RESPONSE TO FREEZE EXPOSURE BY THE WOOD FROG, *RANA SYLVATICA*: INVESTIGATING THE FREEZE INDUCED CHANGES IN TRANSCRIPTION AND TRANSLATION PATTERNS IN HEART AND LIVER

by

JAMES DAYRE McNALLY

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“RESPONSE TO FREEZE EXPOSURE BY THE WOOD FROG, *RANA SYLVATICA*: INVESTIGATING THE FREEZE INDUCED CHANGES TO TRANSCRIPTION AND TRANSLATION PATTERNS IN HEART AND LIVER”

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ABSTRACT

In recent years changes to gene and protein expression have been shown to underlie animal adaptation to environmental stresses. Similarly, recent studies have demonstrated significant changes to transcription and translation in wood frog tissues during freeze and thawing. The primary goal of this thesis was to further investigate these changes through the application of cDNA library screening and 2-D PAGE technologies. In total 7 additional genes and one protein were identified, significantly extending the list of known freeze up-regulated genes and proteins. From this expanded list the contractile protein, MLC2; two identifiable heart transcripts (*hsp27* and *anac*); and the novel liver transcripts (*li16* and *ddx8*) were selected for further investigation. The results implicate the heat shock and hypertrophic gene responses as part of the cellular response to freezing, and reveal that the wood frog heart responds to freezing with changes expected to alter AP-1 mediated gene transcription. Moreover, all of the identified cardiac responses, including specific increases in gene/protein levels and changes to protein phosphorylation states could be linked to the p38 MAPK pathway, confirming that it is the major signaling pathway activated in the wood frog heart following freezing exposure. The two novel liver clones were similarly investigated and studies were carried out to obtain full open reading frames, confirm the protein coding capacity and determine the factors regulating their expression. Although the cellular functions of the novel proteins remain a mystery the results obtained suggest distinctive cellular roles, and on the whole provide further evidence that novel gene development is an important component of acquired freeze tolerance. Secondary to the determination of the specific changes in gene and protein expression patterns following freeze exposure the impact of

freezing on the overall rates of transcription and translation in wood frog heart tissue were evaluated. The data from the nuclear run-on assays and western blots analysis (phosphorylation status of eIF2 α), confirmed the presumed suppression of transcription and translation in the wood frog heart during freezing. This discovery highlights the importance of the aforementioned genes and proteins, as their up-regulation takes place against a background of suppressed macromolecular synthesis.

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LIST OF ABBREVIATIONS

α NAC	alpha subunit of nascent associated complex
2D-PAGE	Two-Dimensional Poly-Acrylamide Gel Electrophoresis
AAT	ADP/ATP translocase
AFPs	antifreeze proteins
ANP	atrial natriuretic peptide
AP-1	activating protein 1
ATF	activating transcription factor
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
cAMP	3', 5' – cyclic adenosine monophosphate
cDNA	complementary DNA
cGMP	3', 5' – cyclic guanosine monophosphate
CPM	counts per minute
ddPCR	differential display polymerase chain reaction
DEPC	diethylpyrocarbonate
EDTA	ethylenediamine tetraacetic acid
EF1- γ	elongation factor-1 gamma
EF-2	elongation factor -2
EGTA	ethylenebis(oxyethylenitrilo)tetraacetic acid
eIF2 α	α subunit of eukaryotic initiation factor 2
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase

EST	expressed sequence tag
EtBr	ethidium bromide
GADD153	growth arrest and DNA damage-inducible protein
GCN2	General control non-repressible protein
GPI	glycophosphatidylinositol
GTP	guanosine triphosphate
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HSF	Heat shock factor
HSP	Heat shock protein
HSPC070	hematopoietic stem/progenitor cell line clone70
INPs	ice nucleating proteins
IP ₃	1,4,5-trisphosphate
JNK	c-jun NH ₂ -terminal kinase
kb	kilobase
kDa	kilo Dalton
KLH	keyhole limpet hemocyanin
MAPK	mitogen-activated protein kinase
MAPKAPK	mitogen activated protein kinase activated protein kinase
MEF2	myocyte enhancement factor
MHC	myosin heavy chain
MLC	myosin light chain
MOPS	2-[N-morpholine] proanesulfonic acid
MPCP	mitochondrial phosphate carrier protein

MRI	magnetic resonance imaging
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NFAT	nuclear factor of activated T-cells
ORF	open reading frame
PCR	polymerase chain reaction
PERK	PKR-like ER kinase
pfu	plaque forming unit
pI	isoelectric point
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethanesulfonyl fluoride
RACE	rapid amplification of cDNA ends
RNasin	ribonuclease inhibitor
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SRP	signal recognition particle
TAE	Tris-Acetate-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
UTP	uridine triphosphate
UTR	untranslated region

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CHAPTER 1

GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

Our planet is a compilation of diverse and constantly changing ecosystems. Each of these ecosystems is associated with a collection of organisms that have evolved and adapted to not only endure, but to flourish, within their habitat. The survival of each organism requires that it maintain homeostasis in the face of constant challenges from the environment including changes in temperature, oxygen tension, UV radiation, salinity, and water and nutrient availability, to name just a few. This problematic assignment is further complicated by the fact that the state of each habitat may fluctuate widely with respect to any one, or sometimes all, of these traits on both daily and seasonal time scales. A potent illustration of this, and the one of interest to our research laboratory and this thesis, is the seasonal changes experienced by those organisms living at high latitudes and altitudes. As the seasons move from spring/summer to the autumn and then winter, these organisms are faced with subzero temperatures, the possibility of freezing, and a reduction in available food supplies.

An examination of the various animals found in seasonally-cold environments reveals that a number of survival approaches have evolved, combining adaptations at behavioural, physiological and biochemical levels. The majority of ectothermic organisms utilize one of two relatively cautious approaches to dealing with temperatures below 0°C: (i) completely avoid such exposures by migrating to warmer climates (some insects) or seeking well-insulated hibernation sites (e.g. under water or deep underground), or (ii) endure subzero exposures but avoid freezing by using adaptations that allow a deep supercooling of body fluids (many arthropods and other terrestrial invertebrates). However, a third, more select, group of organisms has developed the

amazing ability to withstand the freezing of body fluids during winter subzero exposures. This group includes hundreds of species of insects as well as a variety of other terrestrial and marine intertidal invertebrates (Storey and Storey, 1988). As unique as this group of invertebrates may be, an even more exclusive collection is the handful of vertebrate animals capable of performing the same feat. Following the first description of vertebrate freeze tolerance in the early 1980's (Schmid, 1982), 11 vertebrate species have been identified that exhibit ecologically relevant freeze tolerance (e.g. an ability to endure long term freezing at a subzero temperatures that they encounter naturally in their hibernacula). These include 6 species from the Class Amphibia and 5 from the Class Reptilia (Table 1).

The question of why such an exceedingly small number of organisms have developed freeze tolerance can be explained by the series of dramatic physiological changes that take place when ice forms in and around an organ, tissue or cell. Many of the individual stresses that coalesce during the freeze-thaw cycle are by themselves considered to be pathological and in most animals cause widespread cell apoptosis and/or necrosis. Yet even when experienced together these stresses are 100% survivable by this distinct group of animals. If one considers that the problems associated with freeze tolerance are the same obstacles encountered by researchers and health care professionals working in the areas of organ cryopreservation and organ transplantation one can recognize the possible benefits of studying natural freeze tolerance. A greater understanding of the mechanisms and adaptations employed by naturally freeze tolerant organisms could provide insights into novel strategies that could lead to the improvement of current organ cryopreservation protocols.

Scientific investigations are driven by one of two desires: curiosity and demand. We believe that research focused on attaining a full understanding of the various adaptations necessary for freeze tolerance will allow us to not only appreciate a curious biological phenomenon but provide valuable, potentially life-saving, information.

1.2 SELECTION OF A MODEL SYSTEM

Although freeze tolerant animals have been identified in both the Amphibia and Reptilia classes, it is apparent from the existing literature that the freeze tolerance field primarily uses amphibian species as research tools. The 6 amphibians that meet the inclusion criteria established for freeze tolerant vertebrates are the wood frog, *Rana sylvatica*; the gray tree frog, *Hyla versicolor*, Cope's tree frog, *Hyla chrysoscelis*; the chorus frog, *Pseudacris triseriata*, the spring peeper, *Pseudacris crucifer*; and the Siberian salamander, *Salamandrella keyserlingi* (Table 1). Of this group, the wood frog is by far the most common animal used for studies of vertebrate freeze tolerance.

The initial selection of *R. sylvatica* was made following a comparison of the different species with respect to three characteristics: location, freezing competency, and availability. Although it was felt to be important that the animal model be found locally this stipulation did not exclude any of the species as all five frogs are indigenous to Canada. However of the five species, the wood frog has the most northerly distribution, with specimens being found as far north as Labrador and Alaska (Behler and King, 1979). Because the wood frog has the most northerly distribution of any of the freeze tolerant species, it was reasoned that the adaptations permitting survival would be more

fully developed in *R. sylvatica* relative to the other species. This distinction combined with the realization that the wood frog is one of the most available of the five species made it an obvious choice as the primary animal model of freeze tolerance.

Over the past two decades our laboratory has used the wood frog to elucidate the important behavioural, physiological and biochemical adaptations and modifications that contribute to freeze tolerance. However, before one can identify or appreciate these adaptations one must be familiar with the physiological changes that accompany vertebrate whole body freezing and the problems associated with these stresses.

1.3 THE PROBLEMS ASSOCIATED WITH FREEZING

1.3.1 INTRACELLULAR VS. EXTRACELLULAR FREEZING

One of the most prominent pathophysiological problems that can, and commonly does, occur during the freezing of tissues is the crystallization of intracellular water. Intracellular ice formation is highly damaging to subcellular architecture and microcompartments and is virtually always lethal. The only notable exception, to date, can be found with *Panagrolaimus davidi*, a nematode reported to have the capacity to survive intracellular freezing (Wharton and Ferns, 1995; Wharton *et al.*, 2002). For this reason, when considering the potential stresses encountered during a freeze-thaw cycle, intracellular ice formation should be placed near the top of list. Not surprisingly, natural freeze tolerance by the vast majority of organisms means endurance of ice formation in extracellular fluid compartments only, coupled with mechanisms that prevent

intracellular ice growth (Lee, 1991; Karlsson *et al.*, 1993; Wharton and Ferns, 1995). Nonetheless, extracellular freezing, on its own, places a considerable number of stresses on the organs, tissues and cells of a freezing organism, including: ischemia, dehydration/cell volume reduction, and mechanical damage.

1.3.2 ISCHEMIA AND OSMOTIC STRESSES

Two of the major stresses that are placed on the cells of an organism during freezing and that must be addressed in the development of freeze tolerance are osmotic and ischemic stresses. Both are consequences of ice formation in extracellular compartments. As ice grows it excludes solutes from its crystal matrix and, because of this, the remaining extracellular fluids become increasingly concentrated. This places an osmotic stress on cells that leads to an outflow of water and can result in a major decrease in cell volume (Mazur, 1984). The dehydration of cells and the resulting high concentrations of ions and other solutes in the remaining unfrozen fraction can ultimately reach levels that are damaging/denaturing, with the first victims of the osmotic stress being membranes. Cell volume reduction results in membrane compression, and beyond a critical minimum volume, leads to an irreversible collapse of membrane bilayer structure (Mazur, 1970; Wolfe *et al.*, 1986). Upon thawing, the bilayer structure that is key to membrane function and integrity is lost and cells are unable to regulate osmotic balance, ionic composition, transport processes or maintain intracellular compartmentalization of macromolecules. Thus, a key factor in freezing survival is the expression of adaptations that either minimize cell volume reduction or stabilize membranes.

Another consequence of extracellular freezing is that tissues are cut off from their normal deliveries of oxygen and metabolic fuels via the blood and are faced with an ischemic/anoxic state throughout the freeze. Accordingly, in order to survive whole body freezing, all cells of all organs (including the most oxygen-sensitive ones such as nervous tissue) must be able to endure long-term ischemia and derive their ATP throughout the freeze from the anaerobic fermentation of endogenous fuels.

1.3.3 MECHANICAL DAMAGE

Of the stresses encountered during a whole body/organ freeze-thaw cycle the most visible is ice-induced mechanical damage. When intact tissues are frozen, extensive physical damage may be caused by ice formation in extracellular and vascular spaces (Pegg, 1987). In tissues, ice tends to form first in the lumen of blood vessels as water is pulled out of cells and freezes in an expanded vascular space (Rubinsky and Pegg, 1988; Rubinsky et al., 1990). This vascular expansion, if uncontrolled, can rupture the walls of blood vessels so that, upon thawing, the structural integrity of blood vessels is lost and massive internal bleeding occurs. Hence, freeze tolerant organisms should have adaptations that both minimize mechanical damage by ice and promote damage repair during thawing.

1.3.4 OBSERVABLE FEATURES OF FREEZING AND THAWING IN *RANA SYLVATICA*

Some of the problems associated with whole body freezing can be observed in *R. sylvatica* after exposure to subzero temperatures. Following a freezing episode the normal physical and physiological processes of animals are visibly interrupted - limbs are no

longer extendible, and breathing, blood flow, and heart beat are temporarily suspended (Storey and Storey, 1984). The loss of respiration, heart beat, and blood flow gives rise to the described ischemic state, necessitating the need for appropriate adaptations and survival mechanisms to be in place. As well, upon dissection it can be observed that the *R. sylvatica* extracellular compartments are filled with ice, including masses of crystals found under the skin and in the abdominal cavity (Storey and Storey, 1987a). This ice derives from both pre-existing extracellular water and intracellular water that was pulled out of the cells by osmotic forces. Hence, organs of a frozen frog are visibly shrunken. Furthermore, the problems associated with physical damage to organs due to ice-induced rupture in microcapillary beds can occasionally be observed in thawing frogs in the form of hematomas present in leg musculature.

Yet when frozen under appropriate conditions this normally pathological state is 100 % survivable by the wood frog, and our laboratory has and continues to identify the physiological and biochemical adaptations that permit survival. Of the adaptations recognized, some parallel those identified in other model systems of freeze tolerance. Therefore, before we describe, in detail, the systems and modifications that have been implicated by our studies it might be valuable to explore the common adaptations that have been linked to the development of freeze tolerance and relate these to what is known about *R. sylvatica*.

1.4 COMMON ADAPTATIONS FOUND IN FREEZE TOLERANT ANIMALS

1.4.1 CRYOPROTECTANT PRODUCTION

One of the key adaptations supporting natural freeze tolerance is the synthesis and accumulation of low molecular weight cryoprotectants. Cryoprotectants can be divided into two distinct groups (Storey, 1997). The first group of compounds (e.g. trehalose, and proline) interacts with membranes to stabilize the membrane bilayer structure during freezing-induced cell volume reduction. The other class of low molecular weight cryoprotectants, when present, are usually found in much higher concentrations and act in a colligative manner to restrict the loss of intracellular water, allowing a minimum cell volume to be defended. In most cases, the lower threshold for cell volume reduction is associated with the conversion of ~65% of total body water into ice and freeze tolerant organisms pack their cells with sufficient cryoprotectant to prevent ice accumulation from exceeding this value under the normal temperature conditions of hibernation (Storey and Storey, 1992).

Research has established that a variety of different molecules can be used for cryoprotection. For example, among freeze tolerant insects glycerol is a very common cryoprotectant. Other polyhydric alcohols are also employed as cryoprotectants by insects including sorbitol, ribitol, erythritol and ethylene glycol, as well as disaccharides such as trehalose and sucrose. In addition to these carbohydrates, some freeze tolerant animals, such as the midland painted turtle *Chrysemys picta marginata*, elevate their intracellular pools of amino acids to increase cryoprotection in the frozen state (Churchill and Storey, 1991).

The main cryoprotectant mobilized during freezing in the wood frog is glucose. Studies in our laboratory have shown that the assembly of the cryoprotectant production machinery responsible for the release of glucose from the liver during freezing involves a combination of seasonal and freezing-inducible changes to enzyme amounts and activities. Prior to hibernation, glycogen stores in the liver of wood frogs are built up to very high levels - as much as 1000 μmol glucose equivalents per gram weight or 18% of liver fresh weight (Storey and Storey, 1984; Storey and Storey, 1986b). Following the onset of freezing there is an increase in the activity of glycogen phosphorylase that allows for rapid glycogenolysis and the release of glucose from the liver (Storey and Storey, 1984). To distribute this glucose, *R. sylvatica* also show a seasonal increase in the levels of plasma membrane glucose transporters (King *et al.* 1995). This expedites not only the release of glucose by the liver, but the uptake of glucose by other organs (Storey and Storey, 1988). Together these adaptations raise the cryoprotectant levels from <5 mM in control frogs to 200-300 mM in the core organs of frozen animals (Storey, 1984; Storey and Storey, 1984; King *et al.* 1995).

An additional advantage of high intracellular concentrations of glucose was identified using magnetic resonance imaging technology (MRI) (Rubinsky *et al.*, 1994). MRI was used to observe and analyze the freezing process in an intact living animal in real time, and clarified the sequence of organ freezing and thawing. A series of MRI images taken over a time course established that, as anticipated, the extremities of the animal freeze first and then ice propagates through the body cavity. However, an unexpected delayed onset of freezing was observed in the core organs. As ice penetrates more and more tissues, blood circulation is cut back until ultimately the last two organs to

freeze are those with the highest cryoprotectant levels (the liver, which produces the glucose, and the heart which distributes it). Similarly when a time course of thawing was analyzed it was observed that the process was not directional from the outer surface, but started almost simultaneously in most core tissues. This tactic is believed to prevent the cellular deterioration that could occur in thawed exterior tissues that rely upon functional liver and circulatory systems to supply oxygen and other blood borne factors.

1.4.2 ICE NUCLEATING AND ANTIFREEZE PROTEINS

In addition to the biosynthesis of cryoprotectants, it is well known that many freeze tolerant organisms help regulate the freezing process through the production of ice nucleating proteins (INPs) and antifreeze proteins (AFPs) (Duman, 1982). By definition INPs instigate ice crystallization, whereas AFPs bind to the growing outer edges of ice crystals and prevent them from expanding to sizes that damage cells. The presence of INPs prevents extensive supercooling and helps initiate freezing at temperatures just below the plasma freezing point. Freezing at high subzero temperatures ensures that the rate of ice growth through the body of the animal is slow. Slow ice growth, particularly with larger animals, allows time to initiate various freeze tolerance adaptations (Claussen et al., 1990; Storey and Storey, 1988; Duman *et al.*, 1991). Although both AFPs and INPs have been observed in freeze tolerant invertebrates, only INPs have been found in freeze tolerant vertebrates (Wolanczyk *et al.*, 1990; Storey *et al.*, 1992). Presently, investigations have only been able to approximate the size of the INP in *R. sylvatica*, with the identity remaining a mystery (K. Storey and J. Duman, unpublished results).

1.4.3 METABOLIC DEPRESSION

The suppression of basal metabolic rate through a shift to a hypometabolic state is a common response by many animals following exposure to a variety of environmental stresses. For example, the transition to a hypometabolic, hypothermic state by hibernating mammals allows them to compensate for the limited food supply available in winter and the demanding energetic costs that would otherwise be associated with maintaining a high body temperature in cold weather (Wang, 1989). Another form of metabolic depression, estivation, is a survival strategy exploited by a variety of amphibians in response to dry and hot seasonal conditions (Pinder *et al.*, 1992; Abe, 1995; Land and Bernier, 1995). In the same way submerged turtles deal with oxygen deprivation by lowering their metabolic rate to a level ~15% of that measured during normoxic conditions (Jackson, 1968).

Natural metabolic arrest necessitates the suppression of multiple ATP-consuming processes in the cell. The molecular adaptations involved in this suppression include changes to enzyme activities and biochemical pathways, membrane ion channels, transport processes, and protein and RNA synthesis (Hand and Hardewig, 1996; Hochachka *et al.*, 1996). These changes establish a new homeostasis with a greatly reduced rate of ATP turnover. The adaptations establishing this hypometabolic state have been documented in a variety of species. For example, the rate of protein synthesis in brains of hibernating ground squirrels was reduced to only 0.04% of the mean rate in euthermic brains whereas sodium/potassium ATPase activity was reduced by 40-60% in hibernator tissues (Frerichs *et al.*, 1998; MacDonald and Storey, 1999).

With freeze tolerant animals, such as the wood frog, it has been suggested that the lower body temperature experienced during freezing helps to reduce the metabolic rate and conserve ATP but other "active" measures are also likely involved. Future studies will undoubtedly unravel the exact mechanisms used by *R. sylvatica* to reduce energy consumption during the prolonged state of ischemia resulting from freezing exposure (Storey and Storey, 1985a; Storey and Storey, 1986b).

1.4.4 C_{OLD} A_{CC}LIMATION

An adaptive mechanism that has been shown to play a role in the acquisition of both freeze and cold tolerance in numerous plants, insects and prokaryotes is the ability to cold acclimate (CA). For example, exposure to low but nonfreezing conditions prior to freezing has been shown to contribute significantly to the level of freeze tolerance exhibited by over-wintering plants (Uemura *et al.*, 1995; Karlson *et al.*, 2002). Furthermore, a period of CA has been shown to enhance long- term freeze survival in *Panagrolaimus davidi*, a freeze tolerant nematode (Wharton *et al.*, 2000).

During autumn, the wood frog experiences a series of progressively cooler nights occurring over a period of weeks to months. During this time wood frogs are believed to initiate adaptations that permit or at least enhance freeze tolerance. Studies have confirmed the importance of the CA period, as summer wood frogs have shown restricted freeze survival relative to autumn frogs. Presently only a few of the seasonal adaptations permitting cryoprotectant production have been identified (e.g. accumulation of liver glycogen stores and glucose transporters) and future work will required to ascertain whether the other important changes during the CA period.

1.5 SPECIFIC ADAPTATIONS IN *R. SYLVATICA*

1.5.1 CONTROL OF GLYCOGENOLYSIS

Multiple studies have defined the details of cryoprotectant production in wood frogs including the kinetics of cryoprotectant synthesis and the signaling pathways responsible for triggering and controlling the massive glycogenolysis that takes place. Cryoprotectant synthesis in *R. sylvatica* is derived from the huge reserve of glycogen that accumulates in the liver prior to hibernation. Cryoprotectant output is triggered, not as a seasonal event or in response to cold exposure, but only when ice begins to form in peripheral tissues (Storey and Storey, 1985a). Within the first 5 minutes of freezing, blood and liver glucose levels rise by over 3 and 6-fold, respectively (Storey and Storey, 1986b). Glucose production and distribution continues over the subsequent hours until finally being halted with the freezing of the circulatory system. The prompt initiation of glucose synthesis requires the existence of a similarly rapid method of modifying the necessary enzymes and pathways. The fast-acting regulatory mechanism utilized in the wood frog liver is covalent modification of glycogen phosphorylase. Immediately following peripheral nucleation the percentage of the enzyme in the active form increases from 41% in the liver of control animals to 73% within 5 minutes and to 94% after 70 minutes of freezing (Storey, 1987a). In addition to increasing the percentage of glycogen phosphorylase in the active form, there is also a freezing-induced rise in the total liver phosphorylase content.

Further characterization of hormones, receptors, and the second messengers established that beta-adrenergic receptors in the liver plasma membrane play an

important role in the initiation of the freezing-induced hyperglycemic state (Storey and Storey, 1996). These studies involved intraperitoneal injection of the beta-adrenergic antagonist propranolol, and the beta-adrenergic agonist isoproterenol, prior to freezing exposure. Propranolol administration blocked the activation of liver glycogen phosphorylase, whereas isoproterenol stimulated glucose production. It was further demonstrated that freezing stimulates a rise in liver cAMP levels within two minutes that is sustained for at least 24 h (Holden and Storey, 1996). In summary, then, the current model for freeze-induced glucose production involves an as yet unidentified trigger binding to beta-adrenergic receptors on the surface of hepatocytes, stimulating the production of 3',5'-cyclic adenosine monophosphate (cAMP), leading to the activation of cAMP-dependent protein kinase (PKA) which then activates multiple responses including glycogenolysis (Hemmings and Storey, 1994; Holden and Storey, 1996; Storey and Storey, 1996).

1.5.2 PROTEIN KINASE C, PROTEIN KINASE G, MITOGEN ACTIVATED PROTEIN KINASES

In addition to the confirmation of elevated cellular cAMP levels as a response to freezing by wood frog organs, the involvement of other second messengers including cGMP and D-myo-inositol 1,4,5-trisphosphate (IP₃) has been investigated. Freezing induced tissue-specific changes in cGMP and IP₃ levels have indicated that other types of protein kinases also play roles in mediating the response to freezing. Cyclic GMP levels increased in both liver and heart and IP₃ levels increased almost ten-fold over a 24 hour time course of freezing in the liver (Holden and Storey, 1996).

More recently, activation of the mitogen-activated protein kinase (MAPK) family during freezing survival has been reported (Greenway and Storey, 2000). MAPKs participate in signaling cascades initiated by extracellular signals. The MAPK family members investigated included the ERKs (extracellular signal regulated kinases), JNKs (c-jun NH₂-terminal kinases), and p38. Once activated these three MAPKs phosphorylate specific cytosolic or nuclear targets leading to the up-regulation of specific genes (Karin and Hunter, 1995). The initial gene transcription provoked by cell stimulation leading to activation of the MAPK pathways has been determined to be independent of de novo protein synthesis and often involves the “immediate-early gene response” (Karin, 1995) that produces transcription factors such as c-jun, c-fos, c-myc, and egr-1. The protein products of these genes are capable of binding to the promoter regions of target genes, inducing their transcription (Cohen *et al.*, 1991; Webster *et al.*, 1992).

In the wood frog, p38 activation was observed following freezing exposure. In liver and kidney, p38 was strongly activated within 20 minutes of ice nucleation, but returned to control levels within 60 minutes. Heart p38 activation, however, followed a different time course with the phosphorylated content increasing after 1 hour of freezing and continuing to rise over longer periods of freezing exposure (24 h was the longest time tested). As well, increased JNK activity was observed during thawing, but no response by the ERKs was apparent. Activation of JNK in mammalian systems during reperfusion after ischemia has been previously reported (Pombo *et al.*, 1994; Knight and Buxton, 1996). Possible roles for p38 signaling includes activation of the transcriptional machinery through the phosphorylation of ATF-2 or activation of other enzymes such as the protein MAPKAPK-2, which phosphorylates the small heat shock protein HSP27

(Landry and Huot, 1995). Potential downstream targets of the activated JNK include c-Jun, ATF-2, or Elk-1.

1.5.3 FREEZING INDUCTION OF NEW PROTEIN AND TRANSLATABLE RNA

Up until the last few years the majority of the research studies that identified changes in protein expression and enzyme activity as a result of freezing exposure were largely confirmatory in nature; that is, they provided confirmation of enzyme/protein changes that supported identified adaptations for freeze tolerance. For example, the synthesis of high glucose as a cryoprotectant was supported, not unexpectedly, by very high activities of glycogen phosphorylase in wood frog liver and by seasonally high levels of glucose transporters in all frog organs (King *et al.* 1995). Although this approach allowed for the successful identification of a number of significant adaptations it left other, unanticipated but important, protein changes undiscovered. Furthermore, until the release of two recent studies there was no evidence that freezing involved any additional changes in mRNA and protein expression beyond what had been anticipated.

To investigate the possibility that additional changes in gene expression and *de novo* protein synthesis might play a role in freezing survival, changes to the translatable mRNA pool were analyzed in a number of the organs of wood frogs during freezing and thawing (White and Storey, 1999). The results from the *in vitro* translation of isolated mRNA indicated the presence of specific changes in the translatable populations of mRNA, with differences in translation products being observed in every tissue tested during freezing and thawing. As well, a second study that examined potential differences in protein biosynthesis through the uptake and incorporation of ³⁵S-labeled amino acids

into newly synthesized proteins during a freeze-thaw cycle, was carried out on multiple tissues and organs of *R. sylvatica* (Storey *et al.*, 1997). Isoelectric focusing of the radio-labeled proteins revealed the presence of several ^{35}S -labeled protein peaks, differentially expressed in the liver following a freeze-thaw cycle.

1.5.4 FREEZING INDUCED GENE UP-REGULATION

Although these two studies established that freezing is accompanied by a multitude of changes in the translatable mRNA and newly translated protein pools of wood frog organs, the techniques used did not lend themselves to the actual identification of the proteins involved. Fortunately advances in the fields of biochemistry and molecular biology have provided new and improved tools and approaches for the identification and characterization of genes and proteins. For example, through the construction of cDNA libraries (or the screening of microarrays) it has become possible to identify the specific changes in gene expression that accompany changes in physiological states or in response to external stresses. Furthermore, common biochemical techniques, including 2-dimensional gel electrophoresis and western blotting, now allow researchers to compare the protein content of two samples and identify differentially expressed gene products and changes to protein phosphorylation states. For example, molecular biology techniques have allowed a variety of research groups to identify genes associated with cold hardiness in a variety organisms including plants and animals. To date, our laboratory has identified five genes that are up-regulated in response to freezing in liver of wood frogs (Cai and Storey, 1997a, 1997b, Cai *et al.*, 1997) as well as several others that are up-regulated in brain and skin (Wu, 1999).

Freeze-induced genes in liver were isolated via differential screening of a liver cDNA library with ^{32}P -labelled cDNA probes created using mRNA isolated from control and frozen animals. Two of the five genes identified coded for the alpha and gamma subunits of fibrinogen (Cai and Storey, 1997a). It is currently believed that this up-regulation is initiated either by minor injuries that develop as ice propagates through the frog's body, or is triggered in anticipation of tissue injuries that could become apparent during thawing. A third gene up-regulated during freezing showed homology with alpha amylase pancreatase. The fourth cDNA showed >80% homology with the gene encoding ADP/ATP translocase, AAT (Cai *et al.*, 1997). The role of AAT in freezing survival is not yet fully understood. The function of AAT is to catalyze the transmembrane exchange of cytosolic ADP with mitochondrial ATP generated by oxidative phosphorylation. Studies with anoxic wood frogs have established that AAT is also up-regulated during the transition from a normoxic to anoxic environment, suggesting that freezing-induced ischemia, and resulting hypoxia and anoxia, may be the underlying cause of AAT transcript up-regulation. Elevated levels of the protein may contribute to minimizing net ATP depletion during freezing or facilitate the rapid re-establishment of oxidative metabolism when the frog thaws. Finally, a novel transcript, *fr10*, was cloned from *R. sylvatica* liver, and determined through northern analysis to be up-regulated in a variety of tissues following freezing exposure (Cai and Storey, 1997b). Presently, the role of the *fr10* gene product in freezing survival is unknown, but studies have recently been initiated to gain an understanding not only of the mechanisms leading to the increase in *fr10* transcript levels, but to determine whether the gene product is also up-regulated following freezing exposure.

1.6 PROPOSED RESEARCH AND HYPOTHESIS

1.6.1 EXPERIMENTAL HYPOTHESIS

Over the course of my graduate work I set out to identify those changes in the protein and mRNA profile of the wood frog heart (primarily), and liver (secondarily) that accompany freezing exposure. Surviving total body freezing is a complicated procedure requiring intrinsic adaptations to a variety of stresses, and communication and coordination between organ systems. The wood frog heart and liver are excellent examples. Throughout the early hours as ice propagates through the frog's body, the frog heart must continue to beat in order to distribute the cryoprotectant to the organs of the body. This task requires the heart to manage not only the freezing induced changes in blood volume and viscosity during the early hours of freezing, but the potentially damaging effects that hypoxia, ischemia, and hyperglycemia can have on cardiac tissue. As well, the liver must similarly endure the hypoxic, and ischemic states, but must also be able to respond to the freezing trigger, produce cryoprotectant, and synthesize a variety of proteins (many for export) needed for freeze protection (such as those involved the clotting cascade to augment the frog's ability to respond to freeze-induced damage). These energy-expensive gene expression and protein synthesis responses to freezing in both tissues occur against a background of declining organ energy status (due to progressive ischemia) and, as such, freeze-induced gene up-regulation should predictably be limited to those genes whose protein products have important roles to play in freezing survival.

Therefore, I hypothesized that selected changes in mRNA and protein synthesis will occur in frog heart and liver during freezing and that these changes will represent an organized and specific up-regulation of selected genes/proteins that directly contribute to freezing protection. By establishing the identity of the induced transcripts and through further characterization of these genes and their protein products, advances will be made in our understanding of the important signaling pathways, changes in cellular protein contents, and metabolic adjustments that contribute to freeze tolerance in the wood frog. Based on what is presently understood regarding the cellular response of frog tissue to freezing, it is further proposed that the freeze-induced changes in gene expression identified in the heart and liver will be associated with p38 and PKC signal transduction pathways.

1.6.2 EXPERIMENTAL OUTLINE

Changes in gene and protein expression profiles in frog heart and liver as a result of freezing and thawing will be identified primarily using the techniques of differential cDNA library screening, northern blot analysis, 2-dimensional gel electrophoresis, and western/immunoblot analysis. When necessary the technique of 5' Rapid Amplification of cDNA Ends will be used to obtain full length cDNA clones. As well, each gene will be characterized on a variety of different levels, including transcript expression analysis: (i) over a time course of freezing and thawing, (ii) in different tissues and organs, and (iii) following exposure to similar stresses. In addition to transcript analysis, when available, western analysis will be performed to determine whether the changes in transcript levels are translated into changes in the protein content.

1.6.3 SUMMARY

Briefly, seven identifiable genes as well as 1 protein were determined to be differentially expressed in the liver and heart of the wood frog following freezing exposure. Following a complete characterization of these genes and proteins (expression analysis, comparison of amino acid and nucleotide sequences to gene and protein databases, and exploration into the reported cellular roles of each gene and protein), functions for these genes in the freeze-thaw cycle are discussed; some fit established paradigms of freeze survival whereas others do not. In addition, two novel genes (*li16* and *ddx8*) that are strongly induced in the liver of the wood frogs following freezing exposure are characterized. The complete coding region for each gene was obtained, and the nucleotide and amino acid sequences were fully characterized. A complete transcript expression analysis was performed and techniques were used to further establish the mechanisms and cell signaling pathways responsible for novel gene induction.

TABLE 1.1

Freeze tolerant amphibians and reptiles

<i>Species</i>	Common Name	Reference
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Amphibian

<i>Rana sylvatica</i>	wood frog	Schmid, 1982 Storey, 1984
<i>Hyla versicolor</i>	gray tree frog	Schmid, 1982 Storey and Storey, 1985
<i>Hyla chrysoscelis</i>	Cope's gray tree frog	Constanzo <i>et al.</i> , 1992
<i>Pseudacris crucifier</i>	spring peeper	Storey and Storey, 1986a
<i>Pseudacris triseriata</i>	chorus frog	Storey and Storey, 1986a
<i>Hynobius keyserlingi</i>	Siberian salamander	Berman <i>et al.</i> , 1984

Reptiles

<i>Chrysemys picta marginata / bellii</i>	midland and western painted turtles	Churchill and Storey, 1991, 1992
<i>Terrapene carolina</i>	box turtle	Costanzo and Claussen, 1990 *
<i>Trachemys scripta elegans</i>	red-eared sliders (hatchlings)	Storey <i>et al.</i> , 1988
<i>Lacerta vivipara</i>	European common lizard	Constanzo <i>et al.</i> , 1995
<i>Thamnophis sirtalis</i>	garter snake	Storey and Storey, 1988

CHAPTER 2

EXPERIMENTAL PROTOCOLS

2.1 ANIMAL TREATMENTS

2.1.1 ANIMAL PREPARATION

Male wood frogs, *R. sylvatica*, were collected from breeding ponds in the Ottawa area. Animals were washed in a tetracycline bath and maintained at 5°C without feeding in covered containers with damp sphagnum moss for a minimum of 1 week before use. Control frogs were sampled directly from this condition.

2.1.2 AMPHIBIAN FREEZING PROTOCOL

In the standard freezing protocol wood frogs are transferred from 5°C to closed plastic boxes lined with damp paper toweling and placed in an incubator at -2.5°C for freezing exposure. In preliminary studies, selected individuals were cooled in contact with a thermistor secured to the abdomen to monitor body surface temperature and record the exotherm associated with nucleation. These tests revealed that freezing began within 45-60 min after placing frogs in the incubator (Storey and Storey, 1985a). Hence, the standard freezing protocol allowed 1 h of cooling time before beginning to time the length of the freeze exposure. In this thesis the terms freezing and frozen are used interchangeably to describe animals that have experienced a nucleation event and show evidence of peripheral ice formation, while hours are provided in some instances to approximate the extent of ice accumulation. For example, 2 h after the onset of freezing between 15 and 25% of total body water is ice compared to final value of 65% that accumulates in the end. Under the conditions used frogs take from 12-24 h before arriving at the maximum ice content. Both control and frozen frogs were pithed and organs excised within 30-90 s.

2.1.3 AMPHIBIAN ANOXIA AND DEHYDRATION PROTOCOL

Some animals were exposed to anoxic or dehydration treatments. Animal experiments for both of these stresses followed standard protocols and were 100% survivable by the animals (Churchill and Storey, 1993; Holden and Storey, 1997). For dehydration the animals were held at 5°C in closed jars with a layer of silica gel desiccant on the bottom (separated from the frogs by a sponge pad). At intervals, frogs were removed and weighed to determine water loss and animals were sacrificed when the percentage of body water lost reached a pre-determined value (typically 20 or 40 %). The percentage loss of body water was determined using the equation, $[M_i - M_d]/[M_i \times \% \text{H}_2\text{O}]$ where M_i is the initial mass before dehydration, M_d is the animal's weight at any time during the experiment, and % H_2O is the percentage of total body mass that is water. This last parameter was determined from the initial and final masses of sacrificed control animals that were dried to a constant weight at 80°C for 72 h. The percentage of body mass water was 80.8 ± 1.2 for *R. sylvatica* (Churchill and Storey, 1994).

Anoxic exposure was administered as described by Holden and Storey (1997). Anoxia was imposed by placing frogs in glass jars containing a 1 cm layer of 5°C distilled water that had been previously bubbled for 20 min with 100% N_2 gas. N_2 gas bubbling was continued for 20 min at which time the jars were tightly capped and sealed with parafilm. Animals were kept in a 5°C incubator and frogs in one jar were sampled at each time point. Frogs were sampled after 4 or 24 h of anoxia; during sampling N_2 gassing was reinstated to maintain the N_2 atmosphere at 5°C. For recovery time points, animals had received 24 h of anoxia exposure were removed to fresh jars containing 1 cm layer of 5°C distilled water and normal air and were sampled at various time points.

2.2 COMMON MOLECULAR BIOLOGY PROTOCOLS

2.2.1 GENERAL PRECAUTIONS

All plastic and glassware, including bottles and pipette tips, were autoclaved and gloves were worn at all times during operations involving nucleic acid manipulation. For RNA preparation and manipulation, diethylpyrocarbonate (DEPC) was added to double-distilled water at a concentration of 0.1% v/v, to destroy any RNAses that may have been present. Treated water was left overnight at room temperature (RT), then autoclaved to eliminate any lingering DEPC. DEPC-treated water was used to make up all stock solutions and to dissolve RNA samples.

2.2.2 TOTAL RNA ISOLATION

Tissue samples of approximately 50-100 mg were weighed and placed in 2 mL Eppendorf tubes. Trizol reagent (Gibco-BRL, Bethesda, MD) was added, 1ml per 50-100 mg tissue, and the samples were homogenized and allowed to sit for 5 min at RT. Chloroform was then added (0.2ml/ml of Trizol reagent), and after mixing for 15 s, samples were incubated for 2-3 min at RT. Samples were then centrifuged at 12,000 x g for 15 min. The upper aqueous layer was removed and transferred to autoclaved 1.5 mL Eppendorf tubes containing an equal volume of isopropanol. After gentle shaking, the RNA was precipitated at room temperature for 10 min. Centrifuging the samples at 12,000 x g for 10 min pelleted the RNA. The supernatant was discarded and 70% ethanol was used to remove lingering chemicals from the RNA pellet. After gentle shaking and then centrifugation at 12,000 x g for 5 min, the RNA was dissolved in DEPC-treated

water. The concentration of RNA in the prepared samples was determined spectrophotometrically by absorbance at 260 nm.

2.2.3 MESSENGER RNA ISOLATION

Messenger RNA (mRNA) was isolated and purified using the OligotexTM Mini Kit (Qiagen) according to the manufacturers protocol. Oligotex is an affinity reagent consisting of polystyrene-latex particles of uniform size (1.1 μ M diameter) with dC₁₀T₃₀ oligonucleotide covalently linked to the surface. The Oligotex reagent takes advantage of the fact that most eukaryotic mRNAs end in a poly-A tail of adenosine nucleotides. These stretches of adenosines bind to the thymidine oligonucleotide coupled to the bead, whereas genomic DNA, ribosomal RNA, and transfer RNA do not. mRNA was isolated using the following steps.

First, 250 μ g of total RNA was diluted to 250 μ L with RNase free water. Each sample was treated with an equal volume of OBB buffer (20 mM Tris-HCl, pH 7.5, 1 M NaCl, 2 mM EDTA, 0.2% SDS), and 10 μ L of Oligotex suspension (10% w/v suspension, 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.1% NaN₃). The suspensions were incubated at 70°C for 3 min, and then allowed to cool to RT over 10 min. The Oligotex:mRNA complexes were pelleted by centrifuging at 12,000 x g for 2 min, and the supernatant was discarded. The beads were resuspended in 400 μ L of OW2 buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) and pipetted onto a small spin column, placed into a 1.5 mL microcentrifuge tube, and centrifuged for 1 min at 12,000 x g. The flow through was emptied from the microcentrifuge tube and 400 μ L additional OW2 buffer was used to wash the beads. Following centrifugation at

12,000 x g, 35 μ L of OEB buffer (5 mM Tris-HCl, pH 7.5) heated to 70°C was used to elute the mRNA from the Oligotex beads. The spin column was transferred to a sterile microcentrifuge tube and the mRNA solution was collected by centrifuging at 12,000 x g for 1 min.

To quantify the mRNA, 1 μ L of each sample was spotted onto nitrocellulose (pore size 0.2 μ M, Hybond-NTM, Amersham). For comparison 1 μ L of prepared solutions containing 1, 0.5, 0.25, 0.125, and 0.0625 μ g/ μ L of mRNA (Gibco-BRL) were spotted onto the same nitrocellulose. The RNA was cross-linked to the blot, followed by baking for 30 min at 80°C (BioRad Gel Dryer, model 583). The blot was rinsed in 10% acetic acid for 10 min, and the mRNA was then visualized by incubation in a 0.2% methylene blue (Sigma-Aldrich, ON) solution. Following a comparison of the sample intensities to the standards, the typical concentration of mRNA was 0.15 μ g/ μ L.

2.2.4 ALKALINE LYSIS ISOLATION AND DIGESTION OF PLASMID DNA

Gene specific probe synthesis for northern blot analysis and sequencing requires that plasmids containing the cDNA insert be purified from their bacterial hosts. A single colony from a master plate was used to inoculate 4 mL of LB-ampicillin broth (12.5 μ g/mL) and incubated overnight at 37°C with shaking. A 2 mL aliquot of this culture was then centrifuged at 13,000 x g for 3 min. The cell pellet was resuspended, and allowed to sit at RT for 5 min in 100 μ L of GTE (50 mM glucose, 25 mM Tris-HCl, pH 8, 10 mM EDTA). A 200 μ L volume of lysis buffer (0.2 M NaOH, 1% SDS) was then added, and after gentle shaking and a 5 min incubation on ice, neutralization solution was added to a final volume to 0.45 mL. The neutralized solution remained on ice for 5 min, prior to

centrifugation for 3 min at $13,000 \times g$ at 25°C . The supernatant (~ 0.4 mL) was transferred to a new tube and 0.8 mL of 95% ethanol was added. Following a thorough mixing and 5 min incubation at RT, the Eppendorf tube was centrifuged at $13,000 \times g$ for 3 min. The supernatant was discarded and the pellet air-dried for a few minutes before resuspension in 100 μL TE buffer (1 mM Tris-HCl, pH 8, 1 mM EDTA).

The cDNA of interest was digested out of the plasmid using specific restriction enzymes. The main enzymes used for digestion from pBluescript plasmids were Eco RI, Bam HI, and Xho I (Gibco-BRL). A 30 μL aliquot of isolated plasmid was removed from the stock and added to a 0.5 mL Eppendorf tube containing 4 μL of 10 x reaction buffer 2 (Gibco-BRL) and 1 μL of each of the required restriction enzymes. The reaction mixture was maintained at 37°C for 1 h prior to separation in a TAE gel.

2.2.5 TAE GEL VISUALIZATION, AND ISOLATION OF DNA

Following plasmid digestion or PCR, the cDNA samples were mixed with 0.2 volumes of DNA loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 40% w/v sucrose). Samples were loaded onto 1 % TAE agarose gels (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) and separated by electrophoresis at 100 V. A 1 μL volume (per 25 ml of gel volume) of ethidium bromide solution (10 mg/mL) was added to the gel prior to pouring, and the DNA samples were visualized using UV transilluminator (Foto/UV 26, Fotodyne Inc.) at a wavelength of 300 nm and photographed with a Polaroid DS-34 camera loaded with Polaroid instant pack films (667).

The DNA bands were then excised from the TAE gel using sterile razor blades. The gel slices were placed into modified aerosol filter pipet tips (VWR, Cat No. 53510-

058). The modified pipet tip and gel slice were placed at -20°C for 1 h. The pipet tip was then placed into a microcentrifuge tube and the cDNA was separated from the agarose by centrifuging at 8000 x g for 8 min. The eluant was stored at -20°C until required for preparation of gene-specific DNA probe.

2.3 CONSTRUCTION OF cDNA LIBRARY

2.3.1 OVERVIEW OF cDNA LIBRARY CONSTRUCTION

The construction of the cDNA library was carried out using the Lambda ZAP-CMV XR Library Construction Kit (Stratagene) following manufacturer's instructions, and unless stated otherwise all of the reagents were supplied by Stratagene.

The main points of the procedure were as follows. Poly(A)⁺ mRNA was primed with an oligo(dT) linker-primer containing the protective GAGA sequence plus *Xho* I site and transcribed using StrataScript Reverse Transcript in the presence of dATP, dGTP, dTTP and 5-methyl dCTP. Synthesis using 5-methyl dCTP protects the cDNA from digestion by certain restriction endonucleases such as *Xho* I. During second-strand synthesis, RNase H nicks the RNA bound to the first-strand cDNA to produce a multitude of fragments, which serve as primers for DNA polymerase I. Normal dCTP was used for incorporation into the second strand, which ensured that the *Xho* I sites in the linker-primer were susceptible to *Xho* I digestion (see below). The uneven termini of the double-stranded cDNA were repaired using cloned *Pfu* DNA polymerase, and *Eco*R I adapters were then ligated to the blunted ends of the double-stranded cDNA *via* T4 DNA

ligase. The end of *EcoR* I was phosphorylated *via* T4 polynucleotide kinase. cDNA fragments were obtained by *Xho* I digestion to release the *EcoR* I adapter and the residual linker primer from the 3' end of the cDNAs. The cDNA fragments were then size fractionated on a Sepharose CL-2B spin column. Finally, the directional cDNA was ligated into the vector arms in a sense orientation (*EcoR* I - *Xho* I) with respect to the *lacZ* promoter and packaged into phage with Gigapack II Gold packaging extract. The titer of the library [number of plaque-forming units per mL (pfu/mL)] was determined to ensure that more than 1 million individual cDNA were packaged.

2.3.2 FIRST AND SECOND STRAND SYNTHESIS

In an RNase-free 0.5 mL Eppendorf tube, 5 μ L of 10 x first strand buffer, 3 μ L of first-strand methyl nucleotide mixture (10 mM each of dATP, dGTP, dTTP, plus 5-methyl dCTP), 2 μ L of linker-primer (1.4 μ g/ μ L), 1 μ L of RNase block ribonuclease inhibitor (40 U/ μ L), 6 μ L of DEPC treated water and 5 μ g of mRNA in 30 μ L of DEPC treated water were mixed. The primer and mRNA were allowed to anneal for 10 min at RT. Following incubation, 1.5 μ L of StrataScript RT (50 U/ μ L) was added to the first strand synthesis reaction to produce a final volume of 50 μ L. The sample was incubated at 42°C for 1 h and then chilled on ice. A 45 μ L aliquot was removed to a new tube and the following reagents were added: 20 μ L of 10 x second strand buffer, 6 μ L of second-strand dNTP mixture, 114 μ L of sterile distilled water, and 2 μ L of radio-labeled [32 P] dCTP (3000 Ci/mmol). Following gentle mixing, 2 μ L of Rnase H (1.5 U/ μ L) and 11 μ L of DNA polymerase I (9.0 U/ μ L) were used to initiate second strand synthesis. The reaction was incubated for 2.5 h at 16°C, and terminated by placing the tube on ice.

2.3.3 cDNA TERMINI BLUNTING AND ADAPTOR LIGATION OF ECORI ADAPTORS

After second strand synthesis the cDNA ends were blunted by the addition of 2 μL of *Pfu* DNA polymerase, followed by 23 μL of blunting dNTP, and incubation at 72°C for 30 min. A 200 μL aliquot of phenol-chloroform (1:1) was then added to the tube, and following a thorough mixing the tube was centrifuged at 12,000 x g for 2 min. The upper aqueous layer, containing the cDNA was moved to a new tube and an equal volume of chloroform was added. The tube was vortexed and centrifuged at 12,000 x g for 2 min. The upper aqueous layer was collected and placed in a 0.5 mL Eppendorf tube containing 20 μL of 3 M sodium acetate and 400 μL of 100% ethanol. The reaction was allowed to precipitate overnight at -20°C and then the precipitate was collected by centrifuging at 12,000 x g for 60 min at 4°C. The pellet was washed with 500 μL of 70% ethanol, centrifuged again for 2 min, aspirated, and allowed to air dry. The pellet was resuspended in 9 μL of EcoRI and incubated at 4°C for 30 min to allow complete cDNA resuspension. Next, 1 μL of 10 x ligase buffer, 1 μL of 10 mM rATP, and 1 μL of T4 DNA ligase (4 U/ μL) were added followed by incubation overnight at 8°C. The following day the ligase was inactivated by heat treatment at 70°C for 30 min.

2.3.4 PHOSPHORYLATION OF ECORI ENDS AND XHOI DIGESTION

Following heat inactivation of the ligase, the reaction was cooled at RT for 5 min. The adaptor ends were then phosphorylated by addition of 1 μL of 10x ligase buffer, 2 μL of 10 mM rATP, 6 μL of sterile water, and 1 μL of T4 polynucleotide kinase (10 U/ μL). Following incubation for 30 min at 37°C, the kinase was heat inactivated at 70°C

for 30 min. The reaction mixture was allowed to cool for 5 min at RT. Digestion of the cDNA required the addition of 28 μ L of Xho I buffer, and 3 μ L of Xho I (40 U/ μ L) with incubation for 1.5 h at 37°C. The cDNA was then precipitated overnight following the addition of 5 μ L of 10x STE buffer and 125 μ L of 100 % ethanol.

2.3.5 SEPHAROSE CL-2B SPIN COLUMNS AND FRACTIONATION OF cDNA

Following precipitation, the cDNA was collected by centrifuging at 12,000 x g for 60 minutes at 4°C, the ethanol was discarded, and the pellet was air dried. The cDNA pellet was resuspended in 14 μ L of STE buffer (100 mM NaCl, 20 mM Tris-HCl, 10 mM EDTA, pH 7.5) and 3.5 μ L of column loading dye was added to each sample. Sepharose Cl-2B spin columns were prepared using 1 cc syringes (Becton-Dickinson). The syringe tips were blocked with cotton, and 1 mL of Sepharose CL-2B gel filtration medium was loaded into the syringe, and the column was placed into a 15 mL Falcon tube and centrifuged in a clinical centrifuge (Model CL, International Equipment Co.) at maximum speed for 2 min. The column was washed with 10 mL of 1x STE using multiple 1 min spins of 1mL. The cDNA sample was then loaded onto the top of the column and fractions were produced by repeated washing of the column with 30 μ L portions of 1 x STE, followed by 2 min centrifugation at maximum speed in the clinical centrifuge. The fractions were collected in sterile 1.5 mL Eppendorf tubes placed at the bottom of the 15 mL Falcon tube. The size of the cDNA in each fraction was determined using native polyacrylamide gel electrophoresis.

2.3.6 NATIVE POLYACRYLAMIDE GELS

A 6 % polyacrylamide gel was poured without the addition of SDS, and native Tris-glycine running buffer (3 g Tris base, 19 g glycine, 1 liter ddH₂O) was used to separate the DNA bands. A stock of labeled DNA markers was created by mixing 1 µL of 1 kb DNA ladder (Gibco-BRL) with large fragment polymerase (Gibco-BRL), in the presence of ³²P-dCTP (Amersham). A 5 µL aliquot of each cDNA fraction was mixed with 10 µL of autoclaved water, and 3 µL of loading buffer (25 mL of 5x loading buffer containing: 62.5 mg bromophenol blue, 62.5 mg xylene cyanol, 6.25 g Ficoll 400) prepared in TEN-S solution (10 mM Tris, pH 7.5, 1 mM EDTA, 10 mM NaCl). The samples and DNA markers were loaded into the lanes and run at 80 V until the dye reached the end of the gel. The gel was then removed from the glass plates and dried using a gel drier. The dried gel was exposed to X-ray film overnight to visualize the radioactivity incorporated during the second strand synthesis, and estimate the size of cDNA in each fraction relative to the DNA markers.

2.3.7 LIGATING CDNA INTO THE LAMBDA ZAP -CMV XR VECTOR

The fractions containing cDNA estimated to be 0.5 kilobases and larger were processed for ligation into the phage vectors. An equal volume of phenol-chloroform (1:1) was added to each cDNA fraction, vortexed briefly, and centrifuged at 12,000 x g for 2 min. The upper layer was transferred to a new tube and an equal volume of chloroform was added. Following vortexing and centrifuging again, the aqueous layer was again collected and an equal volume of 100 % ethanol was added to precipitate the cDNA overnight at -20°C. The precipitated cDNA was collected by spinning at 12,000 x

g for 60 min at -4°C. A further wash was performed using 80 % ethanol, and following drying the DNA was dissolved in 5 µL of ddH₂O.

The concentration of DNA in the pooled, precipitated and resuspended cDNA fraction was then determined using an ethidium bromide plate assay. Briefly, 1 µL aliquots of a serial dilution set (1, 0.5, 0.25, 0.125, and 0.0625 µg/µL) of a 1 µg/µL solution of cDNA were spotted out on a 1 % agarose TAE plate (1 µg/µL EtBr) and compared to 1 µL of the cDNA sample. Resuspended cDNA (2.5 µL) was added to a 0.5 mL centrifuge tube containing 0.5 µL of 10 x ligase buffer, 0.5 µL of 10 mM rATP, 1.0 µL of Lambda ZAP-CMV XR (1 µg/µL). The ligation mixture was completed with the addition of 0.5 µL of T4 DNA ligase (4U/µL) and was incubated at 12°C for 12 h.

2.3.8 PACKAGING PHAGE DNA INTO GIGAPACK III GOLD

Two days before the packaging a small fraction of the glycerol-XL1 Blue MRF⁺ cell stock provided was streaked on an LB plate (15 × 100 mm Petri dishes; LB agar medium: 10 g NaCl, 10 g tryptone, 5 g yeast extract pH 7.0, 15 g agar per liter) supplemented with tetracycline (12.5 µg/ml) and incubated overnight at 37°C. A 5 mL culture of XL1-Blue was prepared by inoculating a single colony into LB Broth supplemented with tetracycline (12.5 µg/ml) and was allowed to grow overnight with shaking at 37°C. Following a spin at 500 x g for 10 min the supernatant was discarded and the cells were resuspended in autoclaved 10 mM MgSO₄ and diluted to an OD₆₀₀ of 0.5. Immediately before use the packaging extracts were removed from -80°C and thawed on ice. The *R. sylvatica* cDNA was added, mixed gently, and allowed to incubate at RT for 2 h. Following incubation the reaction was diluted with 500 µL of SM buffer (50 mM

Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM MgSO₄, 0.01% w/v gelatin), and 20 µL of chloroform. The precipitated debris was removed by centrifuging and the supernatant was collected and the titer determined.

2.3.9 TITERING AND AMPLIFICATION OF THE cDNA LIBRARY

A series of 10-fold dilutions of the constructed phage library was prepared in SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM MgSO₄, 0.01% w/v gelatin). A 10 µl aliquot of each serial dilution of the library was added to 200 µl of XL1-blue MRF⁺ cells at an OD₆₀₀ of 0.5 and incubated at 37°C for 15 min with gentle shaking. Following incubation, 4-5 ml of melted NZY top agar (0.7% agar, maintained at ~48°C) was added to the mixture and then poured onto a pre-warmed NZY plate (5 g NaCl, 2 g MgSO₄·7H₂O, 5 g yeast extract, 10 g NZ amine, 15 g agar, pH 7.5, per liter) and incubated at 37°C for 8-14 h. Plaques were counted and the titer was determined.

The primary cDNA library was amplified to make a large, stable quantity of a high-titer stock (usually ~10⁹–10¹² pfu/mL). The volume of library suspension containing 2×10^6 plaque-forming phage was mixed with 2.5 ml of the host cells at an OD₆₀₀ of 0.5, incubated at 37°C for 15 min, and then 20 ml NZY top agar (~48°C) was added and the final mixture was poured onto a pre-warmed (37°C) NZY plate (22.5 × 22.5 cm). The plate was incubated at 37°C for 6-10 h (no plaques allowed to grow larger than 1-2 mm) and was then overlaid with 15-20 ml SM buffer, and stored at 4°C overnight with gentle rocking. The pooled phage suspension was stored in a sterile polypropylene tube with chloroform added to a final 5% v/v. After incubation at room temperature for a few minutes, the mixture was centrifuged at 500 g for 10 min to remove cell debris, and the

aqueous phase with amplified phage suspension was removed and chloroform was added to a final 0.3% v/v. Titration of the amplified library was performed as described. Both primary and amplified cDNA libraries were stored in aliquots in 7% DMSO at -80°C.

2.4 DIFFERENTIAL SCREENING OF cDNA LIBRARY

2.4.1 SYNTHESIS OF FIRST STRAND cDNA PROBES

This procedure began with 1 µg Poly(A)⁺ mRNA isolated at the same time from both control and freezing-exposed tissue. The volume of each sample was adjusted to 10 µl with sterile DEPC-treated water, followed by heating at 70°C for 3 min and chilling on ice. To this was added 5 µL of 5x first strand buffer, 1.5 µL of 5 mM dNTPs (without dCTP), 1 µL of 200 ng/µL oligo(dT) primer (Bio S&T, Montreal), 1 µL of 5 U/µL RNasin and 2.5 µL of 0.1 M dithiothreitol (DTT). After a quick spin, the mixture was annealed at RT for 10 min. Then 1 µL of 200 U/µL Superscript[™] RT was added, followed by 5 µL of [α -³²P]dCTP (3,000 Ci/mmol, Amersham, Cleveland, Ohio) and the reaction was allowed to incubate at 37°C for 1 h. Following first strand synthesis the RNA was hydrolyzed by adding 1 µL of 0.5 M EDTA (pH 8.0), 1 µL of 10 % SDS, and 3 µL of 3 M NaOH (68°C for 30 min). The cDNA mixture was then neutralized by adding 10 µL of 1 M Tris-HCl (pH 7.4) and 3 µL of 2 N HCl and passed through a Sephadex G-50 spin column equilibrated in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to remove unincorporated nucleotides. The eluant was diluted to 500 µL with TE buffer and

radioactivity was counted (5 μ L) in a scintillation counter Packard1900 CA (TRI-CARB).

2.4.2 PRIMARY AND SECONDARY SCREENING

Two or three 3 NZY agar plates (22.5 $\times$ 22.5 cm) were prepared at a time containing phage plaques interspersed at the desired density (usually 8-10,000) throughout the bacterial lawn. The plaques on each plate were lifted onto a Hybond-N nylon membrane (20 $\times$ 20 cm, Amersham) after adsorption for 1 min. The membrane was then gently placed in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 1 min, moved to neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) for 5 min and rinsed in 2 $\times$ SSC solution (from 20 $\times$ SSC stock: 175.3 g NaCl, 88.2 g sodium citrate, pH 7.0, per liter). A second lift from the same plate was performed exactly as above with the adsorption time increased by 1 min. The two duplicated membranes were placed on Whatman blotting paper and UV cross-linked (CL-1000 ultraviolet cross-linker, UVP, Inc. USA) at 120,000 μ J/cm² (254 nm) for 1 min to fix phage DNA, followed with baking at 80°C for 1-2 h with a gel dryer (Model 583, Bio-Rad, Hercules, CA).

The two membranes were then placed in different hybridization tubes (4 $\times$ 30 cm, VWR Canlab), and 20-25 mL hybridization solution (50% formamide, 0.125 M Na₂HPO₄, pH 7.2, 0.25 M NaCl, 2% SDS, 1 mM EDTA, adapted from Bio-Rad) was added as well. The tubes were slowly rotated at 65°C in a hybridization incubator (Model 308, LAB-line Instruments, VWR Canlab, Mississauga, ON) for 2-3 h. After pre-hybridization, the solution was replaced with 20 mL hybridization solution containing radiolabeled cDNA probe. Prior to use, probes were treated in boiling water for 3-5 min,

chilled on ice, and then added to pre-warmed (~44°C) hybridization solution. An equivalent amount of radioactive probe was used in each of the two solutions ($2-4 \times 10^6$ cpm per mL of hybridization solution). After hybridization, the membranes were washed in solutions containing SSC (20 x SSC: 175 g NaCl, 88 g sodium citrate, pH 7.0, per liter) and SDS. Excess (background) cDNA probe was washed off the blots by either: reducing the concentration of SSC in the washing solution, increasing the amount of SDS, or heating the washing solution (or combinations thereof). The membranes were then moved to tin foil, covered with saran wrap, and placed on X-ray film (Kodak XAR5) in a cassette and held at -70°C for 1-2 days (for counts between 1000-2000 cpm) or 3-5 days (counts of 500-1000 cpm). The film was developed manually with Kodak reagents.

2.4.3 IN VIVO EXCISION OF PLAQUES FROM UNIZAP CMV XR LIBRARY

Identified clones (phage) from the library screening were selected and the single plaque was isolated. The phage was eluted from the agar and titered. The phages (Uni-ZAP CMV XR vectors) were excised in *E. coli* strains using a helper phage (ExAssist, Stratagene) as follows. In a 1.5 mL tube, 0.1 mL of the XL1-blue MRF' cells ($OD_{600} = 1$ in 10 mM $MgSO_4$), 0.1 mL isolated phage suspension ($>5 \times 10^5$ pfu/mL) and 0.5 μ L ExAssist helper phage ($>1 \times 10^6$ pfu/mL) were added, and the mixture was incubated at 37°C for 15 min. Then 3 mL of LB broth was added and incubated at 37°C for 3 h with gentle shaking. The mixture was then heated at 70°C for 15 min and centrifuged at 1,000 x g for 5 min. The supernatant containing the excised pCMV-Script EX phagemid vector was stored at 4°C, where it is stable for a few months. Before the excised phagemids could be plated, XL0LR cells were grown (with a single colony from a freshly streaked

plate or glycerol stock) in LB broth at 30°C overnight and then kept at room temperature until use. An aliquot of 200 µL XL0LR cells ($OD_{600} = 1$ in LB broth) was mixed with 100 µL of the above phagemid suspension and incubated at 37°C for 15 min. On separate plates 10 and 100 µL samples of the cell mixture were plated on LB-kanamycin agar plates (50 µg/mL) and incubated overnight at 37°C. A few colonies were then streaked on a new LB-kanamycin agar plates, and a single colony was picked and inoculated into 10 mL of LB broth with kanamycin (25 µg/mL) overnight at 37°C. Sterile glycerol stocks were prepared of each excised clone.

2.5 NORTHERN BLOTTING

2.5.1 PREPARATION OF FORMALDEHYDE GEL

The denaturing formaldehyde gel was prepared as follows. For a 125 mL gel, 1.5 grams of agarose (electrophoresis grade, GIBCO-BRL) was dissolved in 95 mL of DEPC treated water with heating to ~80°C. The solution was allowed to cool to ~60°C and then 12.5 mL of 10x MOPS (0.2 M MOPS, 1 mM EDTA, 50 mM sodium acetate, pH 7.0) were added along with 7 mL of a 37% formaldehyde (w/v) solution and 1.5 µL of a 10 mg/mL ethidium bromide solution. The agarose solution was then poured into a tray and allowed to solidify. The gel was covered with 1x MOPS running buffer.

2.5.2 PREPARATION OF RNA SAMPLES AND GEL ELECTROPHORESIS

For each sample, a volume containing 16 µg of total RNA was placed in a 0.5 mL Eppendorf tube. This was supplemented with 3 µL of RNA loading buffer (0.72 mL formamide, 0.16 mL 10x MOPS, 0.26 mL formaldehyde, 0.1 mL DEPC H₂O, 0.2 mL 40% v/v glycerol, 0.08 mL 0.5% w/v bromophenol blue) and 12 µL of sample buffer (1.5 mL 10x MOPS, 5.25 mL 37% w/v formaldehyde solution, and 15 mL formamide). The samples were then heated to 65°C for 5 min and chilled on ice. RNA samples were loaded onto the formaldehyde gel (pre-run at 90 V, 10 min) and the gel was run at 90V.

The quality of RNA obtained was assessed by viewing the gel with a UV transilluminator (310 nm). Destaining required soaking the gel in 400 mL of ddH₂O for 30 min, followed by a destain in 400 mL of 10x SSC (20x SSC: 3 M NaCl, 0.3 M trisodium citrate, pH 7.0). The gel was then photographed on a UV light transilluminator (Foto/UV 26, Fotodyne Inc.) at a wavelength of 300 nm by a DS-34 camera loaded with Polaroid instant pack films (667).

2.5.3 CAPILLARY TRANSFER OF RNA TO MEMBRANE

The RNA on the gel was blotted to 0.2 µm nylon membrane by the neutral transfer method (Sambrook *et al.*, 1989). The gel and a piece of NYTRAN 0.2 µm nylon membrane (Amersham, Schleicher & Schuell) were washed in 10 x SSC, then stacked as follows (bottom to top): a Whatman (VWR) filter paper wick (soaked in 20x SSC), the gel, the nylon membrane, 2 pieces of Whatman filter paper (soaked in 10x SSC), a 15 cm stack of dry paper towels, and a weight (0.5-2 kg). The ends of the paper wick, at the bottom of the stack, were lowered into trays filled with 20x SSC. The transfer was

allowed to take place for at least 14 h, after which the nylon membrane was washed for 2 min in 5x SSC. The RNA was cross-linked to the blot, followed by baking for 30 min at 80°C (BioRad Gel Dryer, model 583)

2.5.4 SYNTHESIS OF ^{32}P –LABELED CDNA

Labeled probe for hybridization of northern blots was created using the random priming method of Sambrook *et al.* (1989). Approximately 50-250 ng of template DNA was diluted to 10 μL with sterile ddH₂O, mixed with 2 μL of 0.5 $\mu\text{g}/\mu\text{L}$ DNA Random hexamers (Gibco-BRL) and heated in boiling water for 3-4 min. The sample was then allowed to chill on ice for at least 2 min. Following annealing of the primer to denatured cDNA, 2 μL a dNTP mix (1 mM dCTP, 1 mM dTTP, 1 mM dATP), 2 μL of Gibco-BRL Reaction Buffer (100 mM Tris-Cl, 50 mM MgCl₂, 75 mM DTT, pH 7.5), 1.0 μL of a 6.19 U/ μL Large Fragment of DNA Polymerase I stock (Gibco-BRL) and 5 μL of ^{32}P -dCTP (3000 Ci/mmol, Amersham) were added and the mixture was incubated at 37°C for 30 min. Unincorporated nucleotides were removed from probe by passing the mixture through a Sephadex G50 (Sigma) spin column prepared in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The labeled cDNA product was diluted to a final volume of 250 μL in TE and stored, if necessary, at -20°C.

2.5.5 PROBING, WASHING, AND VISUALIZATION OF NORTHERN BLOTS

The nitrocellulose membranes were placed in hybridization tubes (4 x 30 cm, VWR) containing 25 mL of Church's solution (7% w/v SDS, 0.5 M orthophosphate, pH 7.5). The tubes were pre-hybridized by rotating at 65°C in a hybridization incubator

(Model 308, LAB-line instruments, VWR Canlab) for 1 h. Radiolabeled DNA probe was boiled for 5 min, chilled on ice and then added to the hybridization solution. Hybridization was carried out at 44°C using Church's buffer in a hybridization oven for a minimum of 12 h. After hybridization the blots were washed in successive dilutions of SSC: 10x SSC, 6x SSC, then 2x SSC, with or without the addition of 0.2% SDS until distinct bands were visible after exposing blots to X-ray film at -70°C.

Densitometric analysis of the developed autoradiograms entailed the use of computer imaging software. A Scan Jet3C scanner (Hewlett-Packard, Palo Alto, CA) was used in conjunction with Deskscan II v2.2 software (Hewlett-Packard, Palo Alto, CA) to convert the autoradiogram into a form recognizable by Imagequant v3.22 (Innovative Optical Systems Research). Imagequant was used to measure relative band intensity, reflecting transcript levels, across the various lanes of the autoradiogram image. The corresponding 18 S ribosomal RNA bands on the ethidium bromide stained formaldehyde gel were used to ensure equal loading. Northern band intensities were scanned and normalized to their respective rRNA bands; control values were set to 100 and band intensities in other lanes were expressed relative to their control. Histograms show mean relative transcript levels, and the standard error of the mean, calculated from at minimum three separate northern blots prepared using tissues sampled from different animals.

2.6 REVERSE TRANSCRIPTION – POLYMERASE CHAIN REACTION

2.6.1 cDNA FIRST STRAND SYNTHESIS

A volume of mRNA containing 1.0 µg of mRNA was diluted to a total volume of 10 µL with DEPC treated water. Each mRNA sample was then mixed with 1 µL of RNase inhibitor (RNA guardTM, Amersham) and 1 µL of 0.2 µg/µL oligo(dT)₁₈ primer (Bio S&T, Montreal) and heated to 68°C for 5 min followed by rapid chilling on ice. To synthesize cDNA from primed mRNA, 4 µL of 5x first strand buffer (Gibco-BRL), 2 µL of 0.1 M DTT (Gibco-BRL), 1 µL of 10 mM dNTP mix, and 1 µL of SuperscriptTM RT (200 U/µL) were added and the final mixture was incubated at 37°C for 1 h. The cDNA:mRNA hybrid was stored at 4°C until needed.

2.6.2 POLYMERASE CHAIN REACTION AMPLIFICATION OF cDNA

For PCR amplification from the cDNA the first strand samples were diluted 1:100 in DEPC treated water. Each PCR reaction contained 10 µL of the diluted cDNA fractions, 30 µL of DEPC treated water, 5 µL of 10x PCR buffer (Gibco-BRL), 2.5 µL of 50 mM MgCl₂ (Gibco-BRL), 1.0 µL of 10 mM dNTP mix (Gibco-BRL), 0.25 µL of *Taq* DNA polymerase (5 U/µL, Gibco-BRL) and 1.5 µL of gene specific primer mix (forward and reverse, 30 µM). A drop of mineral oil (20-30 µL, Sigma) was placed over the top of the reaction to prevent evaporation. The PCR reaction was then carried out in a PTC-100 thermocycler (MJ Research Inc.) with the following cycle repeated 35 times: denature at 94°C for 1 min, anneal at 55°C for 1 min, and extension at 72°C for 1 min. The program

terminated following a 7 min elongation step at 72°C. Amplification of the correct PCR product was confirmed first by comparing the size of the product to the expected size on a TAE gel, and ultimately by sequencing (Canadian Molecular Research Services Inc., Orleans, ON).

2.7 5' RAPID AMPLIFICATION OF CDNA ENDS (RACE)

2.7.1 FIRST STRAND SYNTHESIS

First, 1 µg of mRNA was combined with 1 µL of cDNA synthesis primer (10 µM) and adjusted to a final volume of 5 µL. The mRNA and primer mixture was heated to 70°C for 2 min, and then cooled on ice for 2 min. After cooling, 2 µL of 5x first strand buffer (250 mM Tris, pH 8.5, 40 mM MgCl₂, 150 mM KCl, 5 mM DTT), 1 µL of dNTP mix (10 mM), and 1 µL of AMV reverse transcriptase (20 U/µL) were added. First strand synthesis took place at 42°C for 1 h, and was terminated on ice.

2.7.2 SECOND STRAND SYNTHESIS

The 10 µL of first strand sample was transferred to a fresh tube containing 48.4 µL of sterile H₂O, 16 µL of 5x second strand buffer (500 mM KCl, 50 mM ammonium sulfate, 25 mM MgCl₂, 100 mM Tris, pH 7.5, 0.25 mg/mL BSA), 1.6 µL of dNTP mix (10 mM), and 4 µL of 20x second-strand enzyme cocktail (6 units/µL *E. coli* DNA polymerase I, 1.2 units/µL *E. coli* DNA ligase, 0.25 units/µL *E.coli* RNase H). The mixture was allowed to stand for 1.5 h at 16°C, and then 2 µL of T4 DNA polymerase was added to the sample. Following incubation for a further 45 min at 16°C, 4 µL of an

EDTA/glycogen mix was used to terminate second strand synthesis. The sample was then mixed with 100 μ L of phenol:chloroform:isoamyl alcohol (25:24:1) and vortexed thoroughly. Following centrifugation at 12,000 x g for 10 min the aqueous layer was removed and placed in a tube containing 100 μ L of chloroform:isoamyl alcohol (24:1). The tube was mixed thoroughly and centrifuged at 12,000 x g to separate the phases. The synthesized second strand cDNA was precipitated from the collected aqueous phase by the addition of one-half volume of 4 M ammonium acetate, and 2.5 volumes of 95 % ethanol. The tube was centrifuged at 12,000 x g for 20 min and the pellet was washed with 80% ethanol (300 μ L). The pellet was air dried for 10 min and dissolved in 10 μ L of sterile water. The second strand synthesis product was stored at -20°C until required.

2.7.3 ADAPTOR LIGATION TO CDNA

A 5 μ L aliquot of the double stranded cDNA was moved to a 0.5 mL Eppendorf tube and 2 μ L of Marathon cDNA Adaptor (10 μ M), 2 μ L of 5 x DNA ligation (250 mM Tris-Cl, pH 7.8, 50 mM MgCl₂, 5 mM DTT, 5 mM ATP, 25 % w/v polyethylene glycol 8000), and 1 μ L T4 DNA ligase (400 units/ μ L) were added in order. The sample was incubated at 16°C overnight, and the enzyme was heat activated at 70°C for 5 min. A 1 μ L aliquot of the sample was diluted to 250 μ L with tricine-EDTA buffer prior to PCR.

2.7.4 5'-AMPLIFICATION OF CDNA ENDS

Each 50 μ L PCR reaction was prepared by mixing together the following: 36 μ L of H₂O, 5 μ L of 10x Advantage 2 PCR Buffer (400 mM Tricine-KOH, pH 8.7, 150 mM KOAc, 35 mM Mg(OAc)₂, 37.5 μ g/mL BSA, 0.05 % Tween-20, 0.05 % Nonidet-P40), 1

μ L dNTP mix (10 mM, Gibco), 1 μ L of Advantage 2 Polymerase Mix (Clontech), 1 μ L of the Clontech AP1 primer (10 μ M) and 1 μ L of a 30 μ M stock of gene specific nested primer (Bio S&T, Montreal). The PCR reaction was then carried out in the PTC-100 thermocycler (MJ Research Inc.) with the following cycle repeated 35 times: denature at 94°C for 1 min, then anneal and elongate at 68°C for 4 min. The program terminated following a 7 min elongation step at 72°C.

2.8 TWO DIMENSIONAL PROTEIN GEL ELECTROPHORESIS (2D-PAGE)

2.8.1 PREPARATION OF PROTEIN SAMPLES

2D-PAGE was performed using the Modular Mini-Protean II System (Bio-Rad). Total soluble protein was extracted from frozen samples of frog tissues by homogenizing (1:10 w/v) in buffer containing 250 mM sucrose, 25 mM HEPES, 25 mM KCl, 1mM EGTA, and 1mM EDTA. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added immediately prior to homogenization. After centrifugation at 5000 rpm for 5 min in a Biofuge 15 (Baxter Canlab) the supernatant was removed and used immediately (or stored at -20°C).

2.8.2 ISOELECTRIC FOCUSING OF PROTEINS

Separation of total soluble protein based on isoelectric point (pI) was performed in a capillary tube containing first dimension gel monomer solution (9.2 M urea, 4% acrylamide, 1.6% pH 5-8 ampholytes (Sigma), 0.4% pH 3.5-10 ampholytes (Sigma),

0.01% ammonium persulfate, 0.1% TEMED). The upper chamber running buffer was 100 mM NaOH, and lower chamber running buffer was 10 mM H₃PO₄. Following polymerization the capillary gels were pre-electrophoresed for 10 min at 200V, 15 min at 300 V and 15 min at 400 V (current was typically 1-3 mA). First dimension sample buffer contained 9.5 M urea, 5% β-mercaptoethanol, 1.6 % ampholytes (pH 4-6 range), and 0.4 % ampholytes (pH 3.5-10 range). Soluble protein supernatants were mixed with the first dimension sample buffer (1:1 v/v) and allowed to incubate at RT for 15 min. Equal volumes of supernatant were loaded into capillary sample reservoirs and overlaid with first dimension sample overlay buffer (9 M urea, 0.8% pH 5-8 ampholytes, 0.2% pH 3.5-10 ampholytes, 0.005% w/v bromophenol blue). Separation generally occurred over 8 h at 750 V. Following isoelectric focusing the gels were removed from the capillary tubes and incubated for 10 min in SDS equilibration buffer containing 62.5 mM Tris-HCl, pH 6.8, 2.3% w/v SDS, 5.0% β-mercaptoethanol, 10% w/v glycerol and 0.00125% w/v bromophenol blue.

2.8.3 SDS–POLYACRYLAMIDE GEL ELECTROPHORESIS

The proteins in the first dimension gel were further separated based on molecular weight using 12 or 15% acrylamide gels (acrylamide:bis-acrylamide 29.2:0.8; w/w) and the discontinuous buffer system of Laemmli (1970) with a Bio-Rad mini-gel apparatus. Prior to second dimension electrophoresis the first dimension gels were sandwiched between a 15% acrylamide separating gel, and a 4% stacking gel. Running buffer contained 3.03 g Tris base, 14.4 g glycine, and 1.0 g SDS per liter, pH ~8.3. Separation generally required ~1 h when run at 200 V.

2.8.4 ACRYLAMIDE GEL SILVER STAINING

The two dimensional protein profile was visualized through silver staining. Before staining, the gels were placed in fix solution containing 50% methanol and 10% acetic acid for a minimum of 1 h. Fixed gels were rinsed twice in ddH₂O for 5 min and incubated in a solution of 0.01% w/v sodium thiosulphate for 1 min. Gels were then rinsed 3 times with ddH₂O for 30 s and placed in a 0.2% w/v silver nitrate solution for 10 min. Following a quick rinse with ddH₂O the gels were developed using 100 mL of a solution containing 12 g sodium carbonate, 100 µl of 37% formaldehyde solution and 8 µl of 10% w/v sodium thiosulphate. The reaction was terminated by rinsing the gels in a solution of 5% acetic acid and then gels were stored in ddH₂O.

2.9 NUCLEAR RUN-OFF ASSAY

2.9.1 ISOLATION OF NUCLEI

Intact nuclei were isolated from fresh tissue samples of control and 24 h frozen *R. sylvatica* liver and heart. Tissue was homogenized 1:10 w/v in a ice-cold buffer containing 250 mM sucrose, 50 mM HEPES, pH 7.5, 25 mM KCl, 1 mM EGTA, and 1 mM EDTA. The homogenate was filtered through cheese cloth and 4 mL of eluant was placed on top of a 10 mL sucrose cushion prepared in a 15 mL Falcon tube. The cushion consisted of 2.0 M sucrose, 10% glycerol, 50 mM HEPES, pH 7.5, 25 mM KCl, 1 mM EGTA and 1 mM EDTA. Nuclei were separated from other organelles and soluble

components by centrifuging the cushion at 13,000 g for 30 min. Following the removal of the cushion and remaining cell debris, the nuclei were resuspended in a storage buffer containing 75 mM HEPES, pH 7.5, 60 mM KCl, 15 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, and 40% v/v glycerol and stored at -80°C.

2.9.2 ELONGATION OF NASCENT CHAINS

Elongation of nascent chains and isolation of RNA were performed using modifications to the protocol of Birch and Schreiber (1986). Transcription was performed in a final volume of 800 µl containing 500 mM Tris, pH 7.9, 100 mM NaCl, 20 mM MgCl₂, 20 mM MnCl₂, 1 mM DTT, 2.5 mM GTP, 500 µCi [α -³²P] UTP, 2.5 mM CTP, 7.5 mM ATP, 20 U/ml RNasin, 25% v/v glycerol and nuclei equivalent to ~20 mg of protein. After a 30 min incubation period at 26°C, 8 units of DNase (8 µl) were added and incubation was continued for 15 min. After this 150 µg/mL of proteinase K (15 µl of 10 mg/ml solution) and 0.5% SDS (30 µl of 14% SDS) were added and the incubation was continued at 37°C for 30 min. Total RNA was isolated using TRIZOL reagent and resuspended in 200 µl of DEPC treated water. A 20 µl aliquot of the RNA solution was passed through a 0.5 mL G-15 Sephadex spun column, and the incorporation of [α -³²P]UTP was measured by liquid scintillation and expressed per mg nuclear protein. Protein concentration was measured using the Bio-Rad assay (Bradford, 1976).

2.9.3 HYBRIDIZATION OF ELONGATED RNA TRANSCRIPTS

Plasmids containing cDNA for both a control gene and the gene of interest were isolated from bacterial stocks. Plasmids were digested with EcoRI, boiled for 5 min, cold-

snapped on ice, and blotted to Nytran nitrocellulose. The membranes were hybridized at 42°C in Church's buffer for a minimum of 12 h. Following hybridization, blots were washed with increasing stringency, using successive dilutions of SSC, and increasing amounts of SDS and then placed into cassettes with X-ray film. Transcript levels were quantified by scanning the developed autoradiogram using a Scan Jet3C scanner (Hewlett-Packard, Palo Alto, CA) with Deskscan II v2.2 (Hewlett-Packard, Palo Alto, CA) and an Imagequant v3.22 (Innovative Optical Systems Research) software.

2.10 R. SYLVATICA LIVER SLICE INCUBATIONS

2.10.1 PREPARATION OF AMPHIBIAN TISSUE CULTURES

Methods were essentially as described by Balls *et al.* (1976). Dissection and preparation of organ cultures was carried out under sterile conditions in a laminar flow hood. The skin was sterilized prior to dissection with absolute ethanol and all scissors and tweezers were rinsed with ethanol and flame sterilized. Once removed the livers were separated, washed and stored acutely in petri dishes containing culture medium for washing. The culture medium used consisted of 50% minimum essential medium (Sigma, M-4655), 40% autoclaved ddH₂O, 10% heat inactivated controlled process serum replacement (CPSR, Sigma, C-0766) along with 100 U/mL penicillin, 100 µg/mL streptomycin and 2 µg/mL Fungizone. Before transfer to fresh culture medium in 15 mL sterilized Falcon tubes, the organs were cut into 5-8 mm³ size slices.

2.10.1 IN VITRO SIMULATION OF FREEZING ASSOCIATED STRESSES

Organ slices were allowed to equilibrate at 15°C for 2 h in culture medium. Incubation was carried out in 15 mL Falcon tubes with 4-5 mL of culture medium; tubes were laid on their sides with horizontal shaking at 5-10 rpm. Tissue slices were treated with a variety of protocols meant to simulate a physiological condition that occurs during freezing. The first treatment, serving as the control, involved incubation at 15°C for a further 6 h. The second treatment, simulating the increase in glucose that occurs during freezing, required the addition of glucose to a final concentration of 500 mM. Anoxia was reproduced by bubbling nitrogen gas through the medium for the final 6 h of the experiment. In vitro dehydration was accomplished by adding polyethylene glycol (8000) at a final concentration 1 % to cause osmotic withdrawal of water from cells. Freezing was performed by placing the samples in a freezer at -4°C (after 2 h any samples not frozen were manually nucleated). In the final treatment the organ cultures were moved to medium containing an additional 0.5 M glucose and nucleated as described above to analyze for synergy in *fr10* up-regulation. At the end of the 8 h total incubation the medium was removed and liver slices were frozen in liquid nitrogen.

2.10.2 SECOND MESSENGER STUDIES, IN VITRO TISSUE SLICE EXPERIMENTS

A series of second messengers were applied to the tissue slices including N⁶-2'-O-dibutyryl-adenosine-3':5'-cyclic monophosphate (db-cAMP), N²-2'-O-dibutryl-guanosine-3':5'-cyclic monophosphate (db-cGMP), phorbol 12-myristate 13-acetate (PMA). Stock solutions (20 mM) of each second messenger were made by dissolving the sodium salt of cGMP or cAMP in ddH₂O, and the PMA in DMSO. Following equilibration for 2 h at

15°C the medium was supplemented with one second messenger (10 µl/mL of medium) and then samples were allowed to incubate for a further 2 h. Tissues were then removed from the medium and immediately frozen in liquid nitrogen.

2.11 SDS-PAGE AND WESTERN BLOTTING

2.11.1 PREPARATION OF PROTEIN EXTRACTS

Total soluble protein was extracted from frozen samples of frog tissues by homogenizing (1:10 w/v) in buffer containing 250 mM sucrose, 25 mM HEPES, 25 mM KCl, 1mM EGTA, and 1mM EDTA. The protease inhibitor PMSF was added immediately prior to homogenization. Samples were centrifuged at 5,000 rpm for 5 min at 4°C in a Biofuge 15 (Baxter Canlab), supernatant was collected, and the protein concentration was determined using the Bio-Rad protein assay (Bradford, 1976).

2.11.2 SDS-PAGE AND IMMUNOBLOTTING

Stock solutions for electrophoresis were as follows. The 2× sample buffer contained 100 mM Tris-HCl (pH 6.8), 4% SDS (w/v), 20% glycerol (v/v), 5% β-mercaptoethanol (v/v) and 0.2% bromophenol blue (w/v). The 10x stock of running buffer contained 30.3 g Tris base, 144 g glycine, and 10 g SDS per liter, pH ~8.3. Protein samples were mixed with 2x SDS-PAGE sample buffer (1:1, v/v) and boiled 3-5 min. Equal amounts of protein were loaded into each well. Proteins were separated using 7.5-15% gels (acrylamide:bis-acrylamide 29.2:0.8; w/w) using the discontinuous buffer system of Laemmli (1970). Separation generally required ~1 h at 200 V for the Bio-Rad

Mini Protein II, and 4 h at 200 V with the Hoeffer apparatus. Proteins were blotted to polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer Membrane, Millipore Corp. Bedford, MA) by wet transfer with pre-chilled solution containing 25 mM Tris (pH 8.5), 192 mM glycine, and 10% v/v methanol at 4°C overnight at 40 V.

2.11.3 IMMUNOBLOTTING AND PROTEIN VISUALIZATION

The general immunoblotting procedure was as follows. The protein blot was first rinsed with TBST buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20) and then incubated in blocking buffer (TBST with 5% non-fat dry milk) for 1 h at RT or overnight at 4°C. The blot was then added to blocking buffer containing the primary antibody at the required dilution and incubated at RT for 1 h or at 4°C overnight. Following 3-5 washes in TBST (5 min, 25°C), the blot was then incubated in blocking solution (TBST, 5% non-fat dry milk) with an appropriate dilution (1:3000) of the corresponding secondary antibody. The target protein was visualized by the enhanced chemiluminescence assay (ECL) (NEN Life Science Products, Boston, MA).

CHAPTER 3

**CDNA LIBRARY SCREENING AND 2-DIMENSIONAL GEL ELECTROPHORESIS:
IDENTIFICATION OF FREEZE UP-REGULATED GENES AND PROTEINS**

3.1 INTRODUCTION

3.1.1 AIM OF THE CHAPTER

As previously described, whole body freezing is a complicated process involving changes in temperature, cellular osmolality, oxygen partial pressure, and nutrient availability. These stresses develop over the 12-24 hours that can pass from the time of initial ice nucleation at the extremities to the completion of the freezing process by attainment of equilibrium ice content. If one considers the time frame involved and number of stresses that are accommodated during a freeze-thaw cycle the wood frog would be expected to respond to freezing with specific and important changes in mRNA and protein content. Recent studies with *R. sylvatica* have confirmed the presence of as yet unidentified changes to mRNA and protein synthesis following freeze exposure (Storey *et al.*, 1997; White and Storey, 1999). In an attempt to further understand the overall response to freezing stress, studies were carried out with the liver and heart of wood frogs using techniques including differential cDNA library screening and two-dimensional protein electrophoresis. The initial findings from these studies are presented in this chapter, and selected results are followed up in succeeding chapters with more in-depth analysis of specific freeze-responsive genes. The remainder of this introduction outlines the basics of gene and protein expression, and reviews the various techniques available for analysis.

3.1.2 BASICS OF GENE EXPRESSION

The majority of the transcript content of the cell is located in the cytoplasm, but is encoded by DNA located on chromosomes within the nucleus. The typical gene consists of a promoter region, which regulates how frequently RNA copies are made, followed by the transcription start site. The nucleotide where transcription begins is referred to as base 1, and sites in the promoter region are referenced using negative values (e.g. the TATA box is typically located at -25 to -40 in most genes). The promoter regions of genes contain both common regions of DNA (e.g. the CAAT and TATA boxes), and other distinct DNA sequences in their promoter regions that act to either encourage (enhancers) or suppress (silencers) transcription. These sites are important for initiating the binding of RNA polymerase II (and other proteins required for general transcription), the enzyme that converts the DNA template into mRNA (Knowlton, 1995). RNA polymerase II is large (~500 kDa) and consists of many subunits (Young, 1991). Transcription initiation causes a local unwinding of the double-stranded DNA template and allows RNA polymerase II to move along the template in a 3' to 5' direction, producing a mRNA precursor (pre-mRNA) in a 5' to 3' direction (Roeder, 1996; Uptain *et al.*, 1997).

Following synthesis of the transcript, the cell modifies the structure of the mRNA prior to translation. Almost all pre-mRNA transcripts are “capped” with a modified guanosine residue (7-methylguanosine) shortly after transcription initiation. As well, the majority of eukaryotic mRNA (and their nuclear precursors) have a structure called a polyadenylation tract at their 3' ends, which is not encoded in the DNA, and is synthesized by a template-independent enzyme called poly(A) polymerase. The signal eliciting the addition of the polyA tract is a hexanucleotide (usually 5' AAUAAA 3')

located some 10-30 nucleotides upstream of the tract itself (Wahle and Keller, 1992). Both the 5' cap and 3' polyadenylation tail function to stabilize the transcript and play an important role in translation (Beelman and Parker, 1995). The mRNA at this stage is still a precursor, composed of both exons (coding sequence) and introns (non-coding sequence) that need to be spliced out by small nuclear ribonucleoproteins (snRNPs) to produce the mature mRNA (Dreyfuss *et al.*, 1993; Sharp, 1994).

Following these modifications the mRNA is transported into the cytoplasm and translated at the ribosome. Each mRNA is read in groups of three nucleotides called codons. There are 61 codons for amino acids, one start codon AUG (methionine) that is recognized as the translation initiation point, and three stop codons (UAA, UAG, and UGA) that terminate translation. In general, one mRNA transcript can be re-cycled multiple times, producing many copies of a protein before being degraded. The half-life for an mRNA transcript can vary from minutes to hours, depending on a number of factors (Ross, 1996). The stability of mRNA is one of the key regulatory mechanisms involved in the control of gene expression (Beelman and Parker, 1995).

3.1.4 IDENTIFYING CHANGES IN GENE AND PROTEIN EXPRESSION LEVELS

Over the past decade our laboratory has been attempting to identify changes in protein and gene expression that accompany and contribute to the phenomena of hibernation, estivation, anaerobiosis and freeze tolerance. This research has involved the application and assessment of numerous techniques, included: two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for protein analysis; and differential display PCR (ddPCR), gene arrays and cDNA library screening for mRNA profiling.

3.1.4.A TECHNIQUES FOR PROTEIN ANALYSIS

Over the past two decades, 2D-PAGE has become the technique of choice for analyzing the protein composition of a sample (O'Farrell, 1975; Fey and Larsen, 2001). The method separates proteins in one dimension on the basis of isoelectric point (pI) and in a second dimension by molecular weight (MW). Accordingly, its resolving power is unsurpassed when compared to one-dimensional gel separation techniques. 2D-PAGE technology can be used for applications including the separation of complex protein mixtures into their individual polypeptide components and for comparison of protein expression profiles of sample pairs. Advances in the related areas of protein sequencing and protein identification through mass spectroscopy have greatly expanded the applicability of the 2D-PAGE technique (Lahm and Langen, 2000; Jungblut and Wittmann, 1995).

3.1.4.B TECHNIQUES FOR MRNA EXPRESSION ANALYSIS

cDNA library and cDNA microarray screening, two of the most commonly used techniques for the identification of differentially expressed genes, both rely on basic hybridization technology. cDNA microarray techniques enable the monitoring of cell-, tissue- and process-specific gene expression profiles and simultaneously quantification of the expression levels of thousands of genes (Schena *et al.*, 1995; Duggan *et al.*, 1999). A cDNA microarray consists of small amounts of DNA fixed to a nylon membrane or small glass plate in an organized pattern. Radiolabeled cDNA is then created from mRNA isolated from different samples and hybridized separately to each microarray, revealing genes (often groups) that are more intensely expressed in one condition relative to

another. Complementary DNA libraries have been used for the cloning of genes for a number of decades (Weinstock *et al.*, 1994; St. John and Davis, 1979). In the construction of a cDNA library, copies of expressed genes (mRNA) from an organism or cell type are copied into a more stable form (cDNA) and cloned into a vector that can be propagated within a host microbe (e.g. bacterial, viral). If prepared properly, the final pool of plasmid:cDNA hybrids will contain a copy of each gene expressed in the original system. Each microbe (containing only one cDNA) can then be screened individually for a specific gene (e.g. selected gene sequence, or a clone differentially expressed between samples).

The third mRNA profiling technique that has been used by our lab is ddPCR (Zhang *et al.*, 1998; Liang and Pardee, 1992). ddPCR is a sensitive mRNA fingerprinting technique that enables comparative display of all transcribed genes in a cell or tissue type, and identification of cell- and process-specific differentially expressed genes. This is accomplished through a comparison of reverse transcribed and arbitrarily amplified cDNAs (PCR) from two or more cell or tissue types. The amplified cDNAs are run out on a polyacrylamide gel and differentially expressed genes are located, excised and sequenced.

3.2 MATERIALS AND METHODS

3.2.1 cDNA LIBRARY CONSTRUCTION

A frog heart cDNA library was constructed, as outlined in section 2.3, using a total of 5 µg of mRNA. The mRNA was prepared by combining 1 µg samples isolated from spring-collected frogs that were frozen for 1, 4 or 24 h, and those exposed to anoxia for 4 and 24 h. The motivation for including mRNA isolated from anoxic wood frogs was the realization of a more versatile cDNA library that could be used for future experiments, identifying anoxia induced genes. Total RNA and mRNA were isolated and quantified as described in sections 2.2.2. The only deviation from the manufacturer's protocol involved the use of a spin column, not a drip column, to size fractionate the cDNA (section 2.3.5).

3.2.2 DIFFERENTIAL SCREENING OF THE cDNA LIBRARY

Both the heart cDNA library (Uni-ZAP CMV XR) and a wood frog liver cDNA library (Uni-ZAP XR, previously prepared with mRNA isolated from 4, 12, and 24 h frozen frogs by Dr. S. Wu) were titrated to determine the volume necessary to obtain 10,000 plaque forming units (pfu). For the primary screening of both libraries 10,000 plaques were plated and lifted onto nylon membrane as described in section 2.4.2. Paired nylon membranes were hybridized with ³²P-labeled cDNA probes synthesized from 1 µg of mRNA isolated from heart or liver of control (5°C) versus 24 h frozen animals (section 2.4.2). Conditions of hybridization were 44 °C for 18 h. Removal of excess background counts generally required washing in 10x SSC (10 min), 5x SSC (10 min), and then 1x

SSC (10 min). After exposure of membranes to X-ray film, autoradiograms were developed and examined for the presence in the freeze-exposed hybridization of spots that were either unique (indicating freeze-induced genes) or much darker (indicating up-regulated genes) as compared with the control film.

All of the potentially positive plaques were then excised from the plate using a sterile pipette. The plaques excised were pooled together (~20) and the phage were extracted from the agar. To eliminate false positives, the extracted phage were then replated at a reduced density (500-1000 plaques per plate), transferred to membrane, and probed a second time with radiolabeled cDNA prepared from control and frozen tissue.

3.2.3 *IN VIVO* EXCISION AND IDENTIFICATION OF cDNA CLONES

The phage identified as differentially expressed following secondary screening were excised from the agar plate using a sterile Pasteur pipette tip. The phage were eluted from each plaque using 1 mL of SM buffer. Amplification of each plaque to ensure proper *in vivo* excision was carried out as described in section 2.3.9. *In vivo* excision from the heart cDNA library was performed as described 2.4.3, whereas *in vivo* excision from the liver cDNA library was performed using SOLR cells and required LB ampicillin plates to ensure propagation of only those cells transformed with the UNI-ZAP XR (pBluescript) plasmid. Individual bacterial colonies were used to prepare bacterial glycerol stocks.

3.2.4 NORTHERN BLOT CONFIRMATION OF GENE UP-REGULATION

The plasmids containing cDNA for each potentially upregulated gene were then isolated from bacterial glycerol stocks as described in section 2.2.4. TAE gel electrophoresis following restriction digestion with EcoR I and Xho I was performed to isolate the cDNA for each gene and estimate the size of the cloned transcript. Northern analysis (section 2.5) was then performed on blots prepared with total RNA (16 µg/lane) isolated from heart or liver of control and 24 h frozen *R. sylvatica*. Genes observed to have greater expression in frozen frogs were sequenced by Canadian Molecular Research Service (Orleans, ON), and the sequences were identified using a BLAST analysis at the National Centre for Biotechnology Information website (Altschul *et al.*, 1990). The DNAMAN software package was used to analyze clone structure and prepare gene and protein alignments with sequences obtained from other animals.

3.2.5 TWO-DIMENSIONAL PROTEIN ELECTROPHORESIS

Total soluble protein was extracted (as in 2.8.1) from the hearts of control wood frogs and from animals exposed to 4 and 24 h of freezing. Protein concentration was measured using the Bio-Rad protein assay and equal amounts of protein from each sample were separated by isofocusing in the first dimension and by molecular weight in the second (see sections 2.8.2 and 2.8.3). Silver staining and Coomassie blue staining were used to visualize protein patterns and analyze for differences between experimental groups. Up-regulated protein bands (Coomassie stained) were excised from their gels and mass spectroscopy-microsequencing was performed in the laboratory of Dr. Pierre Thibault (National Research Council, Ottawa, ON).

3.3 RESULTS

3.3.1 CONSTRUCTION OF cDNA LIBRARY

A cDNA library was successfully constructed into the Uni-ZAP CMV XR vector using mRNA pooled from hearts of wood frogs exposed to freezing and anoxia for different lengths of time (1,4 and 24 h freezing, 4 and 24 h anoxia). Figure 3.1 illustrates some of the results obtained at various checkpoints in the multi-step procedure. Incorporation of radiolabeled dATP into the cDNA samples during first strand synthesis allowed for an estimation of the average cDNA size in each fraction eluted from the Sepharose Cl-2B spun column. Figure 3.1A shows the autoradiogram obtained from overnight exposure of the dried native acrylamide gel. Lanes 1 through 5 show 5 μ L samples of the 30 μ L fractions collected for fractions 1, 3, 5, 7, and 9, respectively. Lane 6 contains radiolabeled 1 kb markers from Gibco-BRL. The cDNA eluted in fractions 1 through 3 was determined to be larger than 0.5 kb and the fractions were pooled and used to finish construction of the cDNA library. Prior to cDNA ligation into the Lambda ZAP-CMV XR vector the pooled samples were extracted, precipitated, re-suspended and quantified using an EtBr plate assay (Figure 3.1B). The cDNA serial dilution set used as a standard curve is easily visible at the top of the gel; here 1 μ L samples of 1, 0.5, 0.25, 0.125, and 0.0625 μ g/ μ L samples were spotted on the plate and allowed to dry, prior to photography. Then a 0.5 μ l aliquot of *R. sylvatica* cDNA (total volume of 5 μ L) was added to the plate, and is marked with an arrow in Figure 3.1B. The concentration of cDNA was estimated to be approximately 0.1 μ g/ μ L.

Following ligation and packaging the titer of the library was determined. Twenty μL of the total 500 μL of unamplified library was used to create a set of 200 μL dilutions. Plates were prepared with 10 μL of each dilution set; no plaques were found at the highest dilution of 10^{-6} , 2 plaques were on the 10^{-5} , 12 on the 10^{-4} , and 128 on the plate prepared with 10 μL of the 10^{-3} stock dilution. From these values the number of plaque forming units in the 500 μL primary stock was determined to be approximately 6.5 million. An amplified library prepared from 1 million distinct phage particles was created by plating out 80 μL of the primary stock onto 20 agar plates (22.5 cm x 22.5 cm) with 50,000 pfu per plate. Final titer of the heart cDNA library after amplification was determined to be 2.5×10^8 pfu/mL.

3.3.2 DIFFERENTIAL SCREENING OF CDNA LIBRARY

Genes up-regulated by freezing were isolated through the analysis of 100,000 distinct phage plated out from the heart cDNA library and 50,000 from the liver cDNA library. A representative (primary) differential screen of the *R. sylvatica* heart cDNA library is shown in Figure 3.2 comparing the autoradiogram of a membrane hybridized with ^{32}P -labeled cDNA synthesized from mRNA from control hearts (A) with the duplicate membrane probed with cDNA from heart of 24 h frozen frogs (B). The arrow shows an example of a plaque that was more strongly expressed in the frozen versus control condition. Primary and secondary screening of the heart cDNA library revealed 50 positive plaques with stronger signals on the membranes hybridized with frozen probe whereas twenty clones were identified from the liver cDNA library. These clones were selected, excised *in vivo* into plasmids, and maintained in glycerol stocks. The inserts of

the cDNA clones were separated from the plasmid by double restriction enzyme digestion (EcoR I and Xho I) followed by electrophoresis on a 1% TAE gel. An example of plasmid isolation, digestion and agarose gel visualization is provided in Figure 3.3.

3.3.3 CHARACTERIZATION OF CDNA CLONES

3.3.3.A UP-REGULATED LIVER CLONE 16 – *li16*

Published reports on freeze-specific changes in gene expression have focused mainly on the liver of wood frog (Cai and Storey, 1997a, 1997b; Cai *et al.*, 1997). However, not all of the genes identified in these original investigations, or in a subsequent thesis from our lab (Wu, 1999), have been fully characterized. One such up-regulated clone, liver clone 16 (*li16*), was reported by Wu (1999). This clone was determined to be up-regulated in the livers of frozen wood frogs. The plasmid containing the 300 kb clone (half of full transcript) was obtained in the form of a bacterial stock, and was further investigated in parallel with the clones identified from the cDNA library screens described above.

3.3.3.B NORTHERN ANALYSIS OF HEART AND LIVER CDNA CLONES

Northern blots prepared with mRNA isolated from liver or heart of control and 24 h frozen frogs confirmed that 7 of the heart clones and 4 liver clones, were upregulated during freezing, including *li16* (Figure 3.4). Sequencing of the 11 upregulated cDNA clones revealed that 4 of the 7 heart clones, and 2 of the 3 new liver clones had poly(A) tails, polyadenylation signals (AAUAAA), open reading frames, and definitive stop

codons. One of the three remaining heart clones did not contain an open reading frame or a polyadenylation signal, but was identified as the mitochondrial 16S ribosomal RNA. Following digestion from plasmid, the size of the upregulated liver cDNA clones named *ddx8*, *fl8*, and *lil6* were determined to be 2.2, 1.4 and 0.3 kb, respectively; whereas the 5 heart clones were determined to be 0.8, 0.8, 1.1, 0.7 and 1.4 kb for the clones designated *ba*, *b4*, *h25*, *d1*, and *b2*, respectively. A representative northern blot prepared with equal amounts of control and frozen total RNA hybridized with radio-labeled cDNA prepared with each of the 8 clones is presented in Figure 3.4.

DNAMAN and BLAST (Basic Local Alignment Search Tool) analysis were used to prepare Table 3.1 that summarizes some of the characteristics of the 5 heart and 3 liver genes. Shown are the identities of each cDNA, clone size, transcript size, the size of the largest open reading frame, the predicted open reading frame based on information from literature, and which of the sequences contained full open reading frames. The genes that were freeze up-regulated in heart were determined to be heat shock protein 27 (*hsp27*), elongation factor-1 gamma (*EF1 γ*), ribosomal 16S RNA, the alpha subunit of nascent associated complex (α NAC), and a previously identified mammalian gene coding for a protein of unknown function, *hspc070* (recently re-named Makorin2 so as not to be confused with the heat shock protein 70). The three liver clones were recognized as the mitochondrial phosphate carrier protein (*mpcp*) and two genes of novel sequence and function (*ddx8* and *lil6*). Five of these genes will be characterized and discussed in greater detail in subsequent chapters. However, as only a limited characterization was done with *hspc070*, *16S*, and *mpcp* they will be discussed below.

3.3.4 GENE AND PROTEIN ANALYSIS

3.3.4.A ANALYSIS OF *HSPC070*

cDNA clone *b2* was sequenced and compared to the GenBank database using the BLAST search engine located on the National Centre for Biotechnology Information website. The nucleotide and deduced amino acid sequences are shown in Figure 3.5. A single open reading frame was found coding for 408 amino acids, with a complete C terminal (ending in a stop codon) but an incomplete N terminal. Clone *b2* was identified as the *R. sylvatica* homologue of a gene of unknown function that was originally discovered as an Expressed Sequence Tag (EST) from a human hematopoietic stem/progenitor cell (*hspc*) line (Genbank accession number AF161555) (Zhang *et al.*, 2000). This gene, originally named *hspc070*, has been renamed Makorin Ring Zinc-Finger Protein 2 and has since been cloned from *Mus musculus* (Genbank accession #AF277171) (Carninci and Hayashizaki, 1999).

Northern blot analysis showed a 5.4 ± 2.1 fold increase in transcript levels during freezing and allowed for an estimation of the full length transcript size at 1.5 kb. This is approximately half of the reported size for both the human and mouse *hspc070* genes, which are 3.0 kb in length. However, the longer transcript size in mammalian systems does not translate into a larger final protein sequence since the additional base pairs are primarily found in the 3' untranslated region of the gene. Indeed, an alignment of the open reading frames for the three *hspc070* genes showed that the wood frog sequence may be missing only 7 amino acid residues at the N-terminus compared with the other

species (Figure 3.6). Mouse and human sequences were 100 percent identical, whereas the wood frog amino acid sequence showed 66 % identity with the mammalian protein.

The *hspc070* gene and protein sequences were then compared with the protein domains located in the Prosite and BLOCKS databases maintained on-line (Henikoff and Henikoff, 1994). This comparison revealed the presence of a Zinc Ring Finger domain, C3HC4 type (BLOCKS accession #IPB001841, Prosite accession #PS00518) beginning at amino acid residue 227 (Figure 3.5) (Borden and Freemont, 1996). In addition, BLOCKS identified three additional standard zinc finger domains, C-x8-C-x5-C-x3-H type (or similar, accession #PF00642) beginning at positions 31, 160, and 317. Furthermore, and of interest with regard to cold and freezing survival, BLOCKS identified one of the two motifs that assembles to form a cold shock DNA binding domain, starting at amino acid position 357 (blocks accession number IPB002059).

3.3.4.B ANALYSIS OF THE MITOCHONDRIAL GENES MPCP AND 16S rRNA

A BLAST analysis with the sequence obtained for liver clone *fl8* identified the clone as the mitochondrial phosphate carrier protein. The nucleotide and deduced amino acid sequences are presented in Figure 3.7; the full open reading frame is represented beginning with a start codon at nucleotide 12-14 and running to a stop codon located at 1092-1094. Based on the sequences from other species, the first 45 amino acid residues were identified as a signal sequence that is cleaved from the mature protein. An alignment of the amino acid sequence of the mature *R. sylvatica* mitochondrial phosphate transporter with the rat, human and *Caenorhabditis elegans* sequences obtained from the NCBI website (accession numbers M23984, BC000998, X76113) is presented in Figure

3.8. The wood frog nucleotide sequence showed 70, 70 and 57% identity, whereas the deduced amino acid sequence had 84, 86 and 68% identity with rat, human, and *C. elegans* sequences, respectively. A Prosite scan recognized a “mitochondrial energy transfer proteins signature” (accession #PS00215) at positions 38-46 and 135-144. The pattern required for a positive match is (P-x-[DE]-x-[LIVAT]-[RK]-x-[LRH]-[LIVMFY]-[QGAIVM]). This same domain has been found in other substrate carrier proteins located in the inner mitochondrial membrane including 2-oxoglutarate/malate carrier protein, brown fat uncoupling protein, the phosphate transporter protein and the freeze inducible ADP/ATP translocase (Walker and Runswick, 1993; Cai *et al.*, 1997).

Following the sequencing of heart clone *dI*, a BLAST analysis identified the gene as the mitochondrial ribosomal 16S RNA. The gene sequence for *R. sylvatica* 16S rRNA is presented in Figure 3.9A. A homology tree was created and Figure 3.9B shows that the wood frog sequence shared 84%, 66%, 53%, 51%, 48%, and 53% identity with the sequences of leopard frog (accession number Y10945), African clawed frog, *Xenopus laevis* (M10217), catfish (AF032380), chicken (NC_001323), rat (J01438) and human (V00662) sequences, respectively. Analysis of three separate northern blots prepared with total RNA isolated from control and 24 h frozen *R. sylvatica* hearts showed a 3.58 ± 0.37 (SEM; n=3) fold increase in the number of transcripts following freezing exposure.

3.3.5 TWO-DIMENSIONAL PROTEIN ELECTROPHORESIS

Total soluble protein was extracted from hearts of control, 4 h and 24 h frozen frogs and compared using 2D-PAGE; representative gels (from 3 trials) are shown in Figure 3.10. The gels were silver-stained and approximately 200 proteins were visible. Of

interest was a protein estimated to be 20 kDa in size observed to be up-regulated following freezing exposure. The protein spot was excised from the gel, incubated in acetonitrile and reswollen in digestion buffer containing sequencing grade trypsin for digestion. Tryptic peptides were extracted from the gel slices using acetic acid and acidic acid:methanol solutions; the two extracts were pooled and the solvent was removed using a centrifugal evaporator (Salvant). The peptide fragments were then analyzed by liquid chromatography (LC-MS) and two of the peaks were sequenced through electrospray ionization mass spectrometry. The instrumentation used was a Waters CapLC liquid chromatograph coupled to a micromass Q-TOF 2 mass spectrometer containing an electrospray ionization interface. The instrument was set-up such that detection of a double or triply protonated ion resulted in a switch to MS/MS sequencing mode, using nitrogen as the collision gas.

The amino acid sequences obtained (ILDPNAT and LNVNE) were then used for protein identification, and a BLAST analysis recognized *X. laevis* myosin light chain 2 (MLC2) as the only protein with a primary sequence containing both of the identified peptide fragments (Genbank accession #A53729). To help confirm the identity of the protein, the DNAMAN program was used to predict the molecular weight and isoelectric point of the *X. laevis* protein from its published sequence. The hypothetical molecular weight and pI of the *X. laevis* protein were 19,253 daltons and 4.38, respectively, values that are very similar to those estimated for the *R. sylvatica* protein. Table 3.2 summarizes the findings for both species and shows the *X. laevis* MLC2 primary sequence with the amino acid strings identified from the *R. sylvatica* protein underlined.

3.4 DISCUSSION

3.4.1 GENE DISCOVERY OVERVIEW

Over the past few decades the screening of cDNA libraries has proven to be a valuable technique for the identification and isolation of gene transcripts that are differentially expressed in response to stimulus or stress. The selection of differential cDNA library screening as the appropriate technique for the identification of freezing-induced transcripts in the wood frog model system first required a comparison with the other available techniques: ddPCR and cDNA microarrays.

Recent technical advances have threatened to replace cDNA library screening with quicker, easier, and more thorough procedures for analyzing gene expression, and identifying differentially expressed transcripts. Our laboratory has recently experimented with a number of these approaches including ddPCR and cDNA microarrays. In the last 5 years cDNA microarray technology has started a revolution in gene expression analysis, allowing for the simultaneous analysis of thousands of genes across multiple sample sets. Although extremely powerful, the technique does have a few shortcomings, some of which have been reviewed in the literature (Watson *et al.*, 2000; Wildsmith and Elcock, 2001). The main disadvantages observed by our lab include (i) heterologous probing (sample from one species, microarray prepared from another) can result in low cross-reactivity so that only a fraction of the genes represented on the array can be assessed, and (ii) array screening does not allow for the discovery of novel genes or analysis of genes not present on the array. Due to the difficulties associated with cross-species hybridization, researchers working with non-mammalian vertebrates have yet to

experience the true benefits of microarrays. An additional technique tested by our laboratory for transcript analysis was ddPCR. This technique has proven to be a popular due to the short turn-around time from beginning the experiment to sequencing of the potential clones. However, drawbacks to this technique were soon evident to our lab and to others (Miele *et al.*, 1998; Luce and Burrows, 1998; Liang and Pardee, 1992). The two most important findings were (i) compared to cDNA library screening there was a much higher percentage of false positives and (ii) a large number of the positive clones were either unidentifiable or short, fragmented, unusable ESTs. Consequently, a large amount of time and effort was then required to isolate larger pieces of the fragmented clones through either cDNA library screening or 5'RACE. By contrast, the differential screening of cDNA libraries used in this thesis allowed cDNA clones to be isolated with relative ease in a versatile plasmid form that yielded complete or near-complete sequences and provided for easy identification of genes that were over-expressed during freezing exposure in wood frogs.

3.4.2 CDNA LIBRARY CONSTRUCTION AND SCREENING

In total, ten differentially expressed genes were identified from cDNA library screening and northern blot analysis. Following sequencing, six of the ten genes were identified as true mRNA species containing open reading frames, stop codons, polyadenylation signals, and poly (A) tails. One of the clones was identified as a mitochondrial ribosomal RNA species, and three others were discarded when no open reading frame could be found in the obtained sequence and BLAST failed to identify the transcripts.

The five clones pulled from the heart cDNA library coded for a heat shock protein (Hsp27), a protein and a rRNA associated with protein synthesis (EF1 γ , ribosomal RNA 16S), a protein with proposed dual functionality (the α -subunit of nascent associated complex), and a previously identified mammalian gene (*hspc070*) with unknown function containing zinc finger motifs. The three liver clones identified encoded the mitochondrial phosphate carrier protein and two novel genes, designated *ddx8* and *li16*. Chapters 5, 6 and 7 describe the further investigations performed with *onac*, *hsp27*, *ef1 γ* and the *ddx8* and *li16* genes.

Overall, the cDNA library construction was determined to be successful as 2 of the 4 mRNA species pulled from the heart library contained full open reading frames, 98% of the open reading frame was present in the third clone, and 71% of the open reading frame was obtained for the fourth clone (See Table 3.1). In addition, two of the liver clones (*ddx8* and the mitochondrial phosphate carrier protein) were sufficiently large in size to demonstrate that the *R. sylvatica* liver cDNA library was properly constructed. For *ddx8*, a length of 2.3 kb was obtained, and the entire open reading frame was present in the *mpcp* clone.

3.4.3 TWO DIMENSIONAL PROTEIN ELECTROPHORESIS

Analysis of *R. sylvatica* heart samples via 2D-PAGE represents another way of identifying freeze-responsive genes/proteins, and has the advantage of directly confirming the differential expression of selected proteins during freezing (as opposed to the more involved proofs needed to show that a differentially regulated gene actually results in altered protein expression). Furthermore, unlike the technique of cDNA library

screening 2D-PAGE can provide information regarding the stress-induced changes mediated through post-translation modifications. However, disadvantages of the method can include the fact that some proteins do not enter the gel under the running conditions used, others are masked by high abundance proteins, and the prospect of missing changes in many low abundance proteins. Nonetheless the method was successful in identifying a freeze-responsive protein that was not found by gene screening methods. Approximately 200 different protein spots were visible on the frog heart gels following silver staining. One of these was a protein of ~20 kDa molecular weight with a pI between 4 and 4.5 that increased dramatically following exposure to freezing. The up-regulated protein was assessed by the technique of mass spectroscopy-microsequencing and provided two amino acid strings for protein identification. A BLAST database search revealed that the primary sequence of *X. laevis* MLC2 had both of these amino acid strings. Further analysis of the *X. laevis* MLC2 protein confirmed that the size and pI matched with what was experimentally determined for the *R. sylvatica* protein. Further characterization of MLC2 will be reported in Chapter 4.

3.4.4 GENE EXPRESSION AND FREEZING SURVIVAL

From the current investigations 8 new genes and proteins with increased expression levels following exposure to freezing conditions have been added to the previously existing list of freeze up-regulated genes and proteins. Additional research and analysis will be necessary to fully understand the importance of these genes to freezing survival, and to determine how they fit into a larger picture of environmental stress survival. The first step towards understanding the possible roles of each in freezing

involves understanding the protein structure and function and an evaluation of how each might contribute to cell survival.

3.4.4A *HSPC070*, A ZINC FINGER PROTEIN

One of the freeze-induced genes pulled from the *R. sylvatica* heart library is a previously identified gene of unknown function, *hspc070* (Zhang *et al.*, 2000). Northern blot analysis of the transcript indicated that the gene was up-regulated ~5-fold in the heart following 24 h of freezing exposure. This gene was originally sequenced from a human hematopoietic stem/progenitor cell line, and has since been cloned from mouse (Zhang *et al.*, 2000). To the best of our knowledge this is the first study demonstrating a change in *hspc070* transcript expression as a response to stress or physiological state. Although the cellular function of HSPC070 has yet to be determined, the protein sequence may provide some indication of a role. Analysis of the HSPC070 protein recognized that the protein contains up to 5 zinc binding elements: two in the Zinc RING motif and three standard (Cys₄Zn) zinc finger motifs. The zinc finger was first recognized 16 years ago as a repeated zinc-binding motif, containing conserved cysteine and histidine ligands (Miller *et al.*, 1985). Zinc finger motifs are now known to be very common, with a recent estimate suggesting that 1% of all mammalian genes code for zinc finger proteins (Mackay and Crossley, 1998). However, it is worth noting that only a small fraction of all zinc finger proteins contain the Zinc RING finger motif, a feature that may eventually help to determine the function of the protein. This motif is defined by the pattern: C - X₂ - C - X₍₉₋₃₉₎ - C - X₍₁₋₃₎ - H - X₍₂₋₃₎ - C - X₍₂₋₃₎ - C - X₍₄₋₄₈₎ - C - X₍₂₎ - C, where X is any amino acid; typically two Zn atoms bind per motif (Borden and Freemont, 1996).

The zinc finger motif is an unusually small, self-folding domain in which Zn is a crucial component of its tertiary structure. All zinc finger motifs bind 1 atom of Zn in a tetrahedral array to yield a finger-like projection, which interacts with nucleotides in the major groove of DNA. Zinc fingers have well-recognized DNA-binding motifs and are an integral component of many transcription factors. Sequential arrays of zinc fingers arranged in different combinations can recognize a huge range of DNA sequences. More recently zinc fingers have also been identified as mediators of protein-protein interactions, helping to explain the incredible number of zinc finger containing proteins present in the cell (Mackay and Crossley, 1998). As a result, the function of each zinc finger protein is impossible to predict solely based on sequence data. Future research will be required to determine whether *hspc070* binds DNA, protein or both, the function of the protein in freeze survival, and the mechanisms leading to gene up-regulation.

3.4.4B MITOCHONDRIA ASSOCIATED GENES

ATP is synthesized from ADP and inorganic phosphate by oxidative phosphorylation in mitochondria. Two of the proteins essential for supplying substrates to the mitochondrial matrix for oxidative phosphorylation are the ADP/ATP translocase (AAT) and mitochondrial phosphate carrier proteins (MPCP) (Meijer and van Dam, 1974; La Noue and Schoolwerth, 1979). The phosphate carrier mediates the uptake of phosphate into the mitochondrial matrix, either by proton co-transport or in exchange for hydroxyl ions, whereas AAT exchanges external ADP for internally-generated ATP (Klingenberg, 1985). These two proteins, along with other mitochondrial proteins such as the citrate and oxoglutarate transporters, share a domain (often 2 or 3 copies) called the

mitochondrial energy transfer protein signature (Walker and Runswick, 1993). The homology between these carriers has permitted the development of a carrier family concept, with all of the genes containing the domain believed to have originated from a common gene (Aquila *et al.*, 1987; Runswick *et al.*, 1987).

As mentioned in Chapter 1, the ADP/ATP translocase is known to be up-regulated at both the gene and protein levels following freezing exposure in wood frog tissues (Cai *et al.*, 1997). Comparing the responses of mRNA transcript levels to freezing with the responses to the related stresses of anoxia and dehydration resulted in the suggestion that AAT levels responded to the hypoxic/ischemic conditions that are experienced during and following the onset of freezing. The disruption of oxygen delivery to organs as blood freezes is expected to alter the desired mitochondrial concentrations of various substrates including protons, phosphates, ADP and ATP and could result in a coordinated up-regulation of some or all mitochondrial carrier proteins to provide some stabilization of substrate and ion distribution. The discovery here of a second member of the mitochondrial carrier protein family as being responsive to freeze exposure provides further support for such an idea. Further investigation will be required to determine whether *mpcp* transcript levels are similarly responsive to a hypoxia/anoxia trigger.

The second gene associated with mitochondria that was identified as freeze up-regulated was the mitochondria ribosomal RNA species 16S. This gene is one of a small number that are encoded on the small circular strand of DNA located in the mitochondria. The vertebrate mitochondrial DNA (mtDNA) is a small extrachromosomal genome of approximately 17 kb in size (Boore, 1999). In general, all animal mitochondrial genomes

contain the same 37 genes: two for ribosomal RNA, 13 for proteins and 22 for transfer RNAs. The products of these genes, along with RNAs and proteins imported from the cytoplasm, allow the mitochondria to replicate their mtDNA, transcribe RNA, process mRNA, and translate the mRNA into protein (Boore, 1999).

Considering the findings that the mitochondrial AAT and MPCP are up-regulated during freezing exposure it is not surprising that the wood frog might also respond with the induction of mitochondrial 16S rRNA. A loss or reduction in ATP supply due to the freeze-induced ischemia, could be interpreted by the cell as a loss in the total number of mitochondria, or in the overall mitochondrial activity, and the cells may be attempting to compensate. The heart of wood frogs may react to this loss of ATP by increasing mitochondrial ribosomal RNA and encouraging increased synthesis of mitochondrial proteins, in the hope of creating a more efficient system for ATP synthesis. Further studies will be required to determine how the remaining genes and proteins found in and around the mitochondria (both nuclear and mitochondrial encoded) respond to freezing exposure in the various organs of the wood frog.

FIGURE 3.1

R. sylvatica heart cDNA library construction.

A. Non-denaturing acrylamide gel electrophoresis of selected fractions from Sepharose Cl-2B size fractionation of double stranded cDNA following second strand synthesis and adaptor ligation. Lane 6, radiolabeled 1 kb markers. Lane 5, fraction 9. Lane 4, fraction 7. Lane 3, fraction 5. Lane 2, fraction 3. Lane 1, fraction 1. The cDNA in each lane was visualized by exposing the dried acrylamide gel to X-ray film.

B. Fractions 1 through 3 eluted from Sepharose Cl-2B were pooled, extracted, precipitated and resuspended. A 0.5 μ l aliquot of the pooled cDNA was spotted on an ethidium bromide agarose plate (marker with an arrow). Solutions were prepared with 1.0, 0.5, 0.25, 0.125, and 0.0625 μ g/ μ l of DNA and 1 μ l aliquots of each were used to create a standard curve.

A

B

FIGURE 3.2

Representative primary differential screen of the *Rana sylvatica* heart cDNA library.

A plate of approximately 10,000 phage expressing frog cDNA was lifted onto two membranes, denatured, and cross-linked. The paired membranes were hybridized with radiolabeled cDNA prepared from mRNA isolated from control (A) and frozen (B) hearts. Freeze up-regulated clones (see arrow for example) were identified and the corresponding phage was removed from the plate and the plasmid excised.

A

B

FIGURE 3.3

Representative 1% TAE agarose gel stained with ethidium bromide.

Lane 1, 1 kb molecular weight standards. Lanes 2-7, potential upregulated clones isolated from the *R. sylvatica* cDNA library. Plasmids were isolated from bacterial stocks, and digested with EcoR I and Xho I. The linearized Uni-ZAP CMV XR plasmids are visible in each lane at 3000 base pairs, and the digested cDNA clones range from 500 to 2500 base pairs.

FIGURE 3.4

Transcript expression analysis of select clones excised from the differentially screened heart and liver cDNA libraries.

Northern blots were screened with ^{32}P -labeled probes created using the random primer method. Northern blots were scanned and band intensities were normalized to the corresponding rRNA band intensities obtained from the photographed formaldehyde gel. The control values were set to 100 and the band intensities for the 24 hour frozen samples were expressed relative to their control. The remain relative transcript levels in the frozen sample and the standard error of the mean associated with the fold increase were calculated from three independent trials. The results of the densitometry data have been inserted directly into the text.

- A. Northern blots prepared with total RNA isolated from hearts of control and 24 hour frozen *R. sylvatica*; each lane contains a total of 16 μg of RNA.
- B. Northern blots prepared with total RNA from liver of control and 24 hour frozen *R. sylvatica*; each lane contains a total of 16 μg of RNA.

A. HEART

B. LIVER

FIGURE 3.5

Nucleotide and deduced amino acid sequence of the *R. sylvatica* *hspc070* or *makorin2* gene (clone B2).

Nucleotides and amino acids are numbered on the left. A single open reading frame is predicted to encode a polypeptide with 408 residues. The stop codon (TGA) and polyadenylation signal (AATAAA) have been placed in bold. The presence of 4 putative zinc finger motifs have been identified and are underlined (type C3HC4 and type C-X8-C-X5-C-X3-H, and similar). The stretch of amino acids matching one of the two motifs that make up the cold shock DNA binding (BLOCKS accession number IPB002059) has been highlighted.

1 CGTGCAGATATTTTCATACATGCGCGTGTGCCGAGAAGGAAAATCGCTGTTTATTTTCACAT
1 R A D I S Y M R V C R E G N R C L F S H
61 GATTTAGCCACGAGTAAACCATCCACTGTTTGTCTGATTCTATCAGAAGGGACAGTGCGCC
21 D L A T S K P S T V C R F Y Q K G Q C A
121 TATGGGTCTCGCTGTAGGTATGATCATGTGAAAACCTTCGGCTGCCACAGTTTACATTCCT
41 Y G S R C R Y D H V K P S A A T V Y I P
181 CAAAATATCCCGCCTGCCACTCAAGAAACACCTTCACCTGACCCAGAGGTCCCACCAGTT
61 Q N I P P A T Q E T P S P D P E V P P V
241 GTCACGAAAACCTACCCATCAAAGGCGTGAGAAGAAGAGCCTTGTACTCAGAGATCGTGAT
81 V T K T T H Q R R E K K S L V L R D R D
301 TTATCGGGATTGGCAGAAGATAAAAAATCGAATACATCCTGCTTCTAACCAAAACTACAAT
101 L S G L A E D K N R I H P A S N Q N Y N
361 GGTGATACTCAAGTGACCGATGATGTGTTGAAACCGCACTCTTACCTGGAAGCCATCTGT
121 G D T Q V T D D V L K P H S Y L E A I C
421 ACAGGACTGGAAGAAGTTGAGGCGGCCCTCACCATTTTCTGGGGACCCCCAGCAGCTTTGC
141 T G L E E V E A A S P F P G D P Q Q L C
481 CCCTATGCAGCTGCAGGGGAATGTCACTATGGGGAAAAGCTGTGTCTATCTCCATGGAGAT
161 P Y A A A G E C H Y G E S C V Y L H G D
541 GTTTGTGAGATCTGCAACTTACAAGTGCTTCACCCCTATGACCAGGAACAGAGGAAGTCG
181 V C E I C N L Q V L H P Y D Q E Q R K S
601 CATGAAAAGTTATGTATGGACACGTTTGAATTGGAGATGGAAAAGGCCCTTTGCCTTCCAG
201 H E K L C M D T F E L E M E K A F A F Q
661 GCCAGCCAAGACAAGGTGTGTAGTATCTGCATGGAAAGTCGTGTATGAAAAGCCTTCACCA
221 A S Q D K V C S I C M E V V Y E K P S P
721 TCGGAGCGGAGATTTGGTATCCTGTCCAACCTGCAACCACACGTACTGCCTCTCCTGTATC
241 S E R R F G I L S N C N H T Y C L S C I
781 AGGCAGTGGAGATGTGCCAGACAGTTTGTGAAATCCTGTGCATAAAAAGCATGTCCAGAATGC
261 R Q W R C A R Q F E N P V I K A C P E C
841 CGGGTGATATCAGAGTTTGTATTTCCAGTGTTTACTGGGTGGAAGATCAAAGCAAGAAG
281 R V I S E F V I P S V Y W V E D Q S K K
901 AATGAACTGATTGATGCGTTTAAAGCAGGGAATGGGTAAGAAGGCCTGTAAATACTTTGAT
301 N E L I D A F K Q G M G K K A C K Y F D
961 CAGGGACGTGGCACCTGCCCTTTTGGTGGAAAAGTGCTTTACCTGCATGCATACCCAGAT
321 Q G R G T C P F G G K C L Y L H A Y P D
1021 GGAACCATAGCAGACCCAGAGAAAACCCCGAAACAACCTCGGATCCGAAGGCACAGTACGG
341 G T I A D P E K P R K Q L G S E G T V R
1081 TTCTTTAATGCGGTTAGACTGTGGGATTTTCATTGAAGATCGAGAGCACAGAAGTATTCCT
361 F F N A V R L W D F I E D R E H R S I P
1141 ACCACTGAGGACGATGTAGCCGAACCTGGAGACCTCTTCATGCACCTTGTCTGGAGCAGAT
381 T T E D D V A E L G D L F M H L S G A D
1201 CAGCAAGAAAACCCCTCTTCCAACCTGAATATTTATTAGAAAATTGTAGTATAGAAAACCTGCA
401 Q Q E T P S S N *
1261 TGTATGGGTTAATGAGATGGCAGAACCAAGTGCCTTTCCACTAAGAAAATAAAAAACAAC
1321 ACTATTGGCTAAAAAAAAAAAAAAAAAAAA

FIGURE 3.6

Alignment of the amino acid sequences of the HSPC070/MAKORIN2 homologues from 3 species.

The putative amino acid sequence of wood frog HSPC070 is compared with the human and mouse sequences (Genbank accession numbers AF161555 and AF277171, respectively). For convenience only those amino acids differing from the wood frog sequence are indicated; dashes (-) represent amino acids in the mouse or human sequence that are identical with the wood frog sequence. Periods are present in the alignment to indicate when an amino acid is not present in the coding region of one of the species.

Wood frog	RADISMRVCREG NRCLF SHDLATSKPSTVCRFYQKGQCAYGSR	
Human	MSTKQVTCRYFMHG-----SQ-----N-----I-KY----Y----A-	
Mouse	MSTKQVTCRYFMHG-----SQ-----N-----I-KY----Y----A-	50
Wood frog	CRYDHVKPSAATVYIPQNIPPATQET.....PSPDPEVPPVVTKTTHQR	
Human	-----T--P--AGGAVGPA-NPSPSSGLHSPH----IATSVMRTHSNEPG	
Mouse	-----T--P--AGGAVGPA-NPSPSSGLHSPH----IATSVMRTHSNEPG	100
Wood frog	.REKKSLVLRDRDLSGLAEDKNRIHPASNQNYNGDTQVTDDVLKPHSYLE	
Human	K----T-----N-T-----TPPSKVN-PGGCS-P-TSPEM.-----D	
Mouse	K----T-----N-T-----TPPSKVN-PGGCS-P-TSPEM.-----D	149
Wood frog	AICTGLEEEVEAASPFPGDPQQLCPYAAAGECHYGESCVYLHGDVCEICNL	
Human	--R--DDL--S-SYSNE--.-----RL-DA-----M----R-	
Mouse	--R--DDL--S-SYSNE--.-----RL-DA-----M----R-	198
Wood frog	QVLHPYDQEQRKSHEKLCMDTFELEM EKAFQASQDKVCSICMEVVYEK	
Human	-----F-P----A--M--S--H-----IL--	
Mouse	-----F-P----A--M--S--H-----IL--	248
Wood frog	PSPSERRFGILSNCNHTYCLSCIRQWRCARQFENPVIKACPECRVISEFV	
Human	A-A-----S-----K-----I--S-----	
Mouse	ASA-----S-----K-----I--S-----	298
Wood frog	IPSVYWVEDQSKKNELIDAFKQGMGKKACKYFDQGRGTCPPFGGKCLYLHA	
Human	-----N-----E-----E--K-----S----R--	
Mouse	-----N-----E-----E--K-----S----R--	348
Wood frog	YPDGTIADPEKPRKQLGSEGTVRFFNAVRLWDFIEDREHRSIPTTED.DV	
Human	----RL-E-----S-----S-----N--T-QV-S-D-V--	
Mouse	----RL-E-----S-----S-----N--T-QV-S-D-V--	398
Wood frog	AELGDLFMHL SGADQQETPSSN	
Human	T-----VESSEP	
Mouse	T-----VESSEP	416

FIGURE 3.7

Nucleotide and deduced amino acid sequence of the *R. sylvatica* mitochondrial phosphate carrier protein (liver clone fl8).

Nucleotides and amino acids are numbered on the left. A single open reading frame has been predicted to encode a polypeptide with 362 residues. The amino acids are numbered starting from where the first residue in the mature peptide (following cleavage) should begin, as identified from mammalian homologues. Upper case amino acids represent residues in the mature protein; lower case letters indicate amino acids that contribute to the cleaved signal sequence. The stop codon (TAA) and polyadenylation signal (AATAAA) have been underlined. Two protein mitochondrial energy transfer signature patterns were recognized and have been underlined.

1 TAGCTGCCGAAATGTACTCCACCGTTGCGCACCTAGCTCGGGCGAATCCGTTCAATGCAC
m y s t v a h l a r a n p f n a
61 CACATTTCCAGGTGGGTCAGGAATGTGCGACTTTAAGGAAGAAAAACACTTCAGAGATCC
p h f q v g q e c a t l r k k n t s e i
121 AACCTGTCCGACGCCTTGCTGCTGCAGCTACTGCTGCTGAAGGCGACTACAGTTGTGAAT
1 q p v r r l a a a A T A A E G D Y S C E
181 ATGGCTCAACAAAATTTTATGCTTTTTGTGGCTTTGGTGGCATCCTCAGTTGTGGTATAA
12 Y G S T K F Y A F C G F G G I L S C G I
241 CTCACACTGCAGTTGTACCTTTGGATTTGGTGAAATGTCGTATGCAGGTGGATCCCCAAA
32 T H T A V V P L D L V K C R M Q V D P Q
301 AGTACAAGAGCATTTTCAGTGGGTTTTCTGTAACCTCTTAAGGAAGATGGTGTCCGTGGAC
52 K Y K S I F S G F S V T L K E D G V R G
361 TTGCCAAGGGATGGGCCCCAACATTTATTGGGTATTCCATGCAAGGATTGTGTAAATTTG
72 L A K G W A P T F I G Y S M Q G L C K F
421 GATTTTATGAAGTCTTCAAGGTCTTGTATTCTAACCTTCTAGGAGAGGAAAAATACCTATT
92 G F Y E V F K V L Y S N L L G E E N T Y
481 TGTGGCGCACATCTCTGTACCTGGCTGCATCTGCTAGTGCCGAGTTCTTTGCAGATATTG
112 L W R T S L Y L A A S A S A E F F A D I
541 CACTGGCTCCAATGGAAGCTGCAAAAGTCCGTATTCAAACACAGCCGGGATATGCAAATA
132 A L A P M E A A K V R I Q T Q P G Y A N
601 CTTTGGCACAAGCGGCACCCAAGATGTATGCTGAAGAAGGAATCTGGGCGTTCTACAAAG
152 T L R Q A A P K M Y A E E G I W A F Y K
661 GAGTTGCTCCTCTCTGGATGAGGCAAATTCCTACACCATGATGAAGTTTGCTTGCTTTG
172 G V A P L W M R Q I P Y T M M K F A C F
721 AACGTACAGTTGAAGCACTGTACAAGCATGTTGTACCCAAGCCACGAAGTGAATGTTCAA
192 E R T V E A L Y K H V V P K P R S E C S
781 AGTCCGAACAGTTGGTTGTGACATTTGTTGCTGGCTATATTGCTGGTGTGTTCTGTGCCA
212 K S E Q L V V T F V A G Y I A G V F C A
841 TCGTCTCCCATCCTGCTGATTCAAGTGGTGTCTGTGTTGAACAAAGAAAAGGGAAGCACTG
232 I V S H P A D S V V S V L N K E K G S T
901 CTACGCAAGTCCTTAAACGACTTGGACCAAAAGGAGTCTGGAAGGGGCTGACGGCCSGTA
252 A T Q V L K R L G P K G V W K G L T A X
961 TCATCATGATTGGTACCTTGACTGCTCTGCAGTGGTTCATCTATGACTCTGTGAAGGTCT
272 I I M I G T L T A L Q W F I Y D S V K V
1021 ACTTTAGACTTCCTCGTCCCTCCCCCTGAGATGCCMGAGTCTCTGAAGAAGAAGCTTG
292 Y F R L P R P P P P E M P E S L K K K L
1081 GCTTGACTGAGTAAACATATGGACTTGAATAGTGAAAGTTCCAATGGCACTTCAAACCTGA
312 G L T E *

1141 GAAGTTTTTATAAAATGCAATCGTGTAGGACTTGCCATTTTCTCAGATGTAATTATTGGTT
1201 GTATTCACCTCTGCTTGGTACACCACAACCTGTAATTTGGTTTTTACCACTGTACCTGTCTGT
1261 CTAATAAAACAGAAGACCCCGCGGATG

FIGURE 3.8

Alignment of the amino acid sequence of the mature mitochondrial phosphate carrier protein from 4 species.

The putative amino acid sequence of the wood frog protein is compared with rat, human and *C. elegans* sequences (Genbank accession numbers M23984, BC000998, and X76113, respectively). For convenience only those amino acids differing from the wood frog sequence are indicated; dashes (-) represent amino acids in the other sequences that are shared with the wood frog. Periods are present in the alignment to indicate amino acids that are not present in the coding region of one species.

Wood Frog	ATAAEGDYSCEYGSTKFYAFCGFGGILSCGITHTAVVPLD	40
Rat	..-V-E---D---GR-FIL--L---I---T---L---	
Human	..-V-.E---F--A-Y--L-----V---L-----	
<i>C.elegans</i>	..-VSAPGQV-F--G-Y--Y-AL--V-----I----	
Wood Frog	LVKCRMQVDPQKYKSIFSGFSVTLKEDGVRGLAKGWAPTF	80
Rat	-----F-----	
Human	-----G--N-----	
<i>C.elegans</i>	-----I--N-E--TG-AT--RT-IA-E-A-A-V-----L	
Wood Frog	IGYSMQGLCKFGFYEVFKVLYSNLLGEENTYLWRTSLYLA	120
Rat	----L-----M-----A-----	
Human	L-----M-----	
<i>C.elegans</i>	L---A---G-----I--NV-ADM-----A--Y-----	
Wood Frog	ASASAEFFADIALAPMEAANKVRIQTQPGYANTLRQAAPKM	160
Rat	-----D-----	
Human	-----D-----	
<i>C.elegans</i>	-----L-----T-----S--APP--GC--MI	
Wood Frog	YAEEGIWAFYKGVAPLWMRQIPYTMMKFACFERTVEALYK	200
Rat	-K---LK-----	
Human	-K---LK-----	
<i>C.elegans</i>	-KA--LTG---LP-----K-----Q	
Wood Frog	HVVPKPRSECSKSEQLVVTFVAGYIAGVFCAIVSHPADSV	240
Rat	F-----P-----	
Human	F-----P-----	
<i>C.elegans</i>	Y-----A---A-----T-	
Wood Frog	VSVLNKEKGSTATQVLKRLGPKGVWKGLTAXIIMIGTLTA	280
Rat	-----S-SE-----FR-----F-R-----	
Human	-----S-SL-----F-----F-R-----	
<i>C.elegans</i>	--K--QDSQA-AGGI--K--FAG----LVPR-----	
Wood Frog	LQWFIYDSVKVYFRLPRPPPEMPESLKKKLGLT	314
Rat	-----	
Human	-----	
<i>C.elegans</i>	-----ALN-----A---A--AAQ	

FIGURE 3.9

R. sylvatica 16 S rRNA nucleotide sequence and homology tree.

- A. Nucleotide sequence for *R. sylvatica* heart clone D1 identified as mitochondrial 16 S ribosomal RNA.
- B. A homology tree prepared with DNAMAN following alignment of the wood frog 16S cDNA fragment with corresponding leopard frog, clawed frog (*Xenopus laevis*), catfish, chicken, rat and human sequences.

A

```

1      CGAGGGCGCT TCAGACAAAG TACCGCAAGG GAAAGATGAA
41     ATAGAAATGA AATAACCTTA AAGCCTTAAA TAGCAGAGAT
81     ATTGCCTCGT ACCTTTTGCA TCATGGTCTA GCTAGTCTAC
121    CCAAGCAAAA CGAAACTTTT AGTTTGACAT CCCGAAACTA
161    AGTGAGCTAC TTCAAAACAG CCCTAGGGGC CAACCCGTCT
201    CTGTTGCAAA AGAGTGGGAA GATTTTAAAG TAGAGGTGAT
241    AAGCCTACCG AACTTAGAGA TAGCTGGTTG TTCAGGAAAA
281    GAGTTTTAGC TCTACCTTAA GCTTCCCCAT TTTACCAAAA
321    ATGCCTCAAA GCTTAAGAGC TATTCAAATA AGGCACAGCT
361    TATTTGAAAT AGGGTACAAC CTCCAACATC GGGTAAATTA
401    TAGTAATTAA AATGAAGTGG GCCTAAAAGC AGCCATCTTT
441    AAAAAAGCGT TAAAGCTTAA TTATTATTAC TAAAAAATTT
481    CTTAAATTCT AATTAACCCC TCATTTATAC TGAAGTGCTT
521    TATATTTTAT AAAAGTAATA ATGCTAGAAC TAGTAACAAG
561    AAATCGCTTT TCTCCTAAAT GTAAGCATAA ACCAAAATGG
601    ACCACCTATT GGTAATTAAAC GTAAATGCAA AAACATAAAT
641    AACATAACTA GAAAAACCTA TTATTATTAA CGTTAACCTT
681    AACTAGAAC  ATTATAGGAA AGATT

```

B

FIGURE 3.10

Representative 2D-PAGE of proteins extracted from *R. sylvatica* heart tissue.

Total soluble protein was isolated from control (A), 4 h frozen (B), and 24 h frozen (C) *R. sylvatica* heart tissue. For each gel 25 µg of total soluble protein was isofocused in the first dimension using carrier ampholytes in the pH 4-6 range, and then separated in the second dimension on a 15% SDS denaturing polyacrylamide gel.

TABLE 3.1

Summary of the *R. sylvatica* heart (A) and liver (B) cDNA clones identified by differential screening of the cDNA library

The table identifies each of the clones, provides the size of the cDNA clone, the transcript size as determined from northern analysis, the longest open reading frame predicted from the nucleotide sequence, the open reading frame suggested in literature, and confirms whether the entire open reading frame is present.

A**Heart Clones**

IDENTITY (BLAST)	Clone Size	Transcript Size	ORF Size	Expected ORF	Start Codon
<i>hsp27</i> (B4)	800 bp	900 bp	216 aa	Chicken 193 Human 205 Canine 209	Yes
<i>eflγ</i> (H25)	1081 bp	1600 bp	308 aa	Xenopus 436 Human 437	No
<i>αnac</i> (BA)	798 bp	900 bp	214 aa	Human 215	Yes
16 S (D1)	700 bp	1600 bp	----	----	----
<i>hspc070</i> (B2)	1362 bp	1800 bp	402 aa	Human 416 Mouse 416	No

B**Liver Clones**

IDENTITY (BLAST)	Clone Size	Transcript Size	ORF Size	Expected ORF	Start Codon
<i>mpcp</i> (fl8)	1300 bp	1400 bp	359	Mouse 357 Human 361	Yes
<i>ddx8</i> (novel)	2100 bp	~8000 bp	522	Unknown	No
<i>li16</i> (novel)	300 bp	550 bp	72	Unknown	No

TABLE 3.2

Comparison of the unknown *R. sylvatica* protein identified through 2D-PAGE analysis with *X. laevis* MLC2. (A) Table identifying characteristics shared between the *X. laevis* MLC2 protein and the upregulated *R. sylvatica* heart protein. Both wood frog peptide fragments were determined through a BLAST analysis to be present in the African clawed frog protein. As well, the size and pI of the wood frog protein matched that predicted by DNAMAN for the MLC2 protein. (B) The amino acid sequence for the *X. laevis* MLC2 protein. The amino acid sequences identified for the *R. sylvatica* protein are underlined in the sequence.

A

	<i>R. sylvatica</i> (unknown)	<i>X. laevis</i> (MLC2)
Peptide Fragment 1	ILDPNAT	ILDPNAT
Peptide Fragment 2	LNVNE	MNVNE
Isoelectric Points	4 – 4.5	4.38
Molecular Weight	18 – 22 kDa	19 kDa

B

1	MASRKAAGKAAAKRAQRGSSN
21	VFSMFESQIQEFKEAFSCID
41	QNRDGIISKPDCLKETYMQLGK
61	<u>MNVNE</u> DELDEMLKEGKGPINF
81	TVFLSLFGEKLNKTDPEDSIL
101	SAFK <u>ILDPNAT</u> GNINKDELKL
121	LLMTQADKFSDEEVDQMFAVT
141	PIDVAGNIDYKSLCYIITHGD
161	EKEDS

CHAPTER 4

EVIDENCE FOR INITIATION OF THE HYPERTROPHIC GENE RESPONSE IN THE HEART OF THE FREEZE TOLERANT WOOD FROG

4.1 INTRODUCTION

4.1.1 AIM OF CHAPTER

In chapter 3 the technique of two-dimensional electrophoresis was used to identify changes in the protein profile of the wood frog heart following freezing exposure. This analysis identified an apparent increase in the overall concentration of a 20 kDa protein at 4 and 24 h post-freeze that was putatively identified as the myosin light chain 2 subunit (MLC2) of the myosin motor.

As discussed in chapter 1 the wood frog heart plays an important role in the distribution of cryoprotectant to the organs following freezing-induced glycogenolysis. However, as freezing progresses the work demands on the heart increase due to (i) the rise in blood viscosity associated with the drop in body temperature and the withdrawal of water from the blood into extra-organ ice crystals, and (ii) the difficulties associated with pumping blood through a vasculature that is freezing. These changes give rise to an elevated total peripheral resistance and a rise in blood pressure that increases the workload of the heart. As a result, a number of compensatory adjustments (physiological, biochemical, molecular) by the heart and circulatory system would be expected as the heart attempts to maintain cardiac output and perfuse the tissues for as long as possible.

Myosin restructuring is a commonly observed response to a variety of intrinsic and extrinsic stimuli that stress both cardiac and skeletal muscles. Both subunit concentrations and isoform ratios can be altered. This chapter will begin with a review of the contractile machinery, including the cellular role of MLC2, and the various external stresses, triggers and disease conditions that have been linked to changes in normal cardiac myosin protein expression patterns. Subsequently, the possibility that MLC2 up-

regulation is part of a well-described hypertrophic gene response observed in mammalian systems is investigated. The study uses PCR amplification with primers designed to well-conserved regions of the *mlc2* and atrial natriuretic peptide (*anp*) to retrieve gene fragments. Northern blot analysis is used to investigate *mlc2* gene expression levels following freezing exposure, and analysis of *anp* transcripts tests for the presence of the hypertrophic gene response. Additional studies determine whether the up-regulation of *mlc2* transcript levels can be attributed to triggers derived from either the dehydration or anoxia components of freezing stress.

4.1.2 THE MYOSIN MOTOR

The delivery of blood to the systemic and pulmonary circulations through the contraction of cardiac muscle results from the cyclic interaction of the actin and myosin proteins of the thick and thin filaments. One of the two main components of the contractile machinery is the myosin muscle motor; a hexameric protein (520 kDa) consisting of two heavy chains, approximately 220 kDa each, and two pairs of light chains, each approximately 20 kDa (Schaub *et al.*, 1998). The myosin heavy chain (MHC) subunits can be artificially divided into two domains based on the different roles MHC has in contraction: (i) a globular head domain containing the actin- and ATP-binding sites, and (ii) a tail domain that interacts with the tail of a second MHC to produce an α -helical chain with filament forming properties. Associated with the neck region of each MHC are two types of myosin light chains, MLC1 and MLC2, that can be discriminated based on their structure, function, and location within the myosin motor. The first of the light chains, MLC1, was originally termed the essential light chain

because it was incorrectly believed to carry out ATP hydrolysis; however, it is now known to act as a MHC/actin tether (Wagner and Giniger 1981; Sivaramakrishnan and Burke, 1982; Morano, 1999). The second type of light chain, MLC2, is also known as the regulatory light chain because smooth muscle cell contractile activity (tone) is dependent on the phosphorylation state of the protein (Adelstein *et al.*, 1980). It is now recognized that in skeletal and cardiac muscles the regulatory light chains function to slow the kinetics of tension development between the myosin motor and the actin filament – an ability that is regulated by reversible protein phosphorylation (Sweeney and Stull, 1990; Metzger *et al.*, 1993). Figure 4.1 shows a diagram of the basic scaffolding of the myosin motor. This demonstrates how the myosin crossbridge is formed by the protrusion of the MHC and MLC subunits from the myosin filament. The crossbridge provokes movement by repetitive interaction with the actin filament. The process of crossbridge cycling that leads to the movement of the myosin filament along the length of the actin filament is regulated by cytosolic calcium concentration, ATP availability, and a number of regulatory proteins that are found in association with the thick and thin filaments.

4.1.3 CHANGES TO THE CONTRACTILE PROTEIN EXPRESSION PATTERN

The described myosin motor represents the basic frame onto which adjustments and substitutions are made in different cell and tissue types. Through the expression of specific myosin isoforms, and the ability to reversibly control these proteins via phosphorylation-dephosphorylation, tissues can produce motors with a wide range of characteristics, including differing sensitivities to Ca^{2+} or acidosis, and modified shortening velocity or force production (Schwartz *et al.*, 1981; Metzger *et al.*, 1993; Buck

et al., 1999). As well, the contractility of a cell or tissue can be further modified by increasing or decreasing the overall number of myosin motors, and therefore, the number of crossbridges available for cycling.

However, the existence of a number of genes for each of the subunits does not simply serve as a “store” from which cells can randomly pick and choose. In fact each tissue type has a characteristic myosin isoform expression pattern along with a normal subunit concentration range, both of which are important for proper physiological functioning. The importance of the specific contractile characteristics obtained through selective isoform expression is best illustrated by the strict isoform expression pattern followed throughout development, and the tightly regulated production of specific isoform combinations by the different types of adult contractile tissues (Schuab *et al.*, 1998). Deviations from the normal expression pattern (isoform or concentration level) have been recognized. These can be necessary compensations for dealing with a stress or change in environment, but also occur in association with and as possible causes of disease, including: familial hypertrophic cardiomyopathies (Haase *et al.*, 2002); idiopathic dilated cardiomyopathy (Corbett *et al.*, 1998; Lim *et al.*, 2001); disuse atrophy (Cros *et al.*, 2001); pulmonary hypertension (Morano *et al.*, 1998); pressure overload (Buttrick *et al.*, 1986), infarct (Gidh-Jain *et al.*, 1998); and aging (van der Velden *et al.*, 1998).

Of the reported examples of abnormal or altered contractile protein expression patterns, a number involve animal models that have been exposed to environmental or physiological stresses analogous to those experienced by the wood frog during freezing. For example, carp, when acclimated to water at a warm temperature and then transferred

to a colder environment have been reported to change their myosin alkali light chains subunit isoform ratio (Hirayama *et al.*, 1997). Increases in specific contractile protein transcript levels have also been observed in a number of animal models following periods of ischemia (Lyons *et al.*, 1990; Lyn *et al.*, 2000). Changes in contractile protein expression have previously been reported by our lab; golden-mantled ground squirrels up-regulate the ventricular isoform of the essential light chain 1 during winter hibernation (Fahlman *et al.*, 2000). Another hibernator, the European hamster, shows a change in the expression patterns of the heavy chain isoforms in heart; the heavy chain β isozyme dominated in summer animals (79% of total) and winter-active hamsters at 22°C but during torpor activity of the α isozyme rose to 53% of total.

Of the disease conditions and animal model systems that show myosin remodeling in response to stress, we believe the hemodynamic/ pressure overload and cardiac hypertrophy models to be the most relevant to freeze tolerance in the wood frog. In fact, these two situations are related because cardiac hypertrophy is a well-established adaptational response to hemodynamic and pressure overload secondary to vascular abnormalities, valvular disease and hypertension (Frohlich *et al.*, 1992). With hypertrophy the myocardium adapts to long-term elevations in hemodynamic load, such as hypertension, with the enlargement of individual ventricular myocytes. Both the acute and chronic stages have been linked with the expression of a specific or characteristic subset of genes, including *mlc2*, in what has been termed the hypertrophic gene response (Bouvagnet *et al.*, 1985; Buttrick *et al.*, 1986; Cummins, 1984). The genes activated during the hypertrophic response include some immediate-early genes (Hengstenberg and Maisch, 1993; Slinker *et al.*, 1996; Schnabel *et al.*, 2000), genes coding for contractile

proteins, and the gene for the cardiac hormone, ANP (Izumo *et al.*, 1987; Mercadier *et al.*, 1989; Chein *et al.*, 1991).

If the accepted model of wood frog freezing is accurate, and the associated physiological stresses are correct, the wood frog heart would be expected to experience a progressive rise in pressure, and possibly hemodynamic overload during the freezing process. The investigations in this chapter were designed to (i) investigate whether MLC2 up-regulation by the wood frog during the freezing is an isolated event or is part of a hypertrophic gene response, and (ii) investigate various triggers (e.g. anoxia, dehydration) to determine whether they might be responsible for MLC2 over-expression during freezing.

4.2 MATERIAL AND METHODS

4.2.1 PRIMER DESIGN

Primer design was carried out using Primer Designer (windows version 3.0). Primer Designer selects primers based on a number of characteristics, including melting temperature and GC base pair content, and typically eliminates primers with runs of a single base greater than 3 and those capable of forming dimers or hairpins. The primers were purchased from Bio S&T (Montreal) and diluted to a concentration of 30 μ M. Each possible forward and reverse primer combination (4 combinations in total for each gene) was tested to further increase the chance of gene amplification.

4.2.2 ANP PRIMERS

The primers for *anp* transcript amplification from cDNA were designed from the cDNA sequence of the bullfrog (*Rana catesbeiana*) atrial natriuretic prohormone (Genbank accession number, D01043). The forward primers were selected to begin at the 96 and 123 bp positions and had the sequences, TCCTGCTTCTTCTGGCATT ($T_m = 69^\circ\text{C}$, % GC = 47), and TTCGAGGAGGTCCAGCATACA ($T_m = 74^\circ\text{C}$, %GC = 52), respectively. The reverse primers were selected to begin (reverse complementary) at positions 467 and 483 of the *R. catesbeiana* cDNA and had the sequences GAGCACCGATTCTGTCA ($T_m = 64^\circ\text{C}$, %GC = 52) and CAACCCATGCCGCTTTGA ($T_m = 75^\circ\text{C}$, %GC = 55), respectively.

4.2.3 MLC2 PRIMERS

The primers for amplification of the *mlc2* gene were designed primarily from the submitted cDNA sequence of African clawed frog (*Xenopus laevis*) *mlc2* (Genbank accession number, Z33999). The forward primers were selected to begin at positions 82 and 214 of the *X. laevis* cDNA and had the sequences, TCCAATGTCTTCTCCATGTT ($T_m = 66^\circ\text{C}$, %GC = 40) and CTGAACGTGAATGAGGA ($T_m = 61^\circ\text{C}$, %GC = 47). Two reverse primers were selected with sequences beginning (reverse complementary) at positions 368 and 527 to provide primers of GCGTTGGGGTCAAGAAT ($T_m = 67^\circ\text{C}$, %GC = 52) and TCTCCATGCGTGATGA ($T_m = 63^\circ\text{C}$, %GC = 50), respectively.

4.2.4 PCR AMPLIFICATION OF *ANP* AND *MLC2*

Total RNA and mRNA were isolated from control and 24 h frozen *R. sylvatica* hearts as described in sections 2.2.2 and 2.2.3, and first strand synthesis of cDNA and PCR amplification were performed as described in sections 2.7.1 and 2.7.2. The PCR amplification for both genes occurred over 35 cycles, with an annealing temperature of 55°C. PCR products were visualized and sizes estimated via electrophoresis on a 1.5 % TAE gel as described in section 2.2.5. The PCR products were sequenced by Canadian Molecular Research Services (Orleans, ON) and characterized using BLAST and DNAMAN.

4.2.5 NORTHERN BLOT ANALYSIS

Total RNA was isolated (section 2.2.2) from hearts of *R. sylvatica* exposed to a time course of freezing at -2.5°C (4 h, 8 h, 24 h) or to anoxia (4h, 24 h) or dehydration (20%, 40 %) exposures at 5°C. The RNA was quantified, electrophoresed, and blotted to nitrocellulose, as described in section 2.5. Northern blots were prehybridized at 65°C for 1 h, prior to 12 h hybridization at 44°C with gene-specific radiolabeled probe made with the *anp* and *mlc2* gene fragments. Blots were washed and exposed to film repeatedly until single bands were visible.

4.3 RESULTS

4.3.1 PCR AMPLIFICATION OF ANP AND MLC2 cDNA

Figure 4.2 shows the DNA products generated from PCR amplification of the *R. sylvatica* cDNA using different combinations of primers designed to *anp* and *mlc2*. Previously designed primers to α -tubulin were used as a control to ensure that mRNA was suitable and that first strand cDNA synthesis was successful. As Figure 4.2A illustrates only one of the combinations of *mlc2* primers designed from *X. laevis* MLC2 sequence resulted in a PCR product. The expected size of the product as predicted from the location of the primers on the *X. laevis* sequence, is 445 base pairs. This number agrees with the size obtained during amplification (Figure 4.2A, lane 3), as determined using the Gibco-BRL 1 kb ladder.

Figure 4.2B demonstrates that all combinations of the *anp* forward and reverse primers successfully amplified distinct PCR products. The theoretical sizes of the *R. sylvatica anp* PCR products, as determined from the *R. catesbiana* sequence, were 363, 387, 344, and 360 base pairs for lanes 2 through 5. The size and pattern of DNA obtained with each combination of primers matched perfectly with the expected sizes.

The product from the ANP96 forward and ANP483 reverse primers, and the lone MLC2 PCR product, were sequenced by CMRS (Orleans, ON). The nucleotide sequences for ANP and MLC2, along with the translated amino acid sequences, are shown in Figures 4.3A and 4.4A, respectively. Both nucleotide sequences were compared with those maintained in the Genbank database using a BLAST search on the NCBI web-page.

This analysis confirmed that the amplified products coded for the MLC2 and ANP proteins.

4.3.2 ATRIAL NATRIURETIC PEPTIDE (ANP) SEQUENCE ANALYSIS

Figure 4.3A shows the nucleotide and deduced amino acid sequences for *R. sylvatica anp*. The 390 base pairs of sequence obtained for wood frog gene were compared with the bullfrog (*R. catesbeiana*) and human nucleotide sequences. In this analysis 97 % identity was observed between the bullfrog (accession number D01043) and wood frog nucleotide sequences, whereas the two amphibian sequences showed 55 % identity with human *anp* cDNA (accession number, K02043). Following an alignment of the deduced amino acid sequences the same trend was observed; 95 % identity was observed between bullfrog and wood frog amino acid sequences, whereas only 43 % identity with the human peptide was calculated (Figure 4.3B).

4.3.3 MYOSIN LIGHT CHAIN 2 (MLC2) SEQUENCE ANALYSIS

The 450 base pairs of wood frog *mlc2* gene sequence (Figure 4.4A) were aligned with three *mlc2* nucleotide sequences obtained from the Genbank database following a BLAST analysis. Wood frog *mlc2* showed 84, 77, and 58 % identity with the *mlc2* nucleotide sequences from *X. laevis* (accession number Z33999), *Danio rerio* (zebrafish, accession number AF114428), and human (accession number NM_006097), respectively. Figure 4.4B shows the alignment of the deduced amino acid sequences; the wood frog protein showed 91, 84, 78, and 62 % identity with the amino acid sequences of the corresponding *X. laevis*, zebrafish, and human atrial MLC2 forms (Accession number

NP_067046) and ventricular (Accession number AF020768) isoforms, respectively. As well, a PROSITE scan of the deduced *R. sylvatica* amino acid sequence identified the presence of two EF-hand calcium binding domains (accession number PS00018), which are also found in MLC2 from other species. The pattern required for a match is given as, D-x-[DNS]-{ILVFYW}-[DENSTG]-[DNQGHRK]-{GP}-[LIVMC]-[DENQSTAGC]-x(2)-[DE]-[LIVMFYW]. These domains, located at positions 23-35 and 93-105 in the wood frog amino acid sequence, are underlined (See Figure 4.4A).

4.3.4 *mlc2* NORTHERN BLOT ANALYSIS

Northern blots were used to analyze changes in *R. sylvatica* heart *mlc2* transcript levels over a time course of freezing (Figure 4.5). A representative northern blot is shown along with confirmation of equal loading of total RNA as assessed by 18S and 28S ribosomal RNA band densities (as determined by ethidium bromide staining). Following standardization against corresponding control blots, the mean increases in transcript levels in hearts from frogs frozen for 4, 8, or 24 h were determined to be 1.49 ($\pm$ 0.35, n=4), 2.68 ($\pm$ 0.64, n=4), and 3.77 ($\pm$ 0.60, n=4) fold (Figure 4.5C). A similar analysis of *mlc2* expression in hearts from anoxic and dehydrated frogs was performed, and representative northern blots are shown in Figure 4.6. Following anoxia exposure for 4 and 24 h (at 5°C) *mlc2* transcript levels had increased by 3.03 ($\pm$ 0.54, n=3) and 5.47 ($\pm$ 0.15, n=3) fold in heart, respectively (Fig 4.6C). However, the loss of 40 % total body water did not change *mlc2* transcript levels and the mean transcript intensity value was 0.77 ($\pm$ 0.20, n=4), relative to control animals (5°C).

4.3.5 ANP NORTHERN BLOT ANALYSIS

A comparison of *anp* transcript levels in the hearts of control, frozen, anoxic and dehydrated wood frogs is shown in Figure 4.6. Following quantification, the mean increase in *anp* transcript levels was determined to be 1.88 (± 0.11 , n=5) fold following 24 h freezing. A comparable rise in transcript levels (2.20 ± 0.25 fold, n=4) occurred after 24 h anoxia exposure in heart. However, dehydration had no significant effect on *anp* transcripts; the mean value following 20 % dehydration was 1.16 (± 0.08 , n=4) as compared with the corresponding controls.

4.4 DISCUSSION

4.4.1 RT-PCR, AND SEQUENCE ANALYSIS OF MLC2 AND ANP

The main goal of this study was to establish whether *R. sylvatica* cardiomyocytes alter the steady state concentrations of two well known markers of pressure-hemodynamic overload and cardiac hypertrophy under freezing, anoxia, or dehydration stresses. Before the appropriate northern analysis could be performed *R. sylvatica mlc2* and *anp* gene fragments were obtained by RT-PCR amplification of heart cDNA; sequence analysis was performed with each of the DNA products for identity confirmation.

4.4.1.A ATRIAL NATRIURETIC PEPTIDE (ANP)

Alignment of the amino acid sequences showed that the wood frog and bullfrog ANP prohormone are 95% identical, whereas the wood frog and human sequences share less than half (only 43%) of their amino acid sequence. Inspection of the alignment of the ANP protein sequences (Figure 4.3B) shows that the majority of the conserved residues are located on a short stretch of the polypeptide located at the C-terminal end. This finding is consistent with what is known about the mechanism of synthesis, storage, secretion, and the final structure of the biologically active form of the peptide.

ANP is one of three peptides (ANP, Brain natriuretic peptide, and C-natriuretic peptide) that have been grouped together as the natriuretic peptide family by reason of size, sequence, and shared biological role in salt-water regulation (Matsuo, 2001). Under normal hemodynamic conditions, ANP is predominantly synthesized, stored, and secreted

in a regulated fashion by myocytes of the cardiac atria; whereas BNP and CNP are released by ventricular cardiomyocytes and neural cells (Melo *et al.*, 2000). The prohormone of ANP is stored as a 126 amino acid peptide (proANP₁₋₁₂₆) that is produced by cleavage of a signal peptide located at the amino-terminal end of the original 151 amino acid precursor. The biologically active 28 amino acid portion of the atrial natriuretic prohormone is cleaved from the carboxy end of proANP immediately before release (Clerico *et al.*, 1999). On release ANP exerts its biological effects through interaction with membrane bound guanylate cyclase-linked receptors (NPR-A), stimulating a rise in intracellular cGMP (Brenner *et al.*, 1990). As only the C-terminal portion of the peptide is biologically active, the highest degree of evolutionary conservation is seen in this region (Figure 4.3B).

4.4.1.B MYOSIN LIGHT CHAIN (MLC)

The amplified *mlc2* gene product from *R. sylvatica* coded for 147 amino acids compared with the 173 amino acids that make up the full *X. laevis* sequence. Based on those isoforms with the greatest similarity to the wood frog MLC2 isoform the complete protein is predicted have an additional 14 to 20 amino acids at the N-terminal and an additional 6 residues at the C-terminal. Alignment of the deduced amino acid sequence of *R. sylvatica* MLC2 with the comparable segments of the *X. laevis*, zebrafish, and human atrial and ventricular isoforms of MLC2 showed strongest identity with the other amphibian sequence (91%) and less (84, 78, 62 %, respectively) with the other three isoforms (Figure 4.4B). The presence of two EF-hand calcium binding domains, characteristic of MLC2 proteins, were recognized on a Prosite scan. The MLC2

subfamily, as well as the MLC1 subfamily and the calmodulin and troponin-C subfamilies, belong to a superfamily of intracellular Ca^{2+} binding proteins that characteristically contain four EF-hand domains (Kawasaki and Kretsinger, 1994). However, during evolution all of the EF domains in the myosin essential light chain lost their ability to bind Ca^{2+} , whereas in the regulatory light chains the first of the four domains has retained the ability to bind both Ca^{2+} and Mg^{2+} with high affinity (Watterson *et al.*, 1979). The contribution of this functional EF-hand domain is illustrated by the fact that the rate of force development during crossbridge cycling is influenced by the intracellular concentration of Ca^{2+} , a phenomenon dependent on the presence or absence of the MLC2 subunit (Patel *et al.*, 1996; Metzger and Moss, 1992).

4.4.2 TRANSCRIPT EXPRESSION ANALYSIS

Northern analysis of the two hypertrophic marker genes demonstrated a 4-fold rise in the expression level of the *mlc2* transcripts over a 24 h time course of freezing. The up-regulation of the *mlc2* transcript further confirms the identity of the up-regulated 20 kDa heart protein, described in Chapter 3, as the *R. sylvatica* MLC2 homologue. In addition, *anp* transcript levels rose by 2-fold in wood frog heart after 24 h of freezing. The simultaneous up-regulation of these two transcripts is consistent with the hypertrophic gene response observed in a variety of *in vivo* and *in vitro* model systems of pressure overload and hypertrophy in heart (Yamazaki *et al.*, 1999; Schwartz *et al.*, 1993). To further characterize the trigger for *mlc2* and *anp* overexpression in the wood frog, the influences of anoxia and dehydration stress were investigated. Organ dehydration due to water loss into extracellular ice crystals and anoxia/ischemia due to

plasma freezing are two of the major consequences of whole body freezing. Previous studies by our lab have shown that various metabolic or gene expression responses to freezing by wood frog organs can be linked with one of these component stresses; for example, the synthesis of glucose as a cryoprotectant is stimulated just as strongly by dehydration as by freezing but is not induced by anoxia. Northern analysis indicated that dehydration of *R. sylvatica* did not affect either *mlc2* or *anp* transcript levels. However, anoxia exposure strongly increased the transcript levels of both genes and for both genes the fold-increase after 24 h anoxia was actually greater than in 24 h frozen frogs. This finding parallels the change in ANP metabolism (increased *anp* transcript levels and ANP secretion) that has been observed in mammalian species following exposure to decreased oxygen tension or reduced blood flow (Baertschi and Teague, 1989; Kawashima *et al.*, 1989; Pfeifer *et al.*, 1997). Hence, it appears that the trigger for the hypertrophic gene expression response during freezing might be the hypoxia/ischemia caused by the cessation of breathing and circulation when body ice content rises above a certain level.

4.4.3 THE HYPERTROPHIC RESPONSE

Cells and tissues adapt to increased workload in a number of ways: increased metabolism, hyperplasia, or hypertrophy. Through an increase in metabolism (or metabolic rate) tissues and organs are able to respond without having to make major changes to cell morphology or cell number; instead, the activity of specific biochemical systems are increased via modifications to the existing protein and enzyme complement. This capacity is best demonstrated in heart tissue by the immediate increase in tissue contractility that follows from either an increase in end-diastolic volume (Frank Starling

principle) or sympathetic stimulation. However, when the increase in workload reaches a threshold level or persists over a long enough period of time a tissue/organ will often respond with one of two, more long-term, adaptations. The first is a proliferation or hyperplasia response; by increasing the total cell count of the tissue or organ, the demand on each cell is reduced and the physiological requirements are more easily met. The other possible response is hypertrophy or enlargement of the individual cells, best illustrated by hypertrophy of cardiac myocytes. In fact, it is believed by some that the cardiac hypertrophic gene response, characterized by the re-induction of a fetal or embryonic gene expression pattern, is an attempt by the overworked cardiac cells to re-acquire the proliferative capacity enjoyed during development. However, heart cells are terminally differentiated and proliferation is not possible (Schaub *et al.*, 1998). It is unlikely that significant hypertrophy will occur in the wood frog heart over the relatively few hours (probably no more than about 12 h) that the heart remains beating as the freezing front propagates through the animal. However, over winter, with multiple freeze/thaw episodes and repeated stimulation significant enlargement of the heart could take place. Regardless, increases in cellular MLC2 and serum ANP levels, even in the short term, might be sufficient to provide some benefit to the heart to compensate for the increased pressure and workload associated with the freezing of the vasculature.

4.4.4 ROLE OF MLC2 IN THE FREEZING PROCESS

MLC2 transcript up-regulation has come to serve as a marker for the hypertrophic gene response because it is elevated in both in vivo and in vitro models of hypertrophy, including: in atrial and ventricular muscles of spontaneously hypertensive rats, in aorta-

constricted rat ventricle, and in agonist-treated cardiomyocytes in culture (Kumar *et al.*, 1988; Kwiatkowska-Patzer *et al.*, 1983; Shubeita *et al.*, 1992). One way of compensating to conditions that place increased work load on the heart is to increase the contractility of the heart muscle and overcome the obstacles restricting proper tissue perfusion. The up-regulation of MLC2 would be an important step (possibly the rate-limiting step in the wood frog) in increasing the total cellular concentration of the myosin motors necessary for contraction and force generation. Changes in cellular MLC2 levels have been shown to directly affect contractility under a condition of contractile dysfunction called dilated cardiomyopathy (DCM). DCM involves an idiopathic decrease in MLC2 protein levels that has been suggested to lead to the dysfunctional contractile phenotype (Corbett *et al.*, 1998). If the wood frog was successfully able to increase the number of myosin motors, and therefore the number of cross-bridges available for cycling, it could partially adapt to the increased pressure and workload that accompanies the freezing of the vasculature, allowing for a more sustained period of tissue perfusion and cryoprotectant distribution.

4.4.5 ROLE OF ANP IN THE FREEZING PROCESS

Both the existence and biological function of a cardiac hormone were identified concurrently when the intravascular injection of atrial extracts into rats was observed to cause a dramatic diuresis and natriuresis (de Bold *et al.*, 1981). The main peptide involved, ANP, is now a well-known hormone still being widely studied with respect to (i) the various biological functions of the peptide, and (ii) the mechanisms regulating transcription, translation, storage, and secretion (reviewed in Farrell and Olson, 2000). The main factors regulating the synthesis and release of ANP involve fluctuations in

intravascular volume such that volume expansion and pressure overload lead to increases in ANP expression and secretion (Espiner, 1994). The excessive salt and water excretion that results from elevated ANP serum levels gives rise to a fall in blood volume, diastolic blood pressure, and central venous pressure. The ANP provoked hypotensive state is triggered as the result of peptide binding to receptor sites located on a variety of cell types associated with a number of organ systems. The primary receptor sites are those located in the kidney, where ANP binding increases glomerular filtration rate and inhibits tubular sodium resorption (Farrell and Olson, 2000). Other effector locations have also been identified including the adrenal glands where ANP inhibits aldosterone release, and the vasculature where ANP causes a potent vasodilation (Farrell and Olson, 2000).

Identifying changes in wood frog ANP metabolism following freeze exposure is not unexpected considering the progressive rise in blood pressure that has been predicted to occur as ice propagates inward from the periphery. At first glance increased ANP metabolism might seem to represent a built-in and perhaps futile response by the wood frog cardiomyocytes. However an evaluation of the different biological functions of ANP with respect to the problems associated with whole body freezing demonstrates that increased ANP metabolism might indeed contribute gainfully to freeze survival. For example, by relocating extracellular water to the bladder and reducing blood volume, a freeze induced natriuresis and diuresis would reduce the amount of ice-induced damage to delicate organs. During the thaw, the frogs (unlike humans) could then simply return the water to remainder of the body by resorbing the melting ice from their bladder. The ANP vasorelaxant properties might be of similar benefit: increased intravascular volume would create room for the expanding plasma as it freezes, lessening the amount of

intravascular damage. The expansion of unfrozen blood vessels would also transiently compensate for the progressive rise in peripheral resistance, by reducing the blood pressure and, in at least in part, decreasing the workload and strain on the heart. Studies in mammals have demonstrated that ANP has a significant biological role, protecting the heart during periods of physiological stress. A functional ANP system allows the body to properly compensate for changes in hemodynamics, reducing the need from hypertrophy and protecting against heart failure during times of stress (Oliver *et al.*, 1997; Horio *et al.*, 2000). Finally, other potential benefits of increased plasma ANP might be mediated through as yet unidentified mechanisms. For example, the addition of ANP during hypoxia has been shown to reduce lactate accumulation in rat ventricular myocardium suggesting that ANP might be able to reduce the energy requirements of the heart and decrease the production of toxic substances (Ljusegren and Axelsson, 1993).

4.4.6 SUMMARY

The present study has provided evidence for the activation of the hypertrophic gene response in the heart of the wood frog following freeze and anoxic exposure. Based on what is known about the different biological roles of MLC2 and ANP and the physiological changes that accompany freezing, possible reasons for the up-regulation of these two genes/proteins have been discussed. Future work will be required to determine which of the potentially accountable extracellular trigger(s) and cell signaling pathway(s) are responsible for stimulating the up-regulation of these proteins (reviewed in Hefti *et al.*, 1997; Ruwhof and van der Laarse, 2000).

FIGURE 4.1

Schematic diagram of the myosin motor.

Diagram illustrates the interaction of the myosin motor with the actin filament. The myosin heavy chain globular domain has also been drawn with the ATP binding pocket and the actin binding cleft indicated. The interaction of two heavy chain C terminal domains to form an α -helix that will contribute to the myosin filament has also been illustrated. The described extension of the N-terminus of the essential light chain to form the actin/MLC1 tether can be observed as well as the location of the regulatory light chain (MLC2) at the neck of the heavy chain.

Myosin light chain 1 / Essential light chain

Myosin light chain 2 / Regulatory light chain

Myosin heavy chain (globular domain)

Myosin heavy chain (tail/helical domain)

Actin filament

FIGURE 4.2

PCR amplification of *R. sylvatica anp* and *mlc2* from wood frog heart cDNA.

- A. PCR amplification using MLC2 primers. Primer pairs used were: Lane 1, forMLC82 and revMLC368; Lane 2, forMLC214 and revMLC368; Lane 3, forMLC82 and rev MLC527; Lane 4, forMLC214 and rev MLC527. Lane 5 shows DNA molecular weight standards (1 kb ladder).
- B. PCR amplification using ANP primer combinations. Lanes show: Lane 1, α tubulin primers; Lane 2, forANP96 and revANP467 primers; Lane 3, forANP96 and revANP483 primers; Lane 4, forANP123 and revANP467 primers; Lane 5, forANP123 and revANP483. Lane 6 shows DNA molecular weight standards.

A

B

FIGURE 4.3

Nucleotide, deduced amino acid sequence, and multiple protein alignment of the *R. sylvatica anp* gene product that was PCR amplified from 24 h frozen heart cDNA.

- A. Nucleotide and deduced amino acid sequences for the *R. sylvatica anp* gene and prohormone. Nucleotides and amino acids are number on the left. A single open reading frame was predicted from the nucleotide sequence to code for a polypeptide with 127 residues. Twenty-two of the twenty eight amino acids that form the active hormone were coded for in the PCR amplified gene fragment, and have been underlined.
- B. Alignment of the deduced *R. sylvatica* ANP amino acid sequence with bullfrog (Genbank accession number D01043), and human ANP (Genbank accession number K02043).

A

```

1      TCCTGCTTCTTCTGGCATTACCAAAGTTCGAGGAGGTCCAGCATACTCCCCCTGT
1      L L L L A F T K V R G G P A Y N S P L

61     CTTCAGACTTGTCTGATTTAAAGGGGCTCCTAGAACGTTTAGAAGACAGACTCCCGGTCG
20     S S D L S D L K G L L E R L E D R L P V

121    AAGAAGCCGAGGCTCCAGCTCAAGACATATTCGCTCCAAATTATGATTCCGCTGACTCTT
40     E E A E A P A Q D I F A P N Y D S A D S

181    CAAACTCAGCACCGTCCTTGACTATAGAAGCAGCCCGTCCCGGGGCCGACATGGTGTATA
60     S N S A P S L T I E A A R P G A D M V Y

241    ACAGAGGCTCCTGGACACAGCAAGAGAAATCTTCTCCGCTGAGGAACAACTTCGAGAGT
80     N R G S W T Q Q E K S S P L R N K L R E

301    TGCTGAACGCTCCTCGCAGCATGAGGAGATCTTCCGACTGCTTTGGATCTCGAATTGACA
100    L L N A P R S M R R S S D C F G S R I D

361    GAATTGGCGCTCAAAGCGGCATGGGTTG
120    R I G A Q S G M G

```

B

WOOD FROG	LLLLA.....FTKVRGGPAYNSPLSSDLSDLKGLLERLEDRLPVEEA
BULL FROG	-----.....-----
HUMAN	-----FQLLGQT-AN-M--AVSNA--M-F-N--DH-E EK MPL-DE
WOODFROG	EAPAQDIFAPNYDSADSSNSA.PSLTIEAARPGADMVYN.RGSW
BULL FROG	-T-V-----V-----
HUMAN	VV-P-VLSE--EEAAL-PLPEV-PW-G-VSPAQRDGGALG--P-
WOOD FROG	TQQEKSSPLRNKLRELLNAPRSMRRSSDCFGSRIDRIG AQSGMG
BULL FROG	-----
HUMAN	DSSDR-AL-KS---A--T--L----.---G-M-----L-

FIGURE 4.4

Nucleotide, deduced amino acid sequence, and multiple protein alignment of the *R. sylvatica mlc2* gene product that was PCR amplified from 24 h frozen heart cDNA.

- A) Nucleotide sequence and corresponding amino acids are numbered on the left. A single open reading frame was predicted from the nucleotide sequence, encoding a polypeptide of 148 residues. The two complete EF-HAND calcium binding domains are underlined in the sequence.
- B) Alignment of the deduced *R. sylvatica* MLC2 amino acid protein sequence with *X. laevis* (Genbank accession number Z33999), *Danio rerio* (zebrafish, accession number AF114428), human atrial MLC2 (accession number NP_067046) and human ventricular MLC2 (accession number AF020768).

A

```

1      TCCAATGTCTTCTCCATGTTTGAGCAGTCACAGATCCAGGAATTTAAGGAGGCCTTTAGC
1      S N V F S M F E Q S Q I Q E F K E A F S

61     TGTATAGACCAGAACCGTGATGGTATTATCAGCAAGTCTGACCTCAAAGAGACATACATG
21     C I D Q N R D G I I S K S D L K E T Y M

121    CAGCTAGGTAACTTAATGTAAACGAGAAATGAGCTTGAAGAAATGCTGAAGGAAGGAAAA
41     Q L G K L N V N E N E L E E M L K E G K

181    GGGCCTATTAACCTCACCGTGTCTTGACACTGTTTGGAGAGAAGCTTAATGGGACGGAC
61     G P I N F T V F L T L F G E K L N G T D

241    CCAGAGGACTCTATCGTCAGTGCTTTCAAACCTTTGGACCCAAATGGAACAGGAAATGTC
81     P E D S I V S A F K L L D P N G T G N V

301    AACAAAGATGAGTTAAAGCATCTACTGATGACACAAGCCGACAAATTCACAGCCGAAGAG
101    N K D E L K H L L M T Q A D K F T A E E

361    GTGGAACAAATGTTTGCTGTGACCCCAATTGACGTGGCCGGGAACATTGACTACAAGTCT
121    V E Q M F A V T P I D V A G N I D Y K S

421    CTGTGTTACATCATCACGCATGGAGA
141    L C Y I I T H G

```

B

WOOD FROG	SNVFSMFEQSQIQEFKEAFSCIDQNRDGIISKSDLKETYMQLGK
XENOPUS	-----P-----
ZEBRAFISH	-G-----V-N-----A----
HUMAN MLC2a	-----A-----C-A--R---S----
HUMAN MLC2v	-----TIM-----F-D-N--RD-FAA--R
WOODFROG	LNVNENELEEMLKEGKGPINF TVFLTLFGEKLN GTDPEDSIVS
XENOPUS	M----D-D-----S-----S---
ZEBRAFISH	---SDE---S--T-----ET-LA
HUMAN MLC2a	VS-P-E--DA--Q-----EA-L-
HUMAN MLC2v	V--KNE-ID--I--AP-----M-----K-A---ET-LN
WOOD FROG	AFKLLDPNGTG NVNKDELKHLLMTQADKFTAEEVEQMFAVTPI
XENOPUS	-----A-----L-----SD---D-----
ZEBRAFISH	----F---A--V-----F-R-----D-A---A--
HUMAN MLC2a	--RMF--S-K-V-----F-Q--L-----SPA-----L--M
HUMAN MLC2v	---VF--E-K-VLKA-YVREM-T---ER-SK---D---AF-P
WOOD FROG	DVAGNIDYKSLCYIITHG
XENOPUS	-----
ZEBRAFISH	-----
HUMAN MLC2a	-L-----
HUMAN MLC2v	--T--L---N-VH-----

FIGURE 4.5

Northern analysis of *mlc2* transcript levels in wood frog heart over a time course of freezing exposure.

- A) A representative Northern blot showing samples from heart of control frogs at 5°C (Con) and samples after 4, 8, or 24 h of freezing hybridized with ³²P-labeled *mlc2* probe.
- B) Corresponding 18S and 28S rRNA bands on ethidium bromide-stained formaldehyde gel demonstrate equal loading (16 µg total RNA/lane).
- C) Histogram showing mean relative transcript levels of *mlc2* ($\pm$ SEM) for n = 3 independent blots for tissues samples from different animals. Band intensities on individual blots were scanned and then normalized to their respective 18S rRNA band; control values were set to 100 and band intensities in other lanes were expressed relative to their control.

A

B

C

FIGURE 4.6

Effect of exposure to anoxia or dehydration on *mlc2* transcript levels in wood frog heart.

Hearts were sampled from control frogs (5°C), frogs exposed to 4 or 24 h of anoxia at 5°C, and frogs dehydrated to 40% of total body water lost.

- A) A representative northern blot for 16 µg of total RNA from each heart sample.
- B) Corresponding 18S and 28S rRNA bands on ethidium bromide-stained formaldehyde gel demonstrate equal loading (16 µg total RNA/lane).
- C) Histogram showing mean ($\pm$ SEM; n=3 independent determinations for anoxia, n=4 for dehydration) *mlc2* transcript levels in heart of experimental frogs compared with their corresponding control values set to 100.

A

B

C

FIGURE 4.7

Effect of exposure to freezing, anoxia, and dehydration stresses on *anp* transcript levels in the heart of the wood frog.

Hearts were sampled from control frogs (5°C), and frogs exposed to 24 hours of freeze exposure, 24 hours of anoxia, and animals dehydrated to 20% of total body water lost.

- A) A representative northern blot for 16 µg of total RNA from each heart sample.
- B) Corresponding 18S and 28S rRNA bands on ethidium bromide-stained formaldehyde gel demonstrate equal loading (16 µg total RNA/lane).
- C) Histogram showing mean ($\pm$ SEM, n=4 independent determinations) *anp* transcript levels in heart of experimental frogs compared to their corresponding controls (values set to 100).

A

B

C

CHAPTER 5

**INVESTIGATING THE FREEZING AND THAWING INDUCED CHANGES IN
HSP27 AND EF-1 γ TRANSCRIPT AND PROTEIN LEVELS
IN THE HEART OF THE WOOD FROG**

5.1 INTRODUCTION

5.1.1 AIM OF CHAPTER

Following exposure to harmful environmental conditions organisms often respond with the initiation of a cellular stress response. This cellular stress response involves an increase in transcription, translation, and overall activity of a family of proteins known as the heat shock proteins. The differential screen of the wood frog cDNA library described in Chapter 3 identified the up-regulation of the transcript for *heat shock protein 27* (*hsp27*) in the hearts of frozen wood frogs. To further explore the role of heat shock proteins in freezing, the expression level of *hsp27* over a time course of freezing in the heart and in other organs of the wood frog was evaluated through northern blotting. Western analysis was then carried out on samples prepared from time courses of freezing and thawing to ascertain the changes that take place in both the total protein level and phosphorylated Hsp27 cellular pools. The discordant response observed between *hsp27* transcription and translation was then contrasted with the results from a similar analysis of the eukaryotic Elongation Factor -1 gamma (EF-1 γ). Finally, possible explanations for the observed response of these two genes at the transcriptional and translation levels were explored through an analysis of the impact of freezing on the cellular rates of protein synthesis and mRNA transcription.

5.1.2 THE HEAT SHOCK PROTEINS (HSPs)

The HSPs were first identified as a group of proteins induced by heat stress. Although this initial discovery provided the family name, this designation has been

recognized as inadequate as this well-known protein family is now known to be part of an evolutionarily conserved response to many different stresses, including hypoxia, ischemia, radiation, heavy metals, and oxidative/reactive oxygen species (Benjamin *et al.*, 1990; Knowlton *et al.*, 1991; Bijur *et al.*, 1999; Madamanchi *et al.*, 2001). HSPs are loosely characterized by their up-regulation in response to stress, the presence of weak ATPase activity, and a high degree of sequence identity among evolutionary divergent species (Knowlton, 1997). Furthermore, most of the HSPs are chaperones, capable of preventing either protein denaturation, the aggregation of partially unfolded intermediates, or facilitating the refolding of denatured molecules. This ability was first demonstrated when DnaK, the *Drosophila* homologue of HSP70, was used to re-activate RNA polymerase in an ATP-dependent manner (Skowrya *et al.*, 1990).

HSP gene up-regulation is controlled by the HSF (heat shock factor) family of transcription factors. Although 4 different HSFs have been identified, HSF-1 is the primary factor that mediates the response regardless of the stress (Cotto *et al.*, 1996). HSF-1 is present in the cytoplasm at all times in an inactive form, and following stress is hyper-phosphorylated and trimerizes (Westwood and Wu, 1991). The hyper-phosphorylation is believed to be carried out by MAPK family members (Knauf *et al.*, 1996; Kim *et al.*, 1997). Following activation, HSF-1 moves into the nucleus where it binds to an upstream sequence-specific motif (heat shock element) found in the promoter region of all stress-inducible genes (Amin *et al.*, 1988).

The heat shock proteins can be divided into 5 groups, (i) the small HSPs, 20-30 kDa; (ii) the 50-60 kDa HSPs, (iii) the 70 kDa HSPs, (iv) the 90 kDa HSPs, and (v) the HSPs greater than 100 kDa in size (Hightower and Hendershot, 1997). Although over

twenty HSPs have been identified, the majority of the research has involved Hsp70, and the protective abilities of this protein have been demonstrated repeatedly. For example, the forced expression of Hsp70 conveys a cytoprotective effect in cultured cells, including cardiac myocytes, subjected to simulated ischemia (Williams *et al.*, 1993; Mestril *et al.*, 1994). As well, overexpression of Hsp70 in mice improves myocardial tolerance to ischemia, enhancing metabolic recovery and reducing infarct size (Radford *et al.*, 1996; Suzuki *et al.*, 2000; Marber *et al.*, 1995).

5.1.3 HSP27: A SMALL HEAT SHOCK PROTEIN GROUP MEMBER

Hsp27 protein was first isolated from turkey gizzard muscle as an inhibitor of actin depolymerization and was quickly recognized to have sequence, structural, and functional similarities with many other HSPs (Miron *et al.*, 1988; Miron *et al.*, 1991).

The Hsp27 gene codes for a member of the small heat shock protein (sHSP) family that also includes: $\alpha\beta$ -crystallin, Hsp20, heme oxygenase, and ubiquitin. In addition to molecular weight, this group can be distinguished from other HSPs by a number of shared features (Arrigo, 1998). One of the interesting biochemical properties of the small HSPs (sHSPs) is their ability to form homotypic oligomers ranging in size from 200 to 800 kDa. An additional feature of this group is an ATP-independent chaperone capacity. It is believed that, under conditions that disrupt various cellular processes, the abundant energy-independent sHSPs trap non-native proteins, protecting them from irreversible aggregation until more active, ATP-dependent chaperones can complete the refolding following the restoration of normal physiological and environmental conditions (Patriarca and Maresca, 1990; Ehmsperger *et al.*, 1997).

Protective actions of Hsp27 have been documented against a variety of toxic agents, including heat shock, oxidative stress, anticancer drugs, growth factor withdrawal, and the actions of death receptor agonists such as TNF α or Fas (Charette and Landry, 2000). For example, over-expression of Hsp27 has been demonstrated to reduce radiation-induced apoptosis in L929 cells, protect dorsal root ganglion neurons from apoptosis-induced by growth factor withdrawal, and ND7 neuronal cells from retinoic acid-induced apoptosis (Baek *et al.*, 2000; Wagstaff *et al.*, 1999). More relevant to our model system, Hsp27 overexpression has been reported to protect primary cardiomyocytes from ischemia-induced damage (Martin *et al.*, 1997).

The ability of Hsp27 to provide protection against such an extensive set of stresses, with very different mechanisms of cell injury and death, is feasible only due to the number of protective properties that have been attributed to the protein. First, Hsp27 is a true molecular chaperone, capable of binding denatured proteins, preventing aggregation and facilitating functional protein refolding (Knauf *et al.*, 1994; Erhnsperger *et al.*, 1997; Kawahara *et al.*, 2000). Second, Hsp27 overexpression has also been documented to protect against oxidative stress (Wytenbach *et al.*, 2002; Mehlen *et al.*, 1996, 1997). The Hsp27 generated tolerance to oxidative stress has been associated with increases in intracellular glutathione levels, and reduced levels of reactive oxygen species. Third, Hsp27 has also been described as a F-actin barbed end capping protein capable of inhibiting actin polymerization (Miron *et al.*, 1991). This ability explains the observation that Hsp27 can regulate actin filament dynamics and counteract stress-induced disruption of the actin microfilament network (Miron *et al.*, 1991; Lavoie *et al.*, 1993; Huot *et al.*, 1996). Finally, Hsp27 has recently been shown to prevent apoptosis by

interfering with apoptotic signal transduction. Hsp27 has been reported to protect mitochondrial integrity and reduce cytochrome c release, to bind cytochrome c directly and prevent procaspase-9 activation, and to bind procaspase-3 inhibiting its activation (Gabai and Sherman, 2002). Hsp27 also interacts with the pro-apoptotic protein Daxx, blocking its interaction with both Ask1 and Fas (Charette *et al.*, 2000). Daxx cytotoxicity is linked with its translocation from the nucleus to the cytoplasm and it has been suggested that Hsp27 inhibits this relocation. Consequently Hsp27 can block Fas-induced apoptosis under those conditions where signaling downstream of FADD is blocked.

An interesting feature with regards to Hsp27 is the recent realization that the protective properties associated with the protein are dependent on its state of oligomerization and phosphorylation. Phosphorylation results in a major shift in the quaternary structure of Hsp27 causing the large homotypic oligomers (up to 800 kDa) to dissociate into dimers and monomers (Lambert *et al.*, 1999; Rogalla *et al.*, 1999). The main enzymes responsible for stress-dependent phosphorylation of Hsp27 are MAPKAPK (mitogen-activated protein kinase-activated protein kinase) 2 and 3, both of which are activated by p38 (Stokoe *et al.*, 1992; Kyriakis *et al.*, 1996). Studies have demonstrated that it is the high-molecular weight, unphosphorylated Hsp27 complexes that are responsible for the chaperone and oxidative roles (Lee *et al.*, 1997; Mehlen *et al.*, 1997; Rogalla *et al.*, 1999). Conversely, Hsp27 phosphorylation seems to be a prerequisite for the stress-induced Hsp27 mediated modification of the actin cytoskeleton; similarly only the dimeric phosphorylated form of the protein can bind Daxx and block apoptosis (Charette *et al.*, 2000). A diagram illustrating the different cytoprotective properties of Hsp27 and their regulation is provided in Figure 5.1.

5.2 MATERIALS AND METHODS

5.2.1 GENE AND PROTEIN SEQUENCE ANALYSIS

The initial analysis of the wood frog EF-1 γ and Hsp27 nucleotide and deduced amino acid sequences was performed using DNAMAN and the BLAST database search engine located on the National Center for Biotechnology Information website. DNAMAN was used to align the wood frog sequences with other genes and proteins obtained from Genbank. As well, the Prosite, BLOCKS, and PRINTS programs (located on-line) were used to search the amino acid sequence for recognizable domains.

5.2.2 NORTHERN BLOT ANALYSIS

Total RNA was isolated from hearts of *R. sylvatica* exposed to different lengths of freezing exposure (2, 6 or 24 h). The RNA was quantified, electrophoresed, and blotted to nitrocellulose, as described in section 2.5. Similarly, for the multiple tissue transcript expression studies, northern blots were prepared using total RNA isolated from brain, gut, kidney, liver, lung, skin, and skeletal muscle of both control and 24 h frozen wood frogs. Northern blots were pre-hybridized at 65°C for 1 h, prior to 12 h hybridization at 44°C with gene specific radiolabeled probe made from *ef-1 γ* and *hsp27* cDNA. Blots were washed and exposed to film repeatedly until a single band was visible.

5.2.3 WESTERN ANALYSIS

Total soluble protein was extracted from *R. sylvatica* hearts as described in section 2.11.1. Samples were prepared from animals exposed to different lengths of freezing (2, 6, or 24 h), and from animals allowed to recover from a 24 h freeze for 1, 2, 4, or 8 h. Samples containing 25 µg protein were subjected to electrophoresis on 12% SDS-polyacrylamide gels and transferred to nitrocellulose as described in section 2.11.2.

The Hsp27 protein was detected using a rabbit polyclonal antibody obtained from Stressgen (Vancouver, BC). As well, two antibodies were obtained from Dr. Colleen Brophy (Arizona State University): one that binds human Hsp27 when it is phosphorylated at serine 15, and another that recognizes human Hsp27 when it is phosphorylated at serines 78 and 82. The conditions established for binding of both antibodies were incubation in a 1:5000 dilution of the antibody in 5% non-fat dry milk (TBST) for 1 h at RT. The EF-1γ protein was detected using a rabbit polyclonal antibody obtained from Dr. Jan Dijk (Leiden University). The optimal primary conditions for the EF-1γ antibody binding were established to be a 1:10000 dilution of the antibody in 1% non-fat dry milk (TBST) for 12 h at 4°C. The eukaryotic initiation factor 2 alpha protein subunit (eIF2α) and the phosphorylated form of eIF2α (Ser51) were detected using rabbit polyclonal antibodies obtained from Cell Signaling Technology (Beverly, MA). The primary conditions permitting antibody binding were established to be overnight incubation at 4°C in a 1:1000 dilution (0.25% non-fat dry milk, TBST). Washings and incubation with secondary antibodies were carried out as described in section 2.11.3 for all western blots.

5.2.4 NUCLEAR RUN-ON ASSAY

Intact nuclei were isolated from the hearts of frogs maintained at 5°C and frogs exposed to 24 h of freezing as described for liver in section 2.9.1. The one modification for heart tissue was a decrease in the concentration of sucrose in the cushion to 1.5 M. To compare the RNA transcription rates between physiological states, the elongation and isolation of nascent transcripts was performed as described in section 2.9.2. To ensure that none of the materials were limiting, triplicate reaction mixtures were allowed to incubate for different periods of time (5, 10 or 20 min) prior to reaction termination and sample processing to assess for linearity of ^{32}P -UTP incorporation.

5.3 RESULTS

5.3.1 GENE AND PROTEIN ANALYSIS

Clones *b4* and *h25* were both sequenced and compared to those sequences present in the GenBank database using the BLAST search engine located on the National Center for Biotechnology Information website. The nucleotide and deduced amino acid sequences for the *b4* and *h25* clones are shown in Figures 5.2 and 5.4, respectively.

The only substantial BLAST alignment for the *b4* cDNA sequence indicated that it was the *R. sylvatica* homologue of the chicken inhibitor of actin polymerization/ heat shock protein 25 (Genbank Accession number X59541; Miron *et al.*, 1991). A BLAST analysis of the deduced amino acid sequence indicated that the sequence obtained from the cDNA encoded the entire open reading frame and further confirmed the identity of *b4*

as the *R. sylvatica* heat shock protein 25/27 homologue. Chicken, canine, and human protein sequences were obtained from Genbank (accession numbers Q00649, AAA87172, AAH00510, respectively) and a multiple protein alignment was carried out (Figure 5.3). From the alignment it was determined that the wood frog and chicken amino acid sequences were 77% identical, whereas the frog-canine and frog-human alignments shared 65 and 64 % identity.

A Prosite scan with the Hsp27 amino acid sequence identified a number of common phosphorylation sites, as well as glycosylation and myristylation signatures. It is well established that the sHSPs have conserved serine residues, located within MAPKAP kinase recognition motifs, that are phosphorylated following activation of the MAPK pathway. For example, the mouse and rat Hsp25 proteins can be phosphorylated at two positions (Ser15 and Ser86); whereas the human protein contains three phosphorylatable serines (Ser15, Ser78, and Ser82) (Gaestel *et al.*, 1991; Landry *et al.*, 1992). These same three sites are present in the canine sequence and comparable residues are found at positions 16, 91 and 95 in the wood frog and chicken amino acid sequences (Figure 5.3).

In addition, both the BLOCKS and PRINTS database searches recognized the presence of the α crystallin signature in the putative Hsp27 amino acid sequence (Accession number, PR00299). The α crystallin signature is made up of 7 different elements; 6 elements were recognized by BLOCKS, and 7 elements by PRINTS. As mentioned earlier Hsp27 exists in the cell as one of two distinct populations, as large homotypic multimers or as dimers. The dimers are formed through stable intermolecular interactions at the carboxy-terminal of the α -crystallin domains. The dimers further multimerize through additional intermolecular interactions mediated by an amino-

terminal domain. During stress, phosphorylation of Hsp27 by MAPKAPK destabilizes the amino terminal interactions, resulting in a shift in the equilibrium between dimers and multimers and in a rapid accumulation of dimers as the major species (Charette *et al.*, 2000).

The BLAST assessment of the *h25* cDNA sequence demonstrated that the clone represented the *R. sylvatica* homologue of the gene for eukaryotic Elongation Factor 1 (*ef-1 γ*), a ribosomal protein involved in translation. Alignment of the wood frog nucleotide sequence (Accession numbers AF175982) with that of the *X. laevis*, human and rabbit gene sequences (accession numbers X62508, Z11531, X68142) (Cormier *et al.*, 1991; Sanders *et al.*, 1992) revealed 79, 72 and 71 % sequence identity, respectively. Figure 5.5 shows the alignment of the deduced amino acid sequence of wood frog EF-1 with the *X. laevis* and human proteins (Accession numbers, X62508 and BC007949). A comparison of the amino acid sequence of the wood frog protein to the *X. laevis*, human, and rabbit amino acid sequences demonstrated 88%, 78% and 78% identity, respectively. The EF-1 γ gene and protein sequences were then analyzed with the Prosite, PRINTS and BLOCKS search engines. With the exception of matches for common phosphorylation sites as well as myristoylation and glycosylation signatures, the Prosite scan did not identify any noteworthy motifs. BLOCKs and PRINTS search engines identified only the Elongation factor 1 γ conserved domain (accession number, PF00647A).

5.3.2 HSP27 NORTHERN AND WESTERN ANALYSIS DURING FREEZING

Northern blot analysis was performed on total RNA isolated from hearts of wood frogs frozen for 2, 6 or 24 h at -2.5°C. Figure 5.6A shows a representative northern blot,

demonstrating the change in *hsp27* transcript levels over the freezing time course. Equal loading of total RNA in the different lanes was confirmed by 18S ribosomal RNA band densities, as determined after ethidium bromide staining and photography of each formaldehyde gel (Figure 5.6B). Following standardization against the corresponding control, mean transcript levels in hearts from frogs frozen for 2, 6, or 24 h were determined to be 2.54 (± 0.38 , $n=3$), 2.23 (± 0.36 , $n=3$), and 1.51 (± 0.12 , $n=3$) fold higher than levels in control frogs (5°C acclimated) (Figure 5.5D). In addition, *hsp27* transcript levels in a group of frogs sampled after 24 h thawing (following a 24 h freeze) were 1.90 (± 0.16 , $n=3$) fold higher than control values (data not shown).

Hsp27 protein levels were analyzed by Western blotting. The Western blots prepared with *R. sylvatica* heart homogenates showed one major band with a molecular weight of 30 kDa. Densitometric scanning and quantification of the results revealed that the Hsp27 protein level did not change significantly following freezing exposure (Figure 5.6C). Relative to control values set at 1, the mean Hsp27 protein levels in hearts of wood frogs frozen for 2, 6 or 24 h were 0.85 (± 0.13 , $n=3$), 1.01 (± 0.11 , $n=3$), and 0.88 (± 0.05 , $n=5$) (Figure 5.6D).

5.3.3 EF-1 γ NORTHERN WESTERN ANALYSIS DURING FREEZING

Northern blot analysis was performed on total RNA isolated from *R. sylvatica* heart following a time course of freezing exposure. Figure 5.7A shows a representative northern blot, illustrating the change in *ef-1 γ* transcript levels over the freezing time course. Equal loading of total RNA from each sample was shown by equal 18S ribosomal RNA band densities (Figure 5.7B). Following standardization against corresponding

controls, mean transcript levels in samples from frogs frozen for 2, 6, or 24 h were 1.42 (± 0.19 , $n=3$), 2.04 (± 0.26 , $n=3$), and 2.97 (± 0.34 , $n=3$) fold higher than the levels in control frogs at 5°C (Figure 5.7D).

EF-1 γ protein levels were analyzed by western blottings. Immunoblots prepared with *R. sylvatica* heart homogenates showed a cross-reacting band of ~55 kDa in size. Densitometric scanning and quantification of the results showed a trend towards a slight decrease in cellular levels over a 24 hour time course of freezing exposure (Figure 5.7C,D). Relative to control values set at 1, mean protein contents in hearts of wood frogs frozen for 2, 6 and 24 h were 0.88 (± 0.07 , $n=3$), 0.67 (± 0.13 , $n=3$), and 0.65 (± 0.05 , $n=5$).

5.3.4 HSP27 AND EF-1 γ MULTIPLE TISSUE EXPRESSION ANALYSIS

Northern blots assessed *hsp27* and *ef-1 γ* transcript levels in multiple tissues of control and 24 h frozen wood frogs. Of the seven tissues tested, the heart was the only organ that expressed *hsp27* at detectable levels, either before or after freezing exposure (data not shown). Northern blots detected *ef-1 γ* transcripts in all eight tissues tested but gene expression only changed in the heart following freeze exposure (data not shown).

5.3.5 RECOVERY FROM FREEZING: CHANGES IN HSP27 AND EF-1 γ PROTEIN LEVELS

Possible changes to the cellular Hsp27 and EF-1 γ protein levels during recovery from 24 h of freezing were tested via immunoblot analysis. Figure 5.8 shows that both Hsp27 and the EF-1 γ protein levels increased transiently during thawing protocol. Protein samples prepared from hearts of wood frogs that were thawed for 1, 2, 4, or 8 h had Hsp27 protein levels that were 2.63 (± 0.16 , $n=3$), 2.03 (± 0.39 , $n=3$), 2.02 (± 0.12 , $n=3$),

and 1.37 (± 0.44 , $n=3$) fold higher than levels in 24 h frozen animals (Figure 5.8D). The same protein samples showed EF-1 γ protein levels that were 1.48 (± 0.22 , $n=3$), 2.79 (± 0.17 , $n=3$), 2.42 (± 0.50 , $n=3$), and 1.77 (± 0.22 , $n=3$) fold higher, respectively, than levels in frozen animals (Figure 5.8D).

5.3.6 HSP27 PHOSPHORYLATION DURING THE FREEZE-THAW CYCLE

The sHSPs are subject to phosphorylation at various serine residues in response to different stressors. To assess for changes in the phosphorylation status of Hsp27 during freeze/thaw, two antibodies were used, one that recognizes Hsp27 when it is phosphorylated on serine 15, and another that recognizes phosphoserines 78 and 82. Use of the anti-serine 15 Hsp27 antibody in western blots with samples of *R. sylvatica* heart homogenates showed one major cross-reacting band at a molecular weight of ~20 kDa, about 10 kDa smaller than the size of the cross-reacting protein detected with the anti-Hsp27 antibody. However, application of the anti-serine 78 and 82 phosphorylated Hsp27 antibody showed cross-reaction with one major band of 30 kDa in size, the correct size for the protein. The anti-Hsp27(Ser78/82) antiserum was then used to investigate possible changes in the phosphorylation state of Hsp27 over a freeze-thaw cycle. In agreement with the results shown in Figure 5.6, western blotting showed that the total amount of Hsp27 did not change following exposure to freezing, but analysis with the phospho-specific antiserum showed that the levels of phosphorylated Hsp27 increased in heart during both short and long term freezing (Figure 5.9A). The mean increases in phospho-Hsp27 content were 1.93 (± 0.30 , $n=3$) and 2.35 (± 0.45 , $n=3$) fold following 2 or 24 h of freezing (Figure 5.9B). Analysis of Hsp27 response during thawing (after 24 h frozen)

showed increases in both total Hsp27 and phospho-Hsp27 (Figure 5.9C). Relative to the level of phospho-Hsp27 in hearts from 24 h frozen frogs, thawing elevated phospho-Hsp27 by 4.60 (± 0.66 , $n=3$), 3.72 (± 0.69 , $n=3$), and 2.38 (± 0.81 , $n=3$) fold after 1, 2 or 4 h of thawing, respectively (Figure 5.9D). However, by 8 h thawed, phospho-Hsp27 was reduced to a level, 0.82 (± 0.29 , $n=3$) slightly below that present in 24 h frozen frogs.

5.3.7 RELATIVE PROTEIN AND MRNA SYNTHESIS RATES

To investigate whether freezing exposure actively suppresses the rate of cardiac protein synthesis, the phosphorylation status of the eukaryotic initiation factor 2 alpha (eIF2 α) was analyzed by western blotting. Figure 5.10A shows immunoblots of total and phosphorylated eIF2 α content in heart homogenates from control and 24 h frozen wood frogs. The protein was detected in both instances but the amount of phospho-eIF2 α was considerably higher in frozen frogs. The ratio of phosphorylated eIF2 α to total eIF2 α was determined to be 3.05 (± 0.11 , $n=3$) fold higher in frozen hearts, compared with control animals.

To determine whether freezing was accompanied by a change in the rate of RNA transcription, the nuclear run on assay was performed with nuclei isolated from the hearts of 5°C (control) and 24 h frozen animals. Radiolabeled UTP was used to elongate pre-initiated RNA transcripts from isolated nuclei, and the total amount of ^{32}P -UTP incorporation was determined after Trizol isolation of total RNA. Figure 5.10B shows that the amount of UTP incorporated following incubation of the reaction mixture for different lengths of time was relatively linear. Overall, the nuclei from frozen frogs

incorporated ^{32}P -UTP at a rate that was only 47% ($\pm 9\%$, $n=3$, data from 10 minute incubation) of that observed in the nuclei isolated from control frogs.

5.4 DISCUSSION

5.4.1 CHARACTERIZATION OF *R. sylvatica* HSP27

This chapter assessed the ability of freezing and thawing to elicit a heat shock response. Northern blot analysis of *hsp27* transcripts in wood frog heart sampled over a time course of freezing showed a rapid increase in gene expression over the first 2 hours of freeze exposure; the transcripts remained high at 6 h, but had declined somewhat by 24 h frozen. The expression of *hsp27* is known to vary greatly from organ to organ, and animal to animal, but in general is expressed at the highest level and with the greatest frequency in cardiac tissue (Wilkinson and Pollard, 1993; Miron *et al.*, 1991; Lu and Das, 1993). Our study further confirms this sweeping statement, as the multiple tissue northern blot analysis demonstrated a heart specific expression pattern for *hsp27*, restricting our characterization of the heat shock response to only one tissue of the wood frog.

To further characterize the heat shock response to freezing, Hsp27 total protein content and changes in the amount of phosphorylated Hsp27 were monitored over the freeze-thaw course by western blotting. This analysis showed no change in the total Hsp27 protein over 24 hours of freezing, but identified a significant increase in Hsp27 content during thawing. However, the amount of phosphorylated Hsp27 responded strongly to both freezing (increased 2.4 fold over 24 h) and thawing (rose nearly 5-fold compared with the 24 h frozen value). The response during thawing was very rapid with

phospho-Hsp27 content maximally elevated within 1 h and reduced with longer times of thawing at 5°C. This suggests a probable role for Hsp27 in the metabolic recovery of heart function after freezing. As mentioned previously, MAPKAPK-2 appears to be the primary kinase responsible for stress-induced Hsp27 phosphorylation and this enzyme is, in turn, phosphorylated by p38 MAPK (Stokoe *et al.*, 1992; Kyriakis *et al.*, 1996). Significantly, a recent analysis of MAPK responses to freeze/thaw by wood frog tissues showed a progressive increase in the amount of active phospho-p38 during freezing (rising ~7.5-fold over 12 h) that increased even more during thawing, rising within 4 h to ~13-fold higher than the amount in 12 h frozen frogs (Greenway and Storey, 2000). This parallel increase in p38 and Hsp27 strongly implicates p38 as the regulator of Hsp27 activation during freeze/thaw in wood frog heart.

The observed kinetics of freezing and thawing induced changes to Hsp27 transcription, translation, and phosphorylation are analogous with what has been observed in other systems. A recent summary of the kinetics of Hsp27 metabolism following heat exposure shows a two-phase response (reviewed in Arrigo and Landry, 1994). The initial, or short term, phase is characterized by the phosphorylation of endogenous Hsp27 that begins within a few minutes of the stress and lasts for only a few hours. The second, delayed phase, involves the synthesis of new Hsp27, and generally begins during the recovery after stress. This same pattern, although on a different time frame, was observed during the *R. sylvatica* freeze-thaw cycle.

Thus, we now have good evidence of the operation of a second signal transduction pathway triggered by freezing in the freeze tolerant wood frog. The first is the well-studied pathway for activation of cryoprotectant (glucose) production by liver

that is initiated from beta-adrenergic receptors on the cell surface and transmitted via cAMP and protein kinase A into an activation of glycogenolysis (Storey and Storey, 1992). This second pathway, mediated by the p38 MAPK signal transduction module appears to stimulate, as at least one of its end points, both the phosphorylation of Hsp27 and the increased synthesis of the protein. Further work remains to determine, (i) the role of Hsp27 in freeze survival, (ii) the trigger for the p38/Hsp27 response, and (iii) the contribution of other signaling pathways to the observed pattern of changes to the Hsp27 transcript and protein up-regulation, and phosphorylation.

5.4.2 ELONGATION FACTOR 1 γ

The Hsp27 investigation demonstrated that although the transcript level increases during freezing, no increase in the protein content was detected until thawing. To determine whether this occurrence was specific to Hsp27, or if the same pattern could be observed with other freeze up-regulated transcripts, the EF-1 γ gene and protein levels were also measured over the freeze-thaw cycle. EF-1 γ is one of the four subunits (EF-1 α , EF-1 β , EF-1 β' , EF-1 γ) of the eukaryotic elongation factor 1 (EF-1), a protein that plays a role in the elongation of polypeptide chains during their translation on the ribosome (Kobayashi *et al.*, 2001). EF-1 α catalyzes the GTP-dependent binding of aminoacyl tRNA to the A-site of the ribosome, while EF-1 γ and EF-1 β/β' together function to catalyze GDP/GTP exchange of EF-1 α . Over-expression of EF-1 γ has been observed previously, but mainly in cell culture and cancerous systems including carcinomatous gastric, colon, oesophageal, and pancreatic tissues (Mimori *et al.*, 1996; Sanders *et al.*, 1992).

Northern blot analysis of *ef-1 γ* confirmed the preliminary findings from the cDNA library screening, showing an increase in transcript levels over a time course of freezing. Similar to what was observed with Hsp27, an analysis of the EF-1 γ protein level indicated no increase in the translation rate during the freezing process. However, an analysis of the EF-1 γ protein levels in the hearts of animals allowed to thaw following 24 hours of freezing exposure revealed that the increased number of mRNA are translated during the recovery period, increasing the EF-1 γ protein content in the cells during the first few hours of thawing. The synthesis of EF-1 γ as an early response to thawing could contribute to the reactivation of protein synthesis as animals recover from the ischemic, frozen state.

5.4.3 FREEZING INDUCED CHANGES TO THE RATES OF PROTEIN AND RNA SYNTHESIS

One possible explanation for why the freeze-induced increase in *ef-1 γ* and *hsp27* transcript levels does not translate into increased protein levels might be the suppression of cellular protein synthesis during freezing. Obviously, selective synthesis of some proteins takes place during freezing (Cai *et al.*, 1997; Storey *et al.*, 1997; Chapters 6,7) but a generalized reduction in protein biosynthesis in the frozen state could be an important energy-saving mechanism that ensures long-term survival. Two approaches have been adopted by our laboratory to test for changes in the rates of protein synthesis following exposure to different environmental conditions: a cell-free *in vitro* translation technique, and a western blot approach that analyzes the phosphorylation state of eIF2 α . The *in vitro* system assesses for changes in protein synthesis rates by comparing [³H]-leucine incorporation into acid-precipitable post-mitochondrial supernatants in tissue

extracts from control versus stressed animals. This has been used recently to demonstrate suppressed protein synthesis in *Littorina littorea* during anoxia exposure, and in *Spermophilus tridecemlineatus* during hibernation (Larade and Storey, 2002; Hittel and Storey, 2002). However this technique was not amenable with the wood frog model of freeze tolerance because of the large amount of tissue required.

The second approach, an immunoblot analysis, is based on the fact that translation initiation is controlled by the activity of the translational factor eIF2 α . eIF2 α activity itself is regulated via reversible phosphorylation and increased phosphorylation of eIF2 α has been correlated with a reduction in protein synthesis in a diverse number of systems (Rhoads, 1993). When phosphorylated, eIF2 α serves as a competitive inhibitor of eIF2B, the guanine nucleotide exchange factor responsible for catalyzing the removal of GDP from eIF2. By obstructing nucleotide exchange the alpha subunit prevents the assembly of eIF2, Met-tRNA, and GTP ternary complexes, and the delivery of peptide-initiating methionine to the 40S ribosomal subunit, a key step in translation (DeGracia *et al.*, 2002). Western analysis demonstrated that the amount of phosphorylated eIF2 α was significantly increased following freezing exposure, providing evidence for the active suppression of protein synthesis and an explanation for the lack of synthesis of additional Hsp27 and EF-1 γ during the freezing episode.

Another major energy-consuming processes in most cells is transcription. The dependence of RNA synthesis on ATP and GTP has been demonstrated in both prokaryotic and eukaryotic systems, and has been estimated to account for up to 10 % of total cellular energy consumption (Gaal *et al.*, 1997; Rolfe and Brown, 1997). To determine whether exposure to freezing is associated with a change in transcription rate

the nuclear run-on assay was performed. With this technique it is possible to identify relative differences in the number of transcripts being initiated and synthesized following exposure to environmental or physiological stresses. The nuclear run-on assay that was performed used nuclei isolated from control and frozen frogs, and elongated the mRNA transcripts that were being actively transcribed at the time of isolation at a common assay temperature, 26 °C (Groudine *et al.*, 1981). Assaying both sets of nuclei at the same temperature ensured that any apparent difference was not the result of a temperature-dependent effect on the metabolic rate, but a stable change (e.g. phosphorylation) in the capacity of the transcriptional machinery. Like other anoxia tolerant organisms, *R. sylvatica* demonstrated the ability to regulate the rate of transcription in the heart when faced with conditions (freezing) that reduce oxygen and nutrient availability. During freezing, wood frog heart displayed a significant arrest of nuclear transcription, with a transcription rate estimated to be <50 % of that found in 5°C control frogs.

5.4.4 PERSPECTIVES

The results presented in this chapter show that the cellular rates of transcription and translation are reduced during freezing in the wood frog heart, consistent with an energy-saving strategy that would aid long term survival under the ischemic conditions of the frozen state. Despite the overall reduction in transcription this thesis has identified that the transcript levels of specific genes are increased significantly during freezing. However, the results have also indicated that the translation status or association of these mRNAs with actively translating polyribosomes seems to be gene specific. This stress induced transcription, but not translation, of distinct groupings of genes parallels recent

findings in our own laboratory, and by other researchers, investigating the cellular response following the non-lethal disruption of cellular homeostasis (Hittel, 2001; Larade, 2002). It has been suggested that these mRNA are being sequestered into a monosome/RNP fraction (stress granules, SGs) during periods of stress-induced translational arrest, and are translated once polysomes reform during recovery (Kedersha *et al.*, 1999; Kedersha *et al.*, 2000). A review of the existing understanding of SGs, including their components and the mechanism leading to their formation has been provided by Anderson and Kedersha (2002). Mammalian SGs are described as cytoplasmic domains into which mRNAs are sorted in response to phosphorylation of eukaryotic initiation factor eIF2 α . SGs are believed to contain many, if not all, of the components of the preinitiation complex, but no 60S ribosomal subunits. Phosphorylation of eIF2 α decreases the levels of eIF2-GTP-tRNA(Met), the ternary complex that loads tRNA(Met) onto the small ribosomal subunit and allows the initiation of protein translation. These changes permit the binding of the RNA-binding proteins TIA-1 (T-cell internal antigen-1) and TIAR (TIA-1-related protein) to the preinitiation complex, leading to SG formation, polysome disassembly, and the concurrent routing of the mRNA into a SG. RNA-binding proteins that stabilize or destabilize mRNA are also recruited to SGs.

Some evidence for the occurrence of a similar cellular response in the cardiomyocytes of frozen wood frogs is provided by our findings of eIF2 α phosphorylation, an event that has been linked to the formation of cytoplasmic stress granules. Further studies will be required to confirm the existence of these stress granules in the cells of frozen wood frogs.

FIGURE 5.1

Diagram illustrating the protective properties of Hsp27.

Hsp27 has been reported to have four different cytoprotective properties. These properties have been shown to be regulatable post-translationally, with the p38 MAPK pathway believed to be the primary signaling pathway involved. Under the current model phosphorylation of Hsp27 leads to the de-aggregation of the high molecular weight oligomers, giving rise to Hsp27 dimers. The high molecular weight Hsp27 oligomers have been attributed the chaperonin and cellular oxidative activities, whereas the phosphorylated dimers have the actin binding properties and the ability to prevent signaling through apoptotic pathways.

FIGURE 5.2

Nucleotide and deduced amino acid sequence for *R. sylvatica* clone *b4*, identified as the Heat Shock Protein 27.

Nucleotides and amino acids are number on the left. The complete open reading frame was predicted to encode a polypeptide with 213 residues. The start codon (ATG), stop codon (TAA) and potential polyadenylation signals (AATAAA) are shown in bold. Three MAPKAP kinase recognition motifs containing phosphorylatable serine residues have been underlined. The serine residues are located at positions 16, 91 and 95 in the deduced amino acid sequence.

1 GGCACGAGGGACAGAGCAAAAACAGCTTGTTGAACAGTCCCATAGACCCAGACC**ATG**TCT
 1 M S
 61 CAGAACGTAGAATTCCCTTCACTTTCTTCGCACCCCAAGCTGGGAACCATTCAGAGA
 21 S E R R I P F T F L R T P S W E P F R D
 121 TGGTACCAAGGTACCAACCGACTGTTTGATCAGTCTTTTGGTATGCCAAGAATTCCTGAA
 41 W Y Q G T N R L F D Q S F G M P R I P E
 181 GATTGGTACCAATGGCCTAGCACAAATTGGCCAGGCTATGTCCGTCTTTTTCCAGTCAA
 61 D W Y Q W P S T N W P G Y V R L F P S Q
 241 TCAGTGGATGCTGTTCTCCTACTACTCCAGCTGTAGTTTCTCCTGCAGCAGCAACTGTT
 81 S V D A V P P T T P A V V S P A A A T V
 301 CCTGATTTAAACAGGGCACTAAGTCGGCAACTTAGCAGTGGAATTTTCAGAGATCCGACAA
 101 P D L N R A L S R Q L S S G I S E I R Q
 361 ACAGCCGACTGCTGGAAGATCAGCTTAGATGTCAACCACTTTTCTCCTGAGGAACTGGTT
 121 T A D C W K I S L D V N H F S P E E L V
 421 GTCAAAACTAGAGATGGTATAGTGGAAATCACAGGAAAACACGAAGAGAAACAAGATGAA
 141 V K T R D G I V E I T G K H E E K Q D E
 481 CATGGATTTATCTCAAGATGCTTTACAAGAAAATATACTCTTCCCCCAGGTGTTGATATT
 161 H G F I S R C F T R K Y T L P P G V D I
 541 GCAAGTGTGGCTTCATCTCTTTCTCCGGATGGTGTCTTGACAGTAGAGGCACCACTCCCC
 181 A S V A S S L S P D G V L T V E A P L P
 601 AAGCCAGCAATCCAGTCTGCAGAGATCACTATTCCAGTAAGTTTCCAAAGCTGCGCTGAG
 201 K P A I Q S A E I T I P V S F Q S C A E
 661 ATTGCTAAGAAGCCTGATGAGCCTGCCAAAAA**TAAG**AGGATAAAACCACTGTGTTTTCTT
 221 I A K K P D E P A K K *
 721 TTACTGTGTTTCAGTCACATATTTTTTACACAAAAA**AATAAA**TTTTGAAAA**AATAAA**TGAA
 781 AAAAAAAAAAAAAAAAAA

FIGURE 5.3

Amino acid alignment of the *R. sylvatica*, chicken, canine, and human Hsp27 proteins.

The putative amino acid sequence of the wood frog protein was aligned with the corresponding chicken, canine, and human sequences (Genbank accession numbers Q00649, AAA87172, AAH00510, respectively). For convenience only those amino acids differing from the wood frog sequence are indicated; dashes (-) represent amino acids in the other sequences that are shared with the wood frog. Periods are present in the alignment to indicate amino acids that are not present in the coding region of one species. The putative conservation of the MAPKAP kinase recognition motifs has been indicated (boxes).

Woodfrog	MSSERRIPFTFLRTPSWEPFRDWYQ.GTNRLFDQSFGMPRIPE	DWYQWPS
Chicken	M.A---V-----TS-----	H.-S.-----H-----K---
Canine	M.T---V--SL--S--D-----	PAHS.-----A--L--L---E-A-FG
Human	M.T---V--SL--S--D-----	P.HS.-----A--L--L---E-A-FG

Woodfrog	TN.WPGYVRLFPSQSVD	AVPPTTP....AVVSPAAATVPDLNRALS	SRQLS
Chicken	GSA----F--L--E-ALLPA-GSP....YG.....		E.--
Canine	HSG-----PI-PAVEGPAAAAAAA	PAYS.....	
Human	GSS-----PL-PAAIESPAVAA...PAYS.....		

Woodfrog	SGISEIRQTADCWKISLDVNHFSPEELVVKTRDGIVEITGKHEEKQDEHG
Chicken	-----S--S--VT-----A-----K-N-----
Canine	--V-----R-RV-----A---T---K--V-----R-----
Human	--V---H---R-RV-----A-D--T---K--V-----R-----

Woodfrog	FISRCFTRKYTLPPGV	DIASVASSLSPDGVLTVEAPLPKPAIQSAEITIP
Chicken	-----EATA-R-----M-----S-----	
Canine	Y---RL-P-----PTL-S-----E-T-----M---T-----	
Human	Y-----PTQ-S-----E-T-----M--L-T--N-----	

Woodfrog	VSFQ.....SCAEIAKKPDEPAKK
Chicken	-TVE.....A-KE-----
Canine	-T-EARAQIGGP-AG-SEQSG--
Human	-T-ESRAQLGGP-A--SDETA--

FIGURE 5.4

Nucleotide and deduced amino acid sequence of the *R. sylvatica* clone *h25*, identified as eukaryotic Elongation Factor 1 γ .

Nucleotides and amino acids are numbered on the left. A single open reading frame is predicted to encode a polypeptide with 308 residues. The stop codon (TGA) and potential polyadenylation signals (AATAAA) have been placed in bold.

1 CGGCACGAGGGCCAAGGAGGAGGTGAAGCATGTTCTGGCTACTCTGGATTCCCATCTGCA
1 G T R A K E E V K H V L A T L D S H L
61 GACAAGAACTTTTCTGGTGGGAGAGAGAGTACCCTGGCTGACATCACTGTAGCATGCGC
20 T R T F L V G E R V T L A D I T V A C A
121 CCTGCTTTGGCCGTATAAACAAGTCCTGGAGCCTTCCTTCCGTCAGCCTTATGGTAATGT
40 L L W P Y K Q V L E P S F R Q P Y G N V
181 CACAAGATGGTTTGTGACATGTGTGAACCAGCCCCAGTTCCATGCTGTGCTGGGAGAGGT
60 T R W F V T C V N Q P Q F H A V L G E V
241 GAAACTCTGCGACAAAATGGCTCAGTTTGTATGCCAAAAAATTTGCAGAGTTGCAGCCAAA
80 K L C D K M A Q F D A K K F A E L Q P K
301 GAAGGAGACCCCTAAGAAGGAGAAACCAGCAAAGGAGCAAAAGAAAGAGAAGGAGGAAAA
100 K E T P K K E K P A K E Q K K E K E E K
361 GAAGCCTGCTCCCGCCCCAGCAGCTGCTCCAGAAGATGATTTGGATGAGTCTGAGAAGGC
120 K P A P A P A A A P E D D L D E S E K A
421 TCTTGCAGCAGAGCCAAAGTGAAGGACCCCTATGCTCATCTCCCTAAGAGCTCCTTCAT
140 L A A E P K S K D P Y A H L P K S S F I
481 TATGGATGAGTTTAAACGAAAGTATTCAAATGAAGACACACTTCTGTGGCGCTTCCTTA
160 M D E F K R K Y S N E D T L S V A L P Y
541 CTTCTGGGAGCACTTTGACAAAGAGGGATGGTCCATCTGGTATTACAGGTACCGTTTCCC
180 F W E H F D K E G W S I W Y S E Y R F P
601 AAATGAACCTTTCACAACCCTTTATGAGTTGCAATCTCATTACAGGTATGTTCCAGCGTCT
200 N E L S Q P F M S C N L I T G M F Q R L
661 GGACAAGCTGCGTAAAACAGGCTTTGCTAGCGTCATCCTGTTTGGAAACCAACAATGACAG
220 D K L R K T G F A S V I L F G T N N D S
721 TTCTATCTCCGGCGTATGGGTGTTTCAGAGGACAGGATTTGGCATTACACTGTCTGAGGA
240 S I S G V W V F R G Q D L A F T L S E D
781 TTGGCAAATTGATTACGAGTCCTATGCCTGGAGGAAGCTGGATTTCAGACAGCGAGGAGTG
260 W Q I D Y E S Y A W R K L D S D S E E C
841 CAAGACCCTGGTAAAAGAGTATTTCTGCTGGGAAGGAGACTTCACGCATGTAGGCAAAGC
280 K T L V K E Y F C W E G D F T H V G K A
901 CTTCAACCAGGGCAAGATCTTCAAGTGAGGCCCTCTGGGAGGAAGAGAGGAGCCGTCTTT
300 F N Q G K I F K *

961 CATTTTGAAGAGATCTGGGACATCAATTTCTTTTCTCTTCTCGACCACTCAGACGTTTTA
1021 ACTTAAGGCAGCAAAACAAAA**AATAAAA**TAAAAACCTGAAGTCAAAAAAAAAAAAAAAAAA
1081 A

FIGURE 5.5

Amino acid alignment of the *R. sylvatica*, *Xenopus laevis*, and human eukaryotic Elongation Factor 1 γ proteins.

The putative amino acid sequence of the wood frog protein is compared with the corresponding *X. laevis* and human sequences (Genbank accession numbers X62508 and BC007949, respectively). For convenience only those amino acids differing from the wood frog sequence are indicated; dashes (-) represent amino acids in the other sequences that are shared with the wood frog. Periods are present in the alignment to indicate amino acids that are not present in the coding region of one species.

Woodfrog	GTRAKEEVKHVLATLDSHLQTRTFLVGERVTLADITVACALLWPYKQVLE
Xenopus	TEQ---GIKT--GV--S-----I-----T-S---L-----
Human	TEN---E-RRI-GL--AYLK-----V-T---L-----
Woodfrog	PSFRQPYGNVTRWFVTCVNQPQFHAVLGEVKLCDKMAQFDAKKFAELQPK
Xenopus	-----E-R-----M---
Human	----AFP-TN---L--I----R-----E-----T---
Woodfrog	KETPKKEKPAKEQKKE.....KEEKKPAPAPAA.APEDDLDESEKALAAE
Xenopus	-----P---.....K-APTP-P-----
Human	-D--R---GSR-E-QKPQAER-----A-AP.....EEM--C-Q-----
Woodfrog	PKSKDPYAHLPKSSFIMDEFKRKYSNEDTLSVALPYFWEHFDKEGWSIWY
Xenopus	-----T-----
Human	--A---F-----T-VL-----D---L--
Woodfrog	SEYRFPNELSQPFMSCNLITGMFQRLDKLRKTGFASVILFGTNNDSSISG
Xenopus	A--K--E--T-A-----
Human	-----E--T-T-----NA-----S-----
Woodfrog	VWVFRGQDLAFTLSEDWQIDYESYAWRKLDSDSEECKTLVKEYFCWEGDF
Xenopus	-----N-----G-----A---E-
Human	-----E---P--P---V-----T-----PG---TQ---R---S---A-
Woodfrog	THVGKAFNQGKIFK
Xenopus	KN---P-----
Human	Q---K-----

FIGURE 5.6

Analysis of the Hsp27 transcript and protein levels in *R. sylvatica* heart over a time course of freeze exposure.

Hearts were sampled from frogs acclimated at 5°C (Con) or given freezing exposure at -2.5°C for 2, 6, or 24 h.

- A. A representative Northern blot prepared with total RNA isolated from the hearts hybridized with [³²P]-labeled *hsp27* cDNA.
- B. Corresponding 18S rRNA band on the ethidium bromide stained formaldehyde gel demonstrating equal loading (16 µg total RNA per lane).
- C. A Western blot probed with anti-Hsp27 showing relative protein levels over the freezing time course. Each lane was prepared with 25 µg of total soluble protein.
- D. Histogram showing the relative mean transcript and protein levels of Hsp27 ($\pm$ SEM, n=3 independent blots). Bands intensities on individual blots were scanned, control values were then set to 100, and band intensities in other lanes were expressed relative to their control.

FIGURE 5.7

Analysis of Elongation Factor 1 γ transcript and protein levels in *R. sylvatica* heart over a time course of freeze exposure.

Hearts were sampled from frogs acclimated at 5°C (con) or given freezing exposure for 2, 6, or 24 h at -2.5°C.

- A. A representative Northern blot prepared with total RNA isolated from hearts at different sampling times and hybridized with ³²P-labeled *ef-1 γ* cDNA.
- B. Corresponding 18S rRNA band on the ethidium bromide stained formaldehyde gel demonstrating equal loading (16 μ g total RNA per lane).
- C. Western blot probed with anti-EF1 γ antiserum showing the relative expression level of the protein in hearts over the freezing time course. Each lane was prepared with 25 μ g of total soluble protein.
- D. Histogram showing the relative mean transcript and protein levels of EF1 ($\pm$ SEM, n=3 independent blots). Bands intensities on individual blots were scanned, control values were then set to 100, and band intensities in other lanes were expressed relative to their control.

FIGURE 5.8

Western blots and histograms showing Hsp27 and EF-1 γ protein levels in wood frog heart over a time course of thawing.

Hearts were sampled from wood frogs given 24 h freezing at -2.5°C followed by thawing at 5°C for 1 h, 2 h, 4 h, or 8 h. Each lane was loaded with 25 μ g of soluble protein. Representative immunoblots probed with (A) anti-Hsp27 antibody and (B) EF-1 γ antiserum are shown. (C) Histogram showing relative levels of Hsp27 ($\pm$ SEM, n=3), and EF1 γ ($\pm$ SEM, n=5) protein. Band intensities on individual blots were scanned, frozen values were then set to 100, and band intensities in other lanes were expressed relative to the corresponding 24 h frozen sample.

A

B

C

FIGURE 5.9

Western blots and histograms comparing the total and phosphorylated Hsp27 content in the hearts of wood frogs collected over a freeze-thaw cycle.

Total soluble protein was isolated from the hearts of wood frogs exposed to different lengths of freeze exposure (2 hrs frozen, 24 hrs frozen) and animals exposed to a time course of thawing following 24 hours of freezing (1 hr thaw, 2 hrs thaw, 4 hrs thaw, 8 hrs thaw). Western analysis was performed by incubation of duplicate blots with antibodies to Hsp27 and then to phosphospecific Hsp27. Data presented in the histogram is the mean ($\pm$ SEM) of three independent blots. (A) Representative western blots showing the relative levels of Hsp27 and phosphorylated Hsp27 over a short time course of freezing. (B) Histogram showing the change in total and phosphorylated Hsp27 over the freezing time course. Band intensities on individual blots were scanned; control values were then set to 100, and band intensities in other lanes were expressed relative to the control sample. (C) Representative western blots showing the relative level of Hsp27 and phosphorylated Hsp27 during thawing. (D) Histogram showing the total and phosphorylated Hsp27 content over the thawing time course. Band intensities on the individual blots were scanned, 24 hour frozen values were then set to 100, and band intensities in the other lanes were expressed relative to the 24 hour frozen sample.

FIGURE 5.10

Evaluation of the impact of freezing exposure on the rate of protein synthesis and RNA transcription in the heart of the wood frog.

A. Total soluble protein samples (25 $\mu\text{g}/\text{lane}$) prepared from the hearts of control frogs acclimated to 5°C or frozen frogs held for 24 h at -2.5°C were separated on a 12 % SDS-PAGE gel and blotted. Duplicate blots were probed with an anti-eIF2 α antibody to show total eIF2 α content and with an antibody specific to the phospho-peptide to assess changes in the phosphorylation state of the protein. Accompanying histogram shows the ratio of phosphorylated eIF2 α band intensity to total eIF2 α for three different control and frozen samples.

B. Nuclear Run On Assay. In vitro incorporation of radiolabeled ^{32}P -UTP (per mg protein) into pre-initiated RNA transcripts present in nuclei isolated from the hearts of control (empty circles) and frozen (filled circles) *R. sylvatica*. For each trial, in vitro transcription was allowed to proceed for 5, 10 and 20 minutes at 26 °C prior to total RNA extraction. Each data point represents an average of three independent nuclear isolations ($\pm$ SEM).

A

B

CHAPTER 6

FREEZE INDUCED UP-REGULATION OF α NAC IN THE HEART OF THE WOOD FROG

6.1 INTRODUCTION

6.1.1 AIM OF CHAPTER

One of the freeze up-regulated clones obtained through differential screening of the wood frog heart cDNA library (Chapter 3) was identified as the alpha subunit of the nascent associated complex (α NAC). This chapter takes an in-depth look at α NAC expression and its possible role in freezing survival. α NAC up-regulation is assessed over a complete time course of freeze exposure, and the subsequent analyses searched for freeze-induced changes in the *onac* transcript levels in other tissues of the wood frog and in the heart following exposure to dehydration and anoxia stresses. Western blot analysis was also used to determine the impact of freezing on the protein levels of α NAC and various other AP-1 associated proteins.

6.1.2 OVERVIEW

Although significant progress has been made in many areas of science over the last number of decades, few have had an expansion of the magnitude experienced by the fields of molecular biology and biochemistry. With the advent of new approaches (e.g. cDNA library screening, PCR) and the increased effectiveness of older techniques (2-D analysis, mass spectrometry, sequencing) our understanding of a wide range of cellular processes, and the genes and proteins involved, has been greatly improved. During this time the pace of novel gene and protein discovery has increased to the point that the tools available for protein characterization have become inadequate in comparison and the cellular and physiological functions of most of the recently discovered novel proteins

remain unknown. Interestingly though, the converse holds true for a trivially small group of proteins for which multiple cellular functions have been reported, as is the case for the protein products of the two freeze inducible genes *hsp27* and *αnac* (Arrigo 1998; Arnaud, 1999).

6.1.3 NAC AND PROTEIN TRANSLOCATION

Cellular homeostasis requires the establishment and maintenance of proteins and their associated activities in various subcellular locations and membrane-bound organelles. Proteins, however, do not arrive at their correct subcellular location by chance and a complex system exists to ensure proper protein trafficking. The first step in a proteins journey to its final location begins as it is being translated, when the cell must determine whether the ribosome is synthesizing a cytosolic protein or a polypeptide requiring transfer to the endoplasmic reticulum for secretion, membrane insertion, or post-translational modification. Two of proteins reported to play significant roles in this recognition and targeting process are NAC and the apply named signal recognition particle, SRP. The contribution of SRP was established first; binding nascent chains that possess signal peptides as they emerge from the ribosome and targeting these ribosome-nascent peptide complexes to the ER for further translation and peptide translocation (reviewed in Keenan *et al.*, 2001). However, SRP has since been suggested to only act as a catalyst in this regard, with in vitro studies demonstrating that non-translating ribosomes and ribosomes containing nascent chains (with and without signal peptides) can all interact with ER membranes (Wiedmann *et al.*, 1994; Lauring *et al.*, 1995). It is currently believed that it is the ubiquitously expressed NAC protein that works to prevent

these inappropriate interactions in vivo; the details of which are being worked out by the research laboratory of Dr. Wiedmann in New York (Beatrix *et al.*, 2000).

NAC, a heterodimeric protein consisting of a 33 kDa (α) subunit and a 21 kDa (β) subunit, is the first protein to contact nascent polypeptide chains as they emerge from the ribosome (Wiedmann *et al.*, 1994; Munz *et al.*, 1999). The most recent study investigating the cellular function of NAC evaluated the role of the different subunits in nascent polypeptide sorting (Beatrix *et al.*, 2000). This study reported that both subunits are in contact with the nascent chain on the ribosome, and that both of the subunits are required to prevent SRP targeting of signal-less chains. In contrast however, the β subunit alone appears to be responsible for blocking the ribosomal M-site and preventing the inappropriate targeting of non-translating ribosomes. Based on these and previous findings from the Wiedmann group the following mechanism of nascent chain translocation to the endoplasmic reticulum (ER), has been proposed (Beatrix *et al.*, 2000).

They hypothesize that as the nascent chain lengthens, the interaction of NAC with the ribosome begins to weaken, and for proteins that contain a signal sequence this allows the signal recognition particle (SRP) to more effectively compete for binding. Once SRP displaces NAC, the ribosome is targeted to the ER. At the ER membrane the SRP receptor displaces the SRP, and the M-site previously blocked by NAC is free to bind to the translocon and the nascent chain enters the lumen of the ER. The interaction between SRP and NAC, leading to proper nascent chain targeting is illustrated in Figure 6.1.

6.1.4 NAC AND AP-1 TRANSCRIPTIONAL CO-ACTIVATION

In addition to the body of research outlining a ribosome-associated role for NAC, a series of studies outlining an additional function for α NAC have been published recently from the research laboratory of Dr. St. Arnaud. These reports originated from a single differential display PCR experiment comparing gene expression levels in mineralizing osteoblasts with dedifferentiated, non-mineralizing cells. This first study found that the gene for the murine homolog of α NAC was differentially regulated, and Northern blot analysis both confirmed the ddPCR finding and identified the presence of a differentially spliced muscle isoform (Yotov *et al.*, 1996). Based on sequence similarities with some transcriptional regulatory proteins, and the identification of the transcription factor BTF3b as a α NAC heterodimerization partner, studies evaluating the role of the protein in transcriptional regulation were performed (Yotov *et al.*, 1996). This assessment reported that both α NAC and the skeletal muscle isoform of α NAC (skNAC) were capable of sequence-specific DNA binding, although only skNAC was observed to be transcriptionally active (Yotov *et al.*, 1996). Nevertheless, the movement of the protein into the nucleus following appropriate stimulation encouraged the St. Arnaud group to explore the possibility that α NAC might function as a transcriptional co-activator - a protein not independently transcriptional activity, but capable of enhancing the activity of other transcription factors. Co-transfection experiments eventually found that α NAC could enhance reporter expression levels when used in combination with the transcription factor VP-16 (Yotov *et al.*, 1998; Sadowski *et al.*, 1988). The search for the transcription factor that α NAC interacts with in differentiating bone cells identified the protein as a potent co-activator of AP-1 (Moreau *et al.*, 1998). See Figure 6.1 for illustration.

6.2 MATERIALS AND METHODS

6.2.1 GENE AND PROTEIN SEQUENCE ANALYSIS

The initial analysis of wood frog α NAC nucleotide and deduced amino acid sequence was performed using DNAMAN and the BLAST database search engine located on the National Centre for Biotechnology Information website. DNAMAN was used to align the wood frog with other gene and protein sequences obtained from Genbank. As well, the PROSITE and BLOCKS programs were used to identify domains and signature sequences present in the amino acid sequence.

6.2.2 NORTHERN BLOT ANALYSIS

For the initial gene expression study, total RNA was isolated from hearts of *R. sylvatica* exposed to various lengths of freezing (2, 6, or 24 h), anoxia (4 or 24 h) or dehydration (20% or 40% of total body water lost) exposure, as described in section 2.2.2. The RNA was then quantified, electrophoresed, and blotted to nitrocellulose, as described in sections 2.5.1 thru 2.5.3. Similarly, for the multiple tissue transcript expression study, Northern blots were prepared using total RNA isolated from brain, gut, kidney, liver, lung, skin, and skeletal muscle of both control and 24 h frozen wood frogs. To conclude the gene expression studies, additional Northern blots were prepared from total RNA isolated from skeletal muscle of *R. sylvatica* exposed to a time course of freezing (2, 6, or 24 h). Northern blots were pre-hybridized at 65°C for 1 h, prior to 12 h hybridization at 44°C with gene-specific radiolabeled probe made to *anac* cDNA as

described in sections 2.5.4 and 2.5.5. Blots were repeatedly washed and exposed to X-ray film until a single band was visible.

6.2.3 WESTERN ANALYSIS

Total soluble protein was extracted from hearts and skeletal muscle of wood frogs exposed to different lengths of freezing (Sections 2.11.1). Samples containing 25 µg protein were subjected to electrophoresis on 12 % SDS-polyacrylamide gels followed by transfer to nitrocellulose as described in sections 2.11.2. The alpha subunit of NAC was detected on Western blots following incubation with a 1:3000 dilution of rabbit polyclonal antibody (a gift from Dr. St. Arnaud) prepared in a 1 % blocking solution (1 % non-fat dry milk in TBST), that was allowed to incubate at 4° C for 12 h. Rabbit antibodies capable of detecting the c-Jun, ATF2, and c-Fos AP-1 family members were obtained from Cell Signaling Technology, as were phosphorylation state specific antibodies for c-Jun (Ser63) and ATF2 (Ser69/71). The primary conditions permitting antibody binding for these transcription factors were established to be overnight incubation at 4°C in a 1:1000 dilution (0.25% non-fat dry milk, TBST). Washing and incubation with secondary antibodies were carried out as described in section 2.11.3 for all Western blots.

6.3 RESULTS

6.3.1 GENE AND PROTEIN ANALYSIS

Nucleotide and deduced amino acid sequences of *R. sylvatica* heart clone *ba* are shown in Figure 6.2. These sequences were compared to the GenBank database using the BLAST search engine and clone *ba* was identified as the *R. sylvatica* homologue of the α subunit of Nascent Polypeptide Associated Complex. A full open reading was identified and the size of the transcript that hybridized with radiolabeled wood frog *onac* was estimated to be 0.9 kb in size. At present, only the human (accession number AY034001) and mouse (accession number U22151) gene transcripts have been sequenced, and both are reported to be approximately 0.9-1.0 kb in size. Alignment of the open reading frames for the three *onac* cDNA sequences showed that the mouse and human sequences were 89 % identical, whereas the wood frog sequence showed 74 and 79 % identity with the mouse and human sequences, respectively.

Alignment of the putative amino acid sequence of wood frog α NAC with the human (accession number, AY034001), mouse (accession number, U22151), and *Drosophila melanogaster* (accession number, AAB97513) amino acid sequences is presented in Figure 6.3. The three vertebrate proteins show very high sequence identity; with wood frog α NAC having 96 and 95% identity with the human and mouse sequences, respectively. However, only 55% of the amino acid residues were identical between the wood frog and fruit fly sequences. The BLAST analysis of the wood frog α NAC protein sequence identified two significant domain alignments. The first, designated the NAC domain (pfam accession numbers, pf01849 and pf3674), was

described as a domain found in the archaeal and eukaryotic BTF proteins involved in translation and transcription, and extends through positions 71 to 130 in the amino acid sequence, Figure 6.2 (Makarova *et al.*, 1999). The second signature, the EFTS_N domain, is described as a conserved domain containing 3 helices seen in the N-terminus of a number of EF-TS and eukaryotic NAC proteins (pfam accession number, pssm-Id3664); in the *R. sylvatica* protein this domain stretches from amino acid residues 178 to 213.

6.3.2 α NAC NORTHERN BLOT ANALYSIS

6.3.2.A HEART GENE EXPRESSION ANALYSIS

Northern blot analysis was performed on total RNA isolated from the hearts of wood frogs given different lengths of freezing exposure. Figure 6.4A shows a representative northern blot and the corresponding ribosomal RNA bands demonstrating equal loading are shown in Figure 6.4B. Following standardization, mean transcript levels in samples from frogs frozen for 2, 6, or 24 hours were determined to be 1.21 (± 0.28 , $n=3$), 2.05 (± 0.16 , $n=4$), and 2.93 (± 0.24 , $n=5$) fold higher than levels in 5°C acclimated frogs (Figure 6.4D). A similar analysis of total RNA isolated from heart of anoxic and dehydrated frogs was performed. Following anoxia exposure for 4 or 24 hours *onac* transcript levels had decreased to 82 % (± 14 , $n=4$) and 83 % (± 3 , $n=4$) of the level observed in control animals (data not shown). Furthermore, the loss of 40 % total body water did not substantially change *onac* transcript levels in hearts; the mean transcript level was 110 % (± 4 , $n=3$) compared with control hearts (data not shown).

6.3.2.B MULTIPLE TISSUE EXPRESSION ANALYSIS

The effect of 24 h freezing on *onac* transcript levels in seven other organs was analyzed using total RNA isolated from brain, gut, kidney, liver, lung, skin, and skeletal muscle (Figure 6.5). None of these showed elevated transcript levels following freezing exposure. In skeletal muscle samples, the cross-reacting transcript was observed to migrate more slowly during electrophoresis. Muscle tissue (heart and skeletal muscle) from mammals also express a larger transcript, *sknac*, that codes for a larger isoform of the protein (Yotov *et al.*, 1996). The present data shows that a larger transcript is also found in *R. sylvatica* skeletal muscle, but is predicted to be ~1.8 kb in size, instead of the 7 kb reported for the mammalian isoform.

6.3.2.C SKELETAL MUSCLE GENE EXPRESSION ANALYSIS

Northern blot analysis was also performed on total RNA isolated from *R. sylvatica* skeletal muscle following a time course of freezing exposure. A representative northern blot demonstrating the change in the *R. sylvatica sknac* transcript levels over time during freezing is shown in Figure 6.6A with equal loading verified by equal 18S ribosomal RNA band densities (Fig. 6.6B). Following standardization, mean transcript levels in samples from frogs frozen for 2, 6, or 24 hours were determined to be 72 % (± 13 , $n=3$), 69 % (± 19 , $n=3$), and 49 % (± 4 , $n=3$) of the mean level in control frogs maintained at 5°C (Figure 6.6D).

6.3.3 WESTERN BLOT ANALYSIS

6.3.3A HEART PROTEIN EXPRESSION ANALYSIS

Freezing-induced changes to α NAC protein levels in wood frog tissues were assessed by immunoblotting. Western blots prepared with *R. sylvatica* heart protein extracts and probed with anti- α NAC antiserum showed one major band of approximately 30 kDa (Figure 6.4 C). Densitometric scanning and quantification of the results revealed that α NAC protein levels rose during freezing. Mean protein levels were 2.23 (± 0.42 , $n=3$), 4.07 (± 0.46 , $n=3$), and 2.61 (± 0.42 , $n=5$) fold higher than controls in frogs frozen for 2, 6 or 24 h, respectively (Fig. 6.4D).

6.3.3.B SKELETAL MUSCLE PROTEIN EXPRESSION ANALYSIS

The major immunoreactive band identified on the western blots prepared with *R. sylvatica* skeletal muscle was determined to be approximately 55 kDa in size. Densitometric scanning and quantification of the skeletal muscle blots revealed that, relative to control, the level of the skeletal muscle protein decreased in the frozen tissue samples (Fig. 6.6C). Protein samples prepared from skeletal muscle of wood frogs frozen for 2, 6 and 24 hours revealed a reduction in skeletal muscle α NAC protein content by 37 % (± 5 , $n=3$), 63 % (± 6 , $n=3$), and 56 % (± 10 , $n=3$), respectively (Fig 6.6D).

6.3.4 TRANSCRIPTION FACTOR ANALYSIS VIA WESTERN BLOTTING

Preliminary westerns evaluating the impact of freezing on c-Jun, c-Fos, and ATF2 were also performed. Western blots probed with c-Jun and ATF2 antibodies showed strong

reactivity with proteins of 40 kD and 70 kD in size, whereas primary incubation with the anti-Fos antibody produced a doublet at approximately 55 kD. The initial western blots (provided in Figure 6.7A) indicated that freezing has little to no effect on the c-Jun and ATF2 protein content, but suggested the possible presence of a gradual increase in Fos protein concentration. Various AP-1 family members, including c-Jun and ATF2, are subject to reversible phosphorylation via MAP kinase pathways. To assess freezing-induced changes to the phosphorylation status of these proteins, antibodies that recognize only the phosphorylated peptides of c-Jun and ATF2 protein were tested. The phospho-specific c-Jun and phosphospecific ATF2 antibodies cross-reacted with proteins at the expected molecular weights of 40 and 70 kD, respectively. Representative western blots illustrating freeze-induced changes in the phosphorylation states of c-Jun and ATF2 are shown in Figure 6.7B. The pattern observed with the phosphospecific antibody to c-Jun showed that freezing led to an immediate and dramatic dephosphorylation, whereas the pattern observed with the phospho-specific ATF2 antibody showed a gradual rise in content of the phosphoprotein over time during freezing. Additional work is underway confirming the results from these preliminary studies.

6.4 DISCUSSION

6.4.1 GENE AND PROTEIN EXPRESSION STUDIES

This study characterizes the effect of freezing exposure on the α NAC gene and protein levels in the heart and other tissues of the wood frog. Northern blot analysis indicated that the *onac* gene was strongly up-regulated in wood frog heart with transcript levels rising progressively to reach levels 3-fold higher than controls after 24 hours of freeze exposure (Fig. 6.4A). Immunoblot analysis using antiserum prepared to recombinant α NAC confirmed that the increase in transcript numbers resulted in a comparable rise in α NAC protein content. Protein content rose 2-fold over the first two hours of freezing, reached a peak after 6 hours (4-fold higher than controls), and declined somewhat after 24 hours (Fig. 6.4C). From this data it is difficult to decide which of the two proposed cellular functions for α NAC best agree with the observed expression pattern of the protein in the freeze tolerant frog. It is plausible that the cellular stresses imposed by freezing leads to improper protein translation, folding, and trafficking. Increased levels of NAC (implied by the rise in alpha subunit content) might be part of a cellular response designed to limit the number of such occurrences and the associated damaging effects. However, if the transient rise in α NAC protein levels observed over the time course turns out to be significant then the pattern is more consistent with what would be expected for a protein involved in gene regulation.

General support of a role for α NAC in gene regulation is provided by the results of the sequence analysis (Figure 6.3). Alignment of the putative α NAC amino acid sequence with mammalian homologues indicated that the sequences were 95-96 %

identical. This identity is much higher than what has been observed with other wood frog and mammalian homologues (e.g. Hsp27, EF1 γ , MLC2). On the whole, proteins involved in gene transcription tend to be more highly conserved. Support for the idea that α NAC is specifically up-regulated to alter AP-1 gene transcription can be found in the results of the multiple tissue expression study. To establish whether α NAC gene up-regulation was a common response to freezing, seven additional tissues of the wood frog were analyzed to evaluate *anac* transcript levels. If NAC up-regulation was intended to prevent improper polypeptide trafficking in response to freeze-induced temperature, osmotic, or anoxic stresses, the cellular responses of some of the other tissues would be expected to similarly increase *anac* transcripts. However, no other tissues showed increased cellular *anac* transcript levels following 24 hours of freezing exposure, suggesting that α NAC is providing a heart-specific function.

6.4.2 ANOXIA AND DEHYDRATION EXPRESSION STUDIES

As mentioned previously, studies in our laboratory have shown that various freeze-induced responses can often be linked to either anoxia or dehydration triggers (Storey and Storey, 2001). However, the current data shows that the increase in heart *anac* transcript levels cannot be reproduced by anoxia or dehydration exposures. From these results it would appear that the increased expression of *anac* is not vital to either anoxia or dehydration survival, and that the α NAC protein may have a role that is uniquely linked to freeze survival. A similar finding was recently reported, following an assessment of the MAP kinase responses to freezing, anoxia and dehydration. These studies demonstrated that the freezing induced activation of the p38 MAP kinase cannot

be reproduced by either of the component stresses (Greenway and Storey, 1999). Further work remains to determine the trigger and signaling pathway leading to α NAC gene and protein up-regulation.

6.4.3 CHARACTERIZATION OF THE *R. sylvatica* SKELETAL MUSCLE α NAC

Interestingly, northern blot analysis revealed the presence of an *anac* transcript in skeletal muscle that was estimated to be approximately 1.8 kb in size, double the length of the transcript expressed in other tissues. Similarly, the immunoblot analysis revealed a skeletal muscle α NAC protein band that was, at 55 kDa, approximately double the size of the protein in other tissues, indicating that the additional nucleotide sequence in the *sknac* transcript is located in the coding region of the gene. The gene and protein expression patterns of the skeletal muscle form over the time course of freeze exposure were very different to those seen in heart. Both transcript levels and protein content decreased over time to approximately 50% of the level in control animals after 24 h freezing (Figure 6.6D). The skeletal muscle *anac* transcript in mammals (*sknac*) is also larger (7 kb) than the 0.9 kb transcript found in other tissues (Yotov *et al.*, 1996; Munz *et al.*, 1999). However, unlike the situation in wood frogs, the *sknac* transcript is also found in mammalian heart tissue. Cloning of the mammalian *sknac* transcript revealed that the extra 6 kb was located in the coding region and immunoblot analysis on mammalian muscle confirmed the presence of the predicted 220 kDa NAC isoform (Yotov *et al.*, 1996). Although the isoform can bind DNA and is considered to be transcriptionally active, the cellular and biological roles of the mammalian skNAC protein have not yet been determined. The results of the present study when combined with the mammalian

situation, suggest an interesting evolutionary story for the *sknac* transcript and skNAC protein within the vertebrate phylum.

6.4.4 ACTIVATING PROTEIN-1

Based on the results of the expression studies the up-regulation of α NAC by cardiac cells in response to freezing exposure was suggested to be coupled to the proposed AP-1 co-activating capacity of the protein. α NAC represents one of the more than 50 different proteins that have been reported to interact with and alter AP-1 function (Chinenov and Kerppola, 2001). To determine whether α NAC up-regulation represents just one part of a series of freezing provoked changes intended to modulate AP-1 activity and gene transcriptional patterns, research has been initiated to establish the impact of freezing on some of the more traditional approaches of AP-1 regulation.

AP-1 (activating protein-1) is a term referring to a collection of dimeric transcription factors that bind DNA regulatory elements in the promoter and enhancer regions of numerous genes. Presently, somewhere between 15 and 20 different proteins, capable of forming active AP-1 transcription factors through either homodimerization or heterodimerization, have been identified (Shaulin and Karin, 2001). The presence of many proteins, which have been grouped into c-Jun, c-Fos, ATF, and Maf subfamilies, is significant as each dimer combination exhibits distinctive DNA binding affinities and transactivation capacities (Shaulian and Karin, 2001). For example, proteins from the Jun subfamily form stable homodimers that bind the AP-1 DNA recognition element (5'TGA(G/C)TCA3') known as the TRE; ATF proteins form homodimers as well as heterodimers with Jun proteins that preferentially bind to the cAMP responsive elements

(CRE) (5'TGACGTCA 3'); and Fos proteins form Jun-Fos heterodimers that not only interact very strongly with the TRE, but exclusively recognize a subtype of AP-1 sites (McBride and Nemer, 1998). Considering that different AP-1 proteins have unique transcriptional characteristics it is not surprising that differential expression in response to extracellular stimuli is one of the major mechanisms used to modulate AP-1 activity, and gene expression patterns. The results presented in this chapter also include data from preliminary studies investigating the impact of freezing on the expression patterns of common AP-1 family members. This immunoblot analysis suggested that freezing might lead to increased Fos content, but did not appear to change the c-Jun or ATF2 protein levels.

Differential subunit expression, however, represents only one of the mechanisms used to modulated AP-1 activity. Phosphorylation of AP-1 proteins further modulates their activity and provides another route for extracellular stimuli to regulate AP-1 activity (Shaulian and Karin, 2001). Exposure of cells to a variety of stimuli (e.g. UV irradiation, cytokines, growth factors ischemia, osmotic stresses) often leads to the activation of MAP kinases, many of which have been linked to the transactivation via phosphorylation of AP-1 family members. For example, activation of JNK leads to phosphorylation of the NH₂-terminal activation domain of c-Jun on serines 63 and 73, increasing c-Jun mediated AP-1 transactivation (Smeal *et al.*, 1994). Similar phosphorylation sites have been identified on ATF2, with the two MAP kinases, JNK and p38, both serving as positive regulators of ATF2 transactivation (Karin, 1996). The notion that the different MAPK pathways phosphorylate specific AP-1 family members leading to the transactivation of particular AP-1 regulatory elements in the promoters of gene subsets has been further

entrenched by the recent discovery that the ERK1/p44 (MAPK) pathway is capable of phosphorylating c-Fos and increasing transactivation on specific subtypes of AP-1 sites (McBride and Nemer, 1998).

The availability of antibodies that recognize only the phosphorylated forms of c-Jun and ATF2 has allowed us to begin an assessment of whether freezing induces changes to AP-1 activity by means other than *de novo* transcription and translation. Although the western analysis indicated that the total protein levels of these two AP-1 family members remained constant during freeze exposure, immunoblot analysis with the phospho-specific antibodies to c-Jun and ATF2 showed a dramatic decrease in the fraction of c-Jun phosphorylated as an early response to freezing, followed by a gradual increase in the fraction of ATF2 phosphorylated with long term freezing. The freezing induced phosphorylation of ATF2 and dephosphorylation of c-Jun activity in the heart are consistent with the activation of the p38 MAP kinase pathway. Although both JNK and p38 are capable of activating ATF2, increased JNK activity would have led to a simultaneous increase in phosphorylated c-Jun. This proposed mechanism is consistent with the results reported following a recent assessment of the different MAP kinase pathways during freezing and thawing, which showed a progressive increase in the amount of active phospho-p38 in wood frog heart during freezing (rising ~7.5 fold over 12 h) but no increase in JNK activity (Greenway and Storey, 2000).

Expression of the AP-1 protein subunits has been observed in a diverse range of cell types and tissues, and many studies have attempted to define how modulation of AP-1 activity influences gene transcription and cell physiology. This research has linked increased c-Jun activation to a variety of cellular processes including cell proliferation,

differentiation, apoptosis, and oncogenesis; and ATF2 transactivation to the up-regulation of various stress-responsive genes, including c-jun, beta interferon, transforming growth factor beta, and tumor necrosis factor alpha (Chinenov and Kerppola, 2001; Cho *et al.*, 2001). In spite of all that is known, predicting the downstream effects of the observed changes remains problematic as it is increasingly apparent that the function of the AP-1 transcription factors depends not only on the AP-1 family members involved, but on tissue type and cellular context (Chinenov and Kerppola, 2001).

Currently, the biological relevance and physiological functions of the different AP-1 transcription factors and associated proteins are being elucidated in various cardiac model systems. Although very little attention has been paid to ATF2, a review of the literature finds numerous studies evaluating the role of c-Jun mediated transactivation in myocardial apoptosis following exposure to different stresses, and the involvement of AP-1 in myocardial preconditioning (Maulik *et al.*, 2000; Li *et al.*, 2000; Taimor *et al.*, 2001; Sasaki *et al.*, 1999). Interestingly a significant number of these studies have indicated that the apoptosis induced by NO, oxidants, and ischemic stresses involves c-Jun AP-1 transcription factors and that the fraction of cells killed by these stresses can be reduced following pharmacological treatments and preconditioning protocols that inhibit c-Jun transactivation (Maulik *et al.*, 2000; Taimor *et al.*, 2001; Sasaki *et al.*, 1999). Thus, the decreased phosphorylation of c-Jun following freeze exposure in the heart of the wood frog suggests that c-Jun mediated apoptotic responses are not activated (or perhaps specifically suppressed) during freezing and point to a key difference between the ischemia-tolerant wood frog heart (that naturally endures prolonged disruption of blood flow during freezing) and an ischemia-intolerant mammalian heart. Although the

suppression of c-Jun in wood frog heart during freezing seems explainable by current theories from the mammalian literature, the interesting question that remains is whether the activation of ATF2, c-Fos, and α NAC during freezing stimulates positive or protective gene expression responses and how these contribute to freezing survival.

FIGURE 6.1

Diagram illustrating the proposed cellular roles of α NAC. (Modified from St. Arnau, 1999).

In the cytoplasm, the NAC dimer binds the nascent chains as they emerge from the ribosome. The NAC bound to nascent chains containing signal peptides is out-competed by the SRP, and the ribosome complex is subsequently translocated to the endoplasmic reticulum for further translation and translocation. In those circumstances where the ribosomes are synthesizing nascent chains without signal sequences the NAC prevents SRP binding and the incorrect targeting to the ER.

In the nucleus, α NAC potentiates c-Jun-mediated transcription. The α NAC protein interacts with c-Jun and stabilizes the binding of the c-Jun homodimer with the AP-1 site. Through its interactions with both c-Jun and the TATA Binding Protein (TBP) α NAC is able to strengthen the contacts between AP-1 and the basal transcriptional machinery, and enhance transcription.

FIGURE 6.2

Nucleotide and deduced amino acid sequence for *R. sylvatica* clone *ba*, identified as the alpha subunit of the nascent polypeptide associated complex.

Nucleotides and amino acids are numbered on the left. A full open reading frame was predicted to encode a polypeptide with 214 residues. The start codon (ATG), stop codon (TAA) and potential polyadenylation signals (AATAAA) are shown in bold. The two NAC signature domains (NAC and EFTS_N) have been underlined in the amino acid sequence.

1 GGCACGAGGCCTGAACTCCTCACACAAA**AATG**CCCGGTGAAGCCACAGAACTGTCCCAGT
M P G E A T E T V P V
61 TGCGGAGCAAGAACTCCACCAGCCACAAGCTGAGACGGGGTCAGGAACAGACTCGGACAG
12 A E Q E L H Q P Q A E T G S G T D S D S
121 CGATGATTCTCCTCCAGAGTTGGAACAGGACTCCTCACAAACTTCCACACAGCAGGCACA
32 D D S P P E L E Q D S S Q T S T Q Q A Q
181 GCTTGCCGCAGCAGCAGAAAATAGATGAGGAACCTGTCAGCAAAGCAAAACAAAGCAGGAG
52 L A A A A E I D E E P V S K A K Q S R S
241 TGAAAAAAAAAGCAAGGAAGGCTATGTCAAAGCTGGGGCTTCGCCAGGTTACTGGGGTCAC
72 E K K A R K A M S K L G L R Q V T G V T
301 AAGAGTCACTATCCGAAAATCCAAAAACATCTTATTTGTCATTACAAAACCAGATGTATA
92 R V T I R K S K N I L F V I T K P D V Y
361 CAAGAGCCCAGCATCTGACACTTACATCGTTTTTCGGAGAGGCTAAGATTGAAGATCTCTC
112 K S P A S D T Y I V F G E A K I E D L S
421 ACAACAAGCCCAGCTTGCTGCTGCCGAAAAATTCAAGGTACAGGGAGAAGCAGTATCAAA
132 Q Q A Q L A A A E K F K V Q G E A V S N
481 CATAAGGAAAAACACACAGACTCCACAGTACAAGAGGAGAGCGAAGAGGAAGAGGTTGA
152 I Q E N T Q T P T V Q E E S E E E E V D
541 TGAGAGTGGAGTAGAAGTTAAGGACATTGAGCTTGTAATGTCACAGGCGAATGTGTCTAG
172 E S G V E V K D I E L V M S Q A N V S R
601 GGCGAAGGCTGTGCGCGCTCTGAAGAACAACAGCAATGACATAGTAAATGCTATCATGGA
192 A K A V R A L K N N S N D I V N A I M E
661 GTTGACGATG**TGA**GGATGCCTCAGACTGATCTCCTTTCGTCTTGCTCTTGGGTCGGTGCA
212 L T M *
721 GCTAGAATTGAGATTTATACTATTTCTATTGATTT**AATAAA**CTTGTGGCTTATTGGTGAA
781 AAAAAAAAAAAAAAAAAA

FIGURE 6.3

Amino acid alignment of the *R. sylvatica*, human, mouse and *Drosophila melanogaster* α NAC proteins.

The putative amino acid sequence of the wood frog protein was aligned with the corresponding human, mouse, and fruit fly sequences (Genbank accession numbers AY03400, U22151, AAB97513, respectively). For convenience only those amino acids differing from the wood frog sequence are indicated; dashes (-) represent amino acids in the other sequences that are shared with the wood frog. Periods are present in the alignment to indicate amino acids that are not present in the coding region of one species.

Woodfrog	MP..GEAT.ETVPVAEQELHQPQAETGSGTDSDSDDS..PPEL.EQDSSQT	45
Human	--..----.----AT----P-----E----E-.V---E-----	
Mouse	--..----.----AT----P-----E----E-.V---E----T-A	
<i>D.melanogaster</i>	--ELT-IKS-AA-STSA-AK.-EDVRVEDDG----SDGGM-G-E-AVAAT.	
Woodfrog	STQQAQLAAAAEIDEEPVSKAKQSRSEKKARKAMSKLGLRQVTGVTRVTI	95
Human	A-----	
Mouse	T-----	
<i>D.melanogaster</i>	.--LGGG-TGLP--L..-----G-----I-L----K-IQ--N----	
Woodfrog	RKSKNILFVITKPDVYKSPASDTYIVFGEAKIEDLSQQAQLAAAEKFKVQ	145
Human	-----	
Mouse	-----	
<i>D.melanogaster</i>	-----NN-----N-H-----V-----AP	
Woodfrog	GEAVSNIQENTQTPTVQEESEEEEEVDE.SGVEVKDIELVMSQANVSRKA	194
Human	-----T-----	
Mouse	-----T-----	
<i>D.melanogaster</i>	EA-GAADSVGAT-SVAPIAE-D--DVDDT---E-----IT---TT----	
Woodfrog	VRALKNNSNDIVNAIMELTM	214
Human	-----	
Mouse	-----	
<i>D.melanogaster</i>	IK-----N-----L	

FIGURE 6.4

Analysis of the α NAC transcript and protein levels in *R. sylvatica* heart over a time course of freezing exposure.

Hearts were sampled from frogs acclimated at 5°C (Con) or given freezing exposure at -2.5°C for 2, 6, or 24 hours.

- A. A representative Northern blot prepared with total RNA isolated from hearts, hybridized with [³²P]-labeled *onac* cDNA.
- B. Corresponding 18S and 28S rRNA bands on the ethidium bromide stained formaldehyde gel demonstrating equal loading (16µg total RNA per lane).
- C. Western blot probed with anti- α NAC showing relative protein levels over the freezing time course. Each lane was prepared with 25 µg of total soluble protein.
- D. Histogram showing the relative mean levels of *onac* transcripts and α NAC protein ($\pm$ SEM, n=4 independent blots). Individual blots were scanned, control values were then set to 100, and band intensities in other lanes were expressed relative to their control.

FIGURE 6.5

Organ-specific expression profile of *onac* transcripts in multiple organs of the wood frog comparing 5°C-acclimated controls (C) and 24 h frozen (F) animals. Northern blots used 16 µg of total RNA in each lane.

FIGURE 6.6

Analysis of the transcript and protein levels of the *R. sylvatica* skeletal muscle form of α NAC over a time course of freezing exposure.

Skeletal muscle was sampled from frogs acclimated at 5°C (Con) or given freezing exposure at -2.5°C for 2, 6, or 24 hours.

- A. A representative Northern blot prepared with total RNA isolated from hearts, hybridized with [³²P]-labeled *α*mac cDNA.
- B. Corresponding 18S and 28S rRNA bands on the ethidium bromide stained formaldehyde gel demonstrating equal loading (16 μg total RNA per lane).
- C. Western blot probed with anti- α NAC showing relative protein levels over the freezing time course. Each lane was prepared with 25 μg of total soluble protein.
- D. Histogram showing relative mean levels of *α*mac transcripts and α NAC protein ($\pm$ SEM, n=3 independent blots). Individual blots were scanned, control values were then set to 100, and band intensities in other lanes were expressed relative to their control.

FIGURE 6.7

Preliminary analysis of Activator Protein 1 family members in wood frog heart over a time course of freeze exposure.

Total soluble protein was isolated from the hearts of wood frogs acclimated to 5°C (Con) or given freezing exposure at -2.5°C for 2, 6, or 24 hours. Each lane was loaded with 25 µg of soluble protein. A) Representative western blots, demonstrating the impact of freezing on c-Jun, c-Fos, and ATF2 protein levels. B) Representative western blots probed with antibodies that recognize only the phosphorylated forms of c-Jun and ATF2.

CHAPTER 7

CHARACTERIZATION OF TWO NOVEL FREEZE INDUCED GENES IN THE LIVER OF THE WOOD FROG

7.1 INTRODUCTION

7.1.1 AIM OF CHAPTER

This chapter uses a wide range of molecular biology techniques to characterize the novel freezing inducible genes, *li16* and *ddx8*. Initially a set of techniques was applied to confirm the freeze up-regulation of both transcripts and obtain additional gene sequence from their 5' region, completing their open reading frames and allowing for a proper sequence analysis. The second set of experiments involved a characterization of gene expression in other tissues of the wood frog following freeze exposure, and in the liver in response to other stresses. The third approach to gene characterization involved antibody production to confirm the protein coding capacity of both genes and examine how the protein levels of each respond to freezing, anoxia and dehydration. Finally, two techniques new to our laboratory were used to explore the mechanisms leading to gene up-regulation.

7.1.2 OVERVIEW

The screening of *R. sylvatica* liver and heart cDNA libraries has resulted in the identification of more than ten genes that are differentially expressing during freezing. A number of different types of transcripts were identified including known genes with known functions (e.g. fibrinogen, AAT), identifiable genes with unknown function (e.g. *makorin2*), and novel genes of unknown function (e.g. *fr10*, *li16*, *ddx8*) (Storey and Storey, 2001). The discovery of these three unidentified freeze up-regulated genes strongly suggests that novel gene and protein adaptations contribute to natural freeze tolerance.

The realization that novel genes play a significant role in freeze tolerance is an exciting finding that represents the beginning of a new area of study in the field of cryobiology. Although the discovery of these novel genes does answer a few questions, it raises even more. What are the mechanisms of gene up-regulation? Which of the many freeze-associated triggers and signaling pathways are responsible for transcript up-regulation? Are the novel transcripts protein coding? How does freezing influence the cellular concentrations of the corresponding proteins? What is the biological function of each protein, and how do the functions contribute to freeze survival? To answer these and other related questions an extensive effort will be required, involving the set-up and application of a large number of techniques.

7.1.3 NOVEL GENES: TECHNIQUES AND TOOLS FOR SEQUENCE ANALYSIS

The first clues as to the function of a novel protein can often be found in the nucleotide and putative amino acid sequences. Using a repertoire of available software programs a sequence analysis can be performed to identify protein motifs, domains and signature sequences, regions of hydrophobicity, and predict secondary structure and possible subcellular location. These examinations have provided useful insight into the function of other novel genes (e.g. *fr10*, *kvn*) identified in our laboratory, often suggesting starting points for future investigations (Cai and Storey, 1997b; Larade and Storey, 2002).

Of the three freeze-responsive novel genes, the full sequence has been obtained and considerable work has been done on *fr10* and its putative protein (Cai and Storey, 1997b). The other two, *lil6* and *ddx8*, have received limited characterization of gene

expression (via northern blots) and the sequences excised from cDNA clones were incomplete with only ~50 % (*lil6*) and ~25 % (*ddx8*) of the transcripts present, including complete C terminal ends (Chapter 3). This is a relatively common problem associated with genes discovered using cDNA library screening since the unstable 5' end of mRNA can be fragmented easily. Fortunately, a number of techniques have been developed to obtain the missing 5' sequence. The one applied here is 5' RACE. Briefly, the sequence of steps required to perform 5' RACE is: isolation of mRNA, first and second strand synthesis, followed with adaptor ligation. Gene specific primers are then used in combination with an adaptor primer to generate PCR products containing the 5' region of the gene.

7.1.4 GENE EXPRESSION: MULTIPLE TISSUES AND MULTIPLE STRESS STUDIES

Insight into the function of novel genes/proteins can often be obtained with an understanding of gene/protein expression patterns. For example, an investigation of the tissue expression pattern of *fr10*, identified transcript in all eight organs analyzed, with freeze up-regulation occurring in all tissues except kidney and skeletal muscle. This finding suggests that the cellular function of the protein is not tissue-specific, but that the protein interacts with biochemical pathways, systems, or cellular components common to all cell types.

An additional approach that has been used by our lab to gather information on the potential role of an up-regulated gene or protein in freeze survival is to assess the response of the gene following exposure to the various component stresses of freezing (anoxia, dehydration, and occasionally hyperglycemia). An investigation of the impact of

dehydration and anoxia on *fr10* expression in wood frogs indicated that up-regulation was linked with freeze-induced cellular dehydration whereas the freeze associated up-regulation of other genes (e.g. AAT) were associated with ischemia/anoxia (Cai and Storey, 1997b; Cai *et al.*, 1997).

7.1.5 MECHANISM OF TRANSCRIPT UP-REGULATION: NUCLEAR RUN –OFF ASSAYS

Gene expression can be regulated at both transcriptional and post-transcriptional levels. To determine whether elevated transcript levels are the result of an increase in transcription rate, or other factors (e.g. decrease in mRNA decay) the nuclear run-off assay can be used. As described in Chapter 5, the nuclear run-off assay requires the isolation of fresh nuclei from tissues of control and frozen animals. The nuclei are assumed to have all their transcriptional machinery still intact and in a state that is representative of when they were isolated. Nuclei are then incubated in the presence of NTPs, including radiolabeled UTP and any mRNA that was being transcribed at the time of excision is extended and labeled. The labeled pool of RNA is then isolated and a change in the transcription rate of any gene is indicated by a relative increase in the amount of UTP incorporated into the gene of interest in control versus stressed states.

7.1.6 ORGAN CULTURE: STRESS SIMULATION AND 2ND MESSENGERS STUDIES

A thorough characterization of a novel gene should include an examination of the mechanisms regulating gene expression, including the triggers and signaling pathways involved. These types of investigations have proven to be difficult with our novel freeze inducible genes due to the lack of a cell model system of freeze tolerance or a cell line

known to express our unidentifiable (novel) genes. For this reason an *in vitro* tissue explant system was developed to allow investigation of factors controlling gene expression, based on a long term amphibian organ culture technique (Balls *et al.*, 1976). *R. sylvatica* liver proved to be suitable for *in vitro* tissue maintenance and the system was tested using the *fr10* gene (McNally *et al.*, manuscript in preparation). Subsequent to the success of the system in the characterization of *fr10*, the approach was modified and applied to a number of genes up-regulated in an invertebrate model of anoxia tolerance (Larade and Storey, 2002).

Using the tissue explant system a variety of physiological stresses can be simulated, and second messengers pathways activated. One set of studies possible with this technique involves treating liver slices with protocols that simulate the individual component stresses associated with freezing: dehydration, anoxia and hyperglycemia. These studies are valuable because they allow the investigator to suggest which of the stresses are responsible for the *in vivo* freeze up-regulation of genes, as well as whether gene induction requires a hormonal or blood borne trigger. The second set of liver incubation experiments that were designed uses a series of pharmacological agents capable of simulating the activation of various second messenger pathways. A cAMP analog and PMA were used to activate the protein kinase A (PKA) and protein kinase C (PKC) pathways, respectively. Activation of these two pathways, following freezing exposure in whole animal studies, was established in previous investigations (Holden and Storey, 1996). In addition, a cGMP analog was used to activate cGMP mediated intracellular signaling pathways.

7.1.7 PEPTIDE ANTIBODY PRODUCTION AND IMMUNOBLOTTING

The final set of techniques used in the characterization of the *li16* and *ddx8* genes involved the generation of antibodies to the putative gene products, and immunoblot analysis. Generation of anti-Li16 and anti-DDX8 antibodies was carried out using a standard peptide antibody protocol that involved the selection of an antigenic stretch of amino acids from the putative primary sequence, peptide synthesis, peptide conjugation, followed by a standard rabbit immunization schedule and bleed. The collected antiserum was then used to confirm the presence of *li16* and *ddx8* gene products, and assess their expression profiles over the freeze-thaw cycle, during other stresses, and in various other frog tissues.

7.2 MATERIALS AND METHODS

7.2.1 GENE AND PROTEIN SEQUENCE ANALYSIS

Analysis of the initial and extended wood frog *li16* and *ddx8* nucleotide and deduced amino acid sequences was performed using DNAMAN and the BLAST database search engine located on the National Center for Biotechnology Information website. DNAMAN was used to predict the molecular weight, the amino acid composition, and the isoelectric points of the gene products. The ATGpr program was used to examine each of the potential start codons and eliminate those without the appropriate (consensus) Kozak sequence. The presence of signal sequences, cleavage sites, transmembrane domains and the most likely subcellular location were predicted using the SignalP

PSORT, HMMTOP 2.0 and TopPredII programs. As well, the PROSITE program was used to locate potential phosphorylation, glycosylation and myristylation sites and the BLOCKS and PRINTS search engines were used to search for motifs, domains, and protein signature sequences.

7.2.2 5' RACE OF *LI16* AND *DDX8* TRANSCRIPTS

5' RACE was conducted using the Marathon cDNA amplification kit (Clontech) and the manufacturer's instructions. The mRNA required for cDNA synthesis was isolated from liver tissue as described in sections 2.2.2 and 2.2.3. First strand synthesis, second strand synthesis, and adaptor ligation were performed as detailed in section 2.7. Two sets of antisense primers for amplification were prepared for each gene with the nucleotide sequences: 5'-CTCGCTGGTGCTGGCATAGT-3' and 5'-TGTTCTCAGTGCCGCTCGGA-3' for *li16*; and 5'-CATTCCTGTTCCGGTTGCTC-3' and 5'-ACCCACCTGCTGTGTTATTCG-3' for *ddx8*. The conditions used for PCR amplification of both genes were as outlined in section 2.7.4. Gene products were sequenced by CMRS.

7.2.3 NORTHERN BLOT ANALYSIS

Total RNA was isolated from the livers of wood frogs treated to different lengths of freezing, anoxia and dehydration exposure (section 2.2.2). The RNA was quantified, electrophoresed, and blotted to nitrocellulose, as described in section 2.5. Similarly, for the multiple tissue transcript expression studies, northern blots were prepared using total RNA isolated from brain, gut, kidney, liver, lung, skin, and skeletal muscle of both

control and 24 h frozen wood frogs. Northern blots were pre-hybridized at 65°C for 1 h, prior to 12 h hybridization at 44°C with gene specific radio-labeled probe made with *li16* and *ddx8* cDNA. Blots were washed and exposed to film repeatedly until a single band was visible.

7.2.4 NUCLEAR RUN-OFF ASSAY

Intact nuclei were isolated from the livers of wood frogs maintained at 5°C versus those exposed to 24 h freezing as described in section 2.9.1. Elongation of nascent transcripts and RNA isolation were performed as described in section 2.9.2. To determine whether the transcription rate of *li16* changed following freezing exposure pBluescript containing cDNAs for *li16* and for two genes, eukaryotic elongation factor 1 γ (EF-1 γ) and eukaryotic elongation factor 2 (EF-2) that do not change in transcript number (in liver) following freezing were processed and blotted to Nytran nitrocellulose as described in sections 2.9.3. The membranes were hybridized with the labeled RNA. Following washing and exposure to X-ray film the amount of ³²P-UTP hybridized to the immobilized novel and control gene cDNA was quantified.

7.2.5 LIVER EXPLANT PREPARATION, MAINTENANCE, AND *IN VITRO* EXPERIMENTATION

The protocols used for animal preparation, tissue excision and long-term organ culture are presented in section 2.10. After pre-incubation, liver slices were treated with a variety of protocols designed to simulate some of the physiological stresses that occur during freezing. Glucose-loading of the culture medium was used to reproduce the freeze-induced hyperglycemia; cellular dehydration was achieved using polyethylene

glycol; and freezing was simulated by placing the samples in a freezer at -4°C (details in 2.10.1). The protocols and chemicals used to activate the different second messenger pathways and assess the ability of downstream kinases to regulate novel gene expression are provided in section 2.10.2.

7.2.6 WESTERN ANALYSIS

Total soluble protein was extracted from *R. sylvatica* tissues as described in section 2.11.1. Samples were prepared from tissues exposed to freezing, anoxia or dehydration stresses, and from animals allowed to recover from each of these stresses. Aliquots of these samples containing 25 µg of protein were subjected to electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose as described in section 2.11.2. Rabbit antibodies were produced by Sigma Genosys following the synthesis of peptides corresponding to the C-terminal ends of both the LI16 and DDX8 proteins. The sequences selected for synthesis and conjugation to KLH were DCCQGPLCNA (LI16) and KRLKRRFSTEW (DDX8). Immunoblotting used the technique described in section 2.11. Conditions for binding of the antibody (primary incubation) were the same for both proteins and involved a 1 h incubation at 4°C using a 1:10,000 dilution of the antiserum in 5 % skim milk in TBST.

7.3 RESULTS

7.3.1 5' RACE AND SEQUENCE ANALYSIS

7.3.1.A NOVEL CLONE *LI16*

The *li16* clone isolated from the cDNA library screen was 318 base pairs in length and contained a poly A tail, a polyadenylation signal, and a stop codon in the 3' region (Wu, 1999). With primers designed from the *li16* sequence, RACE was used to extend the 5' end of the transcript and the 280 and 250 bp PCR products are shown in Figure 7.1. Alignment of the nucleotide sequences of the original clone with the sequences of the PCR products confirmed their identity, and increased the length of known *li16* sequence to 446 bp (Figure 7.2A). A larger open reading, complete with start and stop codons extending from base-pair positions 3 to 348 was identified in the new sequence. This sequence was submitted to Genbank (accession number AF175980). Because the sequence contained two possible in-frame methionine translation start sites the ATGpr program was used to predict the most likely start codon. The nucleotides neighboring the ATG codon at nucleotides 171-173 failed to match the general Kozak consensus sequence, (A/G)XXATGG (Salamov *et al.*, 1998) and so this was rejected as a start codon. The final open reading frame (running from positions 3 to 348) codes for a protein of 115 amino acids (Fig. 7.2A) with a calculated molecular weight of 12,755 daltons.

A breakdown of the predicted amino acid composition of *Li16* noted an unusually high cysteine residue content (9%), sixteen strongly basic (lysine, arginine) and eleven strongly acidic (aspartic, glutamic acids) amino acids. When analyzed using a

hydrophobicity plot (DNAMAN), the protein showed strong hydrophobicity near the N-terminal, and the TopPredII program suggested the presence of a transmembrane region extending from amino acids 1-21 (Claros and von Heijne, 1994). A nucleotide search in BLAST indicated that neither the *li16* nucleotide or the Li16 putative amino acid sequence had significant similarity to a known gene or protein. PROSITE recognized only the most common motifs, including possible protein kinase C phosphorylation sites; casein kinase II phosphorylation sites; and a putative N-myristoylation site. Sequence analysis results are summarized in Table 7.1A.

Although a PROSITE and PRINTS analysis failed to identify the presence of any domains or signature sequences, the BLOCKS program recognized the presence of 7 blocks from a total of 5 different domains. In order of best fit, the five domains were the Ly-6/uPAR domain (2 of 3 blocks), the snake toxin domain (2 of 2 blocks), the GS motif preceding kinase domain in TGF β (1 of 6 blocks), the platelet-derived growth factor domain, and the disintegrin domain (1 of 2 blocks). A comparison of the recognition patterns for each domain and the Li16 sequence indicated that the Ly-6/u-PAR, the snake toxin, and the disintegrin domains are all built around a large number of cysteine residues, and that identical stretches of Li16 sequence were aligned with the Ly-6/u-PAR and snake toxin recognition patterns. The presence of two domains with almost identical recognition patterns derives from the fact that the two subfamilies of proteins that were used to arrive at these patterns belong to the same superfamily (Adermann, 1999). However, based on the presence of 10 instead of 8 precisely spaced cysteines in the Li16 primary sequence the wood frog novel protein can be more appropriately grouped with the glycoprotein receptors subfamily, and not with the snake-toxin/cytotoxin subfamily

(Adermann *et al.*, 1999). Chemical analysis of the cysteine rich domain structure from several members of the Ly-6/uPar superfamily has revealed a conserved pattern of disulfide bridging (Ploug *et al.*, 1993; Fletcher *et al.*, 1994). The disulfide pattern is illustrated using the Li16 primary sequence in Table 7.1B. Further support for the idea that the Ly-6/u-PAR domain containing proteins may have all originated from a common ancestor is the presence of a carboxy terminal glycosphosphatidylinositol (GPI) anchoring signal sequence shared by the majority of the Ly-6/u-PAR domain containing proteins (Bothwell *et al.*, 1988). Interestingly though, a number of on-line prediction programs failed to recognize the GPI-anchoring signal sequence in Li16.

7.3.1.B NOVEL CLONE DDX8

Differential screening of the *R. sylvatica* liver cDNA library revealed a clone, named *ddx8*, that was strongly freeze-responsive. The plasmid enclosed an insert of 2073 base pairs that contained a poly A tail, a polyadenylation signal, and a stop codon in the 3' region of the transcript. With primers designed specifically to *ddx8* nucleotide sequence, 5' RACE was used to extend the 5' end of the transcript by an additional 1605 bp to give a final sequence length of 3678 bp (data not shown). This sequence was submitted to Genbank (accession number AY100690). A homology search in BLAST showed that neither the *ddx8* nucleotide sequence nor the deduced DDX8 amino acid sequence had significant similarity to any known gene or protein.

The longest open reading frame in the sequence ran from nucleotides 1227 to 3176 and coded for 649 amino acids to give a protein with a calculated molecular weight of 74,863 Da. However, there were 13 in-frame methionine codons within this sequence

with the potential to be the true translation start site. This list was narrowed down following an assessment of the nucleotides surrounding each (Salamov *et al.*, 1998). Only two (starting at base pair positions 2004 and 2163) proved to have appropriate Kozak sequences. Translation from these two sites would produce proteins of 390 and 337 amino acids in length, respectively (Fig. 7.2B), with calculated molecular weights 45,687 and 39,765 daltons. See Appendix I for complete cDNA with all putative start codons.

A hydrophobicity plot of the 390 amino acid putative DDX8 protein established the presence of a highly hydrophobic region near the C terminal of the putative protein running from amino acid positions 350 to 370. The TopPredII and HMMTOP 2.0 search engines suggested that the hydrophobic region represents a transmembrane spanning segment (Fig. 7.2B). Further analysis of the deduced amino acid sequence with SignalP and PSORT failed to identify any signaling sequences or cleavage sites, and PROSITE recognized only the most commonly identified motifs. The PROSITE findings included cAMP and cGMP dependent protein kinase phosphorylation sites, a possible protein kinase C phosphorylation site, 3 potential casein kinase II phosphorylation sites as well as 6 N-glycosylation and 5 myristylation sites (Table 7.1A). Although a number of individual motifs/blocks were identified with PRINTS and BLOCKS, in all cases the match was for only one portion of a multi-block domain. For example the only hit with BLOCKS was one of the thirteen blocks required for a *Saccharomyces cerevisiae* 175.8 kDa hypothetical protein signature; while by far the best match following PRINTS analysis was the identification of two of the five blocks from the POU DOMAIN, a bipartite DNA binding domain found in some transcription factors.

7.3.2 WOOD FROG NOVEL GENE EXPRESSION DURING FREEZING

Northern analysis confirmed the enhanced expression of both novel genes in response to freezing. Figure 7.3 shows the effect of freezing exposure on *li16* and *ddx8* transcript levels in liver of spring-collected wood frogs over a time course of up to 24 h frozen. Transcript levels of *li16* increased by 1.92 ± 0.21 , 2.67 ± 0.24 and 3.67 ± 0.34 fold after 4, 8, and 24 hours of freezing at -2.5° , respectively. Compared to control frogs *ddx8* transcript levels were 3.21 ± 0.60 and 5.06 ± 0.45 fold higher in liver after 4 and 24 hours of freezing, respectively.

An analysis of gene expression in organs other than liver showed that *li16* transcripts were also up-regulated by freeze exposure in wood frog heart and gut (Fig. 7.4). After a 24 h freeze, transcript levels were 4.09 ± 0.49 fold higher in heart and 3.73 ± 0.26 fold higher in gut than in control tissues. Expression of *li16* was not detected in brain, kidney, lung or skeletal muscle. A similar investigation on *ddx8* indicated that gene expression was liver-specific; northern blots failed to find *ddx8* transcripts in brain, heart, lung, gut, kidney or skeletal muscle of either control or frozen frogs (data not shown).

7.3.3 WESTERN BLOT ANALYSIS OF THE NOVEL PROTEINS DURING FREEZE-THAW

7.3.3.A CHARACTERIZATION OF THE *Li16* AND *DDX8* ANTISERA

Immunoblot analysis of *R. sylvatica* liver homogenates indicated that Li16 antiserum cross-reacted strongly with a protein of ~14 kDa (Fig. 7.5A), consistent with the predicted molecular weight of 12.7 kDa. To confirm that the 14 kDa band was Li16, paired blots were reacted with pre-immune and Li16 antiserum. No cross-reacting 14 kDa

protein was observed with the pre-immunization serum. This preliminary analysis also indicated that the content of the cross-reacting protein increased during freezing and rose to even higher levels during thawing (Fig. 7.5A). To further investigate this, Li16 levels were monitored over a time course of freeze-thaw in the liver of spring frogs (Fig. 7.5B). Li16 protein content increased with increasing time frozen, reaching levels 2.40 ± 0.22 fold higher than control values after 24 h frozen. Li16 continued to accumulate in liver over the early hours of thawing reaching a peak after 2 h of thawing that was 3.51 ± 0.55 fold higher than the value for 24 h frozen liver. Subsequently, protein levels began to fall and after 8 hours of thawing returned to approximately the same level as in 24 h frozen frogs.

Freeze effects on Li16 protein content were also assessed in the two other tissues that expressed *li16* transcripts (Figure 7.6A). Li16 protein content in gut did not change over the course of a 24 h freeze or following 4 h thawing. However, in heart, Li16 responded strongly to freezing with a 4.37 ± 0.30 fold increase in the protein after 24 h of freezing. After a 4 h thaw Li16 content was reduced by about 40% compared with the 24 h frozen peak value but was still more than 2-fold higher than control values.

7.3.3.B CHARACTERIZATION OF THE DDX8 ANTISERUM

Antiserum raised against the DDX8 C-terminal peptide was used to characterize the *ddx8* gene product. Initially, the antiserum was used to confirm the presence of a DDX8 protein, and establish the size of the translated product. The antiserum cross-reacted with one major protein of ~47 kDa (Fig. 7.7A) which closely matched the predicted molecular weight (45.7 kDa) of the 390 amino acid protein shown in Fig. 7.2B.

No immunoreactive bands that would correspond with the putative molecular weight of the smaller ORF (40 kD) or with the largest ORF (75 kD) were found. The antiserum was then used to assess changes in the expression of the 47 kDa protein following freezing exposure (Fig 7.7B). Analysis of soluble protein extracts from liver of control and 24 h frozen frogs revealed a mean 1.56 ± 0.09 fold (n=3) rise in DDX8 protein content in liver of frozen *R. sylvatica*.

Two separate experiments were used to positively identify the cross-reacting protein (47 kDa) as the *ddx8* gene product. Figure 7.7A shows that the 47 kDa cross-reacting protein was not detected following primary incubation with pre-immunization serum, but was detected with DDX8 immunized antiserum. Furthermore, western blots were used to search for the presence of the cross-reacting 47 kDa protein in other organs. The results concurred with the data from northern blot analysis - the 47 kDa cross-reacting protein was found almost exclusively in liver. Over-exposure of the ECL-treated immunoblot revealed the presence of a very minor amount of a 47 kDa protein in kidney, suggesting that DDX8 protein might also be expressed in that tissue at low levels (Fig. 7.6B). Of the remaining proteins detected in the multiple tissue western analysis only the smaller of the two brain proteins was not present when pre-immune was used for primary incubation (data not shown). As the *ddx8* transcript could not be detected in brain tissue the presence of this cross-reacting band suggests the presence of a *R. sylvatica* brain protein with an epitope similar to either, (i) a stretch of amino acids found in KLH (the protein the DDX8 peptide was conjugated with prior to immunization) or (ii) a similar stretch of amino acids to that present in the DDX8 peptide.

The antibody was also used to probe for changes in DDX8 protein levels during recovery from the frozen state. Fig. 7.7C illustrates how the protein accumulated in *R. sylvatica* liver during the early hours of thawing. Peak protein content was reached after 2 h of thawing with amounts that were 2.21 ± 0.29 fold (n=5) higher than the DDX8 content in 24 h frozen liver. DDX8 levels remained high after 4 h thawing but had returned to almost the same level seen in 24 h frozen animals after 8 h of thawing.

7.3.4 NOVEL GENE/PROTEIN EXPRESSION FOLLOWING ANOXIA AND DEHYDRATION

7.3.4.A NOVEL CLONE – *Li16*

Changes in *li16* transcript and Li16 protein levels in response to anoxia or dehydration stresses were also tested in liver (Fig. 7.8A). Levels of *li16* transcripts responded strongly to anoxia, rising by 3.04 ± 0.54 fold after 4 h anoxia and by 7.55 ± 1.59 fold after 24 h. The loss of 20% of total body water had no effect on *li16* transcript levels, however with greater water loss (40%) liver *li16* transcript levels increased by 6.15 ± 1.50 fold. Fig. 7.9A shows that Li16 protein levels also rose in response to anoxia and dehydration stresses. At 20% of total body water lost protein levels were 1.76 ± 0.35 fold higher than controls and this rose to 2.16 ± 0.20 fold by 40% dehydration. However, after rehydration Li16 fell to near control values. Li16 content in liver was strongly responsive to anoxia, with an increase of 1.81 ± 0.38 fold after 4 h of anoxia exposure rising to 4.37 ± 0.55 fold after 24 h under a nitrogen gas atmosphere (Fig. 7.9A). However, within 1 h following re-oxygenation Li16 content fell to near control levels and remained low after 4 h of aerobic recovery.

7.3.4.B NOVEL CLONE – DDX8

The effects of anoxia and dehydration on *ddx8* gene expression in wood frog liver were also assessed (Fig. 7.8B). Northern analysis showed that *ddx8* transcript levels in liver responded strongly to anoxia, rising by 3.93 ± 0.40 fold after 4 h under a nitrogen gas atmosphere at 5°C and by 6.36 ± 1.1 fold after 24 h. The loss of 20% total body water (at 5°C) also resulted in an increase in *ddx8* transcripts in liver by 2.66 ± 0.92 fold. The effects of anoxia and dehydration on DDX8 protein levels in wood frog liver are shown in Fig. 7.9B. In contrast to the strong rise in *ddx8* transcripts during anoxia the protein content decreased relative to control values during anoxia, and only returned to normoxic levels following re-oxygenation. A similar response to dehydration was seen; DDX8 protein levels were low during dehydration but rose again when frogs were rehydrated.

7.3.5 MECHANISMS OF LI16 AND DDX8 TRANSCRIPTIONAL CONTROL

7.3.5.A NUCLEAR RUN-OFF ASSAY

Nuclear run-off assays were used to assess the rate of *li16* transcript synthesis to determine if the increased transcript levels found in frozen frogs were, in fact, due to increased gene transcription. Nuclei were isolated from control and frozen frogs and genes that were being actively transcribed were elongated in the presence of radiotracer. The ^{32}P -labeled mRNA was then isolated and used to probe a dot blot spotted with denatured *li16* cDNA and the cDNA of two genes, EF-1 γ and EF-2, whose transcript levels do not change in the liver following freeze exposure (Fig. 7.10). The results showed an enhanced level of ^{32}P -UTP incorporation into newly synthesized *li16*

transcripts in nuclei from frozen frogs; incorporation was 2.41 ± 0.12 ($\pm$ SEM, n=2) fold higher in nuclei from control frogs.

7.3.5.B *IN VITRO* LIVER INCUBATION EXPERIMENTS

In vitro incubations of liver slices were used to probe the signaling mechanisms, including external stressors and internal second messenger pathways, that might potentially be responsible for novel gene up-regulation during freezing. Preliminary studies established that the preparation of liver slices and their maintenance in the culture medium at 10°C for up to 12 h had no significant impact on *ddx8* or *li16* transcript levels, compared with transcript levels in freshly excised liver (data not shown). In the first series of experiments, liver slices were subjected to *in vitro* freezing, to two elements of freezing stress (hyperglycemia and dehydration) as well as a combination of freezing and hyperglycemia. Analysis of *li16* and *ddx8* transcript levels via northern blotting indicated that none of these treatments changed the transcript levels in liver slices (data not shown).

In a second study, tissue slices were incubated *in vitro* with second messenger analogues capable of activating protein kinases A, C and G to help determine whether any of these pathways were involved in mediating freeze-specific gene up-regulation. Table 7.2 shows transcript levels in liver slices following time-dependent (A) and dose-dependent (B) incubations with second messengers. The tissue explants were exposed to (PMA) at final concentrations of 2, 20 and 200 μ M or to dibutyryl-cGMP or dibutyryl-cAMP at 0.02, 0.2 and 2 mM. After two hours of continuous incubation, total RNA was isolated and northern blotting was used to quantify *li16* transcript levels. Table 7.2B shows that *li16* transcript levels were responsive to db-cGMP with transcript levels

increasing by 1.62 ± 0.22 or 2.53 ± 0.43 fold following treatment with 0.2 or 2 mM db-cGMP, respectively. When incubated with a constant amount of db-cGMP (200 μ M), *li16* transcripts were elevated after 2 h and remained high after 10 h (Table 7.2 A). Neither of the other second messengers, at any concentration or length of incubation, had a significant impact on *li16* transcript levels.

The pharmacological treatments showed a different pattern of effect on *ddx8* transcript levels. Treatments with db-cAMP and db-cGMP had no effect, whereas incubation with PMA increased *ddx8* transcript levels. Transcript levels were dose-dependent, increasing to 2.27 ± 0.26 the level in untreated controls after treatment with 200 μ M PMA (Table 7.2B). PMA treatment also showed time dependent effects, with *ddx8* transcript levels rising significantly within 1 h during incubation with 200 μ M PMA and reaching 2.78 ± 0.26 fold higher than controls after a 10 h incubation.

7.4 DISCUSSION

For the past 5 years the techniques of cDNA library construction and screening have formed the foundation of gene expression studies in our lab. Although this approach is now becoming outdated and other techniques (e.g. array screening) are quickly becoming the standard for mammalian gene expression studies, library screening remains popular in the study of unique biological phenomena in unusual animal model systems. One of the primary advantages of this approach is that it allows for the discovery of novel genes that are either not found or have not yet been identified in the "mainline" model systems. The significance of this feature was immediately realized when the first analysis of freeze-induced gene expression was done; cDNA library screening revealed the prominent up-regulation of a novel gene, *fr10*, in livers of frozen wood frogs (Cai and Storey, 1997b). This approach was further strengthened by the more recent discoveries of *li16* and *ddx8* as freeze-responsive genes in wood frog liver. This chapter describes the characterization of these novel genes and their gene products, to gain a better understanding of their possible role in freeze survival.

7.4.1 SEQUENCE ANALYSIS

Analysis of the *li16* cDNA sequence identified multiple features that showed that it was a true protein-coding transcript: a large open reading frame of 345 base pairs, start and stop codons, a Kozak sequence, a 5' polyadenylation signal (AATAAA) and a poly A tail. The protein coding capacity of the *li16* gene was ultimately confirmed when immunoblotting (using antiserum raised against the C-terminal end of the protein) showed strong cross-reactivity with a liver protein of ~14 kDa, very near to the predicted

size of the *li16* protein product (12.8 kDa). A similar examination of the *ddx8* cDNA sequence also identified multiple characteristics consistent with a true protein-coding transcript, and this was again confirmed when immunoblotting with antiserum raised against the C-terminus of the putative protein, showed strong cross-reactivity with a liver protein of ~47 kDa. This size corresponds well with the predicted size (45,687 kDa) of the 390 amino acid protein.

The Li16 and DDX8 amino acid sequence analysis revealed a number of noteworthy features (Table 7.1). These included the identification of putative transmembrane spanning regions in both proteins, the presence of multiple putative myristylation and glycosylation sites in DDX8, and the recognition of a variety of kinase phosphorylation sites in both proteins, including ones for PKC (Li16, DDX8) and PKA (DDX8), and PKG (DDX8). Previous studies have indicated that all of these protein kinase signal transduction pathways are activated during freezing exposure (Holden and Storey, 1996) and it is possible, therefore, that their cellular activities may be further modified during freeze exposure through one of these protein kinases. Further sequence analysis, using BLAST, failed to find significant sequence identity between either Li16 or DDX8 and any known protein. Interestingly though, BLOCKs suggested that Li16 might be related to a family of proteins containing an evolutionary-conserved 100 amino acid domain, the Ly-6/uPAR domain, that contains 10 cysteine residues that interact in a specific combination giving rise to five intramolecular disulfide bridges (Behrendt *et al.*, 1991). A comparison of the putative Li16 amino acid sequence with the established recognition pattern of the Ly-6/uPAR domain validated the match, and the expected

disulfide bridging pattern is shown in Table 7.1B. No verifiable blocks or motifs were identified for DDX8.

Members of the Ly-6/uPAR domain-containing superfamily include a membrane bound glycoprotein subfamily that includes CD59, the murine and human Ly-6 proteins, and the urokinase-plasminogen activator receptor (U-PAR). A similar complex cysteine-rich structure has also been found in a number of snake venom molecules, a squid protein, and in a CD59-like protein from the herpes virus Saimiri (Petranaka *et al.*, 1996). Although the two subgroups of proteins share similar recognition patterns, Li16 was determined to be more like the members of the membrane bound glycoprotein subfamily based on the presence of 10, instead of 8, cysteine residues. Interestingly, however, in contrast with the majority of the subfamily members, the predicted amino acid sequence of Li16 does not appear to contain a glycoposphatidylinositol (GPI) anchoring moiety. Although uncommon, similar results have been observed with other Ly-6/u-PAR proteins. For example, a Ly-6/u-PAR domain containing gene, *slurp-1*, was recently identified that does not contain a typical carboxyterminal GPI anchoring signaling sequence (Adermann *et al.*, 1999). Although the predicted Li16 sequence lacks the typical GPI anchoring sequence, the protein does have a N-terminal hydrophobic region that is predicted to be a transmembrane spanning region that may provide the same function.

Members of the Ly-6/u-PAR containing protein superfamily exhibit a diversity of biological activities, all of which derive from the capacity to bind both to membranes and to other specific proteins (Petranaka *et al.*, 1996). For example, the CD59 protein inhibits formation of the membrane attack complex, protecting cells from complement-mediated

lysis, by interacting with the C8 and C9 subunits. Furthermore, CD59 is believed to have a role in the signaling of T cell activation through interaction with CD2, and has been suggested to have some role in cell adhesion (Petranka *et al.*, 1996). In the case of the elapid snake venom toxins, the target proteins for binding interactions include the acetylcholine receptor, acetylcholinesterase, platelet integrins, and a variety of other plasma membrane proteins. Thus, the biological activity the Ly-6/u-Par and snake venom toxin domain-containing proteins is expressed through specific binding interactions with a diverse variety of other proteins (Petranka *et al.*, 1996). It has been suggested that the compact, highly constrained disulfide-bonded structure shared among the Ly-6 superfamily of proteins represents a motif that exhibits the remarkable potential to bind a variety of proteins, with the specificity of each particular interaction imposed secondarily through the particular amino acid side chains introduced into the structure (Petranka *et al.*, 1996). Based on its structure, Li16 must have some sort of similar protein-binding role, possibly with a unique action that deals with one of the consequences of freezing. Future experiments will hopefully elucidate its function.

7.4.2 FREEZE-THAW GENE AND PROTEIN EXPRESSION STUDIES

Both *li16* and *ddx8* genes were strongly up-regulated by freezing in wood frog liver with transcript levels rising progressively over time to values 4-5 fold higher in 24 h frozen frogs than in unfrozen controls (Fig. 7.3). The increase in transcript numbers resulted in a comparable rise in Li16 protein content (by 2.4-fold after 24 h freezing), with levels continuing to rise over the first two hours of thawing to reach a peak that was 3.5-fold higher than in the livers of 24 h frozen frogs (Fig. 7.5). DDX8 protein showed a

similar pattern with a 1.6-fold rise in liver after 24 h freezing that continued to reach a peak during thawing that was 2.2-fold higher than in the livers of 24 h frozen frogs (Fig.7.6C) With longer thawing, both Li16 and DDX8 protein contents decreased.

This pattern of gene expression observed for both novel transcripts is similar to that seen for another novel freeze inducible gene, *fr10*, but contrasts with the patterns for AAT and fibrinogen expression in wood frog liver; the transcripts of the latter two genes peaked after 8 hours of freezing and declined with longer freezing times (Cai and Storey, 1997a; 1997b; Cai *et al.*, 1997). The kinetics of transcript and protein levels for both novel species suggest function(s) that become increasingly important: (i) with long term freeze exposure, or (ii) during the early hours of thawing. When frogs thaw at 5°C, recovery is slow and after 1-2 hours only a small percentage of the internal ice has melted. In this state, frog organs would still be under ischemia stress. Furthermore, the marked increase in protein levels during the first few hours of thawing suggests a possible role for the protein in repairing or controlling possible damage associated with extracellular ice formation and/or osmotic stresses associated with the re-uptake of large volumes of water from melted ice.

7.4.3 ORGAN AND STRESS SPECIFIC GENE AND PROTEIN EXPRESSION

7.4.3.A ORGAN-SPECIFIC EXPRESSION

The organ-specific expression of a protein can provide hints as to its function and although Li16 and DDX8 were both strongly expressed in liver, they differed markedly in their expression in other organs. Transcripts of *li16* were detected in three organs of frogs

(liver, heart, gut) and, in all cases, transcript levels showed a 3.5-4 fold increase after 24 h freezing. Li16 protein was similarly present in all three organs but accumulated during freezing only in liver and heart. By contrast, northern analysis detected *ddx8* transcripts only in liver although western blots suggested that very low amounts of the protein might also be present in kidney. The tissue distributions of both Li16 and DDX8 suggest restricted, organ-specific functions for the proteins, primarily in liver but also with roles in selected other tissues. Both patterns are notably different from that of *fr10* which was found in all organs tested. This suggests that the functions of each of these three novel proteins will prove to be quite different (Cai and Storey, 1997b).

The presence and accumulation of Li16 throughout freezing in the liver and heart is interesting because these two organs are the last ones to freeze *in vivo*. Magnetic resonance imaging has shown that ice formation begins at peripheral sites on the skin and spreads inward through the frog's body over several hours (Rubinsky *et al.*, 1994). The extremities freeze first and ice propagates through the body penetrating more and more tissues until ultimately the last two organs to freeze are those with the highest cryoprotectant levels (the liver that produces the glucose and the heart which distributes it). These are also probably the two organs doing the most metabolic work while the frog is freezing: the heart continues to beat against an ever-increasing peripheral resistance and blood viscosity; and the liver synthesizes cryoprotectant and multiple proteins to provide freeze protection to itself and other organs. Furthermore, once freezing begins to impair respiration, this work will be done under increasingly hypoxic and perhaps anoxic conditions, with limited ATP availability. A possible role for Li16 in ischemia/anoxia resistance might, therefore, be proposed. As well, the discovery of a novel protein,

DDX8, that is specific to the liver is not surprising considering the known ability of the wood frog (and other freeze tolerant amphibians) to uniquely regulate glycogen and glucose metabolism following freeze exposure. The liver is the organ responsible for providing cryoprotectant to the whole organism and also produces a variety of proteins, including secreted proteins such as fibrinogen, that are important for freezing survival of the whole organism.

7.4.3.B STRESS SPECIFIC GENE EXPRESSION

The idea that the novel protein Li16 might be linked to freeze-induced cellular anoxia is supported by the responses of *li16* gene and Li16 protein expression to anoxia and dehydration stresses. Anoxia and dehydration are two components of freezing; anoxia (and ischemia) results when blood plasma freezes and remaining tissue oxygen supplies are consumed, and dehydration is a consequence of the loss of intracellular water into extracellular ice masses. Previous studies have shown that various freeze-induced responses are actually linked to either anoxia or dehydration triggers. For example, the massive glycogenolysis that underlies cryoprotectant synthesis is stimulated just as strongly by dehydration as by freezing whereas anoxia exposure has no hyperglycemic effect (Churchill and Storey, 1993; Holden and Storey, 1997). Freeze-induced responses by other genes are also either dehydration (fibrinogen subunits, *fr10*) or anoxia-linked (ADP/ATP translocase) (Cai and Storey, 1997a; 1997b; Cai *et al.*, 1997). The current data show that *li16* transcript levels and Li16 protein content were responsive to both anoxia and dehydration exposure in liver (Fig. 7.8A, 7.9A) but the response to anoxia was more pronounced and more closely mimicked the pattern seen during freezing. Transcript

levels of *li16* rose by 7.5-fold in liver after 24 h of anoxia exposure at 5°C, and protein levels were 4.4-fold higher. Li16 was also very rapidly degraded when frogs were returned to aerobic conditions. This suggests that the protein is highly responsive to changes in tissue oxygenation. A lesser response to dehydration occurred. Transcript levels were not affected by 20% dehydration but rose 6.2-fold after 40% dehydration and the increase in protein content was only 2.2-fold in 40% dehydrated frogs. High levels of water loss (e.g. 40-60% dehydration), but not low or medium dehydration, actually impose a hypoxia stress on frogs because reduced blood volume and increased blood viscosity make tissue oxygenation difficult and, as a result, cellular energetics are compromised and lactate accumulates (Hillman, 1987; Churchill and Storey, 1994). Hence, the response of *li16* transcripts to higher levels of dehydration could actually have been triggered by a low oxygen signal.

The comparative analysis of *ddx8* gene and protein content in wood frog liver showed that neither anoxia nor dehydration stresses adequately mimicked the freeze-induced up-regulation of this novel gene. Although *ddx8* transcript up-regulation occurred during both short and long term anoxia exposure, and to a lesser extent during dehydration, DDX8 protein levels actually fell during anoxia and dehydration, returning to control levels following their removal. From these results it would appear that increased levels of DDX8 protein expression are not vital to either anoxia or dehydration survival; hence, DDX8 may have a function that is uniquely tied to freezing survival. The discordant change in expression levels observed between transcription and translation for DDX8 indicates that control over cellular levels of this protein is, at least in part, exerted at a post-transcriptional level. The suggestion of post-transcriptional processing of the

ddx8 transcript also fits well with the presence of very large 5' and 3' untranslated regions (UTR) of the *ddx8* transcript. The 5' UTR of *ddx8* is predicted to be over 5000 nucleotides in length, and the 3' region is ~500 nucleotides long. By contrast, the 5' UTRs of over 90% of characterized mRNAs are generally between 20 to 100 nucleotides long (Kozak, 1987; Kozak, 1991). A very long 5' UTR is usually associated with those messages where translation initiation is tightly regulated (Pain, 1996).

7.4.4 MECHANISMS OF TRANSCRIPT REGULATION

7.4.4.A NUCLEAR RUN-OFF ASSAYS

Stress-induced changes in gene transcript levels are most often the result of a specific increase in gene transcription but can also be caused by transcript stabilization. The nuclear run-off assay showed that *li16* transcription was indeed up-regulated during freezing (Fig. 7.10). Liver nuclei isolated from freeze-exposed frogs showed substantially higher (nearly 2.5-fold) incorporation of the radiotracer into elongating *li16* transcripts than did nuclei from control frogs. This indicates that the freeze-stimulated increase in *li16* transcript expression in the liver, is due to an increased rate of gene transcription.

7.4.4.B IN VITRO LIVER GENE EXPRESSION EXPERIMENTS

Influences on *li16* and *ddx8* gene expression using isolated liver tissue explants were explored in an attempt to identify signal(s) that mediate freeze-stimulated expression of the genes. Neither *li16* nor *ddx8* transcript levels responded to any of the externally-applied stresses that were applied in vitro: freezing, dehydration (incubation

with polyethylene glycol to pull water out of cells), or hyperglycemia (mimicking high cryoprotectant levels). Similarly, previous studies of cryoprotectant production using isolated wood frog hepatocytes demonstrated that glycogenolysis is not stimulated as a direct effect of freezing on liver cells but instead a peripherally-detected freezing signal (probably on the skin) triggers a nervous/hormonal response that activates beta-adrenergic receptors on the liver and transmits a message via cAMP and protein kinase A (Mommensen and Storey, 1992; Storey and Mommensen, 1994; Storey and Storey, 1996). The lack of direct responsiveness by the liver to freezing and associated stresses is not surprising because biosynthetic responses by the liver need to be fast (during the early hours of freezing) whereas *in vivo* the liver is a core organ that is one of the last organs to be penetrated by ice when frogs freeze. For example, the cryoprotectant biosynthesis response is triggered in less than five minutes after freezing begins on the skin and glucose output reaches maximal rates within ~1 hour whereas ice penetration into the liver is probably doesn't begin until at least 6 hours into the freeze (Storey and Storey, 1985a). The rapid responses by *li16* and *ddx8* transcripts to freezing may suggest that their up-regulation is also triggered by the same nervous or hormonal signals that link peripheral freezing and the activation of cryoprotective responses by liver.

The role of different second messenger in regulating cellular gene expression was addressed by treating liver slices with second messenger analogues capable of activating protein kinase A (PKA), protein kinase G (PKG) and protein kinase C (PKC). The results indicated that two separate pathways, a different one for each gene, may be responsible for the freeze-induced increase in novel gene transcript levels. The *li16* transcript responded to cGMP, indicating a PKG-mediated pathway for its up-regulation, whereas

ddx8 expression appears to be activated via PKC. Activation of both cGMP and IP3 mediated pathways in liver following freezing exposure has been previously established in whole animal studies with wood frogs (Holden and Storey, 1996). In contrast to the well-described role of cAMP and PKA in mediating cryoprotectant synthesis, no downstream effects of freeze-induced increases in cellular IP3 or cGMP levels have been reported to date (Holden and Storey, 1996; Storey and Storey, 1996; Hemmings and Storey, 1994). Hence, the present studies identify, for the first time, some of the potential downstream effects of PKG and PKC activation during freezing. It is interesting to note that similar studies in our laboratory have demonstrated that a significant number of genes that are up-regulated in response to anoxia in an invertebrate model of anoxia tolerance are also regulated by cGMP (Larade and Storey, 2002). This may lend further support to the idea that *Li16* may have a role, possibly protective, in the liver response to anoxia/ischemia during freezing.

FIGURE 7.1

Ethidium bromide stained TAE agarose gel of *lil6* 5' RACE products.

Lane 1 shows the 280 base pair product obtained using one of the two gene specific primers (5' CTCGCTGGTGCTGGCATAGT 3') and the commercial AP1 primer; lane 2 shows the 250 base pair product obtained using the second gene specific primer (5' TGTTCTCAGTGCCGCTCGGA 3') in combination with the AP1 primer. Molecular weight standards are shown in lane 3 (1 kb ladder, GIBCO-BRL).

Figure 7.2

cDNA nucleotide and deduced amino acid sequences of the novel freeze-responsive genes, *li16* and *ddx8*.

A. cDNA nucleotide sequence and deduced amino acid sequence (in bold) of the *R. sylvatica* liver gene, *li16*. The open reading frame encoding 115 amino acids runs from nucleotides 3 to 348. The putative polyadenylation signal and short poly (A) tail are underlined; the stop codon is marked with an asterisk. The sequence has been submitted to Genbank under the accession number AF175980.

B. Deduced amino acid sequence of the novel freeze-responsive gene, DDX8. The *ddx8* cDNA sequence of 3678 nucleotides has been placed into Genbank with the accession number, AY100690. Inspection of the *ddx8* gene for segments matching the Kozak sequence identified two potential translation start sites (at nucleotide positions 2004 and 2163 in the submitted sequence) that would produce proteins of 390 and 337 amino acids in length. The 390 amino acid gene product is shown, and the alternative translation start site beginning at amino acid number 54 is specified (boxed). The potential protein kinase C phosphorylation and cAMP/cGMP-dependent protein kinase phosphorylation sites, located at residues 223-225 and 384-387, respectively, are underlined. The hydrophobic, suspect transmembrane spanning, region starting at position 350 and ending at position 370 is double underlined.

A

1 AGATGGCGATCGTACTGAGCGCACTGCTGGCCATCGTCCTGATTTCAGGTGGTGGAGCCC
 1 M A I V L S A L L A I V L I Q V V E P
 60 AACTCGAAAAATGACAGATTGAGATGCCATTCCCTGTACGTCCCCACCCGCATGTAAAATA
 20 N S K N D R L R C H S C T S P P A C K I
 120 CGCAATGAAGTAGAATGTCGCAGTGGTCAGGACACCTGTCAGAGGACTCACATGCCCATA
 40 R N E V E C R S G Q D T C Q R T H M P I
 180 ACGGATCCGAGCGGCACTGAGAACAGAAACAACACTACTATGCCAGCACCAGCGAGGGCATT
 60 T D P S G T E N R N N Y Y A S T S E G I
 240 ATAGTTTTGGAGCGTTTGTGTACAACATAAAAAACGATGTGAGAACGCCAAGAAGAGGATG
 80 I V L E R L C T T K K R C E N A K K R M
 300 CTGAGCCGTATGTCTGTAGATTGCTGCCAGGGGCGCTCTGCAATGCCTGAGACCATCAC
 100 L S R M S V D C C Q G P L C N A *
 360 CAGGAGGCGACAAACAAGAACGTCCCCCAACTCATAAAACATTCTATACAATAAAACATT
 420 TCCATAGATCAAAAAAAAAAAAAAAAAA

B

1 MGVMGSIQMEMIQNKWKHAGGHIADGLIITSQWLPTTFETQISQVQLTKYLNAMVNYTII
 61 ASLELWRRDQKFINLTQCAFSTLSYHIQVNRAREELQSGNYHTWLKYFKGLDLENTWFEV
 121 PGREIECEEQWCAGRFNIYMVEGIEVMCKLIVMPLLVGEDPPEFWYPEIYGHYADTQDRT
 181 HDLCLLKHQS NICKFQRM PVHIGNRFMEIGPQHICLITNDNDTMRSLNRTAPFSGCIMNV
 241 KVFKWLNDTFIFESDADKTFNREWA VDNLTDTTPFVISLEPLLQVLKESEILRKYIETHE
 301 HYLRNNLLSAIIDKGKLIHLSSQIKEETTHHWYDVFSGWSPTATKTFSWIFSPILILILG
 361 LAIVTVINCCIIYARIKRRVKRLKRRFSTEW

FIGURE 7.3

Northern blot analysis showing freeze-induced changes to *li16* and *ddx8* transcript levels in wood frog liver over a time course.

Representative northern blots were prepared with total RNA isolated from the livers of frogs exposed to different lengths of freeze and hybridized with ^{32}P labeled *li16* (A) or *ddx8* (B) cDNA. Also shown are the corresponding ribosomal RNA bands from the ethidium bromide stained formaldehyde gels demonstrating equal loading. Histograms show the mean relative transcript levels ($\pm$ SEM) for $n=3$ independent blots using tissue samples from different animals. Band intensities on individual blots were scanned and normalized to their respective rRNA bands; control values were set to 100 and band intensities in other lanes were expressed relative to their individual controls.

A

B

Figure 7.4

Organ-specific expression profile of *li16* in six organs of control and frozen wood frogs.

Northern blots were run using 16 µg of total RNA from each organ: brain (B), gut (G), heart (H), kidney (K), lung (L), and skeletal muscle (M). (A) Representative northern blot. (B) Corresponding 18S ribosomal bands from the ethidium bromide stained formaldehyde gel demonstrating equal loading of samples from each organ. (C) Histogram showing the mean ratio of *li16* transcript levels in tissue from frozen versus control frogs (as described in Fig. 7.3). NS - no detectable *li16* transcript signal.

A

B

C

Figure 7.5

Characterization of the Li16 antiserum and Li16 protein levels in *R. sylvatica* liver following freezing and thawing exposure.

A. Western blot characterizing the rabbit Li16 antiserum. Equal amounts of soluble liver protein (25 µg) isolated from 5°C acclimated control animals (C), 24 h frozen animals (F), and animals allowed to recover for 4 h following 24 h of freezing (R) were loaded in each lane. Duplicate blots were incubated with either the polyclonal antiserum prepared against the Li16 peptide, or serum collected from the rabbit prior to immunization (Pre-Immune). The cross-reacting band at ~14 kDa was identified as Li16.

B. Immunoblot characterization of the Li16 protein content in liver of wood frogs over a freeze-thaw cycle. Each lane was loaded with 25 µg of soluble protein. Band intensities were quantified using Imagequant. Representative immunoblots accompany histograms showing mean band intensities ($\pm$ SEM) for n=3 independent trials using tissue extracts from frogs exposed to either different lengths of freeze exposure (0, 2, 6, and 24 h frozen at -2.5°C) or allowed to recover for different lengths of time following 24 h of freezing (1, 2, 4, or 8 h thaw).

A **B**

Figure 7.6

Western blot analysis of Li16 and DDX8 protein contents in wood frog tissues.

(A) Li16 protein levels in heart and gut over a time course of freeze exposure. Representative immunoblots of wood frog heart and gut show cross-reacting material in tissues of wood frogs frozen for 0 (Con), 2, 6, or 24 h and from frogs allowed to thaw for 4 h (following 24 h freeze). The histogram shows the mean protein levels ($\pm$ SEM, n=3 independent trials) relative to controls for heart (dark bars) and gut (light bars). Other information as in Figure 7.5. (B) DDX8 content in wood frog organs. Apart from liver a minor band of cross-reacting material at 47 kDa is seen in kidney

Figure 7.7

Characterization of the DDX8 antiserum and DDX8 protein levels in *R. sylvatica* liver following freezing and thawing exposure.

A. Western blot establishing the size of the translated *ddx8* gene product. Protein molecular weight standards (lane 1) and 25 µg of soluble *R. sylvatica* liver protein (lane 2) were probed with the *ddx8* antiserum. Lane 3 shows a duplicate blot, prepared with the same liver sample that was probed with pre-immunization serum. DDX8 was identified as a cross-reacting protein ~47 kDa in size.

B. Representative western blot prepared with liver protein extracts (25 µg protein per lane) from 5°C-acclimated (Con) and 24 h frozen (Froz) wood frogs. The histogram shows the mean ($\pm$ SEM) relative increase in DDX8 protein content in wood frog liver following freeze exposure. Data was collected from n=3 independent blots using tissue sampled from different animals.

C. Western blot analysis of DDX8 protein levels over a time course of thawing in wood frog liver, following a 24 h freeze. A representative immunoblot prepared with protein extracts from animals frozen for 24 h, and from animals allowed to thaw at 5°C for 1, 2, 4, or 8 h after 24 h frozen is shown. Each lane was loaded with 25 µg of soluble protein and band intensities were quantified using Imagequant. The histogram shows the mean protein intensities ($\pm$ SEM) in liver of thawed frogs (relative to frozen animals) for n=5 independent trials using tissue extracts from different frogs.

Figure 7.8

Effect of anoxia and dehydration exposure on *li16* and *ddx8* transcript levels in wood frog liver.

Representative northern blots, hybridized with ^{32}P labeled *li16* (A) and *ddx8* (B) cDNA. The northern blots were prepared with total RNA isolated from liver of control frogs (5°C), frogs exposed to 4 or 24 h of anoxia, and animals dehydrated (Dehyd) until 20 or 40 % of total body water was lost. The corresponding 18 S ribosomal RNA bands on the photographed ethidium bromide stained formaldehyde gel are presented as proof of equal loading. The histogram shows mean relative transcript levels of *li16* and *ddx8* ($\pm$ SEM) for n=3 independent blots using tissue samples from different animals. Band intensities on individual blots were scanned and normalized to their respective rRNA bands; control values were set to 100 and band intensities in other lanes were expressed relative to their control.

Figure 7.9

Immunoblot characterization of Li16 (A) and DDX8 (B) protein levels in wood frog liver in response to dehydration or anoxia stresses.

Representative western blots illustrate the relative levels of the novel proteins in response to dehydration to 20 or 40% of total body water lost and full rehydration (after 40% dehydration), or anoxia exposure for 4 or 24 h and re-oxygenation for 1 and 4 h (after 24 h anoxia). Each lane was loaded with 25 μ g of soluble protein. Histograms show the mean band intensities ($\pm$ SEM, n=3 independent trials), following normalization of data to control samples.

A

B

Figure 7.10

Nuclear run-off assay to assess *li16* transcription in nuclei isolated from liver of control and 24 h frozen wood frogs.

³²P-UTP labeled transcripts isolated from nuclei from control and frozen frogs were hybridized to nylon membranes spotted with *li16* probe and the cDNA of two genes, EF-1 γ and EF-2, whose transcript levels did not change in liver during freezing. (B) Histogram showing mean relative increase in the transcription rate of *li16* ($\pm$ SEM, n=2 independent trials) in nuclei isolated from frozen frogs relative to control frogs.

TABLE 7.1

Summary of Li16 and DDX8 amino acid sequence analysis.

A. Summary of the results obtained with the DNAMAN program and various on-line analysis programs using the putative Li16 and DDX8 sequences. For both novel proteins the bracketed numbers indicate the amino acid position illustrated in Figure 7.2.

B. Alignment of Li16 amino acid sequence with other Ly-6/u-PAR domain containing proteins. The predicted cysteine-cysteine disulfide bonding pattern of the Ly-6/u-PAR domain is also shown in comparison with the Li16 primary sequence.

A

Molecular Weight Isoelectric Point Dominant amino acid	Li16	12.8 kD, 8.29, (10 Cysteines, 16 basic, 11 acidic residues)
	DDX8	45.7 kD, 6.74, (41 basic and 43 acidic residues)
Phosphorylation sites	Li16	PKC (87, 88), Casein kinase2 (21, 47, 63, 74, 87)
	DDX8	PKA/PKG (384), PKC (223), Casein kinase 2
Myristylation Glycosylation	Li16	Myristylation (48-53)
	DDX8	Glycosylation (56, 74, 221, 228, 247, 268) Myristylation (2, 20, 26, 99, 235)
Signal Sequence (SS) Cleavage Site (CS) Hydrophobicity (H) TransMembrane (TM)	Li16	SS (1-19), CS (19-20), H(19-20), TM (1-17)
	DDX8	H(140-158, 350-370), TM (140-158, 350-370)
Subcellular location	Li16	Nuclear > Cytoplasmic > Mitochondrial, Extracellular
	DDX8	Cytoplasmic > Nuclear > Mitochondrial
BLAST Amino Acid Analysis (Accession No.)	Li16	Mammalian IP3 receptors type 2 (NP_002214) Drosophila unknown gene CG2813 (AAL28761) Drosophila unknown gene CG13102 (AAF52740)
	DDX8	Bonasa umbellus, env protein (AAK14068) V. cholera putative sensor kinase citA (NP_231245)
Domains Motifs Signatures (Interpro No.)	Li16	Ly-6/u-PAR domain (2 of 3 blocks, IPB001526) Snake toxin (2 of 2 blocks, IPB003571) Disintegrin (1 of 2, IPB001762)
	DDX8	Poudomain (2 of 5 blocks, IPR000327) S. cerevisiae 175.8kDa hypothetical protein signature (1 of 13 domains, IPR10547)

B

QCYNcInp...agsCtt....amNCShnqdaCifve (13) deC..	CD59 (PIG)
ECYqCygvpfetsCps.....iTCPypdgvCvtq (18) lpiC..	LY6-A (MOUSE)
RCHvCtss...snCkh....svVCPassrfCktn (15) esC..	LY6-D (HUMAN)
RCYtCeqptainsCkn....iaQCKmedtaCktv (16) trsC..	SLURP (MOUSE)
QCYScegnng.ahrCsseetfliDCRgpmnqClea (18) pswC..	U-PAR (BOVINE)

TABLE 7.2

Time-dependent (A) and dose-dependent (B) effect of second messengers on *li16* and *ddx8* transcript levels in vitro in *R. sylvatica* liver explants.

The effect of second messengers on novel gene transcript levels in *R. sylvatica* liver explants are shown as a function of varying incubation time (at 200 μ M second messenger) or varying second messenger dose (2 h incubation). Transcript levels of *li16* and *ddx8* were determined via northern blotting; band intensity for each sample was normalized against its own 18S ribosomal band, then control (Con) values were set to 100, and experimental band intensities were expressed relative to their corresponding controls. Data for *li16* are means $\pm$ SEM, n=3 except for n=4 for 2 h and 200 μ M treatments with db-cGMP. Data for *ddx8* are means $\pm$ SEM, n=3 except for n=2 for 1 h and 10 h treatments with 200 μ M db-cAMP.

A	<i>li16</i> transcript level			<i>ddx8</i> transcript level		
	db-cAMP	db-cGMP	PMA	db-cAMP	db-cGMP	PMA
Con	100	100	100	100	100	100
2 uM	-	-	123 ± 63	-	-	138 ± 12
20 uM	107 ± 11	101 ± 17	111 ± 34	101 ± 11	76 ± 16	169 ± 16
200 uM	95 ± 20	162 ± 22	85 ± 25	150 ± 30	117 ± 19	227 ± 26
2000 uM	121 ± 23	253 ± 43	-	144 ± 15	80 ± 30	-

B	<i>li16</i> transcript level			<i>ddx8</i> transcript level		
	db-cAMP	db-cGMP	PMA	db-cAMP	db-cGMP	PMA
Con	100	100	100	100	100	100
1 hr	64 ± 33	100 ± 10	89 ± 17	155 ± 11	113 ± 16	180 ± 13
2 hr	95 ± 20	162 ± 22	85 ± 25	150 ± 30	117 ± 19	228 ± 21
10 hr	64 ± 17	176 ± 30	74 ± 25	117 ± 21	122 ± 30	278 ± 22

CHAPTER 8

General Discussion

8.1 Overview

The natural ability to survive the freezing of body fluids during winter hibernation occurs in a small number of amphibian and reptile species. The capacity to withstand the conversion of 50-70% of total body water into extracellular ice, and fully recover following days, weeks and even months without lung, heart, muscle or brain activity represents a truly unique phenomenon. However, natural freeze tolerance represents more than just an interesting biological phenomenon, and a greater understanding of how these animals survive a process lethal to humans and most other vertebrates is acknowledged to have valuable applications for the development of tissue and organ cryopreservation technology. Using the wood frog as the primary model system, our laboratory studies the amphibian solutions to the fundamental problems associated with whole body freezing, including the physical damage commonly caused by extracellular ice crystals; the major reductions in cell volume and increased intracellular osmolality that accompany the freezing of the extracellular fluids; and the prolonged periods of freezing-induced ischemia.

8.2 METABOLIC DEPRESSION AND MACROMOLECULAR SYNTHESIS

As discussed in Chapter 1, the freezing of body fluids requires the cells and tissues of the wood frog to survive for extended periods of time without a supply of oxygen: shutting down the mitochondrial electron transport chain, halting ATP synthesis via oxidative phosphorylation, and subsequently endangering cellular ionic and osmotic equilibrium. To forestall the potentially lethal consequences of a compromised

metabolism, organisms capable of surviving long-term oxygen deprivation actively reduce the demands of the major ATP consuming cellular processes - a capacity that sets them apart from intolerant organisms (reviewed in Hochachka *et al.*, 1996). Consequently, the repertoire of adaptations permitting natural freeze survival has been presumed to include an ability to coordinate the suppression of various ATP-consuming processes. However, little attempt has been made to determine which of the major energy sinks are targeted for down-regulation, or to evaluate the extent of their suppression.

As the primary focus of this thesis was to identify changes in gene and protein expression patterns in the heart and liver of the wood frog, it was logical to also assess how freezing influences the overall rates of transcription and translation in wood frog organs. Gene expression and protein synthesis are two costly cellular processes requiring not only large amounts of ATP but also supplies of nucleotides and amino acid substrates, all of which could quickly become limiting under conditions of freeze-induced ischemia. Indeed, recent research has shown for both anoxia-tolerant animals (turtles, snails) and hibernating mammals that one of the key elements of stress-induced metabolic rate depression is a strong suppression of overall rates of transcription and translation (Hochachka *et al.*, 1996; Storey, 2002; Larade and Storey, 2002). The results reported in Chapter 5 suggest that the same principles are utilized in freeze tolerant frogs. Analysis of nuclei isolated from *R. sylvatica* heart showed an overall decrease in transcription rate in freeze-exposed frogs to less than half of that calculated for control frogs. These results indicate that a stable reduction in the activity of the transcriptional machinery of cells (possibly mediated by some form of reversible covalent modification of key enzymes or

regulatory factors) is one of the energy-saving mechanisms that contributes to long term freezing survival.

In addition, the active suppression of translation during freeze exposure was also implicated by other data from frog heart. A reduction in the rate of protein synthesis can provide substantial ATP savings as the costs associated with protein turnover are estimated to be 18-25 % of total cellular ATP turnover, even higher than the 1-10% of cellular ATP output that supports RNA turnover (Hawkins, 1991; Rolfe and Brown, 1997). A large body of evidence has indicated that the rate of cellular protein synthesis is regulated primarily at the level of initiation (reviewed in Merrick, 1992; Clemens, 2001). In eukaryotes, translation initiation is coordinated by the eukaryotic initiation factors (eIFs); proteins that orchestrate the assembly of the 40S ribosomal subunit, together with the messenger RNA to be translated and the methionine-charged initiator transfer RNA (Pain, 1996). Reversible covalent modification of the eIFs represents a fundamental mechanism for controlling their activity, with the phosphorylation/activity status of the smallest subunit (α) serving as a conserved mechanism of translational control in many organisms (reviewed in Clemens, 2001; DeGracia *et al.*, 2002). As discussed in Chapter 5, the increase in the amount of phospho-eIF2 α in heart of frozen wood frogs is consistent with a strong suppression of global protein synthesis, implicating translation inhibition as a key part of a freeze-induced metabolic rate depression. Subsequent studies (data not show) have also shown an increase in the amount of eIF2 α in the phosphorylated state in liver of frozen wood frogs. Further studies will be required to determine the mechanisms leading to activation of the eIF2 α kinases and the phosphorylation of eIF2 α in the various tissues of the wood frog. The four mammalian

eIF2 α kinases that have been identified to date include (i) the hemin-regulated inhibitor of protein synthesis that is expressed in reticulocytes and is proposed to coordinate globin polypeptide synthesis with hemin availability; (ii) the general control of amino acid biosynthesis kinase (GCN2) that is activated upon amino acid deprivation; (iii) the PKR that protects cells on viral infection; (iv) the recently identified PEK/PERK, that is believed to respond to ER stress and is part of the unfolded protein response (Kaufmann, 1999). As will be discussed below, another line of evidence suggests that either GCN2 or PERK may be the kinase activated in response to freezing.

8.3 FREEZING INDUCED CHANGES IN GENE AND PROTEIN EXPRESSION LEVELS

Following two decades of research by our laboratory we now know that the acquisition of freeze tolerance involves a series of adaptations that include both, (i) modifications to the structure, function, and expression of common genes and proteins as well as (ii) the development of novel genes and proteins. Previous studies have focused on freeze-induced gene/protein expression in wood frog liver and brain (Cai and Storey, 1997a; 1997b; Cai *et al.*, 1997; Wu, 1999) whereas a major focus of this thesis has been the identification of genes/proteins that are important to freezing survival by heart as well as additional studies of liver gene expression. The discovery of 7 genes and one protein that are up-regulated following 24 hours of freeze exposure in heart and liver significantly extends the list of freeze up-regulated genes and proteins. Of those identified, the role of the one protein (MLC2) identified by 2-D electrophoresis, 2 of the

identifiable transcripts (*onac* and *hsp27*), and 2 of the novel transcripts (*li16* and *ddx8*) were selected for further investigation.

8.3.1 NOVEL GENES

The probable existence of novel protein products that aid wood frog freeze tolerance has been presumed for many years, but the only studies directly supporting this contention were those that demonstrated the presence of proteinaceous ice nucleating agents in wood frog blood (Wolanczyk *et al.*, 1990). However, the development and application of cDNA library screening technology to this problem has led to the recent identification of multiple novel genes/proteins that appear to be important to freezing survival. The first of these was the freezing inducible transcript, *fr10* in liver (Cai and Storey, 1997b) followed by another liver gene, *li16* (Wu, 1999). With the discovery of the novel liver gene, *ddx8*, in the present work, coupled with the characterization of both *li16* and *ddx8*, this thesis solidifies the idea that novel genes play a role in the development of freeze tolerance. Although the actions of these three novel proteins in freezing survival has not yet been determined, their importance is highlighted by the strong rise in transcript and protein levels that takes place over the freeze-thaw cycle. As discussed earlier, freezing is accompanied by a decline in organ energy status due to progressive ischemia, and both heart and liver have been shown to suppress energy expensive cellular processes during freezing, including transcription and translation. If ATP availability is restricted, it makes sense that macromolecular synthesis at such times would be limited to only those genes and proteins that had specific functional or protective roles in the stress state and hence, the up-regulation of *fr10*, *li16*, and *ddx8*

during freezing and synthesis of their proteins supports the idea that they have important roles in freezing survival.

The work on *li16* and *ddx8* that is described in Chapter 7, including sequencing and a variety of expression studies, was performed to gather information that could suggest the role of each protein. Although these studies did not ultimately define the cellular function of the proteins, the results did indicate that the cellular roles of Li16, DDX8 and Fr10 will be quite different. For example, as summarized in Chapter 7, Li16 was characterized by the presence of a possible Ly-6/u-PAR domain in its amino acid sequence as well as by its responsiveness to anoxia/hypoxia. Outstanding features of DDX8 were its liver-restricted expression profile and the occurrence of post-transcriptional regulation of DDX8 translation. In addition, a new multi-species analysis is showing that *ddx8* expression may occur in all freeze tolerant frogs (the protein is found in *R. sylvatica*, *Hyla versicolor* and *Pseudacris crucifer*) but not in two freeze intolerant species (*Rana pipiens*, *Scaphiopus couchii*). This further emphasizes the probability that DDX8 has an important role to play in freeze tolerance.

8.3.2 IDENTIFIABLE GENES AND PROTEINS

Chapter 4 described the freeze-stimulated up-regulation of *mlc2* and *anf* transcripts, two established genetic markers of cardiac hypertrophy, and suggested that the freeze-induced state of ischemia may serve as the trigger for their up-regulation. The over-expression of both genes in frog heart during freezing strongly suggests the presence of the hypertrophic gene response, a response commonly observed in cardiomyocytes following exposure to a variety of potentially pathophysiological conditions. The possible

benefits of increasing MLC2 and ANF levels during freezing were discussed thoroughly in chapter 4. Briefly, increased MLC2 was suggested to represent an important, possibly rate-determining, step towards increasing the number of myosin motors present in each cell. By increasing the amount of force generated by each cell the heart could partially compensate for the freezing induced changes in the vasculature, and maintain tissue perfusion and cryoprotectant distribution for longer periods of time. The possible benefit of increased serum ANP levels in freeze survival was also evaluated, and was determined to be mediated through both natriuretic/diuretic and vaso-relaxant properties. Both of these properties are predicted to lessen the freeze induced damage to delicate organs and vasculature via relocation of extracellular fluid to the bladder and dilation of blood vessels.

8.3.3 Hsp27

The role of Hsp27 in freeze tolerance could be derived from one of the several known cytoprotective functions that have been attributed to the protein (Arrigo, 1998). In fact, a task for each of the different Hsp27 activities could easily be identified in the repertoire of freeze-induced stresses. For example, Hsp27 might contribute to freeze survival through its ascribed chaperonin capacity by acting to refold proteins that are freeze/cold denatured or its role might be in blocking apoptosis signaling pathways, preventing the loss of heart tissue and ensuring optimal cardiac functioning following thawing. Hsp27 has also been characterized as an inhibitor of actin polymerization, capable of regulating actin dynamics and improving cell survival following exposure to stresses that disrupt the cytoskeleton. This feature might be significant because long term

ischemia represents one of the major problems associated with prolonged freezing and one of the best characterized sources of microfilament and cytoskeletal disruption (Molitoris *et al.*, 1997). Finally, the unexplained decrease in reactive oxygen species and increase in cellular oxidative status observed following Hsp27 expression might help to prevent cell injury due to oxidative stress in frogs by reducing the free radical induced damage that is generally associated with O₂ reperfusion events (e.g. thawing).

The proposal that the expression of Hsp27 by wood frogs is in anticipation of freezing-provoked oxidative stress is supported by the fact that the freeze-induced increase in *hsp27* transcript levels is not translated into increased protein levels until thawing. Thus, Hsp27 levels are highest during thawing when oxidative stress would also be at a peak. Previous studies have established that wood frogs do not suffer from any of the customary cellular injuries associated with reperfusion oxidative stress, indicating that appropriate mechanisms are in place to prevent and/or repair such damage (Joanisse and Storey, 1996). Based on the results presented in Chapters 3 and 5, it would appear that the absence of free radical damage following thawing could be explained not only by the previously described overabundance of cellular glutathione and antioxidant enzyme activities (Joanisse and Storey, 1996), but also by the high constitutive levels of Hsp27 in wood frogs and the further increases in Hsp27 levels during thawing. It is interesting to note that Hsp27 over-expression has also been reported to increase cellular glutathione and antioxidant enzyme concentrations (e.g. glutathione reductase and glutathione peroxidase) through an as yet undefined mechanism (Arrigo, 1998; Baek *et al.*, 2000).

Although this information strongly supports the idea that Hsp27 is functioning to prevent oxidative damage during freeze/thaw, this property of Hsp27 is a

phosphorylation-independent effect and the results from Chapter 5 also show a dramatic rise in Hsp27 phosphorylation during thawing (10-fold above control), suggesting that a fraction of the Hsp27 cellular pool is performing a phosphorylation-dependent function. In addition to the ability of phosphorylated Hsp27 to increase microfilament stability during periods of ischemia and following oxidative stress, Hsp27 has also been reported to accelerate cell recovery following filament damage (Liang and Macrae, 1997). Therefore, the increase in Hsp27 protein content and the fraction phosphorylated might work to simultaneously prevent ROS accumulation, reduce ROS mediated damage to the cytoskeleton of cardiomyocytes during thawing, and accelerate recovery following microfilament disruption that could occur during prolonged periods of freeze-induced ischemia. That said, the ability of phosphorylated Hsp27 to block apoptosis signaling pathways and, thereby, prevent the loss of heart tissue cannot be ignored.

8.4 FREEZING INDUCED ACTIVATION OF CELL SIGNALING PATHWAYS

8.4.1 α NAC

The third freeze-induced gene selected for further investigation was *onac*, that codes for the alpha subunit of the Nascent Polypeptide Associated Complex. The α NAC protein has been proposed to have two cellular functions: (i) a ribosome-associated role in signal-sequence-specific sorting and translocation, and (ii) a more recently established role in the regulation of gene transcription (St. Arnaud, 1999). The expression studies performed in Chapter 6 showed a tissue specific up-regulation of the protein following

freeze exposure, suggesting that the increase may be intended to trigger the AP-1 co-activating activity. Subsequent (preliminary) investigations identified changes in the cellular concentration of c-Fos (increase), and in the phosphorylation status of Jun (decreased) and ATF2 (increased) providing further evidence that freezing leads to changes in AP-1 mediated transcription. Furthermore, an ongoing evaluation of the responses of other AP-1 associated proteins in wood frog organs has established that the transcriptional co-activators, JAB1 (Jun activation domain binding protein 1) and GADD153 (growth arrest and DNA damage-inducible protein) are induced following freeze exposure (data not shown). On the whole these findings demonstrate that freezing conditions give rise to seemingly complex yet specific changes in AP-1 mediated transcription.

The identification of GADD153 (also known as CHOP) as freeze-induced adds another interesting piece to the puzzle. *Gadd153* encodes a small ubiquitous nuclear protein that heterodimerizes with members of the CCAAT/Enhancer-binding protein family of transcription factors (Ron and Habener, 1992). Under most physiological conditions GADD153 is not expressed at levels sufficient to influence the activity of the other C/EBP factors present in the cell (Ubeda *et al.*, 1996). However, the protein is highly induced following exposure to a number of cell stresses, including: glucose/nutrient deprivation, alkylating agents, oxidative stresses, hypoxia, protein misfolding in the endoplasmic reticulum, and other growth-arresting situations (Fawcett *et al.*, 1999; Ubeda *et al.*, 1999). Once induced, GADD153/CHOP activity appears to significantly influence the cell cycle, giving rise to growth arrest in certain model systems and programmed cell death in others (Kaufmann, 1999).

Initial analysis of the signaling pathways responsible for stress-induced increases in CHOP and a number of other related stress-inducible genes like BiP/GRP78, paradoxically demonstrated the phosphorylation of eIF2 α as a necessary component. Further investigations focusing on the different mammalian eIF2 α kinases established that PERK and GCN2, when activated by their cognate upstream stress signals, repress translation of most mRNAs but also selectively increase the translation of Activating Transcription Factor 4 (ATF4) which induces the downstream gene *Gadd153* (Harding *et al.*, 2000). Therefore, the detection of GADD153 during freezing, via the investigation of AP-1 modifying factors, may have inadvertently helped to narrow down the list of eIF2 α kinases that could be responsible for the phosphorylation of eIF2 α (discussed in Chapter 5 and above), leading to a generalized suppression of translation during freezing.

8.4.2 P38 AND THE CELLULAR RESPONSE TO FREEZING IN THE WOOD FROG HEART

In addition to the reported ability of the eIF2 α kinase / ATF4 pathway to regulate *Gadd153* expression, studies with the different MAPK pathways have shown that a number of the *gadd* genes, including *Gadd153*, can be induced, at least in part, by the p38 pathway (Kultz *et al.*, 1998). This finding is interesting because CHOP activity is also believed to be regulated post-translationally by p38 (Wang and Ron, 1996). As mentioned in earlier chapters, an analysis of the MAPK responses to freezing and thawing in heart of the wood frog showed a progressive increase in the amount of active phospho-p38 during freezing (rising ~7.5-fold over 12 h) that continued during thawing, rising within 4 h post-thaw to levels ~13-fold higher than the amount in frozen frogs (Greenway and Storey, 2000). For this reason, freezing-induced phosphorylation and

further enhancement of GADD153 activity might be predicted. Support for this idea can be found in (Chapters 5 and 6) the results obtained during the characterization of the freeze inducible *hsp27* and *onac* transcripts. Characterization of the phosphorylation status of Hsp27 over the freeze-thaw cycle showed a greater than two-fold increase in the phosphorylation status of Hsp27 following 24 h freeze exposure, and an even more dramatic increase during the first few hours of the thaw. As well, preliminary data also indicated that freezing increases the fraction of ATF-2 phosphorylated. Both ATF-2 and Hsp27 are well-established downstream targets of the p38 pathway. Recognizing that a significant portion of the identified freeze-induced changes in cellular composition could be linked to the activation of the p38 pathway, I decided to search for evidence that some of the other freezing-induced changes, mainly the hypertrophic gene response and *onac* gene up-regulation, might be similarly regulated.

Although there is no literature defining the factors controlling *onac* gene expression, some evidence of regulation by the p38 pathway can be found in the results of studies performed in our laboratory. The *onac* gene expression studies described in Chapter 6 indicated that neither of the component stresses (anoxia or dehydration) were able to simulate the freeze-induced up-regulation of *onac* transcript levels. This pattern matches the one obtained for p38 following a similar assessment of the MAPK cascades in wood frog heart in response to freezing, anoxia and dehydration (Greenway and Storey, 2000). This finding is significant in that it is quite unusual; most other freeze-induced changes in wood frog liver could be linked to either freezing-induced anoxia or dehydration (Storey and Storey, 2001).

Finding evidence that the p38 pathway might play a role in the hypertrophic gene response was comparatively easy. Numerous studies assessing the mitogen-activated protein kinase cascades in cultured cardiomyocytes and animal models of heart disease have suggested that both JNK-dependent and p38 dependent pathways are capable of eliciting the hypertrophic response (Zechner *et al.*, 1997; Wang *et al.*, 1998a; 1998b). It is interesting to note that these two pathways share the same downstream target, ATF2. In addition, other downstream targets of the p38 pathway have also been linked with the development of cardiac hypertrophy: the MEF2 transcription factors. Genomic studies have established that the promoter regions of the various hypertrophy-associated genes contain MEF2 binding sites, and DNA binding studies have shown increased MEF2 activity following exposure to hypertrophic conditions (Kolodziejczyk *et al.*, 1999; Passier *et al.*, 2000; Molkentin and Markham, 1993). While the current evidence from frogs points strongly at p38 as key to freeze-induced signal transduction, other intracellular signaling pathways (e.g. NFAT) have also been implicated in the hypertrophic response and their involvement in a freeze-induced hypertrophic response remains to be evaluated (reviewed in Sugden, 2001; Wilkins and Molkentin, 2002).

In conclusion, our review and discussion of the observed changes in the heart of the wood frog following freezing exposure strongly suggests a role for the p38 MAPK pathway in the majority of the cellular responses.

8.4.3 NOVEL GENES: TRIGGERS AND 2ND MESSENGER PATHWAYS

To similarly assess those factors influencing *li16* and *ddx8* gene expression I designed an in vitro tissue explant system that uses isolated liver slices to evaluate the effects of different external stressors and second messengers on the regulation of expression of these genes. One of the major findings with this system was that although freezing, anoxia, and dehydration exposures all increased *li16* and *ddx8* transcript levels when administered to the whole animal, none of the external stresses were capable of altering gene expression levels when applied to individual liver slices. These results indicate that the observed changes in wood frog liver gene expression patterns following freezing exposure are not a direct response of the liver to freezing conditions, but are transmitted to the liver via a nervous or hormonal signal in a manner similar to what has been described previously for the activation of freeze-induced glycogenolysis. A number of signal transduction pathways that might link the freezing signal to the expression of novel genes were then explored by treating liver slices with second messenger analogues that stimulate protein kinase A (PKA), protein kinase G (PKG) or protein kinase C (PKC). These studies indicated that the freezing provoked up-regulation of *ddx8* was possibly regulated by protein kinase C, whereas *li16* up-regulation was most likely controlled by a cGMP-mediated pathway. Activation of both PKC and PKG pathways in liver following freezing exposure has been previously established in whole animal studies (Holden and Storey, 1996) and *ddx8* and *li16* represent the first downstream targets of these pathways to be identified.

8.5 SUMMARY AND FUTURE STUDIES

In summary, this thesis utilized a variety of molecular biology and biochemistry techniques to assess the changes in gene and protein expression patterns in the heart and liver of wood frogs during freezing. With these techniques I was able to show that the response to freezing includes the up-regulation of select genes and proteins against a background of overall suppression of macromolecular synthesis and ATP consumption. The results presented in this thesis indicate the presence of three different types of gene responses to freezing. The first response type encompasses most genes, and involves a suppression of transcription and translation. The second category would be made up of those genes that demonstrate increased transcription during freezing, with no translation until the recovery phase. The third and final response type, characterized by increased transcription and translation, would be expected to include those genes and proteins whose role is protective or regulatory during the stress period itself.

To date, investigations surrounding the various intracellular signaling pathways have only been able to identify the activation of one pathway, the p38 MAPK pathway, in the heart of the wood frog following freezing exposure. Interestingly, all of the identified cellular responses, including specific increases in gene/protein levels and changes to protein phosphorylation states could be linked, on some level, to the p38 pathway, supporting the idea that this is the major signaling pathway activated in the wood frog heart during freezing exposure. By contrast, however, previous studies that characterized the intracellular signaling pathways in the liver found that freezing increases the activity of cAMP, cGMP, PKC, and various MAPK pathways. Following cDNA library screening analysis of liver, I identified a number of freeze-specific changes to gene

expression, mainly involving the induction of unidentifiable/novel genes. Over the course of my thesis, I performed various gene expression studies on whole animals and developed an in vitro tissue explant system as a means of evaluating the triggers and signaling pathways responsible for freeze-induced gene up-regulation in wood frog liver. Presently these novel genes are believed to play an important role in freeze survival, and the gene and protein expression studies that have been performed with *li16* and *ddx8* represent important first steps in attaining an understanding of their role in freeze tolerance. The results presented in this thesis have laid the groundwork for the second generation of studies, intended to (i) better quantify the cytoprotective capacity of each novel protein, (ii) establish the cellular function of each, and (iii) determine more precisely the regulatory factors controlling freeze activated genes and proteins.

APPENDIX I

CAGGGTATCGGCACACCAAGTCAAAAAACAATTGAAGCAAAAAGGAAATATATTTGCAGATTTAACTGCGAGACAAGCAGCTCTAA
 AGGAAGTAAGCCCTGAGACTGTAGCAGCAGTACAGGTAAGTAGCCGAGAACCAACATGGCAGAAGCAGAGGTGAGCGAAAAGGATAG
 GTGGGTACAAATGGGAGCAGCCGACCCCTGACCAAGTATGGAGAGGGGGGACAAGGTTTGTCCACCAGCTTCTCTGTACCCGGC
 AATGGCGGCAGCAGTTACCTTCCCGCATATGACAGCTCAATTCTGATAGGATGTGAGCAGAGATGGCTCGCCCTGGATT
 CAGCAGCATTGCCAGAACTACTGTGCAGCCTGCCAAATCTGTCTCCTGAATAGCCGAGACAGAAAGTTAAACACCCCGGAGACA
 TCAGGCACAGTACCTCCTTCAGAGACTGCAAAATTGACTATATACAAATGCCTAAGAGCGGCCCTTTTGAGTTTGTCTCGTGTGTAT
 CGATCTGTTTTCCGTTTGGGCGGAAAGTTACCTGTTAAATCAGCCAGAGCCAAAGCCACAGCCAGAAACTGATCAGTGAGTTAAT
 ACCTAGGTTTGGTCTCCCCGAAACTATGAATCAGATAGGGGGAACACATTTCCAGGGGAGAAATCATGCAAAATGTGATGGCAATGT
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