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Gene and protein regulation in liver of the freeze tolerant wood frog, *Rana sylvatica*.

by

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A Ph.D. thesis submitted to the Department of Biology, Faculty of Graduate Studies in partial fulfillment for the requirements of the Doctorate of Philosophy.

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Joseph Nicholas Amritha De Croos

In partial fulfillment of the requirements for the degree of Doctor of Philosophy

Chair Department of Biology

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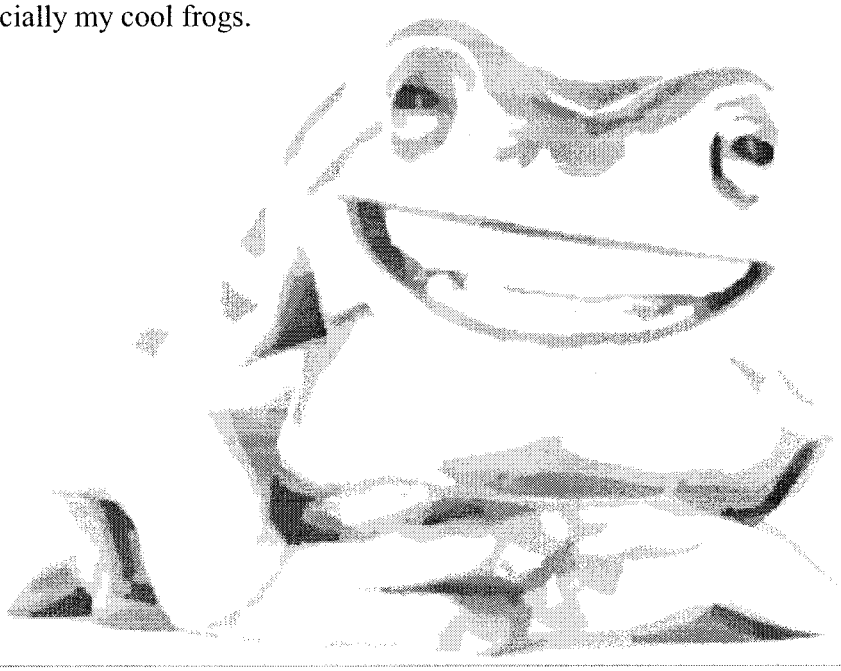
Abstract

The wood frog *Rana sylvatica* has the ability to tolerate whole body freezing in nature. Biochemical mechanisms supporting freezing survival have been widely studied in this species, but much remains to be explored. The present research demonstrates the involvement of specific gene and protein level changes in the response to freezing by liver. Screening of a cDNA library prepared from liver of *R.* sylvatica identified a freeze-responsive clone containing a 1370 nt sequence with an open reading frame of 360 amino acids. Sequence analysis revealed 84-86% identity with the mammalian inorganic phosphate carrier (PiC) that spans the inner mitochondrial membrane. Northern blot analysis showed that *pic* transcript levels increased over time during freezing, reaching >70-fold up-regulation after 24 hours frozen. Transcript levels were also assessed under freezing-related stresses with results showing a strong increase in *pic* transcript levels during dehydration (9.0-fold in 40% dehydrated frogs) but not under anoxia. Western blotting revealed elevated PiC protein over a time course of freeze-thaw whereas other mitochondrial carriers of the same family were not affected. Southern blot analysis showed that the increase in PiC was not due to an increase in mitochondrial numbers. Analysis of polysome profiles revealed disaggregation of polysomes into translationally less active monosomes upon freezing. PiC transcripts segregated into monosomes in control liver, but would aggregate into actively transcribing polysomes during freezing. Western blot analysis showed that the activity of specific transcription factors in frogs is temporally controlled. The transcription factors CREB and NFκB were significantly activated after two hours of freezing exposure whereas protein kinase B (Akt) was activated after 24 hours of freezing. Screening of a cDNA microarray identified an anti-

apoptotic factor that is upregulated during freezing in liver. Western blot analysis showed time dependent changes in anti-apoptotic activity mitigated by significant changes in AP-2α, BAX-inhibitor and p53 protein levels. These results suggest that freeze tolerance in *R. sylvatica* involves control of gene and protein levels through the upregulation of selected transcripts and the activation of kinases and transcription factors. This gene and protein control, or regulation, combined with specific anti-apoptotic actions, allows hepatocyte survival of the multiple stresses imposed by freezing.

Acknowledgements

My doctoral research was an experience I will never forget and the lessons about "life, the universe, and everything" learned in the Storey-Lab will undoubtedly shape my career as a scientist. This journey could not have happened without Ken Storey my supervisor. I really want to thank him for accepting me into the lab, allowing me to work on frozen frogs and especially for listening to all my crazy/expensive/bonehead ideas. I also owe a great deal of thanks to Jan Storey for all her editing, advice in writing and for being so patient with me during the dissections. A thanks also goes out to my committee members Dr. Carmody and Dr. Hickey whose comments and criticisms helped in shaping my research. I also would like to thank the Storey boy(z) and the Storey girl(z) for help with dissections, coffee breaks and laughing at all my lame jokes (were you laughing with me or at me?). And finally I would like to thank my immediate and extended family for all their support and for putting up with my late hours, talking only about science and especially my cool frogs.



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List of Abbreviations

AA	amino acid
AAT	ADP/ATP translocase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
CREB	cAMP response element binding protein
DNA	deoxyribonucleic acid
DEPC	diethylpyrocarbonate
DiC	dicarboxylate carrier
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
eIF2α	eukaryotic initiation factor 2 - alpha
gDNA	genomic deoxyribonucleic acid
kb	kilobase
kDa	kilodalton
MOPS	3-[N-morpholine] propanesulfonic acid
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
MW	molecular weight
NFκB	nuclear factor kappa B
nt	nucleotide
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PiC	inorganic phosphate carrier
PVDF	polyvinylidine fluoride
RT-PCR	reverse-transcriptase polymerase chain reaction
RNA	ribonucleic acid

rRNA	ribosomal ribonucleic acid
tRNA	transfer ribonucleic acid
SDS	sodium dodecyl sulfate
TAE	tris-acetate- ethylenediamine tetraacetic acid buffer
TAIL-PCR	thermal asymmetric interlaced polymerase chain reaction
TEMED	N,N,N',N'-tetramethylethylenediamine
UTR	untranslated region

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CHAPTER ONE

General Introduction:

The ecophysiology and molecular biology of freeze tolerance in the wood frog *Rana sylvatica*.

The ability to tolerate freezing has been reported in five species of North American frogs, *Rana sylvatica*, *Hyla versicolor, H. chrysoscelis, Pseudacris crucifer* and *P. triseriata* (Schmid, 1982; MacArthur and Dandy, 1982; Storey, 1984; Costanzo *et al.*, 1992a). The wood frog, *R. sylvatica*, is widely accepted as a model system for studies of vertebrate freeze tolerance and more is known about the molecular mechanisms of natural freezing survival in this species than in any other. Studies presented in this thesis continue the exploration of the biochemical adaptations and molecular mechanisms that underlie vertebrate freeze tolerance with a particular focus on the roles of mitochondrial transporters, signal transduction mechanisms and anti-apoptotic mechanisms.

1.1 The biology of freeze tolerance

To survive the freezing temperatures that many species must endure over the winter several strategies of cold hardiness have been developed, including: (A) anhydrobiosis – an extreme desiccation whereby all free water that can freeze is removed, (B) vitrification – water solidifies into an amorphous glass-like state rather than changing into ice crystals, (C) freeze avoidance – lowering the temperature at which body water freezes, allows a liquid state to be maintained even at very low subzero temperatures, and (D) freeze tolerance – regulation of ice formation in extracellular spaces while preserving the liquid state of the cytoplasm. Anhydrobiosis is commonly seen in nematodes and other microfauna, and is also a defense against other environmental insults including desiccation and heat (Crowe and Crowe, 1992). Vitrification occurs in many plants (Hirsch, 1987) and is a technique used in artificial tissue and cell cryopreservation (Pegg, 2001). Freeze avoidance strategies are used by many insects and other invertebrates; these animals synthesize antifreeze proteins and

cryoprotectants to lower the temperature at which body water will freeze to a value far below the normal environmental minimum temperatures (Storey and Storey, 1992). The majority of animals that survive freezing temperatures employ a freeze avoidance strategy (Storey and Storey, 2001). A smaller group of animals, which includes many insects, some intertidal invertebrates, and various terrestrially hibernating amphibians and reptiles, tolerate the freezing of extracellular body fluids.

To survive freezing a frog has to contend with multiple stresses including: A) potential physical damage caused by ice crystal formation, B) severe hypoxia (O_2 deprivation) and ischemia (interruption of blood flow) due to the freezing of blood plasma (I will refer to this as anoxia from this point onwards), C) intracellular dehydration and reduced cell volume caused by water exiting to join extracellular ice, and D) the interruption of multiple vital signs including heart beat, breathing, muscle movement, and nerve conductance (Storey, 1999). Multiple adaptive changes at the genetic and protein levels help the freeze-tolerant frog to overcome these obstacles.

Freezing follows a characteristic pattern in frogs (Fig. 1.1). In nature, wood frogs hibernate on the forest floor where they are insulated from rapid temperature change by layers of leaf litter and snow. However, as winter progresses, ambient temperature at the hibernation site slowly drops and the frog's body temperature decreases as well. Wood frogs can supercool to some extent and, on a dry substrate, they will typically cool to between -2.5 and -3°C before freezing begins, apparently initiated by the ice nucleating ability of certain bacteria on the skin surface. However, because frogs have to live in damp sites to keep from dehydrating, freezing probably more commonly begins due to skin contact with ice forming in their hibernaculum. If inoculated in this way, freezing can begin at any temperature below

the equilibrium freezing point of frog body fluids (about -0.5°C). In fact, 98% of frogs that were held in contact with a damp substrate were frozen at –2°C compared with only 20% when the frogs were on a dry substrate (Costanzo *et al.*, 1999a). Freezing at high subzero temperatures allows a slow rate of ice formation to occur that helps to ensure that ice crystals grow only in extracellular spaces and that provides the frog with lots of time to initiate and optimize various metabolic adjustments that aid freezing survival. Indeed, when freezing is at -2.5°C, it can take 12-24 hours for the maximal body ice content to be achieved. The initiation of freezing triggers an immediate jump in body temperature to about –0.5°C (Fig. 1.1) that is called the freezing exotherm. This occurs because the crystallization of water is an exothermic event due to the heat given off by the formation of ice crystals. Body temperature remains at this high value for minutes to hours (depending on body mass and ambient temperature) while ice continues to form and then declines over time as the rate of ice formation slows until body temperature again reaches ambient levels (see Storey and Storey, 1984; Storey and Storey, 1986a). From the skin surface, ice propagates inwards towards the core organs. Proton magnetic resonance imaging has shown the progression of the ice front through the body; in the frog shown in Figure 1.2, freezing is complete within about seven hours (ice appears black under ^{1}H-MRI) (Rubinsky *et al.*, 1994). When *R. sylvatica* are fully frozen, as much as 65-70% of total body water can be locked away as extracellular ice.

As mentioned previously, a freeze-tolerant animal has to contend with other factors besides the conversion of body water to ice. Frogs have the capacity to tolerate low levels of oxygen and the ability to tolerate extensive periods of hypoxia/anoxia aids survival in the frozen state when organs are completely cut off from the oxygen and nutrients that are

Fig. 1.1. Typical cooling and freezing curve for an adult male *R. sylvatica.* In this experiment, the frog was placed in an incubator set at –3°C. A thermistor is taped to the frog's abdomen recorded the temperature change over time. The sharp jump in temperature at about 40 min records the exotherm associated with ice nucleation. Subsequently, body temperature remains at this elevated value while ice accumulates and then body temperature slowly returns to ambient as maximal ice content is reached.

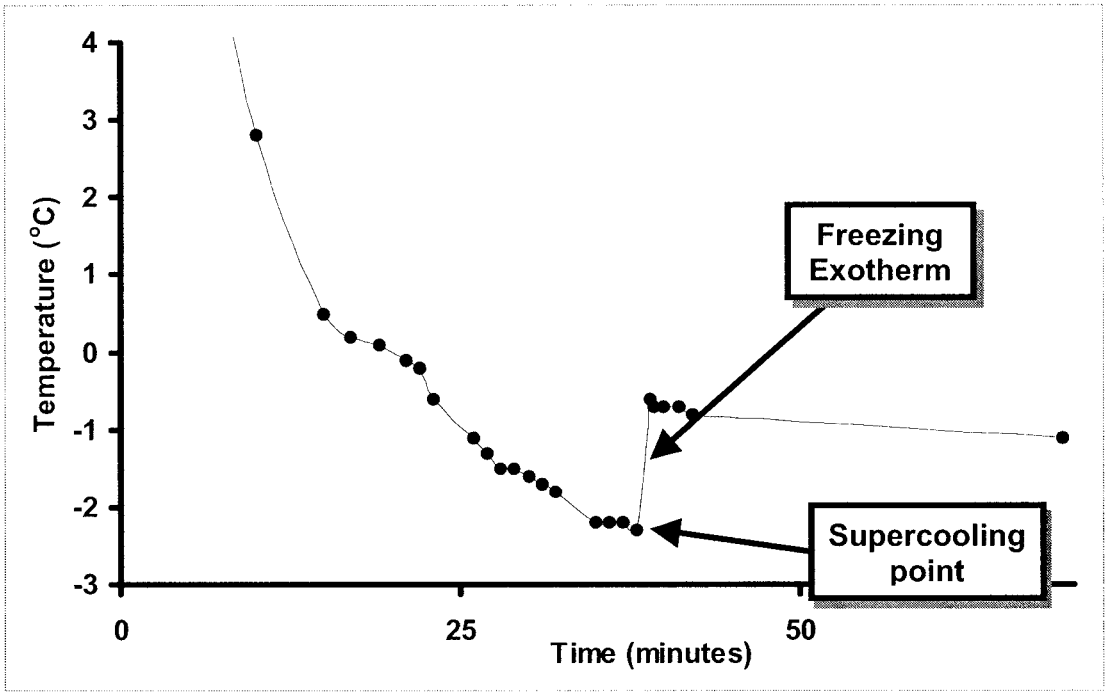
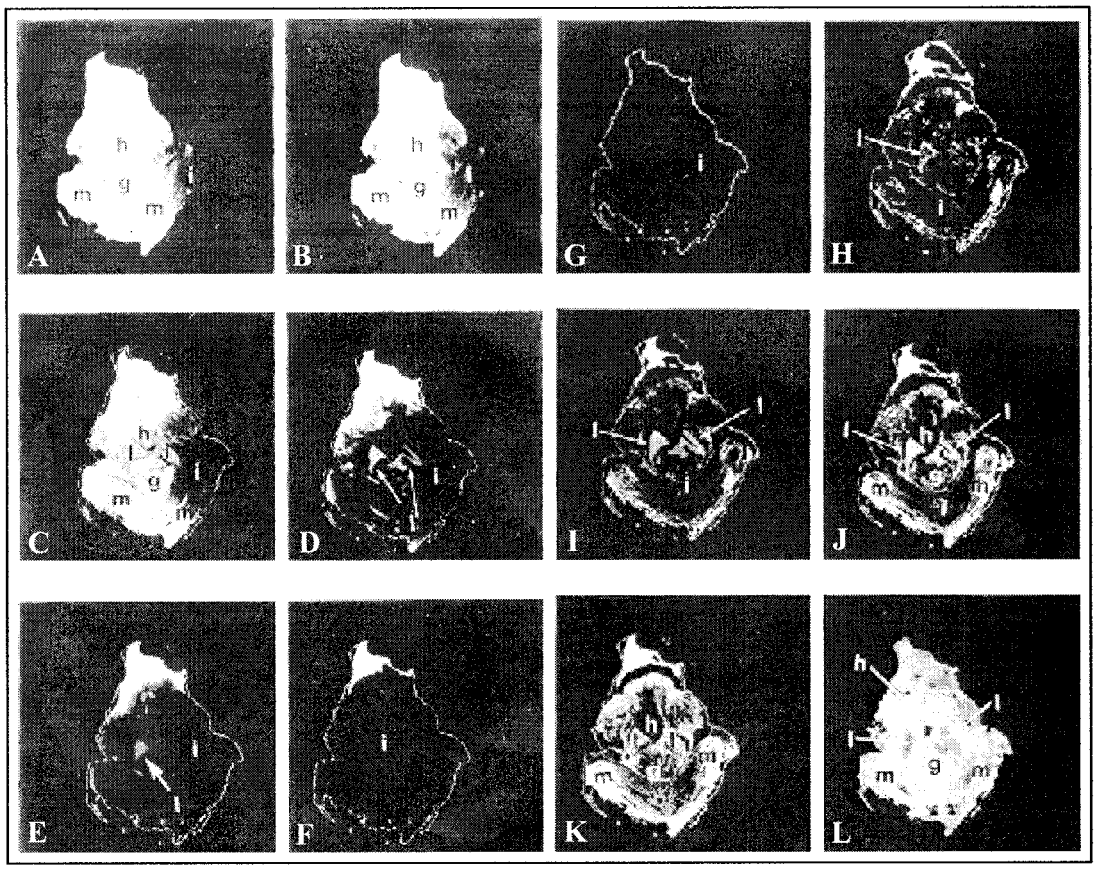

4
3
2
1
0
-1
-2
-3
Temperature (°C)
0
25
50
Time (minutes)
Freezing Exotherm
Supercooling point

Fig. 1.2. Proton magnetic resonance imaging of the freezing (A-F) and thawing (G-L) process. Ice (i) is not visible in ^{1}H MRI and so it appears black as compared to unfrozen areas, which appear white. This is a dorsal cross section of *R. sylvatica* frozen at –7.0°C for A) 10 min, B) 49 min, C) 1 h 59 min, D) 3 h 14 min, E) 3 h 43 min and F) 7 h 5 min. The frog was then thawed at 4.0°C for G) 0 min, H) 34 min, I) 52 min, J) 1 h 8 min, K) 1 h 27 min and L) 3 h 8 min. Tissues are labelled (m) leg skeletal muscle, (l) liver, (h) heart and (g) gut. Data are from Rubinsky et al. (1994).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normally supplied by the blood (Hermes-Lima and Storey, 1996; Storey, 1996). Amphibians as a group also have a high tolerance of extensive water loss because their highly water-permeable skins make it difficult to maintain optimal hydration at all times. (Shoemaker, 1992; Churchill and Storey, 1993). Cellular dehydration occurs during freezing as a result of the high percentage of total body water that moves into extracellular ice. Therefore, we can think of freezing as a form of dehydration. Indeed, the ability of frogs to survive freezing was probably aided by the pre-existing capacity of frogs to tolerate extensive dehydration (Churchill and Storey, 1993; Costanzo *et al,* 1999a; Storey *et al.*, 1996). However, some frogs that are dehydration tolerant are not freeze-tolerant. For example, the leopard frog, *Rana pipiens* is dehydration and anoxia tolerant, but cannot survive freezing. Therefore, there are other factors apart from dehydration and anoxia tolerances that contribute to being freeze-tolerant.

1.2 Freeze tolerance from an ecological view

R. sylvatica has a short generation time of around two to three years (Berven, 1990). There is a high gene flow between neighbouring populations of *R. sylvatica* resulting in similar allele frequencies between neighbouring populations (Newman and Squire, 2001). This species also exhibits strong philopatry, which is a tendency to remain at the established breeding grounds (Berven and Grudzien, 1990). Wood frogs breed in temporary forest ponds formed by spring meltwater and because these ponds dry out quickly as summer arrives, the frogs need to breed as soon as possible in the spring and eggs need to develop quickly. Winter hibernation on land allows species such as *R. sylvatica* to emerge and start breeding as much as a month before frogs that hibernate under water such as *R. pipiens*. However, the

unpredictable nature of snowfall and rainfall each year can mean that both the breeding ponds and the forest sites where adult *R. sylvatica* live vary widely in wetness/dryness from year to year. Hence, because of the philopatric nature of the wood frog, the species has to be well equipped to withstand periods of dryness.

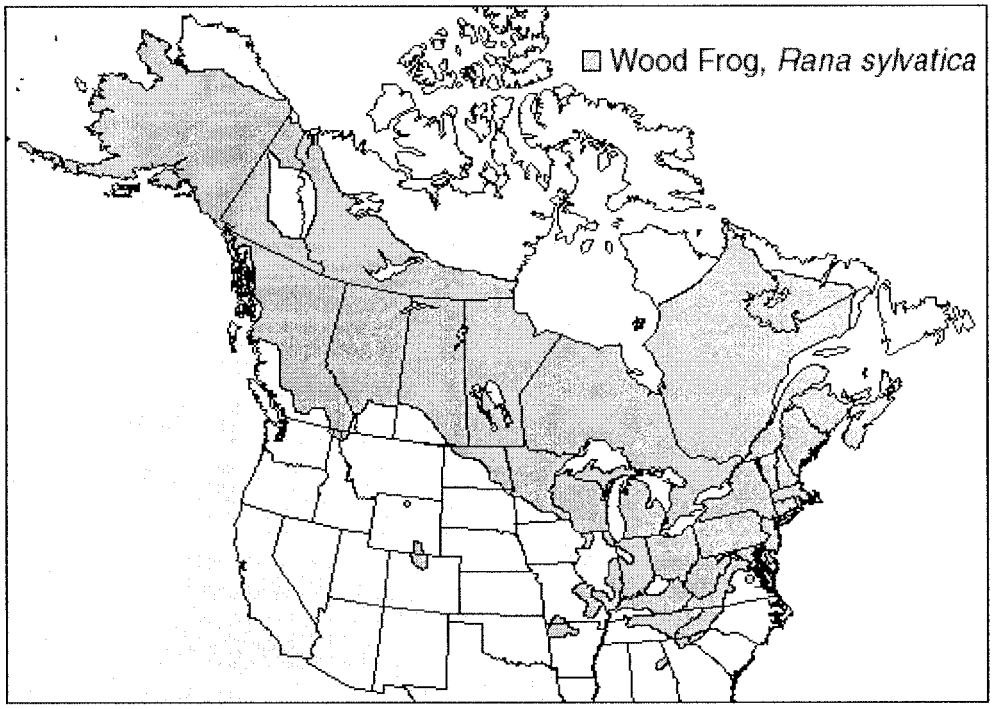
There are about 14 species of frogs living in Canada but only five are freeze tolerant. Of the freeze-tolerant frogs, *R. sylvatica* has the most northerly distribution, with specimens being found at the northern extent of the treeline from Labrador across to Alaska (Fig. 1.3) (Behler and King, 1979). This northerly distribution presumably allows the frog to take advantage of unoccupied niches, as it is the only frog that lives near the Arctic Circle. Other freeze tolerant species do not have such a northerly distribution but are found in areas exposed to freezing temperatures. *P. triseriata* ranges up to Hudson Bay and across the northern prairies and *P. crucifer* is found as far north as James Bay. The gray tree frog *H. versicolor* ranges only to southern Ontario and northern Maine.

All freeze-tolerant frogs have terrestrial hibernation sites. As a result of their water-permeable skin, this choice of hibernation site poses a problem. A terrestrial site under damp leaf litter or under a snow cover gives the frogs little chance of avoiding contact with ice once environmental freezing begins. Inoculative freezing by ice contact, occurring below -0.5°C, will start ice crystal formation. Therefore, a frog must be freeze-tolerant to survive such habitats.

1.3 The physiology of being freeze-tolerant in *R. sylvatica*

The ability to survive freezing is dependent on several factors including the rate of freezing, temperature of freezing, duration of freezing, cryoprotectant concentration and

Fig. 1.3. The North American distribution of the wood frog, *Rana sylvatica*. From: Northern Prairie Wildlife Research Center Home Page, 1997.


Wood Frog, *Rana sylvatica*

season. These factors all influence the degree to which body water is converted into ice. *R. sylvatica* can withstand the conversion of 65-70% of its body water into ice (Costanzo *et al.*, 1993; Layne, 1995).

Survival of frozen *R. sylvatica* was 100% at –4°C for two days, but decreased to 50% at –6°C for eleven days (Storey and Storey, 1986a). Assessments of long-term survival included 100% survival of frogs frozen at –2.5°C at 13 days (Storey and Storey, 1986b). A 100% survival rate was also documented for autumn-collected *R. sylvatica* frozen at –1.5°C for two weeks, but this dropped to 50% when the exposure was increased to 28 days (Layne, 1995; Layne *et al.*, 1998). One hundred percent of freeze tolerant frogs exposed to –30°C had 50% of their body water frozen and did not survive (Schmid, 1982). The ambient air temperature above the snowpack can get as low as –40°C in the regions where *R. sylvatica* lives but frogs cannot survive such temperatures. Hence, a key element of their survival is the insulation offered by layers of snow and leaf litter that typically prevent temperatures in the microenvironment under the snow from dropping below about -6°C.

The lower lethal limit (LLT) can be defined as the threshold temperature at which an animal can survive being frozen. For *R. sylvatica* this temperature is –5 to –6°C in the autumn and rises to around –3°C in the spring (Storey and Storey, 1987; Layne, 1995; Storey *et al.*, 1996). The differences in LLT values for autumn and spring frogs are most likely due to differences in cryoprotectant levels during those seasons (Storey and Storey, 1987).

The rate of temperature decrease also affects the survival of *R. sylvatica*. Spring frogs that were cooled to –2.5°C at -0.16 to -0.18°C/h had 100% survival. Survival decreased to 60-80% when the rate was increased to –0.3 to –1.0°C/h and to 0% survival when the rate was –1.17°C/h (Costanzo *et al.*, 1991). A slow cooling rate would occur in nature. When the

rate of cooling is slow, ice forms first in extracellular compartments and this places an osmotic stress on cells that leads to cellular water loss into growing extracellular ice crystals. If the rate of cooling is too fast, then the osmotic transfer of water out of the cell may not be enough to prevent intracellular supercooling. This could lead to lethal ice crystal formation inside the cell. A slow rate of cooling also ensures adequate time for cryoprotectant distribution and gene regulation. Cryoprotectant distribution was negatively affected when the freezing rate was too high in *R. sylvatica* (Storey, 1987; Costanzo *et al.,* 1992b).

1.4 Current knowledge of molecular adaptations for freeze tolerance

Glucose is the primary cryoprotectant in *R. sylvatica* (Storey and Storey, 1984). Within five minutes after the initiation of freezing at the skin surface, the synthesis of glucose from liver glycogen begins and the sugar is rapidly distributed throughout the body (Storey and Storey, 1984). As a cryoprotectant, glucose acts to retard water loss from cells into extracellular ice masses. Cryoprotectants also aid in stabilizing cell membranes and cellular macromolecules against the stresses associated with freezing (Mazur, 1984). For example, trehalose is a membrane protectant in freeze-tolerant insects that works by forming hydrogen bonds with the hydrophilic phospholipids of the membrane to help protect the bilayer from collapsing into a gel state under the compression stress caused by extensive cell volume decrease (Carpenter and Crowe, 1988 Wolkers *et al.*, 2002). Cryoprotectants should be soluble in water, depress the freezing point, penetrate easily into cells and do all of this with minimal cell toxicity. The glucose used by frogs for cryoprotection is synthesised by the liver, exported into the blood, and transported to the tissues. The average blood concentrations of glucose rise to 185 μmol/ml in the frog during freezing from control levels

of ~3-5 μmol/ml (Storey and Storey, 1984). Organ levels of glucose can reach as high as 390 μmol/g wet weight in liver, about 150-200 μmol/g in heart and kidney and about 30 μmol/g in leg muscle (Storey and Storey, 1984). The gradation in glucose levels with highest values in liver and other core organs, and lower values in peripheral organs arises because of the oppositely directed movement of the freezing front from the periphery inwards These glucose concentrations in the frozen state would be toxic to human cells, but the wood frog has adapted to tolerate these high levels. The mechanisms behind this tolerance are not fully known, but wood frogs represent a good model system for diabetes research.

Glucose distribution as a cryoprotectant is aided by an increase in the number of glucose transporters in the plasma membrane of liver. Transporter numbers are substantially higher in autumn compared with summer frogs, and freeze tolerant frogs also have more transporters than freeze intolerant species (e.g. *R. pipiens*). However, no seasonal effects on transporter numbers were found in skeletal muscle (King *et al*, 1995).

Having a high concentration of glucose in reserve is also beneficial to *R. sylvatica* during recovery from freezing. Because of their high osmolyte concentrations, the melting point of core organs like the liver and heart is lower than in peripheral tissues. Rubinsky *et al.* (1994) showed that during recovery, the core organs like the heart melt first and this aids recovery by restoring circulation to organs as fast as possible after they melt. In fact, heartbeat is re-established even before all body ice is cleared (Layne *et al.*, 1989). Early blood flow, followed by activation of breathing, allows transport of oxygen to anoxic tissues.

Studies of the biochemical mechanisms of freeze tolerance in *R. sylvatica* by our laboratory have centred on the protein and enzymatic changes that take place in wood frogs. Only recently have we begun to assess gene expression changes that support freeze tolerance.

Several genes have now been identified as up-regulated during freezing in wood frog liver (Cai and Storey, 1997a,b, Cai *et al*, 1997, McNally 2002), brain and skin (Wu, 1999) and heart (McNally *et al*, 2002; 2003).

The functional significance of the upregulation of these freeze-responsive genes is not known in all cases but some are readily understandable. For example, upregulation of the alpha and gamma subunits of fibrinogen in liver would increase the synthesis and export of this protein of the plasma clotting cascade (Cai and Storey, 1997a). Enhancement of clotting capability could be key to rapidly dealing with any internal bleeding injuries to organs caused by ice expansion in the microvasculature. The mitochondrial ADP/ATP translocase (AAT) is also upregulated during freezing and anoxia and this may be beneficial to maintaining the functional integrity of mitochondria under stress (Cai and Storey, 1997b). AAT is an inner mitochondrial membrane transporter that belongs to the same class of transporter as the inorganic phosphate carrier (PiC) that is identified in this thesis as another freeze-responsive gene (see Chapter 2). L7, a ribosomal subunit was also upregulated in freezing frogs (Wu, 1999). This subunit forms part of the stalk that connects the large ribosomal subunit (50S) to the small subunit (30S). The reasons for upregulating this gene is not certain as yet and requires further study.

Apart from identified genes that are freeze-responsive in wood frogs, several unknown genes have also been isolated whose function has yet to be determined. At this point, the function of the proteins that these genes encode can only be postulated based on their sequences and what these imply about protein structure, functional groups, sites for posttranslational modification, etc. Furthermore, with the use of isolated tissue incubations, regulatory control of gene expression by various hormone second messengers has been

evaluated with some success in indicating the signals that mediate upregulation of these unknown genes (McNally *et al.*, 2002; 2003).

Other studies have looked directly at the freeze responsiveness of selected second messenger pathways in frog organs including the effects of freezing on the activation of protein kinases A and C and the mitogen-activated kinases ERK, JNK, and p38 (Holden and Storey, 1996; Greenway and Storey, 2000). Except for ERK, all show temporal activation over the course of freezing and thawing in wood frogs. PKA has been definitely linked with the activation of glycogenolysis and cryoprotectant synthesis in frog liver but all of these protein kinases may also affect gene expression.

1.5 Rationale and Hypothesis

The study of animals with abilities to survive extreme conditions provides new insights and approaches to human problems. The wood frog as a model system has the potential to reveal principles and mechanisms that can have applications in a number of fields. For example, the mechanisms of organ cryoprotection that occur naturally in frogs have application for the development of strategies for mammalian organ cryopreservation. The mechanisms that allow frogs to avoid the debilitating effects of extreme hyperglycemia on metabolism and macromolecules may also have important applications for diabetes research. In addition, some of the research presented later in this thesis shows that wood frogs display an anti-apoptotic activity similar to that seen in cancers, and this may also introduce freeze-tolerant frogs as a model for cancer study.

The known gene and protein responses to natural freezing have tissue specific patterns and some organs are more transcriptionally active than others during freezing

(Storey and Storey, 2003). Liver is particularly important to the freezing survival of the whole animal for it produces and exports both the high quantities of glucose cryoprotectant and selected plasma proteins (e.g. fibrinogen) that have cryoprotective functions for the whole body. Hence, I chose to focus on the liver in this project due to its key role in *R. sylvatica* freeze tolerance.

To be tolerant of freezing, animals must respond in multiple ways to cope and survive. Freeze tolerant animals need to withstand dehydration, anoxia, and physical damage imposed by the freezing process. Freeze tolerant organisms must alter their metabolism and utilization of cellular resources in order to deal with the stress of freezing. To date, studies of freeze tolerance have never focussed on the effects of, or responses to, freezing on subcellular organelles. Mitochondria are quite sensitive to severe oxidative and osmotic stress like that experienced during freezing. Much study in recent years has shown that mitochondria are key sensors of overall cell stress. In fact, extensive research has looked at the breakdown of mitochondrial homeostasis, which leads to cytochrome c release and dooms the cell to undergo programmed cell death (apoptosis). Freeze tolerant organisms must be able to prevent or inhibit the tendency for cells to undergo apoptosis in response to stresses of extreme cell volume change and/or ischemia.

Consequently, I have chosen to look at mechanisms of mitochondrial response to freezing. The following two hypotheses provided the starting point for the studies reported in this thesis. Hypothesis 1, as will be shown in Chapter 2, originated from analysis of cDNA frozen liver library. From this cDNA library a clone was isolated that was a potential up-regulated freeze-responsive gene. This clone was an inorganic phosphate carrier (PiC) that resides in the inner mitochondrial membrane and belongs to a family of proteins that

transport ions and molecules across the mitochondrial membrane. Hypothesis 2, as will be shown in Chapter 5, arose from a cDNA microarray analysis. A gene that plays a role in suppression of apoptosis was upregulated in response to 24 hours of freezing in the liver.

Hypothesis 1: Cell water loss during freezing causes a major reduction in mitochondrial volume and a disruption of transmembrane ionic and osmotic balance that will require the upregulation and synthesis of selected mitochondrial membrane transporter proteins to aid in physical or metabolic stability of mitochondria during freezing.

Hypothesis 2: Anti-apoptotic signals will increase during freezing to prevent cellular apoptosis under the stresses of extreme volume reduction and ischemia that would normally trigger mitochondrial damage and initiate apoptosis.

1.6 Experimental Outline

The ability of *R. sylvatica* to survive freezing involves changes at the gene and protein level. As will be shown in Chapter Two, the screening of a cDNA library made from liver of frozen frogs produced a clone that was up-regulated during freezing. Sequence analysis of the clone revealed it to be an inorganic phosphate transporter found in the inner mitochondrial membrane. Chapter Two will show the involvement of mitochondrial membrane transporters in the freezing response.

The role of mitochondria and inner mitochondrial transporters in freeze tolerance is further investigated in Chapter Three. Here I asked the question: does the elevation of transporter levels come from an increase in mitochondrial copy numbers? In addition, the control of the inorganic phosphate carrier was investigated through analysis of the distribution pattern of transcripts between functional polysomes and inactive monosomes

during freezing. As well, the protein levels of PiC were investigated in other stress tolerant frog species.

Transcription factors are mediators of gene expression, and subsequent protein levels, in the cell. Phosphorylation of key amino acid (AA) residues on a transcription factor can affect the influence that the factor has on target genes. Often protein kinase mediated phosphorylation of a transcription factor is necessary before it can bind to and activate the transcription of a gene. The use of phospho-specific antibodies and western blot analysis allows the quantification of transcription factor control in stressed tissues. Chapter Four looks at the control of transcription factors in liver tissue during freezing stress.

Freezing stress can play havoc with cell integrity. Cells and their internal organelles can be damaged by excessive shrinking or swelling during the course of freeze-thaw or by physical injury from ice crystals. Typically damaged cells are identified and destroyed in a process called apoptosis. However, wood frog cells can go through these extreme stresses without being "marked" for apoptosis. I proposed that this could be because these cells exhibit enhanced anti-apoptotic activity as compared with normal vertebrate cells. The changes seen in mitochondria transporter levels in response to freezing suggested that liver cells may be counteracting signals that would cause a cell to undergo apoptosis, a process that is also seen in some cancers. As well, as shown in Chapter Four, transcription factors that are known to control or regulate cell survival genes are activated during stress. Chapter Five examines anti-apoptotic activity in *R. sylvatica* via analysis of several proteins involved in apoptosis. This chapter represents a new avenue of research and introduces *R. sylvatica* as a model animal for cancer research.

CHAPTER TWO

Isolation and characterization of the mitochondrial phosphate carrier upregulated during freezing in the wood frog Rana sylvatica.

2.1 Introduction

Freeze tolerance involves a complex array of changes in gene and protein expression, altered patterns of signal transduction, changes to the regulatory controls on selected enzymes, and the synthesis of high concentrations of glucose as a cryoprotectant (Storey 1999; Storey and Storey 1996, 2001). Recent studies by our lab have documented several examples of freeze-induced gene upregulation including genes encoding fibrinogen subunits (Cai and Storey, 1997a), ADP/ATP translocase (Cai *et al*, 1997), and three genes encoding novel proteins (FR10, FR47, Li16) with different organ-, time-, and stress-specific distributions but as yet unknown functions (Cai and Storey, 1997b, McNally *et al*, 2002, 2003).

Multiple freeze-responsive genes remain to be identified and the present chapter describes one of them. Screening of a cDNA library constructed from liver of frozen wood frogs revealed the freeze-responsive upregulation of the nuclear-encoded gene (*pic*) for the inorganic phosphate carrier protein (PiC). PiC is an inner mitochondrial membrane protein that catalyzes the electroneutral movement of inorganic phosphate from the cytosol across the inner mitochondrial membrane into the matrix of mitochondria with H^+ ions (symport) or for OH^- (antiport) (Kramer, 1996; Stappen and Kramer, 1994). Both *pic* mRNA levels and PiC protein content increase substantially during freezing and also in response to dehydration suggesting that regulation of the transporter is responsive to changes in cellular water content and/or the consequent changes in intracellular or intramitochondrial ionic strength and osmolality.

2.2 Materials and methods

2.2.1 *Animals*

Two-year-old male wood frogs between 5-7g were collected in the first week after spring thaw (water temperature was <8°C) of 2001 from the Ottawa, Ontario area. Animals were washed in a tetracycline bath and placed in plastic boxes with damp sphagnum moss at 5°C for at least 2 weeks before experimentation. Control frogs were sampled directly from this condition. Control and experimental frogs were both killed by pithing and then organs were rapidly excised, frozen in liquid nitrogen, and stored at –80°C until use. Protocols for animal care, experimentation and euthanasia were approved by the Carleton University Animal Care Committee in accordance with the guidelines set down by the Canadian Council on Animal Care.

For freezing experiments frogs were placed in plastic boxes with damp paper toweling on the bottom and then put in an incubator set at -3°C. The damp toweling ensures that all animals are rapidly seeded when ice begins to form on the substrate and initial tests showed that, on average, pre-freeze cooling prior to nucleation required ~45 min (determined from initial trials with animals that had thermistors taped to their abdomens). All frogs were allotted an initial 45 min cooling after which freeze duration was timed for 2, 6 or 24 hours followed by sampling. Other frogs were frozen for 24 hours and then sampled after thawing at 5°C for 1, 2, 4 or 8 hours.

For anoxia experiments, frogs were transferred to 700 ml bottles (5 frogs per bottle) held in crushed ice and containing 5 ml of distilled water, which had been bubbled with N_2 gas (0.2% CO_2-99.8%N_2) for 30 min. Bottles were tightly capped and sealed with a covering of parafilm and N_2 gassing was continued for another 30 min through syringe ports in the caps. Gas lines were then removed and bottles were quickly scaled

and then placed into a 5°C incubator for 4 or 24 hours. At the end of this time, bottles were transferred back to a crushed ice bath, gas lines were quickly reintroduced to maintain the anoxic atmosphere and then animals were quickly sampled. Some frogs were made anoxic for 24 hours and then transferred to a jar with air holes and allowed to recover from anoxia for 4 hours in a 5°C incubator. The pO_2 in jars made anoxic is typically less than 1 torr.

Dehydration experiments were conducted as described by Churchill and Storey (1993). In brief, frogs were placed in closed desiccators at 5°C with a layer of silica gel desiccant on the bottom. Frogs were separated from the desiccant by a sponge pad. Frogs were allowed to dehydrate at an average rate of 0.5% of total body water lost per hour until 20% and 40% of body water was lost. Water loss was confirmed by weighing the frogs at intervals during the experiment. Some frogs were first dehydrated to 40% of total body water lost and were then placed in containers with 1 cm of distilled water on the bottom and allowed to fully rehydrate for 24 h at 5°C.

2.2.2 *cDNA Library Screening*

A cDNA library was constructed using the Lambda ZAP-CMV XR Library Construction kit (Stratagene) with mRNA isolated from liver of 24 h frozen frogs by Dr. Q. Cai (Bio S&T, Quebec). The cDNA library was differentially screened using ^{32}P-labelled single-stranded total cDNA probes made from liver mRNA of control versus 24 h frozen frogs. Plaques that showed a 5-fold or greater signal when exposed to the frozen versus control probes were collected and subjected to further rounds of purification and screening. This selection criterion would undoubtedly miss some upregulated genes,

however, I wanted to decrease the chance of isolating a false-positive clone and purposefully set my threshold high. After selection, potential upregulated clones were rescued by *in vivo* excision and converted to pBluescript circular double-stranded plasmid according to the manufacturer instructions (Stratagene). One clone, termed *frozen liver 8* (*fl-8*), that was subsequently shown to contain the nucleotide sequence (*pic*) of the mitochondrial inorganic phosphate carrier protein (PiC), was chosen for further study.

2.2.3 *RT-PCR*

The reverse transcription-polymerase chain reaction (RT-PCR) was used to assess levels of *pic* mRNA transcripts in liver of control, 24 hour frozen and 8 hour thawed frogs. Tissue mRNA was purified using Oligotex Mini-Prep (QIAGEN) and eluted two times with 40 µl of elution buffer provided with the kit (preheated to 70°C). mRNA was extracted using Oligotex beads (QIAGEN) and the purified mRNA was then reverse transcribed into cDNA using a reverse transcriptase enzyme (Superscript, GIBCO). The cDNA:mRNA hybrids were serially diluted and then RT-PCR was performed using frog specific α-tubulin primers to normalize the amount of mRNA in controlled and stressed samples. The concentrations of control and stressed mRNA were adjusted using distilled-deionized water to ensure that there was the same concentration of starting material in serially-diluted control and stressed samples. The normalized samples were then amplified using primers designed specifically for frog *pic* (5'-GGCATCCTCAGTTGTGGTAT-3' and 3'-ACAAGACACGGTAGCAGAGG-5'). PCR was carried out under conditions of 94°C denaturation (1 min), 55°C annealing (1 min),

and 72°C elongation (1 min), for 35 cycles using an MJ-Research PTC-100 thermocycler.

2.2.4 *Northern Blot analysis*

Total RNA was extracted from tissues using Trizol (Gibco-BRL, Bethesda, MD) and methods previously described (Chomczynki, 1993). Briefly, ~50 µg of frozen tissue was placed in a 2 ml Eppendorf tube and homogenised in 500 µl of Trizol reagent. Chloroform was added (0.2 ml/ml of Trizol) and the samples were incubated at 21°C for 2-3 min. Samples were then centrifuged at 15,000 x g for 15 min to separate the sample into an upper aqueous phase and a lower organic phase. After centrifugation, the upper aqueous phase was removed to fresh 1.5 ml tubes containing an equal volume of isopropanol to precipitate the RNA pellet. RNA was precipitated over 15 min at 21°C and then centrifuged at 12,000 x g for 10 min. The supernatant was discarded and the pellets were washed with 70% ethanol and centrifuged again. The total RNA pellet was resuspended in 200 µl of RNAase free distilled water.

Aliquots (16 µg) of total RNA were separated on a 1.5% denaturing formaldehyde gel for ~2 h at 90V to ensure adequate separation. RNA was transferred onto nitrocellulose (Hybond, AMERSHAM) using a standard capillary transfer method. Blots were hybridized with radiolabeled *fl-8* probe and visualised via exposure to phosphoimaging screens. Figure 2.3 shows a full Northern blot along with the ethidium bromide stained formaldehyde gel. For the rest of the thesis only the relevant sections of each northern are shown. The screens were scanned with a Personal Molecular Imager FX (BIO-RAD) and the images were analysed using ImageQuant (Ver. 4.2) software to provide a densitometric analysis of band intensity.

2.2.5 *Western Blot analysis*

PiC protein levels were quantified using Western blotting. Frozen tissue samples were homogenized (1:9, w/v) in a solution of 25 mM MOPS, 50 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM Na-orthovanadate and 1 mM DTT with a few crystals of phenylmethylsulfonyl fluoride added immediately prior to homogenization. After centrifugation for 10 min at 10,000g, the supernatants were removed and mixed (1:1 v/v) with sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue and 10% (v/v) 2-mercaptoethanol. Protein concentration was measured using the Bradford assay (Bradford, 1976) with the Bio-Rad prepared reagent and 20 μg of total protein was loaded into each sample well. This amount of protein was previously determined by loading different concentrations of protein followed by immunodetection to ensure that quantification of protein bands was in the linear range of detection. Samples were loaded onto sodium-dodecyl-sulfate (SDS) polyacrylamide gels and electrophoresis was carried out for two hours at 150V. Proteins were then electroblotted onto polyvinylidene difluoride (PVDF) (Immobilon-P Transfer Membrane, Millipore, Bedford,MA) by wet transfer using a transfer buffer solution containing 25 mM Tris (pH 8.5), 192 mM glycine, and 10% (v/v) methanol at 4°C for 1.5h at 0.3mA. The membranes were washed in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20) and then blocked for 1 hour at RT in a 1.0% w/v solution of non-fat dry milk (NFDM). Membranes were then probed with primary antibody diluted (1:3000 v/v) in blocking solution (TBST, 1.0% NFDM) overnight at 4°C followed by washing three times in TBST for 5 min at 21°C. The membranes were then incubated in secondary antibody (conjugated with

horseradish peroxidase) diluted 1:2000 v/v in blocking solution for 1 hour at 21°C. Bands were detected using enhanced chemiluminescence (ECL) (DuPont NEN, Boston, MA); the membrane was exposed to X-ray film and the resulting image was scanned on a HP ScanJet 3c using HP Deskscan II (v.2.9) software and quantified using ImageQuant (v.4.2, Innovative Optical Systems Research, Molecular Dynamics). After immunodetection was complete, the membranes were stained with Coommassie blue to reconfirm equal loading of samples and proper transfer by analysis of multiple bands.

Polyclonal antibodies recognizing PiC were raised in rabbits as described previously for PiC (Capobianco *et al.,* 1991). Cross-reaction with frog samples produced a single band of the expected molecular weight (~35 kDa). For anti-PiC, the source antigen was bovine, which shared 86.6% identity with the frog sequence. As well RT-PCR amplification and Northern blot analysis gave a single band so I was quite confident in the validity of the antibodies. Figure 2.5 shows a full western blot with a single cross-reacting band. Subsequent western blots in this thesis will show only the relevant sections. Antibodies for PiC were kindly provided by F. Palmieri. Secondary anti-rabbit antibodies conjugated with horseradish peroxidase and pre-stained protein markers (broad range) were from New England Biolabs (Mississauga, Ontario). Statistical significance of the data was tested using analysis of variance (Model I) followed by the Dunnett's test (two-tailed).

2.2.6 *DNA and Protein Sequence analysis*

DNA sequencing was conducted by Canadian Molecular Research Services (Orleans, Ontario). Alignments of sequence data and prediction of the open reading frame

(ORF) used DNAMAN (v.4.11). Primers for RT-PCR were designed using Primer Designer for Windows (v.3.0). Protein features were predicted based on searches of the Conserved Domain Database (NCBI-BLAST). Predictions of transmembrane regions were done using HMMTOP (Tusnády and Simon, 1998).

2.3 Results

Differential screening of a cDNA library made from liver of frozen wood frogs using ^{32}P-labeled probes made from mRNA of control (5°C-acclimated) versus frozen (24 h at -3°C) frogs revealed several clones that were putatively upregulated (5-fold or greater) in response to freezing. One clone, termed frozen liver 8 (*fl-8*), was chosen for further analysis. Nucleotide sequencing revealed a 1370 bp sequence that contained a potential full open reading frame running from nucleotide 44 to 1126 as well as a polyadenylation signal (nucleotides 1296-1301) and a poly A^+ tail; the sequence was submitted to Genbank with the accession number AF536222 (Fig. 2.1). The sequence encoded a protein of 360 amino acids. BLAST search analysis revealed a high degree of sequence identity with the mammalian mitochondrial inorganic phosphate carrier (PiC), a nuclear-encoded gene (Kramer, 1996). Figure 2.2 shows a comparison of the amino acid sequence of the wood frog protein with PiC from three mammalian species. Sequence differences between the frog and mammalian proteins were primarily limited to a small segment of the protein from amino acids 21-52. Overall, wood frog PiC showed 84.3, 86.2 and 86.6 % sequence identity with the sequences of bovine (Runsiwick *et al.*, 1987), human (Dolce *et al.*, 1991), and rat (Ferreira *et al.*, 1989) PiC, respectively.

Analysis of frog PiC revealed the presence of three repeats of about 100 amino acids each

Fig. 2.1. Nucleotide sequence of the insert from clone *fl-8* and its deduced amino acid sequence. The predicted ORF encodes a protein 361 amino acids in length and nucleotide sequence comparison identified the clone as encoding the mitochondrial phosphate transporter (Genbank accession number AF536222). The stop codon is indicated by an asterisk and the polyadenylation signal is underlined.

```
1          GCACGAGTGAGGGCCGAGTGAGCGCCATCTTCTAGCTGCCGAAATGTACTCCACCGTTGC
1                                                     M  Y  S  T  V  A

61         GCACCTAGCTCGGGCGAATCCGTTCAATGCACCACATTTCCAGGTGGGTCAGGAATGTGC
7            H  L  A  R  A  N  P  F  N  A  P  H  F  Q  V  G  Q  E  C  A

121        GACTTTAAGGAAGAAAAACACTTCAGAGATCCAACCTGTCCGACGCCTTGCTGCTGCAGC
27           T  L  R  K  K  N  T  S  E  I  Q  P  V  R  R  L  A  A  A  A

181        TACTGCTGCTGAAGGCGACTACAGTTGTGAATATGGCTCAACAAAATTTTATGCTTTTTG
47           T  A  A  E  G  D  Y  S  C  E  Y  G  S  T  K  F  Y  A  F  C

241        TGGCTTTGGTGGCATCCTCAGTTGTGGTATAACTCACACTGCAGTTGTACCTTTGGATTT
67           G  F  G  G  I  L  S  C  G  I  T  H  T  A  V  V  P  L  D  L

301        GGTGAAATGTCGTATGCAGGTGGATCCCCAAAAGTACAAGAGCATTTTCAGTGGGTTTTC
87           V  K  C  R  M  Q  V  D  P  Q  K  Y  K  S  I  F  S  G  F  S

361        TGTAACTCTTAAGGAAGATGGTGTCCGTGGACTTGCCAAGGGATGGGCCCCAACATTTAT
107          V  T  L  K  E  D  G  V  R  G  L  A  K  G  W  A  P  T  F  I

421        TGGGTATTCCATGCAAGGATTGTGTAAATTTGGATTTTATGAAGTCTTCAAGGTCTTGTA
127          G  Y  S  M  Q  G  L  C  K  F  G  F  Y  E  V  F  K  V  L  Y

481        TTCTAACCTTCTAGGAGAGGAAAATACCTATTTGTGGCGCACATCTCTGTACCTGGCTGC
147          S  N  L  L  G  E  E  N  T  Y  L  W  R  T  S  L  Y  L  A  A

541        ATCTGCTAGTGCCGAGTTCTTTGCAGATATTGCACTGGCTCCAATGGAAGCTGCAAAAGT
167          S  A  S  A  E  F  F  A  D  I  A  L  A  P  M  E  A  A  K  V

601        CCGTATTCAAACACAGCCGGGATATGCAAATACTTTGCGACAAGCGGCACCCAAGATGTA
187          R  I  Q  T  Q  P  G  Y  A  N  T  L  R  Q  A  A  P  K  M  Y

661        TGCTGAAGAAGGAATCTGGGCGTTCTACAAAGGAGTTGCTCCTCTCTGGATGAGGCAAAT
207          A  E  E  G  I  W  A  F  Y  K  G  V  A  P  L  W  M  R  Q  I

721        TCCCTACACCATGATGAAGTTTGCTTGCTTTGAACGTACAGTTGAAGCACTGTACAAGCA
227          P  Y  T  M  M  K  F  A  C  F  E  R  T  V  E  A  L  Y  K  H

781        TGTTGTACCCAAGCCACGAAGTGAATGTTCAAAGTCCGAACAGTTGGTTGTGACATTTGT
247          V  V  P  K  P  R  S  E  C  S  K  S  E  Q  L  V  V  T  F  V

841        TGCTGGCTATATTGCTGGTGTGTTCTGTGCCATCGTCTCCCATCCTGCTGATTCAGTGGT
267          A  G  Y  I  A  G  V  F  C  A  I  V  S  H  P  A  D  S  V  V

901        GTCTGTGTTGAACAAAGAAAAGGGAAGCACTGCTACGCAAGTCCTTAAACGACTTGGACC
287          S  V  L  N  K  E  K  G  S  T  A  T  Q  V  L  K  R  L  G  P

961        AAAAGGAGTCTGGAAGGGGCTGACGGCCSGTATCATCATGATTGGTACCTTGACTGCTCT
307          K  G  V  W  K  G  L  T  A  X  I  I  M  I  G  T  L  T  A  L

1021       GCAGTGGTTCATCTATGACTCTGTGAAGGTCTACTTTAGACTTCCTCGTCCTCCTCCCCC
327          Q  W  F  I  Y  D  S  V  K  V  Y  F  R  L  P  R  P  P  P  P

1081       TGAGATGCCMGAGTCTCTGAAGAAGAAGCTTGGCTTGACTGAGTAAACATATGGACTTGA
347          E  M  P  E  S  L  K  K  K  L  G  L  T  E  *

1141       ATAGTGAAAGTTCCAATGGCACTTCAAACTGAGAAGTTTTTATAAATGCAATCGTGTAGG
1201       ACTTGCCATTTTCTCAGATGTAATTATTGGTTGTATTCACTCTGCTTGGTACACCACAAC
1261       TGTAATTTGGTTTTACCACTGTACCTGTCTGTCTAAATAAACAGAAGACCCCAGCGGATG
1321       AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 2.2. Comparison of the amino acid sequences of wood frog PiC (Genbank accession number AF536222) with the sequences of rat liver (AAA41634), human heart (CAA42641), and bovine heart (CAA28951) PiC. Dashes replace amino acids that are identical with those in the frog sequence and periods indicate amino acid residues that are not present in some sequences. Lowercase amino acids underneath the frog sequence indicate amino acid changes in other species. The three repeat segments characteristic of mitochondrial membrane carriers are shaded and within each segment the two transmembrane portions are indicated with Roman numerals (I-VI).

```
Frog    MYSTVAHLARANPFNAPHFQVGQECATLRKKNTSEIQPVR      40
Rat     -f-s--------------l-lvhdvsgp-sppgp.....p      35
Human   -f-s-----------t--l-lvhdglgdlrssspgptgqp      40
Bovine  -f-s-----------t--l-lvhdglgdlrssspgptgqp      40

                                              I
Frog    RLAAAATAAEGD..YSCEYGSTKFYAFCGFGGILSCGITH      78
Rat     -rsrhla--aveg.----f--m-y--l-----v----l--      74
Human   -rprnla--aveeq---d---gr-fil--l---i---t--      80
Bovine  -rprnla--avee.----f--a-y--l-----v----l--      79

        ________                              ___
Frog    TAVVPLDLVKCRMQVDPQKYKSIFSGFSVTLKEDGVRGLA     118
Rat     --------------------g--n---i----------      114
Human   --l-----------------g--n--------------      120
Bovine  --------------------g--n--------------      119

            II
        ____________
Frog    KGWAPTFIGYSMQGLCKFGFYEVFKVLYSNLLGEENTYLW     158
Rat     ------l-----------------a----i---------      154
Human   -------l----------------------m---------      160
Bovine  -------l----------------------m---------      159

                 III
        ____________________
Frog    RTSLYLAASASAEFFADIALAPMEAAKVRIQTQPGYANTL     198
Rat     ----------------------------------------      194
Human   ----------------------------------------      200
Bovine  ----------------------------------------      199

                        IV
                ____________________
Frog    RQAAPKMYAEEGIWAFYKGVAPLWMRQIPYTMMKFACFER     238
Rat     -e-v----k---ln--------v-----------------      234
Human   -d------k---lk------------------------      240
Bovine  -d------k---lk------------------------      239

                                    V
                                ____________
Frog    TVEALYKHVVPKPRSECSKSEQLVVTFVAGYIAGVFCAIV     278
Rat     -------f---------t-a--------------------      274
Human   -------f-----------p--------------------      280
Bovine  -------f-----------p--------------------      279

                                          ____
Frog    SHPADSVVSVLNKEKGSTATQVLKRLGPKGVWKGLTAXII     318
Rat     ------------------s---q---fr------f-r--      314
Human   -----------------s-sl------f-------f-r--      320
Bovine  -----------------s-sl------f-------f-r--      319

            VI
        ____________
Frog    MIGTLTALQWFIYDSVKVYFRLPRPPPPEMPESLKKKLGLTE     360
Rat     ----------------------------------------      356
Human   ----------------------------------------q     362
Bovine  ----------------------------------------q     361
```

that are characteristic of the inner mitochondrial membrane protein family (shaded sequences in Fig. 2.2). These span amino acid residues 59-152, 163-246, and 262-338 predicted using HHMTOP (Tusnády and Simon, 1983). Within each of the repeats are two hydrophobic regions (shown by Roman numerals in Fig. 2.2) that are separated by a hydrophilic segment and result in six transmembrane regions.

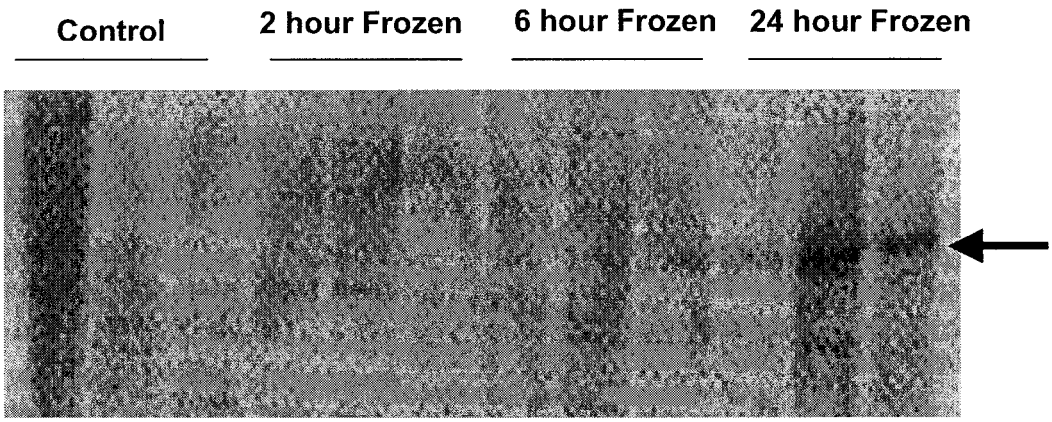
2.3.1 *Northern blotting*

Both Northern blot and RT-PCR analysis using the *fl-8* clone as a probe showed that *pic* transcript levels increased in wood frog liver during freezing. Northern blot analysis of changes in *pic* transcript levels over a time course of freezing is shown in Figure 2.3. Transcripts were virtually undetectable in liver of control frogs and frogs sampled 2 h after freezing began. However, a marked induction of the gene was seen after 6 hours of freezing and transcript levels continued to rise with longer freezing.

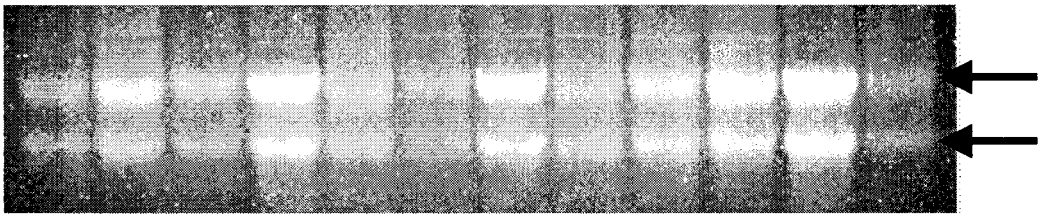
Because of the difficulty of detecting *pic* transcripts in liver extracts of control frogs, I turned to RT-PCR to further assess the changes in *pic* transcripts during freezing and thawing. RT-PCR was carried out using mRNA isolated from liver of control, 24 h frozen, and 8 h thawed frogs and primers specific to the *fl-8* clone (Fig. 2.4). RT-PCR is more sensitive than Northern blotting, allowing detection of minute amounts of transcript. Control, frozen and thawed samples were serially diluted and PCR was performed on each dilution. For each experimental condition, the intensity of the band should decrease as the dilution is increased (higher dilutions have less starting material). The control sample (Fig. 2.4a) shows product up to the 10^{-2} dilution whereas the 24 h frozen sample showed product even at high dilutions (10^{-4}) (Fig. 2.4b). This indicates

Fig. 2.3. (A) Northern blot analysis of *pic* transcript levels (probed with clone *fl-8*) in wood frog liver over a time course of freezing. Arrow points to a band of ~1400 bp which corresponds to the *pic* transcript. (B) Ethidium bromide stained formaldehyde agarose (1.5%) gel of total RNA of wood frog liver over a time course of freezing. Arrows point to 28S (upper arrow) and 18S (lower arrow) ribosomal bands. Densitometric analysis of northern blot showing mean intensity $\pm$ SEM for n=3 separate isolations of mRNA from frog liver under each experimental condition.

A


Control
2 hour Frozen
6 hour Frozen
24 hour Frozen

B



C

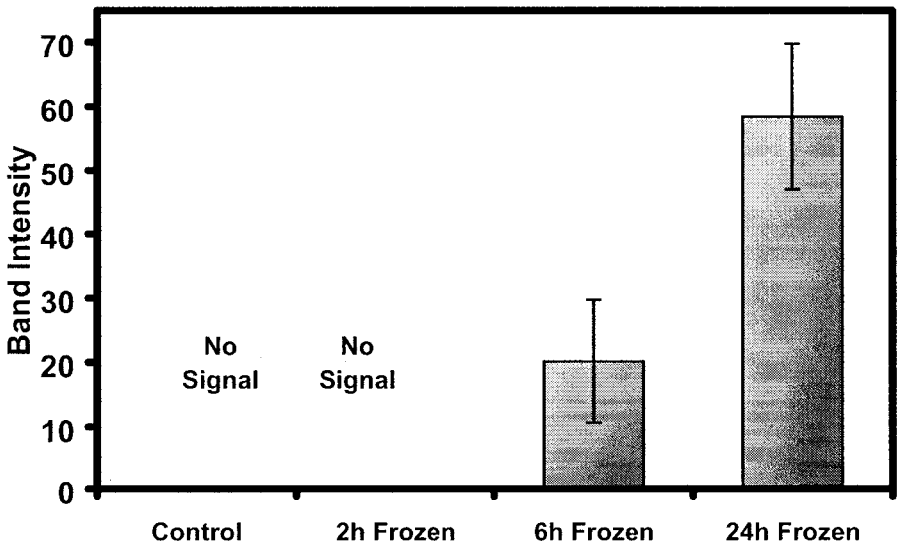
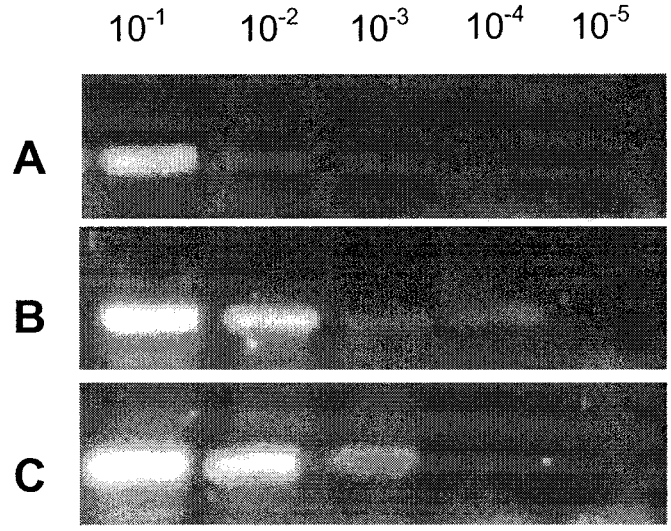
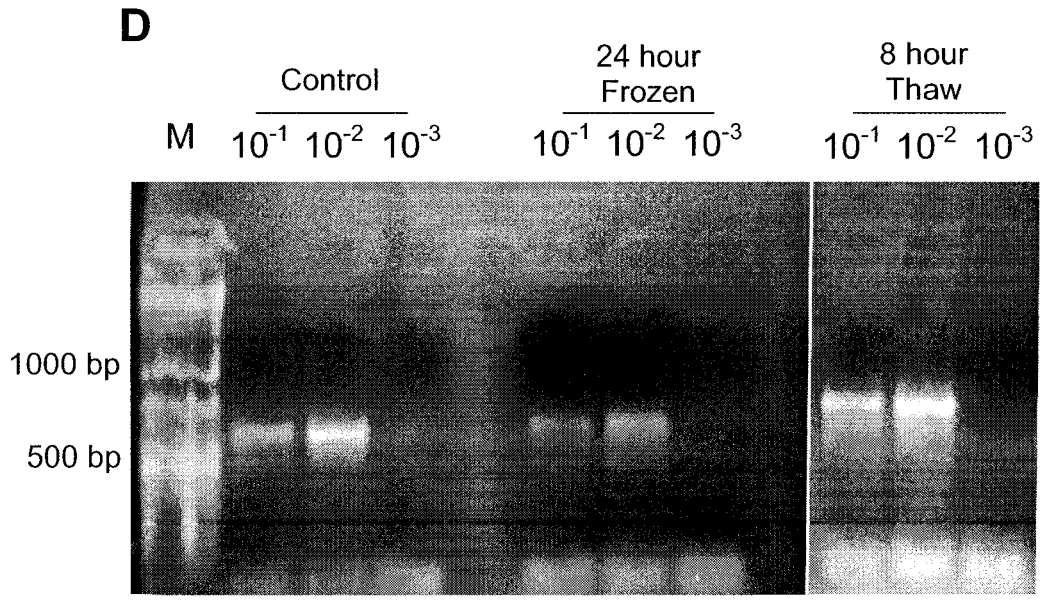

Band Intensity
70
60
50
40
30
20
10
0
No Signal
No Signal
Control
2h Frozen
6h Frozen
24h Frozen

Fig. 2.4. Relative RT-PCR of *pic* amplified from wood frog liver. Dilutions of mRNA isolated from liver of (A) control, 5°C acclimated frogs, (B) 24 h frozen frogs, and (C) 8 h thawed frogs were amplified using the PCR conditions described in the Materials and Methods. (D) Normalization of samples of mRNA using primers for α-tubulin giving a product of 619 bp. Lane M in tubulin normalization is a 1kb DNA ladder (Invitrogen). PCR products were run on a 1.0% TAE agarose gel and stained with ethidium bromide. Product is visible in the 24 h frozen sample even at high dilutions (10^{-4}).


10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5}
A
B
C


D
Control
24 hour Frozen
8 hour Thaw
M
10^{-1} 10^{-2} 10^{-3}
10^{-1} 10^{-2} 10^{-3}
10^{-1} 10^{-2} 10^{-3}
1000 bp
500 bp

much higher levels of *pic* transcripts in extracts from liver of frozen frogs. The thawed sample (Fig. 2.4c) showed product at the 10^{-3} dilution indicating reduced levels compared with frozen liver but greater levels than controls.

Figure 2.5 shows changes in *pic* transcript levels in liver of wood frogs exposed to anoxia or dehydration stresses. Each of these stresses mimics an element of freezing: the anoxia/ischemia induced by plasma freezing versus the strong cell volume reduction caused by water exiting into extracellular ice crystals. The data show that *pic* transcript levels did not respond to anoxia but that transcripts were strongly upregulated by the loss of 20 or 40% of total body water (Fig. 2.5). Mean transcript levels were 3.1-fold higher than control values in liver of 20% dehydrated frogs (although this was not a significant increase) and rose to 9.0-fold higher than controls in 40% dehydrated animals ($P<0.05$).

2.3.2 *Western blotting*

The effects of freeze/thaw on the content of PiC protein in wood frog liver were assessed via Western blotting using a polyclonal antibody raised against bovine PiC. Figure 2.6 shows that the PiC antibody cross-reacted with a single band at the expected molecular weight of ~35 kDa. PiC protein content was not affected by short-term freezing but levels had increased by 3.3-fold after 24 h frozen ($P<0.05$). PiC protein content remained elevated (2.8-fold higher than controls; $P<0.05$) after 1 h of thawing but then gradually decreased until levels similar to control values were re-established after 8 h thawed.

Immunoblotting was also used to assess PiC protein levels in other organs of wood frogs during freeze/thaw, comparing levels in control, 24 h frozen, and 8 h thawed

Fig. 2.5. (A) Northern blot analysis of total RNA isolated from liver of anoxia- or dehydation-exposed frogs probed with *fl-8.* Arrow points to a band of ~1400 bp in size which corresponds to *pic*. (B) Densitometric analysis of northern blots showing mean intensity $\pm$ SEM for n=3 blots using separate isolations of liver mRNA from different animals. [a] - significantly different from controls, $P<0.05$.

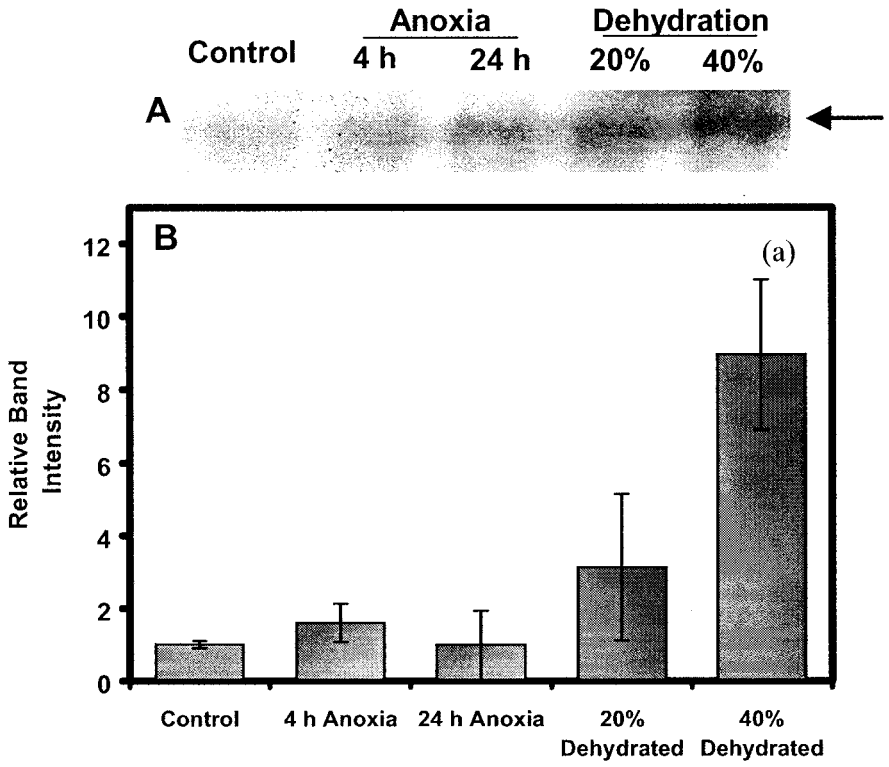
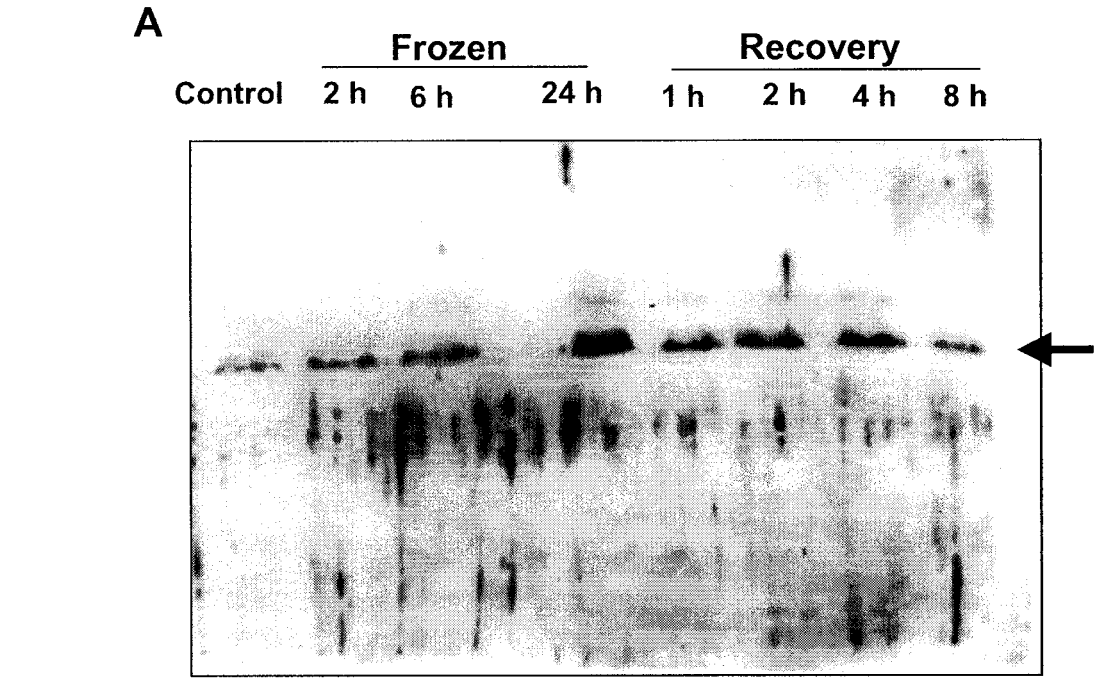
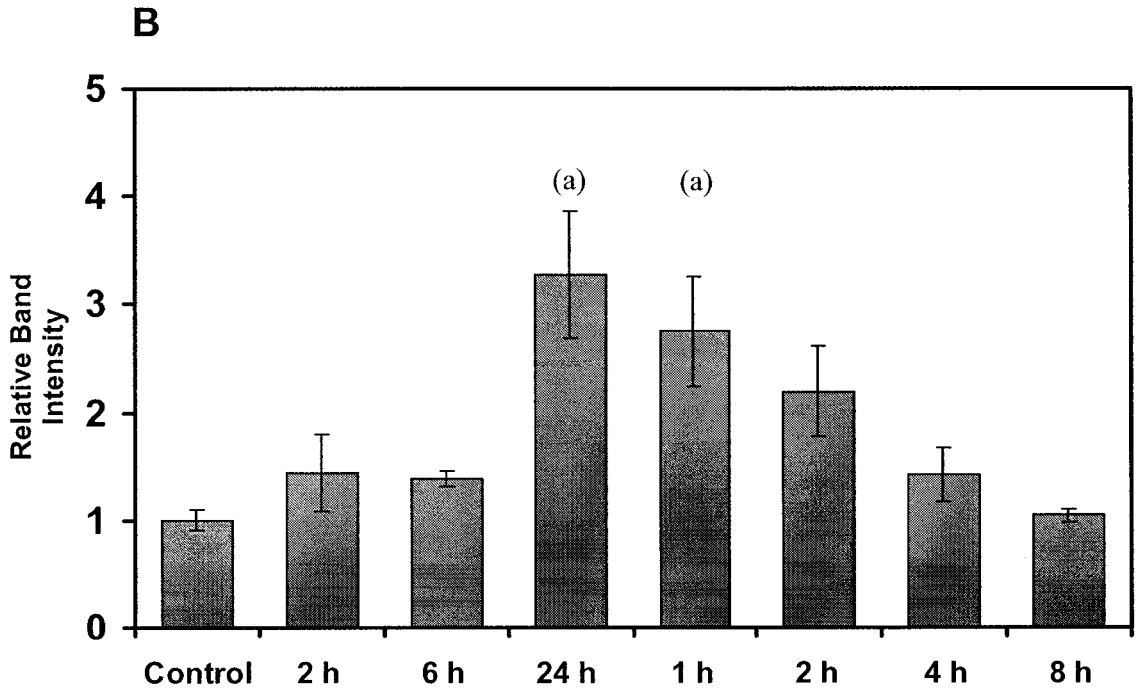

Control
Anoxia
4 h
24 h
Dehydration
20%
40%
A
B
(a)
Relative Band Intensity
0
2
4
6
8
10
12
Control
4 h Anoxia
24 h Anoxia
20% Dehydrated
40% Dehydrated

Fig. 2.6. (A) Western blot showing changes in PiC protein content in *R. sylvatica* liver over a time course of up to 24 h freezing at -3°C and followed by 8 h thawing recovery at 5°C. Proteins were probed using polyclonal antibodies directed against the bovine form of PiC. (B) Histogram shows densitometric analysis of western blots, means $\pm$ SEM for n=3 blots using protein extracts from different animals. [a] - significantly different from the control, $P<0.05$.


A
Frozen
Recovery
Control
2 h
6 h
24 h
1 h
2 h
4 h
8 h


B
Relative Band Intensity
5
4
3
2
1
0
(a)
(a)
Control
2 h
6 h
24 h
1 h
2 h
4 h
8 h

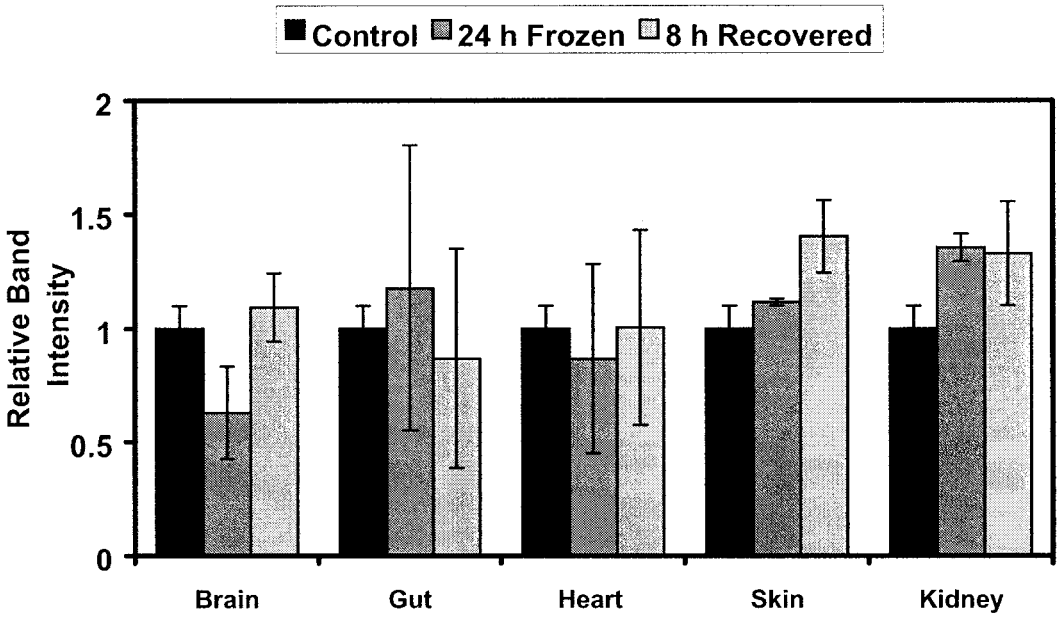
frogs (Fig. 2.7). However, unlike the situation in liver, freeze/thaw had no significant effect on PiC levels in brain, gut, heart, skin, or kidney of *R. sylvatica*.

2.4 Discussion

PiC belongs to a class of structurally similar inner mitochondrial membrane carrier proteins. These are characterized by the presence of three homologous repeats of ~100 amino acids that are found in all members of the family. Each repeat contains two transmembrane α-helical domains for a total of six membrane-spanning domains per protein (Kuan and Saier, 1993; Palmieri 2003).

Wood frog PiC showed these same structural features as well as a high overall sequence identity (84-86%) with the mammalian proteins and a comparable length (360 amino acids compared with 356-362 for the mammalian protein) (Figure 2.2). However, the sequence near the N terminus showed much higher variability in the frog protein compared with the mammals although the mammalian sequences are also variable in this region. This segment of the protein forms the extramembranous region that extends into the mitochondrial intermembrane space (Capobianco *et al.*, 1991). This variability near the N terminus has been reported for all mitochondrial transporters whereas the transmembrane regions are much more highly conserved (Kuan and Saier, 1993; Palmieri 2003). Mammalian PiC does not have any known phosphorylation or glycosylation sites and the frog protein was similar in this regard. Mammalian PiC exists as two splice variants, isoforms A (PiC-A) and B (PiC-B) (Dolce *et al.*, 1994; Fiermonte *et al.*, 1998). Bovine PiC-A was found in muscle mitochondria (heart, skeletal muscle, diaphragm) whereas PiC-B had a wider distribution (Fiermonte *et al.*, 1998). PiC-B showed a higher

Fig. 2.7. Western blot analysis of PiC protein levels in five tissues of *R. sylvatica* after 24 h freezing or 8 h thawing, compared with controls. Bars show densitometric analysis of western blots, means ± SEM, n=3 blots using protein extracts from different animals. Bars are: control (solid), 24 h frozen (open) and 8 h thawed (shaded).


Control
24 h Frozen
8 h Recovered
Relative Band Intensity
2
1.5
1
0.5
0
Brain
Gut
Heart
Skin
Kidney

V_{max} and lower K_m compared to PiC-A (Fiermonte *et al.*, 1998). The present study used a polyclonal antibody that cross-reacts with both PiC isoforms to assess the organ distribution of frog PiC; the protein was found in all 6 organs examined but the present results cannot comment on isoform distribution.

The data demonstrate that *pic* is a freeze-responsive gene in the wood frog. Transcript levels were strongly upregulated after 6 or 24 hours of freezing (Figure 2.3) with RT-PCR analysis suggesting that the increase in *pic* transcript levels may be as much as 100-fold after 24 hours frozen (Figure 2.4). PiC protein also rose during freezing with a maximum increase of 3.3-fold higher than control values seen after 24 h frozen. The marked discrepancy between the fold changes in mRNA transcripts and protein levels is interesting. In some cases we have observed strong increases in the transcript levels of freeze-responsive genes during the freezing exposure but a delay in reaching peak protein levels until the recovery period after thawing (McNally *et al.*, 2002, 2003). The freezing of blood plasma imposes an ischemic state on organs that gradually halts ATP-expensive processes such as protein synthesis. Hence, a high fold induction of transcripts may be necessary to achieve significant protein synthesis before protein synthesis is compromised by ATP restriction. A high fold induction during freezing also makes sense as a means of achieving a rapid increase in the synthesis of the protein product as soon as thawing begins, for example, as is needed for proteins involved in the recovery of cell function after the stress of freezing (McNally *et al.*, 2002, 2003). In the present study, however, Idid not find enhanced PiC protein synthesis during thawing; indeed, PiC levels in liver were beginning to decline even after 1 hour of thawing.

The role of PiC upregulation during freezing appears to be liver-specific since PiC

protein levels did not change during freezing in any of the other five organs tested (Fig. 2.7). Some other freeze-responsive proteins are also liver-specific including the novel protein FR47 and fibrinogen (very minor amounts of fibrinogen transcripts were found in two other organs) (Cai and Storey, 1997a, McNally *et al.*, 2003). In the case of fibrinogen this is because vertebrate liver is typically the sole organ responsible for the synthesis and secretion of this plasma protein. However, the situation with PiC is different because this carrier is found in the mitochondria of all cell types.

Some insights into the reason for PiC upregulation in wood frog liver can be drawn from an analysis of the responses by *pic* transcript levels to anoxia and dehydration stresses. Transcripts of *pic* did not change when frogs were given experimental anoxia exposure but levels increased by 9-fold in frogs that had lost 40% of their total body water (Fig. 2.5). Freezing imposes multiple stresses on cells, two of the most important being (a) anoxia/ischemia due to the interruption of oxygen delivery when plasma freezes, and (b) cell and organ dehydration due to the withdrawal of a high percentage of body water into extra-organ ice masses (Storey and Storey, 1996). When first exploring the regulation of cryoprotectant synthesis in freeze tolerant frogs, the hyperglycemic response to freezing was reproduced when frogs were dehydrated (but not under anoxia stress) and that freeze-intolerant frogs also showed a significant hyperglycemic response to dehydration (Storey and Storey, 1996). From this, it was proposed that the cryoprotectant response to freezing evolved out of a pre-existing volume-regulatory hyperglycemic response by frogs to dehydration. Since then, freeze-responsive genes are categorized as responding to either anoxia (ADP/ATP translocase, FR47, Li16) (Cai *et al.*, 1997; McNally *et al.*, 2002; 2003), or dehydration (fibrinogen,

FR10, PiC) (Cai and Storey, 1997a; 1997b) and this helps us to understand both the cellular signal that triggers their expression and their potential role in freezing survival. The very strong response of *pic* transcripts to dehydration stress, but not to anoxia, suggests that an increase in PiC protein content in the liver mitochondrial membrane may be involved in adjusting ionic, osmotic or volume regulatory parameters between the mitochondrial and cytoplasmic compartments during cellular dehydration. Indeed, during freezing at –2.5°C that converted 65% of total body water into ice, liver lost ~58% of its water (Lee *et al.*, 1992) and accompanying this sustained a large increase in the ionic strength and osmolality of the liver cytoplasm, including an increase in glucose levels of at least 200 mM (Storey and Storey, 1996).

Maintenance of a chemi-osmotic gradient across the inner mitochondrial membrane is essential for cellular health (Huizing *et al.*, 1996; 1998). Disruption of the gradient has been linked to apoptosis (Green and Reed, 1998) and various mitochondrial disorders (Huizing *et al.*, 1996; 1998). Since *R. sylvatica* can survive freezing, they must be able to maintain their mitochondria in an intact and viable state despite the stresses imposed by the freezing or thawing processes. It has been suggested (Huizing *et al.*, 1998) that defects in PiC and AAT result in osmotic imbalances that ultimately affect energy metabolism. Hence, the upregulation of both AAT and PiC during freezing in wood frog liver suggests that both have roles in readjusting mitochondrial metabolism or ionic/osmotic balance during freezing although their roles are different and are undoubtedly responsive to different intracellular signal transduction systems.

CHAPTER THREE

Control and regulation of the inorganic phosphate mitochondrial carrier in the wood frog *Rana sylvatica*

3.1 Introduction

The previous chapter described the identification *R. sylvatica* PiC as a freeze-responsive gene and analyzed changes in transcript and protein expression during freezing and related stresses. The current chapter analyzes further aspects of the regulation of PiC expression in *R. sylvatica* and examines the expression of other mitochondrial transporters in response to freeze-thaw.

The ADP/ATP transporter (AAT) belongs to the same family of inner mitochondrial transporters as PiC. Both of these transporters are upregulated in response to freezing stress in wood frog organs. In freezing-related stresses, AAT was also upregulated during anoxia (but not dehydration) (Cai *et al.*, 1997) whereas PiC was upregulated dehydration (Chapter 2). Other membrane transporters also belong to the same family as PiC and AAT. To determine whether the PiC and AAT responses are part of a concerted change in the levels of all mitochondrial transporters during freezing or are specific responses by selected transporters, I undertook an analysis of the effects of freezing on the levels of two other members of this transporter family, the oxoglutarate carrier (OGC) and the dicarboxylate carrier (DIC).

Furthermore, the upregulation of PiC in response to stress could be a part of a broader stress response seen in multiple stress species of freeze tolerant and/or dehydration tolerant anurans. Apart from *R. sylvatica*, several other species of freeze tolerant frogs are also found in North America. In addition, various other anuran species show good desiccation tolerance. Are the PiC gene and protein changes specific to freeze tolerant species? Do dehydration tolerant species that are not freeze tolerant also up-regulate PiC in response to water loss? Furthermore, is the PiC protein found in all frog

species? To answer these questions, a multiple species analysis of PiC content was under taken to provide further clues as to the nature and function of PiC. Expression was analyzed in three other species, the freeze tolerant grey tree frog, *Hyla versicolor*, the dehydration tolerant spadefoot toad, *Scaphiopus couchii*, from the Arizona desert and the common leopard frog, *Rana pipiens*, a pond frog that is not freeze tolerant but does show substantial anoxia tolerance.

Previous work from our lab has shown several mitochondrially-encoded genes are down-regulated in response to freezing stress in wood frog (Castellarin, 2000). However, most of the proteins in the mitochondria are actually encoded on the nuclear genome. Thus, the response of nuclear and mitochondrially-encoded proteins to freezing stress may be different. The responses of selected mitochondrial proteins to freezing could have several origins: (a) selected individual proteins are specifically up- or down-regulated for individual reasons, (b) there may be a general up- or down-regulation of mitochondrial versus nuclear encoded genes during freezing, or (c) there may be a general increase or decrease in mitochondrial copy number in liver cells during freezing. To differentiate between these possibilities, a general analysis were undertaken to evaluate the responses of mitochondrial proteins to freezing and southern blot analysis was used to compare the amount of mitochondrial DNA to genomic DNA to determine if there was a change in mitochondria copy number in response to stress. An increase in mitochondrial copy number could explain the increased levels of PiC.

During protein synthesis, transcripts that are being actively translated have multiple ribosomes attached to them giving a dense aggregate called a polysome. Transcripts that are less actively transcribed may have only one ribosome attached

forming a less dense complex called a monosome. When stress is imposed on a cell, the energy-expensive process of translation can respond in both a general way (overall increase or decrease in the activity of the translation apparatus), and in specific ways (transcripts of individual genes show an increase or decrease in translation). It would be predicted that the overall response to freezing would be a general suppression of translation, but this has not previously been evaluated. Therefore, studies were undertaken to evaluate both the general response of the translation apparatus to freezing and the individual response of PiC transcripts. The effect of freezing on the distribution of polysomes versus monosomes in a density gradient was evaluated as well as the specific distribution of the *pic* transcripts within the ribosome population.

3.2 Materials and Methods

3.2.1 *Animal experiments*

All whole animal experiments for *R. sylvatica* were carried out as described in Chapter 2. Other species used in these studies (*H. versicolor, S. couchii, R. pipiens*) were exposed to stresses in the same manner as described in Chapter 2. *H. versicolor* is a freeze tolerant frog that was exposed to 24 hour freezing at –3°C. *S. couchii* is a dehydration tolerant toad; animals were dehydrated until 40% of body water was lost. Anoxia tolerant frogs, *R. pipiens*, were given 24 hours anoxia exposure.

3.2.2 *Western blot analysis*

Preparation of protein samples was conducted as described in chapter two. Protein samples were all adjusted so that 25 μg of total protein was loaded per sample well.

Analysis of protein levels used antibodies directed against PiC (see chapter 2) and two other transporters, OGC and DIC. For anti-OGC (Fiermonte *et al.*, 1999) and anti-DIC (Fiermonte *et al.*, 1999) the source antigens were bovine and rat, respectively. Due to high sequence conservation among different species, I was confident that antibodies had a high cross-reactivity with the frog mitochondrial transporters. In fact, all antibodies gave a single band at the expected molecular weight. F. Palmieri kindly provided antibodies for PiC, DIC and OGC.

In addition to *R. sylvatica*, PiC levels were analysed in three other amphibians. The livers from freeze tolerant *H. versicolor*, the anoxia tolerant *R. pipiens*, and the dehydration tolerant *S. couchii* were analysed via western blot analysis for PiC.

3.2.3 *Southern blot analysis*

Genomic DNA was isolated from liver of control and 24 frozen wood frogs using the DNeasy DNA extraction kit (QIAGEN) according to the manufacturers protocol. Isolation of gDNA was done in three separate trials using different animals. After isolation of genomic DNA, the samples were assessed for purity (A_{260}/A_{280}) and their concentration was adjusted to give 5 µg of DNA per sample well in the subsequent southern blot.

DNA samples were electrophoresed on a 0.8% agarose gel in TAE buffer for 8 hours at 30 volts. The gel was stained with EtBr and photographed using a ChemiGenius BioImaging System (Syngene) using GeneSnap (ver. 5.0, Syngene) image capture software. The gel was then depurinated for 15 minutes in 0.25 M HCl, then denatured in 0.5 M NaOH, 1.5 M NaCl for 30 minutes, rinsed in ddH_20 and finally neutralized in two

changes of 0.5 M Tris-HCl (pH 7.2-7.4), 1.5 M NaCl for 15 minutes each. The gel was then equilibrated in two changes of 20X SSC (3 M NaCl, 0.3 M Na_3·citrate) for 15 minutes each. The genomic DNA was transferred to nitrocellulose (Hybond, AMERSHAM) using a standard capillary transfer method. Blots were hybridized with radiolabeled α-tubulin or ATPase6 probe and visualised via exposure to phosphoimaging screens. The screens were scanned with a Personal Molecular Imager FX (BIO-RAD), converted to *.tiff files and then analysed using GeneTools (ver. 3.0, Syngene) analysis software to provide a densitometric analysis of band intensity.

3.2.4 *Polysome profile analysis*

Analysis and generation of polysome profiles was performed as described in Larade and Storey (2002) with slight modifications. The main difference was the use of OptiPrep (MJBiolynx) instead of sucrose to form the gradients for ultracentrifugation. OptiPrep is a solution of iodixanol that can self-generate gradients upon freeze-thaw forming a consistent linear gradient that is quite easily reproduced.

Liver tissue samples (250 mg) from control and 24 h frozen *R. sylvatica* were crushed under liquid nitrogen using a mortar and pestle and then homogenised in 2.5 ml of homogenisation buffer (250 mM Tris-HCl pH 7.6, 25 mM NaCl, 10 mM $MgCl_2$, 30% OptiPrep, 100 μg/ml cycloheximide, 5 U/ml RNAse inhibitor [Promega]). The homogenate was centrifuged at 16,000 x g at 4°C for 15 minutes. The supernatant was removed to a new RNAse free tube and then mixed with 0.1 volume of detergent (5% w/v sodium deoxycholate, 5 %v/v Triton X-100) and stored at -80°C until ultra-centrifugation. The OptiPrep gradient was made by taking the gradient solution (30 %

Optiprep, 300 mM NaCl, 10 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 100 μg/ml cycloheximide and 5 U/ml RNAse inhibitor) and freezing at –20°C for 30 minutes followed by thaw at room temperature for one hour, thus forming a uniform linear gradient. Aliquots (1ml) of the tissue extract were then layered onto the 30% to 0% Optiprep gradient and centrifuged at 4°C for 1 hour at 40,000 rpm in a SW41 rotor. Gradients were drained into eight fractions of approximately 500 μl each and frozen at -80°C.

Distribution of ribosomal RNA (rRNA) profiles for control and 24 h frozen samples were generated by taking absorbance readings of each fraction at 254nm on a Mulitskan Spectrum microplate spectrophotometer (ThermoLabsystems). Total RNA was then isolated from each fraction by TriZol as described in Chapter two. Aliquots of the total RNA from each fraction were electrophoresed and transferred to nitrocellulose. The blots were probed with *fl-8* to assess the distribution of *pic* transcripts between monosome and polysome fractions. Blots were also probed for α-tubulin to assess transcript levels of a constitutive protein as a general control.

3.3 Results

3.3.1 *Western blot analysis*

PiC is one of a family of transmembrane carriers that are responsible for moving metabolites into or out of the mitochondria. Hence, I proposed that freeze-stimulated increases in PiC protein content could be accompanied by similar changes in levels of other mitochondrial carriers. To test this idea, the responses to freezing by two other carriers that belong to the same family were also assessed via western blotting.

Mammalian polyclonal antibodies to the OGC and DIC carriers showed good cross-reaction with the frog liver proteins, in each case showing one strong band that crossreacted with the antibody at the expected molecular weight of each protein. However, as Figure 3.1 shows, there were no significant changes in the amounts of either protein in wood frog liver over the freeze-thaw time course.

To further assess the importance of PiC upregulation to anuran survival of freezing and other stresses, the responses by liver PiC to stresses imposed on other frog and toad species were also assessed using western blots. The stresses used were freezing for *H. versicolor*, dehydration for *S. couchii*, and anoxia exposure for *R. pipiens*. PiC protein was detected in liver of the dehydration tolerant toad *S. couchii* but levels did not change significantly in response to the loss of 40 % of total body water, as compared with controls (Fig. 3.2). In the other two species, *H. versicolor* and *R. pipiens*, no crossreaction with the PiC antibody was detected.

3.3.2 Southern blot analysis

Electrophoresis of the genomic DNA (gDNA) isolated from wood frog liver showed approximately similar amounts of DNA in samples from control and frozen frogs (Fig 3.3a). After electrophoresis, gDNA was transferred to nitrocellulose (Fig. 3.3b) and the gDNA and mitochondrial DNA (mtDNA) were quantified. The amount of gDNA was quantified by probing the DNA with α-tubulin. The same blot was then probed with the mitochondrial gene ATPase6. The amount of mtDNA in control and frozen samples was quantified by expressing the band density of control and frozen samples relative to the corresponding amount of α-tubulin (Fig. 3.3c). There was no significant increase in the

Fig. 3.1. Western blot analysis showing levels of (A) the dicarboxylate carrier protein and (B) the oxoglutarate carrier in *R. sylvatica* liver over a time course of up to 24 h freezing (F) and 8 h thawing (T). Bars show densitometric analysis of band intensities, means $\pm$ SEM, n=3 samples of protein extracts from different animals.

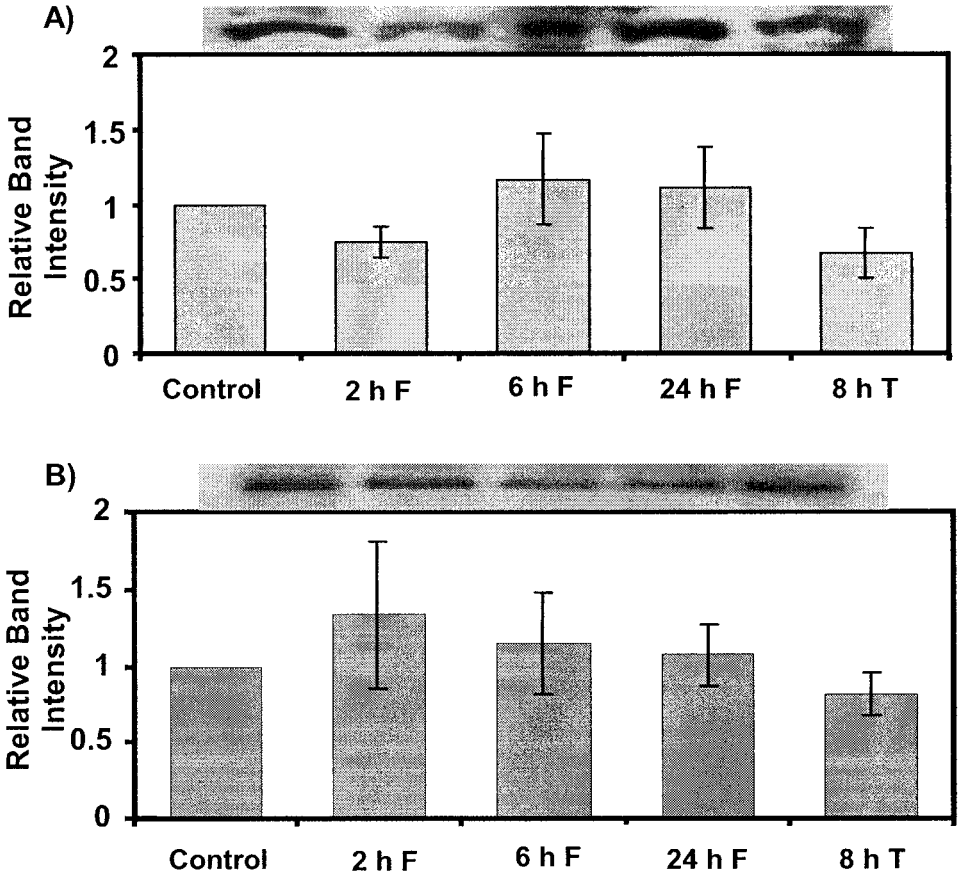

A)
Relative Band Intensity
2
1.5
1
0.5
0
Control
2 h F
6 h F
24 h F
8 h T
B)
Relative Band Intensity
2
1.5
1
0.5
0
Control
2 h F
6 h F
24 h F
8 h T

Fig. 3.2. Western blot analysis showing levels of the inorganic phosphate carrier protein (PiC) in liver extracts from different stress tolerant anuran species: the freeze tolerant grey tree frog, *Hyla versicolor,* the dehydration tolerant spadefoot toad, *Scaphiopus couchii,* and the anoxia tolerant leopard frog, *Rana pipiens*. PiC antibody crossreacted only with *S. couchii* liver extract. Bars show densitometric analysis of band intensities, means $\pm$ SEM, n=3 samples of protein extracts from different animals.

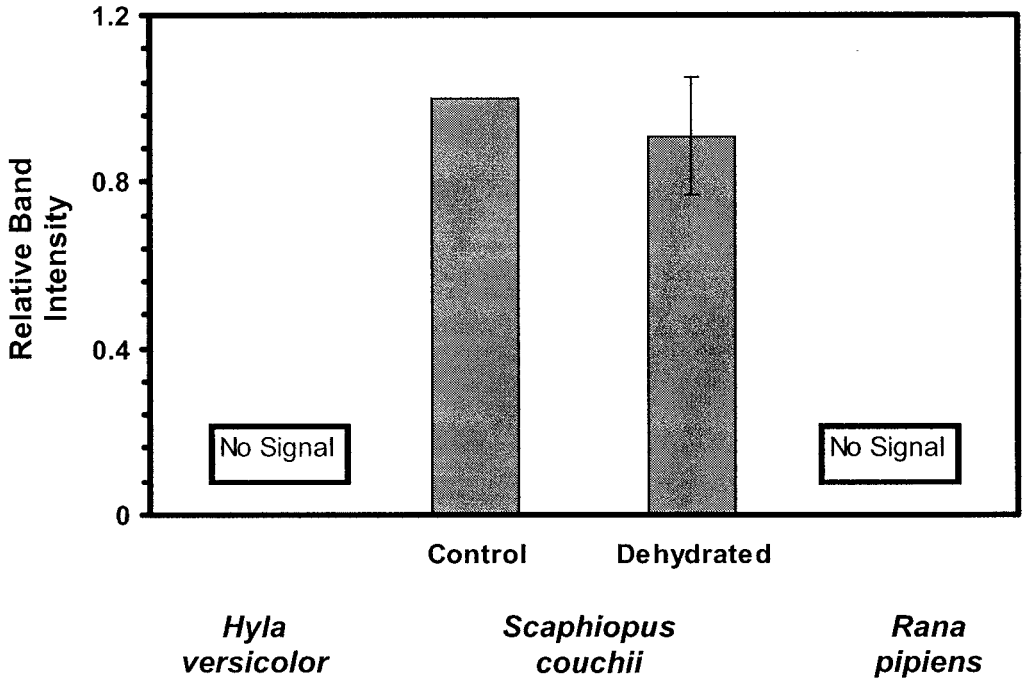
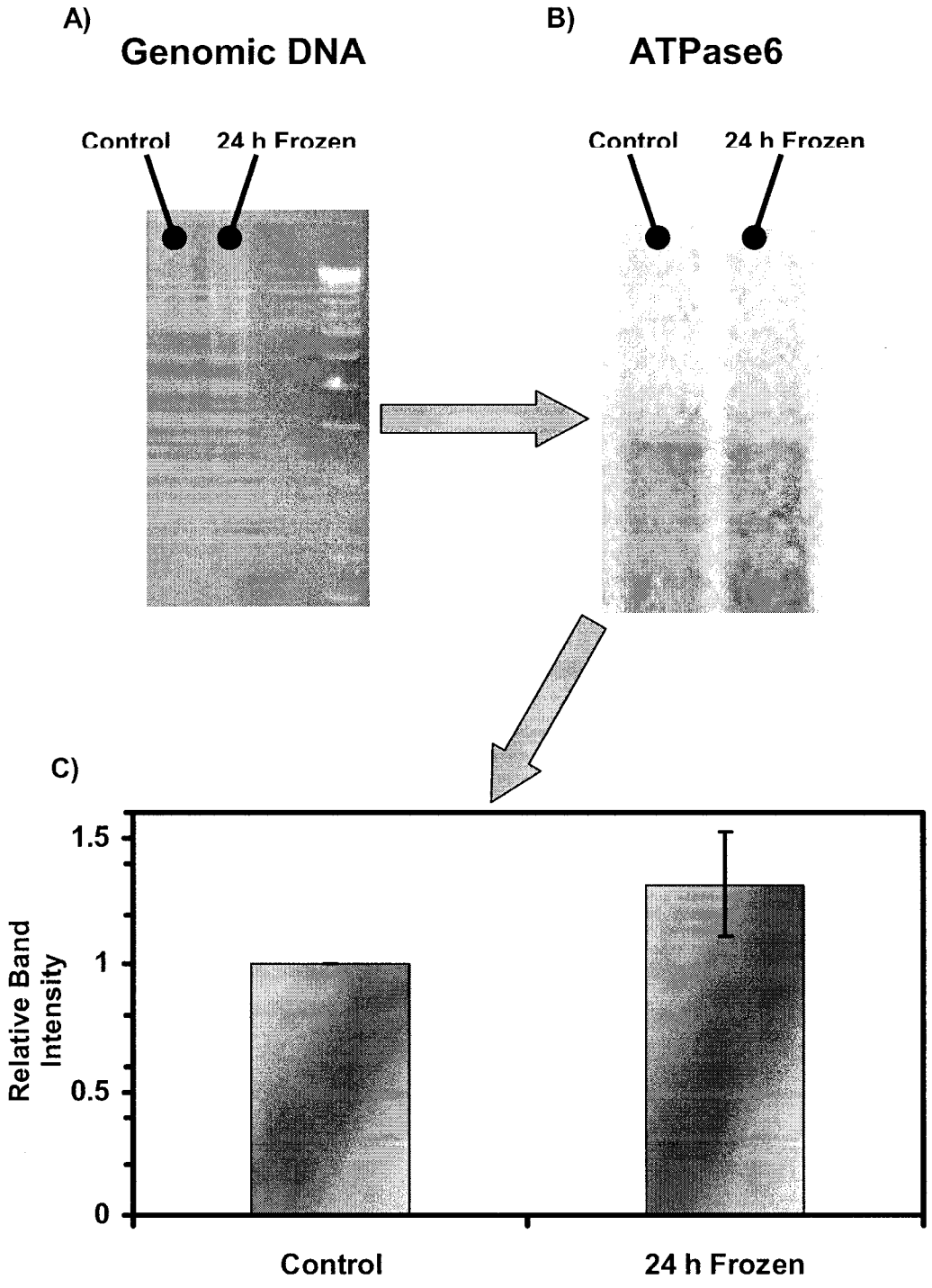

1.2
0.8
0.4
0
Relative Band Intensity
No Signal
No Signal
Control
Dehydrated
Hyla versicolor
Scaphiopus couchii
Rana pipiens

Fig. 3.3. Analysis of mitochondrial DNA copy number in *R. sylvatica* liver from control versus 24 hour frozen frogs. A) 0.8% agarose gel electrophoresis of control and 24 hour frozen liver genomic DNA stained with EtBr. B) Southern blot of control and 24 hour frozen liver genomic DNA probed with *R. sylvatica* ATPase6, a probe for a mitochondrially encoded gene. C) Densitometric analysis of ATPase6 levels relative to α-tubulin showing no significant increase in the copy number of mitochondria in frog liver in response to freeze exposure.


A)
Genomic DNA
Control
24 h Frozen
B)
ATPase6
Control
24 h Frozen
C)
Relative Band Intensity
1.5
1
0.5
0
Control
24 h Frozen

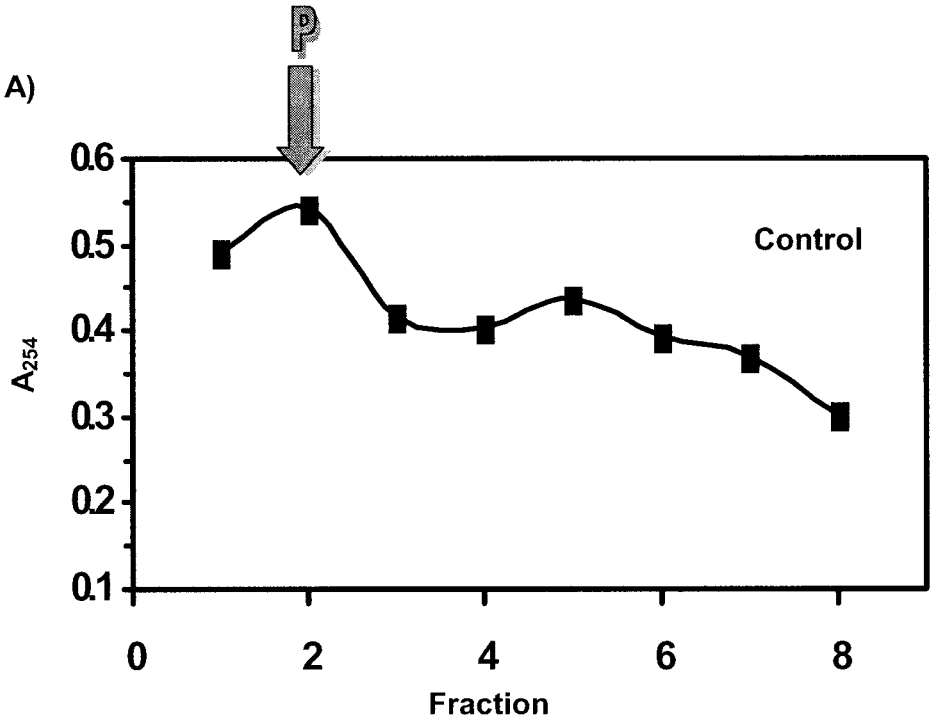
amount of mitochondrial DNA in liver relative to genomic DNA after freezing for 24 hours.

3.3.3 *Polysome analysis*

Extracts from livers of control and 24 hour frozen frogs were separated by centrifugation on a density gradient, drained into eight fractions, and then the rRNA content of each fraction was detected by absorbance at 254 nm. The rRNA profiles are shown in Figure 3.4 with fraction one being the highest density fraction and fraction eight the lightest fraction. Polysomes are heavier than monosomes and, thus, should be found in the heavier (lower numbered) fractions. In the control sample, a peak of RNA absorbance spanning fractions 1-3 was seen representing the polysome fraction. The monosomal fraction, being lighter, was found in the higher fractions. In the control sample, the division between the polysome and monosome fractions was arbitrarily set at fraction 4. In the 24 hour frozen sample, the polysome peak had dissipated indicating a disaggregation of polysomes into translationally less active monosomes.

Total RNA was isolated from each fraction and then northern blots were run and probed with *pic* and tubulin. The relative intensity of probe binding in each lane was determined and profiles of relative transcript levels in each fraction are shown in Figure 3.5 along with the northern blot. The relative intensities of *pic* transcripts in the different fractions showed that in control liver, the probe was mainly associated with monosomes. After freezing for 24 hours, however, there was a shift in distribution with enhanced levels of *pic* transcripts associated with the polysomal fraction. Tubulin was found predominantly in the polysome fraction in both cases. During freezing there was an

Fig. 3.4. Polysome profiles of extracts from liver of control (A) and 24 hour frozen (B) *R. sylvatica*. Samples were centrifuged on a OptiPrep gradient, fractions were drained and absorbance was measured at 254 nm. The A_{254} curve shows the relative amount of total RNA for each fraction, most of which is ribosomal RNA The polysome peak [P] is indicated in the control sample. The figure shows a representative polysome profile from n=3 independent replicates.


A)
P
Control
A_{254}
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
Fraction

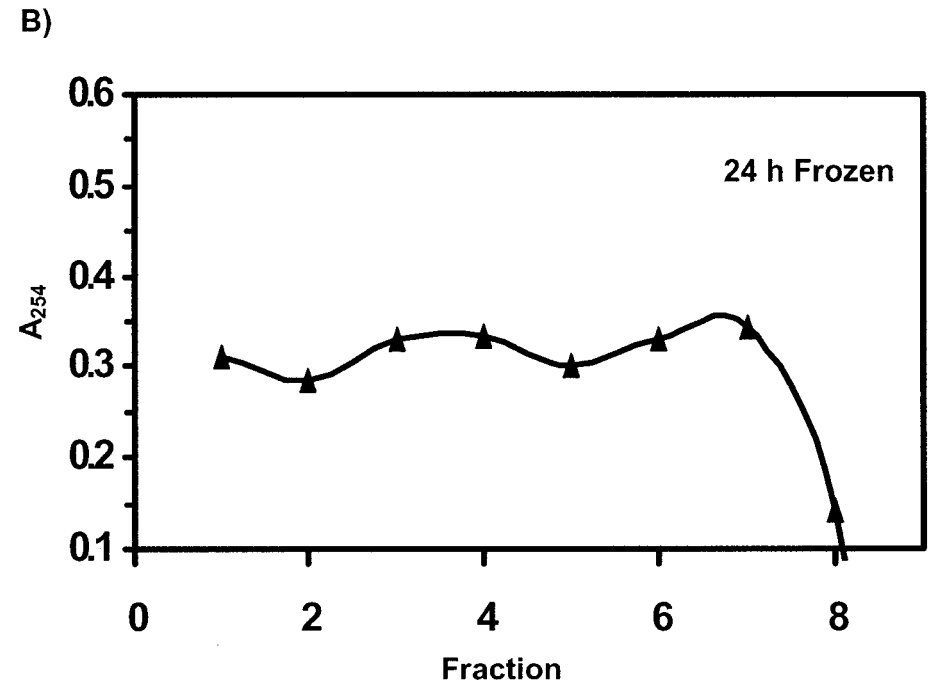
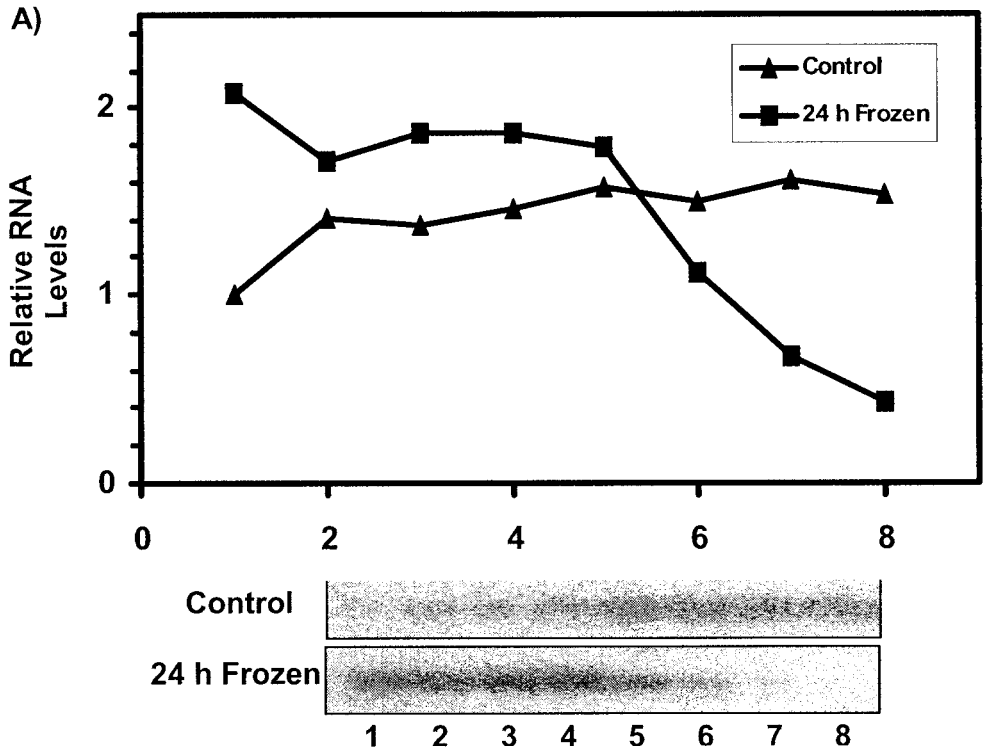
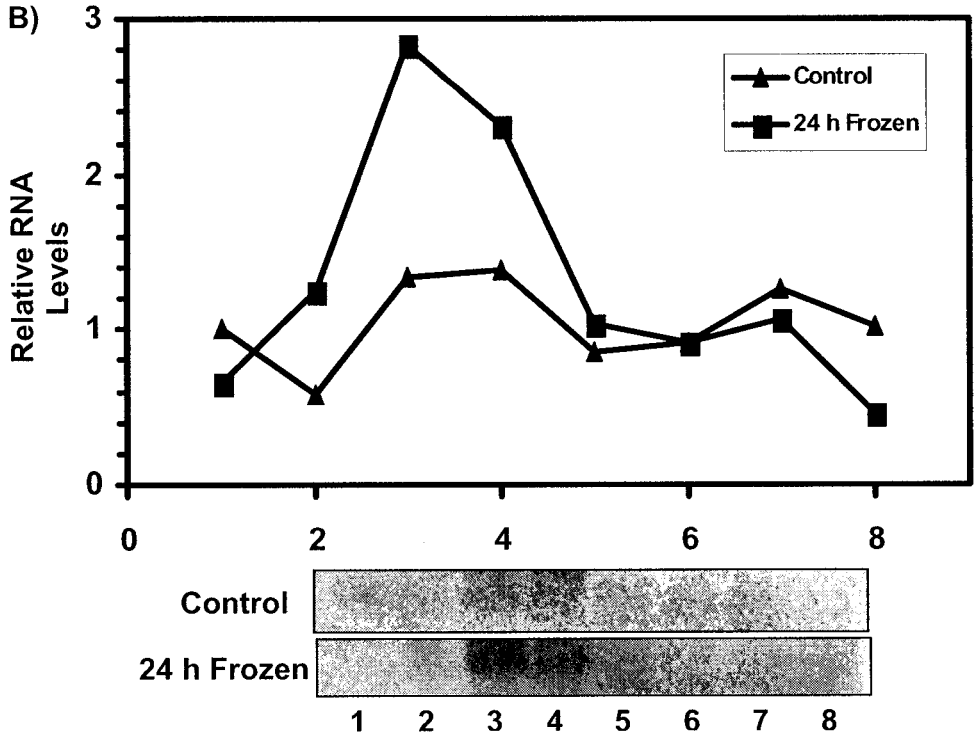

B)
24 h Frozen
A_{254}
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
Fraction

Fig. 3.5. Polysomal distribution of *pic* and *α-tubulin* transcripts in liver extracts of control and 24 hour frozen *R. sylvatica*. Total RNA was extracted from polysome fractions, electrophoresed on a 1.0% formaldehyde agarose gel and probed with (A) *pic* and (B) *α-tubulin*. A representative northern blot of fractions numbered from 1 (heaviest) to 8 (lightest) is shown together with the graphs of the relative band intensity in each fraction. The figure shows a representative profile from n=3 independent replicates.


A)
Relative RNA Levels
Control
24 h Frozen
0
1
2
0
2
4
6
8
Control
24 h Frozen
1 2 3 4 5 6 7 8


B)
Relative RNA Levels
Control
24 h Frozen
0
1
2
3
0
2
4
6
8
Control
24 h Frozen
1 2 3 4 5 6 7 8

increased amount of transcripts in polysome fractions 3-4, but no shift in the pattern of tubulin profiles between control versus 24 hour frozen samples as seen for *pic*.

3.4 Discussion

Mitochondrial transporters such as DIC and OGC belong to the same family of nuclear-encoded transmembrane proteins as PiC and AAT. DIC is a carrier protein that exchanges dicarboxylates like malate or succinate for inorganic phosphate (Kaplan *et al.*, 1985). OGC catalyzes an exchange of oxoglutarate for malate (or certain other dicarboxylates) (Palmieri *et al.*, 1993). All catalyze exchange reactions that are electroneutral (Palmieri, *in press*). Although PiC was strongly upregulated during freezing in wood frog liver, the two other mitochondrial inner membrane carriers, OGC and DIC, showed no significant changes in protein content over the course of freeze-thaw (Fig. 3.1). However, previous work has shown that the ADP/ATP translocase (AAT) (Palmieri 1994) is freeze-responsive in frogs (Cai *et al.*, 1997). *Aat* transcripts levels peaked after 8 hours of freezing exposure and AAT protein content was maximal after 24 hours. Interestingly, however, *aat* transcript levels changed during freezing in six of eight organs tested and, in liver, *aat* transcripts responded to anoxia exposure but not to dehydration (Cai *et al.*, 1997). Hence, expression of *aat* and *pic* genes, although both freeze-responsive, are clearly differentially regulated. This may not be surprising because although both AAT and PiC are involved in energy metabolism in the cell, they respond to different controls *in vivo*. PiC catalyzes Pi and H^+ uptake into the mitochondria using the chemical gradient of the proton motive force generated by the respiratory chain whereas the AAT catalyzes an electrophoretic exchange of cytosolic ADP^{3-} for matrix

ATP^{4-} using the electrical component of the proton motive force (Palmieri, *in press*). In fact, PiC is regulated by the pH gradient and AAT by the membrane potential.

Western blotting to search for PiC protein expression in other anuran species were positive for the presence of PiC in the spadefoot toad, *S. couchii* but failed to locate the protein in the other two species, *H. versicolor* and *R. pipiens*. The lack of a cross-reacting band in *H. versicolor* and *R. pipiens* may be due to low amino acid sequence conservation in PiC of these particular species. *S. couchii* is a desiccation tolerant toad found in Southwest America that spends nine months of the year buried under the desert soil. *S. couchii* alters its metabolism to produce high levels of urea in blood and tissues (about 300 mM in blood), which helps to retard water loss from the body through colligative action (McClanahan 1967; Shoemaker 1992). However, unlike wood frogs that responded to dehydration with an increase in PiC protein, the loss of 40% of total body water by spadefoot toads did not trigger an increase in PiC in toad liver.

The southern blot analysis showed no significant change in the amount of genomic or mitochondrial DNA in control versus 24 h frozen frogs. There was an apparent slight increase in mitochondrial DNA (1.3 fold higher), but this change was not significant. The freeze-induced decrease in mitochondrially-encoded transcripts seen that was reported earlier (Castellarin 2000) is probably due to transcriptional factor control. The increase in PiC protein levels could have arisen as part of a general proliferation of mitochondria, but since there was no significant increase in mtDNA content (hence, no mitochondrial biogenesis) in 24 hour frozen liver, this scenario is unlikely. Oxidative stress to human cells bought on by H_2O_2 treatments resulted in a significant increase in the number of mitochondria per cell after 48 and 72, but not 24 hours of treatment (Lee *et al.*, 2000).

This situation does not, however, occur in wood frogs during winter freezing. In *R. sylvatica* during freezing conditions there is no oxygen getting to the cells, so there is unlikely to be any mitochondrial biogenesis over long freezing exposure. Oxidative phosphorylation under these anoxic conditions could not be occurring and thus there is no need for mitochondrial biogenesis. More likely, however, is that the freeze-induced increase in PiC results in an increased number of transporters per mitochondrion and this could lead to an increased exchange of Pi and H^+ during freezing. The consequence of having increased Pi transport in wood frog mitochondria during freezing has yet to be determined.

The polysome profile analysis showed what appears to be a global suppression of translation in frog liver after 24 hours of freezing exposure as evidence by the disappearance of the polysome peak that was present in control liver extracts. Previous studies in our lab have shown that a disaggregation of polysomes is a frequent response to stress. Polysome disaggregation in liver of frozen frogs concurs with another line of evidence for translation inhibition during freezing. Thus, McNally (2002) found an increase in the amount of phosphorylated (inactive) eukaryotic initiation factor 2 (eIF2α) in frog heart during freezing; elevated levels of phospho-eIF2α has been correlated with inhibition of translation in many systems (Rhoads, 1993). Freezing blocks oxygen delivery to tissues and, therefore, impairs various energy expensive cellular activities such as protein synthesis. Indeed, studies have suggested that there is a hierarchy of sensitivities of cell functions to ATP restriction and protein synthesis is one of the first to be suppressed when ATP is limiting. Hence, a general dissociation of polysomes into monosomes during freezing is consistent with a general suppression of protein

biosynthesis in the frozen state. A suppression of translation documented by both a shift of polysomes to monosomes and an increase in the amount of phosphorylated eIF2α has also been shown during hibernation in the ground squirrel, *Spermophilus tridecemlineatus* (Hittel and Storey, 2002) and in response to anoxia in the marine snail *Littorina littorea* (Larade and Storey, 2002).

Probing the polysome profiles provides a glimpse into the regulation of translation of upregulated transcripts. The strong upregulation of *pic* transcripts during freezing that was documented in the last chapter is supported in the present chapter by the segregation of *pic* transcripts into the polysome fraction of frozen liver. This suggests that despite the overall dissociation of polysomes into monosomes that would result in a reduction of the overall rate of protein synthesis in the liver of frozen frogs, the *pic* transcripts were still actively translated. Indeed, *pic* transcripts appear to be one of a handful of genes that are preferentially translated during freezing. When the same polysome profiles were probed with tubulin (Fig. 3.5b) a different pattern emerged. In both control and 24 hour frozen the intensity of tubulin probe binding was highest in the polysome portion. Both control and 24 frozen profiles had a similar pattern, however in 24 hour frozen profiles there was relatively more tubulin transcripts in the polysome fractions. But, in general, the pattern remained the same with no shift in the translational state of tubulin under stress.

This series of studies investigated PiC upregulation during freezing. The upregulation of PiC seems to be part of a controlled response that targets a specific set of mitochondrial transporters (PiC and AAT), and that occurs only in wood frogs. It remains to be seen if PiC can be detected in other freeze tolerant frogs (perhaps with a redesigned antibody) and if PiC levels are similarly responsive to freezing and related stresses in

those species. Southern blot analysis revealed no increase in mitochondrial copy number during freezing suggesting that there is more PiC protein per mitochondria in frozen wood frogs. As well, the profile of PiC transcripts shifted from less active monosomes to active polysomes after 24 hours of freezing. This shift indicates an active translation of *pic* transcripts and explains the upregulation of PIC protein that was seen during freezing. What controls the transcription of PiC remains to be determined. As will be shown in the next chapter, there are a series of transcription factors that are activated in response to freezing and related stresses in wood frog liver. The promoter region of PiC, which contains potential binding sites for transcription factors, has not been isolated yet and represents an avenue of future study.

CHAPTER FOUR

Transcription factor activity in response to freezing stress in the wood frog *Rana sylvatica*

4.1 Introduction

A central dogma of biology is that DNA is transcribed into single-stranded RNA, which is then translated into proteins. Translation of proteins is dependent on fine control of genes. Through the action of various factors that influence the transcription of a gene, a particular protein is ultimately translated. This fine control allows external factors such as changes in the environment or other stresses to dictate what proteins a cell produces.

The genome of every cell codes for every possible protein needed in any cell type in the entire organism but most cells need only a subset of these proteins to conduct the specific functions of an individual cell type. As a result, a cell will only translate proteins as they are necessary and in response to different cues. There is ample evidence of the upregulation of selected genes in frogs during freezing stress (e.g. Cai and Storey, 1997a, b) and there must be control mechanisms that allow transcription of these genes to respond to freezing stress. There must also be a means of controlling the rate of transcription of these genes in a timely manner.

Previous work in our lab by Greenway and Storey (2000) has shown that the mitogen activated protein-kinase p38 is activated during freezing in *R. sylvatica*. In addition, Holden and Storey (1996) documented the rapid activation (within minutes) of protein kinase A in response to freezing (activating cryoprotectant glucose output) and a later rise in second messenger levels of protein kinase C after several hours of freezing. Holden and Storey (1997) also documented differences in second messenger and protein kinase A and C responses between freeze intolerant (*R. pipiens*) and freeze-tolerant (*R. sylvatica*) frogs under dehydration and anoxia stresses. The MAPK protein kinases,

including p38, are particularly well known for their role in mediating transcription, transmitting environmental signals to the nucleus to initiate appropriate gene expression responses. Key targets of the MAPKs are nuclear transcription factors. The activation of p38 during freezing suggests that the activation of selected transcription factors and their downstream genes would follow.

Recent work in our lab has shown a change in transcription factor activation during hibernation in mammals (Eddy 2003). Similar responses by transcription factors must mediate the gene expression responses by frog organs during freezing. The present chapter looks at transcription factor activity during freezing and related stresses in *R. sylvatica* liver. To begin with, however, the introduction provides some background on transcription and transcription factors. The remainder of the chapter investigates the responses by key transcription factors to freezing in *R. sylvatica* liver. In particular, this chapter looks at possible role of the cAMP response element binding protein (CREB), Protein Kinase B (Akt), CREB binding protein (CBP) and nuclear factor-kappa-B (NFκB) (see Table 4.1) in the response to freezing and related stresses.

4.1.1 *The Process of Transcription*

During transcription a single-stranded piece of RNA is made from a double-stranded piece of DNA. In eukaryotes a gene usually contains coding sequences or exons, interspersed with non-coding sequences or introns. After transcription, the non-coding sequences are removed to form message RNA (mRNA), which is then transferred to the cytoplasm for translation into proteins. A gene also contains specific sequences before and after the transcribed region that are involved in the regulation of transcription. Introns

Table 4.1 – List of transcription factors and associated proteins assessed in *Rana sylvatica* liver during freezing and related stresses. This list shows some of the downstream effects brought on by activation of the transcription factors and associated proteins.

Protein	Function	Reference
CREB	Glucose regulation, cell survival and immune system regulation	Mayr *et al.*, 2001
CBP	Promotes CREB, ATF2 binding	Kawasaki *et al.*, 1998
Akt	Cell survival, proliferation, glycogen and lipid metabolism	Cook *et al.*, 2002
NFκB	Cell survival, proliferation, and immune system regulation	de Martin *et al.*, 1999

and exons along with these regulatory sequences make up the structure of a gene (Fig. 3.1). The transcription process involves numerous proteins that act in a concerted fashion to allow RNA polymerase to work (see Shilatifard, 1998). These proteins are all subject to regulation and are points of control in the transcription of a gene.

Three different RNA polymerases (pol I, II, III) synthesize different types of RNA from a DNA template. Pol I, II, and III transcribe ribosomal RNA (rRNA), message RNA (mRNA), and transfer RNA (tRNA), respectively. The Pol II basal machinery binds to a region called the promoter, which is upstream of the DNA transcriptional start site. The promoter region is an important sequence as the strength of binding of the basal machinery to this region determines how frequently a gene will be transcribed. The frequency of gene transcription, as will be discussed further, is dependent on transcription factors and their control. The transcriptional start site is typically designated as +1 and sequences upstream of the transcriptional start site are given a negative designation. The promotor region is found upstream of the transcriptional start site and includes the TATA box, a highly conserved septamer (5'-TATAAAA-3') that is found at about position –25 (Workman and Roeder, 1987).

Transcription can be broken down into three stages: pre-initiation and initiation, followed by elongation, and then termination. These three steps are summarized in Figure 3.2 and outlined below. Pre-initiation and initiation steps happen close together, do not have clear boundaries and will be discussed here as a single stage. Elongation is the actual synthesis of the mRNA strand by the addition of the ribonucleotides adenine, guanine, cytosine and uracil. Termination involves the release of Pol II from the DNA template allowing it to make another RNA transcript.

Fig. 4.1. Schematic diagram of a gene and the regulatory elements involved in transcription. Numbering of the gene starts at the transcriptional start site (+1) and continues upwards towards the 3' end. Numbers towards the 5' end are negative. See text for discussion of the regulatory elements.

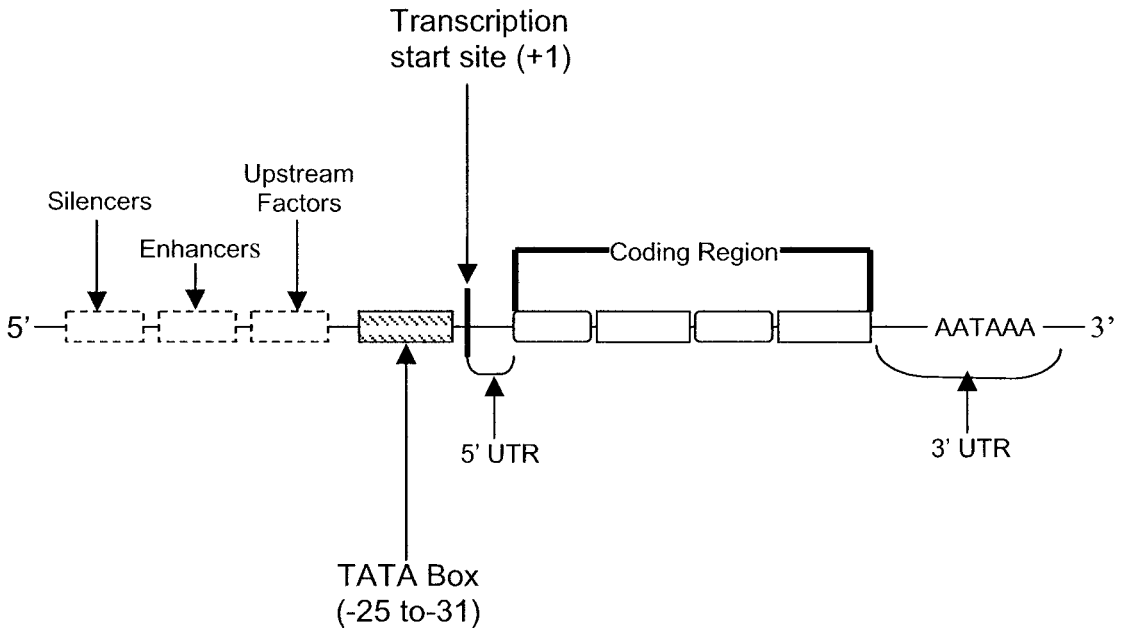
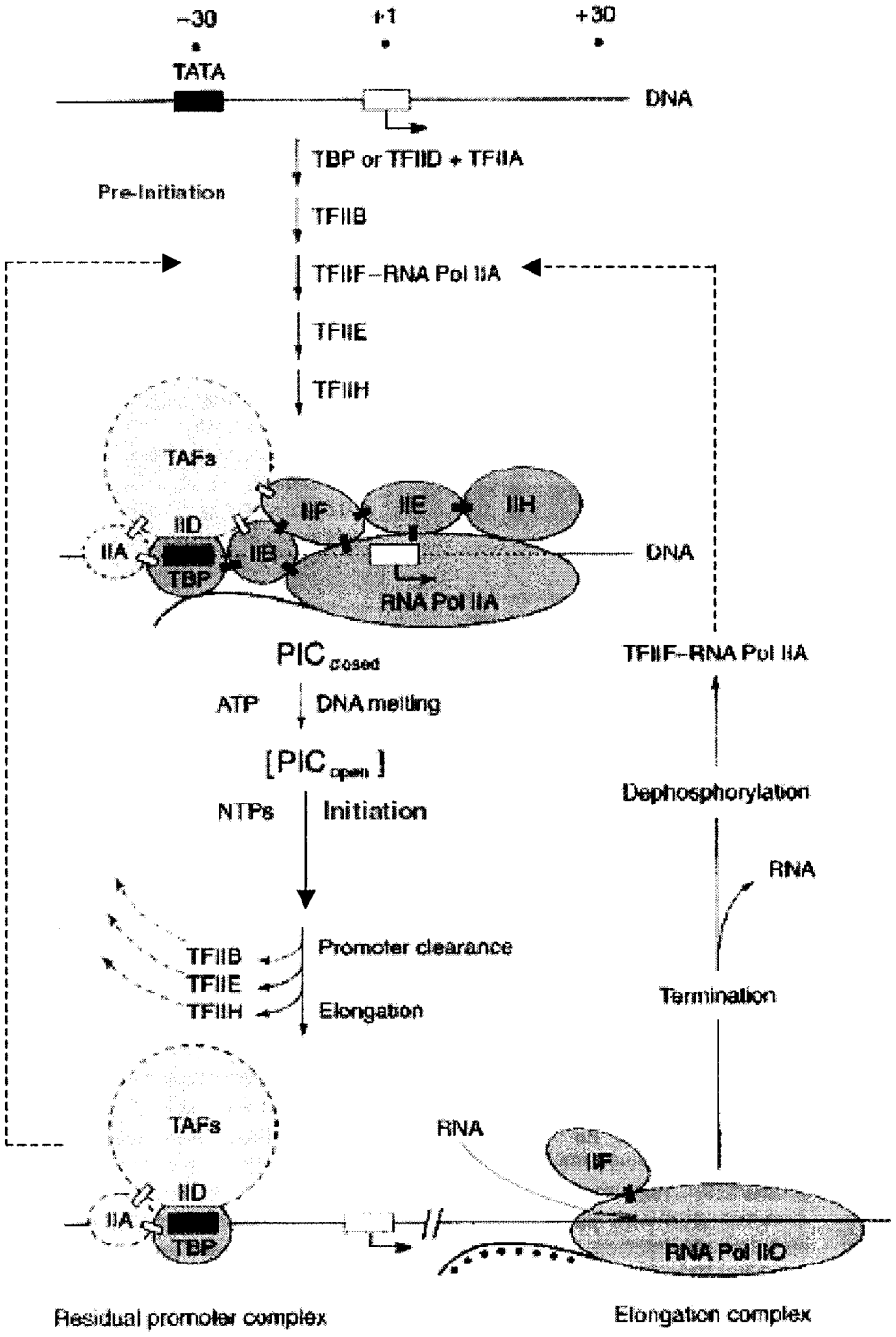

Transcription
start site (+1)
Silencers
Upstream
Factors
Enhancers
Coding Region
5'
AATAAA
3'
5' UTR
3' UTR
TATA Box
(-25 to-31)

Fig. 4.2. The four stages of transcription of DNA to RNA, from pre-initiation to initiation to elongation and termination. This is the currently accepted model. Adapted from http://www.biochem.ucl.ac.uk/bsm/xtal/teach/trans/tata.html, 2002. See text for explanation of the processes and complexes.


−30
+1
+30
TATA
DNA
TBP or TFIID + TFIIA
Pre-Initiation
TFIIB
TFIIF–RNA Pol IIA
TFIIE
TFIIH
TAFs
IID
IIA
TBP
IIB
IIF
IIE
IIH
DNA
RNA Pol IIA
PIC closed
ATP
DNA melting
[PIC open]
NTPs
Initiation
TFIIB
TFIIE
TFIIH
Promoter clearance
Elongation
TAFs
IID
IIA
TBP
Residual promoter complex
RNA
IIF
RNA Pol IIO
Elongation complex
Termination
RNA
Dephosphorylation
TFIIF–RNA Pol IIA

The pre-initiation and initiation steps involve protein interactions that unwind the chromatin DNA and facilitate the interaction of the exposed DNA template with RNA polymerase (Ghosh and Van Duyne, 1996). The pre-initiation step involves the formation of the basal complex from several general transcription factors that allow the binding of Pol II and the initiation of transcription.

After the basal complex is complete, the next step in transcription is elongation. Before this takes place, Pol II must escape from the promoter region. Following formation of the initiation complex the transcription factors release from the complex and can go on to other promoter areas or even be reused for that particular gene (Zawel *et al.*, 1995). After Pol II initiates transcription, about 9-13 bases downstream of the transcriptional start site, there is a good chance of arrest of this growing chain unless Pol II becomes "escape competent" (Dvir *et al.*, 1996). Being escape competent means release of the appropriate attached general transcription factors allowing Pol II to disassociate from the basal complex and move downstream along the DNA template. Once these factors are released Pol II escapes and can elongate the growing RNA strand by the addition of ribonucleotides. Before the 30^{th} nucleotide is added Pol II can stall, aborting transcription. Elongation depends on Pol II escape by the polymerase getting past +30 (Zawel *et al.*, 1995).

Most of the data on the control of elongation has been derived from *in vitro* model systems where the rate of elongation is considerably slower than it is *in vivo* (Ucker and Yamamoto, 1984). *In vivo*, Pol II can add nucleotides at the rate of over 2000 per minute, but under *in vitro* conditions this rate drops to ~300 nucleotides per minute (Ucker and

Yamamoto, 1984). Pol II pauses several times during the *in vitro* elongation process and this contributes to the slower rate. This data indicates both the importance of the general transcription factors for ensuring a fast rate of transcription and suggests that the *in vitro* system, as it is currently understood, must still be missing some element(s) compared with the *in vivo* system that prevents Pol II from pausing. As the elongation process continues, Pol II will eventually reach the end of the gene and terminate transcription. The mRNA transcript gets multiple adenine residues added by the enzyme poly (A) polymerase at the 3' terminus of the transcript in a process called polyadenylation. The polyadenylation signal is AATAA (DNA sense strand) and when Pol II extrudes the resultant mRNA sequence of AAUAA the poly (A) polymerase will then add roughly 250 adenine residues to form the poly (A) tail characteristic of mRNAs (Tran *et al.*, 2001).

The poly (A) tail is added about 18-30 nucleotides downstream of the polyadenylation signal (Zhao *et al.* 1999). Another recognition sequence occurs 1 to 30 nucleotides downstream from the poly (A) site. This site is a weakly conserved G + T or T-rich site and is required in some genes for efficient termination and polyadenylation (McLauchlan *et al.,* 1985). After polyadenylation, this sequence being downstream of the poly (A) site gets cleaved off. As well, other modifications are made to the transcript after elongation is complete. The 5' end of the transcript undergoes a modification known as "capping". A 7-methylguanosine is placed at the 5' terminus. Capping marks the transcript for subsequent processing and eventual transport out of the nucleus (Varani, 1997).

After capping and polyadenylation, the mature mRNA needs to have its introns

spliced out leaving the various exons or coding portions behind. At this point transcription has ended and so have the opportunities for transcriptional control.

4.1.2 *Background on Transcription Factors*

Apart from the TATA box, there are other sequences that proteins (transcription factors) can bind to in order to affect transcription. These transcription factors can enhance or repress transcription of the gene by binding to a specific consensus sequence. For example, CCAAT, GC rich sequences and octamers are upstream factors (Fig. 4.1) that constitutively bind proteins and affect transcription. These promoters help by increasing the strength of binding by the initiation complex and thus increase the speed of mRNA synthesis (Marian and Roberts, 1993).

Further control is exerted by inducible transcription factors that receive environmental cues and can enhance or silence transcription of a gene. Transcription factors bind to specific sequences that can be either upstream or downstream of the gene. They are classified according to their specific DNA binding domains such as the helix-loop-helix, leucine zipper, or zinc finger domains (Marian and Roberts, 1993). There are many other types of domains but most are subsets of the above domains. The amino acid sequences found in these domains bind to the major or minor grooves on the DNA strand.

The helix-loop-helix domain has a basic region and two α-helices of 15-16 amino acids separated by a linker region of 5-20 amino acids (Beyersmann, 2000). The basic region interacts with a specific DNA sequence. Leucine zipper domains are a dimeric structure and are made up of two helical structures on each dimer and interact with DNA at the basic helices (Beyersmann, 2000). Leucine zippers contain a hydrophobic amino

acid every seven residues, most commonly leucine (Beyersmann, 2000). Zinc fingers are the largest class of DNA binding proteins and are split into two groups: Cys_2-His_2-Zn and Cys_4-Zn (Laity *et al.*, 2001). Zinc fingers are roughly 30 amino acids and fold into a loop or finger around a central zinc atom. This loop inserts into the major groove of DNA in a site-specific manner (Beyersmann, 2000).

Before some transcription factors can bind they must undergo a conformational change. Generally transcription factors get phosphorylated at certain protein residues by protein kinases and that induces a conformational change that allows the factor to bind to the consensus sequence and activate transcription (Whitmarsh and Davis, 2000). Other transcription factors like CREB, bind constitutively to the response element but do not induce transcription until they are phosphorylated (for CREB, phosphorylation is at Serine-133) (Shaywitz and Greenberg, 1999).

4.1 Materials and Methods

4.2.1 *Animals*

Frogs were collected in the spring of 2001 and experiments were conducted as described in chapter two.

4.2.2 *Western blot analysis*

Preparation of protein samples was conducted as described in chapter two. Samples were quantified using the Bradford assay (Bradford, 1976) with the Bio-Rad prepared reagent. All protein samples were adjusted with loading buffer to contain 5 µg/µl of protein resulting in exactly 25 µg of total protein per lane after loading 5 µl per

sample well. This accounts for differences in protein content per mL of extract that may be seen in dehydrated and other stressed tissues.

Cross-reacting antibodies gave a single band at the expected molecular weight. Antibodies used were for total CREB, phospho-CREB Ser133 (pCREB), phospho-IκB Ser32 (pIκB), total Akt, phospho-Akt Ser 473 (pAkt) (Cell Signalling) and for CREB binding protein (CBP) (Santa Cruz). The antibodies were all polyclonal and raised in rabbits. Electrophoresis, transfer to polyvinylidene difluoride (PVDF) membranes, and reaction with antibodies was conducted essentially as described in chapter two. Membranes were washed in TBST prior to blocking (if necessary) and then probed with an antibody. Detection of total CREB, pCREB, and CBP was done in TBST with an antibody dilution of 1:2000. For detection of phospho-IκB, total Akt, and phospho-Akt the membranes were blocked in 1% (w/v) non-fat dry milk diluted in TBST for 1 hour at room temperature. The blocked membranes were then probed with anti-phospho-IκB (1:1000), total Akt (1:2000), or phospho Akt (1:2000) overnight at 4°C. After incubation in primary antibody, all membranes were washed three times in TBST for five minutes at room temperature. Blots were then incubated with anti-rabbit secondary antibody at a dilution of 1:2000 in TBST (Cell Signalling). Membranes were then washed four times for five minutes at room temperature and immediately subjected to immunodetection.

Specific bands were detected using enhanced chemiluminescence (ECL) (DuPont NEN, Boston, MA). Immunodetection was performed on a ChemiGenius BioImaging System (Syngene) using GeneSnap (ver. 5.0, Syngene) image capture software. Cross-reacting bands were quantified using GeneTools (ver. 3.0, Syngene) analysis software. Molecular weights were calculated using biotinylated protein markers (7727, Cell

Signalling) and the GeneTools (ver. 3.0, Syngene) analysis software.

4.1.3 *DNA Binding Assay*

Binding of transcription factor pCREB and the p50 subunit of NFκB were assessed by the use of DNA binding assays (ActiveMotif; Carlsbad, CA). The assay is an ELISA-type assay that uses 96-well plates containing an oligonucleotide with the appropriate binding sequence. Assays were run according to the manufacturers instructions (ActiveMotif; Carlsbad, CA) for each of the specific transcription factors. Briefly, a liver tissue extract was lysed using the provided lysis buffer (20 mM Hepes, pH 7.5; 350 mM NaCl; 20% glycerol; 1% Igepal-CA630; 1 mM $MgCl_2$; 0.5 mM EDTA; 0.1 mM EGTA; 5 mM DTT) and the provided protease inhibitor cocktail. The sample was then added to a well in a 96-well plate containing the consensus sequence of the transcription factor of interest. The transcription factor was allowed to bind to its consensus sequence for one hour at room temperature with mild agitation on a rocking platform at 100 rpm. After binding, the sample wells were washed three times with the provided washing buffer (10 mM phosphate buffer, pH7.5; 50 mM NaCl; 0.1% Tween-20) to wash away unbound transcription factor. The wells were then incubated with primary antibody specific to the transcription factor diluted 1:1000 in washing buffer for one hour at room temperature with no agitation. The wells were then washed three times with washing buffer and then secondary antibody (diluted 1:1000 in washing buffer) was added and allowed to bind primary for one hour at room temperature with no agiation. The wells were then washed 4 times with washing buffer and then the provided developer solution was added to each well. After 10 minutes of developing, the provided stop

solution was added and the developed colour was quantified by absorbance at 450 nm.

4.3 Results

4.3.1 *Western blot analysis*

Analysis of CREB showed no change in the total amount of CREB protein present in wood frog liver over a time course of freezing and thawing (Fig. 4.3). Instead, the amount of CREB that is phosphorylated is what changes over a freeze-thaw cycle. The amount of pCREB had risen significantly after 2 hours of freezing exposure to a level that was 2.3 fold ($P<0.05$) higher than the control value. Subsequently, levels of pCREB decreased with longer times of freezing. During thawing, pCREB was again activated, rising to 2.5 fold ($P<0.05$) higher than the control value after 2 hours of thawing before beginning to decline with longer times of thawing.

CREB levels were also tested in freezing related stresses (Fig. 4.4). Under dehydration and anoxic stresses the amount of total CREB did not change significantly. However, as in freezing, the amount of phosphorylated CREB increased under both of these stresses, but with different patterns. The amount of pCREB increased strongly during dehydration rising to 3.0 and 2.6 fold higher than control values in frogs that had lost 20% and 40% of their total body water, respectively ($P<0.05$). However, after rehydration, pCREB content returned to control levels. The response to anoxia was different. Throughout the anoxia exposure, pCREB levels stayed relatively constant but pCREB content increased strongly during aerobic recovery after anoxia. Levels rose to 3.7 fold higher than control values after 2 h of recovery and remained significantly elevated (2.7 fold higher) after 4 h ($P<0.05$).

Fig. 4.3. (A) Western blot showing changes in pCREB protein content in *R. sylvatica* liver over a time course of up to 24 h freezing at -3°C and followed by 8 h thawing recovery at 5°C. Proteins were probed using polyclonal antibodies raised in rabbits giving a giving a band at a molecular weight of ~43 kDa. (B) Histogram shows densitometric analysis of western blots of total CREB (grey bars) and pCREB (black bars), means $\pm$ SEM for n=3 blots using protein extracts from different animals. * - significantly different from the control, $P<0.05$.

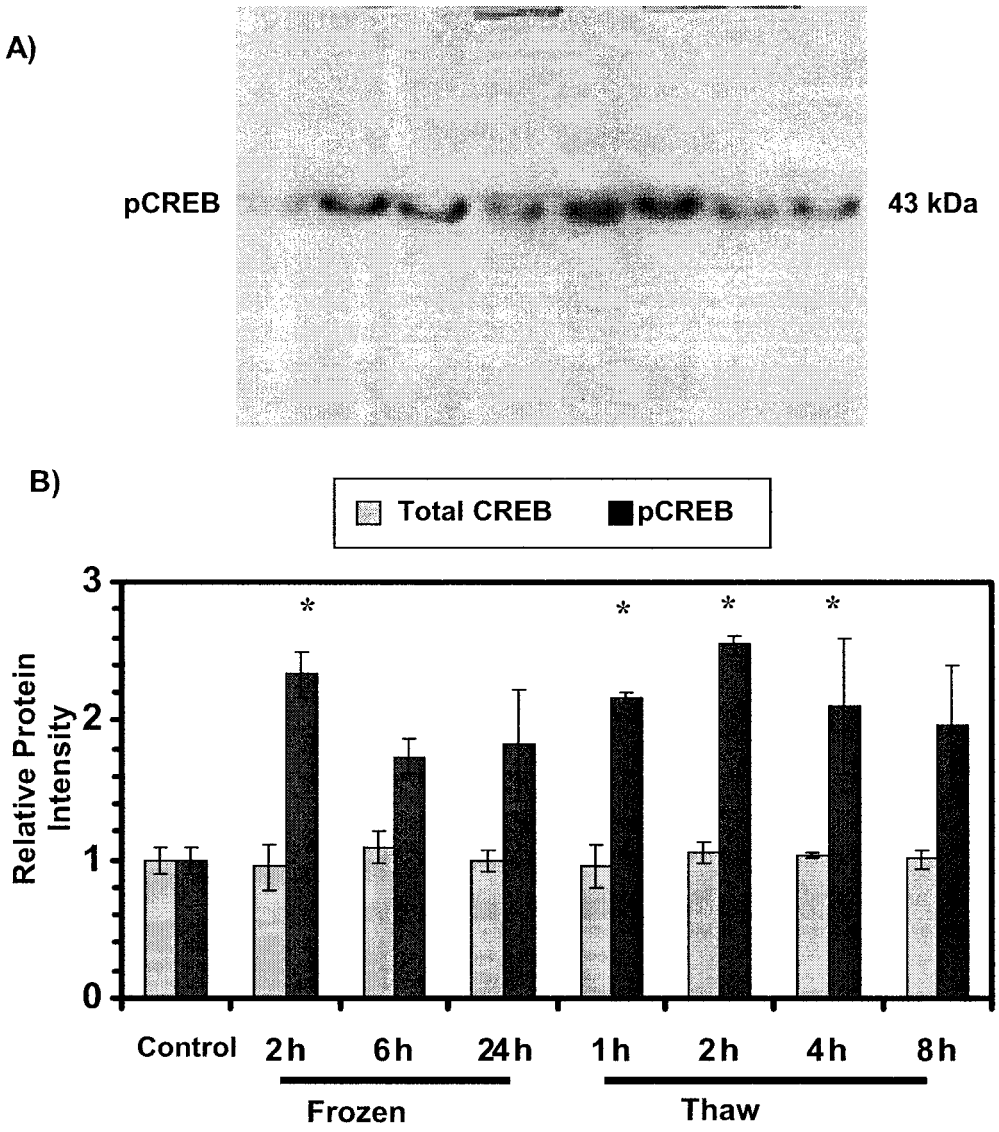
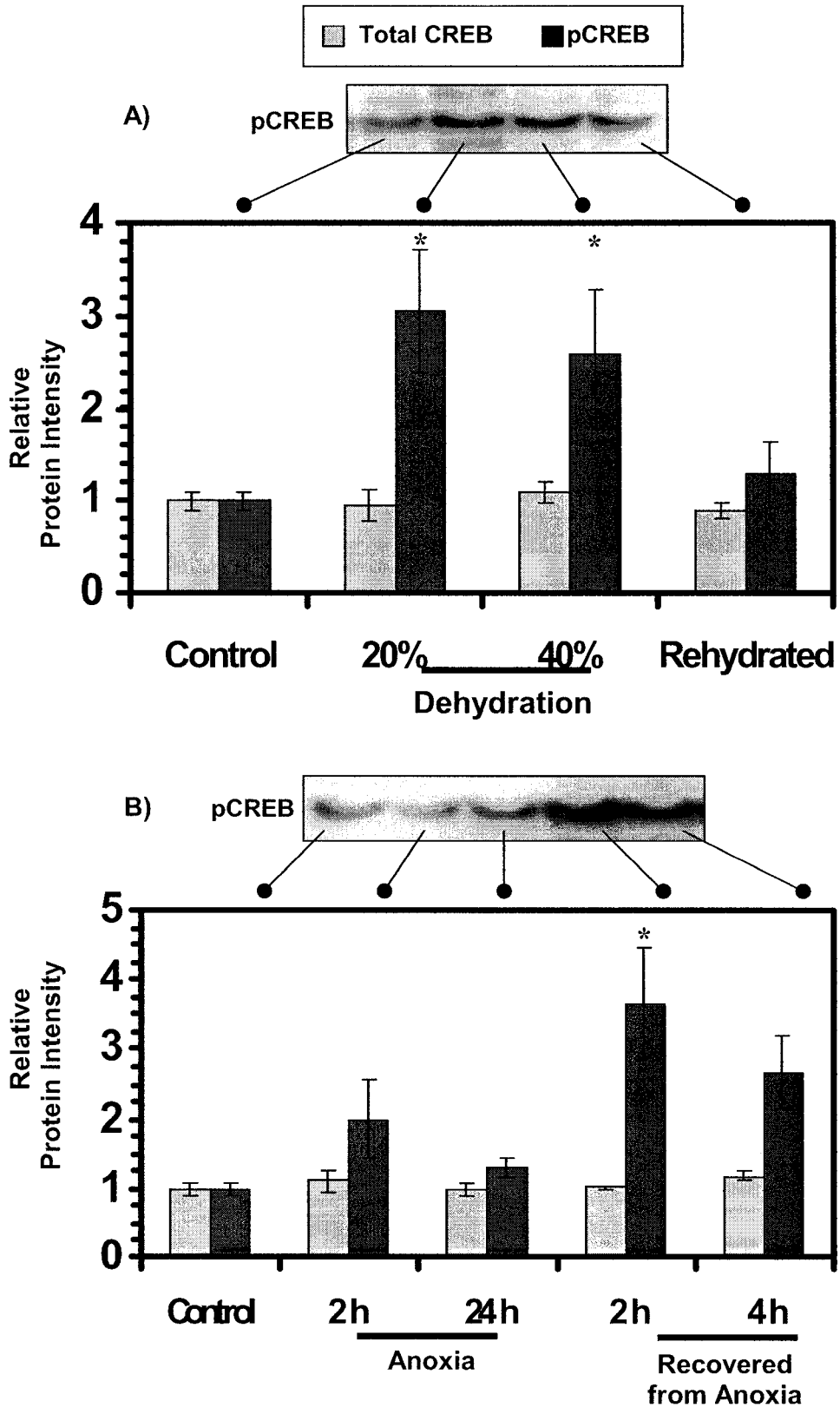

A)
pCREB
43 kDa
B)
Total CREB
pCREB
Relative Protein Intensity
3
2
1
0
*
*
*
*
Control
2h
6h
24h
1h
2h
4h
8h
Frozen
Thaw

Fig. 4.4. Histogram and representative western blots of total CREB (grey bars) and pCREB (black bars) content in *R. sylvatica* liver during (A) dehydration stress and rehydration, and (B) anoxia exposure and recovery. Bars represent means $\pm$ SEM for n=3 blots using protein extracts from different animals. Total protein extracts were probed using polyclonal antibodies raised in rabbits giving a giving a band at ~43 kDa. * - significantly different from the control, $P<0.05$.


Total CREB
pCREB
A)
pCREB
Relative Protein Intensity
0
1
2
3
4
*
*
Control
20%
40%
Rehydrated
Dehydration
B)
pCREB
Relative Protein Intensity
0
1
2
3
4
5
*
Control
2h
24h
2h
4h
Anoxia
Recovered from Anoxia

CBP binds pCREB as a cofactor in order to increase the transcription rate of the target gene. Changes in CBP levels were monitored over a time course of freezing (Fig 4.5). CBP content in liver was unchanged after 2 hours of freezing exposure but had risen significantly after 6 hours freezing to levels that were 2.5 fold higher than controls ($P<0.05$). With longer freezing, CBP content declined to control levels. During thawing, CBP did not change and remained at control levels (data not shown).

To assess the levels of active NFκB in the liver, liver extracts were probed using anti-phospho-IκB (pIκB) (Fig. 4.6). The NFκB complex is made up of two DNA binding subunits complexed with an IκB molecule. To activate NFκB, the IκB subunit is phosphorylated and dissociates, allowing the DNA binding subunits to translocate to the nucleus. Therefore, the levels of free phosphorylated IκB correlate with the amount of active NFκB in a cell. The data in Fig. 4.6 show that pIκB content increased strongly (by 2.7 fold over control levels, $P<0.05$) within 2 hours after the start of freezing but with longer freezing exposure, pIκB was again reduced. During thaw, there was a slight increase in the pIκB after two hours and a down-regulation of pIκB after 8 hours but this was not significantly different from controls. Interestingly, although pIκB levels responded to freezing, pIκB levels did not change in response to either anoxia or dehydration stresses (data not shown).

Protein kinase B (Akt) levels in wood frog liver were assessed under freeze/thaw, anoxia and dehydration stresses. None of these stresses affected the total Akt protein content. However, freezing for 24 h caused a significant 3.1 fold increase ($P<0.05$) in pAkt content (Figure 4.7a). Levels had returned to near control values after 8 hours of

Fig. 4.5. Western blot showing changes in CBP protein levels in *R. sylvatica* liver over a time course of up to 24 h freezing at -3°C. (A) Representative western blot of CBP. Total protein extracts were probed using polyclonal antibodies raised in rabbits giving a giving a single band at 300 kDa for CBP. (B) Histogram of relative CBP band intensities. Bars represent means $\pm$ SEM for n=3 blots using protein extracts from different animals. * - significantly different from the control, $P<0.05$.

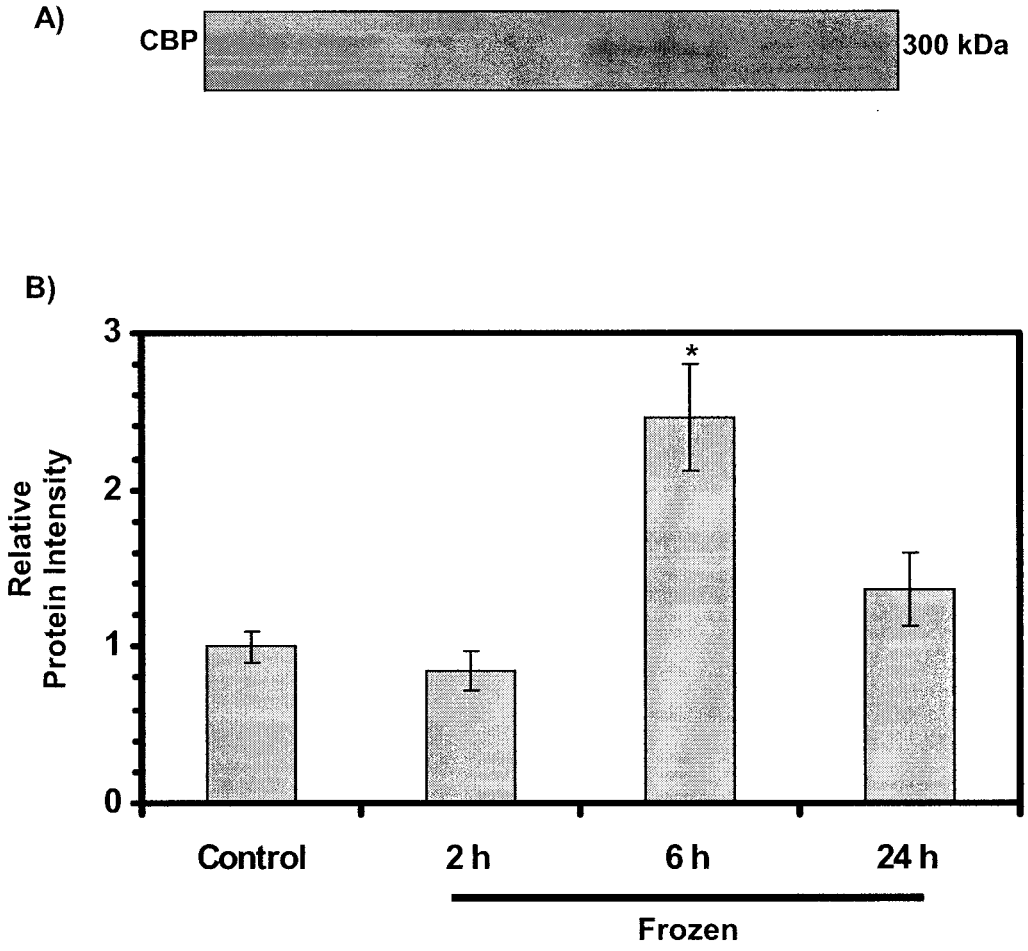

A)
CBP
300 kDa
B)
Relative Protein Intensity
3
2
1
0
*
Control
2 h
6 h
24 h
Frozen

Fig. 4.6. (A) Western blot showing changes in pIκB protein content in *R. sylvatica* liver over a time course of up to 24 h freezing at -3°C and followed by 8 h thawing recovery at 5°C. Proteins were probed using polyclonal antibodies raised in rabbits giving a band at ~41 kDa. (B) Histogram shows densitometric analysis of western blots of pIκB, means ± SEM for n=3 blots using protein extracts from different animals. * - significantly different from the control, $P<0.05$.

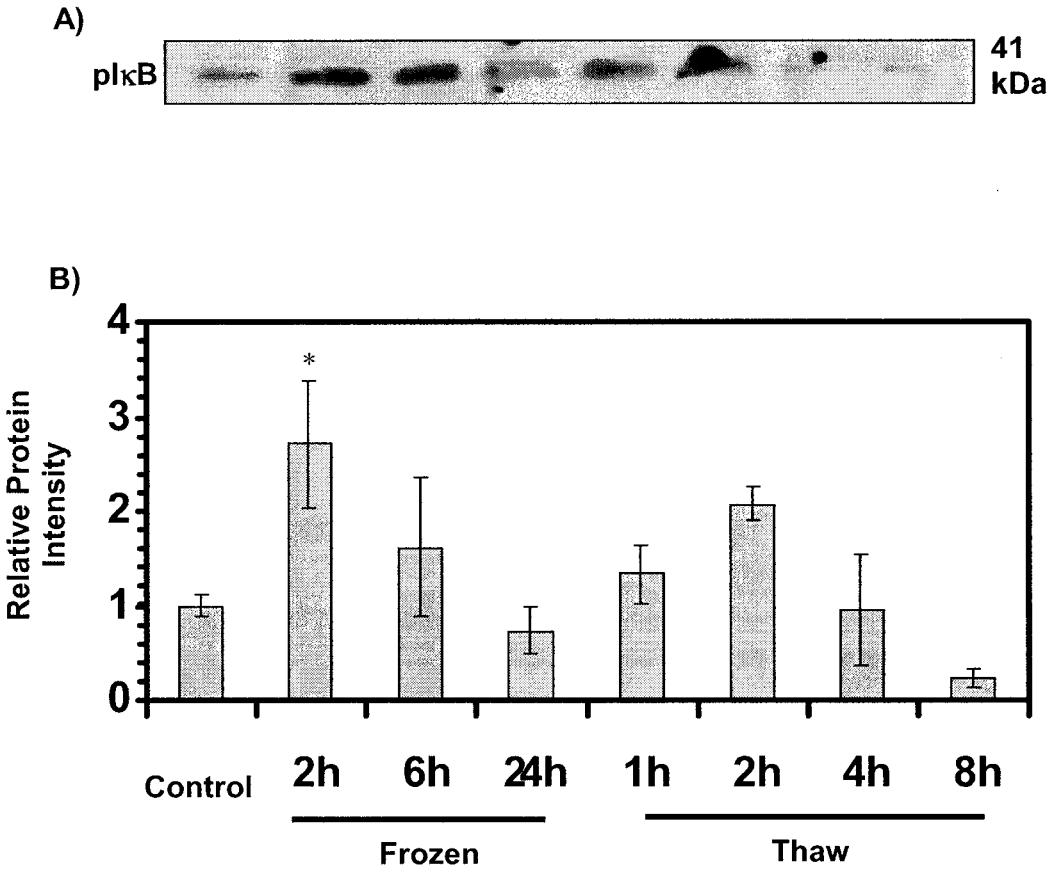
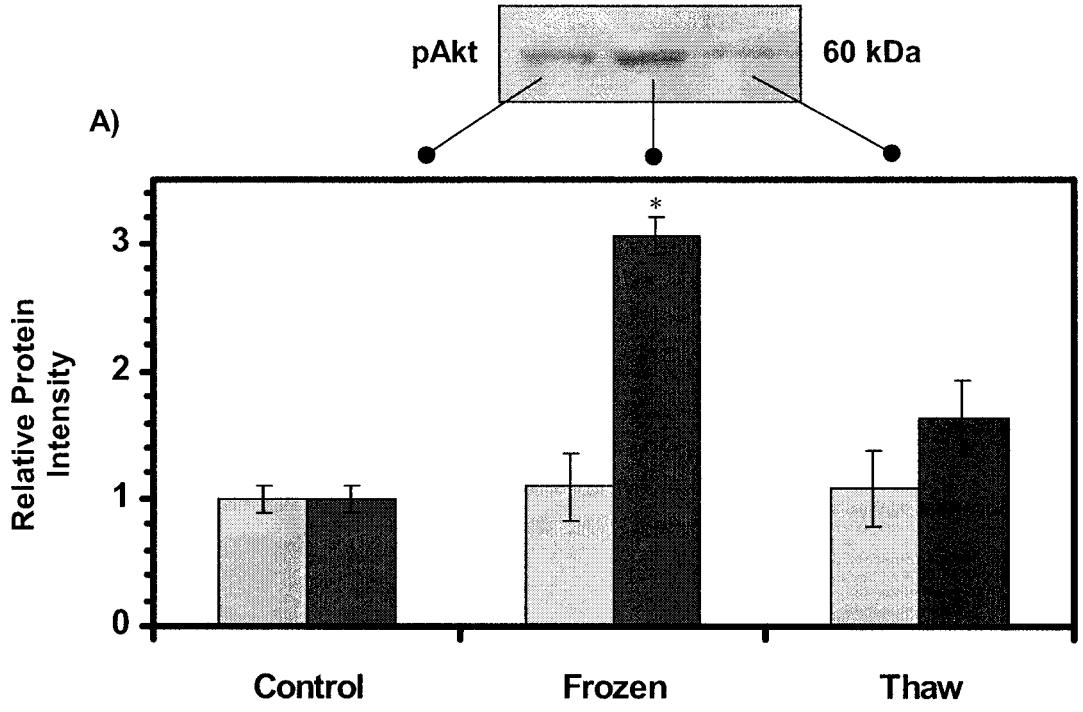
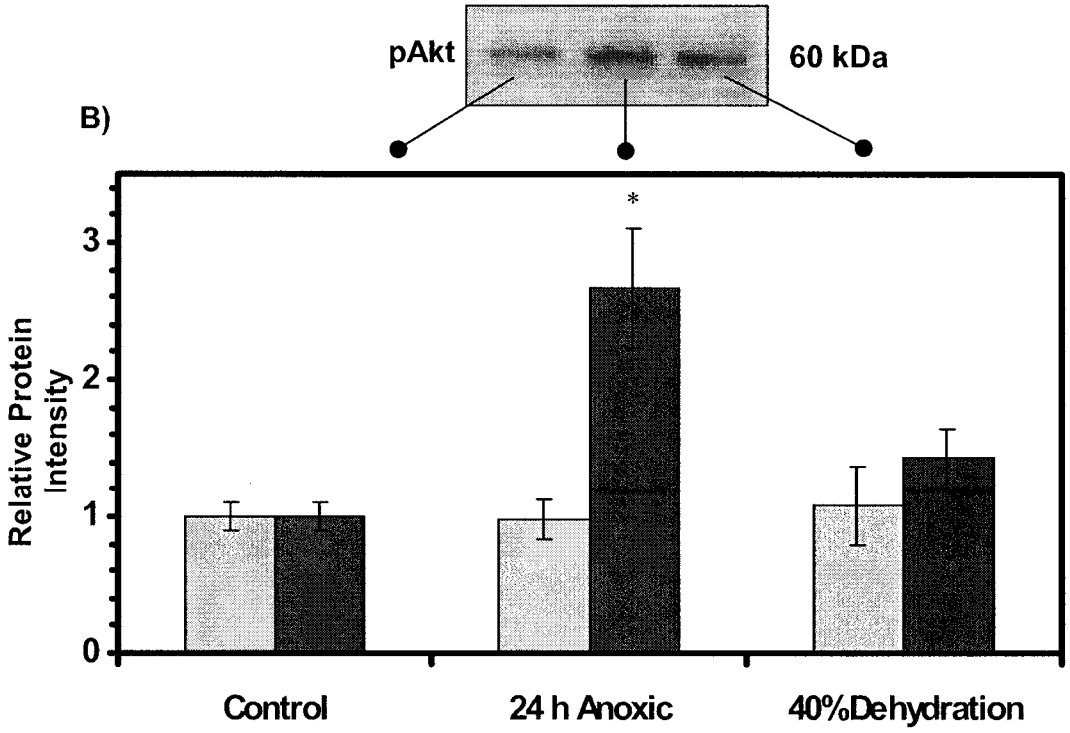

A)
pIκB
41
kDa
B)
Relative Protein Intensity
4
3
2
1
0
*
Control
2h
6h
24h
1h
2h
4h
8h
Frozen
Thaw

Fig. 4.7. Western blot showing changes in total Akt and pAkt protein content in *R. sylvatica* liver. (A) Representative blot of pAkt and histogram showing densitometric analysis of western blots of total Akt (grey bars) and pAkt (black bars) in liver of control, 24 h frozen, and 8 h thawed frogs. (B) Representative blot and histogram showing densitometric analysis of western blots of total Akt (grey bars) and pAkt (black bars) in liver of control, 24 h anoxic, and 40% dehydrated frogs. Total protein extracts were probed using polyclonal antibodies raised in rabbits giving a band at ~60 kDa.


pAkt
60 kDa
A)
Relative Protein Intensity
0
1
2
3
*
Control
Frozen
Thaw


pAkt
60 kDa
B)
Relative Protein Intensity
0
1
2
3
*
Control
24 h Anoxic
40%Dehydration

thaw. Anoxia exposure also stimulated a strong increase in pAkt, with a 2.7 fold increase ($P<0.05$) in pAkt after 24 h of anoxia exposure (Fig 4.7b). However, 40% dehydration had no effect on pAkt.

4.3.2 *DNA binding assay*

Both CREB and pCREB can bind the CREB response element, but only pCREB can activate transcription of the downstream gene. The results of western blots detecting pCREB indicated increased amounts of pCREB in response to freezing and thawing (Fig. 4.3) which suggests increased DNA binding and activation of transcription at these times. However, the DNA binding activity of pCREB can also be measured directly by measuring pCREB binding to an immobilized oligonucleotide sequence containing the CREB response element. Changes in the DNA binding activity of pCREB over the time course of freeze/thaw are shown in Fig. 4.8. DNA binding activity increased significantly after 2 hours of freezing to a value that was 2.0 fold ($P<0.05$) higher than control levels. Subsequently, binding activity decreased gradually with longer freezing exposures. The decline continued during early thawing with the lowest value reached after 2 hours of thawing before binding activity returned to approximately control levels. The DNA binding activity of pCREB during thawing is different than that seen in the western blot analysis of pCREB.

The activity of NFκB depends on translocation of the p50 and p65 subunits to the nucleus. The western blot analysis looked at pIκB levels, which may not exactly reflect the amount of free subunits available to bind the response element. The DNA binding assay allows a direct measurement of NFκB activity. Figure 4.9 shows that the DNA

Fig. 4.8. DNA binding activity of pCREB in extracts of *R. sylvatica* liver over a time course of up to 24 h freezing at -3°C and 8 h thawing recovery at 5°C. Samples were assayed according to manufacturers instructions (ActivMotif, Carlsbad, CA) with absorbance of the bound pCREB measured at 455 nm. Bars represent means $\pm$ SEM for n=3 trials using extracts from different animals. * - significantly different from the control, $P<0.05$.

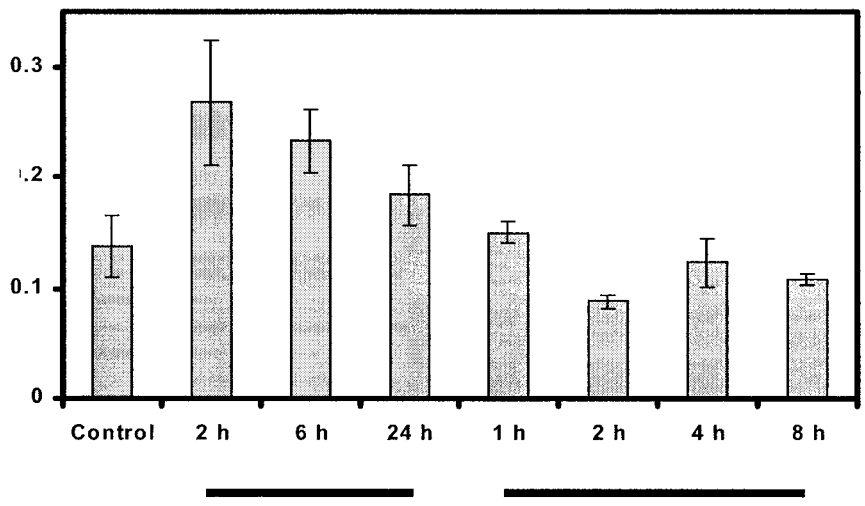
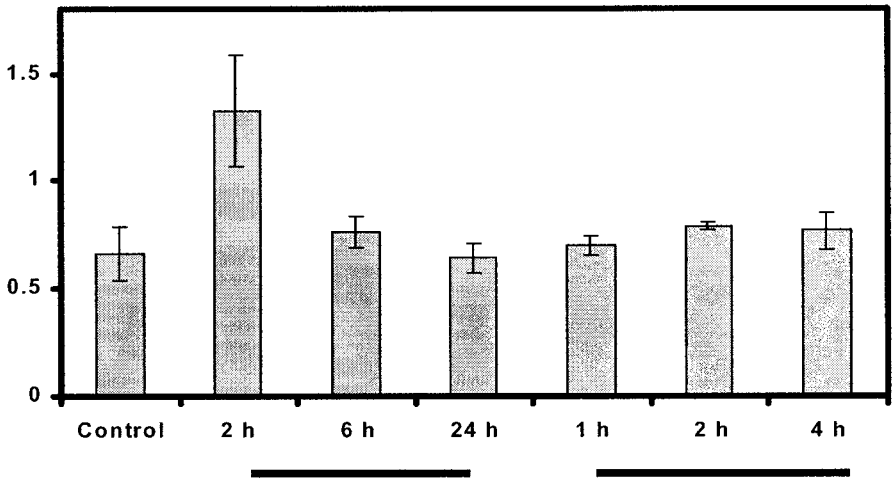

0.3
.2
0.1
0
Control
2 h
6 h
24 h
1 h
2 h
4 h
8 h

Fig. 4.9. DNA binding activity of the p55 subunit of NFκB in extracts of *R. sylvatica* liver over a time course of up to 24 h freezing at -3°C and 4 h thawing recovery at 5°C. Samples were assayed according to manufacturers instructions (ActivMotif, Carlsbad, CA) with absorbance of the bound pCREB measured at 455 nm. Bars represent means ± SEM for n=3 trials using extracts from different animals. * - significantly different from the control, $P<0.05$.


1.5
1
0.5
0
Control
2 h
6 h
24 h
1 h
2 h
4 h

binding activity of NFκB increased significantly after 2 hours of freezing exposure, correlating well with the effect of freezing seen in Figure 4.6. However, during thawing, binding activity remained at control levels.

4.2 Discussion

Controlling transcription factor activity is important for the regulation of gene and protein levels and ultimately for the freezing survival of wood frogs. Selected genes must be induced or upregulated in a coordinated fashion to provide the protein products needed to defend cells against the stresses of freezing. The fastest way to enact and control gene changes is through modifying the activity of transcription factors. This modification can be done in two ways: (a) increasing the amount of the transcription factor present, or (b) changing the activity of the transcription factor via posttranslational modification to enhance the DNA binding capabilities of the transcription factor. For the transcription factors assessed in the present chapter, the general result was that the total amount of transcription factor protein did not change in response to stress. Instead, freezing, anoxia or dehydration stresses altered the phosphorylation state of transcription factors to increase the proportion of the phosphorylated, active form. Phosphorylation of specific amino acid residues on each transcription factor results in a conformational change in the protein that affects binding or activity of the transcription factor (or in the case of NFκB, releases the inhibitory subunit so that the transcription factor can migrate to the nucleus). This chapter shows that the phosphorylation state of transcription factors is regulated in response to stress in wood frog liver.

CREB is a transcription factor that is involved with glucose regulation, cell

survival and learning and memory (Andrisani, 1999). The present results show an early activation of CREB (increase in the amount of pCREB compared to control levels) in response to freezing. The amount of pCREB relative to control levels also increased during dehydration and during thawing. The early response by pCREB to freezing is very interesting because the immediate early response gene *mcl-2* (a member of the Bcl family) is activated by pCREB (Wang *et al*, 1999). This gene is involved in cell survival and its activation is mediated through Akt activation of CREB (Wang *et al.*, 1999). As will be discussed in the next chapter, there appears to be a general suppression of apoptosis during freezing and the early activation of the transcription factors listed here represents a possible mechanism that mediates this response to cell stress.

Comparing the results of the western blotting and the DNA binding assay of pCREB showed a difference in the pattern of pCREB response over a time course of freeze/thaw. Western blot analysis showed increased pCREB during early freezing and during thawing whereas the DNA binding assay only showed increased pCREB during early freezing. The reason for this difference is not yet clear. However, other transcription factors (such as ATF2) can also bind to CRE and could potentially "outcompete" CREB in the binding activity assay. As well, unphosphorylated CREB can also bind to the CRE (but cannot activate the downstream genes) and could also potentially compete with pCREB in the binding assay. The DNA binding assay takes into account the effects of the entire cell so other transcription factors and kinases that can influence pCREB binding to CRE will be seen via the assay. By contrast, Western blot analysis quantifies the amount of pCREB but cannot determine what portion of the pCREB can bind to CRE. Taken together, the results of the two assays suggest that although pCREB is elevated during

thawing, other cellular factors may be interfering or competing with its ability to bind to the CRE during thawing.

CREB can be activated via cAMP or via other signal transduction pathways responding to stress or mitogens. The activation is mediated via the coactivator CBP. cAMP induced activation of CREB requires binding of CBP to pCREB and only a single CRE in the promoter region (Mayr *et al.*, 2001). During a stress, CBP-pCREB interactions are reduced unless the target gene recruits pCREB in a way that allows CBP to bind (Mayr *et al.*, 2001). Thus, CBP activity forms a control over the binding of CREB allowing specific genes to be activated. CBP also responded to freezing but on a slightly longer time scale than did pCREB. CBP protein levels increased significantly in wood frog liver after six hours of freezing. Apart from its binding capabilities with CREB, CBP can bind the transcription factor ATF-2 (which can bind to the CRE like CREB) and enhance transcription of downstream genes (Kawasaki *et al.*, 1998). As well, CBP is not up during thaw like pCREB. Perhaps this is a means by which CBP regulates the regulators.

NFκB is held in an inactive form in the cytoplasm when bound to its inhibitor protein IκB. Phosphorylation of IκB triggers its dissociation and targets it for ubiquitin-mediated proteolysis. The freed NFκB subunits (p50 and p65) then undergo nuclear translocation where they bind to and activate target genes. NFκB responses to freezing were assessed by two methods: (a) levels of phosphorylated IκB suggest the amount of free NFκB available for gene activation whereas (b) analysis of NFκB binding to DNA gives a direct estimate of NFκB effects on gene expression. Both methods indicated an early response by NFκB during freezing showing peaks of pIκB levels and NFκB

binding after 2 hours of freezing exposure. Early activity of NFκB in response to stress has been reported before. In rat hearts that were made ischemic by occlusion of the coronary artery, there was a significant increase in the NFκB activity (Li *et al.*, 2001). The authors suggested that the increase in NFκB was due to an increase in reactive oxygen species (ROS) that occurs during ischemia. However, the binding activity due to ROS depends on the tissue type (Allen and Tresini, 2000).

In the freezing-related stresses, NFκB did not seem to play a role. No increase in NFκB was indicated from western blotting of liver samples from anoxic or dehydrated frogs. Freeze-responsive genes that have been identified to date in wood frogs have typically also responded to either dehydration or anoxia stress (Cai and Storey, 1997a; 1997b, McNally *et al*, 2002; 2003). Hence, the activation of NFκB exclusively during freezing is quite interesting because it suggests that there may still be genes that are specifically freeze-responsive but do not respond to either anoxia or dehydration. NFκB is involved in cell survival and proliferation and the increased activity of this transcription factor during early freezing may upregulate genes to produce proteins necessary to aid late-freeze or post-thaw survival of freezing stress.

Akt is a serine/threonine kinase that modulates the activities of multiple enzymes and functional proteins (Cook et al., 2002). One of the main known actions of Akt is in the regulation of glycogen synthesis. Akt phosphorylates glycogen synthase kinase-3 (GSK3) leading to its inactivation (Cross et al., 1995) and thus preventing GSK3 from phosphorylating and inactivating glycogen synthase. One of the actions of insulin is stimulation of Akt phosphorylation and via this route phospho-Akt mediates the insulin-stimulated activation of glycogen synthesis in liver. During freezing in wood frogs,

glycogen catabolism in liver is strongly increased to support the production of glucose as the cryoprotectant. To facilitate this massive glucose output, liver glycogen synthase activity is strongly suppressed during freezing. Studies showed that the percentage of active glycogen synthase dropped from 34% in control liver to 8% within 20 minutes when freezing began but after thawing this effect was reversed and glycogen synthase had risen to 82% active in 24 h thawed frogs (Russell and Storey, 1995). The observed increase in phospho-Akt content (indicating a more active glycogen synthase) suggests a disruption in the normal signal transduction pathway that links Akt, GSK3 and glycogen synthase. Other aspects of glycogen metabolism in wood frogs are also odd, most notably the fact that homeostatic control over glucose levels is overridden. Feedback controls by high glucose typically shut off glycogenolysis in vertebrate liver when glucose levels rise above ~8 mM whereas wood frogs let liver and plasma glucose soar to >200 mM during freezing. One or more regulatory factors, as yet unidentified, must be intervening to allow the peculiar relationship between glycogen and glucose metabolism in liver of freezing frogs (see Storey and Storey, 1988). Indeed, the present results that so clearly indicate a disruption of the normal link between high phospho-Akt content and suppressed glycogen synthase activity strongly supports the idea that altered regulatory controls on glycogen metabolism must be at work.

However, Akt has many other cellular effects in the areas of cell survival and lipid metabolism (Table 1) and high Akt activity during freezing may be mediating functions other than the control of glycogen metabolism. Akt is a negative effector of peroxisome proliferator activated receptors (PPARs), which are involved in lipid metabolism (Cook et al., 2002). High Akt activity during freezing could be important in

suppressing PPAR activity to inhibit lipid metabolism in the frozen, ischemic state. As well, phospho-Akt has been shown to increase phosphorylation of the pro-apoptotic protein BAD and thereby promote cell survival (Datta et al., 1997). The role of anti-apoptotic activity in freezing survival has not been investigated in the wood frog before, but will be discussed in the next chapter.

As seen in Table 1, there are multiple transcription factors and kinases that have similar effects. For example, both Akt and CREB are involved in glucose regulation (Table 1). As well, all the transcription factors discussed seem to have some effect on cell survival. Due to the potential of multiple signalling effects there needs to be a means of regulating the regulators. For example, perhaps a certain degree of phosphorylation of the total Akt pool may preferentially effect transcription of selected genes. Akt also has two phosphorylation sites suggesting the potential for multiple points of control.

This chapter has shown that a few key transcription factors change their activity levels in response to freezing and freezing-related stresses in liver of wood frogs. Does this mean that all genes that contain a potential binding site for one of these transcription factors in its promoter region are transcribed in response to freezing? Probably not as this would be quite costly to the cell. So there must be other regulatory pathways that impinge on the transcription factors identified here to further regulate transcription of a specific few genes in response to freezing. Promoter analysis for potential binding sites of genes that are upregulated during stress could provide some clues. A gene could contain multiple competing binding sites for transcription factors. Then, if one transcription factor was more active (greater amount of the phosphorylated form), it could displace the other transcription factor allowing for a fine control of gene transcription.

In addition, many more transcription factors (apart from those investigated here) exist and whether or not they are involved in frog freeze tolerance remains to be seen. Recently, transcription factor arrays have been developed that allow the rapid detection of transcription factor activities under different experimental states (Panomics, Redwood, CA). Nuclear extracts of tissues from different states are incubated with labelled oligonucleotide probes containing transcription factor response elements. The DNA-protein complexes are then run out on a gel to purify and isolate the complexes. The DNA probes are separated from the protein and then applied to the transcription factor arrays to provide an indication of which transcription factors have increased activity in different tissue states. Such an analysis could be highly beneficial in sorting out the relevant transcription factors that regulate the gene responses that aid freezing survival.

CHAPTER FIVE

Anti-apoptotic activity during freezing and related stresses: a preliminary look into the suppression of apoptosis in the wood frog *Rana sylvatica*.

5.1 Introduction

Apoptosis, or programmed cell death, is a series of orderly events that culminate in the controlled destruction of a cell. A cell undergoing apoptosis will initially shrink, followed by mitochondrial homeostatic break down and release of cytochrome *c*, DNA degradation, then eventual targeting for phagocytosis by macrophages and other dendritic cells. The fate of cells to enter the apoptotic pathway is under a very fine control of positive and negative effectors. Environmental stresses that impinge on a cell can start the cell towards apoptosis through DNA damage, disruption of the mitochondrial chemiosmotic balance or even through various ligands binding to receptors on the cell surface.

Extremophiles are subjected to stresses that could lead a normal cell to apoptosis. Freezing in the wood frog, as mentioned in Chapter 1, is a deviation from homeostasis. During freezing there is transport of glucose into cells and a loss of water from the cell to the extracellular space. Mitochondria are placed in even greater stress, as glucose cannot penetrate into the mitochondria, yet water can leave the mitochondria. Thus, there is a great osmotic stress place on the mitochondria that would lead a normal cell to apoptosis. In fact, work by Costanzo *et al.* (1999b) showed mitochondrial swelling in wood frog nerve cells as they approached the lethal limit of freeze tolerance. The use of anti-apoptosis techniques like the ones employed by cancerous cells is a way of not "going down that road". The wood frog *Rana sylvatica* is one such extremophile that has the ability to survive freeze-thaw cycles over the winter. Being freeze-tolerant means being able to withstand several environmental stresses such as: a) physical damage caused by internal ice crystal formation; b) ischemia tolerance due to the lack of oxygen delivery to

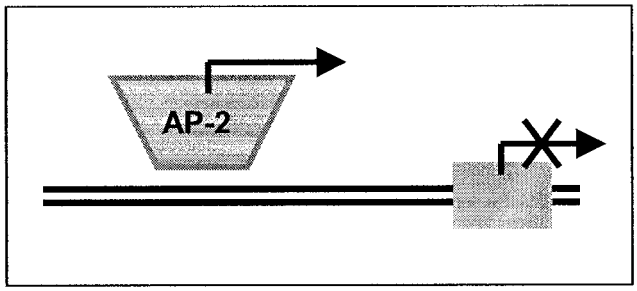
cells; c) dehydration tolerance due to conversion of water to ice. This chapter looks at *R. sylvatica* and the ability of its cells to withstand the tendency to undergo apoptosis despite the stresses place on them.

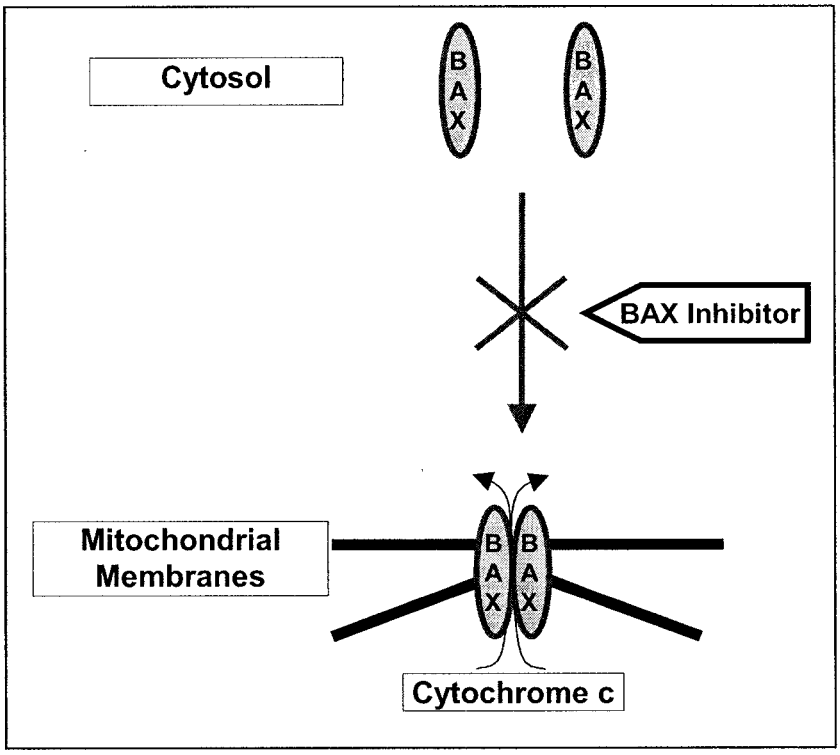
A cDNA microarray was scanned to seek changing patterns of gene expression under freezing stress in the liver. Analysis of the data showed an upregulation of AP-2α, a transcription factor involved in the control of apoptosis. Cells in a frozen frog would have to exhibit some type of anti-apoptotic activity to survive. This discovery propelled me to further investigate instances of anti-apoptotic activity in *R. sylvatica.*

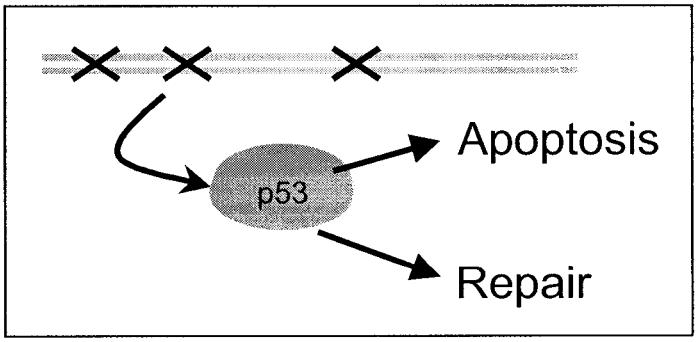
When considering what a cell has to do to prevent apoptosis, different pathways come into play. Apoptotic pathways are typically classified into either death receptor or mitochondrial pathways (Gutpa, 2001). For the purposes of this chapter, I have divided the defence mechanisms for apoptosis in wood frogs into three paths (Fig. 5.1). The first is receptor-induced apoptosis where extracellular signals influence transcription factors like AP-2 to repress apoptosis (Pfisterer *et al.*, 2002). The second is mitochondrial-induced apoptosis where the release of cytochrome *c* is prevented. The third is nuclear-induced apoptosis where DNA damage leads to controlled apoptosis mediated by the cell-cycle control protein p53 (North and Hainut, 2000).

To investigate these three routes I used western blot analysis to look at changes in the levels of selected proteins during freezing and related stresses in wood frogs. Analysis used antibodies directed against AP-2α, BAX-inhibitor 1 (BI-1), and p53 and seven phosphorylated forms of p53. Changes in the levels of each protein will provide clues as to the pattern of regulation that allows frog cells to survive freezing stress. In fact, as in

Fig. 5.1. Possible anti-apoptotic strategies at work during freeze tolerance. in the wood frog, *R. sylvatica*. A) Upregulation of transcription factor AP-2 results in the suppression of transcription of genes promoting apoptosis. B) BAX-inhibitor protein prevents the translocation and homodimerisation of BAX stopping the release of cytochrome *c* into the cytoplasm. C) DNA damage is detected by p53, which can send the cell into apoptosis or repair.


AP-2


Cytosol
BAX
BAX
BAX Inhibitor
Mitochondrial Membranes
BAX
BAX
Cytochrome c


p53
Apoptosis
Repair

Chapter 4, by looking at freezing time courses and freezing-related stresses a preliminary picture of anti-apoptotic activity can be elucidated.

5.2 Materials and Methods

5.2.1 *Animals*

Frogs were collected in the spring of 2001 and experiments were conducted as described in chapter two.

5.2.2 *cDNA microarray analysis*

Total RNA from control and stressed tissues was reverse transcribed with Cyanine 3 (Cy3) and Cyanine 5 (Cy5), respectively, to generate labelled probe for the microarray. The differently labelled probes were hybridized on the same microarray allowing visualization of binding intensity of control and stressed genes. As a result of the inherent differences in binding affinity of the two types of dyes, tissue samples were reciprocally labelled and allowed to hybridize on another microarray. Intensity of binding can be compared from two microarrays where the probes are reciprocally labelled.

Total RNA was isolated from tissue samples stored at –80°C. The total RNA was isolated three different times and an aliquot from each sample was combined to make separate pooled sample of stressed and control RNA. These pooled samples were used for subsequent microarray analysis.

Total RNA from control or stressed samples was divided into two tubes and labelled with Cy3 or Cy5. Approximately 10 µg of total RNA was used for each reaction. The total RNA was incubated with 5X First Strand reaction buffer (Superscript II, Life

Technologies), 150 pmol AncT mRNA primer, 0.1 M DTT, 6.67 mM of dATP, dGTP, dTTP and 2 mM of dCTP and 1 mM of either Cy3 or Cy5 per reaction. The reaction mixtures were incubated at 65°C for five minutes and then 42°C for 5 minutes in the dark. To each mixture, 2 µl of reverse transcriptase (Superscript II, Life Technologies) was added and the reaction was allowed to proceed at 42°C for an additional 2 hours. To stop the reaction and hydrolyse the RNA, 4 µl of 50 mM EDTA (pH 8.0) and 2 µl of 10 N NaOH was added and incubated at 65°C for 20 minutes. Each reaction was neutralized by the addition of 5 M acetic acid and then control and stressed samples were combined together giving two reciprocally labelled reactions. DNA was precipitated with isopropanol on ice for 30 minutes and washed with ice-cold 70% ethanol and resuspended in 5 µl of dH_2O.

For each microarray 100 µl of DIG Easy Hyb solution (Roche) with 10 mg/ml yeast tRNA, and 10 mg/ml of calf thymus DNA was pre-incubated at 65°C for 20 minutes and cooled to room temperature. The hybridization solution was added to each pooled pair of Cy3 and Cy5 labelled cDNA, then pre-hybridised at 65°C for 2 minutes and then cooled to room temperature. The 19K human microarrays (Ontario Cancer Institute) were printed on two glass slides that rest on top of each other with a space in between to pipet 80 µl of the probe and hyb-solution. The slides were placed in a hybridization chamber and allowed to hybridize at 37°C for 16 hours.

After hybridization, the slides were then gently washed 3 times for 10 minutes each at 50°C in pre-warmed 1X SSC, 0.1% SDS. Slides were given a final rinse in 1X SSC and then spun dry at 500 x g for 5 minutes. Slides were stored in the dark until scanned using a Scanarray system at 10 µm resolution at the lab of Dr. R. Walker,

National Research Council (NRC). Analysis of the data was performed using the freeware program Scanalyze2 (http://rana.lbl.gov/EisenSoftware.htm).

5.2.3 *Analysis of protein levels*

Protein level analysis was accomplished by western blotting as described in Chapter 2. The membranes were blocked for 1 hour at RT in a 1.0% w/v solution of non-fat dry milk (NFDM) after electrophoresis and transfer. Membranes were allowed to incubate with an appropriate dilution of primary antibody in TBST overnight at 4°C (AP-2α: 1:500; BI-1: 1:800; p53: 1:2000). Membranes were washed three times in TBST for five minutes at RT and then incubated in an appropriate secondary antibody (conjugated with horseradish peroxidase) diluted 1:2000 in blocking solution for 1 hour at RT. The antibody-protein complex on the PVDF was detected using enhanced chemiluminescence (ECL) (DuPont NEN, Boston, MA). The membrane was scanned using a GEL-DOC system. The image was quantified using GeneTools software. Significant differences ($P<0.05$) were tested using ANOVA followed by a 2-tailed Dunnett's test.

The AP-2α antibody was an anti-mouse monoclonal antibody that was obtained from the University of Iowa Hybridoma bank. The Bax-Inhibitor antibody (BI-1) was a peptide antibody (KNSADKEEKKKKRRN) raised in rabbits (Bolduc and Brisson, 2002). The p53 antibody and antibodies to each of the specific phosphorylated forms of p53 were polyclonal antibodies raised in rabbits and obtained from New England Biolabs (Mississauga, Ontario). All antibodies used gave single bands at the expected molecular weight. As a result of the high sequence conservation for each protein found among different species, I was confident that antibodies had a high cross-reactivity. Secondary

anti-rabbit (1:2000 dilution in TBST) and anti-mouse antibodies (1:1000 dilution in TBST) conjugated with horseradish peroxidase were from New England Biolabs (Mississauga, Ontario).

5.3 Results

5.3.1 *cDNA microarray analysis*

Analysis of the cDNA microarray located several clones that were potentially upregulated. Spots on the array that were at least 2-fold upregulated were further analysed. Like cDNA library screening analysis in Chapter 2, this threshold may result in missed upregulated genes, however the chances of finding a gene that is upregulated is increased.One of the clones was AP-2 (clone N63770) which showed a putative 2.3 fold upregulation in liver of 24 h frozen frogs (Fig. 5.2). The fold upregulation was obtained by determining the ratio of binding by labelled control cDNA versus 24 h frozen cDNA. The ratios obtained for each cDNA was averaged with the reciprocally labelled counterpart run on a duplicate microarray. Confirmation of the results from the microarray requires further transcript analysis to prove transcript upregulation. Other putative upregulated genes (2-fold or greater) identified from the microarray, but not investigated in this thesis, are listed in Appendix A.

5.3.2 *Western blot analysis*

Changes in AP-2 protein levels were measured over a time course of freezing (Fig 5.3) using liver tissue. Protein levels had increased significantly compared to controls

Fig. 5.2. Heterologous screening of a human cDNA microarray with *Rana sylvatica* liver. The microarray was probed with liver cDNA prepared from control and 24 h frozen frogs and labelled with either Cy3 or Cy5. Each pair of spots represents a different cDNA. Binding by Cy3 versus Cy5 labelled cDNA to each spot on the microarray was quantified, the ratio of Cy3 vs Cy5 binding was calculated, and data were colourized for easy interpretation: Red - less binding by cDNA from 24 h frozen frogs compared to controls (down-regulation), Green – more binding by cDNA from 24 h frozen frogs than controls (up-regulation), Yellow - equal amounts of control and 24 h frozen cDNA. Arrow points to spots indicating potential upregulation of the cDNA for AP-2α. Figure shows one section containing 108 cDNAs of the 19,000 human cDNA microarray.

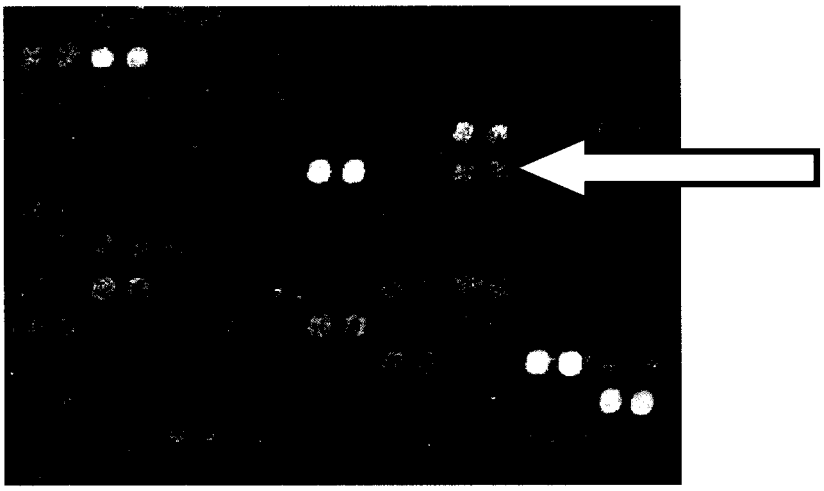
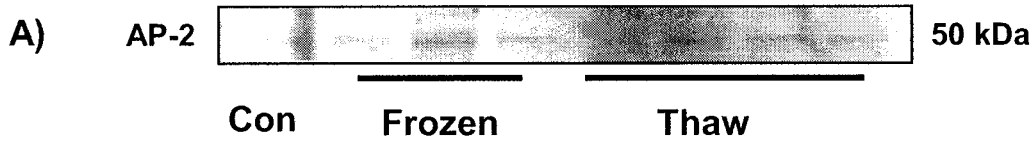
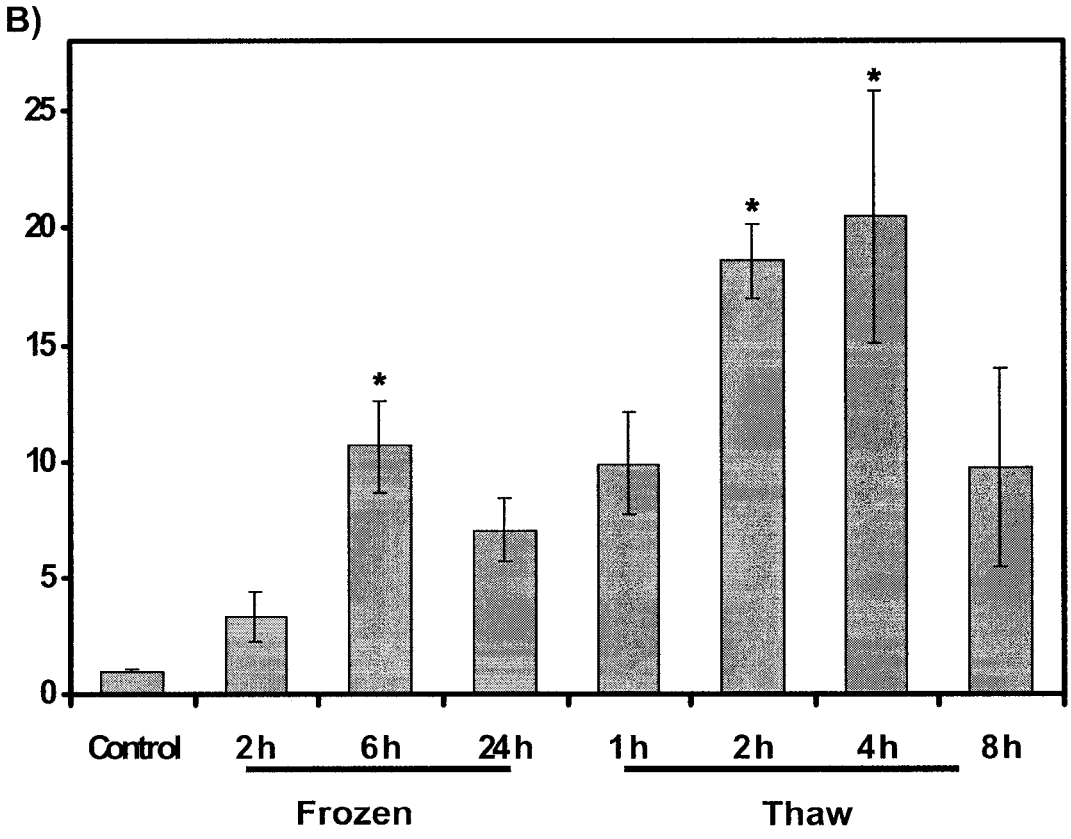


Fig. 5.3. Western blot analysis of AP-2α protein levels in *Rana sylvatica* liver over a time course up to 24 h freezing at -3°C and followed by 8 h thawing recovery at 5°C. (A) Representative western blot showing a single band at ~50kDa which cross-reacts with a monoclonal antibody directed against AP-2α. (B) Histogram shows densitometric analysis of AP-2α protein levels. Bars are means ± SEM for n=3 blots using protein extracts from different animals. * - Significantly different from control value, $P < 0.05$.


A)
AP-2
50 kDa
Con
Frozen
Thaw


B)
25
20
15
10
5
0
*
*
*
Control
2h
6h
24h
1h
2h
4h
8h
Frozen
Thaw

after 6 h of freezing (10.6 fold, $P<0.05$). Protein levels remained 7.1 fold higher than controls after 24 h of freezing, but rose again during thawing. Within 2 h AP-2 protein content in frog liver was 18.6 fold higher than control values ($P<0.05$) and remained high after 4 h thaw (20.5 fold). After 8 h thawed, AP-2 content was reduced by about half (9.7 fold higher than controls) from the peak value at 4 hours of thaw.

In freeze-related stresses, the levels of AP-2α actually decreased significantly (Fig. 5.4). Anoxia exposure for 24 hours resulted in a decrease in AP-2α protein levels to 59% of control values. Loss of body water (40% dehydration) also reduced AP-2α levels to just 33 % of control values ($P<0.05$). After 4 hours of recovery from anoxia and full rehydration (rehydration for 24 hours) AP-2α levels remained suppressed below control levels (at 42 and 20% of control values, respectively), and in both cases were also somewhat lower than their corresponding stress situations.

Analysis of BI-1 protein levels showed a pattern different than that seen for AP-2. BI-1 was significantly increased in frog liver after 6 hours of freezing exposure, rising to 2.8-fold higher than controls ($P<0.05$) (Fig. 5.5). However, with longer freezing, levels were reduced and levels of BI-1 were 1.5-2.0 fold higher than control values over the thaw time course (not significantly different from controls). In freezing related stresses, anoxia and dehydration, BI-1 protein decreased significantly under all conditions ($P<0.05$) (Fig. 5.6). After 24 hours of anoxia exposure, BI-1 protein levels had dropped to 51% of control values and after 40% dehydration BI-1 levels were also down-regulated (to 40% of control levels). BI-1 protein levels remained low after recovery from anoxia or dehydration stresses; values were 43 and 51%, respectively, of control levels in 5°C acclimated frogs.

Fig. 5.4. Western blot analysis of AP-2α protein levels in *Rana sylvatica* liver in freeze-related stresses. (A) Representative western blot showing a single band at ~50 kDa, which cross-reacts with a monoclonal antibody directed against AP-2α. (B) Histogram shows densitometric analysis of AP-2α protein levels. Bars are means ± SEM for n=4 blots using protein extracts from different animals. * - Significantly different from control value, $P < 0.05$.

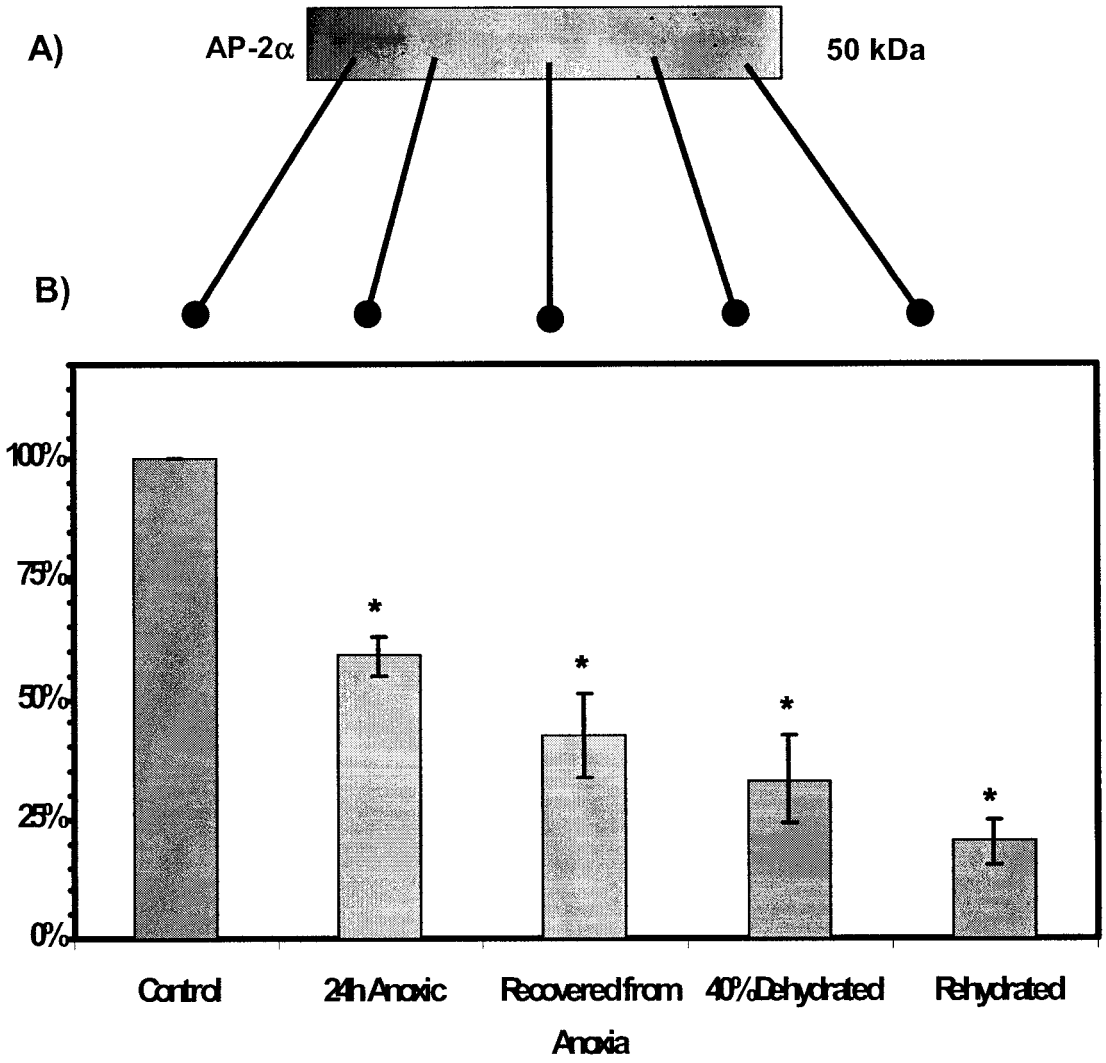
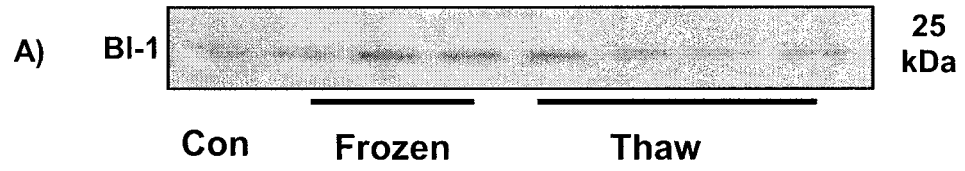

A)
AP-2α
50 kDa
B)
100%
75%
50%
25%
0%
*
*
*
*
Control
24h Anoxic
Recovered from Anoxia
40% Dehydrated
Rehydrated

Fig. 5.5. Western blot analysis of BI-1 protein levels in *Rana sylvatica* liver over a time course up to 24 h freezing at -3°C and followed by 8 h thawing recovery at 5°C. (A) Representative western blot showing a single band at ~25 kDa, which cross-reacts with a peptide antibody directed against BI-1. (B) Histogram shows densitometric analysis of BI-1 protein levels. Bars are means ± SEM for n=3 blots using protein extracts from different animals. * - Significantly different from control value, $P < 0.05$.


A)
Bl-1
25
kDa
Con
Frozen
Thaw

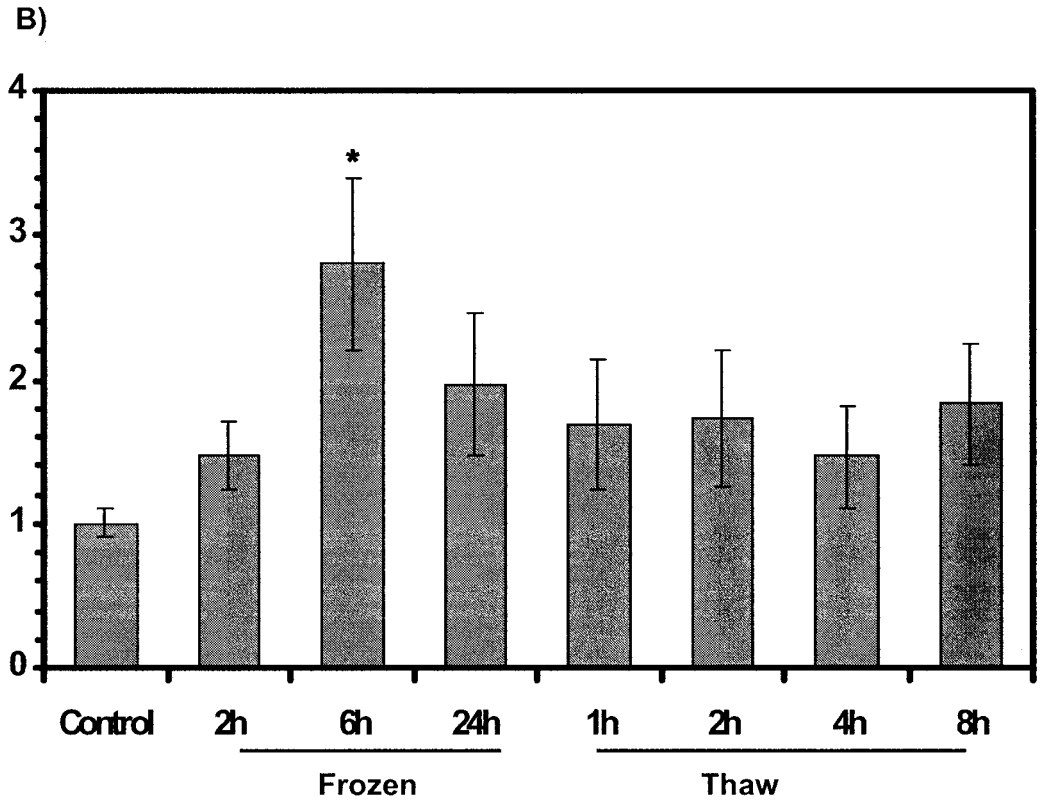
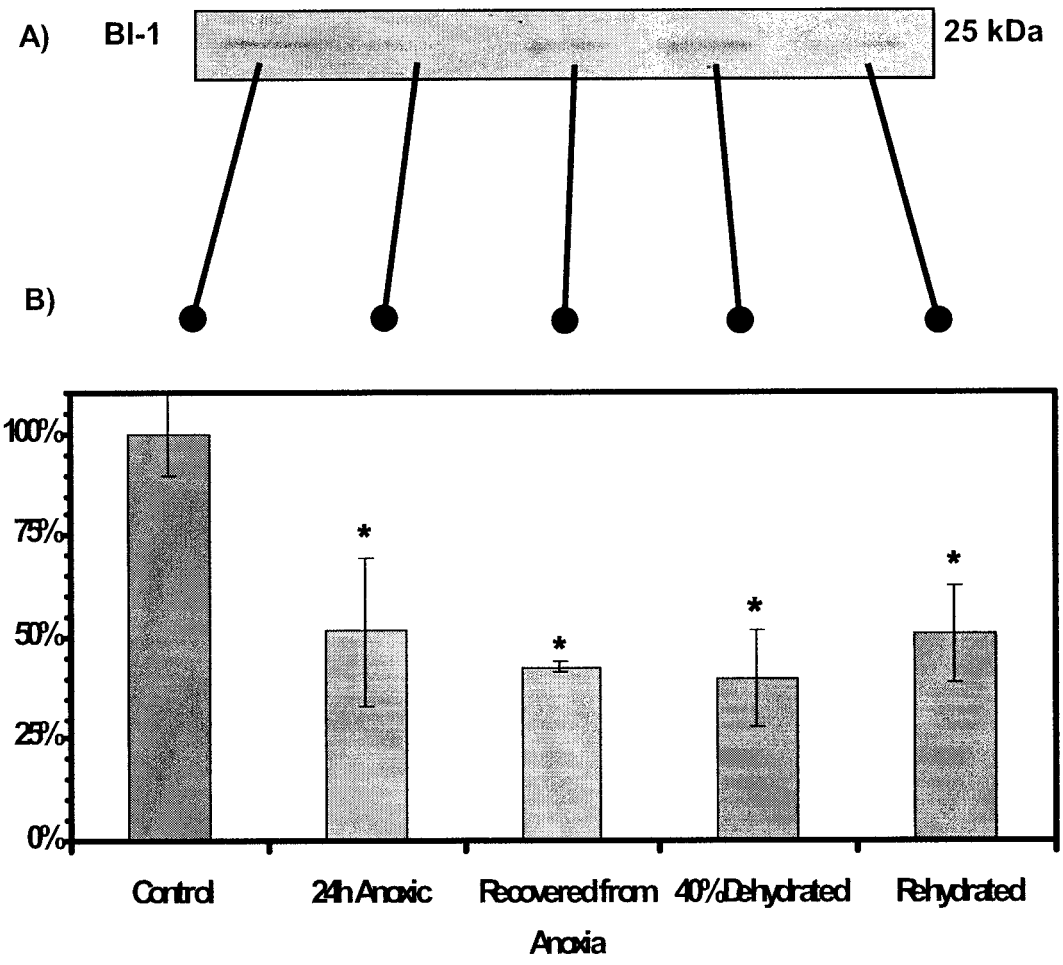

B)
4
3
2
1
0
*
Control
2h
6h
24h
1h
2h
4h
8h
Frozen
Thaw

Fig. 5.6. Western blot analysis of BI-1 protein levels in *Rana sylvatica* liver in freeze-related stresses. (A) Representative western blot showing a single band at ~25 kDa, which cross-reacts with a peptide antibody directed against BI-1. (B) Histogram shows densitometric analysis of BI-1 protein levels. Bars are means $\pm$ SEM for n=3 blots using protein extracts from different animals. * - Significantly different from control value, $P < 0.05$.

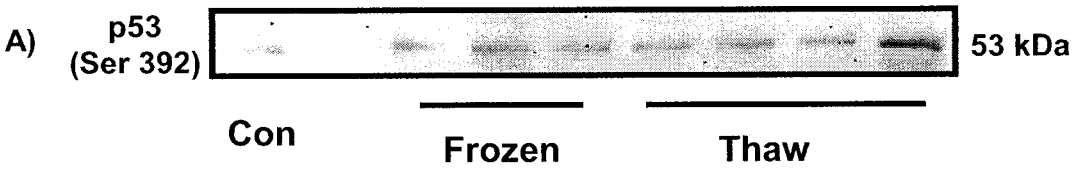

A)
BI-1
25 kDa
B)
100%
75%
50%
25%
0%
*
*
*
*
Control
24h Anoxic
Recovered from Anoxia
40% Dehydrated
Rehydrated

Analysis of total p53 protein levels showed no change in protein content over the course of the freeze-thaw cycle (Fig. 5.7b). However, p53 activity is modified by protein phosphorylation at several sites. Antibodies specific for different phosphopeptides of p53 (Serine 6, 9, 15, 20, 37, 46 and 392) were tested. Only the phospho-specific antibody for the Ser 392 phosphopeptide showed any binding to the frog protein. There were no significant changes in phospho-p53 Ser 392 levels during freezing exposure (Fig. 5.7a) but an increase in the amount of phosphoprotein occurred over time during thawing. Levels of phospho-p53 Ser 392 rose to a peak that was 2.4 fold higher than control levels after 8 h thawed ($P<0.05$). In freezing-related stresses the total amount of p53 was affected in two situations: total p53 was significantly reduced (to 56% of control values) after 24 h anoxia exposure whereas during rehydration after 40% dehydration total p53 content rose to 2.7 fold higher than control values ($P<0.05$) (Fig. 5.8). Anoxia exposure also significantly reduced the amount of phospho p53 Ser 392 to about 44% of the control value but phospho-p53 was not affected under the other experimental conditions.

5.4 Discussion

The freeze-thaw process in wood frogs seems to involve a change in expression of proteins associated with apoptosis. This chapter provides an assessment of proteins that are associated with the suppression of apoptosis and the responses of these proteins to freeze-thaw and associated stresses. The initial cDNA array screen showed that levels of the AP-2 protein increased in liver during freezing. I realized that heterologous screening of a human cDNA microarray might not be entirely valid, which necessitated follow-up studies to confirm the expression patterns seen (see Eddy and Storey, 2002). To this end,

Fig. 5.7. Analysis of total p53 and phospho-p53-Ser392 protein levels in *Rana sylvatica* liver over a time course up to 24 h freezing at -3°C and followed by 8 h thawing recovery at 5°C. (A) Representative western blot showing a single band at ~53 kDa, which cross-reacts with a polyclonal antibody directed against phospho-p53-Ser392 (B) Histogram shows densitometric analysis of total p53 (open bars) and phospho-p53-Ser392 (grey bars) protein levels for control and thawing time points (freezing time points did not change and are not shown). Bars are means $\pm$ SEM for n=3 blots using protein extracts from different animals. * - Significantly different from control value, $P < 0.05$.


A)
p53
(Ser 392)
53 kDa
Con
Frozen
Thaw

B)

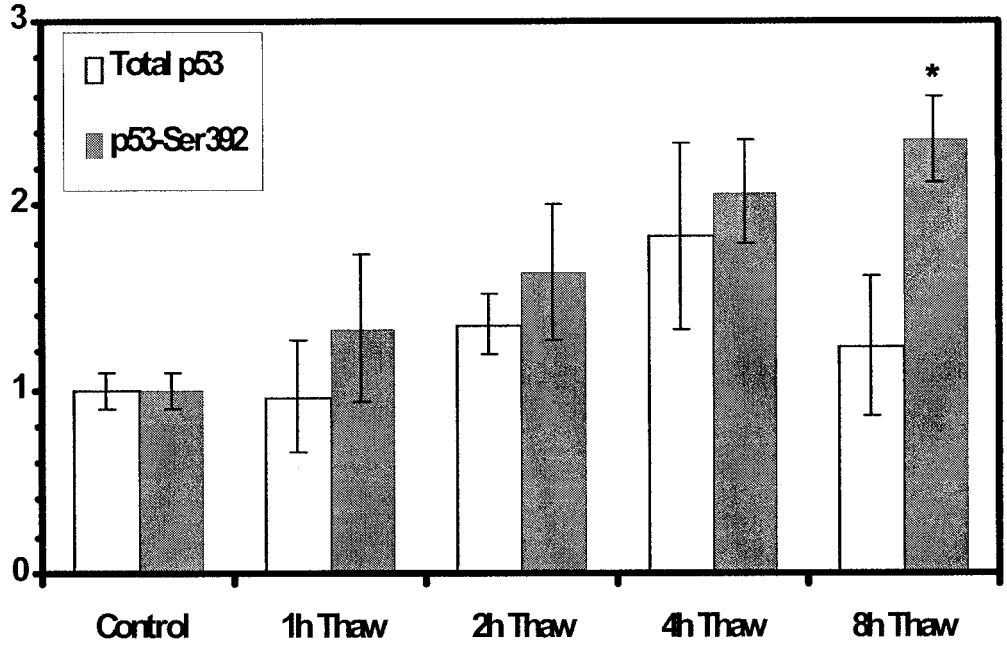
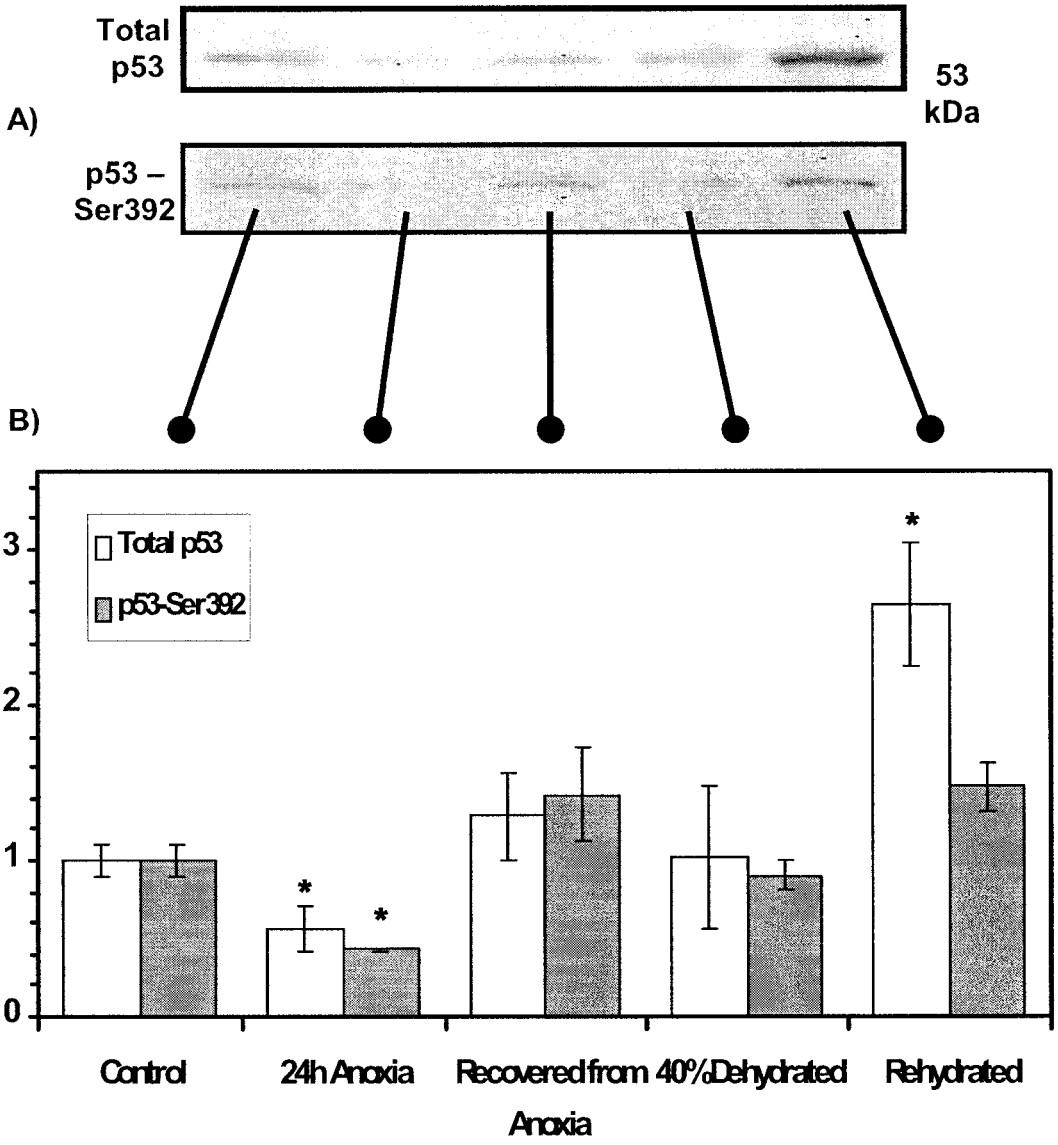

Total p53
p53-Ser392
3
2
1
0
*
Control
1h Thaw
2h Thaw
4h Thaw
8h Thaw

Fig. 5.8. Analysis of total p53 and p53-Ser392 protein levels in *Rana sylvatica* liver in freeze-related stresses. A) Representative western blots showing single bands at ~53 kDa, cross-reacting with either the polyclonal antibody directed against total p53 or directed against phospho-p53-Ser392. B) Histogram shows densitometric analysis of total p53 (open bars) and p53-Ser392 (grey bars) protein levels. Bars are means $\pm$ SEM for n=3 blots using protein extracts from different animals. * - Significantly differences from corresponding control values $P<0.05$.


A)
Total p53
p53 – Ser392
53 kDa
B)
Total p53
p53-Ser392
3
2
1
0
*
*
*
Control
24h Anoxia
Recovered from Anoxia
40%Dehydrated
Rehydrated

I performed an assessment of the AP-2α expression pattern during stress. Given that AP-2 expression is elevated in certain cancers, the possibility that frogs undergo certain anti-apoptotic activities to defend their cells from the stresses associated with freeze/thaw presented itself as a new avenue of research.

AP-2α content was markedly increased after 6 hours of freezing exposure, remained high after 24 h freezing and then rose to even higher levels over the early hours of thawing. Physiologically, the frog after 1 h returned from the freezer to 5°C is still largely frozen although the skin is thawed. By 2 hours more than half of the ice is gone but there are still no vital signs. Often, however, liver and heart have melted due to their high cryoprotectant content even though the abdominal cavity still contains much ice. After 4 hours all ice has melted, the heart has resumed a slow beat and liver appears to be fully rehydrated but the animal shows little or no muscle movement. By 8 hours thawed considerable muscle tone has returned. Hence, the peak of AP-2α protein content in liver correlates with the time when the frog is starting to regain physiological functions of the core organs. The suggested role of AP-2α is in suppression of genes involved in apoptosis and genes that inhibit cell proliferation (Pfisterer *et al.*, 2002). The fact that there is an increase in AP-2α during thaw suggests that the liver may be in a repair mode during these early hours post-thaw to fix any physical damage that was caused by freezing. Alternatively, perhaps AP-2α is allowing the liver to regenerate cells damaged during freeze/thaw. Normally a quickly regenerating liver involving rapid cell proliferation would doom hepatocytes to apoptosis. However, AP-2α may circumvent cellular reflexes to proliferation by inhibiting the genes responsible for this apoptosis.

BAX inhibitor-1 has been shown to suppress apoptosis in mammals (Xu and Reed, 1998), and plants (Kawai *et al.*, 1999; Bolduc and Brisson, 2002). With regard to frog BI-1 expression, levels were elevated only after 6 hours of freezing exposure and then returned to control levels over the remainder of the freeze/thaw time course. After 6 hours of freezing, vital signs (breathing, heart beat) have halted and a high percentage of body water is sequestered into ice so that internal organs are undergoing dehydration. Anti-apoptotic activity at this time may help liver cells to endure stresses that might under other circumstances trigger programmed cell death.

During freeze-related stresses, the levels of AP-2α dropped significantly from control levels and remained low even during recovery. This expression pattern was also seen for BI-1 under freeze-related stresses. This suggests that the signal that stimulates BI-1 and AP-2α production during freezing is neither the progressive ischemia nor the dehydration that accompanies freezing. Furthermore, since *R. sylvatica* can readily survive the anoxia and dehydration stresses to which they were exposed, these stresses either do not trigger apoptosis in wood frogs or an alternative to the these two modes of suppression of apoptosis must exist that responds to these stresses.

In response to a death signal BAX proteins translocate from the cytosol to the mitochondrial membranes, dimerize and permit cytochrome *c* release thereby promoting apoptosis (Gross *et al.*, 1998). BAX forms dimers at contact sites between the outer membrane and inner membrane of mitochondria to allow release of cytochrome *c* (Capano and Crompton, 2002). The translocation seems to target BAX to the voltage dependent anion channel (VDAC) and the ATP/ADP translocase (AAT) in mitochondria. Interestingly, AAT transcript and protein levels have been shown by our lab to increase

during freezing in wood frogs (Cai *et al.*, 1997). An increase in AAT in mitochondria has been shown to increase the translocation of BAX to the mitochondria and thus promote apoptosis (Bauer *et al.*, 1999). The increase in BI-1 protein may be the result of increased BAX translocation to the mitochondrial membrane bought on by increased AAT.

The exact mechanism by which BI-1 prevents dimerization of BAX from occurring is not fully known. It has been shown that BI-1 interacts with Bcl-2 and Bcl-X_l but not BAX or BAK as determined by coimmunoprecipitation studies (Xu and Reed, 1998). As well, BI-1 is associated primarily with intracellular membranes like the endoplasmic reticulum (ER) and nuclear envelope and only minimally found in mitochondrial membranes (Xu and Reed, 1998). Given the above observations of BI-1 anti-apoptotic activity, the inhibition imposed by BI-1 on BAX is indirect. Xu and Reed (1998) showed that BI-1 has several transmembrane sequences suggesting a possible role for BI-1 as a receptor or an ion channel. It has been shown that release of Ca^{2+} from ER is a precursor for apoptosis and has been shown to increase mitochondrial permeability and pore formation (Bernardi *et al.*, 1994). BI-1 binding to Bcl-2 (which is found in ER) may control the release of Ca^{2+} and thus prevent BAX induced apoptosis.

The patterns of p53 expression in wood frog liver in response to stress were also quite intriguing. p53 is a transcription factor like AP-2α and exists in the cell bound to an inhibitor protein called Mdm2 that marks p53 for ubiquitination and subsequent degradation (for a review see Oren, 2003). However, upon activation of p53, there is a marked increase in its abundance and Mdm2 is unable to adequately inhibit p53. Thus, p53 is able to bind to its response element in the promoter regions of various genes and promote that genes transcription. At least twelve phosphorylation and four acetylation

sites are known to play a role in p53 functionality (Kohn 1999; Apella and Anderson, 2001). These potential phosphorylation sites of p53 can be independently phosphorylated resulting in differential binding affinities of p53 to its response element or to its inhibitor protein Mdm2 (Hecker *et al.*, 1996). In the present study I was able to test the stress responsiveness of six different phosphorylation sites on p53.

Depending on which p53 residues are phosphorylated, p53 can bind to consensus sequences on different gene promoter regions, resulting in differential gene upregulation (Hecker *et al.*, 1996). The protein also possesses two recognition sites: one that can bind to sequence specific DNA and another site near the C-terminus that recognizes damaged DNA (Hecker *et al.*, 1996). With at least twelve sites of phosphorylation in the mammalian form of p53, a huge range of responses and binding affinities of p53 are possible depending on which residues are phosphorylated. The Ser392 residue (found in the C-terminal) was phosphorylated during thawing in wood frog liver, but not during freezing. Of the six antibodies to p53 phosphopeptides tested, this was the only site that cross-reacted with the frog protein which suggests either (a) that there is considerable amino acid sequence variation around the various phosphorylation sites in the frog protein, as compared to mammalian p53, or (b) that frog p53 lacks some of the phosphorylation sites found in the mammalian protein. However, overall sequence variation between frog and mammalian p53 cannot be that great because the mammalian antibody detecting total p53 functioned well with the frog extracts. Mutations in p53 are commonly seen in cancers, particularly in the central codons 120-290, which make up the sequence specific DNA binding domain of p53 (North and Hainut, 2000). Ullrich *et al.*, (1993) found that p53 mutant molecules, isolated from human cancers, having a single

AA change at Ile^{237} and Ala^{143} had increased phosphorylation at the Ser392 residue. The authors did not mention if this mutant p53 molecule was deficient in recognizing and responding to damaged DNA. Sequencing the *R. sylvatica* form of p53 would be the next step in determining if there are structural differences that can account for the observed p53 changes.

During the thawing process the heart is one of the first organs to thaw, establishing an early resumption of heartbeat (see Fig 1.2). This serves to establish an early blood flow that will deliver oxygen to cells that have been oxygen deprived throughout the freeze. The reintroduction of oxygen to organs that have been under long term ischemia typically results in reperfusion damage caused by the production of a burst of oxygen free radicals. If damage to macromolecules by free radicals is extensive, apoptosis can be triggered. Since there may be oxidative damage bought on during thawing, then there must be control mechanisms to ensure that cells do not undergo apoptosis as a result of oxidative stress. This warrants further study as to how p53 is involved in prevention of apoptosis in thawing wood frogs.

Integrating what has been learned so far in freezing frogs with respect to apoptosis is difficult at this point given the infancy of this field. However, the proteins that I have looked at so far are intimately involved with each other.

Bcl-2 is important to tumour progression by promoting cell survival (Hockenberry *et* al., 1990). The retinoblastoma (RB) gene activates the transcription of *bcl-2* through AP-2α (Decary *et al.*, 2002). The RB gene product has been implicated in numerous cancers through its anti-apoptotic activity. RB interacts with AP-2α directly and binds in the promoter region of the cell survival gene *bcl-2* (Decary *et al.*, 2002). However, this

interaction promotes cell survival and differentiation, but not proliferation. During thawing, there may be regeneration of damaged cells, but an over-proliferation may be prevented by the huge increase in AP-2 seen during thaw. In fact, AP-2α has been shown to increase when p53 was stimulated to increase (McPherson *et al.*, 2002). Given the role of p53 in preventing cell over-proliferation, this is not surprising. As well, an abundance of p53 can drive a cell towards apoptosis so perhaps the mode of controlling proliferation while preventing apoptosis is through a big increase in AP-2α and a modest increase in p53.

p53 can also bind to the promoter region of BAX and promote transcription. However studies have not looked at the phosphorylation state of p53 during its activation of BAX (Basu and Haldar, 1998). In fact most studies of p53 have not adequately measured the phosphorylation state during activation of a gene. The ability of any transcription factor to bind can be regulated and so knowing the status of phosphorylation can affect future interpretation of data. Studies by Pise-Masison *et al.* (2000) suggest that phosphorylation at the Serine 392 residue can prevent p53 activation and transcription. Interestingly, their study also pointed out that this p53 inactivation depended on NFκB activity, further linking findings from Chapter 4 to what was seen for p53-Ser392 phosphorylation in wood frogs (Fig. 5.7).

Another interesting finding is the role of Akt in cell survival. As shown in Chapter 4, there is an increase in phosphorylation of Akt in *R. sylvatica* liver after 24 hours of freezing and 24 hours of anoxia exposure. Phosphatidylinositol 3-OH kinase phosphorylates Akt and this keeps BAX in the cytosol preventing mitochondrial pore formation and subsequent apoptosis through cytochrome *c* release (Tsurata *et al.*, 2002).

How pAkt prevents BAX translocation is unknown. As well, Akt influences cell survival through regulation of p53. Akt phosphorylates Mdm2, which promotes Mdm2 mediated ubiquitination and subsequent degradation of p53 (Ogawara *et al.*, 2002). During anoxia in *R. sylvatica*, phospho-Akt content increased significantly (Chapter 4, Figure 4.8), which may account for the decrease in p53 levels seen during anoxia (Fig. 5.8). The situation in freezing is a bit more complex. There was no significant change in total p53 levels over the freeze-thaw time course so Akt may not have as much of an influence on p53 levels during freeze-thaw. However, as reviewed by Kohn (1999), there are many different signals that influence p53 so other pathways may supersede the Akt influence on p53 during freeze-thaw.

This chapter presents a brief introduction and lays the groundwork for future research and directions into the study of apoptosis in wood frogs. The wood frog is an underutilized resource in the study of apoptosis and could be further used as a model system in apoptotic studies. Analysis of frog anti-apoptotic activity would provide an alternate strategy in the study of how cells can survive major stresses that are lethal to non-tolerant cells. As well, this model system can be applied to the field of organ cryopreservation. A major problem with achieving freezing survival of mammalian organs is that whereas you might achieve physical preservation of the cells, the stresses involved may trigger apoptotic pathways that then irreversibly limit the functional recovery of the organ after freezing.

CHAPTER SIX

General Discussion and Conclusions

My objective for this thesis was to look at the control of gene and protein levels in the wood frog, *R. sylvatica*. Survival of freezing constitutes a unique ability that occurs in only a few vertebrates. Extremophiles such as *R. sylvatica* represent an underutilized resource in the exploration of mechanisms of stress tolerance. The cellular and biochemical changes that *R. sylvatica* use to survive freezing are not only of great interest for our understanding of this amazing natural phenomenon but can provide new insights into our general understanding of multiple biochemical and physiological problems and suggest new approaches for applied goals such as organ cryopreservation.

To this end I undertook the research seen in this thesis. Analysis of a mitochondrial phosphate carrier, PiC, showed that this particular gene was strongly up-regulated in the frog during freezing. Further analysis looked at the activity of transcription factors associated with cell survival during freezing and related stresses. This led finally to an investigation into anti-apoptotic activity in the wood frog during freeze-thaw.

6.1 Summary and Implications of Findings

Tolerance of freezing is a unique adaptation that allows *R. sylvatica* to survive over the winter. Techniques like northern, RT-PCR, and westerns used in this thesis allow us to quantify the changes in transcript and protein levels that occur in response to stress. Upregulation of genes during a stress such as freezing is quite remarkable, as you would expect a global suppression of transcription and translation in order to conserve energy in ischemic and dehydrated cells. Indeed, a general suppression of the synthesis of most proteins does occur as it has been shown in *R. sylvatica* that freezing stimulates a

strong increase in the phosphorylation of the eukaryotic initiation factor 2 alpha (eIF2α), one of the key proteins involved in translation control (McNally, 2002). When eIF2α is phosphorylated there is an overall decrease in protein translation (Rhoads, 1993). Yet despite this general decrease in translation capacity, selected proteins are actively translated during freeing and related stresses in wood frogs.

Screening of a cDNA library identified the mitochondrial phosphate carrier, PiC, as one of a few selected genes that was strongly upregulated during freezing in *R. sylvatica* liver. Further analysis showed that PiC was also significantly upregulated in response to dehydration stress but not under anoxia exposure. A related carrier protein, AAT, was previously shown to respond positively during freezing (Cai et al., 1997), but two other carriers of the same family, DIC and OGC, were not altered during freezing. This suggests a specific function for the PiC and AAT carriers in dealing with the stresses imposed on mitochondria during cell freezing. Increased translation of PiC during freezing was supported by the redistribution of a high proportion of PiC transcripts into the polysome fractions in frozen frogs, despite the overall shift of polysomes into monosomes that indicated a general suppression of translation. Despite previous studies showing a decrease in levels of mitochondrially-encoded transcripts in frozen frogs (Castellarin, 2000), southern blot analysis revealed that mtDNA levels remained constant during freezing indicating no change in mitochondria numbers. Taken together, this data indicated that an overall increase in PiC content per mitochondrion occurred during freezing.

Control of gene expression, and therefore of protein synthesis, is dependent on signals transmitted via the actions of protein kinases and phosphatases and by a host of

transcription factors and co-activator/co-repressor proteins. The present data showed that a temporal activation of multiple transcription factors occurs in response to freezing and related stresses. The use of DNA binding assays corroborated these findings to show increased DNA binding capacity by both pCREB and NFκB, and hence increased capacity for gene up-regulation, during freezing. The transcription factors investigated are all involved in various aspects of cell survival. The results of the Chapters 2 and 3, along with the findings about transcription factors in Chapter 4, point to an effort by the cell to regulate or control itself during freezing, thus allowing for cellular survival.

As a result of this activation of transcription factors involved in cell survival the possibility of anti-apoptotic activity in liver presented itself. In fact, a screen of a cDNA microarray revealed that a transcription factor involved in prevention of apoptosis (AP-2α) was upregulated in wood frog liver after 24 hours of freezing. Western blot analysis of key proteins involved in anti-apoptotic activity showed that AP-2α, BI-1 and p53 proteins are regulated in wood frogs, apparently to promote cell survival during freezing and related stresses.

6.2 Future Studies and New Directions

After translation PiC is transported to the mitochondria and is inserted into the mitochondrial inner membrane (Truscott and Pfanner, 1999). This insertion process can be stalled or prevented and this may control PiC functionality in mitochondria. Hence, the increase in PiC protein during freezing that was documented by my studies may not necessarily represent an increase in the functional product present in the inner mitochondrial membrane. Immunolocalization studies using *R. sylvatica* liver slices

would allow analysis of the actual subcellular localization of PiC and provide further clues into the functionality of PiC during stress. For example, Trippett *et al.* (2001) using a combination of immunhistochemistry and immunogold labelling were able to localize a human reduced folate carrier protein to predominantly mitochondrial membranes. Assessment of time-dependent changes in PiC incorporation into the mitochondrial membrane would also be important in determining the reason why PiC content is supplemented. Peak levels of PiC at 24 h frozen do not necessarily mean peak functionality of the protein at that time. Multiple studies in our lab have indicated that certain stress-induced proteins are upregulated in an anticipatory manner to serve a function that occurs not during stress but during the recovery period after stress.

The transcription factors CREB and NFκB are both involved in cell survival. These factors were both activated during early freezing suggesting the possibility of transcription of genes necessary for cell survival. The promoter regions of the *R. sylvatica* genes known to be upregulated during freezing have not yet been studied and represent an interesting area for future research. By identifying the response elements present in freeze responsive genes it will be possible to suggest which signal transduction pathways mediate the control of these genes. It is possible that a common "freeze responsive element" may be found in freeze responsive genes that provides for coordinated upregulation of multiple genes. A cold-responsive element is known to coordinate the expression of multiple genes in freeze tolerant plants (e.g. *Arabidopsis*) during cold-hardening. In particular, promoter analysis of several novel genes (*Li16*, *fr10*, *fr47*) that are freeze-responsive in wood frogs could not only identify the possible

transcription factors and signal transduction pathway involved in their control but also potentially identify the area of cell functionality that the encoded protein serves.

Studying the transcription of a particular gene is usually done by several methods. Typically this involves an initial sequence of the promoter area of a particular gene. One method that can be used to isolate the promoter region is a relatively new method called thermal asymmetric interlaced PCR or TAIL-PCR (Nakayama *et al.,* 2001). This process uses nested primers that are specific to the sequence together with shorter degenerate primers with a lower melting temperature. Three successive rounds of PCR are performed using a sequence specific nested primer and the same set of degenerate primers. PCR is performed in alternating cycles with different annealing temperatures so that a specific or a degenerate primer will bind. This method favours the amplification of target molecules over non-specific molecules as high and low stringency cycles are alternated (Nakayama *et al.,* 2001). The resultant PCR product should be enriched for target molecules that can be sequenced and analysed. There are other methods that can be used to isolate the promoter sequence as well such as screening a genomic library but after the clone containing the sequence is obtained, then methods like TAIL-PCR have to be used to obtain the full sequence.

Once the sequence is known then the region can be analysed for the presence of a consensus sequence where transcription factors can potentially bind. Various websites offer search engines that assess sequences for potential transcription factor binding sites. This then suggests which transcription factors are involved in the control of a particular gene and that information, in turn, suggests the possible signal transduction pathways that may regulate gene expression. However, to fully assess the functionality of a promoter

region, deletion studies must be done. This process involves some type of reporter gene activity that indicates transcription of the gene. Sections of the promoter region are deleted or mutated to show which section is essential for gene transcription. A recent study by Palmer *et al.* (2001) looked at the promoter region of a human and a mouse phosphate transporter. Using a luciferase gene assay, the phosphate transporter promoter region of each species was assessed by inserting a fragment of the promoter region into a luciferase expression vector. Palmieri *et al.* (2001) found that despite similar structure and binding capabilities, of the promoter region of human and mouse phosphate transporter that transcription was influenced by different transcription factors. This study also points out the importance of getting a wood frog specific sequence for the genes of interest and subsequent analysis of the promoter region.

Most studies concerning apoptosis investigate the central role p53 plays in regulation. However, these studies often do not address the activation state of p53 and instead focus on total p53 levels. p53 is subject to regulation by phosphorylation and acetylation (Kohn 1999) and thus studies of p53 need to take into account the activation state of p53. To determine where potential sites could be would involve sequence isolation and analysis. This process would involve primer design based on multiple alignments of known sequences p53 sequences. Then a suitable product would be made by RT-PCR using the designed primers. After this product is amplified and sequenced, then the 3' and 5' regions would have to isolated and sequenced in order to obtain a full-length p53 sequence. This technique is called rapid amplification of cDNA ends (RACE)-PCR. This technique is similar to TAIL-PCR but it is performed on a cDNA. RACE-PCR is slightly different depending on whether the 3' or 5' region of cDNA needs to be

sequenced. In 5'-RACE-PCR, an arbitrary primer that binds in the 5' region is used along with a gene specific primer to amplify a sequence in the unknown 5' region of the cDNA. After the sequence is obtained a new gene specific primer may need to be designed if the 5' end is not completed yet. By proceeding in this fashion you "walk" along the sequence towards the 5' end to get a complete sequence. To isolate and sequence the 3' end requires a primer to the poly-A tail and a gene specific primer to amplify the unknown 3' end. Once these sequences are obtained the full p53 sequence can be assembled using the sequenced fragments. At this point sequence analysis would reveal potential phosphorylation sites to which peptide antibodies could be designed. This would allow us to further elucidate the control that p53 dictates during freezing stress in *R. sylvatica*.

6.2 Final Thoughts

Studies into freeze tolerance in *R. sylvatica* have typically been concerned with cryopreservation and diabetes research. However, the results of my studies suggest that the ability to allow internal organs to freeze and thaw must also take into account the suppression of apoptosis. Cells need to suppress the urge to self-destruct under the extreme stresses imposed by freezing (long term ischemia, extreme cell volume reduction, large changes in osmolality and ionic strength). The mechanisms utilized by the frog to prevent apoptosis will provide us with further strategies for enhancing cryopreservation of cells and organs.

Further studies into the nature of apoptosis suppression in wood frogs will undoubtedly add to the work started here. Can the suppression of apoptosis seen in the wood frog be used in cryopreservation or perhaps even combating cancer? The BI-1

protein has been shown to protect yeast cells from apoptosis (Kawai *et al.*, 1999) and evidence from my research suggests that BI-1 may play a role in the prevention of apoptosis in freezing wood frogs. The large increase in levels of the AP-2α transcription factor is also intriguing and studies into its functional significance will help develop further strategies.

In summary, my thesis looked at freeze-tolerance from a gene and protein regulation standpoint. The studies undertaken herein show that there is a concerted change in gene and protein expression in wood frogs in response to freezing. These changes indicate that frog organs are working to overcome the stresses imposed upon them during freezing. Model animal systems allow us to approach problems from different angles. Suppression of apoptosis has not been investigated in wood frogs before so this is a new field of research with a lot of potential. As well, the use of freeze-tolerant frogs as a model system allows us to approach the study of cancer, cryopreservation and diabetes from a unique perspective.

CHAPTER SIX

General Discussion and Conclusions

My objective for this thesis was to look at the control of gene and protein levels in the wood frog, *R. sylvatica*. Survival of freezing constitutes a unique ability that occurs in only a few vertebrates. Extremophiles such as *R. sylvatica* represent an underutilized resource in the exploration of mechanisms of stress tolerance. The cellular and biochemical changes that *R. sylvatica* use to survive freezing are not only of great interest for our understanding of this amazing natural phenomenon but can provide new insights into our general understanding of multiple biochemical and physiological problems and suggest new approaches for applied goals such as organ cryopreservation.

To this end I undertook the research seen in this thesis. Analysis of a mitochondrial phosphate carrier, PiC, showed that this particular gene was strongly up-regulated in the frog during freezing. Further analysis looked at the activity of transcription factors associated with cell survival during freezing and related stresses. This led finally to an investigation into anti-apoptotic activity in the wood frog during freeze-thaw.

6.1 Summary and Implications of Findings

Tolerance of freezing is a unique adaptation that allows *R. sylvatica* to survive over the winter. Techniques like northern, RT-PCR, and westerns used in this thesis allow us to quantify the changes in transcript and protein levels that occur in response to stress. Upregulation of genes during a stress such as freezing is quite remarkable, as you would expect a global suppression of transcription and translation in order to conserve energy in ischemic and dehydrated cells. Indeed, a general suppression of the synthesis of most proteins does occur as it has been shown in *R. sylvatica* that freezing stimulates a

strong increase in the phosphorylation of the eukaryotic initiation factor 2 alpha (eIF2α), one of the key proteins involved in translation control (McNally, 2002). When eIF2α is phosphorylated there is an overall decrease in protein translation (Rhoads, 1993). Yet despite this general decrease in translation capacity, selected proteins are actively translated during freeing and related stresses in wood frogs.

Screening of a cDNA library identified the mitochondrial phosphate carrier, PiC, as one of a few selected genes that was strongly upregulated during freezing in *R. sylvatica* liver. Further analysis showed that PiC was also significantly upregulated in response to dehydration stress but not under anoxia exposure. A related carrier protein, AAT, was previously shown to respond positively during freezing (Cai et al., 1997), but two other carriers of the same family, DIC and OGC, were not altered during freezing. This suggests a specific function for the PiC and AAT carriers in dealing with the stresses imposed on mitochondria during cell freezing. Increased translation of PiC during freezing was supported by the redistribution of a high proportion of PiC transcripts into the polysome fractions in frozen frogs, despite the overall shift of polysomes into monosomes that indicated a general suppression of translation. Despite previous studies showing a decrease in levels of mitochondrially-encoded transcripts in frozen frogs (Castellarin, 2000), southern blot analysis revealed that mtDNA levels remained constant during freezing indicating no change in mitochondria numbers. Taken together, this data indicated that an overall increase in PiC content per mitochondrion occurred during freezing.

Control of gene expression, and therefore of protein synthesis, is dependent on signals transmitted via the actions of protein kinases and phosphatases and by a host of

transcription factors and co-activator/co-repressor proteins. The present data showed that a temporal activation of multiple transcription factors occurs in response to freezing and related stresses. The use of DNA binding assays corroborated these findings to show increased DNA binding capacity by both pCREB and NFκB, and hence increased capacity for gene up-regulation, during freezing. The transcription factors investigated are all involved in various aspects of cell survival. The results of the Chapters 2 and 3, along with the findings about transcription factors in Chapter 4, point to an effort by the cell to regulate or control itself during freezing, thus allowing for cellular survival.

As a result of this activation of transcription factors involved in cell survival the possibility of anti-apoptotic activity in liver presented itself. In fact, a screen of a cDNA microarray revealed that a transcription factor involved in prevention of apoptosis (AP-2α) was upregulated in wood frog liver after 24 hours of freezing. Western blot analysis of key proteins involved in anti-apoptotic activity showed that AP-2α, BI-1 and p53 proteins are regulated in wood frogs, apparently to promote cell survival during freezing and related stresses.

6.2 Future Studies and New Directions

After translation PiC is transported to the mitochondria and is inserted into the mitochondrial inner membrane (Truscott and Pfanner, 1999). This insertion process can be stalled or prevented and this may control PiC functionality in mitochondria. Hence, the increase in PiC protein during freezing that was documented by my studies may not necessarily represent an increase in the functional product present in the inner mitochondrial membrane. Immunolocalization studies using *R. sylvatica* liver slices

would allow analysis of the actual subcellular localization of PiC and provide further clues into the functionality of PiC during stress. For example, Trippett *et al.* (2001) using a combination of immunhistochemistry and immunogold labelling were able to localize a human reduced folate carrier protein to predominantly mitochondrial membranes. Assessment of time-dependent changes in PiC incorporation into the mitochondrial membrane would also be important in determining the reason why PiC content is supplemented. Peak levels of PiC at 24 h frozen do not necessarily mean peak functionality of the protein at that time. Multiple studies in our lab have indicated that certain stress-induced proteins are upregulated in an anticipatory manner to serve a function that occurs not during stress but during the recovery period after stress.

The transcription factors CREB and NFκB are both involved in cell survival. These factors were both activated during early freezing suggesting the possibility of transcription of genes necessary for cell survival. The promoter regions of the *R. sylvatica* genes known to be upregulated during freezing have not yet been studied and represent an interesting area for future research. By identifying the response elements present in freeze responsive genes it will be possible to suggest which signal transduction pathways mediate the control of these genes. It is possible that a common "freeze responsive element" may be found in freeze responsive genes that provides for coordinated upregulation of multiple genes. A cold-responsive element is known to coordinate the expression of multiple genes in freeze tolerant plants (e.g. *Arabidopsis*) during cold-hardening. In particular, promoter analysis of several novel genes (*Li16*, *fr10*, *fr47*) that are freeze-responsive in wood frogs could not only identify the possible

transcription factors and signal transduction pathway involved in their control but also potentially identify the area of cell functionality that the encoded protein serves.

Studying the transcription of a particular gene is usually done by several methods. Typically this involves an initial sequence of the promoter area of a particular gene. One method that can be used to isolate the promoter region is a relatively new method called thermal asymmetric interlaced PCR or TAIL-PCR (Nakayama *et al.,* 2001). This process uses nested primers that are specific to the sequence together with shorter degenerate primers with a lower melting temperature. Three successive rounds of PCR are performed using a sequence specific nested primer and the same set of degenerate primers. PCR is performed in alternating cycles with different annealing temperatures so that a specific or a degenerate primer will bind. This method favours the amplification of target molecules over non-specific molecules as high and low stringency cycles are alternated (Nakayama *et al.,* 2001). The resultant PCR product should be enriched for target molecules that can be sequenced and analysed. There are other methods that can be used to isolate the promoter sequence as well such as screening a genomic library but after the clone containing the sequence is obtained, then methods like TAIL-PCR have to be used to obtain the full sequence.

Once the sequence is known then the region can be analysed for the presence of a consensus sequence where transcription factors can potentially bind. Various websites offer search engines that assess sequences for potential transcription factor binding sites. This then suggests which transcription factors are involved in the control of a particular gene and that information, in turn, suggests the possible signal transduction pathways that may regulate gene expression. However, to fully assess the functionality of a promoter

region, deletion studies must be done. This process involves some type of reporter gene activity that indicates transcription of the gene. Sections of the promoter region are deleted or mutated to show which section is essential for gene transcription. A recent study by Palmer *et al.* (2001) looked at the promoter region of a human and a mouse phosphate transporter. Using a luciferase gene assay, the phosphate transporter promoter region of each species was assessed by inserting a fragment of the promoter region into a luciferase expression vector. Palmieri *et al.* (2001) found that despite similar structure and binding capabilities, of the promoter region of human and mouse phosphate transporter that transcription was influenced by different transcription factors. This study also points out the importance of getting a wood frog specific sequence for the genes of interest and subsequent analysis of the promoter region.

Most studies concerning apoptosis investigate the central role p53 plays in regulation. However, these studies often do not address the activation state of p53 and instead focus on total p53 levels. p53 is subject to regulation by phosphorylation and acetylation (Kohn 1999) and thus studies of p53 need to take into account the activation state of p53. To determine where potential sites could be would involve sequence isolation and analysis. This process would involve primer design based on multiple alignments of known sequences p53 sequences. Then a suitable product would be made by RT-PCR using the designed primers. After this product is amplified and sequenced, then the 3' and 5' regions would have to isolated and sequenced in order to obtain a full-length p53 sequence. This technique is called rapid amplification of cDNA ends (RACE)-PCR. This technique is similar to TAIL-PCR but it is performed on a cDNA. RACE-PCR is slightly different depending on whether the 3' or 5' region of cDNA needs to be

sequenced. In 5'-RACE-PCR, an arbitrary primer that binds in the 5' region is used along with a gene specific primer to amplify a sequence in the unknown 5' region of the cDNA. After the sequence is obtained a new gene specific primer may need to be designed if the 5' end is not completed yet. By proceeding in this fashion you "walk" along the sequence towards the 5' end to get a complete sequence. To isolate and sequence the 3' end requires a primer to the poly-A tail and a gene specific primer to amplify the unknown 3' end. Once these sequences are obtained the full p53 sequence can be assembled using the sequenced fragments. At this point sequence analysis would reveal potential phosphorylation sites to which peptide antibodies could be designed. This would allow us to further elucidate the control that p53 dictates during freezing stress in *R. sylvatica*.

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Appendix A – List of putative genes upregulated (2-fold or greater) in *Rana sylvatica* liver after 24 hours of freezing found through microarray analysis.

Accession Number	Putative Upregulated Gene	Fold Increase
R91244	ESTs	4.573605
T99769	ESTs	3.974647
T91423	ESTs	3.448962
N73749	ESTs	2.92367
H27520	ESTs	2.884538
AA101827	ESTs	2.850964
AA126254	ESTs	2.732669
H89874	ESTs	2.447194
T93598	ESTs	2.250891
R98773	ESTs	2.240307
N21336	ESTs	2.144251
N71428	ESTs	2.142056
T85475	ESTs	2.121558
N81000	ESTs	2.109623
AA136838	ESTs	2.074682
R06464	ESTs	2.058687
N71402	ESTs	2.048991
R92834	ESTs	2.028778
AA156853	ESTs, Weakly similar to AF187016_1 myosin regulatory light chain interacting protein MIR [H.sapiens]	2.017758
H82982	zinc finger protein 275	3.413693
T79928	zinc finger protein	2.386299
AA028911	ring finger protein 23	2.051438
N70593	ribosomal protein L10	3.341138
AA039258	ribosomal protein L31	2.868619
H45241	ribosomal protein L41	2.764906
T62916	ribosomal protein S29	2.658339
W79689	ribosomal protein L15	2.594292
T48772	ribosomal protein L12	2.463298
R05957	ribosomal protein L31	2.383681
N95530	ribosomal protein L32	2.375142
N93499	ribosomal protein L8	2.31071
R72766	ribosomal protein S15	2.052992
T93526	KIAA0630 protein	4.708779
R91263	KIAA0205 gene product	3.241706
H65910	KIAA0381 protein	2.812022
AA151290	DKFZP566B1346 protein	2.717148
N48174	KIAA0332 protein	2.420146

AA044097	KIAA0569 gene product	2.301085
T79362	KIAA0220 protein	2.254701
N74019	KIAA0265 protein	2.00179
AA032148	cytochrome c	4.329176
AA131465	signal transduction protein (SH3 containing)	3.551071
AA054151	oxidase (cytochrome c) assembly 1-like	2.530843
N63770	transcription factor AP-2 alpha (activating enhancer-binding protein 2 alpha)	2.304676
AA210782	signal transducer and activator of transcription 6, interleukin-4 induced	2.280596
N50688	heat shock protein hsp70-related protein	2.111512
N33439	eukaryotic translation elongation factor 2	2.07318
N74225	Homo sapiens clone 25061 mRNA sequence	2.702335
N42943	Homo sapiens cDNA FLJ22479 fis, clone HRC10831	2.168205
AA033838	Homo sapiens cDNA FLJ11588 fis, clone HEMBA1003729	2.127007
N42184	Homo sapiens clone 24606 mRNA sequence	2.066032
H69538	Homo sapiens clone 23860 mRNA sequence	2.046624
N89592	tubulin, beta, 2	2.214128
H27908	tubulin, beta, 4	2.119385
W96066	actin, alpha, cardiac muscle	2.039388
W46370	hypothetical protein, similar to (U06944) PRAJA1	2.792694
W93580	hypothetical protein	2.691702
H11052	kinesin family member 3C	4.768112
R17436	trophinin	3.425335
AA031503	CGI-20 protein	2.855033
AA136271	CD58 antigen, (lymphocyte function-associated antigen 3)	2.829311
N95177	replication factor C (activator 1) 1 (145kD)	2.443199
W52798	emerin (Emery-Dreifuss muscular dystrophy)	2.421115
W04832	hemoglobin, alpha 2	2.368725
W92270	Fc fragment of IgG, low affinity IIa, receptor for (CD32)	2.336786
AA045829	tumor-associated calcium signal transducer 2	2.23694
AA010191	heterogeneous nuclear ribonucleoprotein A/B	2.14203
R05288	acid phosphatase 5, tartrate resistant	2.137902
W40511	protease, serine, 1 (trypsin 1)	2.125899
W95228	cathepsin G	2.098014
AA125865	lymphotoxin beta receptor (TNFR superfamily, member 3	2.095852
N91541	non-metastatic cells 1, protein (NM23A) expressed in	2.08839
AA053984	H2B histone family, member Q	2.051158
AA114919	nuclease sensitive element binding protein 1	2.015647
AA047406	translocase of outer mitochondrial membrane 70 (yeast) homolog A	1.99887

N/A1	Spot 8395	20.7433
N36834		4.39355
N70895		2.716729
AA136161		2.484607
Spot #14383		2.464477
AA131800		2.409351
N30952		2.406092
R50162		2.364524
N31736		2.354483
AA156605		2.15784
N66253		2.092561
R92719		2.038046
R16392		2.020689
AA210886		2.019907
R98516		2.009598
H67193		2.009443
H18697		1.996363