the cDNA library prepared from liver poly (A)⁺ RNA of frozen frogs provided nineteen potential positive clones which were then subjected to secondary screening. This reduced the number of potentials to fifteen. A tertiary screen of these fifteen clones indicated that all were potential positives. The clones were labeled 4A, 4B, 5A, 11A-C, 18A-D and 19A-E. The phagemids containing these potential clones were isolated on a large scale. The purity of the phagemid preparation and size of each insert was determined by agarose gel electrophoresis after digestion of the phagemid with the appropriate restriction enzymes. Figure 16 shows the ethidium bromide stained gel of these digested phagemids. Linearized pBLUESCRIPT (vector) is approximately 2.95 Kb

in size whereas insert sizes ranged from 0.6 - 2.6 Kb. Multiple bands in a lane suggest that the insert contained sites for one or both of the restriction enzymes used.

Partial nucleic acid sequence was obtained for all fifteen potential positive clones. The possible identity of these sequences was then determined using the GenoBase BLAST server available on the World Wide Web. None of the clones were identified as a glucose transporter protein. However, two clones did result in strong possible identities. One clone (18B) was strongly identified ($P(N)$ of 4.3×10^{-118}) as mitochondrial H^+ -transporting ATP synthase (beta chain of F_1 subunit) whereas the other (18C) was suggestive of fibrinogen, alpha chain of ($P(N)$ of 9.2×10^{-107}). Note that $P(N)$ refers to the probability that the match is due to chance alone; therefore, the smaller the number, the more likely the match is not random. $P(N)$ is a relative probability given by the search program and is used to rank homology between the query sequence and database sequences. Other clones revealed some homology to retinol binding protein ($P(N)$ of 1.9×10^{-35}), DTDP-4-dehydrorhamnose-3,5-epimerase ($P(N)$ of 5.0×10^{-53}), NADPH cytochrome P450 reductase ($P(N)$ of 3.1×10^{-29}), cytochrome c oxidase ($P(N)$ of 8.6×10^{-22}).

To re-check the results of the tertiary screen, an agarose gel containing digested vector and inserts (gel seen in Figure 16) was used to produce a Southern blot which was then probed with radiolabeled human GLUT2 cDNA fragment. The results indicated that the probe was binding only the vector, and not insert, which suggested that none of the fifteen potential clones was a glucose transporter (Figure 17). With this result, further pursuit of the glucose transporter by this method was halted and experimental studies were directed to concentrate on the two identified clones.

The possibility that the two identified clones (18B and 18C) were regulated during freezing was investigated by northern analysis. Total RNA isolated from the liver of control, 4 hour frozen, 24 hour frozen and recovered frogs was separated electrophoretically in duplicate and then transferred to a 0.2 μ m nylon membrane. One

blot was probed with radiolabeled 18B insert cDNA (ATPase) whereas the other was probed with radiolabeled 18C insert cDNA (α fibrinogen). The autoradiogram of the blot probed with clone 18B (not shown) displayed non-specific binding to the 18S rRNA band and therefore was eliminated from any further study. The autoradiogram of the blot probed with clone 18C, despite appearing to indicate the up-regulation of α fibrinogen, could not be trusted as there was a discrepancy with the position of the 28S rRNA band (i.e., it appeared smaller than 5 kb as compared to the molecular size standards). This discrepancy may have been due to degradation of the 28S RNA in this particular liver RNA sample although the 18S rRNA migrated the expected distance in the gel (i.e., ~2 kb). As well, inspection of the RNA gel which was used to produce the northern blots (not shown) revealed that the amount of RNA loaded into each of the lanes was not uniform, i.e., the ribosomal bands appeared darker in the four hour frozen sample lane. Therefore, the apparent enhancement of the α fibrinogen transcript in response to freezing may be erroneous.

Third approach to finding wood frog glut2

A final approach to cloning a wood frog glut2 DNA fragment involved attempted amplification of glut2 from the frozen frog liver cDNA library using degenerate primers complementary to well conserved regions of the protein. Degenerate primers derived from amino sequence vary at the 3' codon position due to the degeneracy of the genetic code. Therefore, 2/3 bases will match the complementary nucleic acid sequence while one, the last of the triplet, may not. The choice of the base placed at the last position of the triplet can be based on the GC content of other known genes, if available, or be simply a guess. A PCR amplification product (using the universal primers, T₃ and T₇) of ~1000 bp was cloned into a T-vector (pGEM-T vector) and this recombinant plasmid was used to transform competent *E. coli* HB101 cells. Thirteen white colonies were chosen

from LB+ampicillin plates containing X-Gal and IPTG for plasmid isolation and subsequent analysis of that DNA by restriction digestion and agarose gel electrophoresis. Of those, only one suggested the presence of an insert, i.e., the vector alone was ~3 kb and the digested recombinant vector migrated to ~4 kb) (not shown). This plasmid was then isolated using a mini alkaline lysis procedure and prepared for sequencing by alkali denaturation. Sequence analysis of this clone using the GenBank database identified the insert as a possible DNA adenine methylase gene (*dam*), an *E. coli* gene.

Figure 1. Denaturing agarose gel of total RNA isolated from liver, brain and heart

Total RNA was isolated from liver, heart and brain of control (5°C), frozen (24 hours at -2.5°C) and recovered (24 hour thaw at 5°C) wood frogs. Two µg was analyzed on a 1.5% denaturing (formaldehyde) agarose gel. Lane 1, control; Lane 2, frozen; Lane 3, recovered. (a) Total RNA from liver. (b) Total RNA from brain. (c) Total RNA from heart. Ribosomal RNA (28S and 18S) is indicated on the right.

Figure 2. Denaturing agarose gel of total RNA isolated from skeletal muscle, kidney and gut (stomach + intestines combined)

Total RNA was isolated from muscle, kidney and gut of control (5°C), frozen (24 hour at -2.5°C) and recovered (24 hour thaw at 5°C) wood frogs. Two µg was analyzed on a 1.5% denaturing (formaldehyde) agarose gel. Lane 1, control; Lane 2, frozen; Lane 3, recovered. (a) Total RNA from muscle. (b) Total RNA from kidney. (c) Total RNA from gut (stomach + intestine). Ribosomal RNA (28S and 18S) is indicated on the right.

Figure 3. Photograph of the autoradiogram of radiolabeled proteins from *in vitro* translation of total RNA isolated from frog liver

Total RNA isolated from liver of control (5°C), frozen (24 hours at -2.5°C) and recovered (24 hour thaw at 5°C) wood frogs was translated in a cell free system (wheat germ extract). Resulting proteins were separated by SDS-PAGE using a 8-15% linear gradient. Each sample load was 20,000 cpm. The gel was subjected to fluorography, dried and exposed to x-ray film for 10 days at room temperature to produce the autoradiogram. From left to right: Lane 1: control ; lane 2: frozen; lane 3: recovered. New or enhanced proteins present only in the translation of total RNA from liver of frozen frogs are indicated by arrows with their approximate size (kD). Protein molecular weight standards are indicated on the left.

Figure 4. Densitometry scans of the autoradiograms of protein products from *in vitro* translation of liver total RNA

The autoradiogram was scanned into a computer program (Imagequant) to generate a numerical representation of the banding pattern (O.D. = optical density) of each lane which was then plotted against relative migration along the gel. The x-axis also shows the position of the broad range protein molecular weight standards.

(4a) Comparison of scans of autoradiograms from liver of control and frozen frogs.

Proteins found only in the frozen state are indicated by arrows with their approximate size (range of ~>200 - 15 kD).

(4b) Comparison of scans of autoradiograms from liver of frozen and recovered frogs.

Proteins found only in the frozen state are indicated by arrows with their approximate size (range of ~41 - 15 kD).

(4c) Comparison of scans of autoradiograms from liver of control and recovered frogs.

Proteins found only in the recovered state are indicated by arrows with their approximate size (range of ~>200 - 15 kD).

(b)

(c)

Figure 5. Photograph of the autoradiogram of radiolabeled proteins from *in vitro* translation of total RNA isolated from frog brain

Total RNA isolated from brain of control (5°C), frozen (24 hours at -2.5°C) and recovered (24 hours thawed at 5°C) wood frogs was translated in a cell free system (wheat germ extract). Resulting proteins were separated by SDS-PAGE using a 8-15% linear gradient. Each sample load was 100,000 cpm. The gel was subjected to fluorography, dried and exposed to x-ray film for 1 day at room temperature to produce the autoradiogram. From left to right: Lane 1: control ; lane 2: frozen; lane 3: recovered. Proteins present only in the translation of total RNA from brain of frozen frogs are indicated by arrows with their approximate size (kD). Protein molecular weight standards are indicated on the left.

Figure 6. Densitometry scans of the autoradiograms of protein products from *in vitro* translation of brain total RNA

The autoradiogram was scanned into a computer program (Imagequant) to generate a numerical representation of the banding pattern (O.D. = optical density) of each lane which was then plotted against relative migration along the gel. The x-axis also shows the position of the broad range protein molecular weight standards.

(6a) Comparison of scans of autoradiograms from brain of control and frozen frogs. One protein found only in the frozen state is indicated by an arrow with its approximate size (~59 kD).

(6b) Comparison of scans of autoradiograms from brain of frozen and recovered frogs. No changes in the protein banding pattern profile between the two states were observed.

(6c) Comparison of scans from autoradiograms from brain of control and recovered frogs. One protein found only in the recovered state is indicated by an arrow with its approximate size (~59 kD).

(c)

Figure 7. Photograph of the autoradiogram of radiolabeled proteins from *in vitro* translation of total RNA isolated from frog heart

Total RNA isolated from heart of control (5°C), frozen (24 hours at -2.5°C) and recovered (24 hour thaw at 5°C) wood frogs was translated in a cell free system (wheat germ extract). Resulting proteins were separated by SDS-PAGE using a 8-15% linear gradient. Each sample load was 100,000 cpm. The gel was subjected to fluorography, dried and exposed to x-ray film for 1 day at room temperature to produce the autoradiogram. From left to right: Lane 1: control ; lane 2: frozen; lane 3: recovered. Proteins present only in the translation of total RNA from heart of frozen frogs are indicated by arrows with their approximate size (kD). Protein molecular weight standards are indicated on the left.

Figure 8. Densitometry scans of the autoradiograms of protein products from *in vitro* translation of heart total RNA

The autoradiogram was scanned into a computer program (Imagequant) to generate a numerical representation of the banding pattern (O.D. = optical density) of each lane which was then plotted against relative migration along the gel. The x-axis also shows the position of the broad range protein molecular weight standards.

(8a) Comparison of scans of autoradiograms from heart of control and frozen frogs. One protein found only in the frozen state is indicated by an arrow with its approximate size (~6 kD).

(8b) Comparison of scans of autoradiograms from heart of frozen and recovered frogs. One protein found only in the frozen state is indicated by an arrow with its approximate size (~6 kD).

(8c) Comparison of scans of autoradiograms from heart of control and recovered frogs. One protein found only in the recovered state is indicated by an arrow with its approximate size (~40 kD).

Figure 9. Photograph of the autoradiogram of radiolabeled proteins from *in vitro* translation of total RNA isolated from frog muscle

Total RNA isolated from muscle of control (5°C), frozen (24 hours at -2.5°C) and recovered (24 hour thaw at 5°C) wood frogs was translated in a cell free system (wheat germ extract). Resulting proteins were separated by SDS-PAGE using a 8-15% linear gradient. Each sample load was 50,000 cpm. The gel was subjected to fluorography, dried and exposed to x-ray film for 10 days at room temperature to produce the autoradiogram. From left to right: Lane 1: control ; lane 2: frozen; lane 3: recovered. Proteins present only in the translation of total RNA from muscle of frozen frogs are indicated by arrows with their approximate size (kD). Protein molecular weight standards are indicated on the left.

Figure 10. Densitometry scans of the autoradiograms of protein products from *in vitro* translation of muscle total RNA

The autoradiogram was scanned into a computer program (Imagequant) to generate a numerical representation of the banding pattern (O.D. = optical density) of each lane which was then plotted against relative migration along the gel. The x-axis also shows the position of the broad range protein molecular weight standards.

(10a) Comparison of scans of autoradiograms from muscle of control and frozen frogs. Proteins found only in the frozen state are indicated by arrows with their approximate size (~116 and 53 kD).

(10b) Comparison of scans of autoradiograms from muscle of frozen and recovered frogs. Proteins found only in the frozen state are indicated by arrows with their approximate size (~116 and 53 kD).

(10c) Comparison of scans of autoradiograms from muscle of control and recovered frogs. No changes in the protein banding pattern profile between the two states were observed.

Figure 11. Photograph of the autoradiogram of radiolabeled proteins from *in vitro* translation of total RNA isolated from frog kidney

Total RNA isolated from kidney of control (5°C), frozen (24 hours at -2.5°C) and recovered (24 hour thaw at 5°C) wood frogs was translated in a cell free system (wheat germ extract). Resulting proteins were separated by SDS-PAGE using a 8-15% linear gradient. Each sample load was 50,000 cpm. The gel was subjected to fluorography, dried and exposed to x-ray film for 10 days at room temperature to produce the autoradiogram. From left to right: Lane 1: control; lane 2: frozen; lane 3: recovered. Proteins present only in the translation of total RNA from kidney of frozen frogs are indicated by arrows with their approximate size (kD). Protein molecular weight standards are indicated on the left.

Figure 12. Densitometry scans of the autoradiograms of protein products from *in vitro* translation of kidney total RNA

The autoradiogram was scanned into a computer program (Imagequant) to generate a numerical representation of the banding pattern (O.D. = optical density) of each lane which was then plotted against relative migration along the gel. The x-axis also shows the position of the broad range protein molecular weight standards.

(12a) Comparison of scans of autoradiograms from kidney of control and frozen frogs.

One proteins found only in the frozen state is indicated by an arrow with its approximate size ($\sim >200$ kD).

(12b) Comparison of scans of autoradiograms from kidney of frozen and recovered frogs. One protein found only in the frozen state is indicated by an arrow with its approximate size ($\sim >200$ kD).

(12c) Comparison of scans of autoradiograms from kidney of control and recovered frogs. No changes in the protein banding pattern profile between the two states were observed.

Figure 13. Photograph of the autoradiogram of radiolabeled proteins from *in vitro* translation of total RNA isolated from frog gut (stomach + intestine combined)

Total RNA isolated from gut of control (5°C), frozen (24 hours at -2.5°C) and recovered (24 hour thaw at 5°C) wood frogs was translated in a cell free system (wheat germ extract). Resulting proteins were separated by SDS-PAGE using a 8-15% linear gradient. Each sample load was 50,000 cpm. The gel was subjected to fluorography, dried and exposed to x-ray film for 10 days at room temperature to produce the autoradiogram. From left to right: Lane 1: control ; lane 2: frozen; lane 3: recovered. No new proteins were present in the translation of total RNA from gut of frozen frogs. Protein molecular weight standards are indicated on the left.

Figure 14. Densitometry scans of the autoradiograms of protein products from *in vitro* translation of gut (stomach + intestine combined) total RNA

The autoradiogram was scanned into a computer program (Imagequant) to generate a numerical representation of the banding pattern (O.D. = optical density) of each lane which was then plotted against relative migration along the gel. The x-axis also shows the position of the broad range protein molecular weight standards.

(14a) Comparison of scans of autoradiograms from gut of control and frozen frogs. No changes in the protein banding pattern profile between the two states were observed.

(14b) Comparison of scans of autoradiograms from gut of frozen and recovered frogs. One protein found only in the frozen state is indicated by an arrow with its approximate size (~>200 kD).

(14c) Comparison of scans of autoradiograms from gut of control and recovered frogs. No changes in the protein banding pattern profile between the two states were observed.

Figure 15. Restriction digest of pSP64T/HTL with *Bg*/II to release the human glut2 fragment (1-1762 bp)

pSP64T/HTL containing a human glut2 cDNA (1-1762 bp) was digested with the restriction enzyme *Bg*/II to release the insert. The insert was purified from the vector by agarose gel electrophoresis and isolated from the gel. This fragment was then used as a probe to screen the cDNA library made from liver of frozen frogs. Molecular size standards (1 kb ladder from Gibco-BRL, Bethesda, MD) are indicated on the right.

Figure 16. Agarose gel analysis of fifteen potential frog liver glucose transporter clones

Fifteen potential frog liver glucose transporter clones were initially converted into phagemid via *in vivo* excision and the inserts were subsequently released via a double restriction digest (*EcoRI/XhoI*). The purity and concentration of the phagemid preparation and size of the inserts was determined by agarose gel electrophoresis. Lane 1: 1 Kb molecular size standards (Gibco-BRL, Bethesda, MD); lane 2: 4A; lane 3: 4B; lane 4: 5A; lane 6: 11A; lane 7: 11B; lane 8: 11C; lane 9: 18A; lane 10: 18B; lane 11: 18C; lane 12: 18D; lane 13: 19A; lane 14: 19B; lane 15: 19C; lane 16: 19D; lane 17: 19E.

Figure 17. Autoradiogram of Southern blot of potential glucose transporter clones probed with the human glut2 cDNA fragment

The fifteen potential glucose transporter clones were digested to separate the insert from the vector, electrophoretically separated (see Figure 16) and transferred to a 0.2 μ m nylon membrane. The resulting Southern blot was probed (one million cpm/ml solution) with radiolabeled human glut2 cDNA, the membrane was washed with a solution of 2x SSC and 0.1% SDS at room temperature for 2x30 minutes and then exposed to x-ray film at -70°C for two days. Note that the probe bound only the vector. Lane 1: 4A; lane 2: 4B; lane 3: 5A; lane 4: 11A; lane 5: 11B; lane 6: 11C; lane 7: 18A; lane 8: 18B; lane 9: 18C; lane 10: 18D; lane 11: 19A; lane 12: 19B; lane 13: 19C; lane 14: 19D; lane 15: 19E.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 18. Human GLUT2 sequence and position of polymerase chain reaction primers designed to isolate a wood frog glut2 cDNA clone

Mammalian GLUT2 proteins were aligned using the CLUSTALW Multiple Sequence Alignment program on the World Wide Web. Polymerase chain reaction primers were designed from regions of homology to amplify a wood frog glut2 cDNA clone from a frozen frog liver cDNA library. Boxed amino acids indicate those which were conserved in human, mouse and rat protein sequences. Primer locations are noted by lines: lines above the sequence indicate + strand primers; lines below the sequence indicate - strand primers.

MTEDKVTGTLVFTVITAVLG<u>SFOFGYDI</u> <u>GVINAPQQVIIS</u>	40
HYRHVLGVPLDDRKAINNYVINSTDELPTISYSMNP KPTP	80
WAEETVAAAQLITMLWSLSVSS<u>FAVGGMTASFFGGWLGD</u>	120
TLGRIKAMLVANILSLVGALLMGFSKLGPSHILIIAGRSI	160
SGLYCGLISGLVPMYIGEIAPTALRGALGTFHQLAIVTGI	200
LISQIIIGLEFILGNYDLWHILLGLSGVRAILQSLLLFFCP	240
ESPRYLYIKLDEEVKAKQSLKRLRGYDDVTKDINEMRKER	280
EEASSEQKVSIIQLFTNSSYRQPILVALMLHVAQQFSGIN	320
GIFYYSTSIFQTAGISQPVYATIGVGAINMILTAVSVLLV	360
EKAGRRSLFLIGMSGMFVCAIFMSVGLVLLNKFSWMSYVS	400
MIAIFLFVSFFEIGPGPIPWFMVVAIFFSQGPRPAALAIAA	440
FSNWTCNFIVAL<u>CFQYIA</u><u>DFCGPYVFFL</u><u>FAGVLLAFTLFT</u>	480
<u>FFKVPET</u>KGKS<u>FEEIAAEF</u>QKKSGSAHRPKAAVEMKFLGA	520
TETV	524

Table 1. Sequence and location of polymerase chain reaction primers designed from known mammalian GLUT2 protein sequence to amplify a glut2 cDNA fragment from the frozen wood frog cDNA library (see Figure 19 for protein sequence)

Designation	amino acid location	Primer Sequence 5' to 3'
N1a	29-35	GGAGTCATCAATGCACC
N1b	22-28	CAGTTCGGCTACGACA
N2a	113-118	TTCTTCGGAGGATGGCT
N2b	104-110	TTCGCAGTAGGTGGCATG
C1a	453-460	CCGATGTACTGGAAGCA
C1b	463-469	AGGAAAAAGACGTACGG
C2a	481-487	TCCGGCACCTTGAAGAA
C2b	494-499	TTCCGCGGCGATCTC

DISCUSSION

As predicted, the transition into a frozen state by *Rana sylvatica* caused a change in the translatable mRNA population in five of the six tissues investigated. Liver showed the greatest number of protein changes when comparing the control and frozen states, a finding that probably correlates with the central role of the liver as the key tissue responsible for cryoprotectant responses to freezing. Freezing elicited the production of a variety of hepatic proteins, ranging in approximate size from >200 kD to ~14.8 kD (Figure 4a). It may be suggested that these proteins play a role in freezing tolerance. While exact identification of these proteins was not possible from this study, some speculation can be made. Considering the glycogenolytic role of the liver in freezing, it may be expected that enzymes involved in the conversion of glycogen to glucose are up-regulated during freezing. The enzyme glucose-6-phosphatase, a 40.5 kD protein, may be represented by the enhanced ~41.2 kD band seen the *in vitro* translation of frozen liver samples (Figures 3 and 4a). The ~21.5 kD protein may correspond to the smallest of the heat shock proteins, hsp20, as these stress proteins can be induced by ischemia (Morimoto, *et al.*, 1992). Remaining bands may represent other molecular chaperones that assist in maintaining protein structure and stability during stress conditions. Transcription and translation factors or response element binding proteins may even be up-regulated. Some translation products may also correspond to polypeptides of multi-subunit enzymes.

Only one new protein, ~58.9 kD, was observed upon comparison of the *in vitro* translation samples from control and frozen brain (Figure 6a). Based on the molecular weight of the protein and knowing the importance of the glucose for cellular survival, it may be a glucose transporter that is being up-regulated. Since the brain accumulates high levels of cryoprotectant (Storey, 1987a), transport of glucose across the blood/brain barrier and then into neuronal cells may be enhanced. In mammals, GLUT3 is the

primary glucose transporter found in the brain and neurons and has a molecular mass of ~57 kD (Bell *et al.*, 1993; Mueckler, 1994). Little information is available on this member of the glucose transporter family, but research speculates that GLUT3 is similar to GLUT1 and GLUT4. GLUT1 shows enhanced expression during hypoxia and in response to increased cAMP levels; GLUT4 expression is also stimulated by cAMP (Mueckler, 1994). Therefore, GLUT3 expression may also respond to these stimuli, both of which are experienced by the wood frog during a natural freezing episode.

Heart *in vitro* translation samples revealed one peptide (~5.9 kD) that was produced in response to the stress (Figure 8a, b) and one that disappeared during freezing (~39.8 kD) (Figure 8a, c). A protein of ~5.9 kD would be comprised of only approximately 54 amino acids. The possibility that this band represents an incomplete translation product is low since it is absent from the control and recovered samples and yet so obvious in the frozen sample. One possibility for its identity is atrial natriuretic factor (ANF), even though the approximate molecular weight of the wood frog translation product differs from the known mass of the peptide in other organisms. ANF is a polypeptide hormone produced by the atrial tissue in response to hypoxia. This has been documented in rats and it is believed that ANF acts to increase cGMP and decrease lactate levels suggesting a beneficial metabolic effect during oxygen deprivation (Ljusegren, 1994). As a wood frog freezes, oxygen tension is reduced and fuel supplies are increasingly derived from fermentative glycolysis with lactate as the dominant end product in the heart (Storey, 1990). The induction of synthesis of an amphibian ANF during freezing would not, then, be unreasonable.

Because the frog freezes from the periphery inwards, skeletal muscle has a very limited amount of time in which to respond to freezing. Hence, it is not surprising that very few changes were seen between the *in vitro* translation products of control and experimental animals. In fact, the only new product detected was a ~110 kD protein that was expressed in response to sub-zero temperatures (Figure 10a). A ~52.5 kD protein

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was enhanced during the freezing episode and persisted into recovery (Figure 10b). In addition, two large (>200 kD) proteins that were present in control samples had disappeared from experimental samples; therefore those proteins had no apparent importance to freeze tolerance.

Analysis of the *in vitro* translation products produced by kidney extracts revealed only one difference between the control, frozen and recovered states, this being a large (>200 kD) protein that was enhanced in the frozen state (Figure 12a, b). A similarly large protein was the only difference seen in gut (stomach – intestine) translations as well, but here the protein was present in the control and frozen states and not found in samples from thawed animals. Hence, it is probably not of importance to sub-zero temperature survival (Figure 14b, c).

While *in vitro* translation of tissue mRNA is a very useful technique to scan for changes in the overall translatable mRNA pool in response to a stress, its obvious drawback is the inability to identify the specific proteins that are produced. Further techniques that could be used for protein identification include western blot analysis and two-dimensional electrophoresis. In western blot analysis, proteins in translation samples are separated by SDS-PAGE, electroblotted onto a membrane support (i.e., nitrocellulose or polyvinylidene difluoride (PVDF)) and then probed with an antibody to a specific protein of interest. Binding of the antibody to the blotted protein determines its identification (Burnette, 1981). Two-dimensional electrophoresis provides a method to separate the proteins from the mixture as discrete dots which can then be isolated and identified by amino acid sequence analysis. The first dimension separates the proteins on the basis of their isoelectric point (isoelectric focusing) and the second dimension separates by molecular weight (SDS-PAGE) (O'Farrell, 1975). The final gel is electroblotted onto PVDF, stained and proteins of interest can be cut from the membrane and subjected to automated amino acid sequencing for possible identification. This latter

technique may be worth consideration for use with future *in vitro* translation studies as it would strengthen the investigation by allowing identification of those proteins produced in response to freezing. The former technique is less enticing as it requires some prior "guessing" to decide which antibodies to test (and therefore which proteins are scanned for) and, in many instances, antibodies raised against mammalian proteins will not cross react with the equivalent proteins from amphibian tissues.

Another limitation to the interpretation of *in vitro* translation data is inability to quantitate the information. While laser densitometry can make a relative comparison, it is still not possible to compare the protein bands in a quantitative manner. As well, it is difficult to assign an accurate molecular weight to a protein of interest. Only an estimate can be achieved by producing several identical SDS-PAGE gels of the samples including molecular weight standards and then taking an average of those standards to derive a standard error value to apply to the estimated molecular weight.

The search for *R. sylvatica* liver glut2 was unsuccessful. The initial approach of isolating a human glut2 fragment from a human liver cDNA library via the PCR for use as a heterologous probe to screen the frog liver cDNA library was taken as the commercial availability of a glut2 clone was not known. Despite having primers specific for the DNA of interest, PCR amplification was not forthcoming. This may have been due to several factors including inadequate time for denaturation (especially since the template was encapsulated phage taken directly from the cDNA library stock), overly high annealing temperature, rarity of the target sequence or the primer sites were not present in the target sequence (Bangham, 1991). As an alternative procedure, one of the primers (complementary to a middle region of the nucleic acid sequence) was utilized as an oligo probe to screen the human liver cDNA library to find a glut2 clone. After a primary and secondary screening, fifteen clones were identified as potential human glut2 clones. The PCR was used to test the validity of these clones; four of the fifteen potential

clones were amplifiable. One of the four clones was prepared for sequencing; comparison of the partial, deduced amino acid sequence with the GenBank database revealed no homology to human glut2.

The second approach to finding a wood frog glut2 cDNA followed a similar screening strategy as the first approach with an acquired human glut2 cDNA clone. Due to the heterologous nature of the probe, a lower stringency screening protocol was followed as described by Thorens *et al.* (1988) who used this approach to clone rat glut2 using rat glut1 (brain specific isoform). Whereas the results from the primary to tertiary screen of the frozen frog liver cDNA library using the human glut2 fragment seemed encouraging, initial BLAST sequence analysis of the fifteen potential clones raised suspicion as none of the clones were identified as a glucose transporter. Subsequent Southern analysis of the clones revealed the glut2 probe was binding to the vector and not to the inserts. This non-specific binding may have been due to several short, similar sequences shared between the glut2 probe and the vector, pBLUESCRIPT. Alignment of the two sequences using LALIGN from EERIE-Nimes, France (available on the World Wide Web), revealed a 16 nucleotide overlap with 75.0% identity, a 30 nucleotide overlap with 70.0% identity, and a 46 nucleotide overlap with 58.7% identity. This similarity may have been adequate for hybridization given the target sequence (wood frog glut2) was not present. From figure 16 it can be seen that not all vectors were 3 kb in size; this may be due to poor construction of the original cDNA library. It was found during sequence analysis of the clones that most (14/15) clones did not contain a poly(A) signal. Also, some difficulty was encountered with restriction digestion of the clones in that the proper restriction enzymes did not always cut the vector. These reasons may explain the discrepancy in size in some of the vectors.

Of the fifteen clones isolated from the frog liver cDNA library by non-specific hybridization of the glut2 probe, six displayed some homology to known proteins in the GenBank database. Partial sequence analysis (one or more sequencing events in both the

forward and reverse directions) identified potential cDNA clones of: retinol binding protein, DTDP-4-dehydrorhamnose-3,5-epimerase, NADPH cytochrome P450 reductase, cytochrome c oxidase, the β unit of H^+ transporting ATP synthase (ATPase) and the α subunit of fibrinogen (Fg). Full sequence of all fifteen cDNA clones was not possible in the time frame of the project therefore seven clones remain unidentified. Future considerations for this project may include obtaining full sequence of these cDNA clones, identifying the deduced amino acid sequences via database searches and determining whether they are up-regulated in response to freezing by northern analysis. Indeed, this approach was attempted with two clones, one encoding for the β unit of ATPase and the other for α Fg. Unfortunately, there was an unexpected problem with the RNA samples in that the 28S rRNA band did not migrate the proper distance of ~ 5 kb as compared to the molecular weight standards in the denaturing agarose gel. Since the 18S rRNA band did migrate the expected distance (~ 2 kb) it was postulated that partial degradation of the 28S rRNA band within this sample had occurred. This, combined with uneven loading of RNA samples onto the gel, discredits any results which may have been derived from these northern analyses. Future analyses should include an internal control, a gene which is known not to alter in response to the stress (i.e., actin is commonly used for this purpose), to control for uneven loading of the RNA samples. A rRNA probe would also prove useful by confirming the position of rRNA on the autoradiogram when the clone of interest and rRNA migrates similar distances on the gel. Northern blot analysis using poly(A)⁺ mRNA would also solve the problem of potential ribosomal RNA cross hybridization.

The third and final approach to finding the wood frog glut2 involved TA cloning, a PCR based method. This technique relies on the presence of a single A residue overhang on the 3' end of the PCR product left by the *Taq* polymerase during amplification. This sticky end can be ligated easily into a vector containing a complementary T residue overhang. Degenerate primers were synthesized from

conserved (transmembrane) regions of mammalian glucose transporter amino acid sequences. Of the sixteen possible primer pairs, four displayed amplification products when analyzed by agarose gel electrophoresis. One of these products was isolated for TA cloning, ligated into a T-vector and used to transform competent *E. coli* HB101 cells. Colonies suggesting the presense of a recombinant plasmid as determined by blue white screening were selected, grown in broth culture and the plasmid was isolated. Sequence analysis of this apparently successful cloning event revealed an insert with homology to the DNA adenine methylase (dam) gene which is found in bacterial genomes. Further cloning events were not attempted due to time constraints.

Future studies concerning wood frog differential gene expression as seen in response to freezing could involve the isolation of differentially expressed cDNA clones via cDNA library screening followed by intense study of the 5' upstream regulatory regions of these genes (i.e., identify TATAA and CCAAT boxes, response elements). Regulation of genes involved in *R. sylvatica* freeze tolerance is currently unknown; research of this nature would be ground breaking and may lead to insights in the genetic event of natural freeze tolerance. Techniques such as 5' RACE (rapid amplification of cDNA ends) and 5'-RAGE (rapid amplification of genomic DNA ends), PCR based techniques, could produce copies of the 5' region of the gene which could then be subcloned into a vector for sequence analysis. DNA footprinting could be used to identify regions of the 5' upstream area that are being bound by various DNA binding proteins (i.e., polymerases, response element binding proteins). Finally, CAT (chloramphenicol acetyl transferase) assays could be employed to verify the functionality of such upstream regulatory regions. Such investigations may provide insight to the regulatory elements controlling those genes necessary for freeze tolerance in the wood frog.

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