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**DIFFERENTIAL GENE EXPRESSION UNDER ENVIRONMENTAL STRESS
IN THE FREEZE TOLERANT WOOD FROG, *RANA SYLVATICA***

By

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of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Freeze survival of wood frog, *Rana sylvatica*, involves adaptations including control over extracellular ice formation, production of glucose cryoprotectant, and resistance to freezing-caused intracellular dehydration and ischemia. Gene expression associated with stress survival was investigated in this freeze tolerant species. Freeze-inducible genes were found by differential screening of frog brain and liver cDNA libraries; these included mitochondrial genes [encoding ATPase subunit 6 & 8, 16S rRNA and NADH-ubiquinone oxidoreductase subunit 4 (ND4)], the phosphoglycerate kinase 1 (PGK1) gene, and genes whose products are involved in translational processes [acidic ribosomal phosphoprotein (P0) and elongation factor 1 gamma subunit (EF-1 γ)]. Another ribosome-associated gene, encoding ribosomal protein L7 (RPL7), was identified in skin via differential display of polymerase chain reaction (DD-PCR). This gene was up-regulated in skin of cold-acclimated frogs and brain of freeze-exposed frogs. Freezing stimulated the upregulation of the above genes in selected frog organs. Tissue-specific gene expression also occurred in frog brain and liver in response to anoxia or dehydration. Anoxia stimulated *P0*, *PGK1*, *RPL7*, 16S rRNA and ATPase 6 & 8 gene expression and modulated *ND4* and *EF-1 γ* expression, whereas dehydration enhanced the expression of genes such as *PGK1*. Upregulation of genes whose products are directly involved in energy generation (PGK1, ATPase subunit 6 & 8, ND4) and whose products are related to protein biosynthesis suggested that maintenance of minimal ATP levels and functional translation machinery may be critical for freezing survival. Freezing-induced ischemia may be a primary signal that triggers the upregulation of most of the isolated genes. However, low temperature seemed to play a role in the expression of ribosome-

associated protein genes, whereas freezing-related cell water stress may also regulate other selected genes (e.g. *PGK1*). Immunoblotting confirmed that elevated *PGK1* transcripts resulted in increased enzyme protein and showed the potential physiological significance of up-regulated genes in response to the stress. Immunoblotting also showed the elevation of Ca^{2+} /calmodulin-dependent protein kinase and phosphatase under freezing, anoxia and/or dehydration stress which suggests that a Ca^{2+} signaling pathway plays a role in stress-mediated gene expression. These cellular responses may play an important role in survival of environmental stress.

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LIST OF ABBREVIATIONS

Ab - antibody
AMP, ADP, ATP - adenosine mono-, di- or triphosphate
AP-1 - activator protein-1
ATPase - adenosine triphosphatase
BSA - bovine serum albumin
CaM - calmodulin
CaMK - Ca²⁺/calmodulin dependent protein kinase
cAMP - cyclic 3',5'-adenosine monophosphate
CBP - CREB binding protein
cDNA - complementary DNA
cGMP - cyclic 3',5'-guanosine monophosphate
COI, II, III (or COX1, 2, 3) - cytochrome *c* oxidase
cpm - counts per minute
CRE - cAMP-response element
CREB - cAMP-response element binding protein
Csp - cold-shock protein
cyt *b* - cytochrome *b*
DAG - diacylglycerol
DD-PCR - differential display by polymerase chain reaction
DEPC - diethylpyrocarbonate
DMSO - dimethylsulfoxide
DNA - deoxyribonucleic acid
dNTP - deoxynucleoside-5'-triphosphate
DTT - dithiothreitol
ECL assay - enhanced chemiluminescence assay
EDTA - ethylenediamine tetraacetate
EF - elongation factor
EGTA - ethyleneglycol bis (β -aminoethyl ether) tetraacetate
ERK - extracellular signal-regulated kinase
EtBr - ethidium bromide
FP - freezing point
HIF - hypoxia-inducible factor
HLH - helix-loop-helix
HRP - horseradish peroxidase
HSE - heat-shock element
HSF - heat-shock factor
hsp - heat-shock protein
IF - initiation factor
IgG - immunoglobulin G
INP - ice-nucleating protein
IPTG - isopropylthiogalactoside
IP₃ - inositol-1,4,5-trisphosphate
JNK - c-Jun N-terminal kinase

kb - kilobase
kDa - kilodalton
MAPK - mitogen-activated protein kinase
MOPS - 3-[N-morpholine] propanesulfonic acid
mRNA - messenger RNA
mtDNA - mitochondrial DNA
NAD⁺, NADH - oxidized & reduced forms of nicotinamide adenine dinucleotide
ND - NADH-ubiquinone oxidoreductase
nt - nucleotide
OD - optical density
ORF - open reading frame
P0, P1, P2 (protein) - acidic ribosomal phosphoprotein P0, P1, or P2
PAGE - polyacrylamide gel electrophoresis
PBS - phosphate buffered saline
PCR - polymerase chain reaction
pfu - plaque-forming units
PGK - phosphoglycerate kinase
pI - isoelectric point
PI - phosphatidylinositol
PKA - cAMP-dependent protein kinase
PKC - protein kinase C (Ca²⁺ and phospholipid dependent protein kinase)
PLC - phospholipase C
PLD - phospholipase D
PMSF - phenylmethylsulfonyl fluoride
PVDF - polyvinylidene difluoride
Q₁₀ - effect of a 10°C change in temperature on reaction rate
RACE - rapid amplification of cDNA ends
RNA - ribonucleic acid
RNase - ribonuclease
ROS - reactive oxygen species
rRNA - ribosomal RNA
RPL7 - ribosomal protein L7
RT-PCR - reverse transcription and polymerase chain reaction
SCP- supercooling point
SDS - sodium dodecyl sulfate
snRNP - small nuclear ribonucleoprotein
Tris - Tris[hydroxymethyl]aminoethane
THP - thermal hysteresis protein
UTR - untranslated region
X-gal - 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER ONE
General Introduction

Natural Stress Survival

All living organisms possess the ability to respond to changes in environmental factors, such as temperature, pressure, salinity, and the availability of oxygen, water and nutrients. However, beyond certain limits of variation for these parameters, stress occurs. Extreme stress can result in severe injury or even death to organisms. Many species live in habitats where they must deal with wide variations in one or more environmental parameters on daily or seasonal time scales. For example, many organisms must deal with the seasonal cold of winter (e.g. terrestrial ectotherms, polar marine fish, hibernating mammals), others with intermittent oxygen deprivation (e.g. diving animals, intertidal invertebrates), and others with heat and desiccation (e.g. estivating snails and toads) (Cheng and DeVries, 1991; Davies and Hew, 1990; Duman *et al.*, 1991; Storey and Storey, 1988, 1990, 1992). Behavioral, physiological, and biochemical adaptations help organisms to endure stress.

Biochemical studies have shown that the adaptation of cellular metabolism to environmental stress can involve a number of different mechanisms including changes in metabolic rate, amounts or activities of enzymes and functional proteins, intracellular pH, metabolite and ion concentrations, and membrane composition. Metabolic rate depression, an escape into a hypometabolic or dormant state, is a common mechanism that allows many species to adapt into harsh environment conditions such as low oxygen tension, low temperature or low water availability (Hochachka and Guppy, 1987; Storey and Storey, 1990). By lowering metabolic rate, often to <10 % of normal and sometimes to a virtually ametabolic state, organisms can stretch their metabolic fuel reserves to last

for weeks, months or even years until environmental conditions favorable for renewed growth and development return.

Cellular metabolism is made up of hundreds of different biochemical pathways and functions that call upon thousands of different enzymes, and functional and structural proteins, as well as their substrates and cofactors and their interactions with numerous physical parameters (e.g. pH, temperature, ionic strength). For cells and organisms reacting to environmental stress, the metabolic adjustments can often be accomplished by regulating the action of selected enzymes and other proteins. These can be controlled by a wide variety of regulatory mechanisms, including fine controls such as the availability of enzyme substrates and co-factors, the actions of allosteric effectors, changes in physical parameters, covalent modification of proteins (e.g. protein phosphorylation or dephosphorylation), and coarse controls on the amounts of proteins through regulation of the rates of protein synthesis or degradation. Since gene expression plays a vital role in all aspects of cellular activities including the responses to internal signals and external environmental factors and since the level and types of cellular proteins are mainly controlled by the regulation of gene expression, it is clear that gene regulation must be a part of natural stress survival. Analysis of gene expression by modern techniques of molecular biology may not only help to understand the basis of biochemical adaptations under extreme environmental conditions, but may also help to identify the full range of stress-induced cellular adjustments that may not be detectable with classic biochemical and physiological methods (Storey, 1999). Studies of the role of gene expression in animal response to environmental stress are the focus of my research project.

Regulation of Gene Expression

In eukaryotes, transcription and translation take place in different cellular compartments. Transcription is the first step of gene expression and occurs in the nucleus where ribonucleic acid (RNA) precursor is produced from its DNA template by a DNA-dependent RNA polymerase. The messenger RNA (mRNA) is transcribed from its protein-coding gene by DNA-dependent RNA polymerase II (Pol II). The Pol II and its associated proteins (general transcription factors) assemble at a specific DNA sequence (TATA box of the core promoter) for transcription initiation (Roeder, 1996). The TATA box is typically located 25 to 30 nucleotides upstream from the transcriptional start site of the gene. Transcription initiation causes a local unwinding of the double-stranded DNA template and allows Pol II to move along the template to produce mRNA precursor (pre-mRNA) (Roeder, 1996; Uptain *et al.*, 1997). Almost all pre-mRNAs are “capped” with a modified guanosine residue (7-methylguanosine) shortly after beginning the transcription. The majority of eukaryotic mRNAs (and their nuclear precursors) have a structure called a poly(A) (polyadenylation) tract at their 3' ends, which is not encoded in the DNA. The poly(A) tail is added by a template-independent enzyme called poly(A) polymerase. The nascent mRNA precursor is cut by a specific endonuclease that creates the 3' terminus for polyadenylation. In this process, a hexanucleotide (usually 5' AAUAAA 3') located some 10-30 nucleotide upstream from the cut site is recognized as part of the signal essential for the cleavage (Wahle and Keller, 1992). Both the 5' end cap and 3' end poly(A) tail function to stabilize the transcript (Beelman and Parker, 1995). The mRNA precursor, which contains exons (coding sequence) and introns (non-coding sequence), is

then spliced by small nuclear ribonucleoproteins (snRNPs) to remove the introns and produce mRNA (Dreyfuss *et al.*, 1993; Sharp, 1994).

The mRNA is then transported into the cytoplasm and translated at the ribosome. In eukaryotes, ribosomes are composed of 40S and 60S subunits and each contains their ribosomal RNA (rRNA) and associated proteins. The 40S and 60S ribosomal subunits form a functional 80S unit which is the site for protein synthesis (the translation of the mRNA into protein). The mRNA is read in groups of three nucleotides called codons. There are 61 codons for amino acids, one start codon AUG (also the code for methionine) and three nonsense or stop codons (UAA, UAG and UGA). Translation begins at the first AUG codon. Each codon is recognized by a transfer RNA (tRNA) molecule with the complementary anticodon. The tRNA molecule carrying the corresponding amino acid (aminoacyl-tRNA) enters the ribosome at a position known as the A site (aminoacyl site). A peptide bond is then formed between the activated carboxyl group of the growing polypeptide at the P (peptidyl) site and the amino group of the aminoacyl-tRNA by peptidyl transferase. The uncharged tRNA is then released from the P site and the tRNA/peptide is translocated from the A to the P site by translocase, and the mRNA moves a distance of three nucleotides. Translation is terminated at a stop codon by a release factor which liberates the ribosome from the mRNA. Many ribosomes may bind the mRNA to produce multiple copies of the protein before it is degraded. The rate of mRNA turnover can vary from minutes to hours (Wingender, 1993). The synthesized nascent polypeptide can then undergo further processing by post-translational modifications, including processes such as limited proteolysis, phosphorylation,

glycosylation, acetylation, etc. The functional protein is then translocated to its specific destination to take part in cellular activities (Alberts *et al.*, 1994; Stryer, 1995).

Thus, overall, gene expression involves several major steps: DNA transcription, pre-mRNA processing, mRNA stability, translation of mRNA, and post-translational modifications of proteins. The importance of mRNA stability to the regulation of gene expression has been also recognized (Beelman and Parker, 1995). The mRNA decay in eukaryotic cells is triggered by at least three types of initiating events: poly(A) shortening, premature translational termination, and endonucleolytic cleavage (Jacobson and Peltz, 1996). Translational control also modulates gene expression and this mainly involves control over the efficiency of mRNA translation to give a quantitative change in the overall amount of protein synthesized. The efficiency of protein synthesis is mainly regulated at the translation initiation step via reversible phosphorylation of various initiator factors in eukaryotic cells (Hershey, 1991; Mathews *et al.*, 1996; Pain, 1996).

Among the above steps for gene expression, regulation at the level of transcription is the most fundamental and important. In addition to the basal transcriptional machinery as described above, transcription is also modulated by various transcription regulatory proteins that bind to DNA sequences known as enhancers and silencers. Enhancers stimulate transcription, while silencers repress gene expression. Unlike the promoter region, these DNA regulatory elements are highly varied and gene specific. They can be located near to or at a great distance upstream and downstream from the core promoter. The interaction of a specific binding protein (activator or repressor) with its DNA regulatory element can influence the initiation complex assembly at the core promoter and modulate the activity of the RNA polymerase II basal transcription machinery

(Latchman, 1990; Mitchell and Tjian, 1989). Recent studies have indicated that certain regulatory proteins called co-activators and co-repressors can function as signaling intermediates between the DNA binding proteins and the basal transcription machinery (Bjorklund and Kim, 1996; Verrjzer and Tjian, 1996).

There are a wide variety of response elements and their associated binding proteins (activators or repressors) which up- or down-regulate specific genes in response to different chemical and physiological stimuli. The response of cells to a variety of extracellular stimuli, such as growth factors, heat shock, cell volume changes due to osmotic stress, and ATP depletion can activate signal transduction pathways (Cohen, 1997; Kyriakis *et al.*, 1994; Kyriakis and Avruch, 1996). Signal transduction through protein kinase and phosphoprotein phosphatase cascades is initiated by the binding of a ligand molecule, such as a growth factor or hormone, to a specific receptor on the plasma membrane or inside of a cell, which in turn results in changing the activities of certain protein kinases or phosphoprotein phosphatases and the levels of second messengers, such as cyclic AMP (cAMP), calcium ions, etc., which leads to cellular responses. Many of the signal-mediated cascade pathways responding to stress lead to changes in gene expression (Hunter, 1995; Seger and Krebs, 1995). For example, mitogen-activated protein kinases (MAPKs) are activated by osmotic stresses (Matsuda *et al.*, 1995).

Gene expression is an endpoint of signal transduction pathways and continually reshapes the cell in response to metabolic needs and environmental stimuli.

Environmental stress-induced gene expression in organisms is also well documented (see below).

Stress-Induced Gene Expression

Environmental stress can trigger changes in gene expression. To date, the heat shock response has been the best characterized of the stress responses and is often used as an example. In response to elevated temperatures, cells produce a select group of proteins (heat-shock proteins, hsps) in high levels. Some of these hsps are present constitutively in cells; others appear as *de novo* protein products under stress conditions only (Lindquist and Craig, 1988). Most hsps appear to function to protect cellular proteins from stress-induced denaturation. Some act as molecular chaperons to assist protein folding, higher order assembly, translocation and protein degradation (Lindquist, 1986; Parsell and Lindquist, 1993). The heat-shock response is widespread and highly conserved among organisms. Hsps are also induced by a variety of other stress conditions including heavy metals, amino acid analogues, alcohol and oxidants (Hightower, 1991) and even in pathophysiological circumstances such as cerebral and cardiac ischemia (Morimoto *et al.*, 1994). In addition, the glucose-regulated protein (GRP78) in mammalian cells, an analog of hsp70, can be induced by glucose starvation (Pelham, 1989). Regulation of the heat shock response involves a very conserved regulatory region of the genes called the heat shock element (HSE) which is activated by a heat shock transcription factor (HSF). HSF is present in a latent state under normal conditions and it is activated upon heat stress to bind HSE for transcription activation. The stress signal is thought to be transduced to HSF by changes in the physical environment, in the activity of HSF-modifying enzymes, or by changes in intracellular levels of heat shock proteins (Morimoto, 1993; Wu, 1996).

Gene expression in response to an abrupt shift to lower temperature (cold-shock) has also been investigated recently (Jones and Inouye, 1994; Thieringer *et al.*, 1998). Data from studies with *Escherichia coli* have shown that selected cold-shock proteins (Csps) are induced when cells are abruptly shifted to a lower temperature, the magnitude of induction being generally dependent on the size of the temperature shift. Since an inhibition of translation by certain antibiotics (tetracycline, *etc.*) can also induce the cold-shock response, it has been suggested that the state of the ribosome may be the physiological sensor for the induction. The initiation of translation and the ribosome assembly are inhibited at low temperature and, thus, the cold shock response may function to overcome the partial block of protein synthesis (Thieringer *et al.*, 1998).

The heat shock and cold shock responses are transient responses that are intended to help minimize damage to cells from brief exposures to extreme conditions that are generally outside the normal experience of the organism. However, many organisms must deal with prolonged low temperature exposure during the winter months and the role of gene expression in adapting organisms to long term cold stress is of great interest. To date, the subject has been most extensively studied in plants due to interest in improving the cold-hardiness or frost-hardiness of agricultural crops (Thomashow, 1993; Jaglo-Ottosen *et al.*, 1998). Various cold-induced proteins have been identified that act as membrane protectors to enhance freezing survival of plant cells (Artus *et al.*, 1996). Cold-induced gene expression has also been documented in some fish species (Tiku *et al.*, 1996) and in fish cell culture systems (Yamashita *et al.*, 1996).

In addition to gene expression in response to temperature change, gene regulation related to oxidative stress, low oxygen tension, and dehydration has also been studied.

Oxidative stress arises from the harmful effects of reactive oxygen species (ROS) that can damage nucleic acids, protein and lipids. Cells can counteract oxidative stress by expressing proteins with antioxidant activities as well as enzymes which repair damaged macromolecules (Sen and Larcher, 1996). A lack of oxygen (hypoxia and anoxia) is also harmful to aerobic organisms. Low oxygen tension is a feature of many physiologic and pathologic conditions (wound healing, etc.) and induces the transcription of many genes (Bunn and Poyton, 1996). For example, the induction of erythropoietin gene (*Epo*) transcription in response to hypoxia is well known. Erythropoietin is a plasma glycoprotein that stimulates production of red blood cells to increase the oxygen-carrying capacity of the blood. Gene expression in response to hypoxia enables cells to adapt to the low oxygen condition. A hypoxia inducible transcription factor, HIF-1 (Semenza and Wang, 1992), has been identified that activates certain genes (e.g. erythropoietin, some growth factors, phosphoglycerate kinase 1, and glucose transporters) under hypoxic conditions in mammals (Bunn and Poyton, 1996). Dehydration-associated cell volume change, osmotic and water stress also result in specific gene expression (Burg *et al.*, 1997; Matsuda *et al.*, 1995). Although extreme temperature, drought, and salt stress are generally considered to be different forms of osmotic stress, both cold and drought can activate stress signaling mediated by a MAP kinase pathway in plants (Jonak *et al.*, 1996).

Freezing Survival in Vertebrates: Wood Frog as a Model Animal System

Of numerous environmental stress conditions, changes in temperature are probably the most common stress experienced by virtually all living organisms. When

temperature drops below the freezing point (FP) of body fluids, ectothermic organisms are at high risk of freezing. Freezing is probably one of the most serious environmental threats for living organisms in nature. With rare exceptions, intracellular ice formation is always lethal to cells due to the severe damage to cell membranes and the destruction of subcellular architecture that result from the propagation of large ice crystals (McGann *et al.*, 1988). Over vast areas of our planet organisms must have effective strategies for dealing with seasonal exposures to subzero temperatures. Many terrestrial animals choose sheltered hibernacula that allow them to avoid freezing exposures, and most plants enter dormant life stages or overwinter as seeds or spores. Many ectothermic animals endure long exposures to subzero temperatures and have perfected one of two adaptive strategies: freezing avoidance or freezing tolerance (Duman *et al.*, 1991; Storey and Storey, 1989, 1996a).

Freeze avoidance exploits the tendency of all aqueous solutions to cool substantially below their equilibrium freezing points before spontaneous freezing occurs. Using adaptations that suppress the freezing and supercooling point (SCP) of body fluids and stabilize the supercooled state, an organism can remain unfrozen even at very low subzero temperatures. Biochemical adaptations that support freeze avoidance include the use of antifreeze proteins (AFPs) or thermal hysteresis proteins (THPs) (in insects) and the synthesis of high concentrations of low molecular weight carbohydrate antifreezes in order to push the supercooling point of body fluids to a value well below the normal subzero temperatures encountered in the habitat. This strategy is used by many arthropods (Duman, 1982; Storey and Storey, 1992a) and various polar marine fishes (DeVries, 1982; Davies *et al.*, 1999). However, although freeze-avoiding organisms can

maintain normal life activities at subzero temperatures, they run the risk of lethal freezing if environmental temperature ever drops below the supercooling point of their body fluids.

Other organisms have taken a different strategy and regulate the formation of ice in extracellular spaces while maintaining a liquid state in the cytoplasm. Freeze tolerance occurs widely among invertebrates (Zachariassen, 1985; Duman *et al.*, 1991; Storey and Storey, 1992a) and is also known among a few amphibians and reptiles (Storey, 1990; Storey and Storey, 1992b). Freeze tolerance has also been reported in bacteria (Gounot, 1991) and plants (Sakai and Larcher, 1987; Andrews, 1996). The formation of extracellular ice has several difficult consequences for cells and organs and freeze tolerant animals have had to evolve adaptations to deal with these. Among the consequences of internal ice formation are: 1) the potential for physical damage to tissue structure by ice, particularly by ice expansion within capillaries, 2) the sequestering of much water in ice raises the osmolality of extracellular body fluids which places a hyperosmotic stress on cells and these lose water and shrink, and 3) freezing of blood plasma halts circulation and causes ischemia in the tissues.

Various mechanisms that deal with these problems have been described. The role of cryoprotectants has received much attention. Low molecular weight sugars or polyhydric alcohols are the most common. These often accumulate to several hundred millimolar and their colligative effects prevent cell volume from dropping below a critical minimum value. Cryoprotectants also limit the extent to which cellular ionic strength is disrupted and help to stabilize protein and macromolecular structures from cold- or freeze-induced denaturation (Zachariassen, 1980; Ashwood-Smith, 1987; Fahy *et al.*,

1990; Costanzo *et al.*, 1992). Selected cryoprotectants (such as trehalose and proline) also act as membrane protectants to stabilize the lipid bilayer structure during cell shrinkage; these have been extensively described in anhydrobiotic systems (Rudolph and Crowe, 1985; Crowe *et al.*, 1987). In addition to certain sugars as protectants to stabilize proteins and membranes in the dry state, vitrification also plays a role in anhydrobiosis (Crowe *et al.*, 1998).

Freezing also halts the circulation of blood in animals and hence inhibits the delivery of oxygen and nutrients to tissues. Freeze tolerant animals must, therefore, have adaptations that allow them to endure an anoxic state throughout the freeze. Mechanisms of anaerobic ATP-generation are well-developed in these species and metabolic rate depression during freezing can also lower cellular energy requirements so that organisms can survive being frozen for many weeks (Storey and Storey, 1988).

The wood frog, *Rana sylvatica*, is the primary model animal that is used for studying freezing survival in vertebrates (Storey and Storey, 1988, 1992b, 1996a; Costanzo *et al.*, 1993). Laboratory tests have shown that frogs can tolerate the conversion of up to 65% of total body water into ice and can endure several weeks of freezing at -2.5°C , fully recovering all vital functions within about 24 h after thawing (Storey and Storey, 1993). Freezing is a relatively slow and controlled process; at -2.5°C it takes up to 24 h for ice content to reach its maximum and during this time, the frog initiates various adaptations. One of these is cryoprotectant production. Wood frogs use glucose as their cryoprotectant, synthesizing huge amounts of the sugar whenever tissues begin to freeze. In less than 5 min after freezing begins at peripheral sites on the skin, glycogen catabolism in the liver is activated and glucose is mass-produced and rapidly transported

to all other organs by the blood. Cryoprotectant synthesis and distribution is facilitated by adaptations of both glycogenolytic enzymes in liver and membrane glucose transporters (Storey and Storey, 1988; King *et al.*, 1993). Hormonal and nervous influence can also play a role in the synthesis of cryoprotectant in frog liver (Storey and Storey, 1996b).

Glucose concentrations increase from normal values of 1-5 $\mu\text{mol/g}$ wet weight to about 50 $\mu\text{mol/gww}$ in peripheral tissues (such as muscle) and as high as 350 $\mu\text{mol/gww}$ in core organs (Storey and Storey, 1985; Storey, 1990). A gradient of glucose concentrations develops because the outward export of glucose from the liver is progressively cut back by the opposite movement of the freezing front inwards, cutting off circulation as it goes (Rubinsky *et al.*, 1994). After thawing, glucose is returned to the liver and restored as glycogen.

Extensive biochemical and physiological studies have illustrated many cellular adaptations that are involved in frog freeze tolerance. However, the full range of adaptations that are necessary to ensure freezing survival remains unknown. We are interested in searching for novel proteins that may be produced during freezing exposure of frogs to support survival. Studies in our lab using *in vitro* translation to compare the mRNA pools of control versus freezing-exposed frogs showed elevated levels of some proteins as well as several new peptide bands in the tissues of freeze-exposed frogs (Lobsinger, M.Sc. Thesis, 1996). These changes in tissue mRNA pools, suggest that different gene transcripts must be produced during freezing. Other studies of *de novo* protein biosynthesis, using ^{35}S -methionine/cysteine to label proteins, gave similar evidence of the appearance of new protein types in response to freeze/thaw and

dehydration/rehydration in wood frogs (Storey *et al.*, 1997). Neither of the above techniques, however, led to identification of the new proteins.

The role of gene expression in mediating cellular responses to freezing in naturally freeze tolerant animals has only recently begun to be characterized in our lab. Initial studies used differential screening of a cDNA (complementary DNA) library made from liver of frozen frogs to identify genes that were upregulated in liver during freezing (Cai and Storey, 1997a, 1997b; Cai *et al.*, 1997; Wu and Storey, unpublished). Comparable studies have also begun to analyze gene expression in response to cold exposure in both freeze tolerant and freeze avoiding insects (Bilgen, Ph.D. Thesis, 1998) and in the freeze-tolerant marine gastropod *Littorina littorea* (English and Storey, Ph.D. studies in progress). Thus, our initial studies have indicated that gene regulation is an important part of natural freezing survival.

Much more work remains to be done, particularly an analysis of organ-specific gene expression to determine what genes (representing what protective features) are turned on in different organs. Analysis of the role of gene expression in supporting wood frog freezing survival is the focus of this research project.

Outline of Research Objectives

I hypothesized that specific changes in gene expression (upregulation or induction) would occur in response to freezing that would address specific needs for freezing protection in different organs. I focused on three very different organs (liver, brain, and skin), each chosen for specific reasons, and isolated cold or freeze-regulated

genes and analyzed the roles of stress-induced proteins in cold/freezing adaptation of the frogs.

Frog liver is the major site of glycogenolysis for cryoprotectant synthesis, and thus plays a critical role in the freezing survival of the whole animal. Initial studies performed in our lab isolated freeze-responsive genes from liver by screening a custom-made cDNA library (prepared by Stratagene). Several genes that were up-regulated in response to freezing were identified by screening the frog liver cDNA library (Cai and Storey, 1997a, 1997b; Cai *et al.*, 1997). For my initial studies, I tried to isolate additional freezing up-regulated genes from frog liver by screening many more plaque clones from the same cDNA library.

Brain is one of the most sensitive organs in vertebrate animals. The high rate of ATP generation needed to support electrical activity is highly dependent upon oxygen-based metabolism and hence, brain is generally considered to be the most oxygen-sensitive organ. Vertebrate brain is also highly dependent upon blood-borne substrates (generally glucose, but also ketones or other metabolites in some situations) for its metabolic fuels and generally exhibits low endogenous fuel reserves. Both of these factors, then, make brain highly susceptible to metabolic damage caused by low oxygen and/or ischemic events. Freezing is an ischemic event; plasma freezing cuts off all organs from blood-borne supplies of oxygen and fuels. Hence, I suspected that brain would show specific changes in gene expression that would aid its survival of freezing and/or the ischemia that freezing imposed. Furthermore, protection of the brain and nervous system against freezing damage is also very important in order that the animal may resume a normal life after thawing for brain coordinates movement and many other physiological

functions. Freeze-induced changes in gene expression in wood frog liver address not just the cryoprotection of this organ alone but liver is the site of cryoprotectant synthesis and export for the whole organism (Storey and Storey, 1996a) and liver also apparently synthesizes plasma proteins that aid freezing recovery (e.g. expression of the genes for fibrinogen, a clotting protein, is up-regulated by freezing; Cai and Storey, 1997a). Hence, I expected that the types of genes that would be up-regulated by freezing in brain would be very different from those uncovered in liver and that genes induced or up-regulated in brain would probably represent protein products that had specific functions in providing cryoprotection to brain cells themselves. Isolation and analysis of freezing-responsive genes from frog brain have been major objective of my studies.

Frog skin is another good candidate for the isolation of stress-inducible genes. Skin is typically the first organ that experiences the consequences of environmental stress, such as changes in temperature and humidity. Skin is typically the site of ice nucleation in frogs for the skin can come in direct contact with environmental ice and ice crystallizing in the skin then propagates inward through the frog's body. Studies also indicate that ice formation in skin sets off signals that are rapidly transmitted to the liver and trigger a immediate increase in glycogenolysis and cryoprotectant synthesis within 2-5 minutes post-nucleation (Storey and Storey, 1985). Furthermore, skin is the organ with the least physical protection while frozen (it is in direct contact with the environment) but because it is one of the first organs to freeze it also receives only very low levels of glucose cryoprotectant from the liver (glucose levels in abdominal skin of frozen frogs were never more than 25 mM compared with ~200 mM in core organs; Storey, 1987). Hence, skin would be a good candidate for an organ that would show specific

endogenous cryoprotective responses. However, since the skin of the frog freezes very rapidly, cellular activities would be quickly brought to a halt by freezing in this organ. Thus, it seemed possible that skin might not show freeze-responsive changes in gene expression and that freezing protection might come, instead, from genes that were turned on during cold acclimation in order to prepare the skin for later freezing exposures. Thus, I decided to focus on identifying cold acclimation-regulated genes from the skin of wood frogs. The process of cold acclimation is often a critical step for developing freeze tolerance in plants with both cold-induced and freeze-induced genes and their protein products contributing to the final frost hardiness of the plant (Thomashow, 1993; Jaglo-Ottosen *et al.*, 1998). Therefore, I expected to isolate genes that contributed to freeze tolerance but that were induced in cold acclimation (at 5°C). Hence, studies with skin focused on warm versus cold acclimated frogs and searched for genes that were up-regulated by 5°C cold exposure.

Eukaryotic gene(s) that are induced or up-regulated in response to specific stress conditions can be isolated through differential screening of a cDNA library. In addition to screening the customer-made liver library, a cDNA library was constructed from brains of freezing-exposed frogs. The library was differentially screened to isolate freezing-responsive genes in frog brain. An alternative approach, mRNA differential display by polymerase chain reaction (DD-PCR), was also applied in other studies to isolate cold acclimation-induced genes from frog skin. Cloning and characterization of stress-inducible genes are described in detail in the Method sections of Chapter 2 and 3.

To further study the physiological significance of the isolated cold-responsive genes, the following approaches were taken:

1) To determine whether the identified genes were important to freeze survival in other organs or were specific to liver, brain or skin alone, Northern blotting was used to analyze changes in mRNA levels to determine which organs showed upregulation during freezing for the genes that were identified from the three organs.

2) Northern blotting analysis was also used to determine whether the genes that were up-regulated in response to freezing or cold-acclimation were also responsive to other stresses. In particular, responses to anoxia and dehydration stresses were analyzed since each of these is the component of freezing. Studies of gene expression in response to dehydration or anoxia stresses are helpful in elucidating the possible roles of cold-regulated gene(s) and the signaling mechanisms that control them.

3) Tests were also undertaken to confirm that increased mRNA levels did indeed lead to an elevation of the protein product. To confirm this, immunoblotting was used to quantify levels of selected gene products in tissues of control versus stress-treated frogs.

In addition to testing the levels of protein products of isolated stress-responsive genes, the levels of selected well-characterized regulatory proteins were also analyzed in tissues of stress-treated wood frogs by the immunoblotting assay. For example, levels of the hypoxia-inducible factor transcription factor, HIF-1, are widely elevated in mammalian systems in response to low O₂ level and we wished to know if the similar response might take place in frog tissues as a result of freezing-induced hypoxia. Analysis of changes in HIF-1 and other types of regulatory proteins under freezing stress in wood frogs will help to provide more complete picture of the types of stimuli (e.g. low temperature, anoxia and dehydration) that underlie freeze-induced responses and the types of regulatory pathways that are involved in environmental stress survival.

CHAPTER TWO

Differential Gene Expression during Freezing in the Wood Frog *Rana sylvatica*: Approaches by Screening of Frog Brain and Liver cDNA Libraries

INTRODUCTION

Differential gene expression underlies all parts of life including growth and development and responses of cells to many sorts of endogenous signals and environmental stresses. The population of messenger RNA (mRNA) molecules present at any given time in a cell gives a good representation of those genes that are currently being transcribed and those proteins that are currently being synthesized. When a stress is imposed upon the system, differential changes in the levels of certain transcripts can indicate a regulation of specific genes in response to the stress. By identifying these differentially expressed gene transcripts, we can isolate stress-inducible genes and further elucidate general cell functions that are involved in stress-induced cellular responses.

Since RNA molecules are exceptionally labile and difficult to amplify in their natural form, the information encoded by the mRNA is converted into a complementary DNA (cDNA) by reverse transcription and then inserted into a self-replicating lambda phage vector (cDNA library). A representative cDNA library should contain full-length copies of the original population of gene transcripts present in any given tissue or organism. Screening of such a cDNA library is now widely applied as a conventional method for identifying differentially expressed genes when their transcripts are present with relatively high abundance. ³²P-labeled probes derived from total cDNAs of tissue sampled under different states or stress conditions can be used to screen duplicate filters of the cDNA library. The cDNA clones that are up-regulated or down-regulated in response to the change of state/stress can then be selected for sequencing analysis in order to identify the differentially expressed genes.

The λ ZAP insertion vector system (Stratagene, La Jolla, CA) is commonly used for the construction of cDNA libraries. It can accommodate DNA inserts up to 10 kb in length. The vector system combines the high efficiency of lambda library construction and the convenience of a plasmid system with blue-white color selection. The vector can be screened with either DNA probes or antibody probes and can be used for *in vivo* excision to form a phagemid (pBluescript phagemid). The polylinker of the phagemid has 21 unique cloning sites flanked by T3 and T7 promoters and a choice of 6 different primer sites for DNA sequencing. The vector system can also be used for site-directed mutagenesis, unidirectional deletions, *in vitro* transcription to generate riboprobes used for Southern and Northern blotting, and the expression of fusion proteins.

The first studies of freeze-induced gene expression done in our lab used differential screening of a cDNA library constructed from liver to isolate several genes that were up-regulated in freezing-exposed frogs (Cai and Storey, 1997a, 1997b; Cai *et al.* 1997). The cDNA library was constructed in λ ZAP vector (*EcoR* I as cloning site) by Stratagene using mRNA from liver of wood frogs that were given 24 h freezing exposure at -2.5°C. The liver cDNA library (~80,000 plaques clones) was differentially screened with total cDNA probes synthesized from poly(A)⁺ mRNA isolated from liver of control versus 24 h freezing exposed frogs. Genes encoding the α and γ subunits of fibrinogen and the ADP/ATP translocase were found to be up-regulated as well as a novel 10 kDa protein with a nuclear exporting sequence (Cai and Storey, 1997a, 1997b; Cai *et al.*, 1997). These results of the first studies indicated the involvement in freeze tolerance of several metabolic functions that had never before been suspected as contributing to

freezing survival. For example, the upregulation of fibrinogen gene expression during freezing suggests that levels of this plasma clotting protein may be enhanced as part of a damage repair mechanism that could deal with any bleeding injuries to tissues as the result of ice damage.

In order to isolate additional freezing-inducible genes from frog liver, my initial studies screened many more plaque clones ($\sim 4 \times 10^5$) from the same cDNA library with probes also derived from liver mRNA of control versus 24 h frozen frogs. Several new genes that were up-regulated in response to freezing were identified (see Results).

Apart from screening the frog liver cDNA library, my main focus was on freeze-induced gene expression in frog brain to identify genes that aided freezing survival in this organ. A frog brain cDNA library was constructed into Uni-ZAP XR vector (Stratagene) by using tissue from frogs frozen for 8, 12 and 24 h. Since the upregulation of different genes may take place after different lengths of freezing exposure, the stress library was made from mRNA sampled from multiple time points. The new version of the λ ZAP vector has unidirectional cloning sites by *EcoR* I and *Xho* I. The library was used to screen for genes that were differentially induced or up-regulated during freezing in frog brain. A new liver cDNA library derived from mRNAs isolated from liver of frogs frozen for 4, 12, and 24 h was also constructed with this new version vector and this library was used mainly for screening complete cDNA genes with DNA probes isolated from other organs (e.g. DD-PCR clones from frog skin, see Chapter 3).

The up-regulated genes isolated from frog brain and liver were further analyzed for their expression in other tissues of freezing-exposed frogs and their responses under

dehydration and anoxia stresses. These additional studies (see Chapter 4) help to indicate the significance and function of the freezing-inducible genes.

MATERIALS AND METHODS

Animal Care and Tissue Sampling

Wood frogs, *Rana sylvatica*, were collected from breeding ponds in the Ottawa region during April and were held in the lab at 5°C for ~2 weeks before use. Control animals were taken directly from the 5°C acclimated group. For freezing exposure, frogs were transferred to an incubator set at -2.5°C in plastic containers lined with damp paper towels. Frog body temperature cooled to subzero values and animals began to freeze after about 45-60 min; previous studies showed that ice formed on the towels had inoculated the freezing of body fluids of all individuals within a tight time frame (~10-15 min). For initial studies, animals were sampled after 24 h. In additional studies, the lengths of freezing exposure were modified in order to examine a time-course of gene expression or the appearance of protein product. When very short freeze exposures were used (e.g. < 2 h), animals were individually monitored using a thermistor taped to the abdomen so that the initiation of freezing could be precisely monitored; this method is standard in our lab (Storey and Storey, 1985). To investigate the response to thawing, another group of frogs was first exposed to 24 h freezing at -2.5°C and then animals were returned to 5°C and sampled over the course of up to 24 h thawing. After experimental exposure, animals were killed by pithing and tissues were quickly excised, frozen in liquid nitrogen, and then stored at -70°C. Tissues sampled included brain, liver, heart, kidney, hind leg

skeletal muscle, blood (plasma and cells), skin (from frog abdomen, back and hind legs) and gut (stomach and intestine combined).

Chemicals and Special Care for Manipulating RNA

All chemicals were molecular biology grade or equivalent unless specified. Gloves were worn at all times while manipulating RNA. Deionized distilled water (ddH₂O) was treated with 0.05% diethylpyrocarbonate (DEPC) at room temperature overnight and then autoclaved. Non-autoclavable stock solutions, such as those containing sodium dodecyl sulfate (SDS), were prepared with sterile DEPC-treated water. Since tris[hydroxymethyl]aminomethane can inactivate DEPC, all Tris stock solutions were made with DEPC-treated water and then autoclaved.

Total RNA and Polyadenylated Messenger RNA Isolation

Total RNA was isolated from frog tissues with Trizol reagent (GIBCO BRL, Grand Island, N.Y.) according to the manufacturer's instructions. Polyadenylated messenger RNA [Poly(A)⁺ mRNA] was purified from total RNA samples by oligo(dT) cellulose affinity chromatography. The oligo(dT) cellulose was purchased from New England Biolabs (NEB) (Beverly, MA) and the procedure followed manufacturer's instructions. Both total RNA and mRNA were suspended in sterile DEPC-treated water (or in 50% formamide (v/v) for total RNA) and stored in aliquots at -70°C. The quality of total RNA was assessed by formaldehyde agarose gel electrophoresis (see below) and purity and concentration of RNA was evaluated by spectrophotometry at 260 nm and 280 nm (Gilford Spectrophotometer Model 240 or 260). An OD₂₆₀ of 1 corresponds to 40 µg

of RNA per ml. Ratios of OD_{260}/OD_{280} for frog RNA preparations usually ranged between 1.8 and 2.0, an indication that the nucleic acid was relatively pure.

Formaldehyde RNA Agarose Gel Electrophoresis

RNA molecules can be separated on a denatured agarose gel (Sambrook *et al.*, 1989). A 1.2% gel was prepared by melting 2.4 g of solid agarose (electrophoresis grade, GIBCO BRL) in 169 ml DEPC-treated water, then allowed to cool to $\sim 60^{\circ}\text{C}$ by addition of 20 ml 10 \times MOPS buffer [0.2 M 3-N-morpholino propanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM ethylenediamine tetraacetate (EDTA), pH 7.0] and 11 ml of 37% formaldehyde (v/v). This was then poured into a 20 x 35 cm gel mold to set. To prepare RNA samples, 15 or 20 μg of total RNA from each sample was brought up to a volume of 10-20 μl with sterile DEPC-treated water, heated at 65°C for 3-5 min, and then chilled on ice. To this was added 19 μl of a stock mixture (made of 12.5 μl formamide, 2.5 μl 10 \times MOPS buffer and 4 μl 37% formaldehyde) and 2.5 μl of 10 \times RNA loading buffer [0.9 ml of 50% glycerol, 2 μl of 0.5 M EDTA (pH 8.0), 50 μl of 0.25% bromophenol blue, 50 μl of 0.25% xylene cyanol FF]. After a quick spin, the RNA sample was loaded onto the agarose gel which had been pre-run at 90 V (4.5 V/cm) with 1 \times MOPS as the electrophoresis buffer for 10-15 min. An 7 μg aliquot of RNA ladder (1 $\mu\text{g}/\mu\text{l}$, 0.24-9.5 kb, GIBCO BRL) was applied as a size marker in one lane. The gel was run at 90 V for 4-5 h, then removed, rinsed in DEPC-treated water, stained with ethidium bromide (EtBr, 0.5 $\mu\text{g}/\text{ml}$ in ddH_2O) for 10-15 min, destained in ddH_2O for 15-30 min, and then photographed on a UV light transilluminator (Foto/UV 26, Fotodyne Inc.) at a

wavelength of 300 nm by a DS-34 camera with Polaroid instant pack films (Type 667). The bands of RNA ladder as well as ribosomal RNA in the total RNA sample could also be visualized by staining blots with methylene blue (as below under Northern blotting). Blots were treated in 5% acetic acid for 10 min, stained in 0.05% methylene blue (in 0.5 M sodium acetate, pH 5.2) for a few minutes and then destained with ddH₂O until the clearest band resolution was obtained (Herrin *et al.*, 1988; Sambrook *et al.*, 1989).

cDNA Library Construction

We reasoned that during freezing there could be genes that are induced or up-regulated at different times; for example, some might be activated as a rapid response to the initiation of freezing whereas others might not be activated until much later such as when freeze-induced ischemia and intracellular dehydration become severe. Hence, screening of a library prepared from frogs frozen for only one length of time (e.g. 24 h) could fail to detect many freeze-responsive genes. We decided, therefore, to prepare a library made from equal amounts of poly(A)⁺ mRNA isolated from tissues that had undergone different lengths of freezing exposure. A frog brain cDNA library was constructed using equal amounts of mRNA isolated from tissue of spring frogs frozen for 8, 12 or 24 h. A new liver cDNA library was also constructed using mRNA from the tissue of spring frogs frozen for 4, 12 or 24 h. Since cDNA libraries for other wood frog organs are not available, this liver library was used for rescreening with gene probes isolated from these organs (e.g. the DD-PCR clones isolated from frog skin tissues; see Chapter 3) in order to obtain complete cDNA sequences.

The construction of a cDNA library was carried out using the Uni-ZAP cDNA synthesis kit (Stratagene) following manufacturer's instructions. The main points of the procedure were as follows. Poly(A)⁺ mRNA (5 µg) was primed with an oligo(dT) linker-primer containing the protective GAGA sequence plus *Xho* I site and transcribed using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) in the presence of dATP, dGTP, dTTP and 5-methyl dCTP. The use of 5-methyl dCTP during first-strand synthesis hemimethylates the cDNA, protecting it from digestion by certain restriction endonucleases such as *Xho* I. During second-strand synthesis, RNase H nicked the RNA bound to the first-strand cDNA to produce a multitude of fragments, which served as primers for DNA polymerase I to "nick-translate" these RNA fragments into second-strand cDNA. Normal dCTP was used for incorporation into the second strand, which ensured that the *Xho* I sites in the linker-primer were susceptible to *Xho* I digestion (see below). The uneven termini of the double-stranded cDNA were nibbled back or filled in with cloned *Pfu* DNA polymerase, and *Eco*R I adapters were then ligated to both blunt ends of the double-stranded cDNA *via* T4 DNA ligase. The adapter sequence:

5' AATTCGGCACGAG 3'
3' GCCGTGCTC 5'

The end of *Eco*R I was phosphorylated *via* T4 polynucleotide kinase. The cDNA fragments were obtained by *Xho* I digestion to release the *Eco*R I adapter and the residual linker primer from the 3' end of the cDNAs and then the cDNA fragments were fractionated on a Sephacryl S-500 spin column. Finally, the directional cDNA was ligated into the Uni-ZAP XR vector arms in a sense orientation (*Eco*R I - *Xho* I) with respect to the *lacZ* promoter and packaged into phage with Gigapack II Gold packaging extract.

The recombination efficiency of the primary library was checked with blue-white (clear) plaque formation by infecting XL1-Blue MRF' cells (an *Escherichia coli* strain) on an IPTG-X-gal [Isopropyl-1- β -D-galactopyranoside (IPTG); 5-Bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-gal)] agar plate. The insertion of the cDNA in the vector disrupts the β -galactosidase gene, which causes clear plaques on a plate of bacterial cells in IPTG-X-Gal medium. Vectors without insert produce blue plaques.

The titer of the library [number of plaque-forming units per ml (pfu/ml)] was performed by plating as follows. NZY plates (5 g NaCl, 2 g MgSO₄·7H₂O, 5 g bacto yeast extract, 10 g NZ amine pH 7.5, 15 g agar, per liter) were prepared in Petri dishes (15 × 150 mm). Glycerol-XL1 blue MRF' cell stock was streaked on an LB plate (15 × 100 mm Petri dishes; LB agar medium: 10 g NaCl, 10 g tryptone, 5 g yeast extract pH 7.0, 15 g agar per liter) supplemented with tetracycline (12.5 μ g/ml) and incubated overnight at 37°C. Then a single bacterial colony was transferred into LB broth supplemented with 10 mM MgSO₄ and 0.2% (w/v) maltose and grown overnight at 30°C. The bacteria were then pelleted at 500 g for 5-10 min and resuspended with sterile 10 mM MgSO₄. A series of 10-fold dilutions of the phage library were prepared with SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM MgSO, 0.01% w/v gelatin). A 1 μ l aliquot of each serial dilution of the library was added into 200 μ l of XL1-blue MRF' cells at an OD₆₀₀ of 0.5 and incubated at 30°C for 15 minutes with gentle shaking. Then 4-5 ml of melted NZY top agar (0.7% agar, maintained at ~48°C) was added to the mixture, and it was poured onto a pre-warmed NZY plate (37°C) and incubated at 37°C for 8-14 h. Plaques were counted on the whole plate and the titer was determined according to the formula:

$$\text{pfu/ml} = \text{number of plaques} \times \text{dilution factor} \times 1,000$$

The primary cDNA library was amplified to make a large, stable quantity of a high-titer stock (usually $\sim 10^9$ – 10^{12} pfu/ml) using plating and titering procedures similar to above. The library suspension containing 2×10^6 plaque-forming phage was mixed with 2.5 ml of the host cells at an OD_{600} of 0.5, incubated at 37°C for 15 min, and then 20 ml NZY top agar ($\sim 48^\circ\text{C}$) was added and the mixture poured onto a pre-warmed (37°C) NZY plate (22.5×22.5 cm). The plate was incubated at 37°C for 6-10 h (no plaques allowed to grow larger than 1-2 mm) and was then overlaid with 15-20 ml SM buffer, and stored at 4°C overnight with gentle rocking. The pooled phage suspension was stored in a sterile polypropylene tube with chloroform added to a final 5% v/v. After incubating at room temperature for a few minutes, the mixture was centrifuged at 500 g for 10 min to remove cell debris, and the aqueous phase with amplified phage suspension was removed and chloroform was added to a final 0.3% v/v. Titration of the amplified library followed above procedures. Both primary and amplified cDNA libraries were stored in aliquots in 7% dimethylsulfoxide (DMSO) at -80°C .

Differential Screening of a cDNA Library

The cDNA library was differentially screened using ^{32}P -labeled cDNA probes derived from the poly(A)⁺ mRNA isolated from tissues of control vs freezing-exposed frogs (protocols adapted from Sambrook *et al.*, 1989). Through primary and secondary screening, the cDNA clones that were potentially up-regulated in response to freezing stress were selected and excised *in vivo* into pBluescript plasmids. Plasmid DNA was

purified and dot-blotted onto nylon membrane for tertiary screening with the general cDNA probes (control vs freezing) to further eliminate some false positive clones.

Following differential screening, potential up-regulated clones were finally confirmed by Northern blot analysis with the DNA probe derived from the insert of the clone.

Synthesis of Total cDNA Probes: This procedure began with 1 μg Poly(A)⁺ mRNA isolated from each of control vs. freezing-exposed tissue; volume was adjusted to 8 μl with sterile DEPC-treated water, followed by heating at 70°C for 3 min and chilling on ice. To this was added 5 μl of 5x First Strand Buffer, 1.5 μl of 5 mM dNTPs (without dCTP, 5 mM for each nucleotide), 1 μl of 200 ng/ μl oligo(dT) primer, 1 μl of 5 U/ μl RNasin and 2.5 μl of 0.1 M Dithiothreitol (DTT). After a quick spin, the mixture was annealed at room temperature for 10 min. Then 1 μl of 200 U/ μl MMLV reverse transcriptase was added, followed by 5 μl of [α -³²P]dCTP (3,000 Ci/mmol, Amersham, Cleveland, Ohio) and incubation at 37°C for 1 h. The RNA bound with the synthesized cDNA was hydrolyzed by adding 1 μl of 0.5 M EDTA (pH 8.0), 1 μl of 10 % SDS, and 3 μl of 3 M NaOH and treated at 68°C for 30 min. The cDNA mixture was then neutralized by adding 10 μl of 1 M Tris-HCl (pH 7.4) and 3 μl of 2 N HCl and centrifuged (500 rpm, 2 min) through a Sephadex G-50 column saturated in a TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to remove unincorporated nucleotides and <20-mer oligo nucleotides. The eluant was diluted to 500 μl with TE buffer and radioactivity was counted in a 1 μl aliquot in a scintillation counter Packard 1900 CA (TRI-CARB). MMLV reverse transcriptase, the 5x First Strand Buffer, RNasin and DTT were obtained from GIBCO BRL. The oligo(dT) primer and dNTPs were from New England Biolabs (NEB).

Primary Screening: A relatively large number of phage plaques on NZY agar plate were usually required for primary screening so 2 or 5 NZY agar plates (22.5 × 22.5 cm) were prepared and each plate contained approximately $1.5-2 \times 10^5$ pfu. After plating as described previously, the plaques on each plate were lifted onto a Hybond-N 0.45 μm nylon membrane (cut size: ~20 × 20 cm, Amersham) after adsorption for 4-5 min. The membrane was then gently placed on denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 2-3 min, then treated in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) for 5 min and rinsed in 2× SSC solution (from 20× SSC stock: 175.3 g NaCl, 88.2 g sodium citrate, pH 7.0, per liter). A second lift from the same plate was performed exactly as above with the adsorption time prolonged to 10 min. The two duplicated membranes were placed on blotting paper (VWR 238, VWR Canlab, Mississauga, ON) and treated in a UV cross-linking apparatus (CL-1000 ultraviolet cross-linker, UVP. Inc. USA) at 120,000 μjoules/cm² (254 nm) for 1 min. Fixing phage DNA to the membrane was sometimes also accomplished by baking at 80°C for 1-2 h with a gel dryer (Model 583, Bio-Rad, Hercules, CA).

The two membranes were then placed in different hybridization tubes (4 × 30 cm, VWR Canlab), and 20-25 ml hybridization solution [50% formamide, 0.12.5 M Na₂HPO₄, pH 7.2, 0.25 M NaCl, 2% SDS, 1 mM EDTA, adapted from Bio-Rad] as well as denatured and sheared salmon sperm DNA (100 μg/ml, Sambrook *et al.*, 1989) was added to each tube without radiolabeled probes. This was slowly rotated at 42°C in a hybridization incubator (Model 308, LAB-line Instruments, VWR Canlab, Mississauga, ON) for 2-3 h. After this pre-hybridization, the solution was replaced with 20 ml hybridization solution containing radiolabeled probes derived from control or freezing-

exposed tissues. Prior to use, both probes (control or freezing) were treated in boiling water for 3-5 min along with the salmon sperm DNA, chilled on ice, and then added to pre-warmed (~45°C) hybridization solution so that equal counts were in each solution ($2-4 \times 10^6$ cpm per ml hybridization solution). After hybridization in the incubator at 42°C for 12-18 h, the membranes were washed with 200-500 ml of 2× SSC and 0.2% SDS for 10-15 min at room temperature with one change of solution. Radioactivity was then checked with a Geiger counter. If counts were >5000 cpm, the wash was continued using 0.5× SSC and 0.2% SDS for 10-15 min at room temperature. If needed, a third wash was done with 0.2× SSC and 0.2% SDS at 50°C for 5-10 min. Counts were monitored every 5-10 min until radioactivity dropped to 500-2,000 cpm. The wet membrane was then placed on a piece of tin foil and covered with saran wrap, placed on X-ray film (Kodak XAR5) in a cassette and held at -70°C for 1-2 days (for counts between 1000-2000 cpm) or 3-5 days (counts of 500-1000 cpm). The film was developed manually with Kodak reagents. By comparison of the autoradiograms from hybridization with the control vs freeze-exposed probes, spots were identified on the film from the freeze-exposed hybridization that were either unique (indicating induced genes) or much darker (indicating up-regulated genes) compared with the control film.

Secondary Screening: The plaques identified by primary screening represent potential up-regulated clones but due to the high density of plaques on the plate, it is possible that two different cDNA clones may overlap. Therefore, secondary screening was necessary. A plaque containing a potential up-regulated clone was transferred with a Pasteur pipette into a 1.5 ml Eppendorf centrifuge tube containing 1 ml of SM buffer. An aliquot of chloroform (20 µl) was added and the mixture was vortexed and held at room

temperature for 1-2 h (or overnight at 4°C). Samples were then centrifuged for 5 min at 5,000 g and the supernatant containing isolated phages was stored at 4°C. Each sample was titered, then re-plated on an NZY plate (Petri dish, 15 × 100 mm) to 20-100 pfu/plate in order to obtain well-separated plaques. Lifting (onto half-size of Hybond-N disc membrane, 8.2 cm, Amersham) and screening procedures were as described above.

***In vivo* Excision:** This protocol was adapted from the manufacturer's instructions (Stratagene). Potential clones from the secondary screening were selected and single plaque isolation, phage release and titering were performed as described previously. The phages (Uni-ZAP XR vectors) were excised in *E. coli* strains using a helper phage (ExAssist, Stratagene) as follows. In a 1.5 ml tube, 0.1 ml of the XL1-blue MRF' cells ($OD_{600} = 1$ in 10 mM $MgSO_4$), 0.1 ml isolated phage suspension ($>5 \times 10^5$ pfu/ml) and 0.5 μ l ExAssist helper phage ($>1 \times 10^6$ pfu/ml) were added, and the mixture was incubated at 37°C for 15 min. Then 1 ml LB broth was added and incubated at 37°C for 3 h with gentle shaking. The mixture was then heated at 70°C for 15 min and centrifuged at 1,000 g for 5 min. The supernatant containing single-strand Bluescript phagemids can be stored at 4°C for a few months or kept in aliquots with DMSO (0.7%, for long storage at -80°C). SOLR (another *E. coli* strain) cells were grown (with a single colony from fresh streaking plate or glycerol stock) in LB broth at 30°C overnight and then kept at room temperature until use. An aliquot of 200 μ l SOLR cells ($OD_{600} = 1$ in LB broth) was mixed with 100 μ l of the above phagemid suspension, incubated at 37°C for 15 min, and then 100 μ l of the cell mixture (or a 10-fold dilution in LB broth) was plated on LB-ampicillin agar plates (50 μ g/ml) and incubated overnight at 37°C. The colonies appearing on the plate contained the Bluescript double-stranded phagemid (pBluescript plasmid) with the cloned

DNA insert, which can propagate in the SOLR cells and can be used directly for double strand DNA sequencing due to a choice of several primer sites (T3 and T7 primer, etc.). The SOLR colony was then streaked on a new LB-ampicillin agar plate, and a single colony was picked and inoculated into 10 ml of LB broth with ampicillin (25 µg/ml) overnight at 30°C. Sterile glycerol-LB broth solution (4.5 ml, 1:1 mix glycerol:LB broth) was added to the bacterial culture and then it was stored in aliquots at -70°C.

Purification of Plasmid DNA

Splinters of ice from the above glycerol-bacteria stock were inoculated into 10 ml LB-ampicillin (25 µg/ml) and grown at 37°C overnight with vigorous shaking. The purification of plasmid DNA was then performed by alkaline mini-prep (adapted from Sambrook *et al.*, 1989) or Rapid Pure Mini-prep kit (BIO 101, Inc., La Jolla, CA).

DNA Agarose Gel Electrophoresis

The purified Bluescript plasmid DNA was treated with restriction enzymes (*EcoR* I for ZAP cDNA library clones; *EcoR* I and *Xho* I for the Uni-ZAP cDNA library clones) and analyzed on an agarose gel to estimate the size of the insert and the purity of the mini-prep. *EcoR* I, *Xho* I and related enzyme buffer were obtained from NEB. For analyzing frog cDNA clones, a sample of DNA (2-10 µg) was adjusted to 6 µl with sterile ddH₂O, then 2 µl of 10× enzyme reaction buffer, 1 µl of 10 U/µl *EcoR* I and 1 µl of 10 U/µl *Xho* I were added. The mixture was incubated at 37°C for 1-2 h, heated at 70°C for 3-4 min and chilled on ice. A 2 µl aliquot of 6× DNA loading buffer [60 mM

NaCl, 6 mM EDTA, 60 mM Tris-HCl (pH. 8.0), 15% w/v Ficoll-400 (Sigma), 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol FF] was then added and the sample was loaded on an agarose gel. A 1 kb DNA ladder (GIBCO BRL) was also usually loaded onto gels. The gel (0.8-1.2% agarose) was prepared with 1× TAE buffer [made from 50× TAE stock: 242 g Tris base, 20 ml of 0.5 M EDTA (pH 8.0), 57.1 ml glacial acetic acid per liter] and electrophoresis was carried out with a medium-size agarose gel (10 × 20 cm) apparatus (Bio-Rad) at 80 V for about 1.5 h, then nucleic acid was stained with EtBr.

Dot Blot as Tertiary Screening

If many potential up-regulated clones are isolated after secondary screening, it can be helpful to do a dot blot analysis to further eliminate false positive clones. The procedures for dot blotting followed the manufacturer's instructions (Bio-Rad). Purified plasmid DNA (100-200 ng) from each cDNA clone was denatured by alkaline treatment and transferred onto nylon membrane using a Dot Microfiltration Apparatus (Bio-Rad). Duplicate blots were made and hybridized with the control vs freeze-exposed cDNA probes by procedures described above. The dot blotting was also used for cross-hybridization with a specific cDNA probe in order to isolate unique clones.

Northern Analysis

Northern analysis was used to confirm that isolated genes showed increased levels (or induction) of gene transcripts in the tissues of freezing-exposed frogs. In addition, the analysis was also applied for studying the tissue-specific expression of a gene or the response of a gene to other stress conditions (such as anoxia and dehydration, see Chapter

4). Samples of total RNA (15 or 20 µg each) or mRNA (1 µg) from tissues of control or stressed animals were loaded on a formaldehyde agarose gel and run as described above for gel electrophoresis. After electrophoresis, the RNA agarose gel was rinsed with ddH₂O and soaked in 10× SSC and then RNA was transferred onto a nylon membrane (pore size 0.2 µm, Schleicher & Schull, Keene, NH; or Hybond-N, Amersham) by capillary effect with the same buffer (10× SSC) (method from Sambrook *et al.*, 1989). Total RNA was then fixed to the membrane by the protocol described above and the membrane was hybridized with specific DNA probe derived from the cloned insert DNA. As described above, the insert was isolated by restriction enzyme digestion of the plasmid DNA and the restriction fragments were separated by agarose gel electrophoresis. The insert DNA was then purified from the gel with GeneClean III kit (BIO 101).

The synthesis of ³²P-labeled DNA probe was performed with the random priming method by a protocol adapted from Sambrook *et al.* (1989). Approximately 50-250 ng of insert DNA was adjusted to 10 µl with sterile ddH₂O, heated in boiling water for 3-4 min and chilled on ice. Then 1 µl of 1 mM dNTPs (without dCTP), 2 µl of 10× Reaction Buffer (NEB) (100 mM Tris-HCl, 50 mM MgCl₂, 75 mM DTT, pH 7.5), 1.5 µl of DNA random primer d(N)₆ (1 µg/µl, NEB), 0.5 µl of Large Fragment of DNA Polymerase I (Klenow fragment, exonuclease minus, 5 U/µl, NEB), 5 µl of [α-³²P]dCTP (3,000 Ci/mmol, Amersham) were added and the mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 1 µl of 0.5 M EDTA (pH 8.0) and then the radiolabeled mixture was passed through a Sephadex G-50 column. TE buffer was added to the DNA probe sample to 0.5 ml and radioactivity was measured as described above.

The procedure for hybridization of Northern blots was very similar to that for screening a cDNA library although fewer counts of radiolabeled probe were used ($1-2 \times 10^6$ cpm/ml of hybridization solution) and higher stringency washing at 50-65°C was usually required. Each blot could be reused up to 5 times, and probe used for a previous hybridization was stripped off the membrane by treating the blot in a boiling solution of $0.1 \times$ SSC and 0.2% SDS for 1-2 min.

Autoradiography and X-ray film development were carried out as previously described. Measurement of band intensities on autoradiograms in terms of peak area (cm^2) were determined by densitometry and the use of computer imaging software. A Scan Jet3C scanner (Hewlett-Packard, Palo Alto, CA) was used along with Deskscan II v2.2 software (Hewlett-Packard) to render an autoradiogram into a form recognizable by Imagequant software v3.22 (Innovative Optical Systems Research) which then quantified relative band intensity.

Quantifying mRNA by RT-PCR

Although Northern blotting can achieve high sensitivity in the detection of mRNA, it may fail to detect mRNA transcripts that are in very low abundance. Reverse transcription and polymerase chain reaction (RT-PCR) are the powerful method for the detection of RNA species that are present only 1 or 2 copies per cell. RT-PCR was used as an alternative method for comparing mRNA levels under control and stress conditions for certain isolated cDNA clones. The method uses synthesis of total cDNA (control vs stress sample) from RNA (equal amount of RNA isolated from tissues of control and stress-treated animals) by reverse transcription and the DNA sequence of target genes can

then be amplified by PCR with the primers (forward and reverse) derived specifically from the target gene. The PCR products are then analyzed by agarose gel and the expression of the target gene under control and stress condition can be assessed. The synthesis of total cDNA was carried out by reverse transcription as described previously. The oligo-d(T) primer can also be replaced with an anchored primer H-T₁₁G (5'AAGCTTTTTTTTTTTG3') (GenHunter Corp. Nashville, TN) for the RT reaction. The reaction mixture for reverse transcription was incubated at 37°C for 60 min and then terminated by heating at 75°C for 3-5 min. Total cDNA was diluted 10× with sterile water and stored as aliquots at -20°C. The PCR reaction components included: 2 µl of diluted RT reaction mixture, 9.75 µl sterile water, 2 µl of 10× PCR buffer (Perkin-Elmer), 2 µl dNTP (1 mM), 2 µl of gene-specific primer (forward, 25 µM), 2 µl of gene-specific primer (reverse, 25 µM), and 0.25 µl AmpliTaq DNA Polymerase (5 U/µl, Perkin-Elmer). A 15-20 µl aliquot of mineral oil was added on the surface of the PCR reaction. Then the PCR reaction was carried out in the thermocycler (PTC-100, MJ Research, Inc. Watertown, MA) as follows: 94°C, 1 min, → 94°C, 30 seconds, → 40°C, 100 seconds, → 72°C, 45 seconds, repeat step 2-4 for 39 cycles, → 72°C, 5 min, → 4°C. PCR primers were derived from the isolated cDNA clone and were synthesized either at the Core Facility for Protein/DNA Chemistry, Biochemistry Department, Queen's University, Kingston, or in Canadian Molecular Research Services Inc., Orleans, ON.

The PCR product was tested on a 1-3% agarose gel (in 1× TAE buffer) and the DNA fragment was usually re-amplified by PCR. The gel-purified DNA fragments were used for TA cloning (Invitrogen Corp., Carlsbad, CA). The identity of the DNA fragment

was confirmed by sequencing plasmid DNA (see below) or the PCR product directly by Canadian Molecular Research Services Inc., Orleans, ON.

DNA Sequencing Analysis

DNA sequencing was performed by the enzymatic chain-termination method (Sanger *et al.*, 1977) using the kit from United States Biochemical (USB) (Cleveland, Ohio) and [α -³⁵S]dATP (1,000 Ci/mmol, Amersham). The procedures for alkaline denaturation of double strand plasmid DNA, sequencing reactions and sequencing gel electrophoresis followed the instructions of the manufacturer(s). Initial DNA sequencing analysis for isolated clones was done from both ends. Subsequently, complete DNA sequences were obtained and/or confirmed by sending samples for automated sequencing either at the Core Facility for Protein/DNA Chemistry, Biochemistry Department, Queen's University, Kingston or by Canadian Molecular Research Services Inc., Orleans.

Analysis of DNA Sequencing Data

Computer-assisted programs were used to analyze data. Nucleotide sequences and deduced polypeptide sequences were analyzed with the Blast Program on the World Wide Web (WWW) to search for homologous sequences in Genbank. DNASTAR program (DNASTAR Inc., Madison, Wisconsin) and DNAMAN program (Lynnon BioSoft, Vaudreuil, Quebec) were used for sequence comparison and alignment.

Isolation of Full-length cDNA Clones by Re-Screening a cDNA Library

Some isolated inserts may represent only part of the full-length cDNA. The full-length cDNA clone may be isolated by re-screening the cDNA library with a specific probe derived from the insert of the isolated DNA clone. The procedures for re-screening were very similar to those for primary screening of a cDNA library, as described above. The size of library to be screened depends on the abundance of the gene transcript; usually $2.5-5 \times 10^3$ pfu were re-screened for a cDNA clone as an initial test. Since the probe is derived from a specific cDNA (prepared by random primer labeling as described above), fewer counts of radiolabeled probe were used ($5-10 \times 10^5$ cpm/ml hybridization solution) and higher stringency washing at 70-80°C was usually needed.

Isolation of Full-Length cDNA Clones by RACE

An alternative method for obtaining full-length cDNA clones is the rapid amplification of cDNA ends (RACE) by PCR (Zhang and Frohman, 1997). 3' RACE was used with certain cDNA clones (those with a poly(A) tail at their 3' ends but missing the real poly(A) tail portion). The synthesis of total cDNA was carried out by reverse transcription as described previously. The anchored primer H-T₁₁G was used for total cDNA synthesis and another PCR primer was a gene-specific primer and was derived from the original cDNA clone. The protocol for the PCR reaction was the same as previously described except that H-T₁₁G was used as one of the PCR primer set. Cloning, sequencing and further analysis were as described above.

Southern Blotting

Southern blotting was carried out in order to eliminate false clones when searching for the full-length cDNA by using re-screening or RACE techniques (as outlined above). The newly isolated DNA clones were separated on an agarose gel and then the DNA gel was treated with an alkaline solution, followed by neutralization and finally transferred onto membrane by the standard method (Sambrook *et al.*, 1989). The blot was then hybridized with the probe derived from the original clone.

RESULTS

Construction of cDNA Libraries

Both a brain cDNA library and a new liver cDNA library were successfully constructed into the Uni-ZAP XR vector using mRNA isolated from organs of wood frogs frozen for different lengths of time (8, 12, and 24 h for the brain library and 4, 12, and 24 h for the liver library, respectively). The titers of the brain and liver primary cDNA libraries were 4.0×10^6 pfu/ml and 2.1×10^6 pfu/ml, respectively. After amplification, the titers were 6.5×10^9 - 5.9×10^{10} pfu/ml and 1.8×10^{10} pfu/ml, respectively. The efficiency of recombinants for both cDNA libraries was over 98% and the size of the cDNAs was in the range of 0.4-10 kb. The frog liver library was used mainly for isolation of complete cDNA clones from other frog tissues whose libraries were not available (e.g. frog skin tissues). The brain cDNA library was used for differentially screening to search for freezing-inducible genes in wood frog brain.

Isolation of Freezing-Inducible Genes from Frog Brain

Freezing-inducible genes were isolated by screening the brain cDNA library as described above. ³²P-Labeled total cDNA probes were synthesized from poly(A)⁺ mRNA isolated from brain of control frogs (acclimated at 5°C) and from a mixture of equal aliquots of poly(A)⁺ mRNA extracted from brain of 8, 12 and 24 h freezing-exposed frogs. The probes (from control vs. freezing stress) were used for screening the frog brain cDNA library. Primary screening of the cDNA library (~2.5 × 10⁵ plaques) revealed 45 potential positive plaque clones (designated as Br1-Br45) with stronger signals by the probes derived from the freezing stress. These clones were isolated and used for secondary screening, which resulted in 29 candidate plaque clones. These clones were selected and excised *in vivo* into pBluescript plasmids. The plasmid DNA was purified and then used for Dot blotting analysis as tertiary screening to further select potential up-regulated cDNA clones. The duplicated dot blots were hybridized with total cDNA probes (from control vs. freezing stress). The dot blots were also used for cross-hybridization tests with specific probes derived from the inserts of the tertiary-screened clones. The inserts of the cDNA clones were released by double restriction enzyme digestion (*EcoR* I and *Xho* I) and separated on agarose gels. Finally, five putative freezing-induced clones (Br3, Br4, Br15, Br30, Br34) were selected. The estimated insert size for the clones Br3, Br4, Br15, Br30, and Br34 were 0.85, 0.75, 1.05, 1.65, and 0.9 kb, respectively (Fig. 2.1). Further characterization of these frog brain cDNA clones was performed by Northern blotting and sequencing analysis as described below.

Northern Blotting Analysis of Isolated Frog Brain cDNA Clones

Northern blotting analysis indicated that the gene transcripts encoded by the above cDNA clones were all up-regulated in the brain of freeze-exposed frogs.

The gene transcript probed with clone Br3 insert showed a single RNA band on the Northern blot and the RNA size was estimated to be about 1.05 kb in length (Fig. 2.2). The level of the RNA transcript in the brain was about 3.0 fold higher during freezing exposure and recovery as compared with the control group (at 5°C) (Fig. 2.2). The same results were obtained with identical blots tested by Northern analysis in multiple trials ($n \geq 3$). The blot (loaded with mRNA samples) was re-probed with EF-1 γ gene (encoding for elongation factor 1, gamma subunit) as an internal control for assessing the RNA loading and quality. The EF-1 γ cDNA clone was isolated from frog liver and its transcript level in frog brain was consistent during freezing exposures. The RNA loading, transblotting and quality can also be assessed by staining the mRNA blots with methylene blue as described above.

A single band was also detected on mRNA blots with the probe derived from clone Br4 insert (Fig. 2.3A, B). The estimated RNA transcript size was about 1.6 kb in length on the mRNA blots. The transcript levels increased about 3.5-4.5 fold under freeze exposure (Fig. 2.3A, B) and 3.5-fold during recovery (Fig. 2.3B) as compared with the control group.

Northern blot analysis using the clone Br15 cDNA probe is shown in Fig. 2.4. Blot detected a single transcript band and the RNA size was estimated to be about 1.15 kb in length. A greater than 8 or 12-fold accumulation of the transcripts was detected on the

mRNA blot in the 24 h frozen and recovery groups, respectively, as compared with control at 5°C (Fig. 2.4).

Two transcript bands were detected on Northern blots using the clone Br30 cDNA probe (Fig. 2.5). The upper band was the dominant one and its size was estimated at about 1.7 kb in length. The size of lower band was about 0.85 kb in length. For the 1.7-kb transcript, the Northern blot indicated an increase in the gene transcript abundance of about 70% in brain tissue from freezing-exposed frogs in comparison with the controls (at 5°C). For the 0.85-kb transcript, the blot showed about 50% accumulation of the transcripts in the 24 h frozen group, compared with the control (Fig. 2.5).

A specific transcript band was not detected very well on the total RNA blots with the probe derived from the insert of clone Br34. However, two distinct RNA bands were detected with the same gene probe on the blot loaded with mRNA samples (Fig. 2.6A). The sizes of the two transcripts were estimated as 2.2 and 3.5 kb in length, respectively. The RNA transcripts seemed to be induced under freezing exposure as compared with controls (at 5°C). In addition, the 3.5-kb RNA transcript (as dominant band) further accumulated by 4-5 fold in brain after thawing (Fig. 2.6A). The level of the 2.2-kb RNA transcript (as faint band) decreased during recovery as compared with the freeze-exposed group. The upregulation of this particular gene was also tested by RT-PCR analysis. The amplification of a specific DNA fragment of the clone Br34 insert from total cDNA isolated from brain (control at 5°C versus freezing-treated frogs) with 2 specific PCR primers (Br34-T3-1 and Br34-T7-1 derived from clone Br34 insert, see Table 2.1) indicated that the corresponding gene transcripts were up-regulated under freezing

exposure (Fig. 2.6B). The amplified PCR fragment was confirmed as the expected part of Br34 cDNA insert by sequencing analysis.

DNA Sequencing Analysis of Freezing-Inducible Genes from Frog Brain

Clone Br3

Sequencing data indicated the Br3 cDNA had a fragment of 818 nucleotides in length. Blast searching of Genbank databases showed that the cDNA was significantly similar to the sequence of the mitochondrial genes (ATPase 6 and ATPase 8), and revealed a high degree of similarity with the corresponding mitochondrial genes in fish (common carp, *Cyprinus carpio*; Chang *et al.*, 1994), birds (ostrich, *Struthio camelus*; Harlid *et al.*, 1997) and in toads such as *Xenopus laevis* (Roe *et al.*, 1985). Since mitochondria (mt) have a distinctive genetic code, the vertebrate mitochondrial genetic codon system was applied for analyzing the frog genes. In vertebrates, four mitochondrial codons have different meaning as compared with universal ones: AGA and AGG, which normally encode arginine, are stop codons; ATA encodes for methionine instead of isoleucine; and TGA encodes for tryptophan rather than being a stop signal.

Two reading frames were recognized within the Br3 cDNA sequence and the amino acid sequence was deduced using the DNAMAN program. The nucleotide (nt) sequence and the deduced polypeptide sequence of clone Br3 are shown in Fig. 2.7. The first open reading frame (ORF), running from nt 2-118, was predicted to encode a short polypeptide of 38 amino acid residues and terminated with a terminator codon (TAA) at nucleotide 116. There were 4 TGA triplets upstream of this stop codon and TGA is normally recognized as stop codon in nuclear DNA but it is a codon for tryptophan in

mitochondrial DNA (mtDNA). The second ORF potentially encoded a long polypeptide of 227 residues and started with the start codon (ATG) at nucleotide residue 112 and terminated with a stop codon (TAA) at nucleotide 793. The poly(A) tail was right after the stop codon (TAA).

Protein sequence comparison showed that the deduced polypeptide sequences from Br3 were similar to the ATPase subunit 6 and subunit 8 encoded in the mitochondrial genome from other species (Fig. 2.8). The large polypeptide encoded from Br3 showed about 67% of identity with mtATPase subunit 6 from carp (Chang *et al.*, 1994) and *X. laevis* (Roe *et al.*, 1985). The short polypeptide sequence deduced from frog clone Br3 showed about 38% of the residues identical with those of mtATPase subunit 8 from carp and *X. laevis*. The data indicate that frog brain Br3 cDNA probably encodes mtATPase subunit 6 and 8. Both of these genes are compacted together in the mitochondrial genome and even share overlapping base pairs in vertebrates. Such overlapping bases were also found in the sequences of these genes in the wood frog cDNA clone Br3. In addition, Northern blotting indicated that the 1.05 kb RNA band may contain transcripts of both genes. Indeed, the fused transcript was reported by Desjardins and Morais (1990).

The ATPase subunit 8 of *X. laevis* contains 55 amino acid residues whereas the cloned Br3 cDNA encoded only 38 residues. Thus, the DNA insert represented only about two-thirds of the mtATPase 8 gene. In order to isolate a cDNA fragment with a complete mtATPase genome sequence, re-screening of the cDNA library was performed with clone Br3 insert as the specific probe. About 2500-5000 plaques of both the brain cDNA library and the new liver cDNA library were screened with the Br3 probe. The

liver library was used for re-screening mainly because the expression of mtATPase gene(s) was also up-regulated upon freezing exposure in frog liver and it could be valuable to compare cDNA sequences for the same gene in different tissues. Two plaques from the brain library and four from the liver library with strong signal were isolated. All the candidate clones were excised *in vivo* into pBluescript plasmids. The plasmid DNA from each clone was purified and then treated with restriction enzymes (*EcoR* I and *Xho* I) to release the cDNA insert. The insert fragments were separated on agarose gels (Fig. 2.9A) and hybridized with Br3 probe (Fig. 2.9B). Southern blotting indicated that two isolated clones (both from the liver library) with inserts larger than that of Br3 were related to mtATPase gene(s). They were designated as re-screened Br3 clones (RBr3a and RBr3b, respectively). The insert sizes of RBr3a and RBr3b were estimated to be about 1.5 kb and 1.8 kb, respectively. DNA sequencing data indicated that both clones contained sequences of the mitochondrial genes, ATPase 6 & 8 as well as that of COIII gene (encoding cytochrome oxidase, subunit 3). Clone RBr3a encoded a polypeptide that contained 44 amino acid residues of ATPase subunit 8, the complete amino acid sequence of ATPase subunit 6 and part of the COIII protein (Fig. 2.10). The Clone RBr3b encoded a polypeptide of 47 amino acid residues of ATPase subunit 8, the complete amino acid sequence of ATPase subunit 6 and all of the COIII protein (Fig. 2.11). The deduced polypeptide sequence (mtATPase, subunit 6 & 8) of Br3 (the brain clone cDNA) was identical to the corresponding gene products of RBr3a and RBr3b (the liver cDNA clones). Northern blotting has not been performed yet with the probes derived from COIII cDNA. It is unknown if the COIII gene is also up-regulated in the brain of freeze-exposed frogs.

Clone Br4

Sequencing results showed that the insert of clone Br4 was a fragment of 761 nucleotides in length. Blast searching data indicated this cDNA fragment was significantly similar to another mitochondrial gene, which encodes for 16S rRNA. Analysis of nucleotide sequence homology showed that the Br4 cDNA fragment had 88% identity with the corresponding portion of the mitochondrial 16S rRNA gene sequence from bullfrog (*Rana catesbeiana*). The sizes of the mitochondrial 16S rRNA gene from bullfrog and leopard frog (*Rana pipiens*) are 1584 and 1581 bp, respectively (database accession numbers: Y10945 for *R. pipiens*; X12841 for *R. catesbeiana*; Nagae *et al.*, 1988). Thus, the Br4 cDNA represents only about half of the mitochondrial 16S ribosomal RNA gene. The Br4 cDNA showed a short poly(A) tail at its 3' end but the poly(A) sequence was not present in the 16S rRNA gene of the related species such as *X. laevis* (database accession: M10217; Roe *et al.*, 1985) (Fig. 2.12). Since processed rRNAs usually do not contain a poly(A) tail, novel polyadenylation must have taken place in the middle of the mitochondrial rRNA and the polyadenylated incomplete rRNA was then isolated along with the poly(A)⁺ mRNAs with oligo(dT) chromatography.

The major RNA transcript was detected as 1.6 kb in length on the mRNA blots. Since its size was close to that of processed intact mitochondrial 16S rRNA which seemed to be ~1.58 kb. Since polyadenylated RNA can mainly be isolated by oligo(dT) chromatography for the isolation of RNA used for blotting analysis, the gene transcript in

the RNA preparations seemed to be the mitochondrial 16S rRNA precursor with short poly(A) tail or the processed 16S rRNA with a short poly(A) sequence.

Clone Br15

Clone Br15 had a 1098 bp cDNA insert. Data from a similarity search showed that this cDNA clone was similar to a nuclear gene, which encodes for an acidic ribosomal phosphoprotein (P0). Therefore, the universal genetic codon system was applied for translation of the protein sequence encoded from the gene. The cDNA sequence and deduced polypeptide sequence of clone Br15 are presented in Fig. 2.13. The cDNA insert (1098 nt in length) contained a 73-nucleotide 5'untranslated region and a 3'untranslated region of 79 nucleotides terminating with a homopolymeric A tail 22 nucleotides downstream from a canonical polyadenylation signal (AATAAA). This indicates that the sequence covers the complete 3' end of the gene. A single open reading frame was recognized within this sequence. The complete open reading frame starts with the ATG start codon at nucleotide residue 74 and ends with a TAA terminator at residue 1019, which covers 315 amino acids with an estimated molecular weight of 34,046 Da and an acidic pI of 5.37. Analysis of sequence homology demonstrated that this cDNA is similar to an acidic ribosomal phosphoprotein (P0), with about 92% of amino acid residues identical to the sequences in chick (database accession: P47826; Wang, Meury *et al.*, 1995), rat (accession: P199945; Wool *et al.*, 1991) and human (accession: P05388; Rich and Steitz, 1987) (Fig. 2.14). Therefore, clone Br15 represents a cDNA gene, designated as *P0*, encoding the acidic ribosomal phosphoprotein.

Clone Br30

Sequencing data indicated that clone Br30 contained a 1608-bp cDNA insert. Blast searching of databases showed that the cDNA was similar to the sequence of phosphoglycerate kinase 1, the PGK1 gene, another a nuclear-encoded gene. Both cDNA and deduced polypeptide sequences are presented in Fig. 2.15. A single long open reading frame (nt 1-1185) was recognized within the cDNA sequence and contained 395 amino acid residues. The cDNA contained a 423-nucleotide 3' untranslated region (UTR) with a poly(A) tail and a typical polyadenylation signal (AATAAA). The cDNA appeared to cover the complete 3' end of the gene. However, the cDNA insert seemed to be truncated at the 5' end since the 5' untranslated region and a short portion of protein-coding sequence were missing. Comparison of the deduced amino acid sequence of the Br30 insert with the PGK1 sequences of chick (database accession: P51903; Rauen *et al.*, 1994) and human (accession: P00558; Michelson *et al.*, 1983) indicated that Br30 cDNA was significantly similar to the avian and mammalian proteins, with 88% of amino acid residues identical to chicken and human PGK1 (Fig. 2.16). Thus, the frog Br30 cDNA gene encodes phosphoglycerate kinase 1, designated as the frog *PGK1*. Fig. 2.16 also indicates that nucleotides encoding ~22 amino acid residues are apparently missing from the 5' end of the wood frog cDNA.

Clone Br34

The Br34 cDNA insert was a 866-bp fragment as determined by sequencing analysis (Fig. 2.17). The insert had an internal *EcoR* I cutting site (5' GAATTC 3') which can result in 2 similarly sized bands (424 bp and 442 bp). There was no internal cutting site for *Xho* I. Thus, when the cDNA was treated with *EcoR* I and *Xho* I only one band appeared on the 1-1.5% agarose gel (see Fig. 2.1). The cDNA contained a short poly(A) tail but was short of the typical polyadenylation signal (AATAAA), while the 3' end of the cDNA seemed complete as confirmed by 3'RACE (data are not shown). Sequence similarity searching showed that this cDNA may be related to a *pol*-like reverse transcriptase gene. There was no relatively long open reading frame in the plus frames but in a minus frame (-3), a single ORF was determined. Its deduced amino acid sequence (266 residues) was related to the reverse transcriptase (973 residues) encoded by the CR1 gene of the CR1 element in the turtle (*Platemys spixii*) (Kajikawa *et al.*, 1997). The CR1 element functions as a retroposon to insert itself into the genome. Alignment of the potential polypeptide of Br34 with the corresponding portion of the reverse transcriptase showed about 52% identity (Fig. 2.18).

Isolation of Freezing-Inducible Genes from Frog Liver

My initial studies tried to isolate additional freezing-inducible genes from frog liver. The genes were isolated by screening the commercial cDNA library as described above. ³²P-labeled total cDNA probes were synthesized from poly(A)⁺ mRNA isolated from liver of control (acclimated at 5°C) and 24 h freezing-treated frogs. The probes (from control vs freezing stress) were used for screening the cDNA library. About 4.0 ×

10⁵ plaques from the frog liver cDNA library were differentially screened. Through this extensive primary screening, 75 candidate plaques with stronger signals by stress probes were obtained. They were designated as Li1- Li75 (the liver clones). These plaque clones were isolated and used for secondary screening. Thirty candidate plaque clones were selected and excised *in vivo* into pBluescript plasmids. The plasmid DNA was purified and applied to dot blotting analysis as the tertiary screening. The duplicated dot blots were hybridized with the total cDNA probes (from control vs freezing stress). The cDNA insert from each clone can be purified on agarose gel by the restriction digestion of plasmid DNA with *EcoR* I. The dot blot can also be used for cross-hybridization tests with the specific DNA probes derived from the tertiary screened clones (including the up-regulated liver cDNA clones previously isolated in our lab). Finally, 2 unique cDNA clones (Li16 and Li39; Fig. 2.19) were identified and the corresponding genes were confirmed as up-regulated by freezing in wood frog liver by Northern blot analysis.

The insert size of clone Li16 was estimated as 350 bp. A single transcript band was detected by Northern blotting and the RNA size was estimated to be about 0.5 kb (Fig. 2.20). Fig. 2.20 shows that the gene transcript in frog liver was up-regulated upon freezing exposure. Compared with controls (at 5°C), transcript levels appeared to be 2-2.5 fold higher in liver from frogs frozen for 12-24 h as well as in the recovery group (24 h freeze, 24 h thawed at 5°C). DNA sequencing indicated the insert of clone Li16 was a fragment of 308 bp. The nucleotide sequence is shown in Fig. 2. 21. It contained a polyadenylation signal (AATAAA) and a short poly(A) tail, which indicated that the cDNA probably contained a complete 3' end of the gene. A single open reading frame

(encoding 72 amino acid residues) was recognized within this cDNA sequence (nt 2-220). However, the 5' end of the cDNA insert did not contain either a 5' untranslated region or sequence coding for the N-terminus. In addition, since the insert size (308 bp) was shorter than that of the estimated gene transcript (0.5 kb by Northern blotting), the Li16 cDNA clone is clearly not complete. Blast searching of Genbank databases did not show any significant homologous match; however, the putative protein sequence (from residue 1 to 50) showed over 50% similarity with a portion of the cytoplasmic domain of the inositol 1, 4, 5-trisphosphate-binding protein type 2 receptor (Typ Insp3 receptor), a protein with 2,701 amino acids (accession: Q14571). A short protein sequence (residue 40-72) in the same reading frame had near 60% similarity with a peptide protein, xenoxin, produced by skin of *X. laevis* (accession: P38951). However, since the overall score for the homologous match was very low, the gene identity of clone Li16 is still unknown.

Clone Li39 had a very large insert which was estimated to be about 3.0 kb and 2 fragments of ~1.4 and ~1.6 kb were found after *EcoR I/Xho I* digestion (Fig. 2.19). Two distinct RNA bands (1.4 and 1.7 kb) (Fig. 2.22) were detected by Northern blots with the cDNA probes derived from the 1.4 and 1.6 kb fragments. DNA sequencing data showed the insert of clone Li39 was a fragment of 2935 bp (Fig. 2.23). There are 2 *EcoR I* recognition sites (GAATTC) for the cDNA insert and could result in 3 fragments (36, 1326 and 1573 bp) by *EcoR I* digestion. Since Li39 was isolated from the commercial liver cDNA library (*EcoR I* as cloning site), 2 *EcoR I* sites flanked at both ends of cDNA assumed to be present. However, the guanine of *EcoR I* recognition sequence in the vector adjacent to the 3' end of the insert seemed deleted. Therefore, double restriction

enzyme digestion (*EcoR* I/*Xho* I) was needed to release the insert from the vector. The *Xho* I was used for isolating the insert along with *EcoR* I because there is no *Xho* I cut site within the cDNA fragment. The enzyme digestion resulted in 2 fragments (~1.4 and ~1.6 kb) on 1.2% agarose gel, while the 36 bp fragment can not be seen on the gel. Sequencing analysis indicated that clone Li39 had an extremely high match with the sequences of a mitochondrial gene encoding for NADH-ubiquinone oxidoreductase, subunit 4 (ND4) and a nuclear gene encoding for translation elongation factor 1, gamma subunit (EF-1 γ). This combination of a nuclear and mitochondrial gene is not possible in nature and indicates, therefore, that two different cDNA fragments were joined by end-end ligation at the *EcoR* I cloning site, which may have resulted during the construction of the commercial cDNA library. The large insert (nt 1362-2935 of clone Li39) and the smaller insert (nt 1-1325 of clone Li39), were cloned to the pBluescript and pCR[®]2.1 vector, respectively and were designated as Li39l and Li39s, respectively. Both cDNA and deduced polypeptide sequences are presented in Fig. 2.24 and Fig. 2.26 for Li39s and Li39l, respectively. Comparison of the deduced amino acid sequence of the Li39s insert with the ND4 sequences of *X. laevis* (Roe *et al.*, 1985) and carp (Chang *et al.*, 1994) indicated that wood frog ND4 had about 47% identity with the *Xenopus* and carp proteins (Fig. 2. 25). The wood frog ND4 gene was designated as *ND4*. Comparison of the deduced amino acid sequence of the Li39l insert cDNA showed that the wood frog EF-1 γ had 90% and 78% identity with the proteins from *X. laevis* (accession: Q91375; Morales *et al.*, 1993) and humans (accession: P26641; Sanders *et al.*, 1992), respectively (Fig. 2.27). The wood frog EF-1 γ gene was designated as *EF-1 γ* . When the larger band on the

RNA blot was probed with Li39l only (Fig. 2.22B), levels of the larger gene transcript in frog liver showed about 2 fold increase upon freezing exposure for 4-12 h. However, transcript levels decreased again to the same level as in controls during later stages of freezing (24 h) as well as during recovery (Fig. 2.22A, B). The levels of the smaller gene transcript showed about 50% increase upon freezing exposure for 4-12 h as compared with the control at 5°C (Fig. 2.22A).

Summary of cDNA Clones Isolated from Frog Brain and Liver Tissues

Through extensive differential screening of cDNA libraries, 5 frog brain clones (Br3, Br4, Br15, Br30 and Br34) and 3 frog liver clones (Li16, Li39s and Li39l) were isolated and the expression of corresponding gene(s) for each clone was up-regulated in response to freezing exposure in the freeze-tolerant wood frog. Characterization of the isolated frog cDNA clones showed that both mitochondrial genes (ATPase 6 and 8 as well as 16S rRNA gene from brain; *ND4* from liver) and nuclear genes (*P0*, *PGK1* and a reverse transcriptase gene from brain; *EF-1 γ* from liver) were identified. In addition, a novel gene (the liver Li16 clone) was obtained.

The size of cDNA insert and gene transcript, the gene identity and database accession number for each analyzed clone are summarized in Table 2.2.

DISCUSSION

Modern techniques of molecular biology have been applied as powerful tools to elucidate the biochemical mechanisms of cold adaptation in organisms. Genes

responsible for cold hardiness in plants (Cherry, 1994), including the genes coding for embryogenesis abundant protein (Baker *et al.*, 1988), dehydrin (Close *et al.*, 1989), heat-shock proteins (Neven *et al.*, 1993), and antifreeze proteins (AFPs) (Griffith *et al.*, 1992) have been identified. Genes encoding for desaturase in fish (Tiku *et al.*, 1996), for ferritin H subunit in rainbow trout cells (Yamashita *et al.*, 1996), and for AFPs in cold water fish species (Davis *et al.*, 1999) and cold-hardy insects (Tyshenko *et al.*, 1997) were also cloned and characterized. Previous studies in our lab using differential screening of a wood frog liver cDNA library led to the discovery of new types of genes (encoding for fibrinogen, ADP/ATP translocase and a 10 kDa protein with a potential nuclear-exporting signal sequence) that were up-regulated under freezing exposure and may be involved in cold adaptation in this freeze-tolerant species (Cai and Storey, 1997a, 1997b; Cai *et al.*, 1997).

In current studies, additional genes that were up-regulated in frog liver in response to freezing exposure were identified by more extensive screening of the cDNA library. Included were the genes for EF-1 γ (elongation factor 1, γ subunit), ND4 (NADH-ubiquinone oxidoreductase, subunit 4) and a novel gene from clone Li16. The gene identity and function of clone Li16 is as yet unknown but the upregulation of EF-1 γ and ND4 genes are of interest.

EF1 is responsible for bringing aminoacyl-tRNA to the A site (aminoacyl site) of the ribosome during protein synthesis. EF1 consists of three subunits (α , β and γ). The functional recycling of EF-1 α (conversion of EF-1 α GDP to EF-1 α GTP) is assured by the EF-1 β and EF-1 γ complex (Morales *et al.*, 1993). The upregulation of the EF-1 γ gene

in frog liver may be a response to freezing or freezing-mediated stress (such as ischemic hypoxia/anoxia or intracellular dehydration) to help maintain the elongation process for protein synthesis. This implies that the translational machinery might be affected by the stress and that adjustments to one or more proteins involved in the translational machinery may be required to keep protein synthesis functioning optimally, particular for the synthesis of selected stress proteins used for protection from freezing injuries in wood frog. Indeed, the levels of EF1 protein are increased in the livers of cold-adapted toadfish (Plant *et al.*, 1977).

The upregulation of *ND4* further supports the idea that differential gene expression is involved in freezing survival. *ND4* is a mitochondrial gene and our studies have suggested that mitochondria may play an important role in endurance of various environmental stresses.

Mitochondrial DNA (mtDNA) is a double-helical and circular molecule. The structure and organization of the mitochondrial genome is strongly conservative through phylogeny (Boore, 1999). In vertebrates, the mitochondrial genome usually encodes 2 rRNAs (12S and 16S rRNA), 22 tRNAs and 13 proteins including 2 ATPase subunits (ATPase subunit 6 and 8), a cytochrome reductase subunit, 3 cytochrome oxidase subunits (COI, II and III) and 7 subunits of NADH-ubiquinone oxidoreductase (ND1-6, ND4L) (Wolstenholme, 1992). The mitochondrial genome displays exceptional economy of organization, with tRNA genes interspersed between the rRNA and protein-coding genes with zero or few non-coding nucleotides between the coding sequences. Two non-coding portions of the genome contain the origins of DNA replication. The larger portion is in the displacement-loop (D-loop) region of the heavy-strand DNA (H strand) and the

smaller one involves the replication of light-strand DNA (L strand) (Shadel and Clayton, 1997). Mitochondria maintain a complete protein-synthesis system that is physically and genetically distinct from the cytoplasmic system. The mtDNA encoded rRNAs and tRNAs are the components of the mitochondrial translation system. The mtDNA-encoded proteins are translated on mitochondrial ribosome and are the components of their enzyme complexes on the inner mitochondrial membrane. Mitochondria provide most of the ATP for eukaryotic cells through the process of oxidative phosphorylation (OXPHOS). Five multimeric enzyme complexes are involved in OXPHOS. These are assembled from approximately 100 subunits, 13-encoded by mtDNA and the remainder by nuclear DNA. The 13 proteins encoded by mtDNA contribute to four of the five OXPHOS complexes, including ND1-ND6 for Complex I, cytochrome b (cyt b) for Complex III, COI, II and III for Complex IV, and ATPase subunit 6 and 8 for Complex V (Wolstenholme, 1992; Moyes *et al.*, 1998). Only Complex II is entirely nuclear-encoded.

The isolated ND4 gene encodes one of the subunits of Complex I. This complex transfers the electrons from NADH to ubiquinone on the mitochondrial membrane. The bovine heart Complex I is composed of 41 polypeptide subunits (Ohnishi, 1993). Six of the 41 subunits are encoded by mitochondrial DNA and they are ND1 to ND6 as described above. Among the 13 mtDNA-encoded proteins, only the ND6 gene is located on the light strand (L strand) and the rest of the mitochondrial protein-coding genes as well as 2 rRNA genes are located on the heavy strand (H strand). The upregulation of *ND4* in frog liver may respond to declining oxygen as freezing progresses and may help to optimize the use of limited remaining oxygen supplies as the animal makes a transition to the ischemic state imposed by freezing.

Upregulation of other mitochondrial genome-encoded genes in response to stress has also been reported. Other studies in our lab showed that genes encoding NADH-ubiquinone oxidoreductase, subunit 5 and cytochrome oxidase subunit 1 (COI) were both up-regulated by oxygen deprivation in the heart of anoxia-tolerant turtles (*Trachemys scripta elegans*) (Cai and Storey, 1996). Northern blotting analysis indicated that both gene expression were also up-regulated by freezing in the freezing tolerant hatchlings of the turtle *Chrysemys picta marginata* (Cai and Storey, 1996). Accumulation of selected mitochondrial gene transcripts have also been reported in mammals in acute myocardial stress in cold environment. The upregulation of mtATPase 6 & 8, COI, II, III and cyt b genes under global ischemia and reperfusion was found in the rat heart (Das *et al.*, 1995). The mitochondrial 16S rRNA, and ATPase 6 & 8 gene transcripts were accumulated in high levels in human myoblasts and increased progressively as myotubes matured (Webster *et al.*, 1990). The accumulation of mitochondrial COII transcript and 16S rRNA was also reported in the liver of rats exposed to cold stress (at 4°C) (Martin *et al.*, 1993). In contrary, oxidative stress leads the down-regulation of mammalian mitochondrial RNAs (such as the 16S RNA) (Crawford *et al.*, 1997).

The regulation of energy metabolism is a common phenomena among naturally stress-tolerant organisms and metabolic rate depression is a common mechanism that is used by many species to survive harsh environmental conditions such as low oxygen tension, low temperature or low water availability. Upregulation of mitochondrial genes may be adaptive for environmental stress tolerance by helping to maintain generation of ATP to be used for cellular adjustments even under extreme adverse conditions.

The upregulation of several other mitochondrial genes isolated from wood frog brain further suggested that mitochondria may play an important role in natural stress survival. As discussed above, mitochondria have their own transcription and translation system. The upregulation of the mitochondrial 16S rRNA gene from frog brain may be a response to freezing exposure, and may be required for maintaining mitochondrial translational machinery.

The regions coding for the two rRNAs are located immediately downstream of the H strand transcription initiation site. Mitochondrial transcription can be terminated following the synthesis of the rRNA transcripts. Alternatively, it can continue until the entire DNA strand is transcribed. In general, mtDNA transcription is asymmetric and produces polycistronic transcript. The rate of transcription of mitochondrial rRNA is an order of magnitude faster than that of other mitochondrial mRNAs (Gelfand and Attardi, 1981), which mainly results from the premature termination of the primary transcripts beyond the rRNA species relative to that of the mRNAs or most of tRNAs. Since the rRNAs are major components of mitochondrial ribosomes, the higher level of rRNAs may be required for the mitochondrial translational activity. Frog brain cDNA clone Br4 represented part of the 16S rRNA gene. The cDNA contained a short poly(A) tail, which may result from a novel polyadenylation processing within the rRNA transcript.

Mitochondrial pre-rRNAs and mRNA transcripts are polyadenylated. In the processing of mitochondrial primary transcripts, the tRNA sequences provide the punctuation and serve as recognition sites for endonucleases to excise tRNAs from the polycistron, which is quite different from the polyadenylation processing of nuclear gene-coded mRNAs.

The poly(A) tail for the pre-rRNA may be related to the maintenance of its stability and it is removed during generation of the mature rRNA. For the frog brain 16S rRNA gene, it is unclear why the pre-rRNA transcripts accumulate during freezing stress and its significance for freeze tolerance remains undetermined.

The expression of mitochondrial ATPase 6 and 8 genes further suggested freezing-induced gene regulation may be related to the control of ATP generation. Mitochondrial ATPase subunit 6 and 8 along with nuclear gene-coded ATPase subunits are components of the F_0F_1 ATPase complex of the OXPHOS Complex V. The function of F_0F_1 ATPase is to synthesize ATP using a proton gradient on the mitochondrial inner membrane (Boyer, 1997). The increased levels of mtATPase transcripts, presumably leading to increased amounts of the protein product, may help to optimize the use of declining oxygen supplies during freezing for the generation of ATP for stress survival.

In addition to the above isolated mitochondrial genes, several nuclear genes were also identified as up-regulated during freezing exposure in frog brain. Another gene whose product associated with ribosomal activity, the acidic ribosomal phosphoprotein (P0) gene, was isolated and is apparently involved in the response to freezing stress. The protein P0, together with protein P1 and P2, forms the stalk of eukaryotic ribosomes. The P0, P1 and P2 complex interacts at the GTPase domain in the large subunit of ribosome and involves protein synthesis. The cytoplasmic ribosomes of eukaryotes contain over 80 different proteins. The amount of each protein species as well as the level of each RNA component in the ribosome must be regulated strictly and adjusted to cell growth conditions and demand for protein synthesis (Mager, 1988). Coordinated expression of ribosomal genes is well known for *E. coli*. The cluster organization of the operon is

exploited and stringent control over RNA and protein synthesis is exerted. Moreover, translational regulation occurs through binding of ribosomal protein to its own mRNA. Much less is known about the regulation of ribosomal genes in higher eukaryotes. The isolation of ribosomal genes of the higher eukaryotes and the study of their expression are fundamental steps for the elucidation of the complex mechanism of co-regulation.

The protein P0 is essential for cell viability. The highly conserved protein P0 is analogous to bacterial ribosomal L10 but carries an additional carboxyl domain showing a high sequence homology to the acidic proteins P1 and P2, including the terminal peptide DDDMGFGLFD. The P0 carboxyl end is essential for ribosome activity in the absence of P1 and P2. In the absence of P0, deficient 60S ribosomal subunits are assembled which are inactive in protein synthesis resulting in cell lethality (Santos and Ballesta, 1994, 1995; Remacha *et al.*, 1995). Interestingly, the upregulation of P1 was reported in a freeze-tolerant insect by our lab (Bilgen, Ph.D. Thesis, 1998). The upregulation of the P0 gene may be a response to the low temperature (or other freezing-associated stresses) which inhibits protein synthesis. Indeed, induction of ribosomal protein genes or genes related to the translational system in response to cold shock has been well documented in several model organisms including *E. coli*, yeast and fruit fly (Jones and Inouye, 1994).

Another gene directly involved in ATP production, the phosphoglycerate kinase 1 (PGK1) gene, was identified from frog brain. PGK1 (EC 2.7.2.3) is one of two glycolytic enzymes that generate ATP under anoxic conditions. Recently it was reported that the enzyme can be transported into the nucleus and can function as a transcription factor in regulating gene expression (Ronai, 1993). The activation of PGK1 gene expression has

also been reported as a response to hypoxia in mammalian cells and is regulated by a newly identified transcription factor, hypoxia-inducible factor (HIF1) (Semenza and Wang, 1992; Semenza *et al.*, 1994; Li *et al.*, 1996). The upregulation of the PGK1 gene in frog brain may be related to the ischemia that develops when plasma freezes.

Finally, the clone Br34 may encode a *pol*-like reverse transcriptase that may belong to a family of retroposons. The retroposon elements generated by the reverse flow of genetic information can be inserted into a genome (Weiner *et al.*, 1986). The inserted retroposon elements can be repeated hundreds or even thousands of times such as the CR1 element that was found in chicken genome (Burch *et al.*, 1993). The CR1-like elements appear to exist in all classes of vertebrates. CR1 elements have common 3' end but most of them are extensively truncated at their 5' end. Recently members of CR1 family encoding an ORF segments were isolated and these segments contained *pol*-like ORF (Kajikawa *et al.*, 1997). The sequence of clone Br34 showed high similarity with a ORF of turtle CR1 element that encodes a reverse transcriptase (Kajikawa *et al.*, 1997). The clone Br34 may represent part of the transcriptase gene whose expression was induced during freezing exposure. However, the significance of the upregulation of this gene for stress survival remains unknown.

The identification of the brain genes has provided the first evidence that this central organ shows active responses to freezing exposure. Along with the frog liver, genes isolated from current studies, can be divided into 3 groups:

1. Genes related to translational machinery (e.g. ribosomal proteins or rRNA).
2. Genes directly associated with ATP generation (e.g. mtATPase and PGK1).

3. Genes encoding proteins related to interactions with nucleic acids (RNA or/and DNA) (e.g. clone Br34 and probably also PGK1 gene).

Further studies are required to answer the following questions. Is the expression of these genes brain- or liver-specific in the freeze-exposed frogs or are they up-regulated in multiple organs? *In vivo* gene expression in other tissues of freeze exposed frogs was tested by Northern blotting with the probes derived from the isolated genes (see Chapter 4). What signals are involved in the upregulation of the isolated genes? The upregulation of mtATPase 6 & 8, *ND4* and PGK1 gene suggested that the O₂ level may trigger expression of these genes. Therefore, we were interested in analyzing gene expression from the frogs treated under other stresses (such as anoxia and dehydration) with the isolated genes as probes (also see Chapter 4).

The freezing-induced upregulation of P0 and EF-1 γ gene may result from low temperature or freezing-associated other stresses. I assumed that the cold acclimation stage might also be critical for frog freeze survival. I tried to isolate cold acclimation-related genes from frog skin as described previously. Unlike the quick responses under freezing, heat shock or cold shock, cold acclimation may take days or even weeks. Therefore, it is difficult to define the time-points needed to make a suitable cDNA stress library for differentially screening stress-induced genes. Instead, via the new technique of mRNA differential display PCR (DD-PCR), the construction of a cDNA library can be avoided and transcripts with relatively low abundance can possibly be isolated. The use of DD-PCR to identify cold acclimation-regulated genes from frog skin is described in the next Chapter.

Table 2.1. Primers used for DNA cloning and sequencing

Primer	Sequence
T3	5' AATTAACCCTCACTAAAGGG 3'
T7	5' GTAATACGACTCACTATAGGGC 3'
M13 forward	5' GTAAAACGACGGCCAGT 3'
M13 reverse	5' GGAAACAGCTATGACCATG 3'
SK	5' CGCTCTAGAACTAGTGGATC 3'
KS	5' TCGAGGTCGACGGTATC 3'
(primers from cDNA clones)	
Br30-T3-1	5' CTAAGGTAGAAGCTTTCC 3'
Br30-T3-2	5' GACTGTGGGCCAGAAAGC 3'
Br30-T7-1	5' CCAGTACTGACATGGCTG 3'
Br30-T7-2	5' GATCAGCTGGATCTTGTC 3'
Br34-T3-1	5' CACTGATAACCTTAGACC 3'
Br34-T7-1	5' CGCCAAGCTATGCAGTGG 3'
Li39-T3-1	5' GCTCTATAATCTTAGCTG 3'
Li39-T3-2	5' ACTGATCTCCTGTGCCTTCTTATTTGTT 3'
Li39-T7-1	5' GATGACGCTAGCAAGCCTG 3'
Li39-T7-2	5' TGGGGCAGGAGCAGGCTTCTTTTC 3'

Note: The primers derived from the frog cDNA clones were made either in the DNA Synthesis Lab at Queen's University or in the Canadian Molecular Research Services Inc. Ontario.

Table 2.2. Summary of wood frog brain and liver cDNA clones

Clone	Size of Insert (nt)	Size of Transcript (kb)	Gene Identity and GenBank Accession Number
Br3	818	1.05	mitochondrial (mt) ATPase 6 & 8 (AF175984)
RBr3a	1543		mt ATPase 6 & 8, COIII (AF175985)
RBr3b	1851		mt ATPase 6 & 8, COIII (AF175976)
Br4	761	1.6	mt 16S rRNA (AF175977)
Br15	1098	1.15	acidic ribosomal phosphoprotein (P0) (AF176302)
Br30	1608	0.85, 1.7	phosphoglycerate kinase 1 (PGK1) (AF175978)
Br34	866	2.2, 3.5	<i>pol</i> -like reverse transcriptase gene (AF175979)
Li16	308	0.5	unknown (AF175980)
Li39s	1325	1.4	NADH-ubiquinone oxidoreductase subunit 4 (ND4) (AF175981)
Li39l	1573	1.7	elongation factor 1, gamma subunit (EF-1 γ) (AF175982)

Note: Br: designated for frog brain clones; Li: for frog liver clones. kb: kilobases; nt: nucleotides; RBr3a and RBr3b were the re-screened cDNA clones probed with clone Br3.

Figure 2.1. Agarose gel analysis of wood frog brain cDNA clones. The inserts of the cDNA clones were isolated by restriction enzyme digestion of plasmid DNA with *EcoR* I and *Xho* I and separated on 1 or 1.5% agarose gel (in 1× TAE buffer). The gels were stained with ethidium bromide. M: molecular weight standards (1 kb DNA ladder from GIBCO-BRL). **Note:** DNA sequencing analysis showed that the Br34 cDNA insert had an internal *EcoR* I cutting site (5'GAATTC3') (see Fig. 2.17) which can result in 2 similarly sized bands (~0.45 kb). Thus the estimated insert size for the clone Br34 was ~0.9 kb.

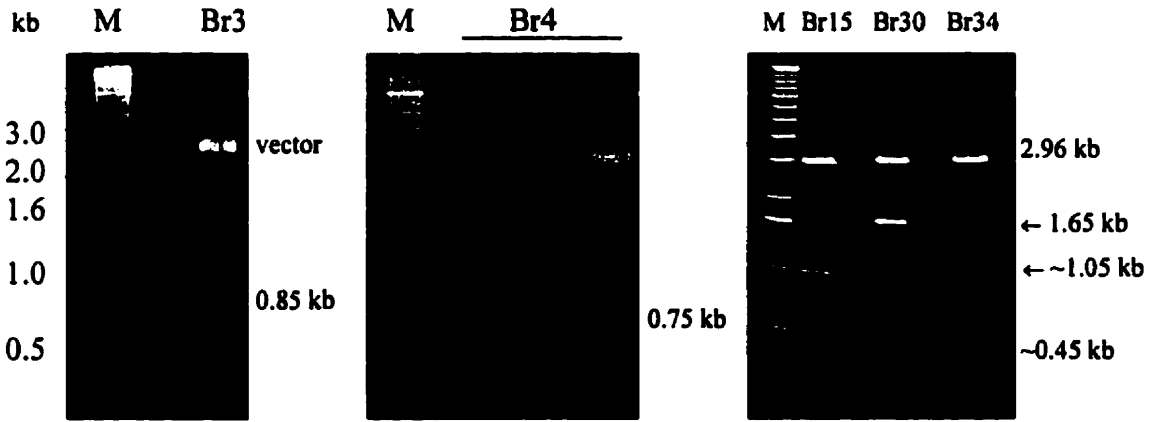


Figure 2.2. Representative Northern blot of brain RNA from wood frog *Rana sylvatica* probed with the insert of clone Br3. An equal amount of mRNA (1 μ g) was loaded in each lane. Equal loading was confirmed by re-probing with EF-1 γ gene (encoding for elongation factor 1, gamma subunit) as an internal control for assessing the RNA loading and quality. The EF-1 γ cDNA gene was isolated from frog liver and was not up-regulated in frog brain. The RNA transcript size was estimated based on the molecular size standards (RNA ladder from GIBCO-BRL) against the migration distance on the gel. C: control, cold acclimated frogs (at 5°C for 2 weeks); F: freezing at -2.5°C for 24 h; R: freezing for 24 h and recovery at 5°C for 24 h. The RNA blotting and hybridization were repeated with multiple trials and the data shown in the graph represent the average value from the repeats (n = 3). Analysis of band intensities using Imagequant. The target RNA band intensity in each lane is normalized to the intensity of the band of EF-1 γ in the same lane and the value of relative RNA band intensity for the test groups is normalized to the control group.

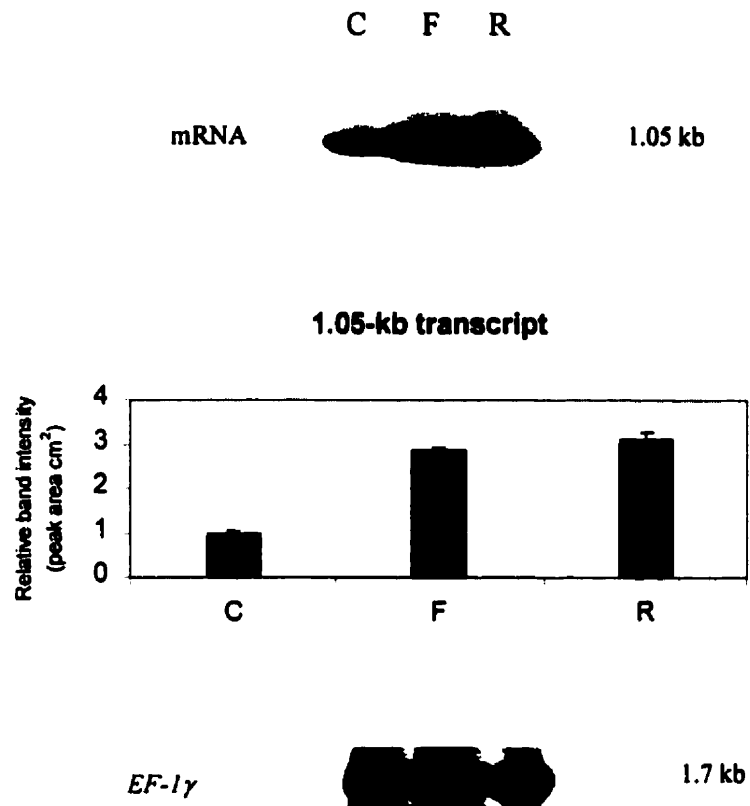
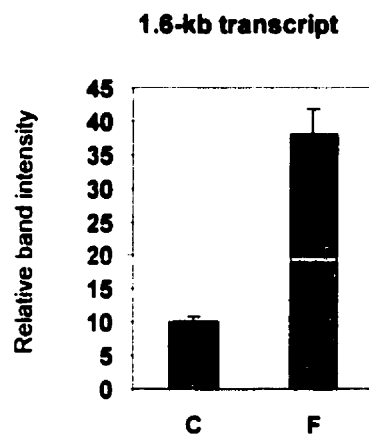
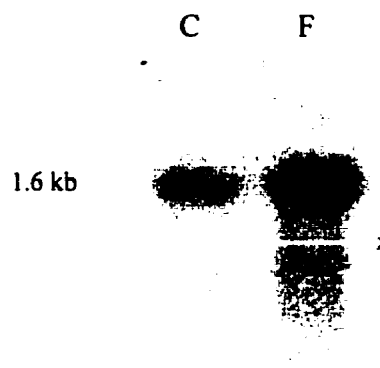


Figure 2.3. Frog brain RNA samples probed with clone Br4. Both blots were loaded with mRNA samples (1 μ g mRNA per lane). The RNA blotting and hybridization were repeated with identical blots and the data shown in the graph represent the average value from the repeats ($n = 2$). The value of the relative RNA band intensity is normalized to control.

A. C: control at 5°C; F: freezing for 24 h. The RNA loading was also assessed by staining the mRNA blot with methylene blue.

B. The mRNA blot was the blot used for probing by clone Br3, Fig. 2.2. The blot was re-probed with EF-1 γ gene (encoding for elongation factor 1, gamma subunit) as an internal control. C: control at 5°C; F: freezing for 24 h; R: freezing for 24 h and recovery at 5°C for 24 h.

A.



B.

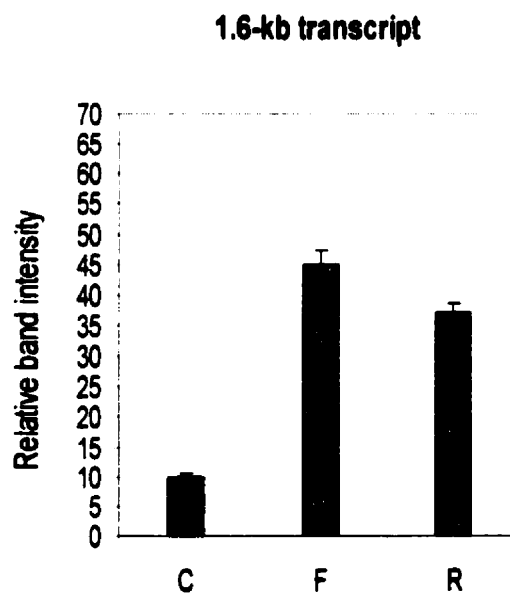
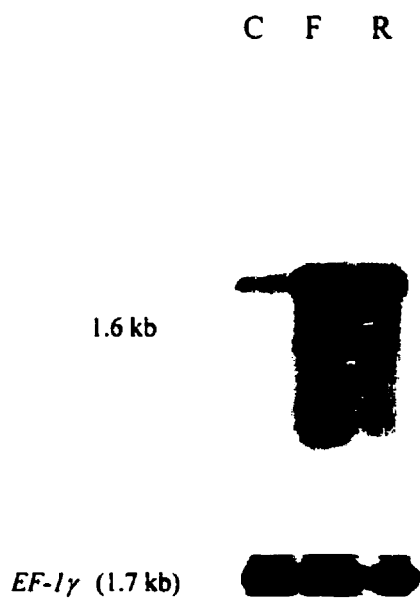
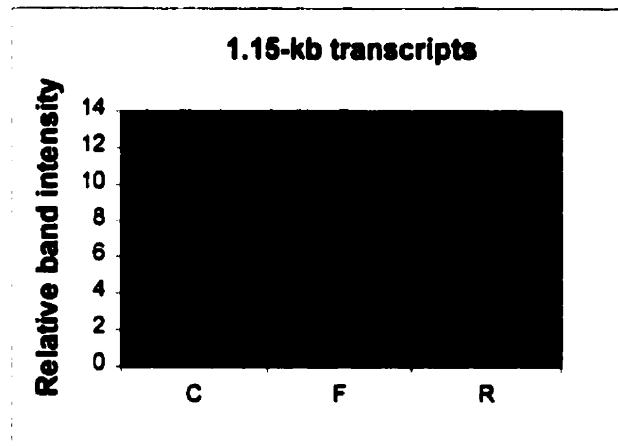
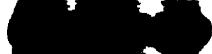


Figure 2.4. Representative Northern blot of brain RNA from wood frog *Rana sylvatica* probed with the insert of clone Br15. The mRNA (1 μ g) was loaded for each lane and the blot was re-probed with EF-1 γ gene as an internal control for assessing the RNA loading and quality. C: control at 5°C; F: Freezing for 24 h; R: freezing for 24 h and recovery at 5°C for 24 h. The mRNA blot was same as the blots used for probing by clone Br3 (Fig. 2.2) and clone Br4 (Fig. 2.3B). The RNA blotting and hybridization were repeated with multiple trials and the data shown in the graph represent the average value from the repeats (n = 3).

C F R



EF-1 γ  1.7 kb

This figure shows a Northern blot for the *EF-1 γ* gene. It has three lanes labeled C, F, and R. A single, dark, prominent band is visible in all three lanes, indicating the presence of a 1.7 kb transcript. The band is positioned to the left of the '1.7 kb' label.

Figure 2.5. Frog brain RNA samples probed with clone Br30.
The RNA blot was identical to that used for probing with clone Br4 gene as shown in Fig. 2.3A. C: control at 5°C; F: freezing for 24 h. The value of relative RNA band intensity is normalized to the 0.85-kb RNA transcripts of the control group.

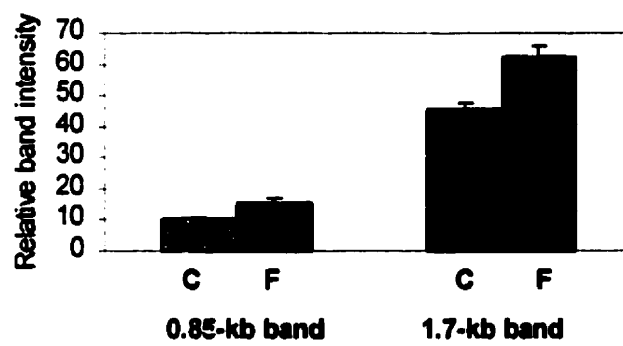
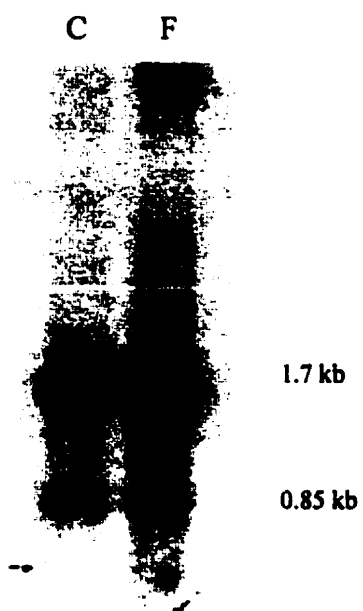



Figure 2.6. Frog brain RNA samples probed with clone Br34 and RT-PCR for quantifying the transcripts with primers derived from the clone Br34.

A. Northern analysis. The identical mRNA blot were also used for probing with clone Br3, Br4 and Br15, as shown in Fig. 2.2, Fig.2.3B and Fig. 2.4, respectively. C: control at 5°C; F: freezing for 24 h; R: freezing for 24 h and recovery at 5°C for 24 h. The value of relative RNA band intensity is normalized to control.

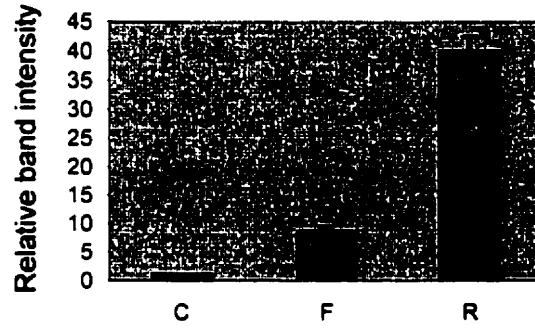
B. RT-PCR. C: control at 5°C; F: freezing for 24 h. Equal amounts of poly(A)⁺ mRNA (1 µg) isolated from brain tissues of control or freeze-exposed frogs were used for reverse transcription (RT). The RT lysates (C as the control group and F as the freezing test group) were then used for the RT-PCR and DD-PCR. Primer Br34-T3-1 and Br34-T7-1 were applied for the RT-PCR. The RT-PCR products were separated on 3% agarose gel (in 1x TAE buffer) and the gel was stained with EtBr. The amplified 0.35-kb fragment was confirmed as the expected part of Br34 cDNA insert by sequencing analysis. The level of the 0.35-kb fragment was much higher in freezing group. The 0.45-kb band was down-regulated and was nonspecific to Br34 cDNA by sequencing analysis. The level of cDNAs in the RT lysates from control and test group can be assessed by the DD-PCR (arbitrary primer: AP4-AP8; anchor primer: H-T₁₁G). The details are described in Chapter 3 and also shown in Fig. 3.12.

A

C F R

RNA  3.5 kb
2.2 kb

3.5-kb RNA transcripts



EF-1 γ



1.7 kb

B

DD-PCR

AP4 AP5 AP6 AP7 AP8
C F C F C F C F C F



RT-PCR

kb M C F

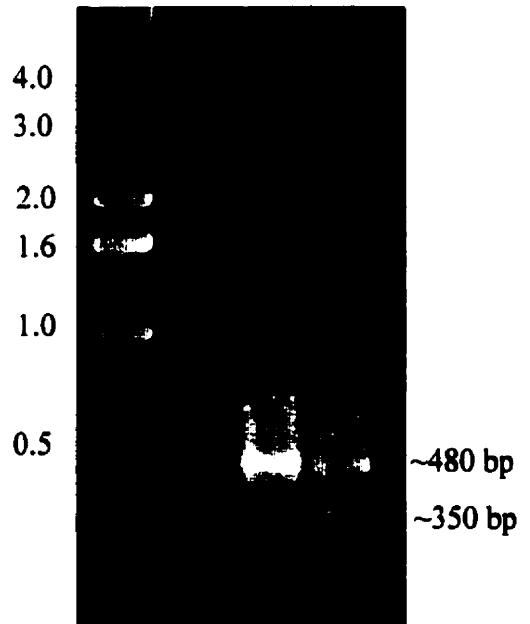


Figure 2.7. Nucleotide and deduced amino acid sequences of clone Br3.

The vertebrate mitochondrial genetic codon system was applied for sequencing analysis. Nucleotides and amino acid residues (in bold) are numbered on the left; the amino acid sequence from the short open reading frame (ORF) is underlined and the potential complete amino acid sequence from the longer ORF is non-underlined. The predicted polypeptide sequences are shown using the 1-letter amino acid code (see Table 2.4). The start codon (ATG) and poly(A) tail are underlined. The stop codon (TAA) is marked with an asterisk.

Figure 2.8. Comparison of the deduced amino acid sequence of frog clone Br3 with mitochondrial ATPase subunits from *Xenopus laevis* and carp (*Cyprinus carpio*).

- A. Comparison of the deduced amino acid sequence of short ORF of clone Br3 with mitochondrial ATPase subunit 8 from *Xenopus laevis* and carp. The amino acid residues as dashed lines are identical to those in the *Xenopus laevis*.

- B. Comparison of the deduced amino acid sequence of the long ORF of clone Br3 with mitochondrial ATPase subunit 6 from *Xenopus laevis* and carp. The amino acid residues as dashed lines are identical to those in the wood frog.

A.

<i>X. laevis</i>	MPQLNPGPWFLILIFSWLVLLTFIPPVKLKHKAFNEPTTQ	40
Carp	-----n--lf-mfm--t-f-flmq--ltsfist-s-snk	40
Br3v--ifilla-a-i-s-tnl---nsk	25
<i>X. laevis</i>	TTEKSKPNPWNWPWT	55
Carp	nkttlt-t--t---	54
Br3	n.t-thkfm-t---	38

B.

Wood Frog	MMTDLFSQFASTTSFGTPIILVAMLVPWLLFPTSPK.WV	39
<i>X. laevis</i>	-nlsf-d--m-pvil-i-l-ai---d-ft-isw-iqsnqf	40
Carp.	-nlsf-d---pqll-i-l--lsl-f-t--l-s-nnr.-i	39
Wood Frog	NNRLLTFQSWFLNLFTKQLLLPLNTPAHKWAFLLASLMVF	79
<i>X. laevis</i>	----i-l-----hn--tifyq.-ts-g----l--t---ll	79
Carp.	----s-l-l---q-i----mm---kag----li-t---t-	79
Wood Frog	LLSMNLLGLLPYTFTPTTQLSINLGLAVPLWLATVLTGFR	119
<i>X. laevis</i>	-m-l-----l-m-----imas.	118
Carp.	--li-----m-ma--f-----l---l-	119
Wood Frog	NQFNHSLAHFLPEGTPAPLIPVLILIIETISLFIPLALGV	159
<i>X. laevis</i>	kpt-ya-g-l-----t-----i-----	158
Carp.	--psi--g-l-----t----a-----t--l-----	159
Wood Frog	RLTANLTAGHLLIHLISSAVSTLFSLSIASVLLTFTVLIL	199
<i>X. laevis</i>	-----q--at-afv-l-impt-ai--si--f-	198
Carp.	-----q--t-tla-lptmpti-v--a---l-	199
Wood Frog	LTILEIAVAMIQAYVFILLLSLYLQENT	227
<i>X. laevis</i>	--l-----v-----v	226
Carp.	----l-----v-----v	227

Figure 2.9. Southern blot test of re-screened clones probed with Br3. Plasmid DNA was digested with with *EcoR* I and *Xho* I and separated on 1% agarose gel (in 1x TAE buffer).

A. The gel was stained with ethidium bromide. M: molecular weight standards (1 kb DNA ladder).

B. DNA blot was probed with Br3 insert. lane 1: clone Br3, lane 2-3: re-screened clones from frog brain cDNA library; lane 4-7: re-screened clones from frog liver cDNA library. Lane 4 is for clone RBr3a and lane 6 is for clone RBr3b.

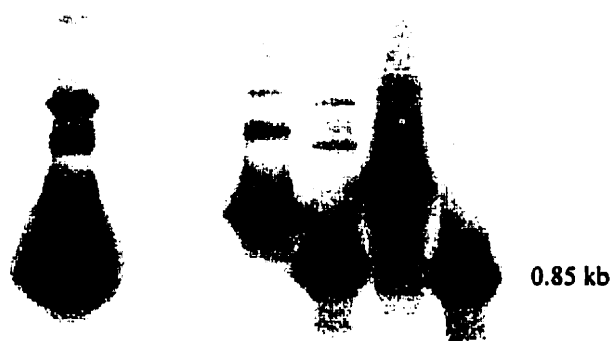
A.*B.*

Figure 2.10. Nucleotide and deduced amino acid sequences of clone RBr3a. Nucleotides and amino acid residues (in bold) are numbered on the left. The start codon (ATG) is in bold. The stop codon (TAA) and poly(A) tail are underlined. An asterisk marks the stop codon for the ATPase 8 gene. Clone RBr3a encoded 3 mitochondrial genes, the partial sequence for ATPase 8 and COIII gene and the complete ATPase 6 gene. The predicted polypeptide of ATPase 6 was identical to the coding sequence of same gene from clone Br3 as shown in Fig. 2.7. RBr3a ATPase 8 contained a polypeptide of 44 amino acid residues and the 6 residues at the upstream of Br3 ATPase 8 (38 residues) are underlined. The cDNA clone RBr3a showed a novel polyadenylation processing appeared within the COIII transcripts.

Figure 2.11. Nucleotide and deduced amino acid sequences of clone RBr3b. Nucleotides and amino acid residues (in bold) are numbered on the left. The start codon (ATG) is in bold. The stop codon (TAA) is underlined. An asterisk marks stop codon for the ATPase 8 gene. Clone RBr3b contained the partial sequence of ATPase 8 and the complete COIII and ATPase 6 gene. The predicted polypeptide of ATPase 6 was identical to the coding sequence of the same gene from clone Br3 and is shown in Fig. 2.7. The ATPase 8 fragment of clone RBr3b encoded a polypeptide of 47 amino acid residues and the 3 residues at the upstream of RBr3a ATPase 8 (44 residues) are underlined.

ATPase 8 (partial)

1 GTTCCATGACTTTATTATCTCCTTCTCGTCTGATTAATCTTTATCCTTCTAGCCCCGCT
 1 V P W L Y Y L L L V W L I F I L L A P A
 61 AAAATTTTAAGCCATACTAATCTTAATGAGCCAAATCTAAAAATACTAAAACACATAAA
 21 K I L S H T N L N E P N S K N T K T H K

ATPase 6

121 TTTATGTGAACTTGACCATGATAACAGACTTATTTAGCCAATTTGCCTCAACAACCTTCAT
 41 F M W T W P W *
 181 TTGGTACCCCCATTATCCTCGTAGCTATATTAGTCCCATGATTATTATCCCTACACCTT
 241 CACCTAAATGAGTAAATAATCGTCTTTTAACTTCCAAAGCTGATTTTTAAATCTGTTTA
 301 CCAAACAACCTTCTCCTACCCCTGAATACGCCAGCCACAAATGAGCTTTCCTCCTAGCCT
 361 CTTTAATAGTATTTCTTCTCAGCATAAATCTCCTGGGATTATTACCCTATACATTCACCC
 421 CAACAACCCAACCTATCAATTAATTTAGGATTAGCCGTACCCTTGGACTCGCAACTGTTC
 481 TTAAGTGGTTCCGCAACCAATTTAACCACTCTCTCGCACATTTCTCCAGAAGGTACTC
 541 CGGCCCTTTAATCCCTGTTTTAATTCTAATCGAAACCATCAGCCTTTTTATTTCGACCTT
 601 TAGCCCTAGGAGTCCGACTTACCGCCAACCTCACTGCCGGACACCTACTTATTTCATCTAA
 661 TTTTCATCAGCTGTATCTACGTTATTCTCCTTATCTATCGCAGTCTTTTATTGACCTTCA
 721 CCGTCCCTCATCCTTCTTACAATCCTAGAAATCGCAGTTGCCATGATCCAAGCCTATGTCT
 781 TTATTCTTCTTTTAAAGCCTGTATCTACAAGAAAATACTTGAGTCCGACTTACCGCCAACC
 841 TCACTGCCGGACACCTACTTATTTCATCTAATTTTCATCAGCTGTATCTACGTTATTCTCCT
 901 TATCTATCGCAGCCTCTTTATTGACCTTACCCTCCTCATCCTTCTTACAATCCTAGAAA
 961 TCGCAGTTGCCATGATCCAAGCCTATGTCTTTATTCTTCTTTTAAAGCCTGTATCTACAAG

COIII

1021 AAAATACTTAAATGGCCCCACCAAGCTCACGCCTATCACATAGTTGACCCCAGCCCTTGACC
 1 M A H Q A H A Y H M V D P S P W P
 1081 TCTCACAGGAGCCGCCGCCGCTCTTCTAGTAACGTCAGGCCTTGCCGCATGATTTTCATTT
 18 L T G A A A A L L V T S G L A A W F H F
 1141 TAACACTTTCATCTTACTAGCCCTAGGACTAACCTTAATGCTATTGACTATATATCAATG
 38 N T F T I L A L G L T L M L L T M Y Q W
 1201 ATGACGAGATGTCGTTCCGGAAGGTACCTTCCAAGGCCATCACACCCACCAGTACAAAA
 58 W R D V V R E G T F Q G H H T P P V Q K
 1261 AGGCCTTCGTTATGGCATAATTTTATTATTACCTCTGAAGTATTCTTCTTCGTCGGCTT
 78 G L R Y G M I L F I T S E V F F F V G F
 1321 TTTCTGAGCATTCTATAACTCCAGTCTTGCCCCAACCCAGAACTAGGAGAATGCTGACC
 98 F W A F Y N S S L A P T P E L G E C W P
 1381 ACCAACAGGAATTACTCCATTAAACCCATTTGAAATCCCCCTTCTAACACAGCAGTCCT
 118 P T G I T P L N P F E I P L L N T A V L
 1441 ACTAGCATCAGGTGTTACCGTTACATGAGCCCATCACAGTATTATACAAGCTGACCGTAA
 138 L A S G V T V T W A H H S I M Q A D R K
 1501 GGGGACTCTTCAAGCCCTTACCTTAACAATTACCCTCGGCCTTTACTTTACTCTTCTCCA
 158 G T L Q A L T L T I T L G L Y F T L L Q
 1561 AGCTATGGAGTATTATGAAGCCCCTTTTACAATTGCAGATGGAATTTATGGTACAACCTT
 178 A M E Y Y E A P F T I A D G I Y G T T F
 1621 CTTTGTAGCTACAGGCTTCCACGGCCTACATGTAATCATCGGCTCAATCTTCTTATACA
 198 F V A T G F H G L H V I I G S I F L M H
 1681 TTGCCCTCGCTCAAAATCACATATCACTTTACTTCACACCTCCATTTGGCTTTGAGGC
 218 C L L R Q I T Y H F T S H L H F G F E A
 1741 GGCATGATACTGACACTTCGTAGACGTGGTTGACTTTTTCTTTATGTTCAATTTACTG
 238 A W Y W H F V D V V W L F L Y V S I Y W
 1801 ATGAGGATCATAAAAA
 258 W G S *

Figure 2.12. Comparison of DNA sequences from wood frog clone Br4 with mitochondrial 16S rRNA gene of *Xenopus laevis*. The 16S rRNA gene sequence (from nucleotide 1- 1630) of *Xenopus laevis* is shown in the lower line. Dashed lines are identical nucleotides between wood frog and *Xenopus laevis*. The alignment region for the two fragments showed 74% identity. The short poly(A) tail of clone Br4 is in bold and underlined.

Clone Br4	0
<i>X. laevis</i>	tccaaaaacctagcattccaattataataacaataacctcatattctcat	50
Br4CCTAAACAAAACATTTTTATATTTTAGTACAGGCGATCGAAAAAT	45
<i>X. laevis</i>	aaat---tt---c---c-a-a-----t-----a---c---	100
Br4	TCTAAGCGCTTCAGACAAAGTACCGCAAGGGAAAGATGAAATAGAAATGA	95
<i>X. laevis</i>	ca---ta---at---a-----t-----,-----	149
Br4	AATAACCT.TAAAGCCTTAAATAGCAGAGATATTGCCTCGTACCTTTTGC	144
<i>X. laevis</i>	-----ttaact---aac---a-----ac--a-----	199
Br4	ATCATGGTCTAGCTAGTC.TACCCAAGCAAACGAACTTTTAGTTTGAC	193
<i>X. laevis</i>	--a-----c---a---at-----,---c-----	247
Br4	ATCCCGAAACTAAGTGAGCTACTTCAAACAGCCCTA.GGGGCCAACCCG	242
<i>X. laevis</i>	ta-----c---t---c-g-g---tt-tta-a--a-----	297
Br4	TCTCTGTTGCAAAGAGTGGGAAGATTTTAAAGTAGAGGTG.ATAAGCCT	291
<i>X. laevis</i>	-----g-----c---ccg---g---t-c-ga--a	347
Br4	ACCGAACTTAGAGATAGCTGGTTGTTTCAGGAAAAGAGTTTAGCTCTACC	341
<i>X. laevis</i>	-a---g-c---t-----c-----t--a-a-a--t-----	397
Br4	TTAAGCTTCCCCATTTTACCAAAAATGCCTCAA...GCTTAAGAGCTAT	388
<i>X. laevis</i>	c---ata-agatt---a--a-tt--a-taaa--gtcta---g--tt---	447
Br4	TCAAATAAGGCACAGCTTATTTGAAATAGGATACAACCTCCAACATCGGG	438
<i>X. laevis</i>	----tc-g--t-----c-ga-----c-----at--t-ct---	497
Br4	TAAA..TTATAGTAATTAATAATGA.....AGTGGGCCTAAAAGCAGC	478
<i>X. laevis</i>	----ga-----a-ct-c--ggaa-gttgagtc-----	547
Br4	CATCTTTAAAAA.AGCGTTAAAGCTTAATTA.TTATT..ACTAAAAAATT	524
<i>X. laevis</i>	--c--g---g-c-----c-----c-c-c-a-c---ta--ccttt---	597
Br4	TCT.TAAATTCTAATTAACCCCTCATTTTACTGAACTGCTTTATATTTT	573
<i>X. laevis</i>	ag-a---c-aa-tc-a-----ca-aca-----g--at-c---aac-	647
Br4	ATAAAAGTAATAATGCTAGAACTAGTAACAAGAAATCGCTTTTCTCCTAA	623
<i>X. laevis</i>	---g---c-c-t-----tg--t-.a-ga-----	696
Br4	ATGTAAGCATAAACCAAAATGGACCACCTATTGGTAATTAACCTTAAATGC	673
<i>X. laevis</i>	-----tg---t--g-tc-a-ta-atc-c--a-----g-cctcc-	746
Br4	AAAAAC...TATAATAACATAACTAGAAAACCCTATTATTATTAACGTTA	720
<i>X. laevis</i>	tg-g-tcct-gc-----a--a-----a-gcac-----c-----	796
Br4	ACCTTACACTAGAACATT.ACAGGAAAGATT <u>AAAAAAAA</u>	760
<i>X. laevis</i>	-t--a---a-----t-----g-cgc-gaaggaact	846
<i>X. laevis</i>	cggcaaacatgaaccccgctggttaccaaaaacattcgccctcttgctt	896
	aaaaaacattgtataagaggtccagcctgccaagtgacgtatatgtcaa	946
	cggccgcggtatctgaccgtgcaaaggtagcgtaatcactgtcttaataa	996
	gaactggtatgaacggccacgaaggttcaactgtctcctgcatccaatcc	1046
	attaaactgacctccgtgcagagggcgggtagaacatagaacgagaag	1096
	ccctatggagctttaaactaaagaactgccaagttgaacctaacccataa	1146
	ggaaataacaattaa-caagcagaaactgacctaaagtttctcggtgggg	1196
	cgaccacgggagaataaaaaatcctccttgaagaataggcctaccacct	1246
	ttcaccaagaaccaccattctaagtaacaaaatttatgactataattgat	1296
	ccagtcctactgatcaacgaaccaagttaccctagggataacagcgcgaat	1346
	ccatttcaaaagttcctatcgacaaatgggtttacgacctcgatgttga	1396
	atcagggcatcccagtggtgcagccgctactaaaggttcggttgttcaac	1446
	gattaaagccctacgtgatctgagttcagaccggagtaatccaggtcagt	1496
	ttctatctatgaagtatttttctagtcagaaaggaccgaaaaaatgagg	1546
	ccaatgttttaataagcctctctctatatcaatgtagtcactaaattga	1596
	aaatagaactaatactctgccaagattagggct	1630

Figure 2.13. Nucleotide and deduced amino acid sequences of clone Br15.
Nucleotides and amino acid residues (in bold) are numbered on the left; the methionine residue of the start codon, the poly(A) tail and the putative polyadenylation signal (5' ATTAAA 3') are underlined. The stop codon (TAA) is marked with an asterisk.

1 ctcgacsttcggcgatgtgaggtcatcaggtctttaaaccttttgtggcaatccttgg
 61 aagcactgtcaagATGCCAGGGAAGACAGGGCGACATGGAAGTCCAACACTTTTTGAA
 1 M P R E D R A T W K S N Y F L K
 121 AATCATTCAACTGCTTGATGATTACCCAAAATGTTTCATCGTTGGGGCGGACAATGTTGG
 17 I I Q L L D D Y P K C F I V G A D N V G
 181 TTCAAAGCAAATGCAACAGATCCGTATGTCCCTGCGAGGAAAAGCGTTGTGCTTATGGG
 37 S K Q M Q Q I R M S L R G K A V V L M G
 241 AAAGAACCACATGATGCGCAAGGCAATCCGTGGTCACTTAGAAAACAACCTCTGCTCTGGA
 57 K N T M M R K A I R G H L E N N S A L E
 301 GAAGCTTTTGCCTCACATAAAGGGGAATGTTGGCTTCGTTTTCACTAAGGAGGATCTTGC
 77 K L L P H I K G N V G F V F T K E D L A
 361 TGAAGTACGAGATATGCTGTTGGCAAACAAGGTGCCAGCTTCTGCTCGTGCAGGTGCCAT
 97 E V R D M L L A N K V P A S A R A G A I
 421 TGCCCCCTGTGATGTCACAGTGCCTGCTCAGAATACTGGTTTGGGCCCTGAGAAGACATC
 117 A P C D V T V P A Q N T G L G P E K T S
 481 CTTCTTTCAGGCTTTGGGAATCACCACGAAAATCTCTAGAGGAACTATTGAAATCTTGAG
 137 F F Q A L G I T T K I S R G T I E I L S
 541 TGATGTACAGCTGATTAAGACTGGAGATAAAGTAGGTGCCAGTGAAGCCACCCTCCTTAA
 157 D V Q L I K T G D K V G A S E A T L L N
 601 CATGCTCAACATTTCCCCCTTCTCCTTTGGTTTGATCATCCAACAAGTATATGACAACGG
 177 M L N I S P F S F G L I I Q Q V Y D N G
 661 CAGCATCTACAGCCCAGAGGTTCTGGATATCACTGAAGATGCTCTGCGTGCTCGCTTTTT
 197 S I Y S P E V L D I T E D A L R A R F L
 721 AGAGGGTGTTCGCAACATCGCCAGTGTCTGCCTTCAAATGGATATCCCACTGTCGCCTC
 217 E G V R N I A S V C L Q I G Y P T V A S
 781 TGTACCTCACTCTGTCGTCATGGGTATAAACGAGTTCTTGCTCTTGCTGTGGAAACAGA
 237 V P H S V V N G Y K R V L A L A V E T D
 841 TTACAGCTTCCCATTGGCAGATAAGGTCAAAGCTTTCTTGGCAGATCCATCAGCCTTTGT
 257 Y S F P L A D K V K A F L A D P S A F V
 901 GGTGCTGCTCCAGTTGCTGAGACAGCTGCTGCTCCGGCTGCTTCAGCTGCACCAGCCAA
 277 V A A P V A E T A A A P A A S A A P A K
 961 AGAGGAAAAGGAAGAATCTGAGGAATCCGATGATGATATGGGCTTTGGGCTCTTTGACTA
 297 E E K E E S E E S D D D M G F G L F D *
 1021 agtctgttttagtctgaagtctcattaaatgcttacatctttaaacctctagaaaaaaaaa
 1081 aaaaaaaaaaaaaaaaaaaa

Figure 2.14. Comparison of the deduced amino acid sequence of frog clone Br15 with P0 from chick (*Gallus gallus*) and human (*Homo sapiens*). The amino acid residues as dashed lines are identical to those in the frog P0.

Frog P0	MPREDRATWKSNYFLKIIQLLDDYPKCFIVGADNVGSKOMQOIRMSLRGK	50
Chick P0	-----m-----v-----	50
Human P0	-----	50
Frog P0	AVVLMGKNTMMRKAIRGHLENNSALEKLLPHIKGNVGFVFTKEDLAEVRD	100
Chick P0	-----p-----r-----t-i--	100
Human P0	-----p-----r-----t-i--	100
Frog P0	MLLANKVPASARAGAIAPCDVTVPAQNTGLGPEKTSFFQALGITTKISRG	150
Chick P0	-----a-----	150
Human P0	-----a-----e-----	150
Frog P0	TIEILSDVQLIKTGDKVGASEATLLNMLNISPFSGFLIIQQVYDNGSIYS	200
Chick P0	-----v---f-----n	200
Human P0	-----v---f-----n	200
Frog P0	PEVLDITEDALRRARFLEGVRNIASVCLQIGYPTVASVPHSVVNGYKRVLA	250
Chick P0	-----et-hk-----v-----i-----i-----	250
Human P0	-----et-hs-----v-----ii-----	250
Frog P0	LAVETDYSFPLADKVKAFVLADPSAFVVAAPVAETA.AAPAASAAPAKEE.	298
Chick P0	v-----t---e-----a---vvet.---a---ap	299
Human P0	-s-----t---e-----a---aatt---a---v-a	300
Frog P0	KEESEESDDDMGFGLFD	315
Chick P0	-----e-----	316
Human P0	-----e-----	317

Figure 2.15. Nucleotide and deduced amino acid sequences of clone Br30. Nucleotides and amino acid residues (in bold) are numbered on the left; the poly(A) tail and polyadenylation signal are underlined. The stop codon (TAA) is marked with an asterisk.

1 GTTGACTTTAATGTCCCTATGAAAAATAACCAGATTACAAACAACCAAAGGATTAAAGCT
 1 V D F N V P M K N N Q I T N N Q R I K A
 61 GCTGTTCCAGTATCCAGTATTGCCTGGACAATGGTGCTAAATCCGTCGTTCTAATGAGC
 21 A V P S I Q Y C L D N G A K S V V L M S
 121 CACCTGGGGCGGCCGGATGGTGTTCATGCCTGATAAGTACTCTCTGGCTCCAGTCGCT
 41 H L G R P D G V P M P D K Y S L A P V A
 181 GTGGAACCTAAGGCTCTTCTAAAAAGGGATGTAGTGTTCCTGAAGGACTGTGTGGGACCA
 61 V E L K A L L K R D V V F L K D C V G P
 241 GAAGTTGAGGCATCATGTGCTGCTCCAGCCACTGGGACTGTTTTCTCCTGGAGAACCCTG
 81 E V E A S C A A P A T G T V F L L E N L
 301 CGATTCCATGTAGAAGAGGAGGGTAAAGGCAAAGATGCAGCTGGGAACAAGATCAAGGCA
 101 R F H V E E E G K G K D A A G N K I K A
 361 GATGCTAAGGTAGAAGCTTTCCGTGCCTCTCTGTCTAAACTGGTGTGTCTACATC
 121 D A A K V E A F R A S L S K L G D V Y I
 421 AATGATGCTTTTTGGAACCTGCACATAGAGCTCACAGCTCTATGGTCGGTGTAAATTGCCC
 141 N D A F G T A H R A H S S M V G V K L P
 481 CAGAGAGCTGCTGGTTTCTTAATGAAGAAAGAGCTTGAATATTTGCCAAGGCACTGGAA
 161 Q R A A G F L M K K E L E Y F A K A L E
 541 AACCCAGAAAGACCTTTCCCTCGCCATTCTTGAGGGGGCCAAGGTCAAGGACAAGATCCAG
 181 N P E R P F L A I L G G A K V K D K I Q
 601 CTGATCAATAACATGTTGGACCAAGTGAATGAAATGATCATTGGTGGAGGAATGGCATT
 201 L I N N M L D Q V N E M I I G G G M A F
 661 ACCTTTTTGAAAGTGCTGAACAACATGGAGATTGGTACTTCTTGTATGATGATGAAGGA
 221 T F L K V L N N M E I G T S L Y D D E G
 721 GCCAAGATTGTAAAGGACCTGATGGCTAAGGCTGAGAAAAACCTGTAAAGATTACTCTG
 241 A K I V K D L M A K A E K N L V K I T L
 781 CCTGTGACTTCACAACCTGCTGACAAGTTGATGAGAATGCCACTACTGGCAGGCCACA
 261 P V D F T T A D K F D E N A T T G Q A T
 841 GTTGCTGGTGAATCCCAGCTGGATGGATGGGTCTTGACTGTGGGCCAGAAAGCATGAAG
 281 V A G G I P A G W M G L D C G P E S M K
 901 CTGTTTGTGGAAGCTGTGGGAAGAGCCAAGCAGATCGTTTGGAAATGGTCCAGTTGGGGTG
 301 L F V E A V G R A K Q I V W N G P V G V
 961 TTTGAGTGGAAACGAGTTTGCTAAAGGAACTAAAGCGGTAATGGACAAGGTGGTGGAAAGTC
 321 F E W N E F A K G T K A V M D K V V E V
 1021 ACAGCCAAAGGAACCATCACCATCATCGGTGGTGGAGATACTGCAACCTGCTGTGCAAAG
 341 T A K G T I T I I G G G D T A T C C A K
 1081 TGGGACACTGAAGACAAAGTCAGCCATGTCTAGTACTGGAGGAGGCGCTAGCCTGGAAGTC
 361 W D T E D K V S H V S T G G G A S L E L
 1141 CTTGAAGGTAAAGTCCTTCTGGTGTGATGCTCTGAGCAGCGTCTAAtttctttcaccg
 381 L E G K V L P G V D A L S S V *
 1201 gataaagcgatgatcgagaggggaatgtcttgggtcacaatcctgggaaatgaagccagaa
 1261 cctgatgcaaccacaagcactttacttcggcttaactcacagttctctaaaggatttac
 1321 aatgtggtcaggttacctactcagcagcttctgactgtattgatgagtagtaacaacgg
 1381 ttaataagcacgctcatttcccattgcccagctggtagcttctctggacagagtagttg
 1441 taggctttgtgtttttgctcttagatgtatatattgcctgtagctgtctgtgaatctgt
 1501 gttgggtcctcaccaaatggttaatggttacaggaactacatgggacccccagaaata
 1561 tctgctaaaaaaaaaatataataataaaaaaaaaaaaaaaaa

Figure 2.16. Comparison of the deduced amino acid sequence of frog clone Br30 with phosphoglycerate kinase 1 (PGK1) from chick (*Gallus gallus*) and human (*Homo sapiens*). Chicken PGK1 (database accession: P51903) and human PGK1 (database accession: P00558). A small fragment at the N-terminus is lacking from the clone Br30 and the amino acid residues as dashed lines are identical to those in the frog PGK1.

Wood frog PGK1VDFNVPMKNNQITNNQRI	18
Chicken PGK1	m slsnkltldkvdvkgkrvvr-----dhk-----	40
Human PGK1	m slsnkltldkldvkgkrvvr-----	40
Wood frog PGK1	KA AVPSIQYCLDNGAKSVVLM SHLGRPDGV PMPDKYSLAP	58
Chicken PGK1	-----t-kh---h-----f-fs-	80
Human PGK1	-----kf-----e-	80
Wood frog PGK1	VAVELKALLKR DVVFLKDCV GPEVEASCAAPATGTVFLLE	98
Chicken PGK1	-----g-e-s-----ka--n--n-s-i---	120
Human PGK1	-----s-gk--l-----ka--n--a-s-i---	120
Wood frog PGK1	NLRFHVEEEGKGKDAAGNKIKADA AKVEAFRASLSKLG DV	138
Chicken PGK1	-----s-----	160
Human PGK1	-----s--v--ep--i-----	160
Wood frog PGK1	YINDAFGTAHRAHSSMVGKLPQRAAGFLMKKELEYFAKA	178
Chicken PGK1	-v-----h--k-----d-----	200
Human PGK1	-v-----n--k-g-----n-----	200
Wood frog PGK1	LENPERPFLAILGGAKVKDKIQ LINMLDQVNEMIIGGGM	218
Chicken PGK1	--s-----q-----s--k-----	240
Human PGK1	--s-----a-----k-----	240
Wood frog PGK1	AFTFLKVLNNMEIGTSLYDDEGAKIVKDLMAKA EKNLVKI	258
Chicken PGK1	-----q--n--f--e--s-----g---	280
Human PGK1	-----f--e-----s-----g---	280
Wood frog PGK1	TLPVDFTTADKFDENATTGQATVAGGIPAGWMGLDCGPES	298
Chicken PGK1	-----i-----h-q-e--s-----	320
Human PGK1	-----v-----k-----s-----	320
Wood frog PGK1	MKLFVEAVGRAKQIVWNGPVG VFEWNEFAKGTKAVMDKV V	338
Chicken PGK1	v-k--v-----dk-s-----l-----	360
Human PGK1	s-ky a--t-----ea--r--l--e--	360
Wood frog PGK1	EVTAKGTITIIIGGGDTATCCA KWDTE DKVSHVSTGGGASL	378
Chicken PGK1	---g--c-----n-----	400
Human PGK1	ka-sr-c-----n-----	400
Wood frog PGK1	ELLEGKVLPGVDALSSV	395
Chicken PGK1	-----	417
Human PGK1	-----ni	417

Figure 2.17. cDNA sequence of clone Br34. *EcoR* I recognition sequence (5'-GAATTC-3') is shown in bold. The short poly(A) tail sequence is underlined.

1 GGAAGACAAA AAAAAAACA GCAGAGCATG ATCCAATTTG CTACATCCGG GGGATAAAAA
 61 TTCCTTCCTG ATCTACAAA ACCCCCAGAT CCTTCACTAC AGATCCCCC CCCAGTTGTA
 121 ATCCTCCTCC TAGCATGTAT GATGCATGCA TATTCTTGGC CCCCAAATGC ATAACTTTAC
 181 ATTTATCAAT ATTAAACCTC ATCTGCCACA TAGTCGCCCA ATTAGACAGA GCATTGATGT
 241 CGGCTTGTA AATTGGAGACA TCCTGTAAGG ACGTTATTCC ACTGCATAGC TTGGCGTCAT
 301 CTGCAAAGAC AGAAATGTTA CTTTTGATCC CAGACCCAAT ATCATTTATA AAGATATTAA
 361 AAAGTAAGGG TCCCAGCACT GAACCTTGGG GTACACCACT GATAACCCTT AGACCATTCA
 421 GAGTAAGAAT CACTACTCTC TGAATTCTGT CTTTTAGCCA GTTTTCTATC CATTTACAAA
 481 CCGATATTTT CATACTGTA AACTTTACCT TACACATGAG CCGTGTGTGC GGAACGTGTAT
 541 CGAAGGCTTT TGCAAAATAC AAGTACACCA CGCCAACCGC CACCCCTCTG TCAACGGTTT
 601 TACTTACCTC TTCATAAAAA GAAATCCCAT TTGTCTGACA ACTTCTGTCT TTCATGAACC
 661 CATGCTGTCT GTTGCTTAAA ATTGTTTGTT TTTCCAGCAA GAACTCATCA ATGTGGTCTT
 721 TTATTAAACT CTCCAGTATC TTCCCAACTA TAGAAGTTAA ACTAACAGGT CTATAGTTGG
 781 GTAAAGACTT TGTTAAAAAT ATAGGCAACA CGTTCGCCCT GCGCCAATTC CCGTCATTAA
 841 TGAGTCCCTA AAAAAAAAAA AAAAAA

Figure 2.18. Comparison of the deduced amino acid sequences of frog clone Br34 with a segment of a protein related to *pol*-like reverse transcriptase with novel Zn finger motif from *Platemys spixii*. The turtle protein has 973 residues. The amino acid residues as dashed lines are identical to those in the polypeptide deduced from clone Br34.

Br34	GLINDGNWRRANVLPIFLTKSLP...NYRPVSLTSIVGKILESLIKDHID	47
Turtle	lgevpdd-kk--iv---kkgkkedpg-----xp---m-qvl-es-l	50
Br34	EFLLEKQTILSNRQHGFMKDRSCQTNGISFYEEVSKTVDRGVAVGVVYLY	97
Turtle	rh-e-rkv-rxs.----t-gk--l--l-a-----tgs--v-k--d-i--d	99
Br34	FAKAFDTVPHTRLMCKVKFTGMEISVCKWIENWLKDRIQRVVILTTLNGLR	147
Turtle	-s-----s-si-is-l-ky-ldewti-----xh-a-----ngsmssw	149
Br34	..VISGVPQGSVLGPLEFNIFINDIGSGIKSNISVFADDAKLCGITSLQ	195
Turtle	qp-t-----v-----led-vdctl-k----t--ggvvdte	199
Br34	DVSNLQADINALSNWATMWQMRFNIDKCKVMHLGAKNMHASMYLGGGLQL	245
Turtle	grdri-k-l-k-ed--krnl----k---r-l---w--pmh--r-.-tde-	248
Br34	GGGSVVKDLGVLVDQEGIFIP	268
Turtle	-ss-ae-----t--kkldmsq	270

Figure 2.19. Agarose gel analysis of frog liver clones.

The insert of the liver cDNA clone Li16 was isolated by restriction digestion of plasmid DNA by *EcoR* I, the inserts of Li39 were isolated by double restriction enzyme digestion (*EcoR* I and *Xho* I). The inserts were separated with an agarose gel (1.5%, in 1x TAE buffer). The gel was stained with ethidium bromide. M: molecular weight standards (1 kb DNA ladder from GIBCO-BRL).

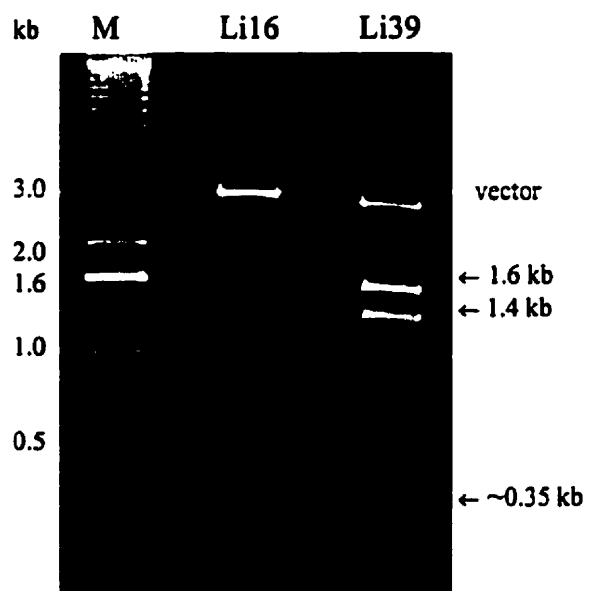
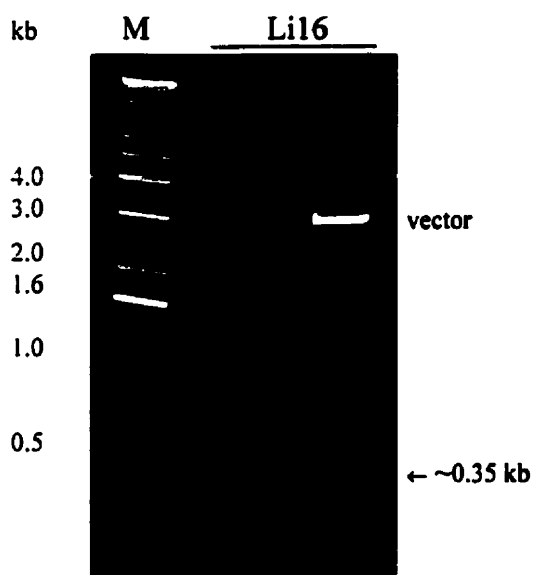


Figure 2.20. Time-course test of wood frog liver RNA probed with frog liver cDNA clone Li16. Each lane was loaded 15 μg total RNA. Lane 1: control at 5°C; lane 2-4: freezing at 4, 12 and 24 h, respectively; lane 5: freezing for 24 h and thawing for 24 h. The 28S rRNA was detected by ethidium bromide staining. The RNA blotting and hybridization were repeated with multiple trials and the data shown in the graph represents the average value from the repeats ($n = 3$). The value of target RNA band in each lane is normalized to the value of the 28S rRNA band in the same lane and the value of relative RNA band intensity for the test groups is normalized to the control group.

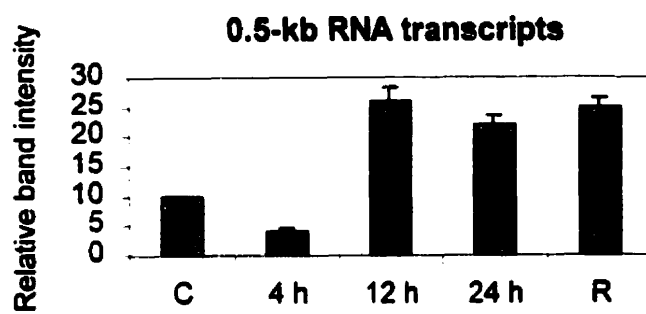
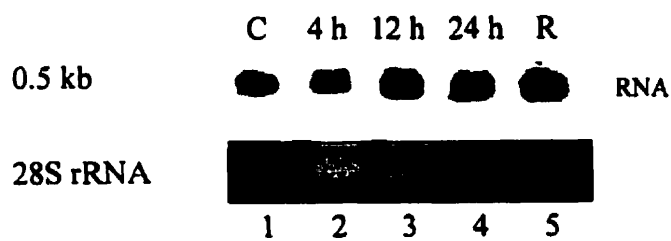


Figure 2.21. Nucleotide and deduced amino acid sequences of clone Li16. Nucleotides and amino acid residues (in bold) are numbered on the left; the putative polyadenylation signal and short poly(A) tail are underlined. The stop codon (TGA) is marked with an asterisk.

1 AGAATGTCGCAGTGGTCAGGACACCTGTCAGAGGACTCACATGCCCATAACGGATCCGAG
1 E C R S G Q D T C Q R T H M P I T D P S

61 CGGCACTGAGAACAGAAACAACACTACTATGCCAGCACCAGCGAGGGCATTATAGTTTTGGA
21 G T E N R N N Y Y A S T S E G I I V L E

121 GCGTTTGTGTACAACTAAAAACGATGTGAGAGCGCCAAGAAGAGGATGCTGAGCCGTAT
41 R L C T T K K R C E S A K K R M L S R M

181 GTCTGTAGATTGCTGCCAGGGGCCGCTCTGCAATGCCTGAgaccatcaccaggaggcgac
61 S V D C C Q G P L C N A *

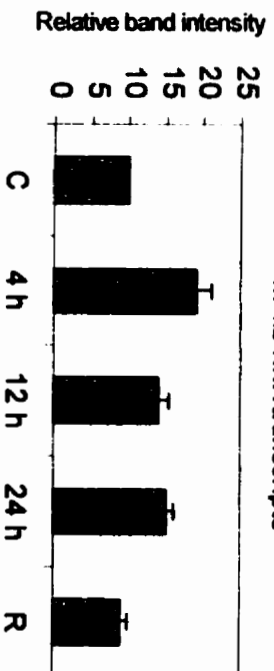
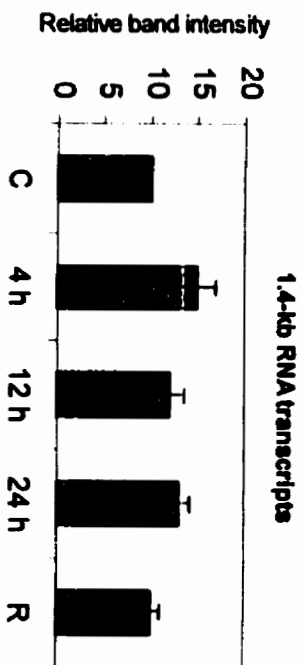
241 agacaagaacgtcccccaactcataaaacattctatacaataaaacatttccatagatca

301 aaaaaaaa

Figure 2.22. Frog liver RNA blots probed with clone Li39. The blots were loaded with total RNA samples (15 μg per lane). The ribosomal RNAs were detected with ethidium bromide staining of the formaldehyde agarose gel. C: control at 5°C; R: recovery (freezing for 24 h and thawing for 24 h at 5°C); 1-24 h: freezing for 1-24 h.

- A.* Probe derived from the total insert (1.4 and 1.6 kb fragments) of clone Li39.
- B.* Probe derived from the 1.6 kb fragment of clone Li39 insert.

A



B

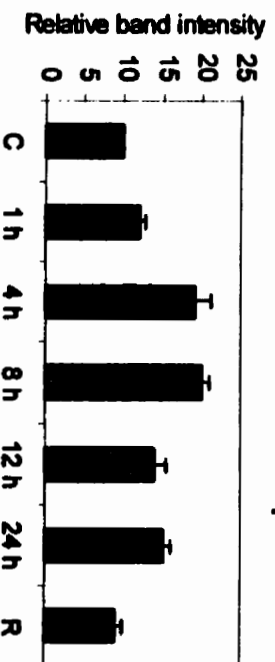
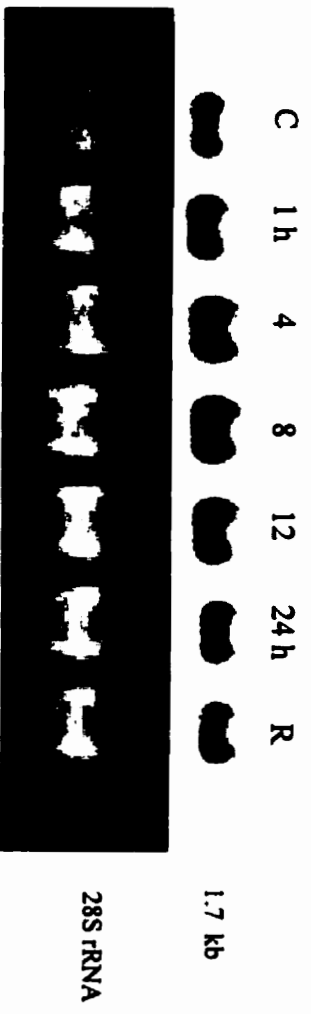


Figure 2.23. cDNA sequence of clone Li39. The *EcoR* I recognition sequence (5' ***GAATTC*** 3') is shown as bold and italic format. The cloning adapter sequence (5' **AATTCGGCACGAG** 3') within the insert of clone Li39 is in bold and the partial adapter sequence (5' AATTCGGCAC 3') is underlined.

1 AACATCCTCT CCCCTCAAAA AACATCTATG AACCTTGTA ACCAGCCAAG GCTTCTTCCT
61 TACATTTTTT TCTCTCGCAT GAATTTTTCT CCAGGACTTT AATTTTGCTA ACTCCCTTTT
121 CCTTATCGAT AAAATTTCTG GCCCTCTAGC CATCTTAACC TGCTGACTAT TCCCATTAAC
181 CATTCTAGCG AGCCAAAGTA AAATATGTCT TGAACCAATC ACTCGCCAAC GAACTTATAT
241 CGCAAACATT ACCTTCTTGC AACTCACCAC CTTTTAGCC TTCACAACCC CCGACTTAAT
301 ATTATTTTTT ATCTTCTTTG AAGCTTCCTT AGTGCCTACT ATAGTTGTTA TTACCCGGTG
361 AGGCGCTCAA GAACGACGAC TGGAAAGCCGG CATATACTTG GCATTTTATA CTATACTAGG
421 AGCTGTTCCCT CTTATAATTT GGATTACTAA ATTTTATTCG ACTCATGGTT CTTTACTCCC
481 ATCTCTGACA CACACCTTGC ACGTAACCCA AGCTTAACT AGCTATCCCG GCCTCTTTTG
541 AGCCACCTAT AATGCAGCAT TCTTAATTAA ACTGCCATC TACTGTCTCC ATCTATGACT
601 CCCTAAAGCC CATGTTGAAG CACCAATTGC AGGCTCTATA ATCTTAGCTG GGACCCTTCT
661 TAAACTTGGA GGCTACGGAA TCCTCCGAAC ATCCCCCTA CTTCAAGAGA ATAACCTAAA
721 CCAAACACTA TTTATTATAC TCATTGCTAT TCTCGGTATC TTAGCTACTG CACTCCTCTG
781 TCTCGACAA ACGGACCTAA AATCACTTAT TGCTATATCA TCTGTTAGTC ATATAAACCT
841 TGTAGTCGCC GCTGCCCTAA TCTCCTCCCC ATGAAGCTAC TCGGGAGCAA TAGCAATAAT
901 AATTGCCAC GGCCTCACCT CCTCAGCCCT CTTTTGCCTC GCTAACACCT CTTATGAACG
961 CACAAACAGC CGCACTATAA TTCTACTCCG AGGAACATTA ACAATTTTCC CTCTTGCTGG
1021 GGCATGATGA TTAATTATTA TATTACTTAA TATAGCTCTC CCTCCTACAA TTAACTTTGC
1081 GGCAGAAGTT CTTATCATAG TCTCACTCTA TAACTGATCT CCTGTTGCCT TCTTAATTGT
1141 TGCCCTAAAT CTCATCTTCA CAACTGCCTA TACTTTATAT GTTCTCTGGT CCACACAACG
1201 AGGCCCACT CCTAATCATA TAAAACCTT CTCCCCTAT CAAATTCGTG AACGCCTCCT
1261 ATTCTTTTTA CATATCTTAC CGGGCTACT TCTCATCCTT ATACCAGAAT TAACTTGGCG
1321 TGCC**GAATTC** GGCACA**AATC** **GGCACGAGAA** CAAAACGCCA **GAATTC**CTAA AGAAATTTCC
1381 TCTGGGGAAG GTTCCAGCAT TTGAGGGAAA TGACGGCTTC TGTCTGTTTG AGAGCGGTGC
1441 CATTGCTCAC TATGTGGGTA ATGATGAACT CCGTGGAAT TCCCGCTTGG ATCAGGCTCA
1501 GATAATCCAA TGGGTCAGCT TCGCAGACAG TCACATTGTC CCTCCGGCCA GTGCCTGGGT
1561 TTTCCCAACT CTGGGGATCA TGCAGTTCAA CAAACAGGCC ACAGAACAAG CCAAGGAGGA
1621 GGTGAAGCAT GTTCTGGCTA CTCTGGATTC CCATCTGCAG ACAAGAACTT TTCTGGTGGG
1681 AGAGAGAGTC ACCCTGGCTG ACATCACTGT AGCATGCGCC CTGCTTTGGC CGTATAAACA
1741 AGTCTGGAG CCTTCCTTCC GTCAGCCTTA TGGTAATGTC ACAAGATGGT TTGTGACATG
1801 TGTGAACCAG CCCCAGTTCC ATGCTGTGCT GGGAGAGGTG AAATCTGCG ACAAAATGGC
1861 TCAGTTTGAT GCCAAAAAAT TTGCAGAGTT GCAGCCAAAG AAGGAGACCC CTAAGAAGGA
1921 GAAACCAGCA AAGGAGCAA AGAAAGAGAA GGAGGAAAAG AAGCCTGCTC CTGCCCCAGC
1981 AGCTGCTCCA GAAGATGATT TGGATGAGTC TGAGAAGGCT CTTGCAGCAG AGCCAAAGTC
2041 GAAGGACCCT TATGCTCATC TCCCTAAGAG CTCCTTCATT ATGGATGAGT TTAACGAAA
2101 GTATTCAAAT GAAGACACAC TTTCTGTGGC GCTTCCTTAC TTCTTCTGGG AGCACTTTGA
2161 CAAAGAGGGA TGGTCCATCT GGTATTCAGA GTACCGTTC CCAAATGAAC TTTACAACC
2221 CTTTATGAGT TGCAATCTCA TTACAGGTAT GTTCCAGCGT CTGGACAAGC TGCSTAAAAC
2281 AGTTGCTAGC GTCATCCTGT TTGGAACCAA CAATGACAGT TCTATCTCCG GCGTATGGGT
2341 GTTCAGAGGA CAGGATTTGG CATTCACT GTCTGAGGAT TGGCAAATTG ATTACGAGTC
2401 CTATGCCTGG AGGAAGCTGG ATTCAGACAG CGAGGAGTGC AAGACCCTGG TAAAAGAGTA
2461 TTTCTGCTGG GAAGGAGATT TCGCGCATGT AGGCAAAGCC TTCAACCAGG GCAAGATCTT
2521 CAAGTGAGGC CCTCTGGGAG GAAGAGAGGA GCCGTCTTTC ATTTTGAAGA GATCTGGGAC
2581 ATCAATTTTT TTCTCTTCTC GACCACTCAG ACTTTTAACT TAAGGCAGCA AACAAAAAAT
2641 AAAATAAAAA CCTGAAGTCA AAACAAAGTG CCACGCATTC TCAGATCAAA TTAGAGACCA
2701 AAGCTATTGG CTACTAGCCT CTACGTAGTT CCGTAGTATG CAACGAATGT CACCACGATC
2761 GGAAGATCA GATTTTCCAA CAACTTTGTC CGACCATGTG TATGCAACAC CAGTTTCGGA
2821 CAAAATCCGT TGGAAAAAAT CCGACGGATT TTGTGGTCGA AATTTCCGAC CGTGTGTACG
2881 GGGCTTAGT GTCCGACTGT CCGACGCAAT ATAGCAGTCC TGCTATATGT TGTT

Figure 2.24. Nucleotide and deduced amino acid sequences of clone Li39s.

Nucleotides and amino acid residues (in bold) are numbered on the left. A single open reading frame (nucleotide 2-1330) is predicted to encode a polypeptide with 443 residues. The *EcoR* I recognition sequence (5'***GAATTC***3') is shown in bold and italic format. The underlined are the partial or complete adapter sequences. The sequence with lowercase was from clone Li39.

1 AACATCCTCTCCCCTCAAAAAACATCTATGAACCTTGGTAACCAGCCAAGGCTTCTTCCT
1 T S S P L K K H L W T L V T S Q G F F L
61 TACATTTTTTCTCTCGCATGAATTTTTCTCCAGGACTTAAATTTTGCTAACTCCCTTTT
21 T F F S L A W I F L Q D F N F A N S L F
121 CCTTATCGATAAAATTTCTGGCCCTCTAGCCATCTTAACCTGCTGACTATCCCATTAAC
41 L I D K I S G P L A I L T C W L F P L T
181 CATTCTAGCGAGCCAAAGTAAAATATGTCTTGAACCAATCACTCGCCAACGAACTTATAT
61 I L A S Q S K M C L E P I T R Q R T Y I
241 CGCAAACATTACCTTCTTGCAACTCACCACCCTTTTAGCCTTCACAACCCCGACTTAAT
81 A N I T F L Q L T T L L A F T T P D L M
301 ATTATTTTTTATCTTCTTGAAGCTTCCCTAGTGCCTACTATAGTTGTTATTACCCGGTG
101 L F F I F F E A S L V P T M V V I T R W
361 AGGCGCTCAAGAACGACGACTGGAAGCCGGCATATACTGGCATTTTATACTATACTAGG
121 G A Q E R R L E A G M Y L A F Y T M L G
421 AGCTGTTCCCTTATAATTTGGATTACTAAATTTTATTCGACTCATGGTCTTTACTCCC
141 A V P L M I W I T K F Y S T H G S L L P
481 ATCTCTGACACACACCTTGACGTAACCCAAGCTTAACTAGCTATCCCGGCCTCTTTTG
161 S L T H T L H V T Q A L T S Y P G L F W
541 AGCCACCTATAATGCAGCATTCTTAATTAACCTGCCATCTACTGTCTCCATCTATGACT
181 A T Y N A A F L I K L P I Y C L H L W L
601 CCCTAAAGCCCATGTTGAAGCACCAATTGCAGGCTCTATAATCTTAGCTGGGACCCTTCT
201 P K A H V E A P I A G S M I L A G T L L
661 TAACTTGGAGGCTACGGAATCCTCCGAACATCCCCCTACTTCAAGAGAATAACCTAAA
221 K L G G Y G I L R T S P L L Q E N N L N
721 CCAAACACTATTTATTATACTCATTGCTATTCTCGGTATCTTAGCTACTGCACTCCTCTG
241 Q T L F I M L I A I L G I L A T A L L C
781 TCTTCGACAAACGGACCTAAAATCACTTATTGCTATATCATCTGTTAGTCATATAAACCT
261 L R Q T D L K S L I A M S S V S H M N L
841 TGTAGTCGCGCTGCCCTAATCTCCTCCCATGAAGCTACTCGGGAGCAATAGCAATAAT
281 V V A A A L I S S P W S Y S G A M A M M
901 AATTGCCACGGCCTCACCTCCTCAGCCCTCTTTTGCCTCGTAACACCTCTTATGAACG
301 I A H G L T S S A L F C L A N T S Y E R
961 CACAAACAGCCGCACTATAATCTACTCCGAGGAACATTAACAATTTCCCTCTTGCTGG
321 T N S R T M I L L R G T L T I F P L A G
1021 GGCATGATGATTAATTATTATATTACTTAATATAGCTCTCCCTCCTACAATTAACCTTGC
341 A W W L I I M L L N M A L P P T I N F A
1081 GGCAGAAGTTCTTATCATAGTCTCACTCTATAACTGATCTCCTGTTGCCTTCTTAATTGT
361 A E V L I M V S L Y N W S P V A F L I V
1141 TGCCCTAAATCTCATCTTCACAACCTGCCTATACTTTATATGTTCTCTGGTCCACACAACG
381 A L N L I F T T A Y T L Y V L W S T Q R
1201 AGGCCCACTCCCTAATCATATTAACCCCTCTTCCCCTATCAAATTCGTGAACGCCTCCT
401 G P L P N H I K T L F P Y Q I R E R L L
1261 ATTCTTTTTACATATCTTACCGGGCCTACTTCTCATCCTTAATCCAGAATTAATTTGGCG
421 F F L H I L P G L L L I L N P E L I W R
1321 TGCCGaattc ggcac aattcggcacgag
441 A E F

Figure 2.25. Comparison of the deduced amino acid sequence of clone Li39s with NADH-ubiquinone oxidoreductase subunit 4 (ND4) from *Xenopus laevis*. WF: wood frog (*Rana sylvatica*); XE: *Xenopus laevis*. The amino acid residues as dashed lines are identical to those in the frog ND4.

WF.ND4TSSPLKKHLWTLVTSQGFFLTFSLAWIFLQD..FN	34
XE.ND4	mkillptlmlips-wltn--w--psl---sliisll--m-f-n-setth	50
WF.ND4	FANSLFLIDKISGPLAILTCWLFPLTILASQSKMCLEPITRQRTYIANIT	84
XE.ND4	--s-y-mt--q--t--l-----l--mli---nhlsn---s-----f-tmlv	100
WF.ND4	FLQLTTLLAFTTPDLMLFFIFFEASLVPTMVVITRWGAQERRLEAGMYLA	134
XE.ND4	----slim--sate-i--y-m--it-i--lii-----n-ae--n--t-fl	150
WF.ND4	FYTMLGAVPLMIWITKFYSTHGSLPLSLTHTLHVTQALTSYPGLFWATYN	184
XE.ND4	---la-sl--lvalisl--st-t-sln-lql-pnhipm-wanysw-lacl	200
WF.ND4	AAFLIKLPIYCLHLWLPKAHVEAPIAGSMILAGTLLKLGGYGILRTSPLL	234
XE.ND4	l--mv-m-l-gt-----v--ai-----i-i-it-	250
WF.ND4	QENNLNQLFIMLIAILGILATALLCLRQTDLKSLIAMSSVSHMNLVVAA	284
XE.ND4	spsmkelaypflilslw--im-ssi-----m--y-----g--is-	300
WF.ND4	ALISSPWSYS.GAMAMMIAHGLTSSALFCLAN.TSYERTNSRTMILLRGT	332
XE.ND4	gnnqt-mkalt---ilntsd---h---c---kyq-----h--all-s--l	350
WF.ND4	LTIFPLAGAWWLIIMLLNMALPPTINFAAEVLIMVSLYNWSPVAFLIVAL	382
XE.ND4	e--l--m-t-----sn-a-----sp-wmg-it--ta-f---swtiiltd-	400
WF.ND4	NLIFTTAYTLYVLWSTQRGPLPNHIKTLFPYQIRERLLFFLHILPGLLLI	432
XE.ND4	gtll-as-s--mflm----mt-e-lnain-tht--ht-mtm-li-iip-m	450
WF.ND4	LNPELIWRAEF	443
XE.ND4	mk-----glf-	461

Figure 2.26. Nucleotide and deduced amino acid sequences of clone Li39I. Nucleotides and amino acid residues (in bold) are numbered on the left. The uplined sequence was from clone Li39 (nt 1346-1361). The Li39I was derived from clone Li39 sequence (nt 1362-2935). The *EcoR* I recognition sequence (5'GAATTC3') is shown in bold. A poly(A) tract, adapter and other vector sequences (in lowercase as well) are underlined. The stop codon (TGA) is marked with an asterisk.

AATTCGGCACGAG

1 AACAAAACGCCGGAATTCCTAAAGAAATTCCTCTGGGGAAGGTTCCAGCATTGGAGGGA
1 N K T P E F L K K F P L G K V P A F E G
61 AATGACGGCTTCTGTCTGTTGAGAGCGGTGCCATTGCTCACTATGTGGTAATGATGAA
21 N D G F C L F E S G A I A H Y V G N D E
121 CTCCGTGGAACCTCCCGCTTGGATCAGGCTCAGATAATCCAATGGGTGAGCTTCGCAGAC
41 L R G T S R L D Q A Q I I Q W V S F A D
181 AGTCACATTGTCCCTCCGGCCAGTGCCTGGGTTTTCCCAACTCTGGGGATCATGCAGTTC
61 S H I V P P A S A W V F P T L G I M Q F
241 AACAAACAGGCCACAGAACAAGCCAAGGAGGAGGTGAAGCATGTTCTGGCTACTCTGGAT
81 N K Q A T E Q A K E E V K H V L A T L D
301 TCCCATCTGCAGACAAGAACTTTTCTGGTGGGAGAGAGAGTACCCTGGCTGACATCACT
101 S H L Q T R T F L V G E R V T L A D I T
361 GTAGCATGCGCCCTGCTTTGGCCGTATAAACAAGTCTCGGAGCCTTCCTCCGTCAGCCT
121 V A C A L L W P Y K Q V L E P S F R Q P
421 TATGGTAATGTCACAAGATGGTTTGTGACATGTGTGAACCAGCCCCAGTTCATGCTGTG
141 Y G N V T R W F V T C V N Q P Q F H A V
481 CTGGGAGAGGTGAACTCTGCGACAAAATGGCTCAGTTTGTATGCCAAAAATTTGCAGAG
161 L G E V K L C D K M A Q F D A K K F A E
541 TTGCAGCCAAAGAAGGAGACCCCTAAGAAGGAGAAAACCAGCAAAGGAGCAAAGAAAGAG
181 L Q P K K E T P K K E K P A K E Q K K E
601 AAGGAGGAAAAGAAGCCTGCTCCTGCCCCAGCAGCTGCTCCAGAAGATGATTTGGATGAG
201 K E E K K P A P A P A A A P E D D L D E
661 TCTGAGAAGGCTCTTGCAGCAGAGCCAAAGTGAAGGACCCTTATGCTCATCTCCCTAAG
221 S E K A L A A E P K S K D P Y A H L P K
721 AGCTCCTTCATTATGGATGAGTTTAAACGAAAGTATTCAAATGAAGACACACTTTCTGTG
241 S S F I M D E F K R K Y S N E D T L S V
781 GCGCTTCTTACTTCTTCTGGGAGCACTTTGACAAAGAGGGATGGTCCATCTGGTATTCA
261 A L P Y F F W E H F D K E G W S I W Y S
841 GAGTACCGTTTTCCCAAATGAACTTTTACAACCCCTTATGAGTTGCAATCTCATTACAGGT
281 E Y R F P N E L S Q P F M S C N L I T G
901 ATGTTCCAGCGTCTGGACAAGCTGCGTAAAACAGTTGCTAGCGTCATCCTGTTTGGAAAC
301 M F Q R L D K L R K T V A S V I L F G T
961 AACAAATGACAGTTCTATCTCCGGCGTATGGGTGTTGAGAGGACAGGATTTGGCATTCA
321 N N D S S I S G V W V F R G Q D L A F T
1021 CTGTCTGAGGATTGGCAAATTGATTACGAGTCCATGCCTGGAGGAAGCTGGATTGAGAC
341 L S E D W Q I D Y E S Y A W R K L D S D
1081 AGCGAGGAGTGCAAGACCCTGGTAAAAGAGTATTTCTGCTGGGAAGGAGATTTCCGCGCAT
361 S E E C K T L V K E Y F C W E G D F A H
1141 GTAGGCAAAGCCTTCAACCAGGGCAAGATCTTCAAGTGAGGCCCTCTGGGAGGAAGAGAG
381 V G K A F N Q G K I F K *
1201 GAGCCGTCTTTCATTTTGAAGAGATCTGGGACATCAATTTTTTCTTCTCGACCACTC
1261 AGACTTTTAACTTAAGGCAGCAAACAAAAATAAAATAAAAACCTGAAGTCAAACAAAG
1321 TGCCACGCATTCTCAGATCAAATTAGAGACCAAAGCTATTGGCTACTAGCCTCTACGTAG
1381 TTCCGTAGTATGCAACGAATGTCACCACGATCGGAAAAGATCAGATTTTCCAACAACCTTG
1441 TCCGACCATGTGTATGCAACACCAGTTTCGGACAAAATCCGTTGGAAAAATCCGACGGA
1501 TTTTGTGGTCGAAATTTCCGACCGTGTGTACGGGGGCTTAGTGTCCGACTGTCCGACGCA
1561 ATATAGCAGTCTGCTATATGTTGTT aattcgatatcaagcttatcgataccgtcgacc

Figure 2.27. Comparison of the deduced amino acid sequence of clone Li391 with elongation factor 1 gamma subunit (EF-1 γ) from human and *Xenopus laevis*. WF: wood frog (*Rana sylvatica*); XE: *Xenopus laevis*; HU: human (*Homo sapiens*). The amino acid residues as dashed lines are identical to those in the frog EF-1 γ .

WF.EF-1γNKTPEFL	7
XE.EF-1γ	maggtylytpdnwraykpliaaqysgfpikvassapefqfgvt-----	50
HU.EF-1γ	maagtlytypenwrafkalialaaqysgaqvrvlsapphfhfgqt-r-----	50
WF.EF-1γ	KKFPLGKVPAFEGNDGFCLFESGAI AHYVGNDELRGTSRLDQAQIIQWVS	57
XE.EF-1γ	-----k-----s-----t--h--v-----	100
HU.EF-1γ	r---a-----d---v---n---y--s-e---stpeaa--vv-----	100
WF.EF-1γ	FADSHIVPPASAWVFPTLGIMQFNKQATEQAKEEVKHLATLDSHLQTRT	107
XE.EF-1γ	-s-----y-----gi-t--gv-----	150
HU.EF-1γ	----d-----t-----hh-----n-----rri-gl--ay-k---	150
WF.EF-1γ	FLVGERVTLADITVACALLWPYKQVLEPSFRQPYGNVTRWFVTCVNQPQF	157
XE.EF-1γ	-----i-----t-s--l-----f-----e-	200
HU.EF-1γ	-----v-t--l-----afp-tn--l--i-----	200
WF.EF-1γ	HAVLGEVKLCDKMAQFDAKKFAELQPKKETPKKEKPAKEQK.....KEKE	202
XE.EF-1γ	r-----m-----p-----	245
HU.EF-1γ	r-----e-----t---d--r---gsr-e-qkpqaer--	250
WF.EF-1γ	EKK.PAPAPAAAPEDDLDESEKALAAEPKSKDPYAHLPKSSFIMDEFKRK	251
XE.EF-1γ	---ka--t--p-----	295
HU.EF-1γ	---...-a-p---eem--c-q-----a--f-----t-vl-----	296
WF.EF-1γ	YSNEDTLSVALPYFFWEHFDKEGWSIWYSEYRFPNELSQPFMSCNLITGM	301
XE.EF-1γ	-----t-----,-----a--k--e--t-a-----	344
HU.EF-1γ	-----,-----d--l-----e--t-t-----	345
WF.EF-1γ	FQRLDKLRKT.VASVILFGTNNDSISGVVFRGQDLAFTLSEDWQIDYE	350
XE.EF-1γ	-----gf-----n-----	394
HU.EF-1γ	-----naf-----s-----e--p--p--v---	395
WF.EF-1γ	SYAWRKLDSDSECKTLVKEYFCWEGDFAHVGKAFNQGKIF	391
XE.EF-1γ	--n-----g-----a--e--kn--p-----	435
HU.EF-1γ	--t-----pg--tq--r--s--a-q-----	436

CHAPTER THREE

**Gene Expression in Response to Low Temperature
in the Skin of Wood Frog: Approaches by
Differential Display PCR**

INTRODUCTION

In eukaryotes, biological processes such as cellular growth, differentiation and stress responses are mediated by differential gene expression. To understand the molecular regulation of these processes, the differentially expressed genes of interest must be identified, cloned and studied in detail. Differential display of mRNA by use of the polymerase chain reaction (DD-PCR) has become a powerful new tool for identifying the differentially expressed gene transcripts in cells of different types or under different biological conditions (Liang and Pardee, 1992; Liang and Pardee, 1995). DD-PCR utilizes reverse transcription of RNA samples (from two or more different cell or tissue types or different states of the same tissue), followed by PCR amplification using one 5' arbitrary primer and one 3' anchored primer. The PCR products (usually radiolabeled for this step) are loaded on a high resolution sequencing gel in adjacent lanes. By comparing the intensity of cDNA bands, the putative up- or down-regulated cDNA fragments can be excised and recovered from the gel, then re-amplified by PCR and further screened by dot or Northern blotting. The differentially expressed cDNA fragments are then cloned and identified by sequencing analysis.

Compared with the previous approach by differential screening of cDNA libraries as described in Chapter 2, DD-PCR appears simple and fast for isolating differentially expressed genes. In addition, the high sensitivity and efficient amplification of cDNA fragments by PCR make this approach suitable for identifying genes when only low amounts of the source tissue are available or when transcripts are in low abundance. Finally, complete or partial cDNAs can be isolated directly via DD-PCR without having to first construct a cDNA library.

As discussed in Chapter 1, wood frog skin plays an important role in freezing survival. Frog skin is very sensitive to environment stresses such as changes in temperature and humidity and, thus, skin is a good candidate organ to be used for isolating stress-inducible genes in response to temperature change. In addition, ice formation in the skin of wood frogs can set off signals to liver that trigger an immediate increase in glycogenolysis and cryoprotectant synthesis within 2-5 minutes post-nucleation (Storey and Storey, 1985). Clearly, the cellular responses of wood frog skin under low temperature must be an important part of freeze survival. However, since the skin of the frog freezes very rapidly, gene expression and other cellular activities would be quickly brought to a halt by freezing in this organ. Thus, it is possible that the gene regulation needed for freezing survival by skin may actually take place during a period of cold acclimation in order to prepare the skin for later freezing exposures. In fact, the process of cold acclimation is often an important step for developing freeze tolerance in many plants (Thomashow, 1993) and insects (Duman *et al.*, 1991). Therefore, studies with skin focused on cold versus warm acclimated-frogs and tried to identify genes that were up-regulated at low temperature.

Since a frog skin cDNA library was not available and the process of cold acclimation may take days or even weeks, DD-PCR seemed to be an ideal approach to use for identifying cold acclimation-regulated genes from different stages of cold exposed frogs.

Summer frogs live in a warm environment and they should be more suitable for isolating genes that are up-regulated in response to cold exposure when these frogs are treated at low temperature. However, they are more difficult to capture. The RNAs isolated from skins of spring frogs (cold versus warm acclimation) were utilized for DD-PCR and Northern analysis. Thus down-regulated genes under

warm-exposure were selected first and these genes potentially up-regulated in cold acclimation were then tested by Northern analysis with skin RNA samples isolated from summer frogs (warm versus cold exposure).

MATERIALS AND METHODS

Tissue Sampling and RNA Isolation

The collection of wood frogs, animal care and tissue sampling were as described in Chapter 2. In addition to tissue sampling from spring frogs that were cold-acclimated at 5°C for 2 weeks, or exposed to freezing or freezing/thawing, a few spring frogs (cold-acclimated) were selected and transferred to room temperature (~20°C) for 1 week and then killed for tissue sampling. Skin tissues were from abdomen, back and hind legs of frogs. All tissue samples were stored at -70°C.

Summer wood frogs are more difficult to capture, but a small number of wood frogs were obtained in August 1997 and these were placed in an incubator at 24°C and held for 2 weeks. After this time, some frogs were killed for tissue sampling, whereas others were shifted to 5°C and tissue were sampled after 7 h, 24 h, or 5 d of cold-exposure. All tissue samples were frozen in liquid nitrogen and then stored at -70°C.

The isolation of total RNA and mRNA was as described in Chapter 2. All RNA samples were suspended in sterile DEPC-treated water [or in 50% (v/v) formamide for total RNA samples] and stored as aliquots at -70°C.

The RNAs isolated from spring frogs were utilized for DD-PCR and Northern analysis. Comparisons were made between skin samples from cold acclimated frogs (5°C) and frogs that were warmed to 20°C for 1 week. DD-PCR cDNA fragments that showed much stronger labeling in cold- versus warm-exposed frogs were selected as

candidates for isolating genes that are differentially expressed in response to temperature change. Because this experimental protocol selected for genes that were down-regulated by warm exposure, it was necessary to confirm that the opposite was true - that these genes were also up-regulated by cold exposure. To do this, RNAs isolated from skin of control summer frogs (24°C-acclimated) versus cold-exposed summer frogs (5°C for up to 5 days) were compared by Northern analysis.

Differential Display of mRNA by PCR

Four major steps are needed to perform DD-PCR: 1) Synthesis of cDNA fragments from mRNA through reverse transcription (RT), 2) Amplification of cDNA fragments by PCR, 3) Isolation of specific cDNA fragments on DNA sequencing gels and further screening of the differentially displayed cDNA band, and 4) Analysis of gene identity by cloning and sequencing. The DD-PCR kit (RNAimage™) was purchased from GenHunter and reagents were from GenHunter unless otherwise specified. Procedures were adapted from the instructions of the manufacturer. Unlike the instructions in the kit manual, I used poly(A)⁺ mRNA instead of total RNA for cDNA synthesis and [α -³⁵S]-dATP instead of [α -³²P]-dCTP for radiolabeling during the PCR reaction. The mRNA sample was used because it was much purer and the mRNA transcripts were enriched. In addition, the DNase treatment (required with a total RNA sample) could be avoided. The [α -³⁵S]-dATP was used mainly because ³⁵S had much low energy of radiation as compared with ³²P and was safer for manipulation procedures. Furthermore, the incorporation of [α -³⁵S]-dATP can result in sharp bands and longer PCR products. However, the ³⁵S tends to become volatile at high temperature during PCR and so greater

care is needed in some parts of the procedure in handling the radioisotope (Liang and Pardee, 1995).

Synthesis of cDNA fragments from skin mRNA of frogs from two conditions (warm or cold acclimated) via reverse transcription was described in the RT-PCR section of the Methods in Chapter 2. The PCR reaction mixture contained: 7.8 μ l of sterile ddH₂O, 2 μ l of 10 \times PCR Buffer (Perkin-Elmer), 2 μ l dNTP (1 mM of each of dGTP, dTTP and dCTP; 50 μ M dATP), 2 μ l of H-APn (arbitrary primer, 25 μ M, n:1-8, GenHunter, see Table 3.1), 2 μ l H-T₁₁G (25 μ M; see Table 3.1), 2 μ l of RT-mixture (diluted 10 \times), 0.25 μ l of *Taq* DNA polymerase (5 U/ μ l, Perkin-Elmer) and 2 μ l of [α -³⁵S]-dATP (\geq 1,000 Ci/mmol, Amersham). The DD-PCR program was as described in the RACE methods in Chapter 2. Following the PCR reaction, 5 μ l of DD-Loading Buffer (Stop Solution of DNA Sequencing Kit) was added to the sample and then the sample was loaded onto a DNA sequencing gel.

By comparing the band intensities of PCR samples on the autoradiogram, bands representing up-regulated or induced genes were identified and excised from the dried sequencing gel. These were then resuspended in 100 μ l sterile water and heated at 95°C for 5-10 min. The DNA eluted from the gel was usually used for reamplification by PCR using the same primer set as in RT-PCR but without [α -³⁵S]-dATP. Characterization of the isolated DD-PCR fragments by agarose gels, TA cloning, and sequencing was performed as described in the Methods of Chapter 2.

The radiolabeled products of DD-PCR are mainly short DNA fragments (200-500 bp) and many of the small DNA fragments may be only derived from the untranslated regions of genes (especially at the 3' end). Cloning and sequencing (before

Northern blotting) may be useful for DD-PCR (with its majority of short fragments) because the small fragments can be easily sequenced. The cDNA clones (analyzed as known genes) as well as novel genes (with relatively long open reading frames) can be selected for further analysis (e.g. Northern blotting for confirming the up-regulated or induced genes). Since most DD-PCR fragments are incomplete cDNA clones, the isolation of complete cDNA clones for stress-inducible genes can be carried out by re-screening the cDNA library with the DD-PCR fragments as probes or by 5' or 3' RACE techniques with the specific primers derived from the DD-PCR clones (see Chapter 2).

Alternatively, without the cloning steps, some PCR fragments were sent for direct sequencing by Canadian Molecular Research Services Inc., Orleans, ON.

RESULTS

Isolation of Cold Acclimation-Regulated Genes from Frog Skin by DD-PCR

Amplified cDNA fragments from skin were compared for two experimental groups of spring frogs: cold-acclimated frogs (held at 5°C for 2 weeks after collection) versus warm-acclimated frogs (moved from 5°C to ~20°C and held for 7 days). The initial DD-PCR approach indicated 4 cDNA clones from the cold acclimation group that were potentially down-regulated when frogs were warm acclimated and these were selected for further analysis as candidates for cold acclimation-regulated genes (Fig. 3.1). The PCR fragments were recovered from the sequencing gel and reamplified by PCR (Fig. 3.2). The DD-PCR fragments were very small in size (estimated range 150-350 bp). Fig. 3.2A shows that more than one DNA band was amplified from each excised band. However, a dominant band appeared for each reamplified DNA sample. The dominant

DNA band from each reamplified DNA sample was purified from the agarose gel via a GeneClean kit. The PCR fragment was then cloned into pCR[®]2.1 vector (with *EcoR* I cloning sites flanking both sides of the cloned insert) according to the procedures of TA cloning (Invitrogen Corp.) (Fig. 3.2B, C). The potential cold acclimation-regulated skin cDNA clones were designated as SkC1, SkC2, SkC3 and SkC4. Sequencing data showed that the insert sizes for the DD-PCR clones (SkC1, SkC2, SkC3 and SkC4) were 325, 303, 128 and 125 bp, respectively. Blast homology searches indicated that clone SkC2 had a relatively high match with a conserved mammalian ribosomal protein L7 (RPL7), whereas the other 3 clones did not show any significant match with the sequences in Genbank (data are not shown).

The nucleotide and deduced polypeptide sequences of clone SkC2 are shown in Fig. 3.3. The cDNA insert contained an arbitrary primer (the AP7) at the 5' end and the anchor primer (H-T₁₁G) recognition sequence at the 3' end. The cDNA contained a single open reading frame and it was likely to be a part of cDNA sequence since the cDNA did not contain 5' and 3' untranslated sequences. The gene transcript probed by clone SkC2 occurred in high levels in skin of cold acclimated frogs as assessed by Northern blotting but the gene was down-regulated during warm acclimation as compared with the cold acclimation group (Fig. 3.4A). A single RNA transcript (about 0.95 kb in size) was detected on Northern blots and the RNA level was about 4.5-fold higher in the skin of the cold acclimation group (at 5°C) as compared with the warm group (Fig. 3.4A). When cold-acclimated frogs were given freezing exposure at -2.5°C for 24 h, transcript levels remained at a high level (Fig. 3.4A). Since the freezing of frog skin is probably complete with 1 hour of freezing exposure and there should be no further mRNA synthesis in the

frozen tissues, the high levels of RPL7 transcript in skin of frozen animals probably represents the existing mRNA that was in the cold acclimated animals which seemed stable during freezing-exposure.

The above results show that the isolated RPL7 gene was down-regulated in the skin of warm acclimated frogs. However, we were more interested in knowing if the gene was actually up-regulated in response to low temperature when warm acclimated frogs were exposed into cold. To determine this, we used summer frogs that were collected in August when outdoor temperatures were $>20^{\circ}\text{C}$. Animals were held in the lab at a constant 24°C for 2 weeks and then some were transferred to 5°C and sampled after 7 hours, 24 hours, or 5 days at the low temperature. Northern blotting using total RNA isolated from frog skin indicated that the RPL7 gene transcript was elevated when summer frogs were transferred into the cold. Transcript levels were unchanged after 7 hours of cold exposure but had risen by 65% within 1 day of transfer to 5°C , compared with warm acclimated controls, and remained high after 5 days of cold exposure (Fig. 3.4B).

Since the size of the SkC2 insert was about 0.3 kb, it clearly represented an incomplete sequence and further searching for the complete RPL7 cDNA gene was, therefore, undertaken.

Isolation of Wood Frog RPL7 cDNA Gene

In order to isolate the complete RPL7 cDNA gene, rescreening of the liver cDNA library (Uni-Zap vector, *EcoR* I/*Xho* I as cloning sites) was performed with the specific DNA probe derived from clone SkC2 insert. By screening about 5,000 plaques, 2

candidate plaques with strong signals were selected and *in vivo* excised into pBluescript plasmids. The plasmid DNA was purified and the inserts were released by double digestion with *EcoR* I and *Xho* I. The insert was separated on an agarose gel (Fig. 3.5A) and detected with SkC2 probe (Fig. 3.5B). One clone was a potential candidate for encoding RPL7 by Southern analysis and was designated as clone RSkC2. The insert of clone RSkC2 was estimated to be 0.85 kb in size (Fig. 3.5C). The rescreened clone was sequenced and nucleotide and putative amino acid sequences are shown in Fig. 3.6. The sequencing data indicated that RSkC2 cDNA was 876 bp. The RSkC2 cDNA (accession no. AF175983) contained a single open reading frame encoding 243 amino acid residues and a 3' untranslated region of 145 nucleotides terminating with a long poly(A) tail 16 nucleotides downstream from a polyadenylation signal (AATAAA). Blast searching of Genbank databases showed the protein encoded by RSkC2 was indeed a homologue of mammalian RPL7 (Fig.3.7). A few amino acid residues seemed to be missing at the N-terminus of the predicted protein sequence encoded by the RSkC2 cDNA as compared with the human protein. The cDNA of clone RSkC2 contained internal sequences as the recognition sites for the PCR primers (AP7 and H-T₁₁G) (with a few mismatched base pairs) and the full sequence of the SkC2 insert (Fig. 3.8).

Isolation of Larger DD-PCR cDNA fragment from Frog Skin Tissues

The short cDNA fragments (100-500 bp) isolated by conventional DD-PCR are usually comprised primarily of noncoding sequence and are generally not valued for gene identification. Therefore, the central issue for current DD-PCR technology is how to

obtain larger cDNA fragments in order to isolate differentially expressed genes more efficiently.

Further modification of the DD-PCR procedures was carried out by increasing the final concentration of nonradiolabeled dNTP to 5 μ M for dATP and to 100 μ M for the other three dNTPs, respectively (see details in the method sections). From this, larger DD-PCR cDNA fragments (0.5-2.5 kb) were obtained (Fig. 3.9). Eighteen cDNA candidates that were potentially down-regulated in the skin of warm-acclimated frogs, as compared with cold-acclimated animals, were isolated and recovered from the sequencing gel. These cDNA fragments were designated as Skn(a-d) where n ranges from 1-8, indicating the primer (AP1-AP8) used. Reamplification of the cDNA fragments was performed by PCR and these were analyzed on agarose gels (Fig. 3.10). All the reamplified cDNA fragments were purified from the agarose gel. Twelve of them were selected and sent to Canadian Molecular Research Services Inc. (Orleans, ON) for direct sequencing. The sizes of inserts and gene identity for these cDNAs as well as other isolated frog skin clones are summarized in Table 3.2 (sequencing data for these larger DD-PCR cDNA clones are not shown). Further characterization of these DD-PCR clones is in progress (especially for the cDNA clones that may encode for ATPase δ subunit and mRNA capping enzyme).

Non-radiolabeled DD-PCR can be used to isolate even larger cDNA fragments (Lohmann *et al.*, 1995). Up to 3-5 kb fragments can be obtained and they were confirmed with agarose gels by EtBr staining or conventional SDS polyacrylamide gel by silver staining (Wu and Storey, unpublished data). However, the silver-stained DNA fragments (over 1 kb) cannot be re-amplified efficiently so far. Reamplification worked easily for

DNA fragments <0.7 kb as has also been reported in the literatures (Weaver *et al.*, 1994). A new approach with fluorescence labeling combined with an automated DNA sequencer has been applied for DD-PCR (Ito *et al.*, 1994).

Test of mRNA Profiles in Frog Tissues under Different States by DD-PCR

DD-PCR is also a powerful RNA finger printing technique. The RNA profile tested by DD-PCR is valued for assessment of the overall state of transcribed genes.

Although the cDNA fragments amplified by DD-PCR (with different combinations of arbitrary and anchor primers) only represent less 40% of the mRNA pool, the technique is still a useful tool to assess overall changes in mRNA transcripts in a specific tissue under different stress conditions. Comparison of the cDNA patterns from cold acclimation versus warm acclimation frogs as well as from cold acclimation versus freeze-exposed frogs revealed bands of differential intensities among the different stress conditions in frog liver, skin and muscle tissues (Fig. 3.11).

More detailed studies performed with DD-PCR using different combinations of arbitrary and anchor primers on frog brain mRNA also revealed that certain cDNA bands with higher intensity appeared in freeze-exposed groups as compared with the 5°C cold-acclimated groups (Fig. 3.12). The cDNA bands representing potential up-regulated genes in response to freezing exposure were not used for further analysis since extensive screening of frog brain cDNA library was performed as described in Chapter 2.

Our studies with DD-PCR technology have shown that differential gene expression in wood frogs at low temperature appeared not only in central organs, as was

also indicated by screening of cDNA libraries (see in Chapter 2), but also in peripheral tissues.

DISCUSSION

DD-PCR is another powerful method to screen differentially expressed genes in eukaryotes. The technique allows simultaneous comparison of multiple samples and works fast. In addition, a small amount of starting material can be used and transcripts that are present in relatively low abundance can be detected. However, like conventional differential screening of cDNA libraries, a large number of false positives must be eliminated by selection. Conventional DD-PCR is also hindered by other technical shortcomings, such as the relatively low reproducibility of PCR cDNA patterns and cDNA fragments that are too short for reliable identification. Rescreening of the cDNA library with the isolated DD-PCR cDNA probe is usually required to retrieve full-length sequences. To obtain clones of particular interest, subtractive cDNA hybridization is a more efficient approach. In general, this technique involves hybridization of cDNA from one source (tester) to an excess of mRNA (cDNA) from other source (driver) and then separation of the unhybridized fraction (target) from hybridized common sequences. Thus, the target gene transcript can be greatly enriched and mRNA transcripts in extremely low abundance can be analyzed by this method. This technique was developed in the early 80's and numerous important genes such as T-cell receptors (Hedrick *et al.*, 1984; Yanagi *et al.*, 1984) were identified using it. By the combination of long and accurate PCR techniques (Barnes, 1994; Cheng *et al.*, 1994) subtractive cloning has become the most powerful method for the identification of differentially expressed genes (Sagerstrom *et al.*, 1997). Finally, a complete new technology for searching differentially

expressed genes has recently been developed that analyzes a whole genome on glass “chips” (Lockhardt *et al.*, 1996). As the nucleotide sequence of the entire genome of several model organisms is uncovered, such as for yeast (Goffeau *et al.*, 1996) and *Caenorhabditis elegans* (reviewed by the *C. elegans* Sequencing Consortium, 1998) as well as the human genome which will be sequenced within a few years, it may soon be unnecessary to isolate differentially expressed genes *de novo* and it may then be possible to screen all known genes for differential expression. The new technique relies on the synthesis of large arrays of oligonucleotides on a small glass “chip”. The chips are then probed with labeled cDNAs from different tissue sources, and a comparison between the hybridization of each gene to the different sources is made using computer analysis. However, it is still less clear whether this method will be as sensitive as current subtractive cloning techniques.

By optimizing the PCR conditions for differential display, up to 2.5 kb cDNA fragments were obtained by radiolabeling DD-PCR for the isolation of differentially expressed genes from wood frog skin (Fig. 3.9). More precise work with both central and peripheral frog organs should be performed to isolate cold acclimation-regulated genes by DD-PCR in order to assess the overall significance of cold adaptation in the freeze tolerance. The importance of cold acclimation in wood frog freeze survival can be addressed by inhibiting protein synthesis by treatment with cycloheximide and by using antisense RNA techniques to block the function of stress-induced gene transcripts during cold adaptation. However, this will be difficult to study in intact animals. Cell and tissue culture will be valuable for these approaches, although the responses to low temperature and freezing in a cell culture system may be quite different from those in whole animals.

Alternatively, knockout techniques will be powerful approaches to define the physiological significance of the isolated novel genes in natural stress survival.

In addition to the temperature changes that can result in the differential gene expression, the effect of photoperiod and breeding should also be considered to involve the regulation of gene expression from different season's frogs.

As discussed above, a desire to obtain larger cDNA fragments is the central issue for current DD-PCR technology in order to obtain full-length clones. In current studies, up to 3-5 kb cDNA fragments were achieved by non-radiolabeling DD-PCR (data are not shown). Efforts directed at the reamplification of >1 kb DD-PCR cDNA fragments are in progress. The ability to obtain larger PCR products is also limited by the use of *Taq* DNA polymerase since the enzyme has no proofreading ability. The presence of misincorporated nucleotides at the 3'-terminus is thought to lead to termination of synthesis (Barnes, 1994). Long and accurate PCR can be achieved by using a mixture of *Taq* DNA polymerase and a thermostable polymerase that has proofreading ability to amplify up to 35 kb DNA fragments efficiently (Barnes, 1994).

In addition to the above technical innovations and related applications, the expression of the ribosomal protein L7 (RPL7) gene in response to temperature change in wood frog skin is of particular interest. The RPL7 transcript was maintained at a high level in skin of spring-collected, cold acclimated frogs whereas its expression was down-regulated during warm acclimation (Fig. 3.4A). Oppositely, transcript levels were elevated in skin when warm acclimated summer-collected frogs were shifted to low temperature (Fig. 3.4B), which indicated that RPL7 gene expression was up-regulated in response to low temperature. The upregulation of this ribosomal protein gene along with

the acidic ribosomal phosphoprotein (P0) gene as described in Chapter 2 may add further support to increasing evidence that the state of ribosome may be the physiological sensor to low temperature (Jones and Inouye, 1993; Thieringer *et al.*, 1998).

To my knowledge, cloning and characterization of the RPL7 gene has not previously been reported from any amphibian source. The RPL7 gene has been isolated in humans (Hemmerich *et al.*, 1993; Seshadri *et al.*, 1993) and rodents (Lin *et al.*, 1987; Hemmerich *et al.*, 1993; Meyuhas and Klein, 1990), and homologous genes to RPL7 have been identified in yeast (Murray and Watts, 1990), *Dictyostelium discoideum* (Szymkowski *et al.*, 1989), and fruit fly (database accession no: P32100). The homologous gene to RPL7 in *C. elegans* should also be available in the DNA sequencing database. In *E. coli*, the ribosomal protein L30 is the homologue of RPL7 in higher eukaryotes. The human, rat and mouse RPL7 proteins share 97% identity. In addition, the position of the first intron is conserved for human and rodent genes. Human RPL7 protein is smaller than the rodent protein due to the absence of a few basic repetitive motifs at the amino terminus in the human protein. Human and rodent RPL7 carry sequences at the N-terminus similar to the leucine-zipper motif of DNA-binding transcription factors. That region may mediate RPL7-dimerization and stable binding to RNA, which may be responsible for the interactions with other ribosomal proteins and RNAs (Hemmerich *et al.*, 1993). Frog RPL7 shares about 77% identity with the human and rodent proteins (Fig. 3.7). Although no specific function of RPL7 in the translation apparatus is known as yet, the protein may have a critical role in ribosomal activity because of its high degree of structural conservation during evolution.

The induction of RPL7 mRNA was reported in lipopolysaccharide-stimulated mouse spleen B-lymphocytes (Hemmerich *et al.*, 1993). Down-regulation of RPL7 transcripts was also identified in senescent human fibroblasts as compared with presenescent and quiescent cells. The mRNAs encoding for five other ribosomal proteins (L5, P1, S3, S6, and S10) behaved similarly in the fibroblasts (Seshadri *et al.*, 1993). The above studies suggest that the expression of RPL7 gene can be regulated by extracellular stimuli and can be affected by the cell's physiological state which is consistent with the presently-reported response of the frog skin gene to the change in acclimation temperature.

The upregulation of RPL7 gene transcripts in response to low temperature has not previously been reported. Other types of stress-inducible genes have been identified as responding to low temperature in other organisms. In *E. coli* a specific pattern of gene expression in response to an abrupt shift to a lower temperature was identified. The pattern includes the induction of several cold-shock proteins (Csps), synthesis of proteins involved in transcription and translation, and repression of heat-shock proteins (Jones and Inouye, 1993). Csps are involved in various cellular functions from supercoiling of DNA to initiation of translation. The *E. coli* Csps share sequence similarity with yeast cold shock proteins and with the cold-shock domain of eukaryotic Y-box proteins (Wolffe, 1994) and may have a function in activating transcription or in unwinding or masking RNA molecules as RNA chaperones (Jiang *et al.*, 1997). Unlike the heat shock proteins, *E. coli* Csps are not highly conserved through evolution. However, like the heat-shock response, it is believed that the cold-shock response also serves an adaptive function.

Nothing is yet known about whether cold-shock responses occur in the wood frog. However, our new approaches with DD-PCR show that differential upregulation of RPL7 and a number of other genes occur in frog skin when warm-acclimated animals are given up to one week low temperature exposure. Although the present data do not provide a definite link between the cold acclimation-regulated gene expression and improved freeze tolerance, the upregulation of the frog RPL7 gene during cold acclimation may suggest the involvement of the ribosome in signaling the response at low temperature. It is well known that low temperature can affect ribosomal subunit assembly and inhibit the initiation of translation. A downshift in temperature is not the only inducer of cold-shock responses. The addition of certain inhibitors of translation (e.g. chloramphenicol, tetracycline etc.) also induced the cold-shock response in *E. coli* (VanBogelen and Neidhardt, 1990). Thus, the state of ribosome may be a physiological sensor for the cold-shock response. The response may function to overcome the partial block in translation caused by low temperature and, thereby, increase the translational capacity of the cells. The increased levels of RPL7 gene transcripts in frog skin at low temperature may suggest that a similar signaling response might also appear during cold acclimation in wood frogs. Cold-induced (as opposed to freeze-induced) gene expression may be another important part of adaptation for overwintering.

The isolated frog RPL7 clone appeared to be missing the 5' untranslated region as well as a short sequence coding for a few amino acid residues at the N-terminus of the protein. To obtain the complete cDNA gene, screening of a larger cDNA library or the use of 5'RACE techniques could be performed. Alternatively, an oligonucleotide corresponding to ~30 nucleotides at the 5' end of clone RSkC2 could be used to screen a

liver genomic library (available in our lab) to obtain the 5' sequence of frog RPL7 cDNA gene. Screening of the genomic library with the probe derived from RPL7 cDNA could also be applied to study the structure and organization of the gene as well as the gene regulation at the transcription level.

RPL7 should be constitutively expressed under normal physiological conditions and it is a housekeeping gene. The differential expression of the frog RPL7 gene in response to low temperature may result from transcription controls or perhaps from post-transcription regulations (such as mRNA stability, etc.).

To further study the role of RPL7 gene expression in wood frog freeze survival, the expression of the RPL7 gene should be studied in other tissues in response to low temperature and the responses of the gene to other stress conditions (such as dehydration and anoxia) should also be analyzed. Along with the genes isolated by differential screening of cDNA libraries, the following chapter will address tissue-specific gene expression in the cold and the possible stress signals (such as oxygen level, temperature, cell volume and osmotic changes) involved in the regulation of genes that respond to freezing or cold acclimation.

Table 3.1. DD-PCR primers

Primer	Sequence
Anchored:	
H-T ₁₁ G	5' AAGCTTTTTTTTTTTTG 3'
Arbitrary:	
AP1	5' AAGCTTGATTGCC 3'
AP2	5' AAGCTTCGACTGC 3'
AP3	5' AAGCTTTGGTCAG 3'
AP4	5' AAGCTTCTCAACG 3'
AP5	5' AAGCTTAGTAGGC 3'
AP6	5' AAGCTTGCACCAT 3'
AP7	5' AAGCTTAACGAGG 3'
AP8	5' AAGCTTTTACCGC 3'

Note: All primers have *Hind* III recognition sequence (5' AAGCTT 3').
The DD-PCR primers were from GenHunter Corp.

Table 3.2. DD-PCR clones from wood frog skin tissues

Clones	Insert Size (bp)	Gene Identity and Comments
SkC1	325	unknown
SkC2	303	ribosomal protein L7 (RPL7)
RSkC2	876	RPL7
SkC3	128	unknown
SkC4	125	unknown
Sk1a	743	unknown (with long ORF)
Sk1b	577	mRNA capping enzyme (low match)
Sk1c	447	like fly pol protein (low match)
Sk2a	798	RNA replication protein (low match)
Sk3b	600	F ₀ F ₁ ATPase δ subunit (high match)
Sk4b	674	unknown
Sk5a	791	unknown
Sk6a	815	receptor-like his kinase (low match)
Sk6b	454	unknown
Sk7c	574	unknown
Sk7c1	400	cystatin (low match)
Sk8b	670	unknown

Figure 3.1. Differential display of mRNA from skin of wood frog *Rana sylvatica*. Primer combination was H-T₁₁G and AP7 and PCR products (³⁵S-labeled) were resolved on a polyacrylamide gel. The DD-PCR followed the procedures as described in the Methods section, while the final concentration of nonradiolabeled dATP and dNTP (for other 3 nucleotides) was 2 μM and 25 μM, respectively. Arrows indicate bands that show up-regulated cDNA gene candidates (e.g. SkC1-SkC4) during cold acclimation. Lane 1 and 3: identical samples from warm acclimation frogs (by shifting cold-acclimation frogs to room temperature at ~20°C for 7 days). Lane 2 and 4: identical samples from cold-acclimation frogs (at 5°C for 2 weeks). Lane 1 and 2: load 5 μl of DD-PCR products; lane 3 and 4: load 3 μl of the products.

1 2



3 4



← SkC1



← SkC2



←



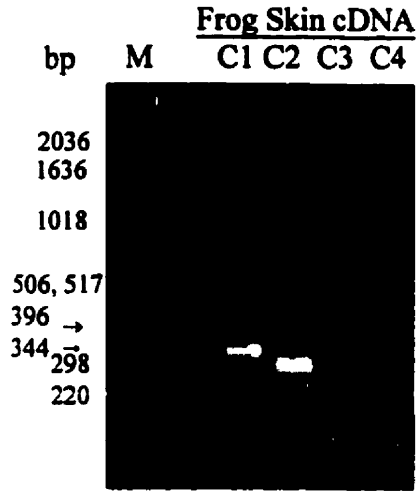
← SkC3
← SkC4

Figure 3.2. Agarose gel analysis of frog skin DD-PCR clones.

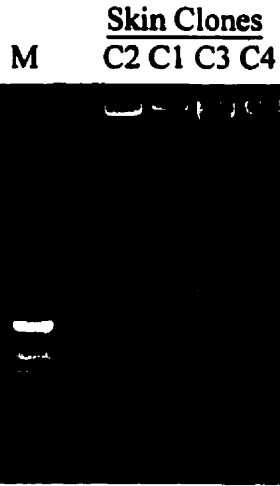
A. Reamplified DD-PCR fragments were analyzed on 3% agarose gel (in 1xTAE buffer). The gel was stained with ethidium bromide (EtBr). M: molecular weight standards (1 kb DNA ladder from GIBCO-BRL).

B, C. Analysis of the skin DD-PCR clones. The dominant cDNA band for each sample was isolated from the agarose gel (Fig. 3.2A) and ligated into pCR[®]2.1 vector via TA cloning. The inserts of the cDNA clones (from SkC1 to SkC4) were isolated by restriction enzyme digestion of purified plasmid DNA with *EcoR* I and separated on 2% agarose gel (in 1x TAE buffer). The agarose gels were stained with EtBr. M: molecular weight standards (1 kb DNA ladder).

A



B



C

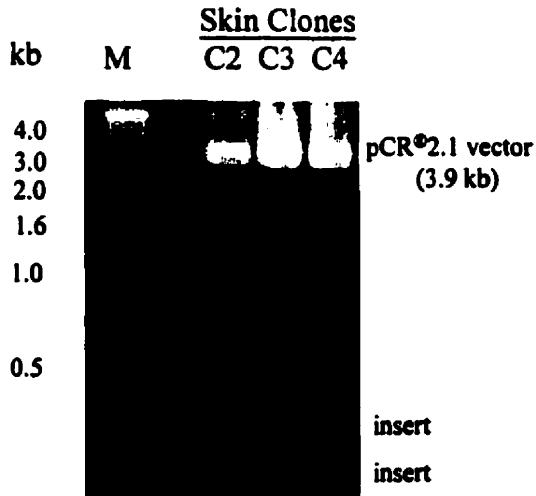


Figure 3.3. Nucleotide and deduced amino acid sequences of DD-PCR clone SkC2. Nucleotides and amino acid residues (in bold) are numbered on the left; the arbitrary primer (AP7) sequence and the anchor primer (H-T₁₁G) recognition sequence are underlined.

(AP7 primer)

1 AAGCTTAACGAGGCTTCTATCAACATGTTGAGATTGGTGGAGCCC
 1 **K L N E A S I N M L R L V E P**

46 TACATCGCTTGGGGGTATCCTAATCTGAAGTCTGTAAAGCAGCTC
 16 **Y I A W G Y P N L K S V K Q L**

91 ATCTACAAGAGAGGATACCTGAAGATCAAGAAGCAGCGTATCCCT
 31 **I Y K R G Y L K I K K Q R I P**

136 CTCACTGACAACTCTCGGATTGAGAAATACCTACGGAAAACGTAC
 46 **L T D N S R I E K Y L R K T Y**

181 ATCATTGTGTTGAAGATCTGATCCAGGAGATCTACACTGTTGGC
 61 **I I C V E D L I Q E I Y T V G**

226 AAAA ACTTCAATGGCGCAAACAATTCCTTTGGCCCTTCAAATTT
 76 **K N F N G A N N F L W P F K F**

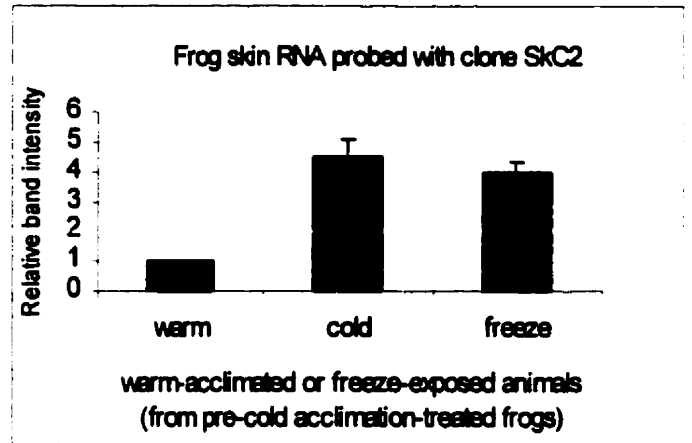
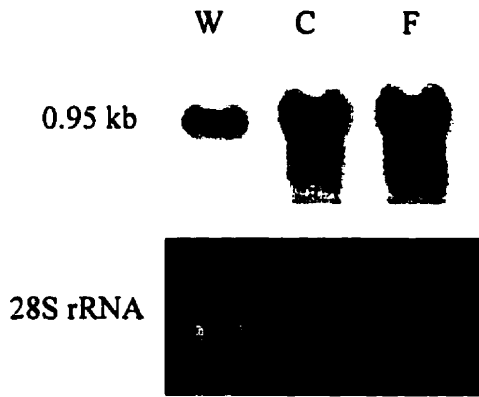
(3'GTTTTTTTTTTTCGAA5', primer H-T₁₁G)
 271 TCCTTTCCCGGGGGTGGCAAAAAAAAAAAGCTT
 91 **S F P G G G**

Figure 3.4. Northern blot analysis of skin total RNA from wood frog *Rana sylvatica* probed with the insert of clone SkC2.

- A.** The total RNA (15 μg) was loaded for each lane and ribosomal RNA was detected with EtBr staining. W: warm acclimation-treated frogs (pre-cold acclimation-treated frogs kept at room temperature ($\sim 20^\circ\text{C}$) for 7 days); C: cold acclimation-treated frogs at 5°C for 2 weeks; F: freezing-exposed frogs (at -2.5°C) for 24 h.
- B.** The total RNA ($\sim 10 \mu\text{g}$) was loaded for each lane and ribosomal RNAs were detected by staining the RNA blot with methylene blue. Lane 1: summer frogs kept at an incubator ($\sim 24^\circ\text{C}$) for 2 weeks; 2: warm acclimated frogs shifted to 5°C for 7 h; 3: warm acclimated frogs shifted to 5°C for 24 h; 4: warm acclimated frogs shifted to 5°C for 5 days.

The RNA blotting and hybridization were repeated with multiple trials and the data shown in the graph represents the average value from the repeats ($n = 3$). The value of target RNA band in each lane is normalized to the value of the rRNA band in the same lane and the value of relative RNA band intensity for the analyzed groups in Fig. 3.4A and 3.4B is normalized to the warm-acclimated frog group, respectively.

A.



B.

Cold acclimation (5°C)

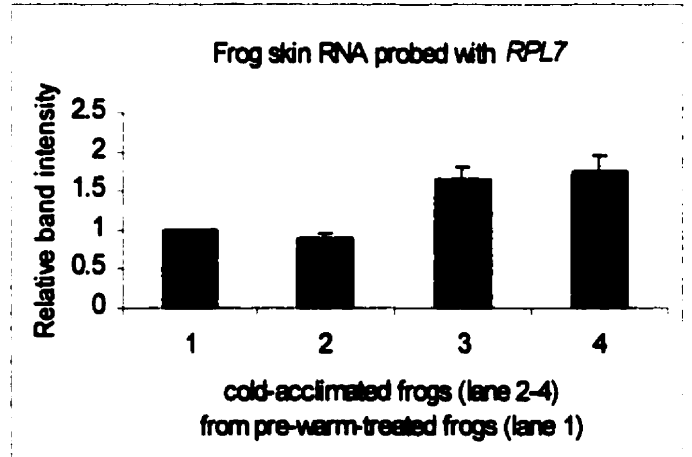
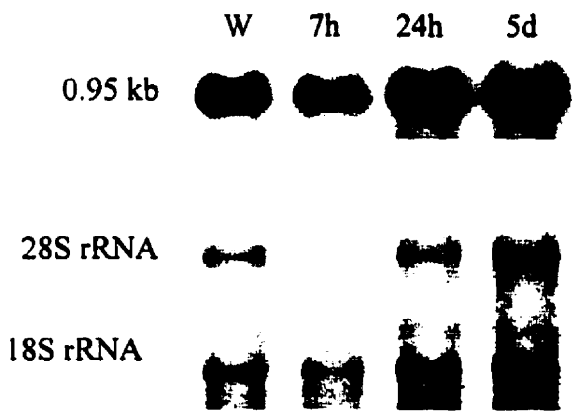


Figure 3.5. Analysis of re-screened clones encoding for frog RPL7 by agarose gel electrophoresis.

A. Agarose gel analysis of re-screened clones. Plasmid DNA from re-screened clone (lane 3-6) was digested with *EcoR* I and *Xho* I. SkC2 plasmid DNA (lane 1-2) was digested with *EcoR* I. Lane 1 and 2: plasmid DNA of clone SkC2 with different loading; lane 3-6: rescreened clones from frog liver cDNA library (Uni-Zap II vector, *EcoR* I and *Xho* I as cloning sites). Lane 3 and 4: different preps of plasmid DNA from same clone. Lane 5 and 6: different preps of plasmid DNA from another clone. The DNA fragments were separated on 1.5% agarose gel (in 1× TAE buffer). The gel was stained with ethidium bromide. M: molecular weight standards (1 kb DNA ladder).

B. DNA blot prepared from above agarose gel was probed with SkC2 insert. The clone (for lane 3 and 4) was tested as positive by SkC2 probe and was designated as clone RSkC2. Another clone (for lane 5 and 6) was false positive by Southern blotting test.

C. Agarose gel analysis of rescreened clone RSkC2. Plasmid DNA of clone RSkC2 was digested with *EcoR* I and *Xho* I. The DNA fragments were separated on 1.0% agarose gel (in 1× TAE buffer). The gel was stained with EtBr. M: molecular weight standards (1 kb DNA ladder). Lane 1-2 and 3-4: duplicate loading, respectively.

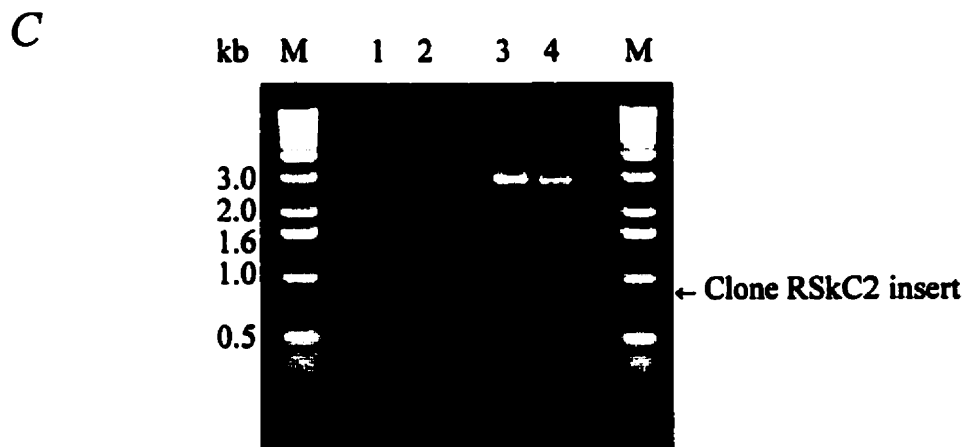
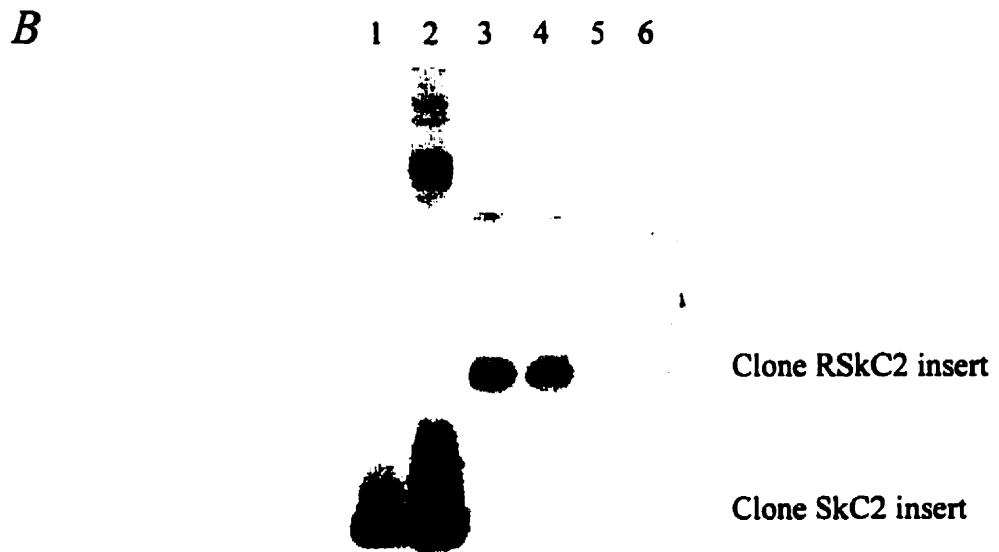
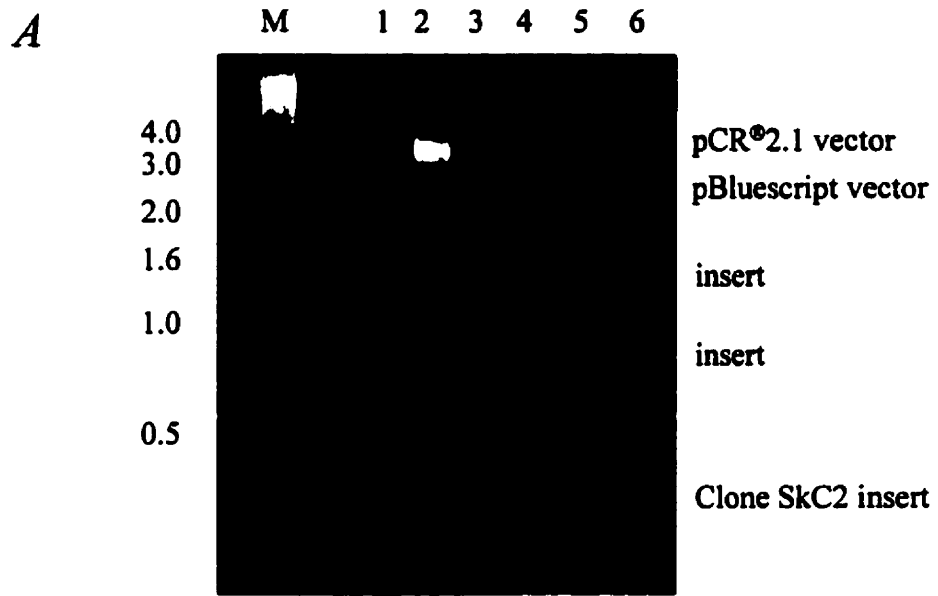


Figure 3.6. Nucleotide and deduced amino acid sequences of clone RSkC2. Nucleotides and amino acid residues (in bold) are numbered on the left; the poly(A) tail and putative polyadenylation signal are underlined. The stop codon (TAA) is marked with an asterisk.

1 CAGAGGAGAAAAAGTTGCCGTCCGTTCCAGAAAGCCTTTTAAAAAGGCGAAAAGCCTTCGCG
 1 E E K K L P S V P E S L L K R R K A F A

 63 GAAGCCAAGGCCAAAAGGATCAAGAAAATCCTAGCTGAAAAGAAGGCCCGCAAAGAAAA
 21 E A K A K R I K K I L A E K K A R K E K

 123 AGGAAGGTCATTTACAAGAGAGCCGAATCCTACTACAAAGAATACAGACAGCTGTACAGG
 41 R K V I Y K R A E S Y Y K E Y R Q L Y R

 183 CGTGAAGTGCCTGCTGGCTAGGATGGCTCGCAAAGCTGGAACTATTATGTGCCTGCTGAA
 61 R E V R L A R M A R K A G N Y Y V P A E

 243 CCAAGTTGGCATTTCGTCATTAGAATCAGAGGTATCAATGGAGTAAGTCCAAAGGTTCCG
 81 P K L A F V I R I R G I N G V S P K V R

 303 AAAGTGTTCAGCTCCTCCGTCTTCGCCAGATCTTCAACGGCACCTTTGTCAAGCTCAAC
 101 K V L Q L L R L R Q I F N G T F V K L N

 363 AAGGCTTCTATCAACATGTTGAGATTGGTGGAGCCCTACATCGCTTGGGGGTATCCTAAT
 121 K A S I N M L R L V E P Y I A W G Y P N

 423 CTGAAGTCTGTAAAGCAGCTCATCTACAAGAGAGGATACCTGAAGATCAAGAAGCAGCGT
 141 L K S V K Q L I Y K R G Y L K I K K Q R

 483 ATCCCTCTCACTGACAACTCTCGGATTGAGAAATACCTACGAAAACGTACATCATTTGT
 161 I P L T D N S R I E K Y L R K T Y I I C

 543 GTTGAAGATCTGATCCAGGAGATCTACACTGTTGGCAAAAACCTCAATGGCGCAAACAAT
 181 V E D L I Q E I Y T V G K N F N G A N N

 603 TTCCTTTGGCCCTTCAAATTTTCCTTTCCCGGGGTGGAATGAAAAGAAAACCACACAC
 201 F L W P F K F S F P G G G M K K K T T H

 663 TTTGTAGAAGGTGGGGATGCAGGCAACAGGGAAGACCAGATCAACAGGCTGTTAAGGAAG
 221 F V E G G D A G N R E D Q I N R L L R K

 723 TTGAATTAAaaggttttccatggatggtttccctgccagtttgttataaaaatttacata
 241 L N *

 783 caaaagtcaa
 843 aa

Figure 3.7. Comparison of the deduced amino acid sequence from wood frog cDNA clone RSkC2 with human and mouse ribosomal protein L7 (RPL7) and yeast YL8.

- A.* The database accession numbers for human and mouse RPL7 are AAA03081 and P14148, respectively. The amino-terminal repetitive motifs of 12 basic amino acids are underlined, six in the mouse protein and four in the human protein. The yeast (*Saccharomyces cerevisiae*) YL8 (accession no: P05737) is a homolog of mammalian RPL7. A few amino acid residues at the N-terminus of the frog ribosomal protein were missing from the clone RSkC2 cDNA and the amino acid residues as dashed lines are identical to those in the frog ribosomal protein.
- B.* Similarities of wood frog RPL7 protein with its counterparts from human, mouse and yeast.

A

FrogEKKK	LPSVPESL LKRRKAFAEA	22
Human	megv---ke.....	...v-a---t- k-k-rn---l	28
Mouse	meavp---kkvatvpgtl kkkvpagpktl kkkv-a---t- k-k-rn---l		50
Yeastm	aaekilt---q --ks--qqkt	22
Frog	KA KRIKKILAEKKA RKEKRKVIYKRAE SYYKEYRQLYRREVRLARMARKA		72
Human	-i --lr-kf-q-ml --ar--l--ek-k	h-h-----m--t-i-m-----	78
Mouse	-v --lr-kf-l-tl --ar--l--ek-k	h-h-----m--t-i-m-----	100
Yeast	ae qvaaera-r-a n----ai-le-na	a-q---etae-niiqak-d-ka-	72
Frog	GNYYPVPAEPKLA FVIRIRGINGVSPKVRKVLQLLRLRQIFNGTFVKLNKA		122
Human	---f-----		128
Mouse	---f-----		150
Yeast	-s---e-gh--v--v--k---kip--p-----tr-ns-----vt--		122
Frog	SINMLRLVEPYIAWGYPNLKSVKQLIYKRGYLKIKKQRIPLTDNSRIEKY		172
Human	-----i-----ne-----g--n-k--a-----l-ars		178
Mouse	-----i-----ne-----g--n-k--a-----l-ars		200
Yeast	tl-el-k-i---v-y---systir--v----fg--n---v--s--ai--an		172
Frog	LRKTYIICVEDLIQEIYTVGKNFNGANNFLWPFKFSFPGG..GMKKKTTH		220
Human	-g-yg---m---h-----r-ke-----l-s-r..-----		226
Mouse	-g-fg---m---h-----r-ke-----l-s-r..-----		248
Yeast	-g-yg-lsid---h--i---ph-kq-----l-n-s-gw-vpr-fk-		222
Frog	FVEGGDAGNREDQINRLRLKLN		242
Human	-----i-rm-		248
Mouse	-----i-rm-		270
Yeast	-iq--sf----ef--k-vksm-		244

B

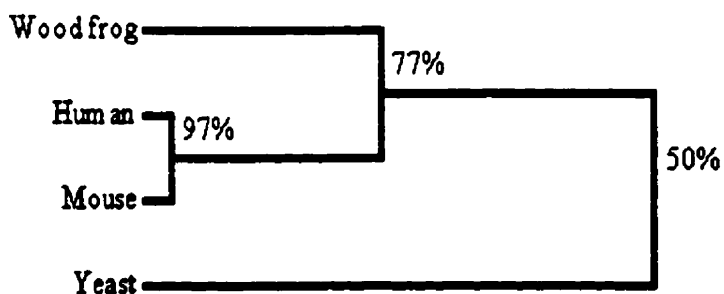


Figure 3.8. Comparison of the nucleotide sequences of clone SkC2 and RSkC2. Nucleotides are numbered on the right. SkC2 nucleotide sequence is shown in bold. The arbitrary primer (AP7) sequence and the anchor primer (H-T₁₁G) recognition sequence are underlined. The Nucleotide residues as dashed lines are identical to those in the clone SkC2.

Figure 3.9. Differential display of mRNA isolated from skin of wood frog *Rana sylvatica*. Primer combination was H-T₁₁G and APn (n: 1-8) and PCR products (³⁵S labeled) were resolved on a DNA sequencing gel. DD-PCR followed the procedures as described in the Methods section. Arrow-indicated bands show up-regulated cDNA gene candidates [e.g. bands (1a-1d, from AP1 primer reaction) were designated as clone candidates (Sk1a-Sk1d)]. C: samples from the cold-acclimated frogs (at 5°C for 2 weeks); W: DD-PCR samples from the cold-acclimated frogs (at 5°C for 2 weeks) shifted to warm condition (at ~20°C) for 7 days.

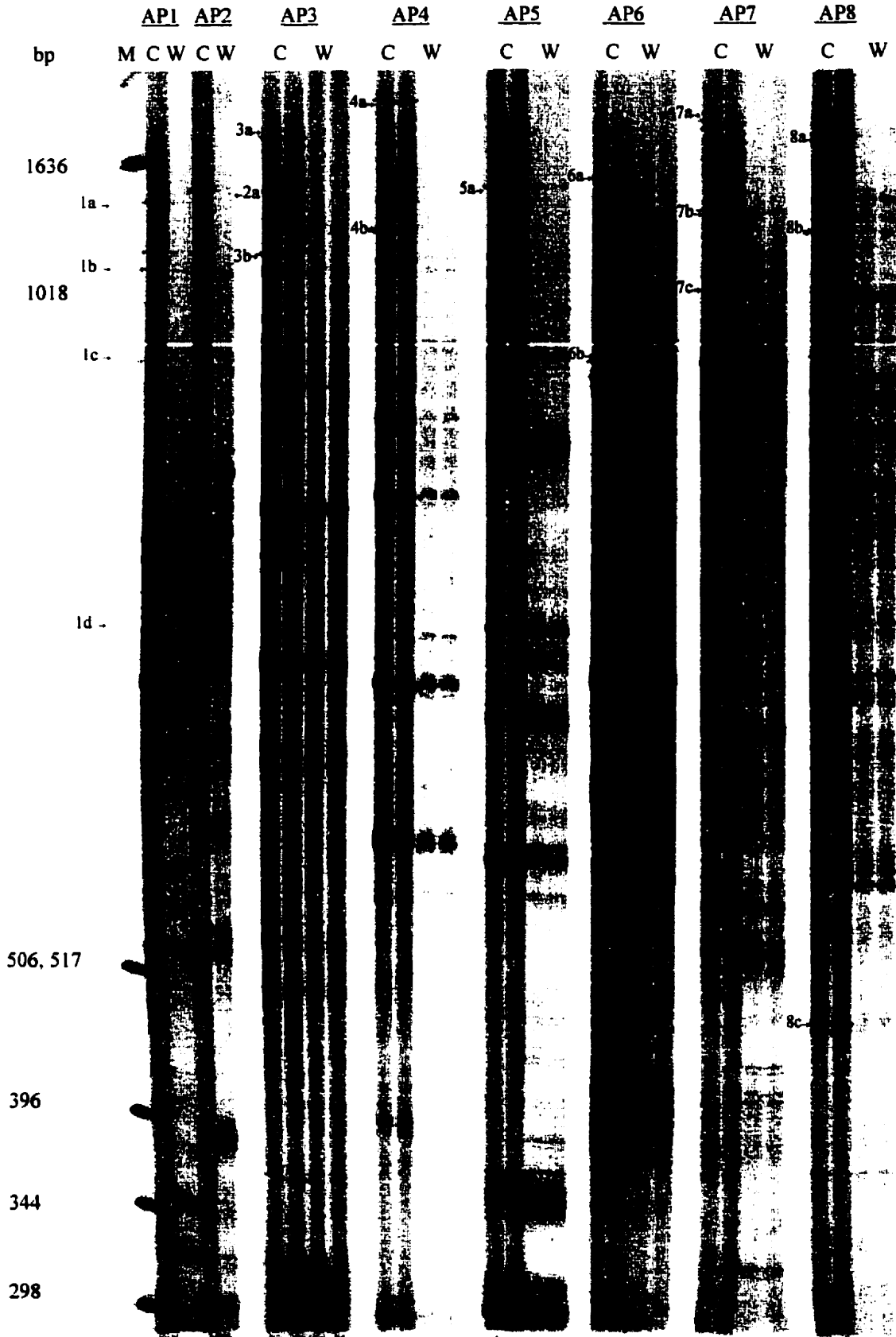


Figure 3.10. Agarose gel analysis of re-amplified DD-PCR fragments from skin of wood frog. Re-amplified DD-PCR fragments were analyzed on 1.5% agarose gel (in 1x TAE buffer). Gels were stained with ethidium bromide. M: molecular weight standards (1 kb DNA ladder)

A. Lane 1, 2, 3, and 4: skin clone Sk1a, Sk1b, Sk1c, and Sk1d, respectively. Lane 5: Sk2a. Lane 6 and 7: Sk3a and Sk3b; lane 8 and 9: Sk4a and Sk4b. More than one PCR fragment appeared in samples from lane 5, 7 and 9. The larger and dominant band from lane 5, 7 and 9 was selected to represent for clone Sk2a, Sk3b and Sk4b, respectively.

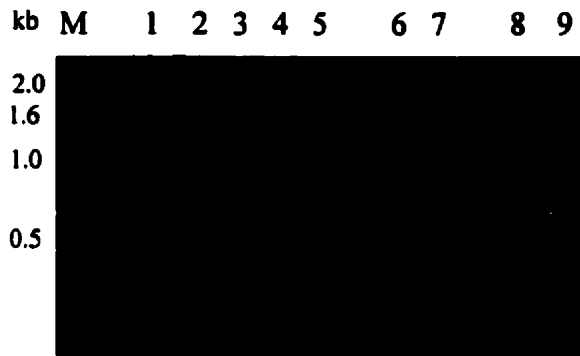
B. Lane 1: clone Sk5a. Lane 2: the larger PCR fragment as for clone SK6a. Lane 3: the dominant cDNA band as for Sk6b. Lane 4: Sk7a. Lane 5: Sk7b (very faint band). Lane 6: two distinct bands and the large and the small DNA band was designated as Sk7c and Sk7c1, respectively. Lane 7: Sk8a. Lane 8: clone Sk8b. Lane 9: Sk8c.

Note: Compared with the estimated sizes for the selected DD-PCR fragments on the DNA sequencing gel (Fig. 3.9), the re-amplified PCR products fragments for these DD-PCR fragments were much smaller in size (Fig. 3.10A, B). It is unknown the reasons.

Following DD-PCR clones (with relatively large size or distinct band on the above gels) were selected for sequencing analysis:

Clone Sk1a, 1b, 1c, 2a, 3b, 4b, 5a, 6a, 6b, 7c, 7c1, and 8b (see Table 3.2).

A



B

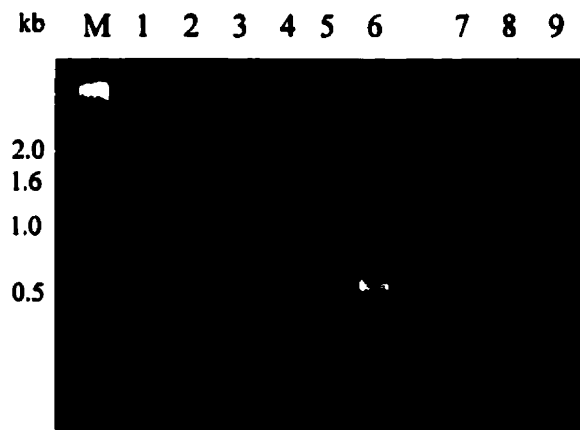


Figure 3.11. Differential display of mRNA from multiple tissues of wood frog *Rana sylvatica*. Part of the results were shown in Fig. 3.1 (DD-PCR of frog skin mRNA). C: cold acclimation-treated frogs (at 5°C for 2 weeks); W: warm acclimation-treated frogs (cold acclimation-treated frogs shifted to ~20°C for 7 days); F: cold acclimation-treated frogs exposed at -2.5°C for 24 h.

Liver

W C



Skin

W C



Muscle

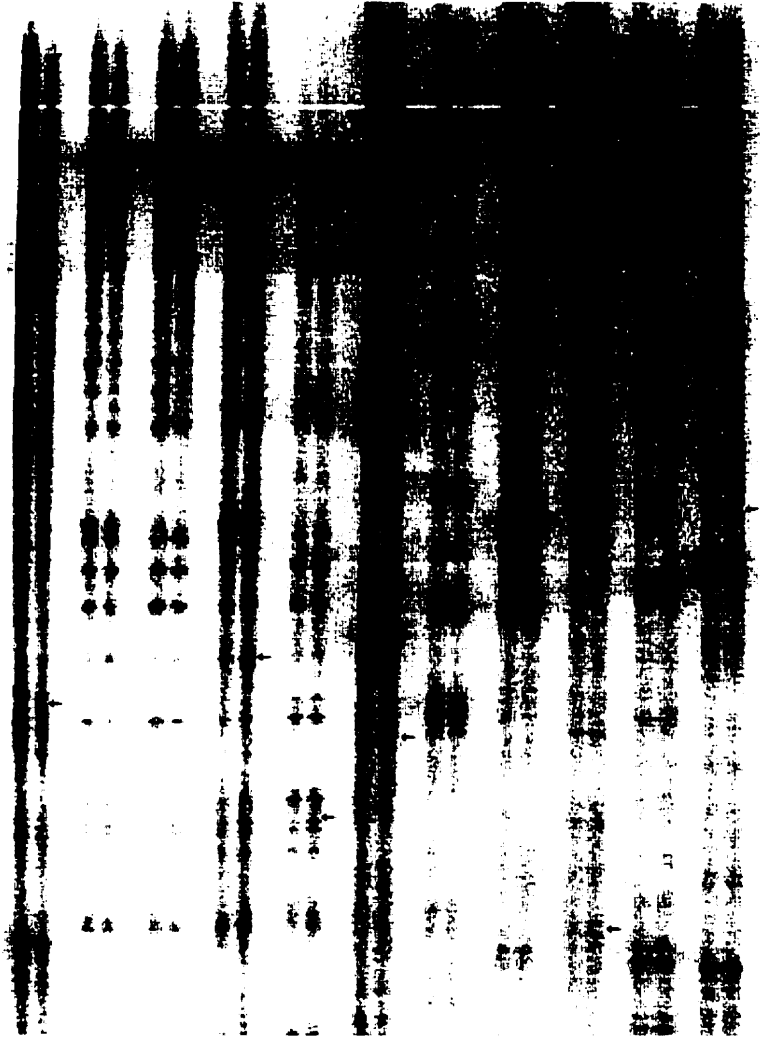
W C F



Figure 3.12. Differential display of mRNA from brain of wood frog.

Primer combination was H-T₁₁G and AP_n (n: 1-8) and PCR products (³⁵S labeled) were resolved on a DNA sequencing gel. DD-PCR followed the procedures as described in the Methods section. The method of test 1 for AP₂ primer reaction was the same as that of other primers. Test 2: adding 2.5× more RT (reverse transcription) mixture. Test 3: adding 2.5× more AP₂ primer concentration. Test 4: applying 2.5× more [³⁵S]α-dATP. C: DD-PCR of brain mRNAs isolated from cold-exposed frogs at 5°C for 2 weeks. F: DD-PCR of brain mRNAs isolated from freeze-exposed frogs at -2.5°C for 24 h. Arrows indicate bands that show up-regulated cDNA gene candidates under freezing exposure.

AP1 AP2 AP3 AP4 AP5 AP6 AP7 AP8
CF CF CF CF CF CF CF CF CF CF CF CF
Test 1 2 3 4



CHAPTER FOUR

**Tissue-Specific Gene Expression in Response to
Freezing, Anoxia and Dehydration Stress**

INTRODUCTION

Via the approaches of differential screening of cDNA libraries and DD-PCR, genes that are differentially expressed in response to freezing or cold acclimation have been identified in frog brain, liver or skin (Chapters 2 and 3). To further study the roles of these genes in frog freezing survival, the influences on the expression of these genes need to be assessed. This includes analysis of the organ-specific patterns of gene expression to determine if the gene product is critical to stress endurance by one, some or all organs and, thus, whether the gene product addresses a highly specific organ need or contributes to cold or freezing survival by all organs. Furthermore, we can gain additional information about the specific trigger that stimulates gene expression and/or the function of the gene product by looking at gene expression responses to two other stresses which are each components of freezing stress -- anoxia and dehydration. Northern blotting analysis provides an effective way to determine if the genes that were up-regulated during freezing or cold-acclimation were also responsive to anoxia or dehydration stress.

As described in Chapter 1, both anoxia and dehydration are the components of freezing. While plasma is frozen, tissues experience oxygen deprivation and products of anaerobic glycolysis accumulate (Storey, 1987). Extracellular ice formation also strongly dehydrates and shrinks cells. Each of these stresses alone appears to trigger some of the cryoprotective responses of wood frogs. For example, glucose synthesis by liver is stimulated when frogs are dehydrated at 5°C, just as in freezing, but anoxia exposure does not stimulate hyperglycemia (Churchill and Storey, 1993; Holden and Storey, 1997). Genes for fibrinogen subunits in liver are also up-regulated when frogs are dehydrated at 5°C, just as they are during freezing, but do not respond to anoxia stress (Cai and Storey,

1997). However, freeze-induction of ADP/ATP translocase gene expression in wood frog liver also occurred during anoxia exposure (N₂ gas atmosphere at 5°C) but not during dehydration (Cai *et al.*, 1997). Hence, studies of gene responsiveness to dehydration or anoxia stresses are helpful in elucidating the possible roles of freeze-responsive gene(s) in cells and in illuminating the signaling mechanisms that control them.

As discussed in Chapter 2, the upregulation of the mitochondrial genes (encoding ATPase subunit 6 & 8, ND4 and 16S rRNA) suggested that low O₂ levels may trigger the differential expression of these genes. Analysis of the expression of these genes under other stress conditions such as anoxia and dehydration will help to define the possible stress signals involved in the regulation of genes that respond to freezing or cold acclimation. In addition to the isolated mitochondrial genes, cDNA clones encoding for two ribosomal proteins (P0 and RPL7) and a regulatory protein (EF-1 γ) involved in protein synthesis as well as the glycolytic enzyme PGK1 (which can also potentially interact with DNA) were selected for further study.

MATERIALS AND METHODS

Animal Care and Tissue Sampling under Anoxia and Dehydration Stresses

Wood frogs were obtained in Ottawa region in the spring season. Tissue sampling from frogs after cold-acclimation at 5°C, or freeze/thaw treatments was as described in Chapter 2. Other groups of frogs were given anoxia or dehydration exposures. Animal experiments for both of these stresses are standard in the lab and are 100% survivable by the animals (Churchill and Storey, 1993; Holden and Storey, 1997). For dehydration at 5°C, frogs were sampled after the loss of 20 or 40% of total body water or after

rehydration at 5°C following 40% dehydration. For anoxia exposure, frogs were sampled after 1, 4, 12 or 24 h of anoxia under a nitrogen gas atmosphere at 5°C.

The expression of selected genes was also tested in organs of two other species, leopard frogs, *Rana pipiens* and adult red-eared slider turtles (*Trachemys scripta elegans*). Leopard frogs are not freeze tolerant but have well developed anoxia and dehydration tolerances similar to wood frogs. Turtles have excellent anoxia tolerance and can endure 3-4 months of submergence in oxygen-depleted cold water while overwintering. Turtles were given anoxic submergence and samples were prepared for Northern blotting as described in detail by Cai and Storey (1996). Anoxia exposure of leopard frogs was conducted exactly as for wood frogs.

Experimental dehydration was conducted as in Churchill and Storey (1993). Frogs were held at 5°C in closed jars (5 animals in a container) with a layer of silica gel desiccant on the bottom (separated from the frogs by a sponge pad). The rate of water loss was approximately 0.5-1% of total body water lost per hour. The equation used to calculate the percentage change in body water content was:

$$\% \text{ change} = [M_i - M_d] / [M_i \times \% \text{ H}_2\text{O}]$$

where M_i is the initial mass of the frog before dehydration, M_d is the weight of the frog at any given time during the experiment, and % H_2O is the percentage of total body mass of control frogs that was water. This last parameter was determined from initial and final masses of control frogs that were sacrificed and then dried to a constant weight at 80°C for 72 h; % H_2O was $80.8 \pm 1.2\%$, mean \pm SEM, $n=6$. For rehydration at 5°C, animals that were first dehydrated by 40% were moved to plastic containers with about 1 cm of distilled water in the bottom and were allowed to fully rehydrate for 24 h.

Anoxic exposure was administered as described by Holden and Storey (1997). Anoxia was imposed by placing four frogs in 0.5 L glass jars containing a 1 cm layer of 5°C distilled water that had been previously bubbled for 20 min with 100% N₂ gas. N₂ gas bubbling was continued for 20 min at which time the jars were tightly capped and sealed with parafilm. Animals were kept in a 5°C incubator and frogs in one jar were sampled at each time point.

RNA Isolation and Precautions for Manipulating RNA

Special care for dealing with RNA was taken as described previously (Chapter 2). For analysis of organ-specific mRNA levels, total RNA was extracted from multiple organs of 5°C acclimated frogs or freeze-exposed frogs at -2.5°C for 24 h. Total RNA was also isolated from brain and liver of animals sampled at selected time points from the experimental courses of freezing, anoxia or dehydration. Total RNA extractions were in sterile DEPC-treated water (or in 50% formamide) and stored as aliquots at -70°C.

Differential Gene Expression by Northern Analysis

Northern blotting was performed to test for differential gene expression in multiple organs when frogs were under freezing exposure or in brain and liver under anoxia or dehydration stresses. The details of procedures for Northern analysis were described in Chapter 2.

The genes isolated from frog brain and liver by screening cDNA libraries (Chapter 2) and from skin by DD-PCR (Chapter 3) were used as probes for the Northern analysis. Table 4.1 shows the identified cDNA genes in wood frogs.

RESULTS

Differential Expression of Mitochondrial Genes in Wood Frog *Rana sylvatica*

As reported in Chapter 2, frog ATPase 6 & 8 and 16S rRNA genes were isolated from a brain cDNA library and their transcripts accumulated in brain during freezing exposure. The ND4 gene was originally isolated from a liver cDNA library and the gene was up-regulated upon freezing exposure in frog liver. Probes for these genes were used to evaluate the expression of these genes in other tissues of frogs and in response to alternative stresses (anoxia, dehydration).

ATPase 6 & 8

As in frog brain, ATPase 6 & 8 transcripts were also elevated in wood frog liver during freezing exposure (Fig. 4.1-4.4). As assessed on a blot run with isolated mRNA, transcript levels in frog liver were about 7-fold increase during freezing exposure or after 24 h recovery than in liver of control animals (Fig. 4.1). Comparable blots run using total RNA indicated elevated transcripts levels during freezing in liver and brain, with freeze-induced increases of about 80% to 2-fold (Fig. 4.2-4.3). The somewhat lower fold increase in transcript levels observed in liver and brain using total RNA blots, compared with mRNA blots, may indicate that the total RNA blots are somewhat less sensitive than the mRNA blot for Northern analysis. ATPase 6 & 8 genes were also up-regulated in kidney during freezing, rising about 3-fold (Fig. 4.3). ATPase 6 & 8 transcript levels were relatively constant in heart, muscle, skin and blood (blood cells and sera) during freezing exposure, as compared with controls but in the lung and gut, transcripts were down-regulated by about 30% and 2.5 fold, respectively, during freezing. As described

in Chapter 2, the dominant RNA band hybridizing with the ATPase 6 & 8 gene pair was about 1.05 kb in length. However, Fig. 4.2 and 4.3A show that two distinctly larger RNA bands can be observed in autoradiograms from over-exposed X-ray film. Their sizes were estimated to be 1.91 and 3.31 kb, respectively, and these RNA may represent ATPase 6 & 8 RNA precursors or other longer segments (with part of ATPase 6 & 8 RNA sequences) derived from the mitochondrial primary transcripts. A few very faint bands (0.79, 0.55, ~0.28 kb) can also be seen in Fig. 4.1, 4.3 and 4.4 and these may represent degraded ATPase 6 & 8 transcripts.

Fig. 4.4 shows how the ATPase 6 & 8 transcripts responded to anoxia exposure in frog brain and liver. Within 1 hour, transcript levels in brain had risen to the same extent as was seen in tissue from freeze exposed animals and transcript levels remained elevated in 4 h anoxic exposure group (Fig. 4.4A). Gene expression was also up-regulated in liver of anoxic frogs. Transcript levels in frog liver increased about 2-fold even after 24 h anoxic exposure, compared with the control (Fig. 4.4B). The response of these genes to dehydration stress was also tested in frog brain. ATPase 6 & 8 transcript levels decreased by about 50% in the brain of 40% dehydrated frogs as compared with the control group (Fig. 4.4A).

The frog ATPase 6 & 8 cDNA probe was also used to test for the expression of these genes in the brain and liver of anoxia tolerant turtles (*Trachemys scripta elegans*) but as can be seen in Fig. 4.4, only a rather faint band of hybridization could be seen in 2 test samples. The general lack of response suggests that the frog probe did not cross-react well with the turtle mRNA transcripts.

16S rRNA

Under short X-ray film exposure and quick development of the film, a single band for mitochondrial 16S rRNA can be observed on Northern blots (Fig. 2.3A). The size was estimated at ~1.6 kb. However, in addition to this dominant rRNA band, a smear region (0.24-1.6 kb) can be seen on most RNA blots (Fig. 4.5-4.7). By using a mRNA blot, the 16S rRNA level in the brain was shown to be about 4.5-fold higher during freezing exposure and about 3.5-fold higher after thawing recovery than in controls (Fig. 4.5), whereas the 16S rRNA level in the liver was also about 4-fold higher in both freeze-exposed and thawed groups than in their controls. However, much less sensitivity was found when total RNA blots were used for Northern analysis. Under freezing stress, the rRNA level was only about 40% higher in brain and over 50% higher in the liver tissues (Fig. 4.6) as compared to controls. For other tissues (skin and heart), the RNA levels were relatively constant among the control, freezing and recovery groups. However, the RNA levels were elevated about 2-fold in muscle tissues as compared to control on the total RNA blot (Fig. 4.6B).

Under anoxia stress, the level of 16S rRNA dramatically increased (about 4-5 fold) in the brain of anoxic wood frogs, while there was no significant change in content under dehydration conditions (Fig. 4.7) Expression of the mitochondrial 16S rRNA gene in response to anoxia stress was also examined in brain of the anoxia-tolerant leopard frog, *R. pipiens* (Fig. 4.7). The 16S rRNA gene seemed up-regulated under longer anoxia exposure (up to 24 h) as compared to the 1 h anoxia-exposed group of leopard frogs (Fig. 4.7).

ND4

Analysis of organ-specific responses by ND4 gene transcripts to freezing exposure are shown on total RNA blots in Fig. 4.8. Transcript levels were relatively low in frog liver as compared with in skin and brain. However, ND4 transcripts were up-regulated in both the liver (up to 50% increase) and brain (2-fold increase) during freezing; levels remained high during thawing recovery in brain but were reduced again in liver. In skin, the level of ND4 transcripts was constant in control, frozen and recovery groups. Freeze upregulation of ND4 transcripts in liver can also be seen in Fig. 4.9 but, by contrast, transcript levels were reduced under both anoxia and dehydration stresses in liver. In particular, ND4 transcript levels were almost undetectable in liver after 1 h anoxia exposure (Fig. 4.9, lane 8), however, the transcript levels elevated again under deep anoxia (4-12 h) but with longer anoxic exposure (up to 24 h) ND4 gene expression was repressed again (Fig. 4.9, lane 9-11).

Expression of Ribosomal or Translational Regulatory Protein Genes

The ribosomal protein (P0) and the translational regulatory protein (EF-1 γ) genes were isolated from frog brain and liver cDNA libraries, respectively. Another ribosomal protein (RPL7) gene was isolated from frog skin by DD-PCR approaches. Tissue-specific gene expression in response to low temperature, anoxia and dehydration stresses were studied for the 3 genes.

P0

The pattern of *P0* gene expression in the liver of freeze-exposed frogs was very similar to the pattern seen in brain as shown on mRNA blots (Fig. 2.4 and 4.10).

Transcript levels were 8-10 fold higher during freezing or thawing recovery as compared with control groups. The blots loaded with total RNA showed a similar gene expression pattern but the test sensitivity was lower (Fig. 4.11). The *P0* gene was highly expressed in brain, muscle and liver, but transcript levels were much lower in skin (Fig. 4.11).

Transcript levels remained constant in the skin and muscle during freezing. The pattern of *P0* gene expression under anoxia and dehydration stresses was similar to that of 16S rRNA gene as described before. The *P0* transcript level was higher at 4 h anoxia but not changed by dehydration. However, in *R. pipiens* it seemed the RNA levels went down a bit at 12 h anoxia but increased at 24 h anoxia.

EF-1 γ

The *EF-1 γ* gene was up-regulated in frog liver upon freezing exposure as described in Chapter 2 (Fig. 2.22A, B). The *EF-1 γ* transcript was about 1.7 kb in length. In the brain and skin of freeze-exposed frogs, the intensity of the dominant band (1.7 kb) was constant during freezing and thawing (Fig. 4.8 and Fig 4.13). However, levels of a minor band (2.0 kb) in Fig. 4.8 rose during freezing in liver, brain and skin, which did not appear in Fig. 2.22B and Fig. 4.13 (probed with the larger insert of clone Li39). The 2.0-kb transcript may be probed by ND4 gene (the smaller insert of clone Li39). Under dehydration stress, *EF-1 γ* transcript levels were significantly reduced; levels fell by about one-half in the 40% dehydration group as compared with the 20% dehydration group

(Fig. 4.9). Furthermore, EF-1 γ gene expression was completely suppressed within 1 hr when frogs were exposed to an anoxic atmosphere. Transcript levels rose again under deep anoxia (4-12 h) but with longer anoxic exposure (up to 24 h) EF-1 γ gene expression was reduced again (Fig. 4.9).

RPL7

The frog RPL7 gene was isolated by DD-PCR. The gene was up-regulated in the skin during cold acclimation as described in Chapter 3. Upon freeze exposure, RPL7 transcript levels remained constant in skin (Fig. 4.14) and liver (Fig. 4.14, 4.15). Accumulation of the transcripts can be shown in the freezing and recovery groups of frog muscle tissues (Fig. 4.14). However, transcript levels were about 2-fold higher in frog brain in frozen or thawed recovery groups as compared with the control group (5°C cold acclimation) (Fig. 4.14, 4.15). Fig. 4.15 also shows that the gene transcripts were elevated in the skin of cold-acclimated frogs as compared with warm-acclimated frogs as control. Fig. 4.16 compares the effects of freezing, dehydration and anoxia on RPL7 gene expression in frog brain. Freezing (for 24 h) and short anoxia (for 4 h) exposures greatly increased the level of gene transcripts (about 2.5-3.5 fold increase), and dehydration also raised transcript levels somewhat. However, long anoxia (24 h) strongly reduced gene transcript levels (Fig. 4.16).

Expression of Genes Encoding Products Potentially Interacting with Nucleic Acids

The 16S rRNA should also interact with other rRNA and/or mRNA in mitochondrial translation machinery, and P0 and RPL7 proteins must interact with

cytoplasmic rRNAs involving ribosomal assembly and protein synthesis. These genes should also belong to the gene group that the gene products interact with nucleic acids. The following description will focus on the differential expression of *PGK1* (its protein product potentially as a transcription factor) and *pol*-like reverse transcriptase-related gene.

PGK1

The frog *PGK1* gene was isolated by differential screening of the brain cDNA library. A major RNA band (1.7 kb) was detected on total RNA blots in the brain and liver tissues. It was highly expressed in brain and skin tissues of freeze-exposed frogs. The level of *PGK1* gene transcripts in frog liver and brain was about 50% or 2-fold higher during freezing (12-24 h), respectively, as compared with the control group and the transcript level was further elevated even during recovery (2-2.5 fold increase) (Fig. 4.17). In skin the *PGK1* probe hybridized with 2 distinct bands (1.7 and 3.4 kb) but bands showed no significant changes in intensity during freezing or thawing.

PGK1 gene transcripts also accumulated in frog brain under anoxia exposure, being increased after just 1 h anoxia exposure (Fig. 4.18). Anoxia seemed to enhance *PGK1* expression in frog liver during short exposure. However, longer anoxia exposure appeared significantly to reduce the level of gene transcripts (Fig. 4.18). Unlike the results with mtATPase 6 & 8 probes, frog *PGK1* cDNA could be applied for probing turtle tissues due to the high conservation of the gene sequence through evolution. In turtles, anoxia exposure resulted in a strong increase in transcript levels in brain by 5 hours of anoxia exposure and these remained elevated through 20 hours anoxia and also

after 5 hours of aerobic recovery (Fig. 4.18). Fig. 4.19 also shows the responses of PGK1 transcripts to dehydration in frog tissues. Transcript levels in brain increased about 2.5-fold over control values when frogs were dehydrated to either 20 or 40 % of total body water lost.

Pol-Like Reverse Transcriptase Gene

This gene was also isolated from the frog brain cDNA library. Northern blot analysis of brain and liver mRNA samples from control, 24 h frozen, and 24 h thawed frogs are shown in Fig. 4.20. As was also seen for brain in Fig 2.6, gene expression was induced during freezing and rose higher still in thawed frogs. The gene showed stronger expression in frog liver than in brain under all conditions and the dominant band (3.5 kb) in liver increased greatly in intensity during freezing (~30-fold greater than control) and further elevated even in the recovery group (~45-fold greater than control). It was less successful to detect the transcripts on Northern blots loaded with total RNA samples (in some trials there was no signal or multiple non-specific bands). The gene transcript level may be extremely low *in vivo*. Gene expression in other tissues during freezing or under other stresses has not yet been assessed.

DISCUSSION

The present chapter provides a broader examination of the expression of those genes that were identified as differentially up-regulated in brain or liver under freezing stress or in skin under cold exposure in Chapters 2 and 3. The results show that these genes are also differentially expressed under freezing exposure in other tissues of wood

frogs. Furthermore, differential gene expression in frog liver or brain was also stimulated by stresses that are components of freezing, anoxia exposure and/or dehydration stress, and this information will aid our understanding of the triggers involved in gene expression and/or the metabolic functions supported by the gene products.

The four mitochondrial genes (ATPase 6 & 8, 16S rRNA, *ND4*) were all up-regulated in both brain and liver of freeze-exposed frogs. Freeze upregulation of mitochondrial ATPase 6 & 8 genes was also shown in frog kidney (Fig. 4.3B), whereas down-regulation of ATPase 6 & 8 gene expression occurred in lung and gut of freeze-exposed frogs. ATPase 6 & 8 were highly expressed in frog organs under control conditions, especially in the heart, muscle, brain and liver, which suggests high rates of mitochondrial gene expression under normal aerobic conditions. Expression was particularly strong in heart, a mitochondria-rich organ that requires high rates of aerobic ATP production.

For the mitochondrial gene encoding for 16S rRNA, the gene was up-regulated during freezing in frog brain and liver but transcript levels were relatively constant in other organs during freezing. Since the 16S rRNA gene transcript is extremely abundant (~10 fold more than that of most mitochondrially encoded mRNAs), a high intensity band (~1.6 kb) and dark smear area (size below 1.6 kb) were detected on RNA blots (Fig 4.5, 4.6 and 4.7). The smear area may result from the cleavage or degradation of 16S rRNA. Since mitochondria have their own transcription and translation system, the upregulation of the 16S rRNA gene from frog brain may be a response to freezing exposure, which may be required for maintaining mitochondrial translational machinery.

Like 16S rRNA and ATPase 6 & 8 genes, levels of ND4 transcript were also up-regulated in liver and brain but were constant in the skin of freeze-exposed frogs as compared with controls (at 5°C). Both central organs may need to stimulate expression of these genes during freeze exposure in order to maintain a certain level of mitochondrial activity for generating ATP used for cellular adjustment during the stress. Since skin of frogs is typically the first tissue to freeze and can be fully frozen in less than 1 hour, differential gene upregulation in this tissue may not be a major part of the cryoprotective response by skin to freezing due to the short time frame available for response. Furthermore, the transcripts of the 4 mitochondrial genes were constant in the skin tissues during freezing and recovery stages, which suggests that pre-existing gene transcripts may be stable even under the stress conditions.

Under anoxia or dehydration stress, mitochondrial genes responded quite differently. The expression of ATPase 6 & 8 genes was enhanced in both brain and liver even under short anoxic exposure (1-4 h) and transcripts remained high in both organs throughout the anoxic exposure, even after for 24 h in liver (Fig. 4.4). By contrast, however, dehydration reduced transcript levels in brain. These different effects of anoxia and dehydration on ATPase 6 & 8 may suggest that the upregulation of these genes by freezing is linked with a low oxygen trigger.

Because of this low oxygen link, it was decided to test the response of these genes to anoxia in a well-known anoxia-tolerant vertebrate, the turtle *Trachemys scripta elegans*. However, the frog probe for ATPase 6 & 8 did not hybridize well with RNA from either turtle brain or liver (Fig. 4.4). This may be because the ATPase 6 & 8 genes are not highly conserved in phylogeny. For example, the homology of ATPase subunit 8

is about 27% between chicken and mammals and 32% between the toad (*Xenopus laevis*) and mammals. The homology for ATPase subunit 6 is about 55% between chicken and mammals and 53% between *X. laevis* and mammals (Desjardins and Morais, 1990). Although the heterologous gene probing did not work out for turtle tissues, a single faint band (also 1.05 kb in size) was observed on the blots with turtle samples, which may suggest that turtle mitochondrial ATPase subunit 6 & 8 may also be produced from a single transcript with two different reading frames.

Under anoxic exposure, the 16S rRNA significantly increased in frog brain after 4 h anoxia as compared with the control group. The gene transcript also increased about 3-fold in the brain of anoxia-tolerant frog *R. pipiens* after prolonged anoxic exposure (for 24 h) as compared the control of the leopard frogs. Levels of the 16S rRNA transcript in brain did not respond when wood frogs were dehydrated and, hence, as for ATPase 6 & 8 transcripts described above, it appears that a low oxygen signal may be the stimulus initiating upregulation of these genes under both anoxic and freezing conditions.

By contrast with the responses of the above genes, *ND4* expression, which increased over the early hours of freezing exposure, was sharply repressed in the brain within 1 h of anoxia (Fig. 4.9). Transcript levels rose again at 4 h and 12 h anoxia but were low again after 24 h anoxia, which indicated that *ND4* expression can be modulated by the O₂ level. Furthermore, mitochondrial *ND4* genes also appeared to be down-regulated in frog brain during dehydration so it appears that with respect to *ND4* dehydration stress may not mimic the response of the gene to freezing.

The above results indicated that dehydration alone may not enhance mitochondrial gene expression whereas anoxia alone can modulate the expression of the

4 isolated mitochondrial genes. The ischemia/hypoxia that is induced by freezing may be an important trigger that stimulates the expression of certain genes. Of note are other studies that show that other freeze-induced genes that are related to energy metabolism in frog tissues (mitochondrial ADP/ATP translocase) (Cai *et al.*, 1997) were also up-regulated by anoxia whereas mitochondrially-encoded genes (*CoxI* and *ND5*) were up-regulated by anoxia in the anoxia-tolerant turtles (*Trachemys scripta elegans*) and were also up-regulated in response to freezing in freeze-tolerant turtles (*Chrysemys picta marginata*) (Cai and Storey, 1996). So there seems to be a general link between stress that imposes oxygen limitation and the upregulation of mitochondrially-encoded genes in species that are tolerant of anoxia and/or freezing stress.

As discussed in Chapter 3, the accumulation of gene transcripts is generally regulated by the rates of transcription or/and other gene expression steps. For mitochondrial genes, the levels of gene transcripts are determined by mtDNA replication (i.e. controlling the gene copy number), transcription, processing of the primary transcripts and RNA stability. It is generally thought that increases in mtDNA gene products under physiological stimuli may primarily result from mtDNA replication (Williams, 1986). It would be valuable to analyze mtDNA content during stresses (freezing or anoxia) with the isolated mitochondrial genes as probes. If mtDNA replication or activation of mtDNA at the transcriptional level takes place during freezing or anoxic treatment, it could be predicted that other mitochondrial genes should also up-regulated under the stress conditions.

Tissue-specific gene expression was also demonstrated for the genes encoding ribosomal proteins (P0 and RPL7) and the translational regulatory protein (EF-1 γ). Under

freezing exposure, the P0 gene was up-regulated in both brain and liver (Fig. 4.10 and 4.11) whereas P0 transcripts did not change significantly in muscle and skin of freeze-exposed frogs. Unlike the P0 gene, the EF-1 γ gene transcripts (1.7 kb) showed equal levels in control, frozen and thawed samples from frog brain (Fig. 4.8 and 4.13). As expected, the EF-1 γ transcript (1.7 kb) was constant in skin from freeze-exposed frogs (Fig. 4.8). Like the P0 gene, RPL7 was also up-regulated in frog brain during freezing. The RPL7 gene was originally isolated from frog skin by DD-PCR. RPL7 gene transcripts were elevated during cold acclimation as comparing with warm-acclimated frogs. The levels of RPL7 transcripts were not changed in frog skin, liver and heart under freezing exposure (Fig. 4.14 and 4.15) but in muscle the intensity of RPL7 transcripts had increased after 24 h freezing and were maintained at a high level during thawing recovery (Fig. 4.14). Although the specific role of tissue-specific expression of these ribosome-associated protein genes is unknown, the upregulation of these genes under freeze exposure in selected frog organs may compensate for freezing-caused impairment to the translational machinery. The freeze tolerant frog needs to maintain functional ribosomal activity in selected organs and may produce tissue-specific proteins to endure the freezing stress and to do this, selected changes may have to be made to proteins involved in the translation process.

Under anoxia or dehydration stress, P0 gene expression in the brain showed a similar pattern to that of the mitochondrial 16S rRNA gene as described above (Fig. 4.7 for 16S rRNA gene, Fig 4.12 for P0 gene), being up-regulated by 4 h anoxia exposure but less affected by dehydration stress. However, the gene expression pattern of EF-1 γ to anoxia and freezing was similar to that of the mitochondrial ND4 gene. Unlike the P0 and

EF-1 γ gene, both anoxia and dehydration (as well as freezing) can result in the accumulation of *RPL7* transcripts (Fig. 4.16). The upregulation of *RPL7* gene expression under anoxia occurred after a short period of anoxic treatment (~4 h) whereas prolonged anoxic exposure dramatically decreased the level of gene transcripts (Fig. 4.16). The results suggested that the *RPL7* expression is also regulated by O₂ level.

Finally, analysis of the expression of the gene encoding *PGK1*, a glycolytic enzyme that can also potentially function as a transcription factor in the nucleus, revealed that freezing, anoxia and dehydration can all enhance the gene expression in frog brain. This suggests that freezing-induced ischemia/hypoxia and freezing-caused intracellular dehydration may both contribute to *PGK1* gene upregulation in the brain of freeze-exposed frogs. For the *pol*-like reverse transcriptase gene (its product involving interaction with nucleic acids), freezing can induce gene expression both in brain and liver. The results also implied that activation of this gene at the transcription level must take place in the freeze-exposed frogs.

Anoxia can promote the expression of all of the analyzed genes although each of them may respond to anoxic stress differently. By contrast, dehydration stress enhanced only *PGK1* and *RPL7* expression among the genes analyzed. Thus, current studies suggest that ischemia/hypoxia may be the major stimulus that triggers upregulation of genes when frogs freeze whereas freezing-caused changes in cell volume play a role in the regulation of only selected genes (such as *PGK1*). Cryoprotectant production (glucose accumulation in wood frogs) may compensate for some the severe consequences of cellular dehydration during tissue freezing. Since freezing-caused cellular dehydration is correlated with the freezing state of the tissues and transcription processes should cease

in the frozen tissues, this may explain why dehydration-regulated genes were generally not isolated from organs of freeze-exposed frogs. Under freeze exposure, frog peripheral tissues freeze first and freezing of blood plasma halts circulation which causes ischemia in tissues and may activate certain gene expression in the central organs (still in non-frozen state). It is possible that the ischemia-induced gene expression in the liver and brain may be the early freezing event. This is also in agreement with the observation that isolated anoxia/hypoxia-regulated genes did not respond to freezing exposure in skin since this organ froze first (within 1 h in freezing-treated frogs). As for the accumulation of the gene transcripts, the post-transcriptional modification and RNA stability should also be considered. For example, cold-specific mRNA stability appears in type I antifreeze protein gene in winter flounder (Duncker *et al.*, 1995).

As discussed in Chapter 2, hypoxia-induced PGK1 gene activation was also reported in mammalian cells and tissues (Li *et al.*, 1996). Freezing of blood plasma limits the availability of O₂ supply for the tissues, necessitating cells to depend more on glycolysis for their energy. In addition to the Pasteur effect that relates to an acute increase in the intracellular phosphofructokinase activity resulting mainly from changes in the concentration of low molecular-mass allosteric effectors, enzymatic activities of glycolytic components are enhanced by chronic hypoxia. Steady-state mRNA levels of several glycolytic enzymes (pyruvate kinase 1, PGK1, etc.) are up-regulated by hypoxia (Semenza *et al.*, 1994). PGK1 mRNA transcripts were elevated in the brain of freeze- or anoxia-exposed frogs, which suggests that the induction of PGK1 may contribute to the regulation of glycolytic flux under reduced O₂ tension and to adaptation of cells to ischemia/hypoxia. Recent studies have indicated that PGK1 and several other glycolytic

enzymes can also function as DNA binding proteins in the nucleus (Ronai, 1993). It would be interesting to test if freezing stress also leads to the accumulation of PGK1 protein in the nucleus of frog brain cells where it could affect transcription regulation.

Accumulation of selected mitochondrial gene transcripts has also been reported in mammals in acute myocardial stress, in differentiation of myoblasts or in cold environment. The upregulation of mtATPase 6 & 8, COI, II, III and cyt b genes under global ischemia and reperfusion was found in the rat heart (Das *et al.*, 1995). Mitochondrial 16S rRNA, and ATPase 6 & 8 gene transcripts were accumulated in high levels in human myoblasts and increased progressively as myotubes matured. When partially differentiated myotubes were switched to hypoxic conditions, mRNAs coding for glycolytic enzymes increased whereas mRNAs for respiratory enzymes declined (Webster *et al.*, 1990). The accumulation of mitochondrial COII transcript and 16S rRNA was also reported in the liver of rats exposed to cold stress (4°C) (Martin *et al.*, 1993). The cold environment can induce a rapid (a few hours) increase in the liver COII mRNA and 16S rRNA levels. High levels of both RNAs are present under cold acclimation. The cold-induced mitochondrial gene activation is regulated at the level of transcription. The mtDNA content is constant during cold acclimation and thus unlikely to play a role in the elevation of cold-induced COII mRNA and 16S by changing the gene copy number via mtDNA replication (Martin *et al.*, 1993). However, cold exposure of mice can stimulate both mtDNA replication and expression of mtDNA-coded genes in brown fat and skeletal muscle (Puigserver *et al.*, 1998).

EF-1 protein was elevated during cold acclimation in the liver of toadfish as mentioned in Chapter 2 (Plant *et al.*, 1977). It is generally considered that EF-1 is the

major controlling element in the changes in protein synthesis associated with temperature acclimation. To my knowledge, anoxia-regulated EF-1 γ gene expression has not been reported yet. Cold acclimation also led to the accumulation of RPL7 transcripts in the skin of wood frog (Fig. 3.4 and 4.15). Freezing increased the levels of EF-1 γ , P0 and RPL7 gene transcripts in certain organs of frogs, however, the signals (low temperature and/or low O₂ level) involved in the gene regulation remain undetermined.

Overall, the differentially-expressed frog genes can be classified into two groups: 1) Genes whose products involving energy generation (such as mtATPase subunit 6 & 8, PGK1 etc.), 2) Genes whose products are the components of protein translation machinery (such as EF-1 γ , P0, RPL7 *etc.*). The expression of these genes in the selected tissues under freeze, anoxia or dehydration stress is shown in Table 4.2.

The *EF-1 γ* , *P0*, *RPL7*, *PGK1* and the isolated mitochondrial genes should be highly expressed in cells under normal conditions, as they are all housekeeping genes. They are essential for cell growth and survival. It is unclear what the significance of the accumulation of their gene transcripts is under freezing, anoxia or dehydration stress in selected frog organs. Under freezing exposure, it is undetermined if the accumulated frog gene transcripts result from changes in RNA synthesis or/and RNA stability. To further explore this, nuclear run-on assays might be applied to test stress-induced gene activation. Other questions that remain to be explored are whether the elevated levels protein-coding gene transcripts (mRNAs) actually lead to enhanced synthesis of these proteins. The following chapter will address gene expression at the translational level and possible signals involving cellular adaptation under the stress conditions.

Table 4.1. Stress-inducible genes in wood frog *Rana sylvatica*

Energy Generation	Translation Machinery
<p>Mitochondrial ATPase subunit 6 & 8 (mtATPase 6 & 8 genes)</p> <p>NADH-ubiquinone oxidoreductase (<i>ND4</i>)</p> <p>Phosphoglycerate kinase 1 (<i>PGK1</i>)</p>	<p>Mitochondrial 16S rRNA</p> <p>Elongation factor 1, gamma subunit (<i>EF-1γ</i>)</p> <p>Acidic ribosomal phosphoprotein (<i>P0</i>)</p> <p>Ribosomal protein L7 (<i>RPL7</i>)</p>

Table 4.2. Tissue-specific gene expression in response to freezing, anoxia or dehydration stress in wood frog

Gene	Brain			Liver			Skin			Muscle	Heart
	F	A	D	F	A	D	F	W	C-T	F	F
<i>ATPase 6 & 8</i>	↑	↑	↓	↑	↑		→			→	→
<i>mt 16S rRNA</i>	↑	↑	↓	↑			→			↑	→
<i>ND4</i>	↑			↑	↘ ^Δ ↘	↓	→				
<i>PGK1</i>	↑	↑	↑	↑	↑	↘ ^Δ ↘	→				
<i>EF-1γ</i>	→			↑	↘ ^Δ ↘	↓	→				
<i>P0</i>	↑	↑	↘ ^Δ	↑			→			→	
<i>RPL7</i>	↑	↑	↑	→			→	↓	↑	↑	→

Note: F: freezing; A: anoxia; D: dehydration; W: warm-acclimation; C-T: cold acclimation.

↑: Up-regulated gene expression.

↓: Down-regulated gene expression.

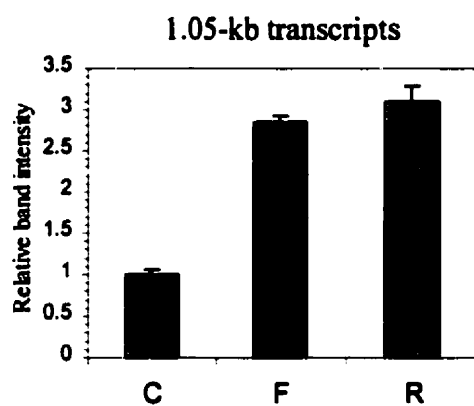
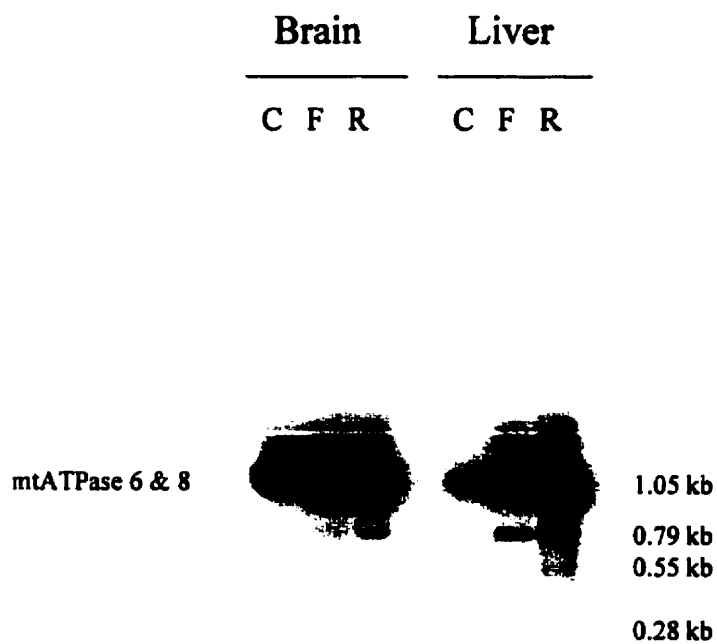
→: Consistent level of transcripts during stress.

↘^Δ: Down-regulation under 20% dehydration treatment and then upregulation under 40% dehydration.

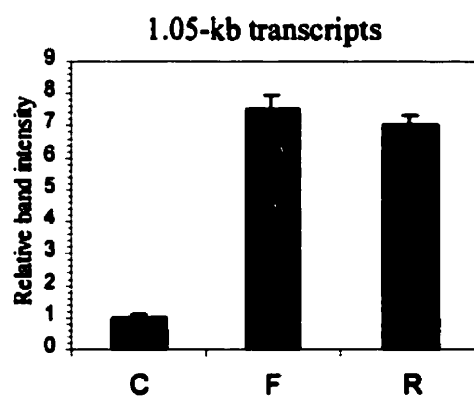
↗^Δ: Up-regulated gene expression under 20% dehydration and then down-regulated under 40% dehydration.

↘^Δ↘: Down-regulated gene expression under initial anoxic exposure (1-4 h), then up-regulated under 4-12 h anoxia and finally down-regulated under longer anoxia exposure (up to 24 h).

Figure 4.1. Representative Northern blot of brain and liver mRNA probed with mitochondrial ATPase 6 & 8 genes in the freeze-exposed wood frog *Rana sylvatica*. Equal amounts of mRNA (1 μ g) were loaded for all samples. The RNA loading and quality were assessed by staining the mRNA blot with methylene blue. Identical blots were prepared for multiple Northern tests and the same blot can be reused for 3-5 times by stripping the hybridized probes. The results of frog brain mRNA from the same blot probed with the mitochondrial ATPase 6 & 8 genes were shown in Chapter 2 and the EF1- γ gene was used as an internal control for the brain RNA samples (Fig. 2.2). The gene probe was derived from the insert of frog brain clone Br3. C: control, from cold-acclimated frogs (at 5°C for 2 weeks); F: freezing the cold-acclimated frogs at -2.5°C for 24 h; R: freeze-exposed frogs at -2.5°C for 24 h and then thawed at 5°C for 24 h. The RNA blotting and hybridization were repeated with multiple trials and the data shown in the graph represent the average value from the repeats (n = 3). Analysis of band intensities was performed by using Imagequant. The value of RNA band intensity of test groups from frog brain and liver tissues is normalized to their control groups, respectively.



Frog Brain



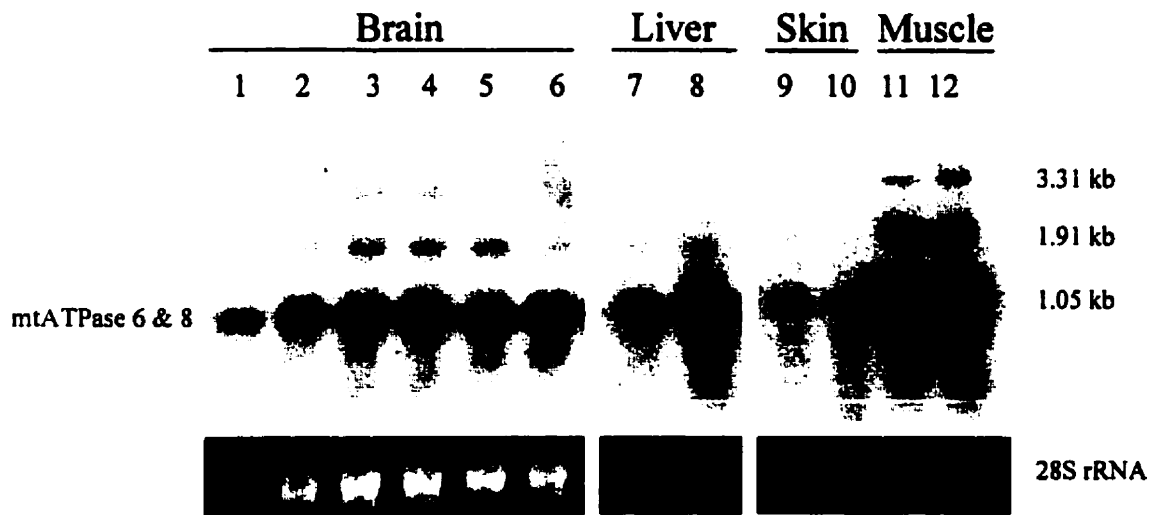
Frog Liver

Figure 4.2. Expression of mitochondrial ATPase 6 & 8 genes under freezing stress in frog brain, liver, skin and muscle tissues by Northern blotting analysis.

A. Equal amounts of total RNA (15 µg) were loaded for all samples. The rRNAs were detected by staining the agarose gel with ethidium bromide. All blots were carried out under the same conditions for Northern analysis. Lane 1: control frog brain from cold-acclimated frogs at 5°C for 2 weeks; lane 2, 3, 4, and 5: freezing-treated groups from the cold-acclimated frogs exposed at -2.5°C for 4, 8, 12 and 24 h, respectively; lane 6: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h; lane 7 and 8: frog liver, control and 24 h freezing group, respectively; lane 9 and 10: frog skin, control and 24 h freezing group, respectively; lane 11 and 12: frog muscle, control and 24 h freezing group, respectively.

B. The RNA blotting and hybridization were performed with multiple trials and the data shown in the graphs represent the average value from the multiple trials (n = 5 for frog brain, liver and skin; n = 3 for muscle). The value of RNA band intensity of freezing test groups from the frog tissues is normalized to their control groups, respectively. C: control, from cold-acclimated frogs (at 5°C for 2 weeks); F: freezing the cold-acclimated frogs at -2.5°C for 24 h.

A



B

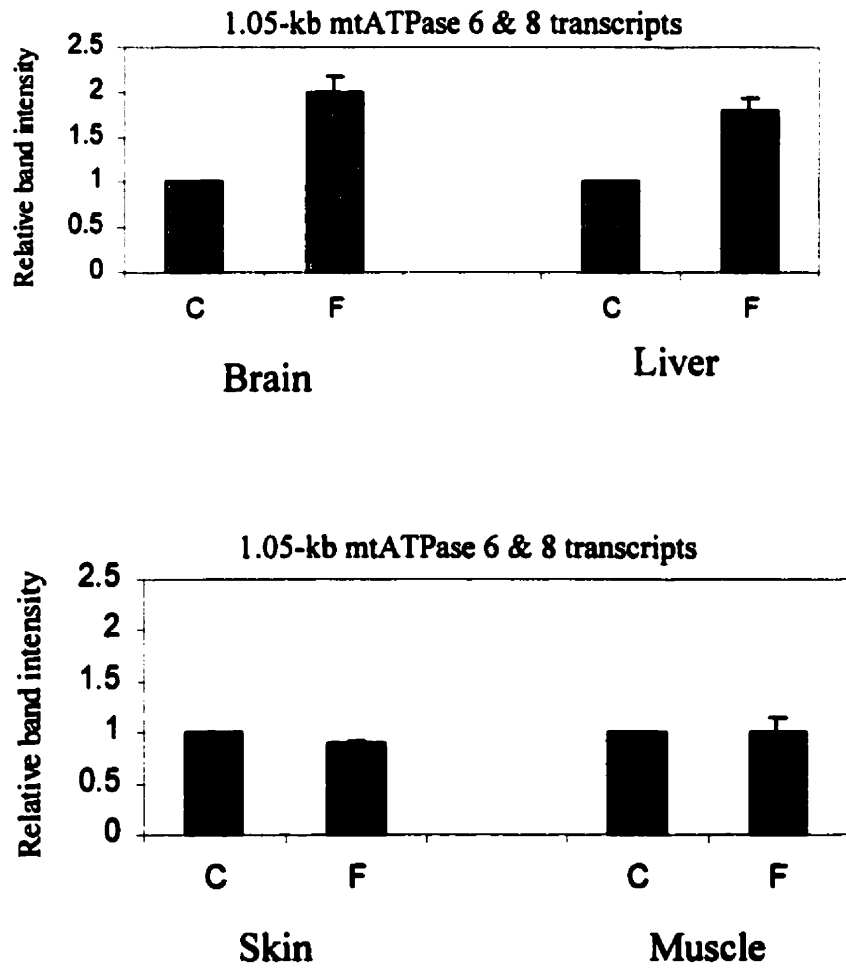


Figure 4.3. *In vivo* gene expression of mitochondrial ATPase 6 & 8 in multiple tissues of freeze-exposed wood frog *Rana sylvatica*. Same amount of total RNA (15 µg) was loaded for each lane. The ribosomal RNAs shown in Fig. 4.3A were detected by staining the RNA blot with methylene blue. The ribosomal RNAs shown in Fig. 4.3B were detected by staining the agarose gel with ethidium bromide (EtBr). C: control groups (cold acclimated frogs at 5°C for 2 weeks); F: freezing-treated groups (freeze-exposed frogs at -2.5°C for 24 h). The data shown in the graphs (Fig. 4.3C) represent the average value from the multiple trials (n = 3). The value of RNA band intensity of the freezing test groups from the frog tissues is normalized to their control groups, respectively.

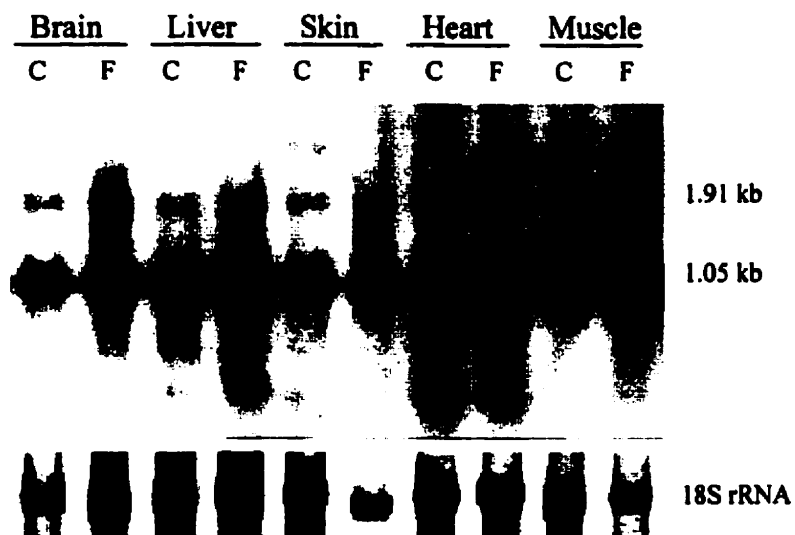
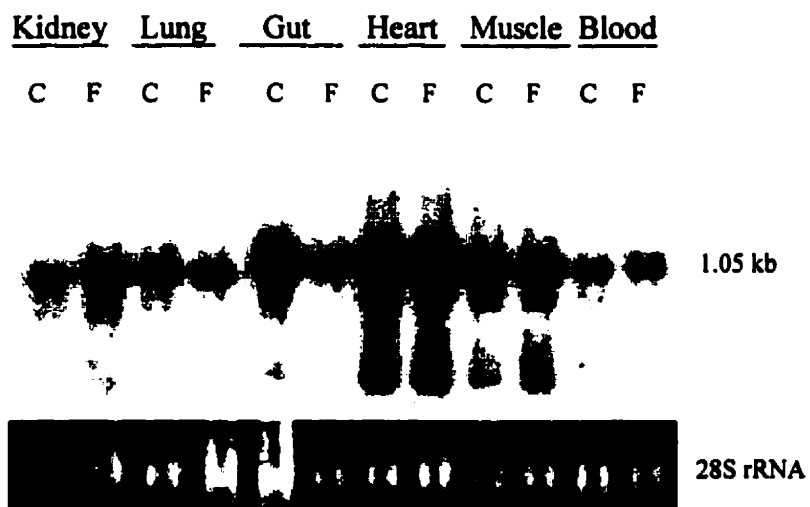
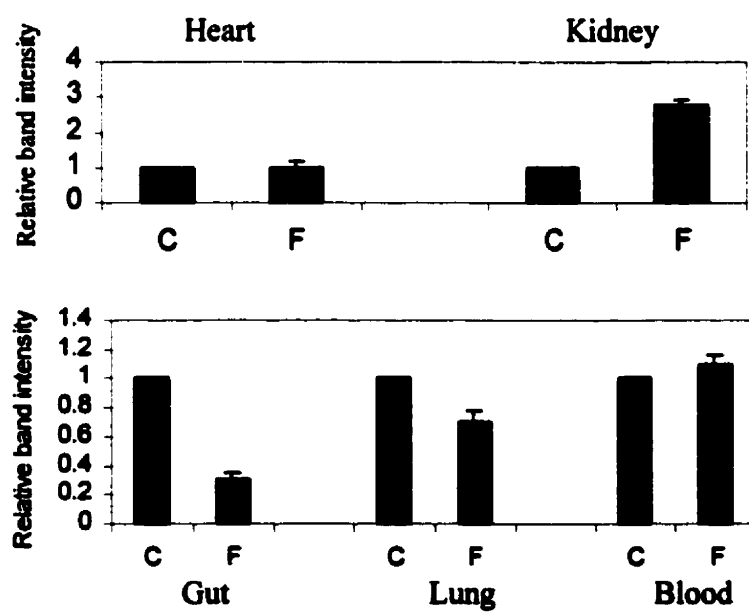
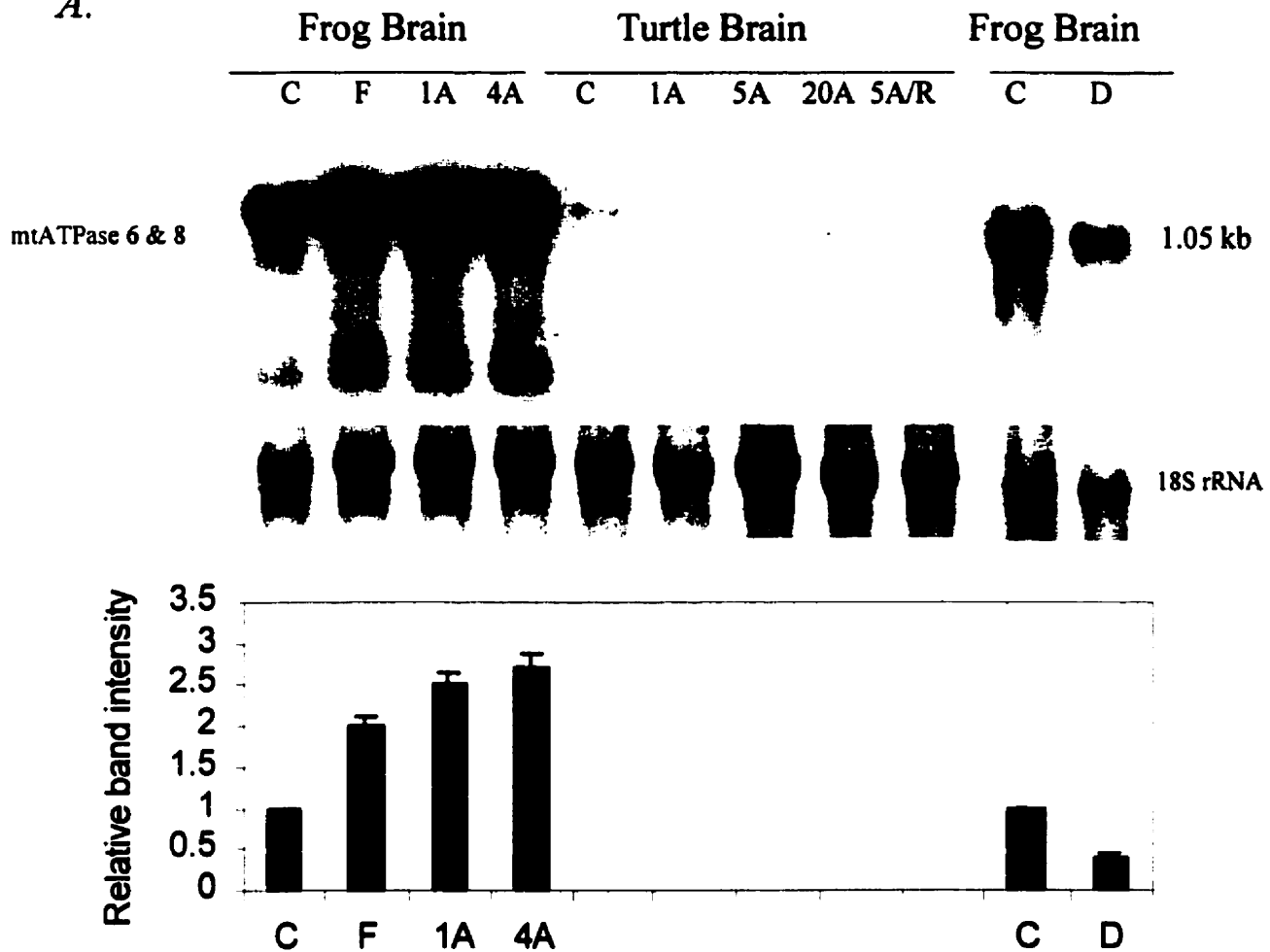
A*B**C*

Figure 4.4. Differential expression of mitochondrial ATPase 6 & 8 genes in the brain and liver in response to freezing and anoxia (or dehydration) stress in wood frog *Rana sylvatica* and in anoxia-exposed adult turtle *Trachemys scripta elegans*. The total RNA (15 µg) was loaded for each lane. The ribosomal RNAs were detected by staining the RNA blots with methylene blue. The gene probe was derived from the insert of frog brain clone Br3. C: control groups (cold acclimation at 5°C for 2 weeks for frogs, or for turtles by keeping them in large tanks of dechlorinated water at 7°C for at least 3 weeks); F: freezing-treated groups (freeze-exposed frogs at -2.5°C for 24 h); 1A, 4A, 5A, 12A, 20A and 24A: anoxia for 1, 4, 5, 12, 20 and 24 h, respectively. 5A/R: anoxia for 5 h and recovery by reexposing oxygen in control conditions for 24 h in turtles. D: brain sample from dehydrated frog with 40% of total body water loss. The RNA blotting was performed with multiple trials and the data shown in the graphs (Fig.4.4A for frog and turtle brain samples and Fig. 4.4B for the liver samples) represent the average value from the multiple trials (n = 3). The value of RNA band intensity of test groups from the frog tissues is normalized to their control groups, respectively.

A.



B.

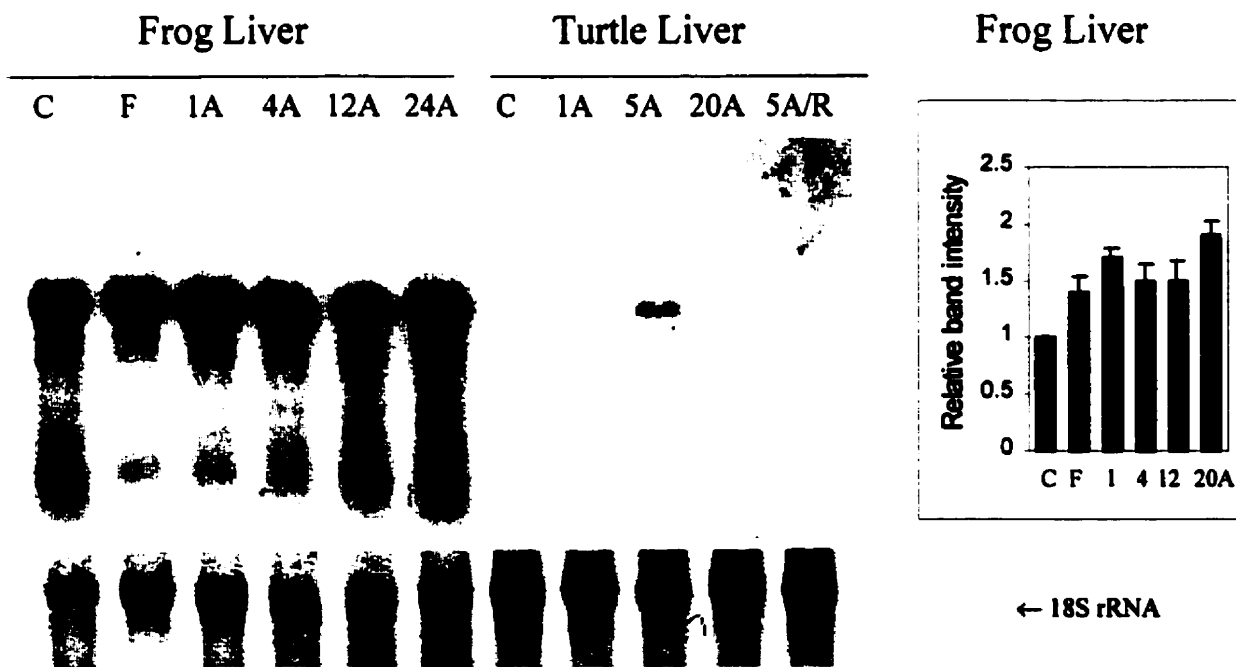


Figure 4.5. Representative Northern blot of brain and liver mRNA probed with mitochondrial 16S rRNA gene in freezing-exposed wood frog *Rana sylvatica*. Same blot was used for Northern as shown in Fig. 4.1. The gene probe was derived from the insert of frog brain cDNA clone Br4. The data shown in the graphs represent the average value from two different trials. The value of RNA band intensity of test groups from the frog tissues is normalized to their control groups, respectively.

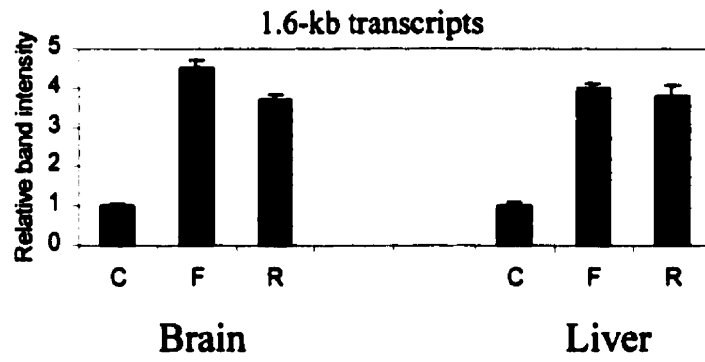
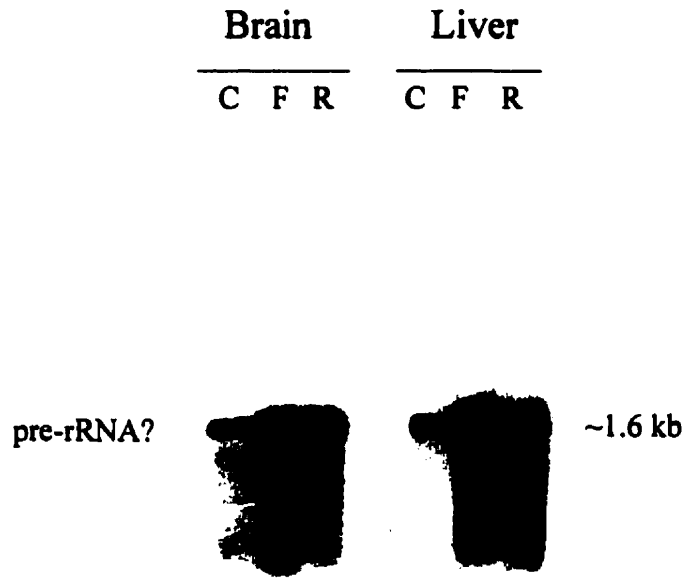


Figure 4.6. Expression of mitochondrial 16S rRNA gene under freezing exposure in multiple tissues of wood frogs. Equal amounts of total RNA (15 μg) were loaded for all samples. The rRNAs were detected by staining the agarose gel with ethidium bromide. All blots were carried out under same conditions for Northern analysis. C: control groups (cold acclimated frogs at 5°C for 2 weeks); 12F or 24F: freezing-treated groups (freeze-exposed frogs at -2.5°C for 12 or 24 h, respectively); F: same as the 24F group; R: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h; M: molecular weight standards (0.24-9.49 kb RNA ladder, from GIBICO-BRL) were detected by staining the RNA blot with methylene blue and shown in Fig. 4.6B. The data shown in the graphs (Fig. 4.6C) represent the average value from different trials (n = 3). The value of RNA band intensity of test groups from the frog tissues is normalized to their control groups, respectively.

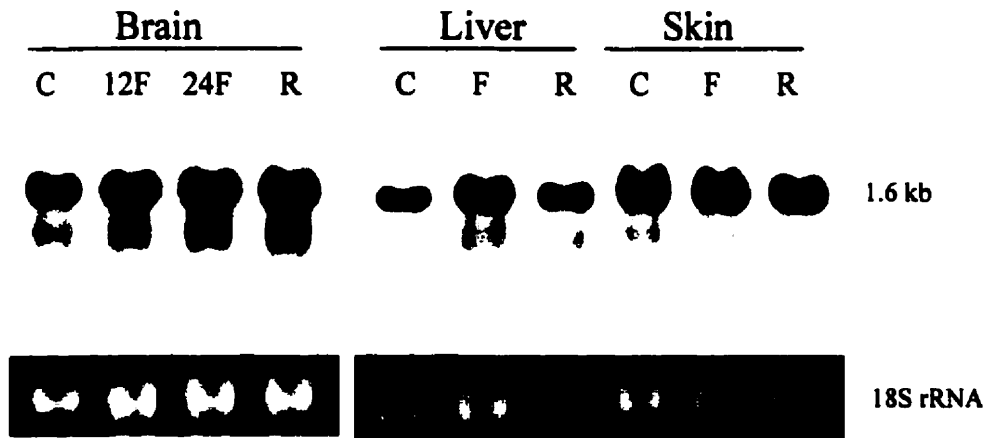
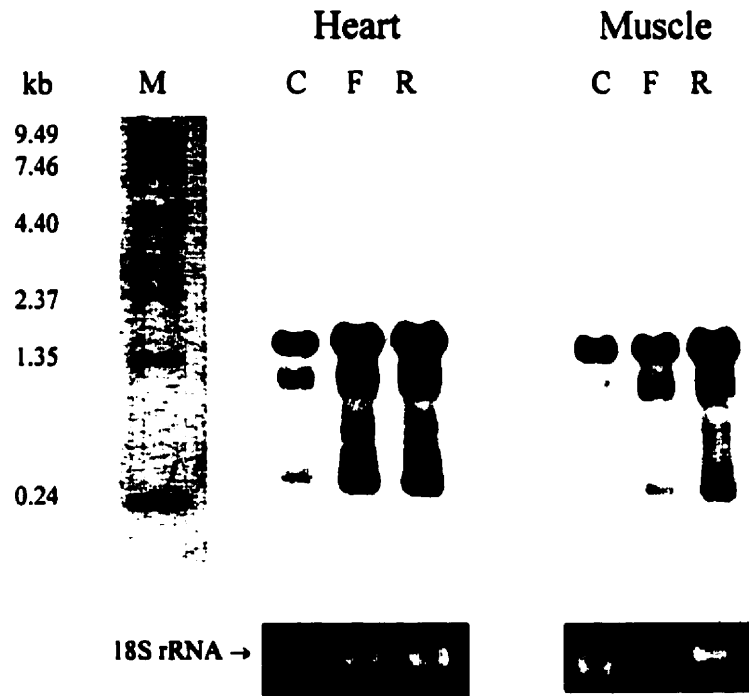
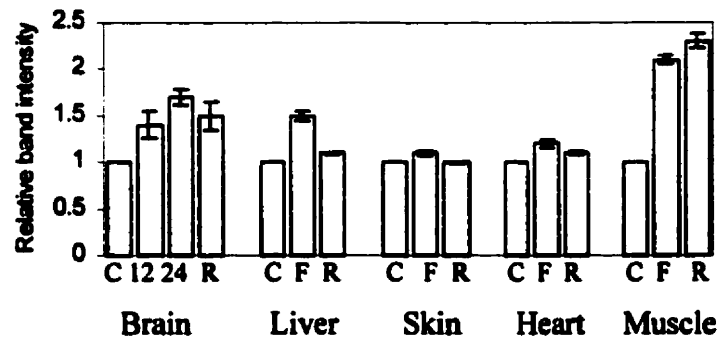
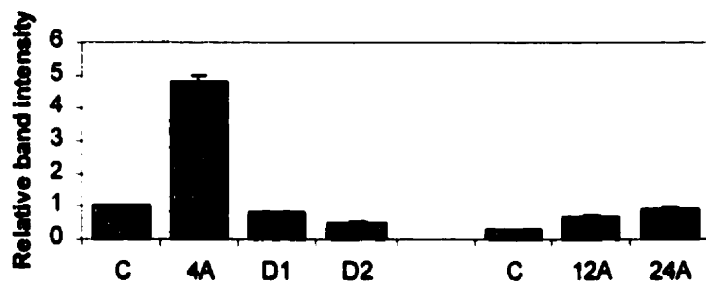
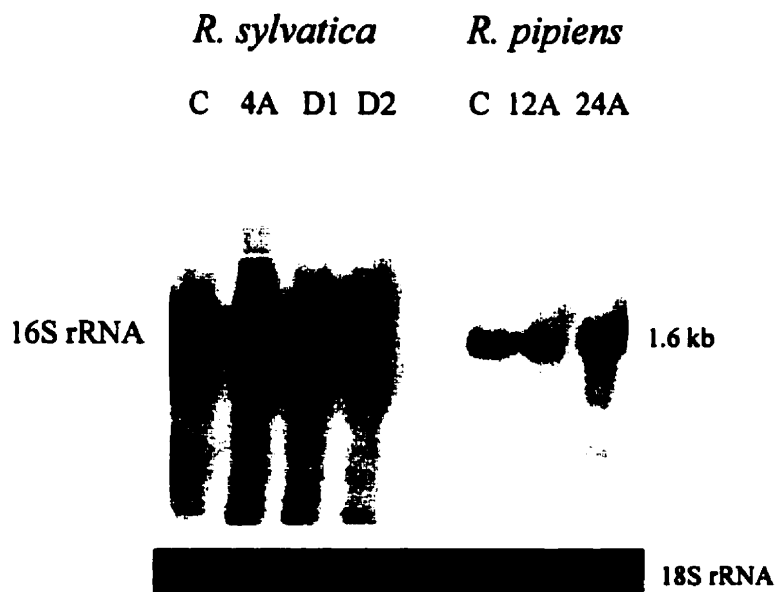
A*B**C*

Figure 4.7. Differential expression of mitochondrial 16S rRNA gene in the brain from anoxia or dehydration-treated wood frog *Rana sylvatica* and from anoxia-exposed leopard frog *R. pipiens*. The total RNA (15 µg) was loaded for each lane. The ribosomal RNAs were detected by staining the agarose gel with ethidium bromide. The isolation of total RNA for the brain tissues of *R. pipiens* was performed by the same methods as described in the Methods section of Chapter 2. The anoxic treatment for the leopard frogs was similar to that described for wood frogs in the Methods section in this chapter. The gene probe was derived from the insert of frog brain cDNA clone Br4. C: control groups (cold acclimation at 5°C for 2 weeks for both frogs; 4A, 12A, and 24A: anoxia for 4, 12, and 24 h, respectively; D1, D2: 20% and 40% dehydration, respectively. The data shown in the graph represent the average value from two different trials. The value of RNA band intensity is normalized to wood frog control group.



R. sylvatica

R. pipiens

Figure 4.8. Expression of ND4 and EF-1 γ gene under freezing exposure in multiple tissues of wood frogs. The blots were identical for the Northern in Fig. 4.6A. C: control groups (cold acclimated frogs at 5°C for 2 weeks); 12F or 24F: freezing-treated groups (freeze-exposed frogs at -2.5°C for 12 or 24 h, respectively); F: same as the 24F group; R: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h. The gene probe was derived from whole insert of frog liver cDNA clone Li39 and thus the transcripts of *ND4* (~1.4 kb) and *EF-1 γ* (~1.7 kb) were both detected by the Northern analysis. The data shown in the graphs represent the average value from different trials (n = 3). The value of RNA band intensity of test groups from the frog tissues is normalized to their control groups, respectively.

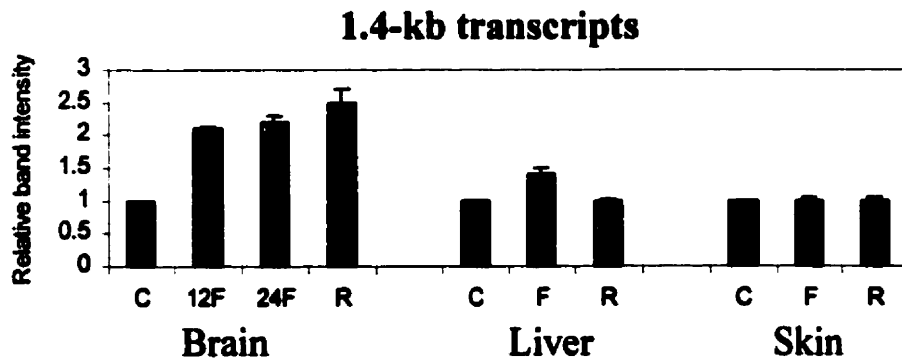
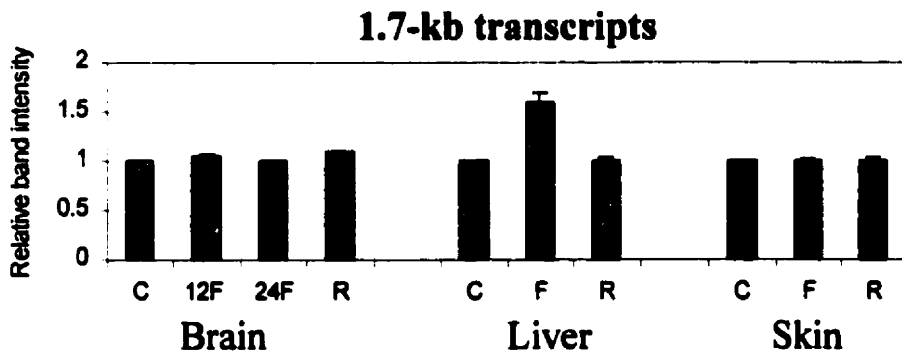
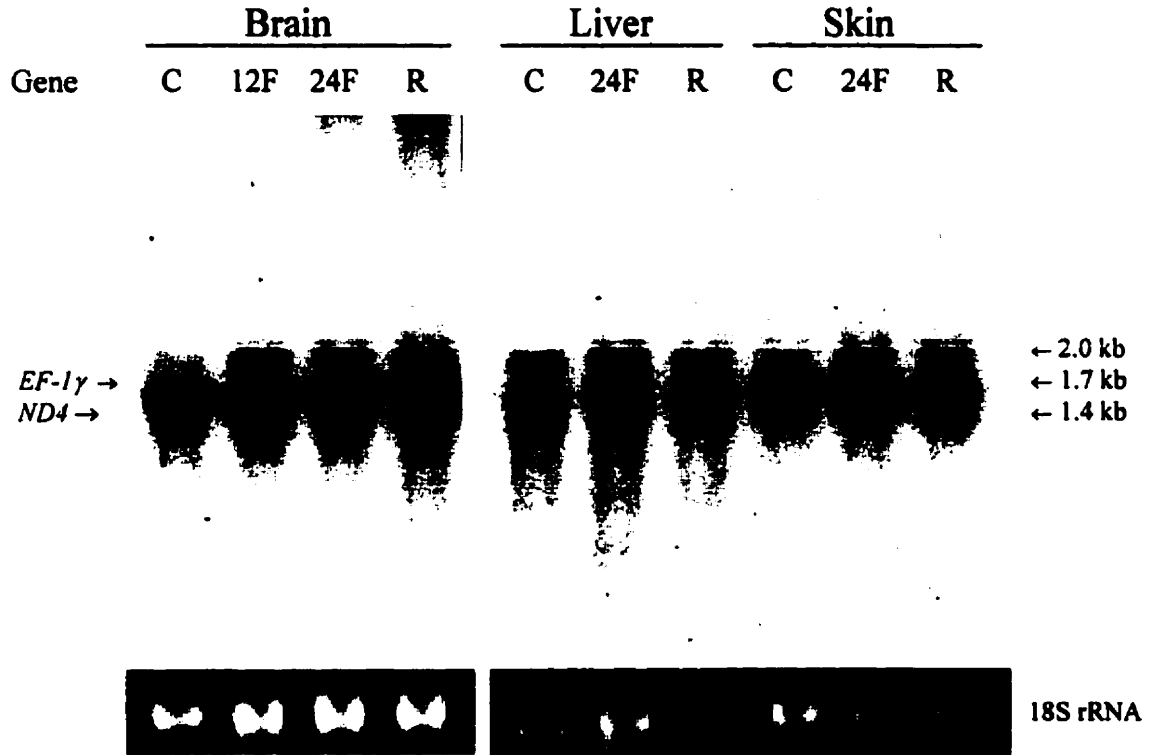


Figure 4.9. Differential expression of ND4 and EF-1 γ gene in response to freezing, anoxia and dehydration stresses in the liver of wood frog *Rana sylvatica*. The total RNA (15 μ g) was loaded for each lane. The ribosomal RNAs were detected by staining the agarose gel with ethidium bromide. The gene probe was derived from the insert (containing both the 1.5 kb and the 1.6 kb fragment) of frog liver cDNA clone Li39. Lane 1, control group (cold acclimated frogs at 5°C for 2 weeks); lane 2, 3 and 4, freezing the cold-acclimated frogs at -2.5°C for 4, 12 and 24 h, respectively; lane 5: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h; lane 6 and 7: 20% and 40% dehydration, respectively; lane 8, 9, 10 and 11: anoxia for 1, 4, 12, and 24 h, respectively. The data shown in the graph represent the average value from the multiple trials (n = 3). The value of RNA band intensity of freezing or dehydration-treated groups is normalized to the control, while the relative band intensity of anoxia groups is normalized to the 1 h anoxia group.

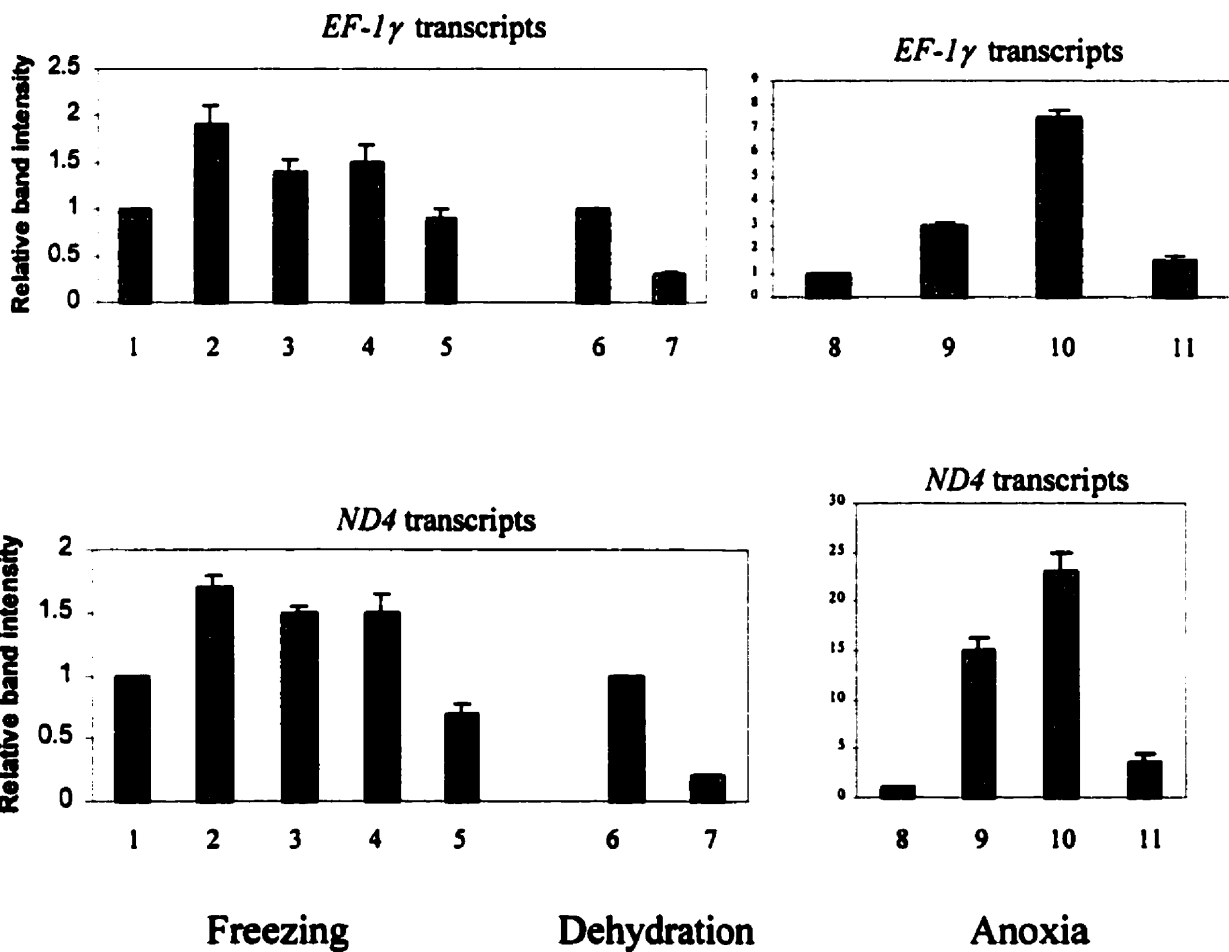
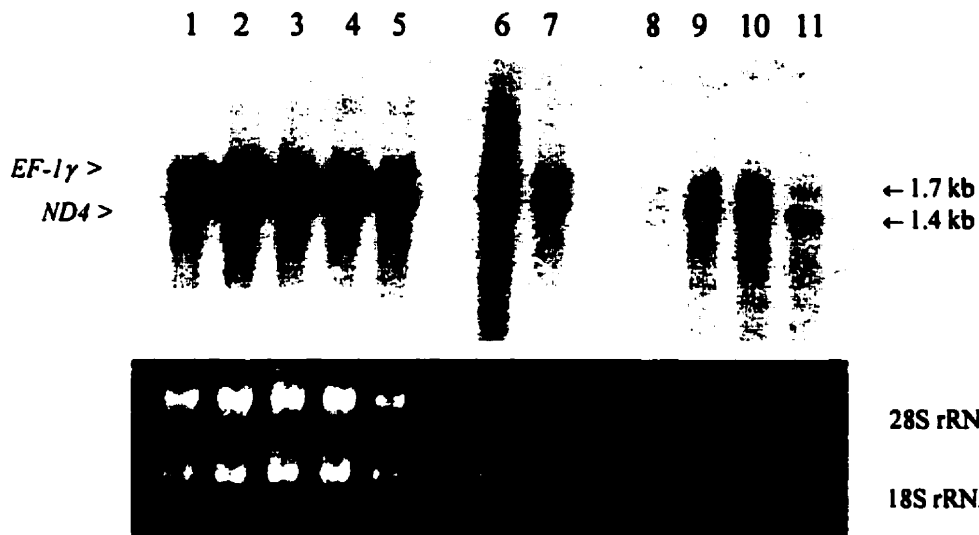


Figure 4.10. Representative Northern blot of brain and liver mRNA probed with P0 gene under freezing-exposure in wood frog *Rana sylvatica*. The same mRNA blot was used for Northern as shown in Fig. 4.1 and 4.5. The gene probe was derived from the insert of frog brain cDNA clone Br15. C: control, from cold-acclimated frogs (at 5°C for 2 weeks); F: freezing the cold-acclimated frogs at -2.5°C for 24 h; R: freeze-exposed frogs at -2.5°C for 24 h and then thawed at 5°C for 24 h. The RNA blotting and hybridization were repeated with multiple trials and the data shown in the graphs represent the average value from the repeats (n = 3). The value of RNA band intensity of test groups from frog brain and liver tissues is normalized to their control groups, respectively.

Brain			Liver		
C	F	R	C	F	R

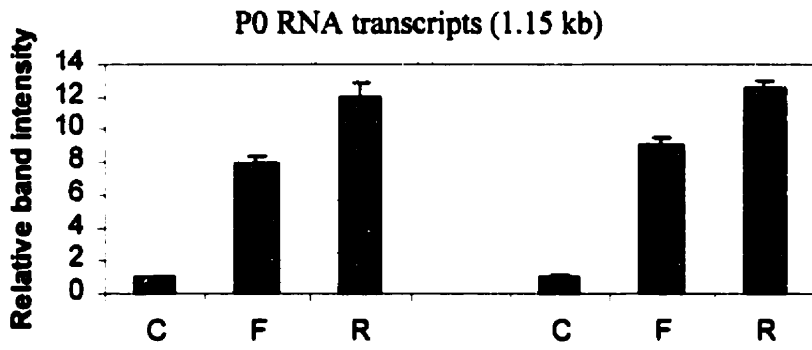


Figure 4.11. Expression of P0 gene under freezing exposure in multiple tissues of wood frogs. The total RNA (15 µg) was loaded for each lane. The ribosomal RNAs were detected by staining the agarose gels with ethidium bromide. The gene probe was derived from the insert of frog brain cDNA clone Br15. C: control, from cold-acclimated frogs (at 5°C for 2 weeks); F: freezing the cold-acclimated frogs at -2.5°C for 24 h; R: freeze-exposed frogs at -2.5°C for 24 h and then thawed at 5°C for 24 h. The RNA blotting and hybridization were repeated with multiple trials and the data shown in the graphs represent the average value from the repeats (n = 3). The value of RNA band intensity of test groups from frog tissues is normalized to their control groups, respectively.

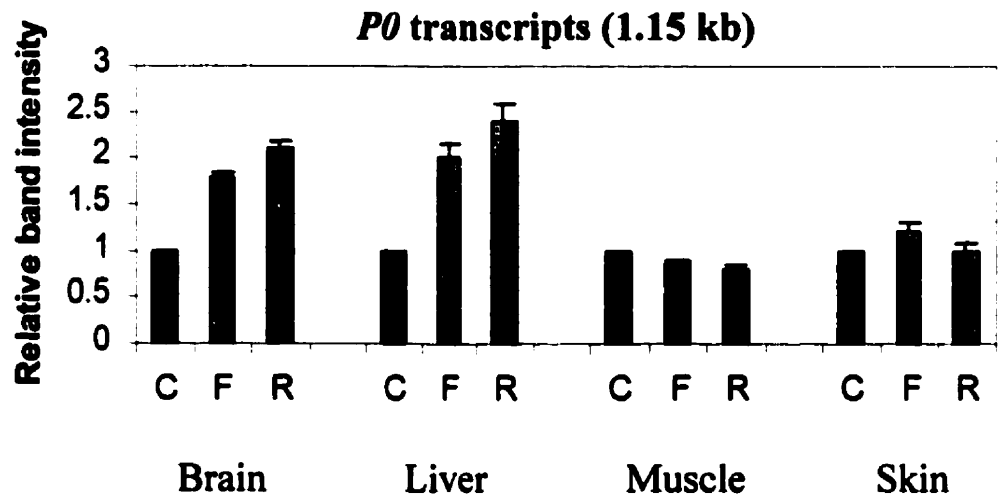
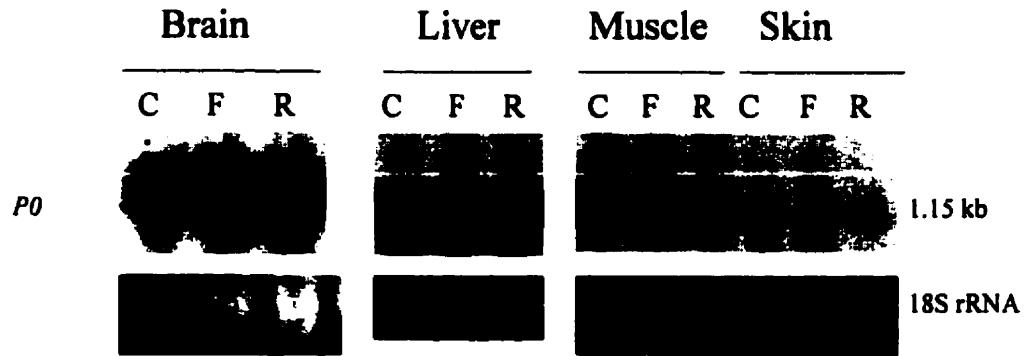


Figure 4.12. Differential expression of P0 gene in the brain from anoxia or dehydration-treated wood frog *Rana sylvatica* and from anoxia-exposed leopard frog *R. pipiens*. Same blot was used for the Northern as shown in Fig. 4.7. C: control, from cold-acclimated frogs (at 5°C for 2 weeks); F: freezing the cold-acclimated frogs at -2.5°C for 24 h; R: freeze-exposed frogs at -2.5°C for 24 h and then thawed at 5°C for 24 h. The gene probe was derived from the insert of frog brain cDNA clone Br15. The data shown in the graph represent the average value from multiple trials (n = 3). The value of RNA band intensity is normalized to the control group of wood frog.

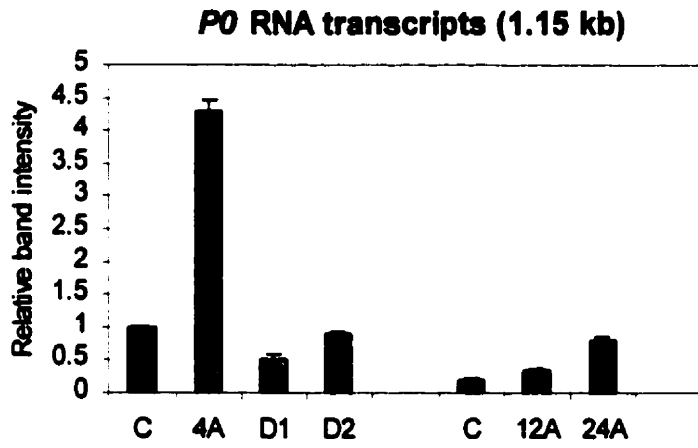
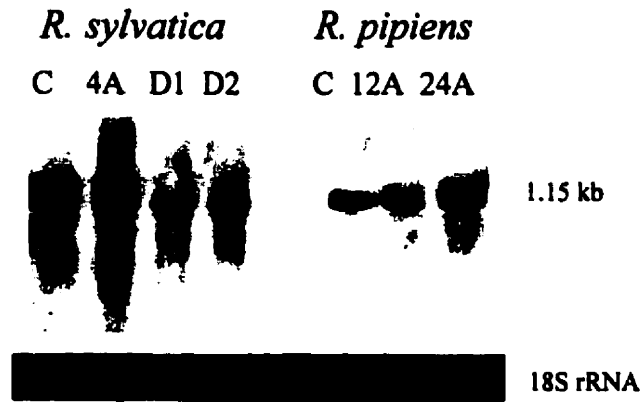


Figure 4.13. Expression of EF-1 γ gene in the brain of freeze-exposed wood frogs. The total RNA (15 μ g) was loaded for each lane. The ribosomal RNAs were detected by staining the agarose gel with ethidium bromide. The gene probe was derived from the insert of frog liver clone Li39l. C: control groups (cold acclimated frogs at 5°C for 2 weeks); lanes for 1h-24 h: freezing the cold-acclimated frogs at -2.5°C for 1, 4, 8, 12 and 24 h, respectively; R: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h. EF-1 γ transcript level was consistent in wood frog brain tissues as shown on the mRNA blot (in Fig. 2.2, 2.3B, 2.4 and 2.6) and on the total RNA blot (in Fig. 4.8) detected with the probe derived from the large fragment (~1.6 kb) of liver clone Li39 insert.

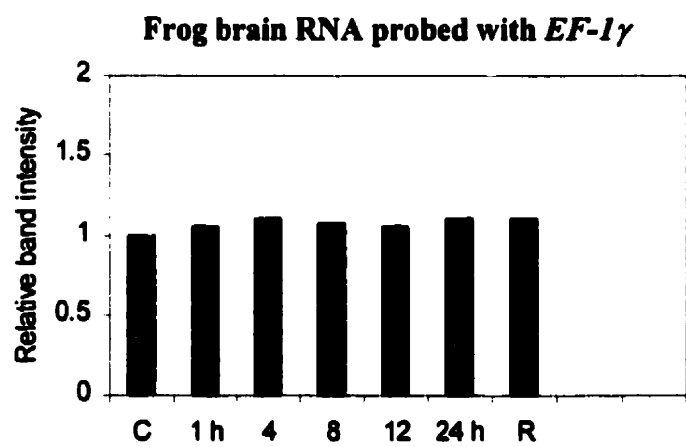
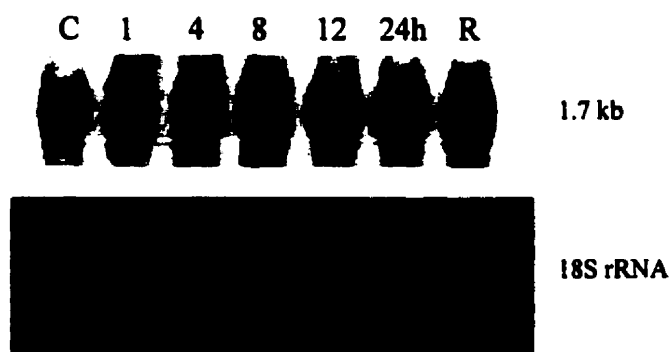


Figure 4.14. Expression of RPL7 gene under freezing exposure in multiple tissues of wood frogs. The blots were identical to those used for the Northern blots as shown in Fig. 4.6. The gene probe was derived from the insert of frog skin DD-PCR clone SkC2. C: control groups (cold acclimated frogs at 5°C for 2 weeks); 12F or 24F: freezing-treated groups (freeze-exposed frogs at -2.5°C for 12 or 24 h, respectively); F: same as the 24F group; R: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h. The data shown in the graphs represent the average value from different trials (n = 3). The value of RNA band intensity of test groups from the frog tissues is normalized to their control groups, respectively.

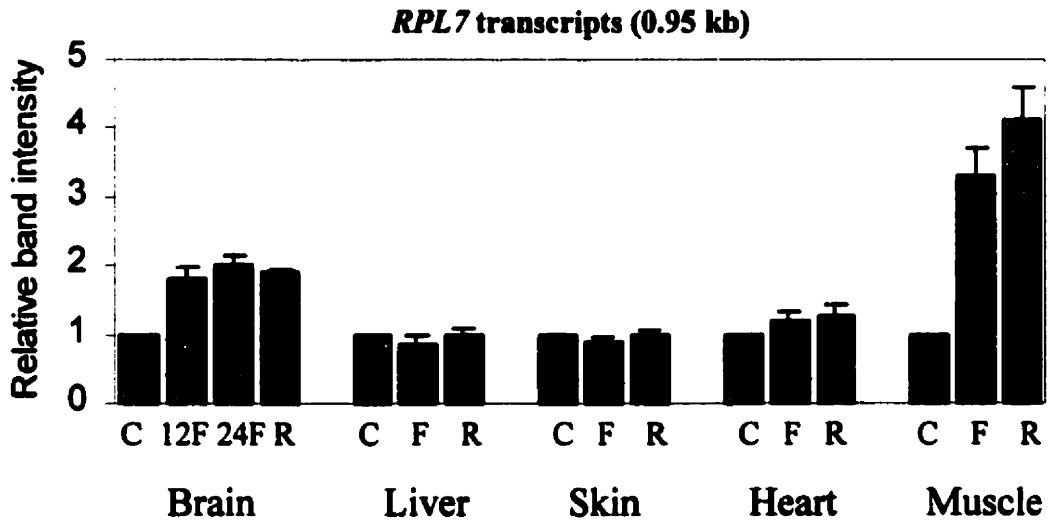
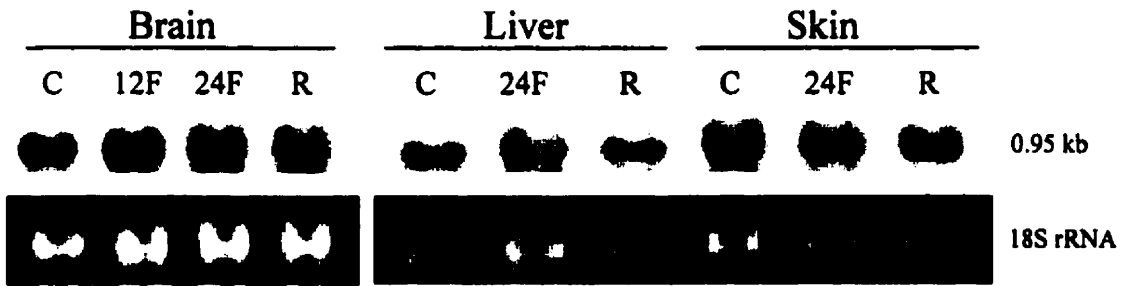
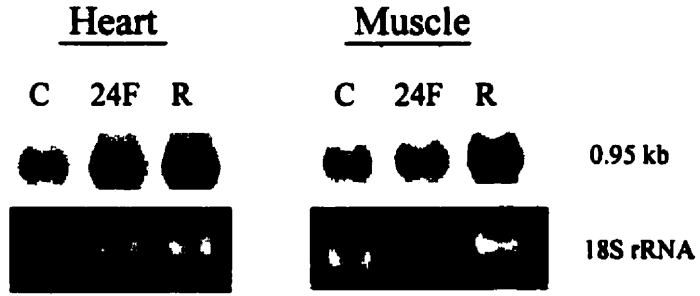


Figure 4.15. Differential gene expression of RPL7 in response to temperature change in multiple tissues of wood frogs. The total RNA (15 μ g) was loaded for each lane. The ribosomal RNAs were detected by staining the agarose gel with ethidium bromide. For frog brain and liver tissues: C: control groups (cold acclimated frogs at 5°C for 2 weeks); 12F or 24F: freezing-treated groups (freeze-exposed frogs at -2.5°C for 12 or 24 h, respectively); R: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h. W: skin tissues from warm-acclimated frogs by keeping the summer frogs in an incubator (~24°C) for 2 weeks; C-T: skin tissues from cold acclimation-treated frogs by shifting the warm-acclimated frogs to 5°C for 5 days. The RNA blotting and hybridization were repeated with multiple trials and the data shown in the graphs represent the average value from the repeats (n = 3). The value of RNA band intensity of test groups from frog brain and liver tissues is normalized to their control groups, respectively. The value of RNA band intensity of cold acclimation-treated group for frog skin sample is normalized to the warm-acclimated frog group.

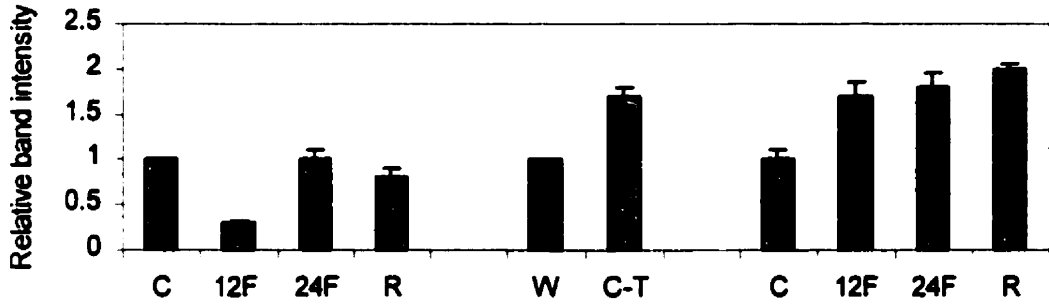
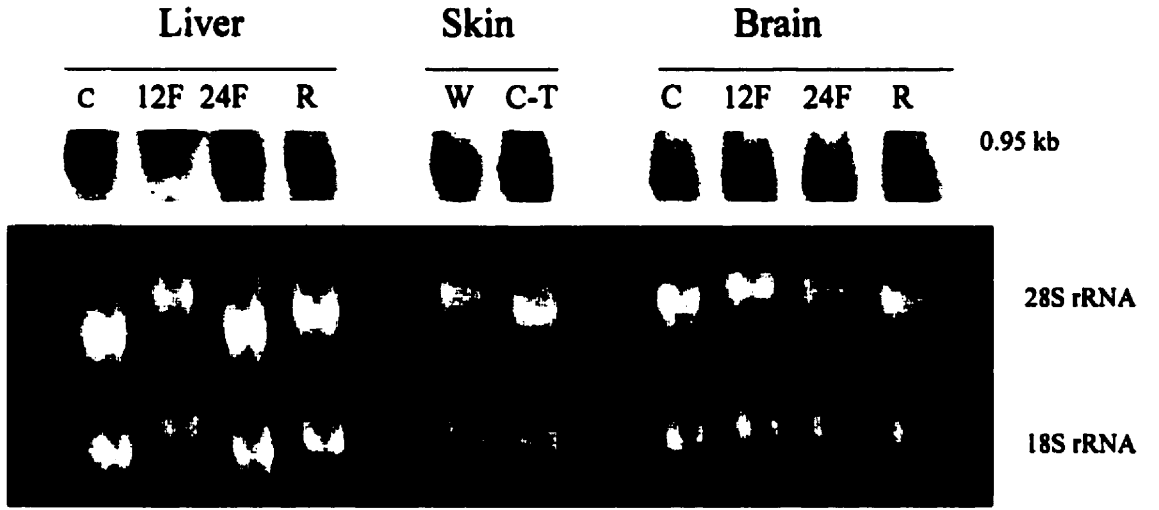


Figure 4.16. Differential expression of RPL7 gene in response to freezing, anoxia and dehydration stresses in the brain of wood frog *Rana sylvatica*. The total RNA (15 μ g) was loaded for each lane. The ribosomal RNAs were detected by staining the agarose gel with ethidium bromide. The gene probe was derived from the insert of frog cDNA clone RSkC2. C: control groups (cold acclimated frogs at 5°C for 2 weeks); F: freezing the cold-acclimated frogs at -2.5°C for 24 h.; D: 20% dehydration; 4A and 24A: anoxia for 4 and 24 h, respectively. The data shown in the graph represent the average value from multiple trials trials (n = 3). The value of RNA band intensity is normalized to the control.

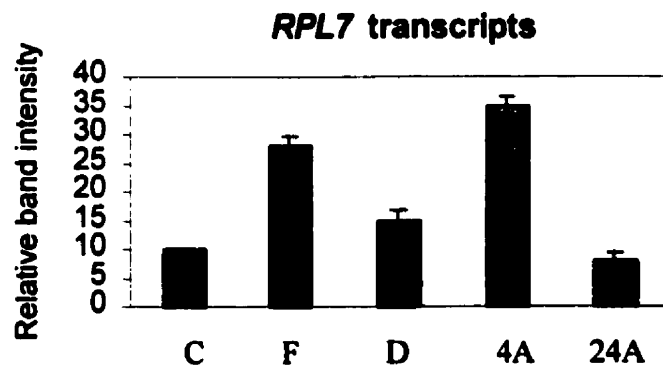
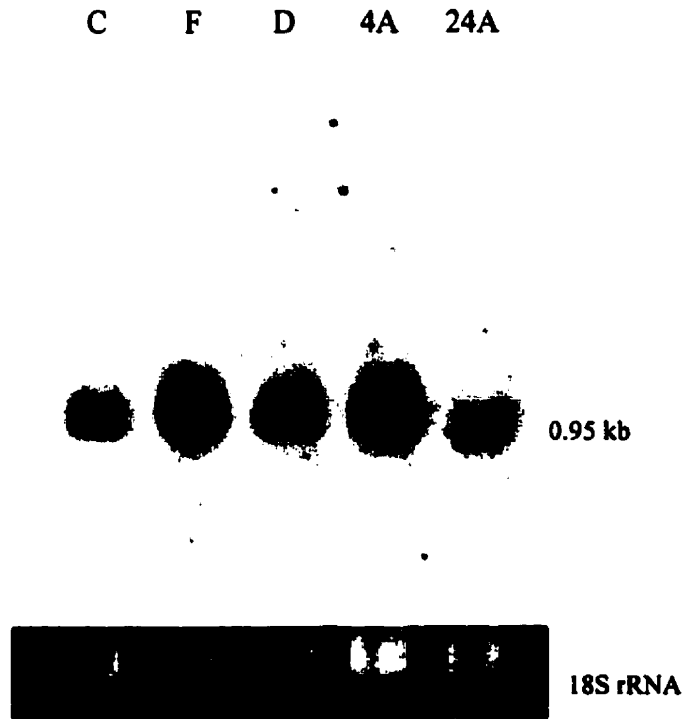


Figure 4.17. Expression of PGK1 gene under freezing exposure in multiple tissues of wood frogs. The blots were identical to those used for the Northern blots as shown in Fig. 4.6A. The gene probe was derived from the insert of frog brain cDNA clone Br30. C: control groups (cold-acclimated frogs at 5°C for 2 weeks); 12F or 24F: freezing-treated groups (freeze-exposed frogs at -2.5°C for 12 or 24 h, respectively); R: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h. The data shown in the graphs represent the average value from different trials (n = 3). The value of RNA band intensity of test groups from the frog tissues is normalized to their control groups, respectively.

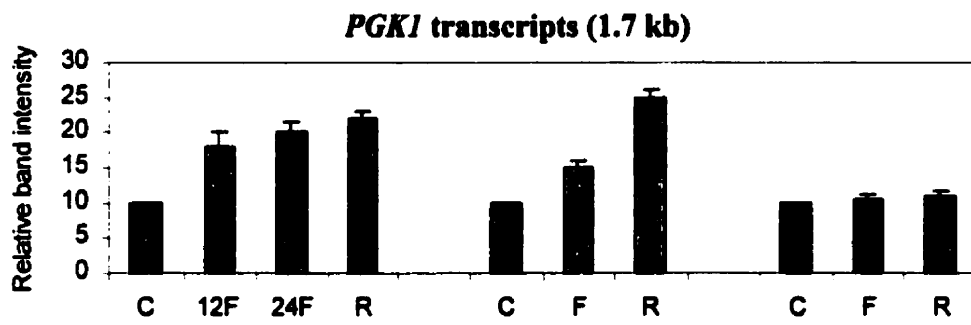
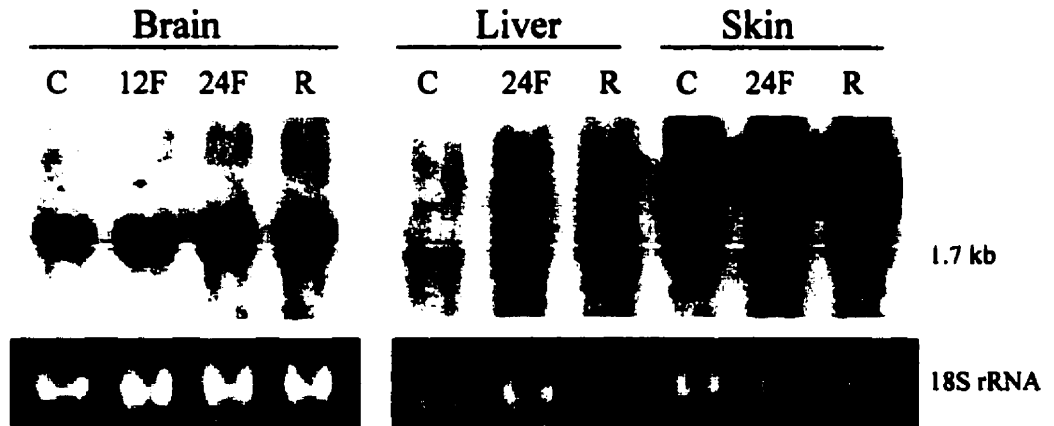
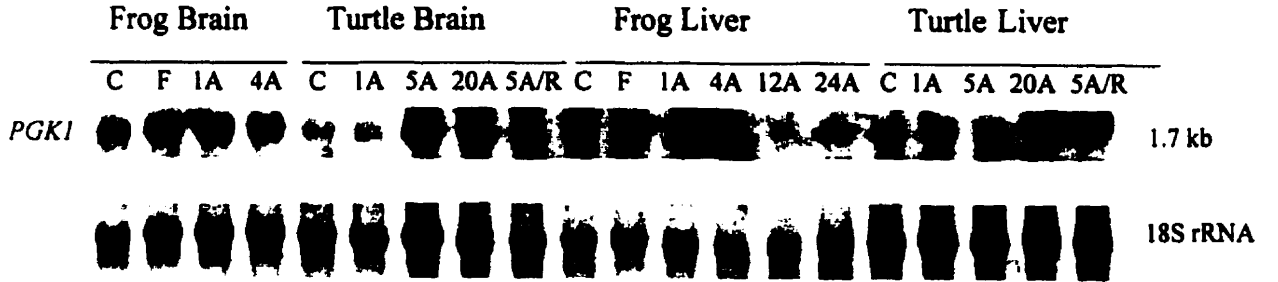


Figure 4.18. Differential expression of PGK1 gene in the brain and liver under freezing and anoxia exposure in wood frog *Rana sylvatica* and under anoxic treatment in adult turtle *Trachemys scripta elegans*. The total RNA (15 µg) was loaded for each lane. The ribosomal RNAs were detected by staining the RNA blot with methylene blue. The gene probe was derived from the insert of frog brain cDNA clone Br30. C: control groups (cold acclimation at 5°C for 2 weeks for frogs, or for turtles by keeping them in large tanks of dechlorinated water at 7°C for at least 3 weeks); F: freezing-treated groups (freeze-exposed frogs at -2.5°C for 24 h); 1A, 4A, 5A, 12A, 20A and 24A: anoxia for 1, 4, 5, 12, 20 and 24 h, respectively. 5A/R: anoxia for 5 h and recovery by reexposing oxygen in control conditions for 24 h in turtles. This blot was used for probing with the mitochondrial ATPase 6 & 8 gene as shown in Fig. 4.4. The data shown in the graphs (Fig. 4.18B) represent the average value from multiple trials (n = 3). The value of RNA band intensity of test groups from frog brain and liver tissues is normalized to their control groups, respectively.

A



B

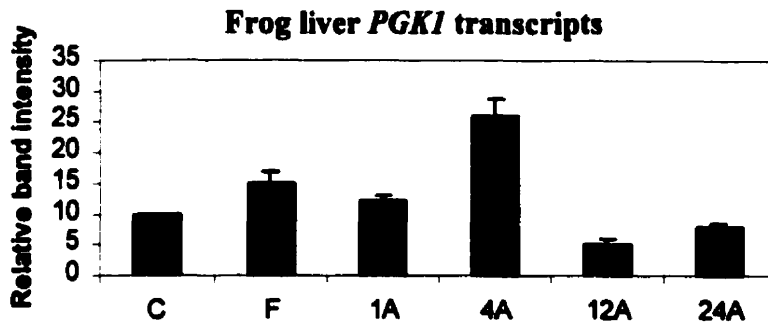
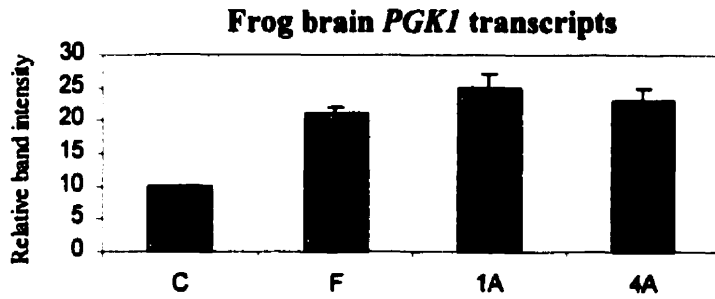
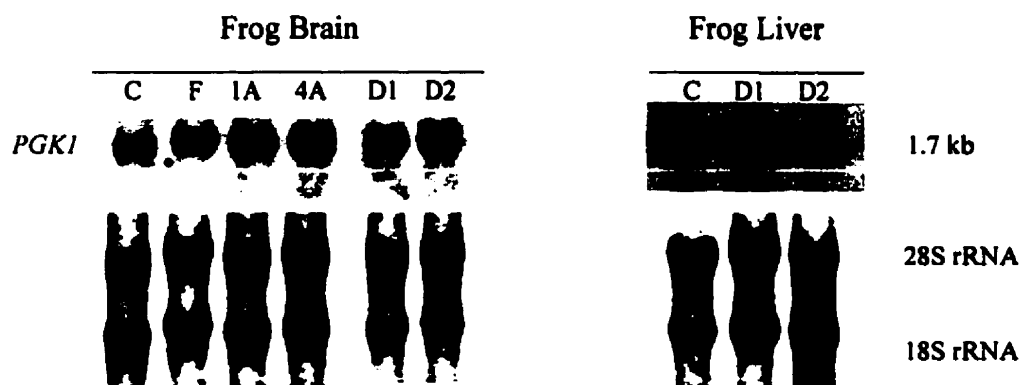


Figure 4.19. Differential expression of PGK1 gene in the brain under freezing, anoxia and dehydration stresses and in the liver under dehydration stress in wood frog. The total RNA (15 μg) was loaded for each lane. The ribosomal RNAs were detected by staining the RNA blots with methylene blue. The gene probe was derived from the insert of frog brain cDNA clone Br30. C: control groups (cold acclimation at 5°C for 2 weeks for frogs; F: freezing-treated groups (freeze-exposed frogs at -2.5°C for 24 h); 1A and 4A: anoxia for 1, 4 h, respectively. D1 and D2: 20% and 40% dehydration, respectively. The data shown in the graphs represent the average value from different trials (n = 3). The value of RNA band intensity of test groups from frog brain and liver tissues is normalized to their control groups, respectively, and the relative values for freezing and anoxia groups for frog brain samples were also used in Fig. 4.18.

A



B

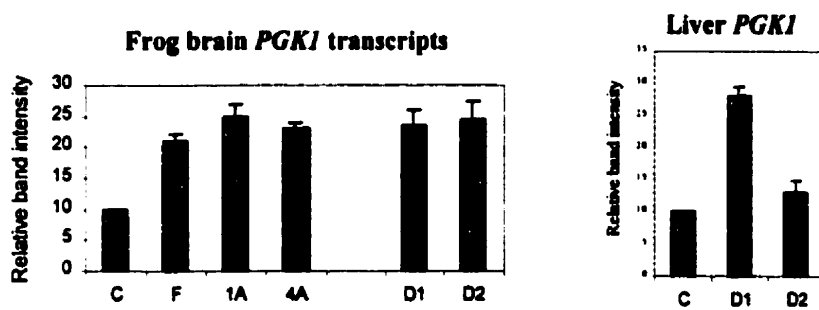
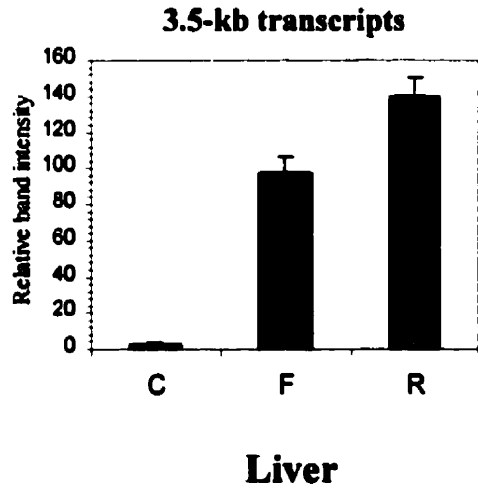
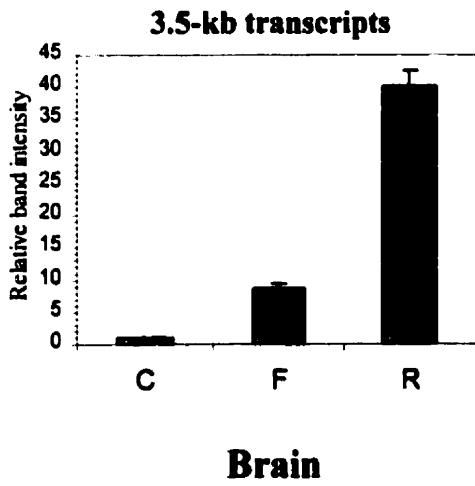


Figure 4.20. Representative Northern blot of brain and liver mRNA probed with pol-like reverse transcriptase gene under freezing-exposure in wood frog *Rana sylvatica*. Same blot was used for Northern as shown in Fig. 4.1, 4.5, and 4.10. The gene probe was derived from the insert of frog brain cDNA clone Br34. C: control groups (cold-acclimated frogs at 5°C for 2 weeks); F: freezing-treated groups (freeze-exposed frogs at -2.5°C for 24 h); R: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h. The RNA blotting and hybridization were repeated with multiple trials and the data shown in the graphs represent the average value from the repeats (n = 3). The value of RNA band intensity of test groups from frog brain and liver tissues is normalized to their control groups, respectively.

Brain			Liver		
C	F	R	C	F	R



CHAPTER FIVE

Stress-Induced Elevation of Proteins Involved in Signal Transduction and Gene Transcription in the Freeze Tolerant Wood Frog

INTRODUCTION

The previous chapters describe the differential expression of selected genes in wood frog tissues in response to low temperature, freezing, anoxia or dehydration stress. The accumulation of mRNA transcripts for these genes suggests, but does not prove, that levels of the protein products are also elevated under the stress conditions. Further studies are needed to confirm whether the increased mRNA levels also result in an elevation of the protein products. To do this, immunoblotting can be applied to quantify protein levels in tissues of control versus stress-treated frogs. The present chapter focuses on immunoblotting analysis of protein levels in frog tissues. Of the genes identified as up-regulated in previous chapters, only anti-PGK1 antibody (Ab) was readily available and so studies focused on PGK1 protein levels in the brain of cold acclimated, control frogs versus freeze-exposed, dehydrated, or anoxia-treated frogs.

In addition to analyzing PGK1 protein levels, I also chose to analyze levels of selected regulatory proteins that are involved in signal transduction and gene transcription, also using immunoblotting assays. In recent years, a large number of proteins/enzymes involved in signal transduction have been identified including multiple types of protein kinases, protein phosphatases, and transcription factors. For example, the hypoxia-inducible factor (HIF-1), a heterodimer of two basic helix-loop-helix (HLH) proteins (HIF-1 α and HIF-1 β), is activated in mammalian cells under hypoxia stress (Wang, Jiang *et al.*, 1995). HIF-1 α represents a newly detected protein, whereas HIF-1 β is identical to the heterodimerization partner of the dioxin receptor/aryl hydrocarbon receptor (AhR), called AhR nuclear translocator (ARNT). HIF-1 activates the transcription of a variety of genes that coordinate cellular responses to hypoxia including

of genes that encode erythropoietin (Wang, Jiang *et al.*, 1995), vascular endothelial growth factor (Levy *et al.*, 1995) and several glycolytic enzymes including PGK1 (Firth *et al.*, 1994, 1995; Li *et al.*, 1996). HIF-1 is recognized as a key regulator of oxygen homeostasis and of adaptation to hypoxic conditions. Therefore, given the role of HIF-1 in increasing PGK1 gene transcription in other species and the fact that freezing is an ischemic stress that could stimulate compensatory responses to the oxygen limitation of the frozen state, it seemed reasonable to hypothesize that HIF-1 might be activated in frog organs during freezing-induced hypoxia. Hence, immunoblotting analysis was also used to examine HIF-1 levels in frog organs under freezing and other stresses.

As discussed in Chapter 1, we were also interested in determining whether mitogen-activated protein kinases (MAPKs) are involved in modulating stress responses in frogs, particularly the kinase called p38. MAPKs phosphorylate specific cytosolic or nuclear target proteins leading to the activation of selected transcription factor genes such as *c-jun*, *c-fos* and *c-myc*. The potential importance of p38 is that it is activated by osmosis stress (Raignaud *et al.*, 1995) and, hence, could be involved in mediating responses to the large changes in cell volume that occur during freeze/thaw. Two other types of MAPKs, ERKs (extracellular-signal-regulated kinases) and JNKs (c-Jun NH₂-terminal kinases) are also involved in stress responses. ERKs can be activated by osmotic or hypoxic stresses (Matsuda *et al.*, 1995), whereas JNKs can respond strongly to heat shock, osmotic shock, ultraviolet (UV) irradiation, and reperfusion following ischemia (Kyriakis *et al.*, 1994).

We were also interested in exploring whether Ca²⁺ signaling was involved in freeze-mediated cellular responses in wood frog tissues. Calcium is essential for many

physiological processes. It is required, for example, in neuronal activity, bone growth, blood clotting, maintenance of the transmembrane potential, and cell growth and differentiation. Ca^{2+} is an important signal and functions as a second messenger. An increase in cytoplasmic Ca^{2+} levels can activate a wide variety of enzymatic processes, including smooth muscle contraction, exocytosis and glycogen metabolism. Increases in cytosolic Ca^{2+} can be derived from either external or internal sources. Ca^{2+} can enter from outside the cell by passing through channels on the plasma membrane. It can also be released from Ca^{2+} reservoirs inside the cell, through channels in the endoplasmic or sarcoplasmic reticulum (Berridge, 1993; Clapham, 1995). Several second messengers can modulate intracellular Ca^{2+} levels including cAMP and IP_3 (inositol-1, 4, 5-trisphosphate) which activate Ca^{2+} channels. cAMP opens the Ca^{2+} channel on the plasma membrane, whereas IP_3 binds receptors on the ER and then activates the channel. Elevation of intracellular Ca^{2+} can activate Ca^{2+} /calmodulin dependent kinases (CaMK) and protein phosphatases (e.g. calcineurin) or protein kinase C (PKC) along with DAG (diacylglycerol), which further leads to cellular responses. Calcium-mediated cellular processes have been identified that signal the responses to drought and cold in plants (Sheen, 1996). More recently, cyclic ADP-ribose has been confirmed as a universal signal in response to cold and drought in plants (Wu *et al.*, 1997). Cyclic ADP-ribose is a second messenger that can increase Ca^{2+} levels by activating Ca^{2+} channels in animal cells (Gerasimenko *et al.*, 1996). Activation of certain CaMKs by cyclic ADP-ribose via changing the level of Ca^{2+} was assumed to induce specific gene expression (Pennisi, 1997). Therefore, by testing the levels of selected Ca^{2+} -binding or Ca^{2+} -regulated proteins,

we could evaluate whether Ca^{2+} -mediated signal transduction pathways can also be an important part of cellular adaptations under environmental stress conditions in wood frogs.

The present chapter uses immunoblotting techniques to evaluate the responses of PGK1 and a variety of signal transduction proteins to freezing, dehydration, and anoxia stress in wood frog brain tissues.

MATERIALS AND METHODS

Animals and Tissue Sampling

The collection of wood frogs was outlined in Chapter 2. Animal care and tissue sampling under freezing, anoxia and dehydration stress were described in previous Chapters.

Chemicals and Reagents

Electrophoresis reagents were mainly obtained from Bio-Rad Laboratories. Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or the equivalent, unless specified. Chemicals were of molecular biology grade or their equivalent and of the highest purity.

List of Antibodies

Antibodies used were: anti-PGK1 rabbit polyclonal antibody (a synthetic peptide derived from the C-terminus of PGK1 was used as immunogen), kindly provided by Dr. John A. Bryant (University of Exeter, Exeter, UK); anti Ca^{2+} /calmodulin-dependent protein kinase IV CT rabbit polyclonal antibody (Upstate Biotechnology Inc.); anti-p38

MAPK rabbit polyclonal antibody and anti phospho-specific p38 (Tyr¹⁸²) MAPK rabbit polyclonal antibody (NEB); anti-calcineurin mouse mAb and anti- β 1-Ca²⁺ channel mouse mAb (Transduction Laboratories); anti-HIF-1 α chicken polyclonal antibody and anti-HIF-1 α mouse mAb (from Dr. M. Gassmann, University of Zurich-Irchel, Zurich) or anti-HIF-1 α rabbit polyclonal antibody (from Dr. F. Bunn, Harvard University, MA). Secondary antibodies were: goat anti-rabbit IgG, horseradish peroxidase (HRP) conjugated (Santa Cruz Biotechnology); goat anti-mouse IgG, HRP conjugated (Transduction Laboratories); and rabbit anti-chicken IgY HRP conjugated (Promega Corp.). Rat brain lysates were from Transduction Laboratories and used as positive control for anti-calcineurin and anti- β 1 Ca²⁺-channel antibodies.

Western Immunoblotting

In addition to testing the mRNA levels of isolated stress-responsive genes, changes in the levels of the corresponding protein were evaluated by immunoblotting for the frog brain gene *PGKI*. The levels of certain well-characterized regulatory proteins that modulate gene expression (such as HIF-1 α , p38 and CaM kinases, etc.) were also analyzed for the brain tissues of stress-treated frogs by immunoblotting with appropriate antibodies (see the list of antibodies).

Total soluble protein was extracted from frozen samples of frog tissues by homogenizing (1:10 w/v) in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 1mM EGTA, 1mM EDTA with protease inhibitors (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride [PMSF]) were added immediately prior to

homogenizing. After centrifugation at 14,000 rpm for 15 min at 4°C in a Biofuge 15 (Baxter Canlab), supernatants were removed and centrifuged again at the same speed for 15 min. The final supernatant was removed and stored at -70°C. Supernatant protein concentration was determined by the Bio-Rad protein assay (Bradford, 1976). SDS-polyacrylamide gel electrophoresis was used to separate proteins. Stock solutions for electrophoresis were as follows. The 2× protein sample buffer contained 100 mM Tris-HCl (pH 6.8), 4% SDS (w/v), 20% glycerol (v/v), 5% β-mercaptoethanol (v/v) and 0.2% bromophenol blue (w/v) whereas the stock 10× running buffer contained 30.3 g Tris base, 144 g glycine, and 10 g SDS per liter, pH ~8.3. Aliquots of supernatant were mixed with 2x SDS-PAGE protein sample buffer (1:1, v/v) and boiled 3-5 min. Equal amounts of protein (15 or 20 μg per lane) were loaded into each sample well on a mini-gel. Proteins were separated using 7.5-15% gels (acrylamide:bis-acrylamide 29.2:0.8; w/w) using the discontinuous buffer system of Laemmli (1970) and with a Bio-Rad mini-gel apparatus. Separation generally required ~1 h at 180 V. Proteins were blotted to polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer Membrane, Millipore Corp. Bedford, MA) by wet transfer with pre-chilled solution containing 25 mM Tris (pH 8.5), 192 mM glycine, 0.05% SDS and 20% v/v methanol at 4°C for 1.5-2 h at 350 mA. Western immunoblotting was performed as recommended by the antibody supplier. The general procedures were as below. The protein blot was first rinsed with TBST buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) and then incubated in blocking buffer (TBST with 1~2% bovine serum albumin [BSA] or 5% non-fat dry milk) for one hour. The blot was then added to blocking buffer containing the primary antibody at an appropriate dilution and incubated at room temperature for one hour or at 4°C for

overnight. Following 3-5 washes in TBST, the blot was then incubated in blocking solution (TBST with 1~2% BSA or 5% non-fat dry milk) with an appropriate dilution of the corresponding secondary antibody. The target protein was visualized by the enhanced chemiluminescence assay (ECL) (NEN Life Science Products, Inc. Boston, MA).

RESULTS

Stress-Induced Elevation of PGK1 Protein in Wood Frog Tissues

Western blotting showed that a protein band of ~45 kDa in frog brain extracts was recognized by the anti-PGK1 antibody (Fig. 5.1). The intensity of the protein band increased under freezing, anoxia or dehydration stress and was particularly pronounced under anoxic conditions. An 3.5-5 fold increase in PGK1 protein level was observed in brain extracts from 4 or 8 h frozen frogs as compared with control groups (5°C cold-acclimated group) but protein level declined with longer freeze-exposure (12-24 h) (Fig. 5.1). PGK1 protein levels also increased by about 6-fold when frogs were dehydrated but levels were reduced to the level of control group after rehydration (Fig. 5.1). However, anoxia exposure (4 or 24 h) strongly stimulated PGK1 production with protein content increasing by 8.5-10 fold (Fig. 5.1).

p38 and HIF-1 in Frog Tissues

Mammalian p38 (a MAP kinase) is a protein with molecular size about 38 kDa. In frog brain, a single faint band (~38 kDa) was detected with an anti-p38 antibody (data are not shown). Western blotting indicated that the level of frog p38 was not significantly changed during freezing, anoxia and dehydration exposures. In addition, there was no

cross-reaction was detected when trials were conducted with antibodies to the phosphorylated form of p38.

Western blots with antibodies to mammalian HIF-1 α were unable to detect any cross-reacting material in frog brain samples of either control or stressed animals. This negative result could mean one of two things. Either this stress tolerant organism may not possess a HIF-1 α -like protein or the frog protein may not cross-react with the mammalian antibody.

Elevation of Selected Proteins Involved in Ca²⁺-Mediated Signaling Pathway

Immunoblotting indicated that the β 1-calcium channel protein subunit in frog brain tissues was a 52 kDa protein, the same size as its counterpart in rat brain (Fig. 5.2). However, the levels of the Ca²⁺-channel protein in frog brain did not change significantly among the control and stress (freezing or anoxia) groups (Fig. 5.2).

Western blotting was also used to analyze selected Ca²⁺-binding proteins in frog brain with interesting results. Ca²⁺/calmodulin dependent protein kinase IV (CaMKIV), a 60 kDa protein, was elevated in the brain of stress-treated frogs (Fig. 5.3). The amount of CaMKIV increased during freezing reaching a maximum in brain of 8 h freeze-exposed frogs (about 5-fold greater than control). The intensity of CaMKIV decreased slightly after 12 and 24 h freezing exposure but rose again in 24 h thawed animals (about 6-fold greater than control). High levels of CaMKIV were also found in 40% dehydrated, rehydrated and anoxia-exposed frogs, with highest levels in 24 h anoxic animals (8.5-fold greater than control).

Calcineurin is a Ca^{2+} /calmodulin-dependent protein phosphatase. It is a heterodimer consisting of a 61-kDa catalytic subunit and a 19-kDa Ca^{2+} -binding regulatory subunit. The protein was also abundant in frog brain. Western blotting showed that the level of calcineurin in frog brain responded to freezing, dehydration and anoxia stresses (Fig. 5.4). Calcineurin levels increased by about 60-70% in frozen frogs (8-14 h) and were about 2 fold higher than control values in the thawed recovery group. Short anoxia (4 h) exposure suppressed calcineurin levels but with longer anoxia exposure (24 h) the protein level increased again by ~2.5 fold as compared with the 4 h anoxia group but still slightly lower than control values (Fig. 5.4). Dehydration stress had the opposite effect, significantly increasing calcineurin levels by 150% as compared with controls. Levels appeared to decrease slightly following rehydration.

DISCUSSION

Analysis of protein levels is essential to demonstrate the physiological significance of differentially expressed mRNA transcripts. Proteins are the final products of gene expression and they take part in cellular activities. Protein analysis is another powerful approach to study molecular mechanisms of stress responses. For example, recent developments in two-dimensional (2-D) gel electrophoresis and the techniques associated with appropriate computer analysis of gel images and 2-D gel-purified protein identification can enable a wide range of protein changes to be analyzed in a biological system (Pennington *et al.*, 1997). The 2-D gel technology should also be a powerful method to directly identify proteins that change under environmental stress responses.

However, the current 2-D gel technology is only suitable for analyzing proteins with high-moderate abundance.

Immunoblotting can be used for the analysis of protein change in wood frog tissues under stress conditions but to do this, appropriate antibodies are required. Of the genes up-regulated by stress in wood frogs, antibodies were available for only one of their protein products, PGK1. Western blot assays showed that PGK1 protein levels in frog brain were elevated during freezing, anoxia and dehydration stresses. PGK1 was particularly strongly induced by anoxia exposure which is consistent with its known behavior in mammalian systems where PGK1 is one of the glycolytic enzymes whose levels rise under hypoxic stress, mediated by HIF-1 (Semenza *et al.*, 1994; Li *et al.*, 1996). PGK1 protein content was also elevated in brain of 4-8 h frozen frogs. Hence, the immunoblotting assay indicated that stress-induced accumulation of PGK1 mRNA in frog brain led to the elevation of the protein product. Furthermore, the anoxic stress test suggested that freezing-inducible PGK1 gene expression might be triggered by low O₂ tension that results from freezing-induced ischemia/hypoxia. PGK1 content was also elevated in brain of 40% dehydrated frogs which could suggest that its expression is also responsive to cell water or volume signals but at very high water loss values amphibians also experience hypoxia stress due to low blood volume and high blood viscosity which impairs oxygen delivery to tissues. Hence, it appears that PGK1 protein content (and PGK1 mRNA levels) are regulated in response to low oxygen signals and that freeze-induction of PGK1 can probably be linked with freeze-induced oxygen limitation. However, further studies using a greater range of anoxia and/or dehydration sampling times would be necessary to fully confirm this. In addition, changes in PGK1 content

may result not only from increased gene expression but also from stress-induced changes in the rates of protein synthesis and/or degradation which could also be assessed. It would also be of value to measure changes in PGK1 maximal activity in the brain of control versus stress-treated frogs and assess any changes in the subcellular distribution of the enzyme.

PGK1 is one of two reactions in glycolysis that generates ATP from substrate levels phosphorylation and thus is key to cellular energy production under oxygen-limiting conditions. Elevation of this enzyme under stresses that create low oxygen conditions (e.g. freezing) could, therefore, be an important part of maintaining cellular homeostasis and energy levels under stress conditions. However, recent studies have also indicated that PGK1 can have multiple roles, acting in the cytoplasm as an enzyme of glycolysis but PGK1 present in the nucleus has been reported to act as a transcription factor (Ronai, 1993). One or both of these roles may be enhanced during freezing or other stresses in frogs and further studies will have to analyze the subcellular distribution of PGK1 in frog brain and the relative changes in enzyme amount and activity in different cellular compartments in response to stress.

For the other protein-coding genes that were identified by my work, a corresponding analysis of changes in the levels of their protein products could not be performed due to a lack of appropriate antibodies from commercial or other sources. However, a possible avenue for further research would be to express the protein products of these up-regulated genes in appropriate systems in order to produce recombinant protein that could then be used to raise antibodies. These could then be used in analyzing both the amounts and the subcellular distribution of these proteins. By using eukaryotic

expression systems we could also produce sufficient amounts of proteins for use in structural and function studies of the protein products.

In order to define the signaling mechanisms involving in environmental stress responses in wood frogs, analysis of HIF-1 α and p38 was carried out with frog brain. As discussed before, the transcription factor, HIF-1, is well-known to mediate hypoxia-induced gene transcription in mammalian and other systems that are largely intolerant of low oxygen stress. However, the role of HIF-1 in species that are anoxia tolerant is as yet completely unknown, even including whether or not the transcription factor is present in these species. Therefore, the present study represented one of the first trials by our lab in determining whether HIF-1 has any role to play in lower vertebrate species with good anoxia tolerance. Surprisingly, there was no cross-reaction in immunoblotting assays between frog brain protein samples and the mammalian anti-HIF-1 α . There are several possibilities for this lack of reaction including: (a) frog HIF-1 (if it exists) may be present only in extremely low levels, (b) frog HIF-1 protein may not crossreact with antibodies to its mammalian counterpart, and (c) HIF-1 mediates compensatory responses to low oxygen in oxygen-sensitive species but it may not be the key regulatory protein that mediates adaptive responses to low oxygen in hypoxia- or anoxia-tolerant species.

As mentioned before, p38 is a MAPK involved in mediating responses to osmotic stress in some species (Raingeaud *et al.*, 1995), and could possibly be involved, therefore, in dealing with freeze-induced cell volume change. However, the amount of p38 in frog brain, as detected by immunoblotting, was constant in both control and stressed groups. Thus, the signal transduction mechanism mediating any changes in gene expression that are stimulated by freeze-induced cell dehydration or volume changes remains to be

determined. Other types of MAPKs may respond to the stress in wood frogs.

Immunoblotting analysis also showed changes in the levels of several Ca^{2+} signaling-associated proteins in frog brain under freezing, anoxia or dehydration stress. The amount of $\beta 1\text{-Ca}^{2+}$ channel protein seemed unchanged during stress (freezing or anoxia), but Ca^{2+} /calmodulin dependent proteins (CaMKIV and calcineurin) responded to freezing and thawing, dehydration and rehydration, and anoxia stress. Since the activity of both proteins is Ca^{2+} and calmodulin dependent, the elevation of the two proteins during freezing may related to Ca^{2+} signaling pathways. In other studies of wood frog responses to these stresses, we found evidence that the Ca^{2+} and phospholipid dependent protein kinase C (PKC) was also involved in mediating metabolic responses to these stresses. Levels of one of the PKC second messengers, inositol trisphosphate (IP_3), rose by 75 % within 4 hours of freezing exposure in brain and increased 8-fold in liver after 24 h freezing (Holden and Storey, 1996) suggesting that PKC is activated during freezing in frog organs. IP_3 levels also rose by 8-fold in wood frog liver within 30 min of anoxia exposure and increased to a lesser extent during dehydration (Holden and Storey, 1997). Hence, we now have increasing evidence that Ca^{2+} -mediated signaling pathways have important roles to play in the adaptive responses to stress by frog organs.

CaMKIV plays an important role in Ca^{2+} regulated gene expression (Ghosh and Greenberg, 1995). CaMKIV is localized to the nucleus (Jensen *et al.*, 1991) and it may be critical for transcription activated by nuclear Ca^{2+} . CaMKIV activates the signal-regulated transcription factor, CREB (cAMP response element binding protein), by phosphorylation of CREB on Ser¹³³, which is essential for stimulation of target gene

expression. Recent work indicated that CBP (CREB binding protein, a transcription coactivator) was found to contain a signal-regulated transcriptional activation domain which is controlled by nuclear Ca^{2+} and CaMKIV and by cAMP (Chawla *et al.*, 1998). CBP and its close relative p300 are vital components of the cellular machinery that regulate gene expression. They can connect sequence-specific transcription activators to the components of the basal transcription machinery and may disrupt repressive chromatin structures through the intrinsic or associated histone acetyltransferase activity (Ogryzko *et al.*, 1996). CBP and p300 also play an essential role in the cellular response to hypoxia (Arany *et al.*, 1996).

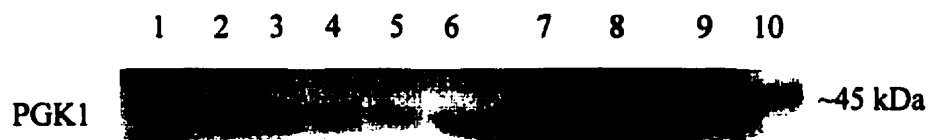
The elevation of CaM kinases has been reported in plants in response to cold and drought stress (Sheen, 1996). It was also suggested that CaM kinases must be involved in the cyclic ADP-ribose-mediated cold stress responses (Pennisi, 1997). The elevation of CaMKIV in frog brain under freezing, anoxia and dehydration stress suggests that Ca^{2+} signaling pathways may be important in mediating cellular adaptations in the freeze tolerant frogs since enzyme action is mainly determined by its level and activity.

Calcineurin is a Ca^{2+} /calmodulin-dependent protein phosphatase. It has been identified as the physiological target of immunosuppressants such as cyclosporin A and acts as an important regulator of signaling pathways in numerous tissues. A rise in intracellular Ca^{2+} (e.g. triggered by ligand binding to a cell membrane receptor) leads to the activation of the phosphatase activity of calcineurin, which dephosphorylates cytoplasmic target proteins. Dephosphorylated target proteins enter the nucleus and cooperatively bind to DNA with certain transcription factors such as AP-1 (activator protein-1), which then stimulates gene expression (Crabtree, 1999). The elevation of

calcineurin in the brain of freezing or dehydration-treated frogs further suggests that Ca^{2+} signaling pathways may mediate the responses to environmental stress in freeze tolerant wood frogs.

Calcium is a vital cellular signaling molecule (Berridge *et al.*, 1998). It would be valuable to test the changes of subcellular Ca^{2+} levels in frog tissues under freezing, anoxia or dehydration stress. Analysis of phosphorylated CREB is also required to further demonstrate the physiological significance of CaMKIV elevation in response to the stress.

Figure 5.1. Representative Western blots of brain extracts from wood frogs probed with an anti-PGK1 antibody. Equal amounts of protein (20 μ g) were loaded for each sample. Lane 1: control frog brain from cold-acclimated frogs at 5°C for 2 weeks; lane 2, 3, 4, and 5: freezing-treated groups from the cold-acclimated frogs exposed at -2.5°C for 4, 8, 12 and 24 h, respectively; lane 6: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h; lane 7 and 8: brain of anoxia-treated frogs for 4 and 24 h, respectively; lane 9: brain of 40% dehydration-treated frogs; lane 10: brain of fully rehydrated frogs after 40% dehydration. The immunoblotting were repeated with multiple trials and the data shown in the graph represent the average value from the repeats (n = 3). Analysis of protein band intensities was performed by using Imagequant. The value of relative protein band intensity for the test groups is normalized to the control group.



PGK1 protein levels in frog brain tissues

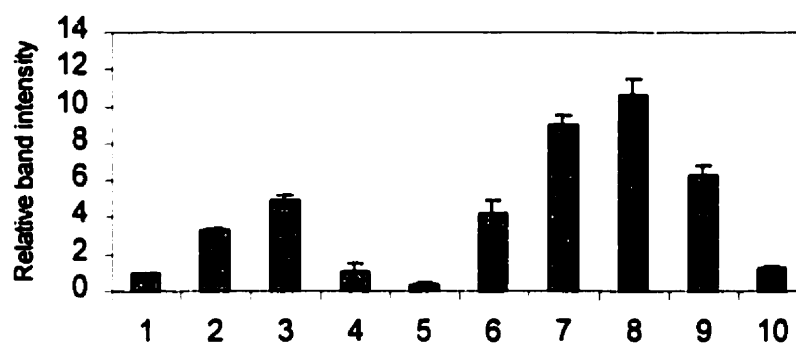


Figure 5.2. Representative Western blots of brain extracts from wood frogs probed with an anti- β 1-Ca²⁺ channel antibody. Equal amounts of protein (15 μ g) were loaded for frog brain samples. Lane 1: rat brain lysates as positive control for detecting the Ca²⁺-channel protein (load ~10 μ g); lane 2: rat brain lysate (load ~5 μ g); lane 3: frog brain from cold-acclimated frogs at 5°C for 2 weeks as control group; lane 4, 5 and 6: freezing-treated groups from the cold-acclimated frogs exposed at -2.5°C for 8, 12 and 24 h, respectively; lane 7: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h; lane 8 and 9: brain of anoxia-treated frogs for 4 and 24 h, respectively. The immunoblotting was performed with identical blots and the data shown in the graph represent the average value from the repeats (n = 2). The value of relative protein band intensity is normalized to control.

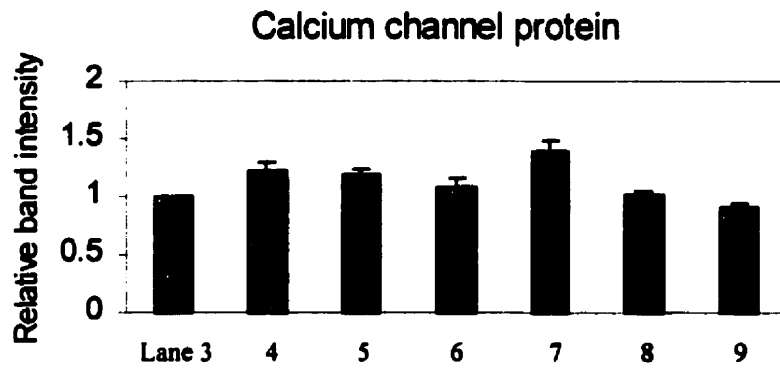
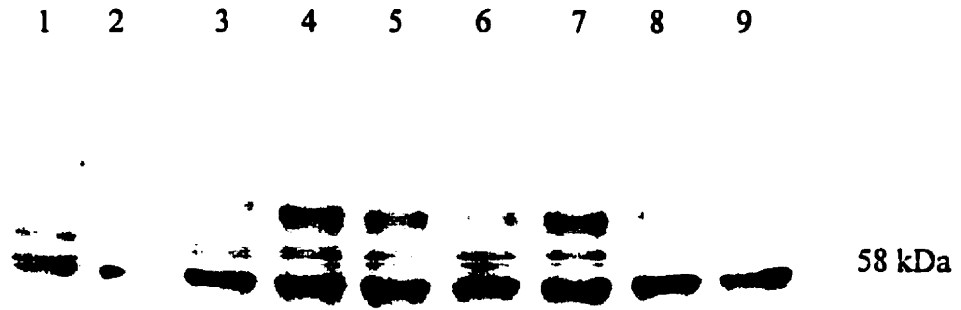


Figure 5.3. Representative Western blots of brain extracts from wood frogs probed with an anti-CaMKIV antibody. Equal amounts of protein (20 μ g) were loaded for each sample. Lane 1: control group, frog brain from cold-acclimated frogs at 5°C for 2 weeks; lane 2, 3, 4, and 5: freezing-treated groups from the cold-acclimated frogs exposed at -2.5°C for 4, 8, 12 and 24 h, respectively; lane 6: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h; lane 7: brain of 40% dehydration-treated frogs; lane 8: brain of fully rehydrated frogs after 40% dehydration; lane 9 and 10: brain of anoxia-treated frogs for 4 and 24 h, respectively. The immunoblotting was repeated with multiple trials and the data shown in the graph represent the average value from the repeats (n = 3). The value of relative protein band intensity is normalized to control.

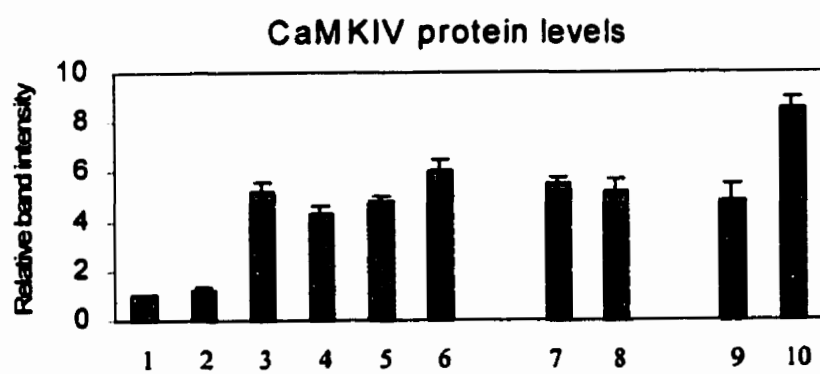
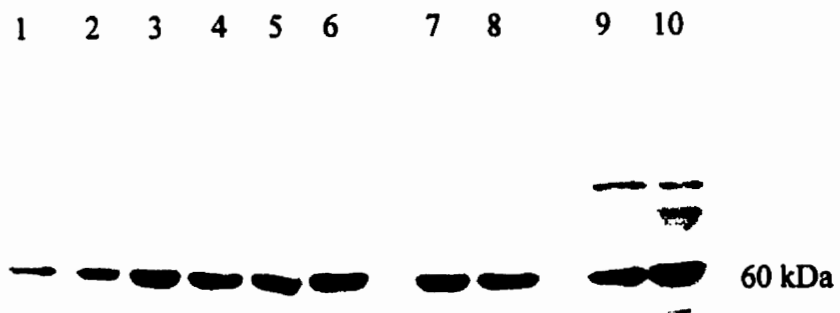
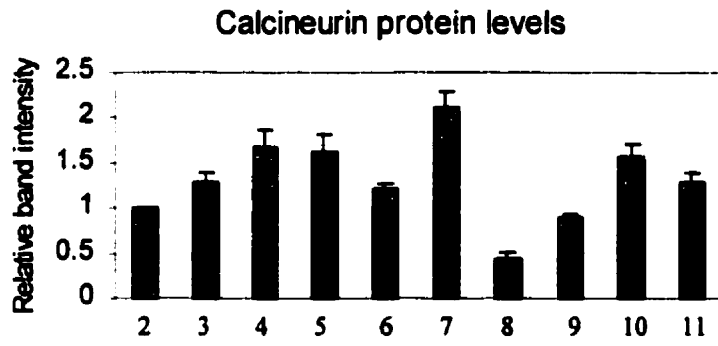
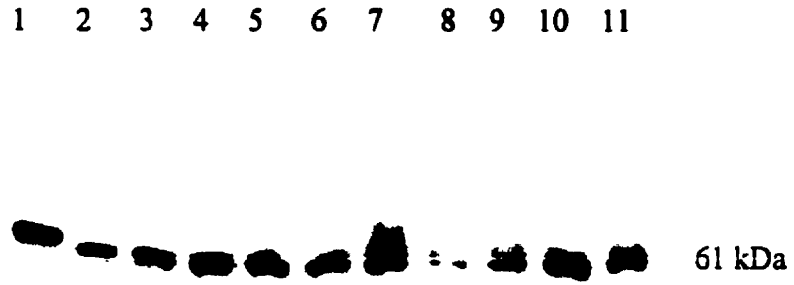


Figure 5.4. Representative Western blots of brain extracts from wood frogs probed with an anti-calcineurin antibody. Equal amounts of protein (20 μg) were loaded for each lane of frog samples. Lane 1: rat brain lysates (load $\sim 10 \mu\text{g}$); lane 2: control group, frog brain from cold-acclimated frogs at 5°C for 2 weeks; lane 3, 4, 5 and 6: freezing-treated groups from the cold-acclimated frogs exposed at -2.5°C for 4, 8, 12 and 24 h, respectively; lane 7: recovery group from the cold-acclimated frogs freeze-exposed at -2.5°C for 24 h and thawed at 5°C for 24 h; lane 8 and 9: brain of anoxia-treated frogs for 4 and 24 h, respectively; lane 10: brain of 40% dehydration-treated frogs; lane 11: brain of fully rehydrated frogs after 40% dehydration. The immunoblotting was repeated with multiple trials and the data shown in the graph represent the average value from the repeats ($n = 3$). The value of relative protein band intensity is normalized to control.



CHAPTER SIX
General Discussion

Among numerous environmental stresses, cold is probably the most common stress for living organisms on Earth. Terrestrial organisms that live at high latitudes or altitudes must have effective strategies to deal with cold and/or freezing stress. When temperature drops below the freezing point of the body fluids, freezing takes place and intracellular ice formation is always lethal to cells and organisms. Many terrestrial animals choose to leave the cold environment (e.g. migration) or stay in insulated hibernacula (e.g. hibernation underwater or deep underground) to escape exposure to freezing temperatures. Many other ectothermic animals can endure subzero exposures with adaptations that allow them to maintain body fluids in a liquid state (freeze avoidance) or survive extracellular ice formation (freeze tolerance). Freeze tolerance occurs widely among invertebrates (Storey and Storey, 1992a) and is also found in several species of terrestrially hibernating amphibians and reptiles (Storey, 1990; Storey and Storey, 1992b).

The wood frog (*Rana sylvatica*) is the model animal that has been most extensively used for studying freezing survival in vertebrates (Storey and Storey, 1996a). This frog can tolerate the conversion of 50-65% of total body water into extracellular ice and live several weeks in a frozen state with no breathing, no heart beat, and no detectable brain activity. However, extracellular ice formation has at least three serious consequences for cells and tissues: 1) potential physical damage by ice expansion in microcapillaries, 2) freezing-induced ischemia halts the delivery of O₂ and nutrients to tissues, and 3) the exit of cell water into extracellular ice masses results in intracellular dehydration creating osmotic stress, an increase in ionic strength and a reduction in cell volume. The frog must develop effective ways to adapt to these stresses.

Extensive biochemical studies to elucidate freeze tolerance adaptations have focused on intermediary energy metabolism, the action of ice nucleating proteins (INPs), the regulation of enzyme function, and the production and distribution of cryoprotectant in wood frogs (Storey and Storey, 1988, 1992b, 1996a; Costanzo *et al.*, 1993). Recently, modern techniques of molecular biology have also been applied to study the role of gene expression in freeze survival by lower vertebrates. The first studies carried out by our lab used differential screening of a wood frog liver cDNA library to identify several genes that were freeze-upregulated (genes encoding the α and γ subunits of fibrinogen, mitochondrial ADP/ATP translocase) (Cai and Storey, 1997a; Cai *et al.*, 1997). The upregulation of fibrinogen transcripts may be involved in tissue damage repair processes during freezing and/or thawing, whereas the upregulation the ADP/ATP translocase gene may have a role in the compensatory strategies needed to deal with depleted ATP levels due to low O₂ in the frozen state. These studies indicated new types of proteins and new cellular adaptations may be involved in frog freeze survival. Thus, the molecular biology approaches opened a new avenue to explore the cellular adaptations to freezing.

This thesis further demonstrates that regulation of gene expression is an important part of natural stress survival. Via extensive screening of frog liver cDNA library, additional up-regulated liver genes (e.g. *ND4* and *EF-1 γ*) were isolated. In addition, more mitochondrial genes (encoding for ATPase subunit 6 and 8, 16S rRNA), a ribosomal protein gene (*P0*), a glycolytic enzyme gene (*PGK1*) and a *pol*-like reverse transcriptase-related gene were identified from frog brain by cDNA library screening. These were up-regulated in the brain of freeze-exposed frogs. Another ribosomal protein gene (*RPL7*) was isolated from frog skin by DD-PCR. This gene was up-regulated in response to cold

acclimation in the skin and was also found up-regulated in the brain of freeze-exposed frogs. Overall, then, the genes that were identified from my studies belong to two general categories: mitochondrial or nuclear genes with links to energy metabolism or genes whose products are involved in protein translation machinery (see Chapter 2, 3 and 4).

The isolated mitochondrial genes (encoding for ATPase subunit 6 & 8, 16S rRNA and ND4) were all up-regulated both in brain and liver of freeze-exposed frogs whereas their expression did not seem to be enhanced in the skin under freezing exposure.

Although the expression of ATPase 6 & 8 genes was also up-regulated in another core organ, kidney, it was down-regulated in lung and gut and was unchanged in other tissues such as blood, muscle, and heart. It is not clear why the individual mitochondrial genes were selectively expressed among the tissues of freeze-exposed frogs. The upregulation of the mitochondrial genes in frog brain or liver could be stimulated by anoxia instead of by dehydration stress. The low O₂ tension may trigger mitochondrial gene expression in selected frog organs, both mtATPase subunit 6 & 8 and ND4 contributing to ATP generation. The elevation of these gene transcripts may be required to optimize the use of declining O₂ supplies as freezing moves through the body tissues, cutting off circulation and oxygen delivery. Since 16S rRNA is major component of mitochondrial ribosomes, an increased 16S rRNA level in selected frog organs during freezing or anoxia may suggest that mitochondrial protein synthesis machinery needs to be maintained in these organs in order to translate mitochondrially-encoded mRNAs under the adverse conditions. Although the physiological significance of up-regulated mitochondrial genes needs to be further defined, mitochondria may play critical role in frog freeze tolerance.

Mitochondria can be defined as "the powerhouse of the cell". The ability of mitochondria to convert the energy provided by the electron transport system into ATP via oxidative phosphorylation is critical to virtually every activity in eukaryotic cells. Mitochondria also appear to play a central role in the regulation of apoptosis (programmed cell death) and necrosis (accidental cell death). These organelles can also respond to numerous physiological and some pathological stimuli to trigger an increase in mitochondrial membrane permeability. The mitochondria release apoptogenic factors (such as cytochrome c) that can further activate caspases (cysteine aspartases) and result in apoptosis (Kroemer *et al.*, 1998; Mignotte and Vayssiere, 1998). It is believed that ROS (reactive oxygen species, mainly generated from mitochondria), ATP depletion and the outflow of matrix calcium also play roles in signaling apoptosis (Kroemer *et al.*, 1998). It is unknown if apoptosis and/or necrosis might play roles in freeze tolerance in wood frogs, for example during the thawing recovery stage when it may be necessary to remove damaged cells. However, the upregulation of mitochondrial genes in frog brain and liver as seen in current studies and in turtle heart (Cai and Storey, 1996) as well as upregulation of the ADP/ATP translocase gene in frog liver (Cai and Storey, 1997) suggests that maintaining a certain level of mitochondrial activity may be required for cell survival under extreme environmental conditions in these stress-tolerant organisms.

Another ATP generation-associated gene, *PGK1*, was highly expressed in frog brain and the transcript levels increased under freezing, anoxia and dehydration stresses in brain. However, *PGK1* seemed less expressed in liver and the mRNA level was increased during freezing, short period anoxia (1-4 h) or mild dehydration (20%) stress (see Chapter 2 and 4). Immunoblotting assays indicated that *PGK1* protein was also

elevated under freezing, anoxia and dehydration with the highest PGK1 production found in the anoxic group.

These studies showed that the elevated levels of PGK1 transcripts under the stresses used did, indeed, lead to more protein synthesis. However, although the elevated mRNA level was similar among stress conditions, a much higher level of induction of PGK1 protein was seen under anoxic conditions. This implies that translational control or posttranslational modification may also play role in PGK1 protein elevation. It is not clear if the increase in PGK1 content affected its function as a glycolytic enzyme or as a nuclear regulator of DNA transcription or both.

As for ribosome-associated proteins, *P0* and *RPL7* are ribosomal proteins, whereas *EF-1 γ* is an elongation factor subunit involved in protein translation machinery. They all play roles in protein synthesis. The accumulation of these gene transcripts in selected frog organs may be a response to stress-caused inhibition of protein translation processes. It is well known that low temperature can affect ribosomal assembly and inhibit protein translation processes (Nomura, 1987). The translation process will eventually cease in frozen tissues. Freeze exposure can stimulate the expression of *P0* in frog brain and liver, *EF-1 γ* in liver and *RPL7* in brain of wood frogs, which suggests that elevation of certain ribosome-associated gene products may be required by the stress-tolerant species to maintain functional protein translation machinery during freezing. Anoxia can also strongly inhibit protein translation (Tinton *et al.*, 1997), but anoxia was found to stimulate the expression of certain ribosome-associated genes in wood frogs. The anoxia-caused translation inhibition may result in the upregulation of these genes (such as the *P0* and *RPL7*). Although the expression of *EF-1 γ* was repressed upon initial

anoxic exposure, the gene expression was enhanced during 4-12 h anoxia exposure. The expression of *RPL7* and *EF-1 γ* were both repressed in longer anoxia (24 h), which indicated that expression of both genes may be sensitive to O₂ level. By contrast, dehydration decreased the expression of *EF-1 γ* and *P0* genes. However, severe dehydration (40% of total body water loss) can increase the *RPL7* transcript level as compared with the control group and the *P0* mRNA level as compared with the 20% dehydration-treated group. Severe dehydration may inhibit translation process that triggers the upregulation of certain protein synthesis-associated genes. Severe dehydration, as compared with 20% dehydration, also has a hypoxic component to it because increased blood viscosity and decreased blood volume leads to impairment of oxygen delivery to the tissues. Hence, it seems possible that the accumulation of the transcripts of the above 3 genes under freeze exposure may be linked with ischemia/hypoxia as the major stress signals, not dehydration stress.

Impaired protein synthesis is one of the major problems for organisms exposed in cold. Most organisms have developed a general adaptive mechanism that alters the protein translation machinery at low temperature. For example, in *E. coli*, at least 3 cold-regulated proteins are ribosome-associated: initiation factor (IF2); an RNA unwinding protein (*CsdA*); and ribosome-binding factor (*RbfA*). Their levels are elevated under cold exposure and are required for efficient ribosomal function at low temperature (Thieringer *et al.*, 1998). In yeast *S. cerevisiae*, a cold-induced protein, *NSR-1*, was shown to be directly involved in ribosome biogenesis (Kondo *et al.*, 1992). Alterations in the ribosome occur during the cold-hardening process in plants. Approximately 17 ribosomal proteins are altered at low temperature in black locust seedlings (Bixby and Brown,

1975). As discussed in Chapter 4, the levels of EF1 protein are also increased in the liver of cold-adapted toadfish. The accumulation of EF1 is believed to help overcome an initial decrease in protein synthesis when shifted to low temperature and maintain steady protein translation during cold acclimation. The above evidence indicates that there may be a switch to a cold-adapted ribosome during freezing in wood frogs, which results in maintaining functional translational machinery at low temperature.

The elevation of 3 ribosome-associated protein genes (*P0*, *EF-1 γ* and *RPL7*) was shown in selected frog organs under freezing stress. Although the specific roles of these ribosome-associated proteins are unknown in freezing survival, the upregulation of these genes may suggest that the translational machinery should be functioning optimally, perhaps to produce the selected stress proteins needed for protection from freezing injuries in wood frogs.

The above genes can be classified into 2 groups (Table 4.1, Chapter 4): (1) genes whose products are related to protein translation machinery (16S rRNA gene, *P0*, *EF-1 γ* and *RPL7*), and (2) genes whose products are directly involved in ATP generation (ATPase 6 & 8 genes, *ND4* and *PGK1*). The importance of elevation of selected ribosome-associated proteins at low temperature is described above. The adaptation of energy generation under adverse conditions is usually found among stress-tolerant organisms. Metabolic rate depression is a common mechanism of survival that is used by many species to survive harsh environmental conditions such as low oxygen tension, low temperature or low water availability. Wood frogs may rely strongly on glycolysis to generate ATP during freezing exposure. The frogs may also utilize the remaining limited O₂ efficiently for ATP production via oxidative phosphorylation. Maintenance of a

minimal ATP level may be needed for cellular adaptations and may be critical for frog survival during stresses. Hence, the upregulation of genes associated with energy generation may help frogs endure freezing exposure.

In addition to the ribosomal changes, another universal response to low temperature is a change in the lipid composition of membranes, e.g. by changing the degree of saturation of the hydrocarbon chains of membrane phospholipids. Unsaturated fatty acids have lower melting points and a greater degree of flexibility, which makes organisms more resistance to cold or freezing. Remarkably, a nonspecific cyanobacterial desaturase was able to impart chilling resistance to transgenic tobacco plants (Ishizaki-Nishizawa *et al.*, 1996). Recently, a lipid desaturase gene was cloned from carp fish (Tiku *et al.*, 1996). The enzyme shows an 8-10 fold increase in activity after cold exposure of the carp. The response is believed to be regulated by both transcriptional and posttranslational mechanisms. So far via differential screening we did not isolate genes whose products are involved in changing membrane fluidity or membrane protection. However, genes involved in such processes would likely be induced by cold acclimation rather than by freezing itself and since our screening used 5°C acclimated frogs as controls, it is not surprising that genes associated with membrane cold adaptation were not identified in wood frogs. We also did not find stress proteins such as heat shock proteins (hsps) in freeze-exposed frogs. In contrast to heat shock, protein folding and denaturation may not be a major problem at low temperature in stress tolerant organisms. Freezing-associated low temperature and anoxia may be the major reasons for impaired protein synthesis and may be one of major problems that frog must overcome during freeze exposure. Indeed, secondary structure of RNA and DNA is more stable at low

temperature. As a result, ribosomes are less efficient at translating mRNA and RNA polymerases are likely to have more difficulty unwinding DNA for normal function. The upregulation of the selected ribosome-associated protein genes in selected wood frog organs may be the cold stress response as discussed above.

The upregulation of the identified genes may take place at the level of transcription or/and other steps of gene expression (such as RNA precursor processing and mRNA stability). The expression of *pol*-like reverse transcriptase-related gene was induced upon freezing exposure in both brain and liver and gene transcription must play a key role in the elevation of the transcripts. It may be possible to define stress-responsive elements by analyzing the structure and organization of stress-induced genes. Since freezing is a multiple stress-associated event, the "freezing-responsive element(s)" may be related to the hypoxia-inducible element (HIE) or other types of stress-responsive elements. Hence, it is also valuable to identify and analyze stress-inducible transcription factors, which will provide more insights into the signaling mechanisms of stress-induced gene expression.

Freezing-induced hypoxia/anoxia may be the major signal that triggers the upregulation of most isolated genes, whereas freezing-associated low temperature or freezing-caused cell volume change and water stress may also play a role in the regulation of selected genes. The exact signaling mechanisms of freezing-inducible gene expression remain unclear, but the elevation of Ca²⁺/calmodulin-regulated protein kinase and phosphatase suggests that a Ca²⁺ signaling pathway may play a role in stress-mediated gene expression.

Wood frogs have developed freeze tolerance to allow them to survive winter on land, the advantage of terrestrial hibernation being that frogs can respond quickly to warming temperatures in the spring and breed very early in the season (Storey and Storey, 1996a). Freeze tolerance also allows frogs to endure lower subzero temperature than other amphibians. Wood frogs are also desiccation-resistant and anoxia-tolerant and are, overall, excellent model animals for studies of natural stress survival. Future approaches with both gene screening and direct protein analysis (2-D gel technology) may provide the full range changes in gene products that support freezing adaptation and could uncover more secrets of natural freeze tolerance that may be beneficial for medical applications, especially for organ cryopreservation and transplantation.

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