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**THE ROLE OF DIFFERENTIALLY EXPRESSED GENES AND THEIR
PROTEIN PRODUCTS IN THE HIBERNATING THIRTEEN-LINED GROUND
SQUIRREL SPERMOPHILUS TRIDECEMPLINEATUS**

By

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AND THEIR PROTEIN PRODUCTS IN THE HIBERNATING THIRTEEN-
LINED GROUND SQUIRREL SPERMOPHILUS TRIDECIMLINEATUS”**

submitted by

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ABSTRACT

The role of differential gene expression in supporting the survival of the hibernation phenotype was investigated using a variety of “gene discovery” techniques. A cDNA library constructed from kidney of the thirteen-lined squirrel, *Spermophilus tridecemlineatus*, was differentially screened for genes that were up-regulated during hibernation. A clone encoding cytochrome c oxidase subunit 1 (*CoxI*) was found and confirmed as up-regulated by Northern and Western blotting. This revealed the differential expression of *CoxI* mRNA in multiple organs during hibernation. It is hypothesized that hibernating mammals increase the expression of the mitochondrial genome in general and *CoxI* specifically during torpor, to prevent or minimize the damage caused by the cold and ischemia experienced during a hibernation bout. The up-regulation of heart and adipose type fatty acid binding proteins (FABPs) was detected during hibernation in brown adipose tissue (BAT) using a commercial rat cDNA array. Full length cDNAs encoding heart and adipose-type FABPs were subsequently retrieved from a BAT cDNA library. H-FABP mRNA transcripts increased in BAT, skeletal muscle and heart of hibernating animals whereas A-FABP transcripts, which are normally expressed exclusively in adipose tissue, increased in both BAT and heart during torpor. The translation status of differentially expressed mRNAs during hibernation was also investigated in kidney and brown adipose tissue. Polysome profile analysis revealed a significant de-aggregation of polyribosomes during hibernation and a shift of housekeeping gene mRNAs and the up-regulated organic cation transporter 2 (OCT2) mRNA to the translationally silent monosome and mRNP fractions of kidney cytoplasmic extracts. *In vitro* translation rate and immunoreactive OCT2 protein were also

significantly decreased in hibernating kidney. By contrast, the increased translation status of H-FABP mRNA, increase in immunoreactive H-FABP protein and unchanging *in vitro* translation rate reflect the important role of active brown adipose tissue during hibernation. The decrease in protein synthesis and de-aggregation of polysomes in kidney but not in brown adipose tissue, is linked to the phosphorylation of eIF2 α . Additionally, redistribution of *Cox4* but not H-FABP mRNA to the monosome fraction in hibernating BAT may indicate a mechanism for the preferential translation of a subset of genes physiologically relevant to the survival of hibernation.

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First and foremost I would like to thank my thesis supervisor Dr. Kenneth B. Storey and his wife Janet for taking a new graduate student (me) into the lab in the middle of the academic year. The dynamic and independent research environment of the “Storey Lab” and editorial prowess of Janet has helped me to better define, achieve and exceed my research objectives. I would also like to thank my lab mates for educating this western Canadian on the finer points of “maritime culture” and more seriously, for their insightful comments and criticisms which have significantly influenced the research presented in this thesis.

Also, for the science fiction authors who shaped my view (at a young age) of scientists as heroic, yet quixotic, protagonists who battle the dark forces of ignorance with the power of rational thought. And finally, for those who have supported me in this notion and my pursuit of a career in the sciences..... with understanding and love.

I wonder why I wonder why I wonder why, I wonder...

Richard, P. Feynman

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List of Abbreviations

AA	amino acid
ATP 6/8	ATP synthase subunits 6 and 8
BAT	Brown Adipose Tissue
cDNA	complementary DNA
COX1	cytochrome c oxidase subunit 1
COX4	cytochrome c oxidase subunit 4
cpm	counts per minute
dpm	disintegrations per minute
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
eIF2α	the α subunit of elongation initiation factor 2
ETBr	ethidium bromide
FABP	fatty acid-binding protein
FFAS	free fatty acids
GAPDH	glyceraldehyde phosphate dehydrogenase
kb	kilobase
kDa	kilo Dalton
MOPS	3-[N-morpholine] propanesulfonic acid
mRNA	messenger RNA
mtRNA	mitochondrial mRNA
MW	molecular weight

NPY	neuropeptide Y
NST	non-shivering thermogenesis
nt	nucleotide
OCT2	organic cation transporter type-2
ORF	open reading frame
PABP	poly(A) binding protein
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque forming unit
PGC1/2	PPAR Gamma coactivator 1 or 2
PPAR	peroxisome proliferator-activated receptor
PVDF	polyvinylidene fluoride
rRNA	ribosomal RNA
SCN	superchiasmatic nucleus
SDS	sodium dodecyl sulfate
TIA-1	T-cell intracellular antigen-1
UTR	untranslated region
WAT	White Adipose Tissue

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CHAPTER 1

GENERAL INTRODUCTION: INVESTIGATING THE ROLE OF DIFFERENTIAL GENE EXPRESSION IN MAMMALIAN HIBERNATION

Winter poses extremely difficult challenges for small mammals. The lower the ambient temperature, the higher the rate of metabolic fuel consumption that is required to maintain a constant high (euthermic, 37°C) body temperature. But winter is also the time when food supplies are scarce or even unattainable and, hence, the replenishment of body fuel reserves becomes problematic. Many small mammals solve the problem of surviving through the winter with hibernation, entering a state of torpor where metabolic rate falls to very low values (typically to <5% of euthermic resting rate) and body temperature drops to near ambient (Wang and Lee, 1996; Boyer and Barnes, 1999; Storey, 2000). In this state they can survive for many months, slowly oxidizing body fuel reserves. It is speculated that hibernation represents the ultimate extension of daily torpor, which evolved from an ancestral pattern of heterothermy. This process is used by a variety of endotherms including birds, to conserve energy stores by actively lowering their body temperature; this ability was apparently maintained in some species and lost in others (Srerer et al., 1992).

Spermophilus tridecemlineatus, the thirteen-lined ground squirrel, is one of a variety of ground squirrel species that hibernate. Ground squirrels of this species populate most of the North American great plains and feed primarily on seeds, flowers, garden vegetables (when available) and insects (Wang and Lee, 1996). In the late summer and early fall this feeding reaches a frenzied pace (hyperphagia) as the animals put on layers of fat in preparation for the winter season (Storey, 1997). Hibernation is a state in which all physiological and metabolic activities are profoundly depressed in a highly controlled and co-ordinated manner. This phenomenon has inspired several generations of research

into the physiological mechanisms that govern this remarkable adaptation. Recently, however, hibernation research has entered a new phase as the tools of molecular biology have been employed to investigate the role of gene expression in hibernation and other forms of animal dormancy in response to environmental stress. This research has produced strong evidence that changes in the expression patterns of known genes, rather than a subset of “hibernation-specific” genes, are intimately associated with the state of hibernation (Srere et al., 1992). Thus, it can be hypothesised that a biochemical reprogramming, mediated by differential gene regulation, plays an important role in mammalian hibernation.

Hibernating ground squirrels surrender to, rather than resist, the winter chill, allowing their body temperatures to plummet to near freezing. Striking decreases in blood flow (ischemia) and respiratory rate that accompany this drop would be irreversibly damaging to the cells, tissues or organs of humans yet ground squirrels endure extended hypothermia and its many consequences with no ill effects (Hochachka, 1986; Frerichs et al., 1998). These animals are, therefore, excellent research models to use to investigate a number of medically important problems including hypothermia resistance, cold or freezing preservation of transplantable organs, and the prevention of damage to the heart, brain and other ischemia-sensitive tissues during heart attack or stroke (Zancanaro et al., 1987; Frerichs et al. 1998; Green, 2000; Storey, 2000). Elucidating the biochemical and genetic mechanisms of hibernation is, therefore, an enchanting prospect with potential implications for several avenues of medical research.

Before stating the objectives of this study, it is important first, to review the achievements and shortcomings of past research on gene expression in hibernating mammals.

Heart

Upon entry into hibernation, the heart rate of a 13-lined ground squirrel plunges 100 fold (from 300 to ~ 3 beats per minute) (Wang and Lee, 1996; Boyer and Barnes, 1999). There is also a profound decrease in body temperature, mean arterial blood pressure and oxygen consumption (Anderson et al., 1990). For the heart to function under these suboptimal conditions a significant biochemical reprogramming must occur. An increase in (left) ventricular mass prior to hibernation is ostensibly the physical manifestation of these changes (Fahlman et al., 2000). Studies to date have revealed selected gene and gene products that are up-regulated during hibernation. For example, the genes encoding pancreatic lipase (PL) and pyruvate dehydrogenase kinase isozyme 4 (PDK4) are up-regulated during hibernation in the heart of the 13-lined ground squirrel and it has been suggested that these gene products play a role in the shift to a lipid-based during hibernation (notably PDK4 turns off pyruvate dehydrogenase, thereby suppressing carbohydrate oxidation) (Andrews et al., 1998). This is also consistent with the widely accepted theory that hibernating ground squirrels use their extensive fat depots almost exclusively to fuel survival during the cold winter months. Research in our laboratory has recently revealed the up-regulation of the ventricular isoform of myosin light chain 1 (MLC1(v)) as well as subunit 2 of NADH-ubiquinone oxidoreductase (ND2), a mitochondrially encoded protein (Fahlman et al., 2000). Hibernation-induced up-

regulation of MLC1(v) may indicate a restructuring of myosin subunit composition to enhance muscle contractility for hypothermic function, whereas changes in ND subunit composition may affect the function of the electron transport chain during hibernation (Fahlman et al., 2000).

White Adipose Tissue

During late summer and early fall, most species of hibernating ground squirrel experience a dramatic weight gain, primarily in the form of white adipose tissue (WAT). This is in preparation for the near exclusive use of a lipid-based metabolism during the hibernation season. Protein and mRNA levels of lipoprotein and hormone sensitive lipase in WAT of the yellow-bellied marmot, *Marmota flaviventris*, increase over the hibernation cycle (Wilson et al., 1992). These enzymes liberate free fatty acids (FFAs) from triacylglycerol depots into the blood where they are distributed to tissues, such as the heart, brown adipose tissue, and skeletal muscle during hibernation.

Skeletal Muscle

Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA and protein decrease in the skeletal muscle of the jerboa (*Jaculus orientalis*) during hibernation (Soukri et al., 1996). These changes are likely responsible for the perceived decrease in skeletal muscle glycolysis during hibernation, preserving limited glucose reserves and facilitating the shift to an entirely lipid-based metabolism. This also raises the possibility of the selective degradation or deactivation of proteins at important metabolic control points during mammalian hibernation (Storey, 2000). During hibernation, all species

experience some form of disuse-associated loss in total skeletal muscle mass. Paradoxically, the oxidative capacity of skeletal muscle is shown to increase during hibernation (Wickler et al., 1991). This suggests the presence of a mechanism which specifically prevents the loss of oxidative enzymes which would compromise the locomotor and thermogenic capabilities of the hibernating animal. Preliminary evidence suggests that the increased expression of mitochondrial-encoded genes in skeletal muscle may in part, explain this adaptive strategy (Wang and Lee, 1996; Fahlman et al., 2000). Notably, shivering thermogenesis in skeletal muscles is an important contributor to heat production during arousal, particularly in the "second half" of the arousal process once tissues have been partially rewarmed due to heat generated by nonshivering thermogenesis in brown adipose tissue.

Intestinal Epithelium

The 75 kDa cytoskeletal protein, moesin, was specifically up-regulated in the intestinal brush border membrane of the hibernating 13-lined ground squirrel (Gorham et al., 1998). Moesin functions as a membrane-cytosol linking protein of the ERM family (erizin/radixin/moesin). It is possible that increased quantities of this protein during hibernation may structurally enhance the plasma membrane but, as yet, no function has been assigned to this up-regulation. More recently, the redox sensitive transcription factor NF-kappa-B was shown to be highly activated in the intestinal epithelium and other tissues of hibernating versus euthermic animals (Carey et al., 2000). NF-kappa-B activates a wide variety of stress response pathways that deal with oxidative stress and ischemia-reperfusion type injuries.

Brain

The suprachiasmatic nucleus (SCN), located in the hypothalamic region of the brain, is responsible for regulating the circadian rhythms of most mammals (Wang and Lee, 1996). Increased gene expression occurs in the SCN of the golden-mantled ground squirrel, *Spermophilus lateralis*, during hibernation (O'Hara et al., 1999). This includes the transcription factors, C-fos, JunB, and JunC, whose up-regulation may subsequently alter long-term gene expression patterns and neuronal activity. More recently, the mRNA levels of neuropeptide Y (NPY) were found to increase in the arcuate nucleus (ARC) of hibernating jerboa (*Jaculus orientalis*) brain. This elevated NPY mRNA expression supports the hypothesis that NPY may be implicated in abnormal feeding behaviour associated with eating deprivation (El Quezzani et al., 2001). An increase in NPY gene expression in hibernating jerboas may be related to both food deprivation and cold exposure since NPY is also known to be a hypothermiant factor (El Quezzani et al., 2001). Thus, it is hypothesized that NPY within neurons of the ARC plays an important role in the control of energy metabolism.

Liver

The expression of alpha₂-macroglobulin increases during hibernation in the liver of the Richardson's ground squirrel, *Spermophilus richardsonii* (Srere et al., 1992). As a broad-spectrum protease inhibitor, alpha₂-macroglobulin may be responsible, in part, for decreased levels of protein degradation in hibernating animals. Alpha₂-macroglobulin also inhibits blood factor X and is responsible in part, for the decrease in clotting activity

that is observed during hibernation. Presumably, a reduced clotting potential helps to protect delicate capillary beds at low temperatures and blood pressures (Srere et al., 1992).

Brown Adipose Tissue

Brown adipose tissue (BAT) regulates the levels of free fatty acids in the blood and more importantly contributes to non-shivering thermogenesis (NST) which is the primary mechanism used by hibernators to rewarm their bodies during arousal (Boyer et al., 1998; Nedergaard et al., 1989). As mentioned previously, the physiological and metabolic depression that accompanies entry into hibernation is a highly co-ordinated process. BAT plays a significant role in controlling the rates of both cooling and rewarming during hibernation (Boyer and Barnes, 1999). An increase in the size of interscapular and subscapular BAT depots occurs prior to the hibernation season (Wang and Lee, 1996). It has not been clearly established whether this change is due to increased adipocyte numbers, mitochondria per adipocyte, or protein content per mitochondrion. An immediate-early response to cold exposure in the BAT of mice is a transient increase in the mRNA levels of c-fos and c-jun (Nedergaard et al., 1989). It is possible, then, that the active transcription factor AP-1, formed as a dimer of c-fos and c-jun, changes long-term gene expression patterns. Extended periods of cold exposure (in rats and mice) also induce the expression of hormone sensitive and lipoprotein lipases (HSL, LPL), vascular endothelial growth factor (VEGF), uncoupling protein (UCP1), and numerous mitochondrial matrix and electron transport chain proteins (Boyer and Barnes, 1999). The protein products of these genes enhance the capacity of BAT to produce heat by NST.

Surprisingly, little analysis of gene expression has been conducted with BAT from hibernating ground squirrels or any other true hibernating species. Instead, the response of BAT to cold exposure has been largely studied in laboratory mice and in cultured brown adipocytes and the results have been extrapolated to hibernators. Because of its increased activity during the hibernation season BAT is a worthy target for studying differential gene expression.

Kidney

The kidney has received remarkably little attention from hibernation researchers. A complex organ, it not only filters nitrogenous waste and toxins from the blood but acts also as an endocrine organ, producing a number of products including erythropoietin, renin and vitamin D (Anderson et al., 1990). The kidney is composed of ~1 million nephrons (a specialized multi-cellular structure that filters and concentrate the urine) and an extensive network of blood vessels, nerves and lymphatics. During hibernation there is a 60% reduction in mean arterial pressure which virtually stops glomular filtration and urine production (Anderson et al., 1990; Harlow and Braun, 1995). Electron micrograph studies of ground squirrel kidney have also shown a thickening of the glomular basement membrane and a decrease in the number of endothelial pores and epithelial filtration slits during hibernation. The production and concentration of urine is an energy-intensive process involving the active transport of salts and water across membranes. One would assume, therefore, that the kidney is highly sensitive to ischemia and to cold temperatures associated with hibernation (Frerichs et al., 1998). However, hibernators must be able to protect their kidneys from damage because urine production begins immediately upon

arousal. As observed in other hibernating tissues, the underlying mechanisms of this physiological adaptation must be regulated, in part, by the differential expression of genes.

Rationale

The metabolic depression associated with hibernation involves a strong, coordinated suppression of the rates of many metabolic enzymes and functional proteins, involved in both ATP-utilising and ATP-generating pathways. In a number of cases, this regulation has been linked to reversible protein phosphorylation via the action of protein kinase A (PKA), eEF-2 kinase and a variety of protein phosphatases (Boyer and Barnes, 1999; Storey, 2000; Chen et al., 2001). In addition, transcription and translation are substantially inhibited during hibernation (Frerichs et al., 1998; Martin et al., 2000; Zhegunov and Mikulinskii, 1987). Whereas the mechanisms of transcriptional inhibition have not yet been elucidated, the inhibition of translation has been linked to the phosphorylation of elongation initiation factor 2 α (eIF2 α) (Frerichs et al., 1998). Interestingly, various enzymes and proteins from hibernating ground squirrels maintain or show proportionately increased activities at temperatures approaching 0°C (Storey, 2000). These adaptations explain, in part, the ability of these animals to survive the challenges associated with living in the cold. It has become increasingly apparent, however, that differential gene regulation plays an important role in the biochemical reprogramming required for hibernation (Srere et al., 1992; Barnes and Boyer, 1999; Storey, 2000). Whereas a molecular biology approach has been whole-heartedly adopted by the hibernation research community, results have been slow to appear and the

responses of relatively few tissues have been investigated. Some of these difficulties may stem from technical problems both in the choice of the appropriate control and experimental conditions for comparison and from the difficulties associated with certain techniques (e.g. the often high rates of false positives associated with differential display PCR) (Debouck, 1995). Problems of the former sort can arise from a commonly held misconception that hibernation is a continuous, uninterrupted cold slumber from fall to spring. In fact, it is not. This deep sleep or torpor of hibernation is punctuated by infrequent, short arousals where the animal rapidly rewarms to $\sim 37^{\circ}\text{C}$, maintains this temperature for a few hours and then sinks into another bout of torpor (Wang and Lee, 1996; Barnes and Boyer, 1999). These inter-bout arousals complicate the picture when trying to determine the appropriate times for sampling animals in order to pinpoint the optimal expression of genes that support hibernation (e.g. is expression optimal during hibernation or during the entry into or arousal from hibernation).

Hypothesis

The thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*), is one of the most thoroughly investigated hibernating mammals. Its abundance, ease of capture and relatively small size also makes it easier (than other hibernating species) to study under controlled laboratory conditions (Frerichs et al., 1998). This species of ground squirrel has been used frequently as a “model hibernator” when describing the physiological and molecular changes associated with this state. The kidney, a highly active and ischemia sensitive organ is practically shut down during hibernation and has received surprisingly little attention from the hibernation research community (Zancanaro et al., 1999). I

hypothesise that the differential expression of genes and their protein products is associated with, and plays an important role in hibernation. To test this hypothesis I will use cDNA library and cDNA array screening techniques to find genes which are preferentially expressed in hibernating kidney.

Summary

As mentioned previously, there has been little analysis of gene expression in BAT from hibernating ground squirrels or any other true hibernating species. Instead, the response of BAT to cold exposure has been largely studied in laboratory mice and the results have been extrapolated to hibernators. BAT is physiologically unique in that it remains active (as a thermogenic organ) throughout the hibernation cycle. Because of this important function, I hypothesized that BAT will show differentially expressed genes and their protein products during hibernation.

In Chapter 2, data are presented from the differential screening of a cDNA library from kidney of hibernating squirrels. The up-regulation of a mitochondrial encoded subunit of cytochrome c oxidase is described in kidney and several other tissues at the mRNA and protein level. This study is currently under review as a submitted manuscript. Supplementary cytochrome c oxidase biochemical data, not present in the original manuscript, is also described.

In Chapter 3, data are presented from cDNA nylon array screening of hibernator BAT. This study represents the first successful use of DNA array screening technology for detecting hibernation-responsive genes, and promises to accelerate the rate at which hibernation specific gene expression patterns are described. This study has been accepted

for publication and is currently in press. Additional figures and data not included in the published manuscript are also presented.

In Chapter 4 I describe how genes which were identified as up-regulated (using cDNA nylon arrays) are translated into protein products in both kidney and brown adipose tissue of hibernating animals using polysome profile, Western blot and *in vitro* translation analysis. In this chapter gene expression is compared and contrasted in the kidney and brown adipose from euthermic and hibernating animals. This study is currently under review as a submitted manuscript. Additional data, which were not included in the original manuscript are also presented.

Chapter 5 describes the physiological role of additional hibernation-responsive clones that were obtained from the differential screening of the cDNA library and cDNA arrays for kidney, BAT and other tissues. Data in this chapter identify hibernation-responsive clones in a wide variety of ground squirrel tissues.

Chapter 6 is a summary and general discussion of the data and presents a model for the integrated regulation of gene expression responses to hibernation.

CHAPTER 2

THE DIFFERENTIAL EXPRESSION OF A MITOCHONDRIAL ENCODED SUBUNIT OF CYTOCHROME C OXIDASE IN THE KIDNEY OF A HIBERNATING MAMMAL

Introduction

Many small mammals living in seasonally cold climates are faced with difficult choices. A huge metabolic energy consumption is needed to defend body temperature against the winter cold while at the same time the animals are challenged with the scarcity or absence of food to provide this energy (Boyer and Barnes, 1999; Storey, 2000). Hibernation provides the solution to this dilemma, allowing animals to profoundly depress their metabolic rate (often to <5% of euthermic resting rate) and drop body temperature (T_b) to near ambient in order to greatly extend the time that their body fuel reserves can support survival (Frerichs et al., 1998; Hochachka, 1986; Storey, 2000). While the organs of most mammals sustain significant metabolic injuries during ischemia and at low body temperatures, hibernators routinely suppress their T_b , heart rate and breathing for several weeks at a time (Frerichs et al., 1998; Storey, 2000). Researchers are therefore interested in mammalian hibernation as a natural model for tolerance to ischemia in light of the poor performance of therapies based upon (non-hibernating) animal ischemia models (Frerichs et al., 1998). Surviving this seemingly lethal combination of low temperature and ischemia is also attractive to those who research the long-term storage of organs at low temperatures for transplantation (Green, 2000; Zancanaro et al., 1999).

The kidney, normally a highly active organ, is practically shut down during hibernation and has received surprisingly little attention from the hibernation research community (Zancanaro et al., 1999). A one-hundred fold decrease in heart rate reduces the mean arterial pressure by 60% in hibernating ground squirrels (Anderson et al., 1990; Harlow and Braun, 1995). This dramatically reduces the rate of glomerular

microfiltration by kidney and therefore the rate of urine production. Because of the greatly reduced or “trickle” blood flow during hibernation it is likely that the kidney and other organs become profoundly ischemic (Frerichs et al., 1998). Infrequent arousals during hibernation cause a rapid warming and re-perfusion of the kidney, and the immediate restoration of blood filtration and urine production (Zancanaro et al., 1999). These physiological extremes endured by the kidney during hibernation are associated with significant adjustments to metabolic rate and glomular ultrastructure (Harlo and Braun, 1995). It is likely that this adaptation is in part, the result of differential gene expression and our contention is that the hibernating kidney is an excellent model for natural resistance to ischemia and hypothermia.

In the present study I investigated the role of differential gene expression in kidney in supporting hibernation in the thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*. The differential screening of a cDNA library constructed from the kidney of hibernating squirrels revealed a gene that is up-regulated during hibernation. The gene was identified as the mitochondrial-encoded cytochrome c oxidase subunit 1 (*CoxI*). Northern and Western blot analysis of kidney and other tissues revealed significant and tissue-specific changes in mRNA and protein levels of mitochondrial-encoded and nuclear-encoded subunits of the electron transport chain during hibernation. It was hypothesized that the increased expression of *CoxI* during hibernation represents a proactive response to protect the kidney from ischemia-reperfusion injury.

Materials and Methods

Animals

Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) weighing 120 to 180 g were captured in Illinois during August by a US Department of Agriculture-licensed trapper (TLC Research, Batlett, IL) and shipped by air to the animal housing facility of the National Institutes of Health campus (Bethesda, MD). Hibernation experiments were conducted by the laboratories of Dr. J.M. Hallenbeck (National Institute of Neurological Disorders and Stroke) and Dr. R.M. McCarron (Naval Medical Research Centre) and I am grateful to them for the supply of hibernator tissue samples. Animals were maintained and cared for essentially as described by Frerichs et al. (1998), as follows. Initially, animals were housed in a holding room in individual shoebox cages maintained at 22-24°C, with 60% relative humidity and 12/12h light/dark cycle; free access was given to food and water. Individuals were weighed weekly to determine when they entered and finished the pre-hibernation phase of hyperphagia that maximised body lipid reserves prior to hibernation. When animals had exhibited a rapid increase in body weight (220-240 g), they were placed in a dark chamber at 5-6 °C and 60% humidity to facilitate hibernation. Some animals were not placed in the hibernaculum at this time but were sacrificed as controls with tissues rapidly excised and flash frozen in liquid nitrogen. After placement in the hibernaculum, most animals entered a state of deep torpor within 3 to 8 days. Animals that failed to enter hibernation within the 8 days were removed and immediately sacrificed, and are referred to as being cold-adapted. Male animals were subjected to experiments as hibernators after 4 days of continuous torpor at 5-6 °C. Hibernating animals were sacrificed, organs harvested and flash frozen. Tissue

samples were transported to Ottawa on dry ice where the remaining experiments were performed.

RNA Preparation and cDNA Library Construction

All materials and solutions used for RNA isolation were treated with 0.1% v/v diethylpyrocarbonate (DEPC) and subsequently autoclaved. Total RNA was isolated from tissues of hibernating and euthermic ground squirrels using Trizol solution (Gibco-BRL), following the manufacturer's protocol. Poly(A)⁺ RNA was isolated from total RNA using an oligo(dT)-cellulose column (Quiagen, Valencia, CA). The concentration of the poly(A)⁺ RNA was determined spectrophotometrically at 260 nm. Poly(A)⁺ RNA from the kidneys of hibernating animals was used to construct a cDNA library using cDNA library and Uni-ZAP unidirectional cloning kits from Stratagene (La Jolla, CA) following manufacturer's instructions.

Differential Screening of the cDNA Library

³²P-Labeled single-stranded cDNA probes were synthesised from poly(A)⁺ RNA isolated from kidney of hibernating and euthermic control animals. Into an autoclaved, DEPC-treated, 1.5 ml microfuge tube, 2 µl poly(A)⁺ RNA template (about 1 µg) and 6 µl DEPC ddH₂O were added. The mixture was heated at 65°C for 5 min and then 5 µL of 5x 1st strand buffer (Gibco-BRL), 1.5 µL dNTPs without dCTP (5 mM for each nucleotide), 1 µL oligo dT primer (200 ng/µL, NEB, Beverly, MA), 1 µL RNasin (5 U/µL, Promega, Madison, WI), and 2.5 µL dithiothreitol (0.1 M, Gibco-BRL) were added. The reaction was mixed well and the primers were allowed to anneal to the poly(A)⁺ RNA at room

temperature for 10 min. Following this, 1 μ L M-MLV reverse transcriptase (200 U/ μ L, Gibco-BRL), and 5 μ L 32 P dCTP (3000 Ci/mmol, Amersham) were added to the reaction, and the mixture was incubated at 37°C for 1 h. The RNA was degraded by adding 1 μ L EDTA (0.5 M), 1 μ L sodium dodecyl sulfate (10% w/v), and 3 μ L NaOH (3 M) to the reaction and the mixture was incubated at 68°C for 30 min. Next, the probe was cooled to room temperature followed by the addition of 10 μ L Tris-HCl (1 M, pH 7.4) and 3 μ L HCl (2 N). Finally, the probe was passed through a Sephadex G-50 column equilibrated in TE buffer, pH 8 (10 mM Tris-HCl, 1 mM EDTA, made with DEPC water) and then brought to a final volume of 500 μ l with TE buffer; a 2 μ l aliquot was removed for scintillation counting. Approximately one million cpm of probe per ml of hybridization solution was used for hybridization of the plaque lifts.

For primary screening approximately 35,000 plaques/plate were grown on 10 agar plates. Two lifts were made from each plate using nylon membranes (Amersham). The membranes were UV-crosslinked, and allowed to air dry. The lifts were then hybridized with 32 P-labeled single-stranded cDNA probes made from kidney of either hibernating or euthermic animals in a hybridization incubator (LAB-Line Instruments) using Denhardt's hybridization solution with 50% formamide (Fahlman and Storey, 2000). Plaques showing a stronger signal with the probe from hibernator kidney compared with euthermic kidney were retrieved and subjected to two more rounds of screening to confirm the stronger signal and purify the clones. After tertiary screening purified clones in Bluescript plasmid vectors were rescued by *in vivo* excision using Exassist as the helper phage.

Northern Hybridization Analysis

Northern hybridization was used to confirm the up-regulation of the putative clones in the kidney of hibernating ground squirrels and to determine transcript abundance in other organs (heart, brown adipose, skeletal muscle). Total RNA was isolated from tissue samples using Trizol (Gibco-BRL), separated in a formaldehyde agarose gel using 16 µg of total RNA/lane, and then blotted onto a Nytran membrane by capillary action. The quality of total RNA was assessed by the identification of well defined 18S and 28S ribosomal bands. The DNA inserts from the tertiary screened clones were cut from the plasmid vector using *Bam*HI and *Xho*I and separated on a 1% agarose gel run in 40 mM Tris-acetate, 2 mM EDTA, pH 8.5. The inserts were purified and labeled with ³²P using a random primer procedure. Northern blots were hybridized at 44°C, using labeled probes (8 x 10⁶ cpm/10 ml hybridization solution) and a sodium phosphate hybridization solution. After hybridization the blots were washed with increasing stringency and then placed into autoradiography cassettes with X-ray film (X-Omat, AR, Kodak). Prior to hybridization the Northern blots were stained with methylene blue (0.03% w/v) in ddH₂O to visualize ribosomal RNA bands, and destained in ddH₂O overnight.

Transcript levels were quantified by scanning the X-ray autoradiogram using a Scan Jet 3C scanner with DeskScan II V2.2 program (Hewlett Packard) and an Imagequant V3.22 program (Innovative Optical Systems Research). The ribosomal bands of the stained Northern blots were quantified and these values were used to evaluate any differences in loading between lanes. RNA transcript sizes were estimated from a plot of

RNA molecular mass (Gibco-BRL standards) versus migration distance in the formaldehyde gel.

DNA Sequencing and Analysis

Isolated clones were sequenced by Canadian Molecular Research Services (CMRS) Inc. (Ottawa, Ontario) using an automated DNA sequencing procedure and a translation program (EditSeq, DNASTAR, Inc.) was used to define the putative protein sequence. The nucleotide sequence and the six possible open reading frames for each clone were loaded into a Blast program at NCBI for a similarity search in GenBank.

Western blotting

Samples of frozen tissues from euthermic and hibernating ground squirrels were crushed under liquid nitrogen, weighed and then homogenized 1:3 w/v in Buffer A (1 % SDS, 1% 2-mercaptoethanol, 50 mM Tris pH 6.8, 1 mM EDTA, 10 mM sucrose). Soluble protein content was measured with the Bio-Rad (Hercules, CA) Coomassie blue dye binding reagent using a microplate reader. An equal amount of protein from each sample was then boiled in 2 X SDS PAGE loading buffer (contains 0.5M Tris-HCl (pH 6.8), 4.4% (w/v) SDS, 20% (v/v) glycerol, and 2% (v/v) 2-mercaptoethanol in distilled/deionized water), centrifuged for 1 minute at 10,000 rpm and loaded onto two identical 12 % SDS-PAGE mini-gels run at 150 V for 45 minutes. Bio-Rad kaleidoscope pre-stained markers were used to track the progress of the proteins through the gel. Protein samples size fractionated on 12 % SDS PAGE gels were transferred to a PVDF membrane by electroblotting. These membranes were blocked and incubated with a 1/1000 dilution of a

mouse monoclonal antibody against either subunit 1 or 4 of cytochrome c oxidase (Molecular Probes, Eugene, OR). Blots were then incubated with a HRP-conjugated anti-mouse secondary antibody (Molecular Probes) and developed with colorimetric immunoblot-staining reagents (Bio-Rad).

Results

cDNA Library Construction and Screening

A cDNA library was constructed with poly(A) RNA isolated from the kidneys of hibernating ground squirrels. Approximately 1,000,000 plaques were differentially screened using ^{32}P -labeled single stranded cDNA probes synthesized from poly(A) RNA isolated from the kidneys of hibernating versus euthermic animals. Two clones showing a stronger signal in the kidney of hibernators were isolated after a second round of differential screening. Clones 9.1 and 13.2 were subsequently identified as the same sequence by cross hybridization experiments (data not shown). Clone 9.1 was used for further studies as it contained the longest insert. Northern blot analysis using the ^{32}P -labeled cDNA insert from clone 9.1 confirmed that mRNA transcripts of this gene were up-regulated during hibernation in the kidney (Fig. 2.1A) and bound strongly to a ~1700 nucleotide band.

DNA Sequence Analysis of Clone 9.1

Sequencing of clone 9.1 produced a 1604 nucleotide cDNA (Fig. 2.4) (GenBank Accession No. AF330007) that was identified as encoding the gene (*Cox1*) for the mitochondrial-encoded subunit 1 of cytochrome c oxidase (COI). Analysis of the putative

amino acid sequence of ground squirrel COI showed that it encoded 508 of the 513 amino acids of the full sequence with an intact C terminal. A comparison of the deduced amino acid sequence of the COI protein with those from other taxa revealed a 90.5 % amino acid identity with human COI and 98% with COI from the Eurasian red squirrel (Fig. 2.5) (Reyes et al., 2000).

CoxI and CoxIV Expression in the Kidney

A plasmid containing the cDNA of mouse cytochrome c oxidase subunit IV (IMAGE ID# 535611), a nuclear encoded subunit of the protein, was purchased from Research Genetics (Huntsville, AL). This cDNA insert was ³²P-labelled and used to probe a Northern blot of kidney mRNA from euthermic and hibernating squirrels. The *CoxIV* probe bound to an ~670 nucleotide band (Fig. 2.1B) which is identical in size in most species described (Carter and Avadhani, 1991). Western blotting using mouse monoclonal antibodies against either subunit 1 or 4 of cytochrome oxidase also showed strong cross-reaction with proteins of the appropriate molecular weight, 57 kDa for COI and 17 kDa for COIV (right columns of Fig. 2.1A,B). Fig. 2.1C shows that whereas the relative levels of *CoxI* mRNA increased 2.04 fold (+/- 0.13 SEM) in kidney from hibernating animals compared with euthermic controls, the levels of *Cox4* mRNA were relatively unchanged. Western blot analysis showed the same response; levels of immuno-reactive Cox 1 protein (COI) were 2.4 fold (+/- 0.2 SEM) higher in hibernators whereas the amount of immuno-reactive Cox 4 protein (COIV) showed an insignificant (~12 %) decrease during hibernation.

Tissue-Specific Expression of Mitochondrial and Nuclear Encoded Genes

The vertebrate mitochondrial genome is transcribed as a large polycistronic transcript which is subsequently processed into distinct mRNA, tRNA and rRNAs (Clayton, 1991). The mRNAs tend to encode the core subunits of the proteins of the mitochondrial electron transport chain whereas the rRNAs and tRNAs constitute the RNA component of the mitochondrial translational machinery. Mitochondrial transcription proceeds, therefore, in an “all or none” fashion, meaning if one transcript increases, they all increase (Clayton, 1991). Because of the dichotomy of response to hibernation by the mitochondrial-encoded *Cox1* and the nuclear-encoded *Cox4* genes, I decided to examine the transcript levels of an additional mitochondrial-encoded and nuclear-encoded gene in parallel studies. Fig. 2.2A shows Northern blot analysis of *Cox1* and *Cox4* together with a previously identified ground squirrel *ATPase 6/8* (Genbank Accession No. AF362073) bicistronic mRNA from the mitochondrial genome and *ATP α* (I.M.A.G.E. ID# 3385828), a nuclear encoded subunit of the mitochondrial ATP synthase. *ATPase 6/8* encodes two overlapping full length open reading frames encoding ATP synthase subunits 6 and 8 (Fig. 2.6). During hibernation, *Cox1* transcript levels increased 2.0 ($\pm$ 0.13 SEM) fold in kidney, 4.51 ($\pm$ 0.5 SEM) fold in brown adipose tissue (BAT), 4.22 ($\pm$ 0.2 SEM) fold in heart and decreased slightly (by 18%) in skeletal muscle (Fig. 2.2B). Transcript levels of the other mitochondrial-encoded gene, *ATP6/8*, also increased by 1.98 fold ($\pm$ 0.2 SEM) in kidney, 3.97 fold ($\pm$ 0.3 SEM) in BAT and 3.98 fold ($\pm$ 0.4 SEM) in heart of hibernators but did not change in skeletal muscle.

However, transcript levels of the nuclear-encoded *Cox4* and *ATP α* genes did not change significantly during hibernation in any of the four tissues.

Cold-Adapted Animals

To determine whether the hibernation-induced upregulation of *CoxI* transcripts was a specific response to torpor or to the cold ambient temperature of the hibernaculum, transcript levels of *CoxI* were compared in kidneys from euthermic, cold-adapted and hibernating ground squirrels. Surprisingly, (Fig. 2.3A) *CoxI* transcript levels were appreciably lower in kidneys of cold-adapted animals compared with euthermic control kidneys (and much lower than in hibernators). Though the functional significance of this observation is not known, it suggests that the expression of the mitochondrial-encoded genes plays a role in the ability to enter hibernation.

Mitochondrial Polycistronic Precursors

Northern blot analysis of *CoxI* expression revealed the presence of an ~3 kb band in kidney and BAT of hibernating, but not euthermic, ground squirrels (Fig. 2.3B). This likely represents an unprocessed mitochondrial RNA precursor. The presence of these precursors have been previously noted in anoxic turtle heart (Cai and Storey, 1996) and ischemic kidney (Van Itallie, et al., 1993) where they are believed to indicate an increase in the transcription of the mitochondrial genome or the inhibition of the mitochondrial RNA processing machinery.

Discussion

The profound decrease in body temperature and cardiac output experienced by most hibernating mammals renders their organs both hypothermic and ischemic (Frerichs et al., 1998). In addition, periodic “interbout” arousals during the hibernation season effect a rapid re-warming and reperfusion of these same organs (Green, 2000). Surprisingly, the organs of hibernating mammals suffer no apparent damage after months of this cold ischemia-warm reperfusion regime (Zancanaro et al., 1999).

Modern medicine has made great strides in the use of organ transplantation as an intervention strategy and the kidney is one of the most commonly transplanted organs. However, the ischemic damage accrued while organs are outside the body and the hypothermic damage caused by the use of low temperatures to retard metabolic decay are still major contributors to post-operative morbidity and mortality (Zancanaro et al. 1999; Green, 2000). Not surprisingly, then, there has been great interest in the mechanisms by which hibernator organs endure the hypoperfusion and hypothermia that characterize torpor. By identifying the biochemical mechanisms used by hibernating mammals to endure these physiological extremes, applications may be developed that could improve the storage and preservation of organ explants destined for transplantation.

Cytochrome c oxidase (COX) is a 13-subunit complex spanning the inner mitochondrial membrane and responsible for the terminal reduction of dioxygen to water in the electron transport chain (Gnaiger et al., 1998; Napiwotzki and Kadenbach, 1998). Given its essential role in the electron transport chain, and its sensitivity to ischemia/reperfusion (IR) damage (Van Itallie et al., 1993; Montagna et al., 1998; Cochrane et al., 1999), it is not surprising that expression levels of cytochrome c oxidase

subunits should respond to stress. An increase in the expression of the mitochondrial genome is known to occur in response to a wide variety of oxidative and cold stresses (Immaculada et al., 1993; Storey, 2000). For example, research in our laboratory has demonstrated an increase in the transcript levels of *CoxI* and other mitochondrial-encoded genes in both hibernating ground squirrels (Fahlman et al., 2000) and anoxic turtles (Cai and Storey, 1996). This indicates perhaps, a common mitochondrial response to anoxia/ischemia and/or cold stress.

The mitochondria-encoded gene *CoxI* was recovered from the differential screening of a cDNA library made from kidney of hibernating *S. tridecemlineatus*. The gene encodes the large transmembrane, oxygen binding subunit of COX (Poyton, 1999). It is one of three core catalytic subunits (COX I, II and III) that are transmembrane proteins encoded by the mitochondrial genome whereas the remaining 10 subunits are nuclear-encoded and expressed in a tissue specific manner (Lenka et al., 1998). The expression of nuclear and mitochondrial subunits of the mitochondrial respiratory chain is thought to be highly coordinated. To determine if this was also true during hibernation, I supplemented the analysis of *CoxI* with studies of the expression of a nuclear encoded subunit of cytochrome c oxidase, *CoxIV*, which is believed to regulate COX activity depending on the extramitochondrial ATP/ADP ratio (Napiwotzki and Kadenbach, 1998). However, the strongly increased levels of *CoxI* mRNA and COI protein in the hibernating kidney (Fig. 2.1A) contrasted sharply with the lack of effect of hibernation on *CoxIV* transcripts and the amount of immuno-reactive COIV protein (Fig. 2.1B). This suggests a shift in the ratio of these two subunits in the enzyme (normally 1:1) and possibly altered enzyme activity during hibernation.

The vertebrate mitochondrial genome is composed of 13 tightly packed open reading frames (ORFs) and two ribosomal RNAs which are punctuated by tRNA clusters (Clayton, 1991). Transcription of the mitochondrial genome produces several large polycistronic RNAs which are subsequently processed into distinct coding and non-coding molecules. The stability and subsequent translation of the protein coding mRNAs is regulated in a tissue specific manner, yet poorly understood (Clayton, 1991). Two commercially available mouse (*Mus musculus*) mitochondrial cDNAs (Cox 4 and ATP α) were used to probe Northern blots of kidney, brown adipose tissue (BAT), heart and skeletal muscle from euthermic and hibernating ground squirrels (Fig 2.2A). Transcript levels of mitochondria-encoded genes were strongly increased (~4 fold) in hibernating BAT and heart, and rose 2 fold in kidney but were unchanged in skeletal muscle. Previous studies have also demonstrated an increase in expression of selected mitochondrial transcripts in BAT during cold exposure (Nedergaard et al, 1989) and in the hearts of hibernating ground squirrels (Fahlman et al., 2000). By contrast, the expression of the two nuclear-encoded genes was not altered during hibernation. The increased expression of mitochondrial-encoded but not nuclear encoded genes in kidney, BAT and heart suggests a global mitochondrial response associated with the expression of the hibernation phenotype. Interestingly, a significant decrease in the transcript level of *Cox1* was observed in the kidney of cold-adapted ground squirrels (Fig 2.3A). Perhaps an inability to up-regulate the expression of the mitochondrial genome may be one of the factors that prevents these cold-exposed animals from entering hibernation.

The special case of mitochondrial gene expression in BAT is interesting. Brown adipose depots flank the major organs and arteries of hibernating mammals (Wang and

Lee, 1996). The mitochondria in BAT are highly uncoupled; this allows them to generate heat by the futile cycling of protons across the inner mitochondrial membrane (Boyer and Barnes, 1999). The increased expression of the mitochondrial genome in BAT of hibernating animals is believed to enhance the thermogenic capacity of the tissue. Interestingly, the upregulation of the mitochondrial genome in BAT does not uniformly increase the levels of all 13 protein products. Specifically, the levels of ATP synthase in BAT mitochondria are quite low since the primary role of BAT is to generate heat. This, in contrast to the relatively high levels of *ATPase 6/8* and *ATP α* mRNA in BAT (Fig 2.2A), suggests a high degree of post-transcriptional control in the regulation of BAT ATP synthase (Tvrđik et al., 1992).

I propose that kidneys of hibernating ground squirrels may “anticipate” the metabolic lesions known to occur at complex IV during ischemia in other mammals (Montagna et al., 1998) by over-producing those subunits that are particularly sensitive to ischemia/reperfusion (IR) damage. In mammalian models of renal IR damage, there is an increase in the expression of mitochondrial encoded genes, presumably to repair some damage which has been done to the organ (Van Itallie et al., 1993). Despite this effort, complex IV activity drops significantly after the IR insult and the organ suffers significant, sometimes fatal injury. This same study demonstrated an increase in the amount of *CoxII* mRNA, as well as of unprocessed mitochondrial RNA precursors, in response to renal ischemia/reperfusion. The presence of these precursors was also documented in hibernator kidney and BAT, parallel to the elevated levels of *CoxI* transcripts (Fig. 2.3B). The presence of these precursors indicates that there is either a breakdown in the mitochondrial RNA processing machinery or that there is an increase in

the expression of the mitochondrial genome (Van Itallie et al., 1993). Since in ground squirrels there is also an increase in the amount of *CoxI* transcript and immuno-reactive COI protein one must assume that the latter hypothesis is more likely.

Ischemic preconditioning is a technique initially developed to enhance the preservation time of hearts harvested for transplant, but has recently been extended to other organs including the liver, muscle and kidney (Cochrane et al., 1999). A preconditioned organ is made briefly ischemic to ameliorate the damage caused by a subsequent extended period of ischemia. Prior to entering hibernation, ground squirrels go through a series of “test drops” during which they briefly depress their metabolic rate (blood pressure, breathing, heart, body temperature) and then rapidly return to euthermia (Fig. 6.1; discussed also in chapter 6) (Wang and Lee, 1996). These test drops may represent an innate sub-acute ischemic preconditioning program which prepares hibernator organs for an extended bout of torpor using pre-existing stress response pathways (one of which is increased mitochondrial gene expression) to prevent subsequent widespread cellular damage under the prolonged ischemia and hypothermia of the hibernating state.

Figure 2.1. Northern-blot hybridization profiles (left) and Western-blot profiles (right) of *Cox I* (A) and *Cox IV* (B) genes and their protein products in kidneys of euthermic (E) and hibernating (H) ground squirrels. Histograms show ratios of scanned band intensities for hibernating versus euthermic values (C). Data are mean $\pm$ SEM, n=3 separately run Northern and Western blots; each containing RNA or protein prepared from separate animals.

Figure 2.2. Northern blots (A) and analysis (B) of 2 mitochondrial-encoded and 2 nuclear-encoded genes whose protein products include subunits of complex IV (cytochrome c oxidase) and V (ATP Synthase) of the mitochondrial electron transport chain in four tissues from euthermic and hibernating ground squirrels. Northern blots of total RNA (16 ug/lane) were scanned, band intensities were quantified and normalized to their corresponding 28S ribosomal bands. The ratios of normalized band intensities for hibernating versus euthermic blots were then calculated. Data are means $\pm$ SEM, n=3 separately run Northern blots; each containing RNA prepared from separate animals.

Figure 2.3. Northern blots of (A) *CoxI* transcript levels in kidney from euthermic (E), cold-adapted (C) and hibernating (H) 13-lined ground squirrels and Northern blots (B) of an additional ~3.0 kb band which cross reacts with the *Cox I* probe in hibernating kidney and BAT.

A.**E****C****H** **B.****Kidney****BAT****E****H****E****H**

Figure 2.4. The partial sequence of the mitochondrial-encoded cytochrome c oxidase subunit 1, from the 13-lined ground squirrel. Shown is the cDNA sequence on top and the translated amino acid sequence below. Sequence was translated using the vertebrate mitochondrial genetic code.

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1      gaTGATTCTTCTCAACTAATCACAAGATATTGGAACACTTTACCTTCTATTTGGCGCTT
1      W F F S T N H K D I G T L Y L L F G A W
61     GAGCTGGAATAGTAGGAAGTGCCTTAGTCTACTAATCCGTGCTGAGCTAGGTCAACCTG
21     A G M V G T A L S L L I R A E L G Q P G
121    GAGCTCTATTGGGTGACGACCAAAATTTACAATGTTATTGTTACTGCTCATGCATTCGTTA
41     A L L G D D Q I Y N V I V T A H A F V M
181    TAATTTTCTTTATAGTTATACCAATTATAATTGGTGGAAATGGAACTGATTAGTACCCC
61     I F F M V M P I M I G G I G N W L V P L
241    TAATAATTGGAGCTCCTGATATAGCATTCCACGAATAAATAATATAAGCTTCTGACTTC
81     M I G A P D M A F P R M N N M S F W L L
301    TTCCCTCTTCTTTCTCTCTTACTCGCTTCTTCTATGGTTGAGGCAGGAGCAGGAAGT
101    P P S F L L L L A S S M V E A G A G T G
361    GCTGAACCGTTTATCCTCATTAGCTGGAATCTTGCCATGCAGGAGCTTCAGTAGACC
121    W T V Y P P L A G N L A H A G A S V D L
421    TAACCATCTTCTCCCTTCACTTAGCAGGAGTTTCATCCATCCTAGGAGCAATTAACCTTA
141    T I F S L H L A G V S S I L G A I N F I
481    TTACAACAATTATTAACATAAAACCACCTGCCATATCTCAATATCAAACCTCCTTTATTG
161    T T I I N M K P P A M S Q Y Q T P L F V
541    TATGATCCGTGTTAATTACAGCAGTACTTCTACTCTTGTCCCTCCGGTCTTGCGGCAG
181    W S V L I T A V L L L L S L P V L A A G
601    GAACACTATATTACTTACAGATCGTAATCTTAATACCACATTTTGGACCTGCTGGAG
201    I T M L L T D R N L N T T F F D P A G G
661    GTGGAGATCCTATCCTCTATCAACATCTATTTGATTCTTTGGACACCCTGAAGTCTATA
221    G D P I L Y Q H L F W F F G H P E V Y I
721    TTCTTATCCTTCCAGGATTGGTATAATTTCTCATATCGTAACATATTACTCAGGAAAAA
241    L I L P G F G M I S H I V T Y Y S G K K
781    AAGAACCATTGCGTTATATAGGAATAGTATGAGCTATAATATCTATTGGTTTCCTTGGAT
261    E P F G Y M G M V W A M M S I G F L G F
841    TTATTGTATGAGCCATCACATATTTACCGTTGGAATAGATGTAGATACTCGAGCTTATT
281    I V W A H H M F T V G M D V D T R A Y F
901    TTACATCCGCAACTATAATTATTGCTATTCCTACAGGAGTAAAGTTTTAGCTGATTAG
301    T S A T M I I A I P T G V K V F S W L A
961    CGACCCTGCACGAGGAATATTAAATGATCACCTGCAATACTATGAGCACTCGGCTTTA
321    T L H G G N I K W S P A M L W A L G F I
1021   TTTTCTTATTCTAGTAGGAGGCTAACAGGTATTGTCTTAGCCAACCTCCTCATTAGATA
341    F L F T V G G L T G I V L A N S S L D I
1081   TTGTCCTACAGATACATATTATGTAGTAGCCACTTTCATATGTATTATCAATGGGAG
361    V L H D T Y Y V V A H F H Y V L S M G A
1141   CTGATTTTGCATTATGGGAGGATTCGTTCACTGATTCCCTCTTTTTCTGGTTACACAC
381    V F A I M G G F V H W F P L F S G Y T L
1201   TAAATGATATATGAGCTAAAATTCATTTTACTGTAATATTTGTTGGAGTAAATTTAACTT
401    N D M W A K I H F T V M F V G V N L T F
1261   TCTTTCCTCAACATTTCTAGGGTTATCAGGTATACCACGTCGATATTCTGATTACCCAG
421    F P Q H F L G L S G M P R R Y S D Y P D
1321   ATGCTTACACAGCATGAAATACTGTATCCTCAATAGGTTCCCTTTATTTCTCTCACAGCTG
441    A Y T A W N T V S S M G S F I S L T A V
1381   TTATAATCATAATTTTATAATTTGAGAAGCATTTCATCAAAACGAGAAGTCCCTCACCG
461    M I M I F M I W E A F A S K R E V L T V
1441   TAGAACTAACGCCAACTAACCTAGAATGACTGCATGGGTGCCCTCCACCCTATCACACAT
481    E L T P T N L E W L H G C P P P Y H T F
1501   TTGAAGAACCTACTTACATTAAGGCTTAAatcaagaaggaagaatcgaacttcctaag
501    E E P T Y I K A *
1561   actagtttcaagccagcctcataaccattatgactttcttcat

```

Figure 2.5. Multiple alignment of cytochrome c oxidase subunit 1 from ground squirrel, human rat and mouse. Sequences in blocks are identical.

13L Squirrel....G	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	46
ER Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	50
Rat	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	50
Mouse	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	50
Human	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	50
13L Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	96
ER Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	100
Rat	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	100
Mouse	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	100
Human	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	100
13L Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	146
ER Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	150
Rat	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	150
Mouse	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	150
Human	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	150
13L Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	196
ER Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	200
Rat	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	200
Mouse	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	200
Human	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	200
13L Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	246
ER Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	250
Rat	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	250
Mouse	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	250
Human	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	250
13L Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	296
ER Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	300
Rat	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	300
Mouse	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	300
Human	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	300
13L Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	346
ER Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	350
Rat	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	350
Mouse	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	350
Human	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	350
13L Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	396
ER Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	400
Rat	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	400
Mouse	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	400
Human	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	400
13L Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	446
ER Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	450
Rat	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	450
Mouse	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	450
Human	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	450
13L Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	496
ER Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	500
Rat	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	500
Mouse	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	500
Human	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	500
13L Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	509
ER Squirrel	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	513
Rat	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	514
Mouse	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	514
Human	MFVNR	MFVNR	MFVNR	MFVNR	MFVNR	513

Figure 2.6. The translated cDNA sequences from the bicistronic ATPase 6/8 transcript. Shown are the cDNA sequences on top and the translated amino acid sequence below. Protein sequences were translated using the vertebrate mitochondrial genetic code.

ATPase 6

```

181      CTAAATATACTAACTAATTGAACATAAACTCCTTGAGAAGAAAAATGAACGAAAATC
1                                     M N E N

241      TATTTGCCTCTTTCATTACCCCTACACTTATAGGTCTCCCTATTGTCCTTCTTATTATTA
5      L F A S F I T P T L M G L P I V L L I I

301      TATTTCCAATTTACTCTTTCCTTCACCTACTCGACTAATAAACAACCGTTTAGTATCAT
25     M F P N L L F P S P T R L M N N R L V S

361      TCCAACAATGACTAATTCAACTTGTACTAAAACAAATAATGGCAATGCACAACCCTAAAG
45     F Q Q W L I Q L V L K Q M M A M H N P K

421      GACGTACCTGATCCTTAATACTAATTTCAATTAATTATATTTATTGGCTCAACTAACCTTC
65     G R T W S L M L I S L I M F I G S T N L

481      TAGGCCTTCTACCTCATTCTTTCACACCTACAACCCAATTATCAATAAACTTAGGAATGG
85     L G L L P H S F T P T T Q L S M N L G M

541      CCATCCCTCTATGAGCAGGAGCCGTAATCACCGGATTCCGTCATAAGACTAAAGCATCAT
105    A I P L W A G A V I T G F R H K T K A S

601      TAGCCCACTTTCTCCCACAAGGAACCCCAATTCCTCTAATCCCTATACTTATTATTATTG
125    L A H F L P Q G T P I P L I P M L I I I

661      AGACAATTAGCCTTTTTATTCAACCTATAGCATTAGCTGTACGATTAACAGCTAATATTA
145    E T I S L F I Q P M A L A V R L T A N I

721      CAGCTGGTCATCTTCTTATACATTTAATTGGAGGAGCAACTCTTGTATTAACATCTATTA
165    T A G H L L M H L I G G A T L V L T S I

781      GCCCTCCACAGCTATTCTTACTTTTATTATTCTCGTCCTATTAACAATACTCGAATTTG
185    S P P T A I L T F I I L V L L T M L E F

841      CAGTTGCATTAATTCAAGCCTATGTTTTACCCTCTTAGTAAGTCTTTATTTACACGATA
205    A V A L I Q A Y V F T L L V S L Y L H D

901      ATACTTAAAAAAAAAAAA
225     N T

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ATPase 8

```

61      GGGCTGCAGGAATTCGGCACGAGATCCACATGATTTATCACAATTCTATCAATGATTCTA
1                                     M I L

121     GCCCTTTTCTTTATATTTCAACTTAAAATCTCAAATCACTCTTATCCGTCTAATCCCTCT
4      A L F F M F Q L K I S N H S Y P S N P S

181     CTAAATATACTAACTAATTGAACATAAACTCCTTGAGAAGAAAAATGAACGAAAATC
24     L K Y T K L I E H K T P W E E K W T K I

241     TATTTGCCTCTTTCATTACCCCTACACTTATAGGTCTCCCTATTGTCCTTCTTATTATTA
44     Y L P L S L P L H L

```

APPENDIX 2.1

Hibernation-induced changes in the activity of the enzyme are also important and, therefore, I assayed cytochrome c oxidase activity in several hibernator tissues to determine if enzymatic activity is affected by hibernation. The cytochrome c oxidase assay was kindly provided and demonstrated by Prof. Bruce C. Hill (Queens University, Kingston, Ontario).

Methods

Reagents (Refrigerated):

100 mM Na_2HPO_4 pH 7.0, 0.2 % w:v deoxycholate (DOC)

25 mM Na_2HPO_4 pH 7.0, 0.2 % w:v DOC

40 mg/ml horse cytochrome C Type III (frozen in ddH₂O)

1M ascorbic acid (pH 6-7)

Sephadex G25 or G-50 in 25 mM Na_2HPO_4 pH 7.0

Tissue Homogenization and Clarification

The multi-subunit nature of COX renders it sensitive to shear stress and other forms of harsh mechanical disruption (ie. sonication); hence, a low-speed homogenizer is used without sonication. Tissue is homogenized in the presence of deoxycholate detergent (0.2 % DOC) to produce submitochondrial particles (SMP) that contain the active enzyme.

A sample of 100-200 mg frozen tissue was washed in 100 mM Na_2HPO_4 pH 7.0, 0.2 % DOC (1ml per 100 mg tissue) to remove blood and/or myoglobin from the sample. Tissue was then resuspended in 100 mM Na_2HPO_4 pH 7.0, 0.2 % DOC (1 ml per 100 mg tissue), 1 crystal of PMSF was added, and the tissue was disrupted using a pre-chilled homogenizer with a firm constant pressure until the homogenate was a pink opaque colour. Crude homogenate was poured into 1 ml microfuge tubes, incubated on ice for ½ hour and then centrifuged at 10,000 x g for 10 minutes. The supernatant was carefully decanted into a new 1 ml microfuge tube and stored at 0°C. Protein concentration of the clarified supernatant was determined by spectroscopic (A_{280}) or colourimetric (BioRad) assay.

Production of Reduced Horse Cytochrome C (RHCC)

A 300 μl aliquot of a 40 mg/ml frozen stock solution of horse heart type III cytochrome c was placed in a sterile microfuge tube and 10 μl of 1.0 M sodium ascorbate (pH 6-7) was added. The solution was mixed by flicking the tube with a finger until the solution turned from a dark maroon to a light red colour. This colour change indicates that cytochrome c is fully reduced (RHCC). The solution was then loaded onto a 3 ml column of Sephadex G-25 or G-50 that was equilibrated in 25 mM Na_2HPO_4 pH 7.0. After the red solution had penetrated the column, an additional 300 μl of 25 mM Na_2HPO_4 buffer was added and then the column was centrifuged in a benchtop centrifuge for 3 minutes. This separates the RHCC from free ascorbate and the RHCC was then placed on ice for immediate use, stored at 4 °C for up to a week, or frozen for use at a later date. The Sephadex beads were regenerated by passing 10 X column volume of 25

mM Na_2HPO_4 buffer through the column and then the material was removed from the column, placed in buffer and stored at 4 °C.

The Spectroscopic Cytochrome C Oxidase Assay

A Cary 3 UV/VIS spectrophotometer was used to obtain visible absorption spectra for reduced and oxidized cytochrome c. The reduced form of cyt c (RHCC) shows two distinct peaks, the one at 550 nm being used to monitor the rate of oxidation of RHCC by COX activity in the clarified tissue extracts.

Activity was assayed in 1 ml glass cuvettes containing 0.8 ml of 25 mM Na_2HPO_4 pH 7.0, 0.2 % DOC and 25 μl of the RHCC solution. After mixing, an initial A550 nm reading was taken and then the assay was started by addition of 10 μl of the clarified tissue extract (containing similar amounts of protein from each tissue extract), followed by mixing and immediate measurement of A550 at time zero. A550 readings were subsequently taken at 30 second intervals up 5 minutes. The rate of activity was calculated from the initial linear portion of the OD versus time relationship and RHCC

consumption was calculated using the molar extinction coefficient for reduced cytochrome C at 550 nm which is 27/nm.cm (Prof. Bruce C. Hill, personal communication). Activity was expressed per mg protein in the clarified tissue extract. To test for non-specific oxidation of RHCC, blanks were run with the addition of a single crystal of sodium azide, a specific inhibitor of COX. As needed, the final end point of the reaction was also tested by adding a single crystal of $K_3Fe_3(CN)_6$ to completely oxidize all of the RHCC.

Results and Discussion

All tissues assayed showed substantial cytochrome c oxidase activity. Maximum enzyme activity did not change in skeletal muscle or BAT during hibernation but activity was strongly reduced in heart (64 %) and kidney (71%) (Fig. 2.7). Reduced COX activity is opposite to the increased expression of subunit 1 of the enzyme described in Figure 2.1. This may indicate that COX activity is specifically inhibited in kidney and heart during hibernation, probably as part of the global depression of metabolism (Storey, 2000) or that the enzyme may be damaged by the cold and/or ischemia associated with the hypometabolic state (Montagna et al., 1998; Van Itallie et al., 1993). Damage to the multi-subunit cytochrome c oxidase may explain in part, the increased transcript and protein levels of cytochrome c oxidase subunit 1 observed in these tissues (Figs. 2.1, 2.2). It is also possible that this subunit has a function in adjusting the enzyme for cold temperature function and for this reason, hibernating animals contain a higher proportion of COI subunits.

Figure 2.7. The activity of *S. tridecemlineatus* cytochrome c oxidase activity in euthermic (eut) and hibernating (hib) kidney, heart, BAT and skeletal muscle. One unit of enzyme activity is defined as the amount that consumes 1 nmol of reduced horse cytochrome c (RHCC) per min at 21°C; activities are expressed per mg soluble protein. Data are means $\pm$ SEM, n =3.

CHAPTER 3

DIFFERENTIAL EXPRESSION OF ADIPOSE AND HEART TYPE FATTY ACID-BINDING PROTEINS IN HIBERNATING GROUND SQUIRRELS

INTRODUCTION

Hibernation is a state of profoundly depressed physiological and metabolic activity that is used by many small mammals to survive long, cold winters (Boyer and Barnes, 1999; Storey, 2000; Wang and Lee, 1996). By hibernating, animals reduce the “burn rate” of stored fat reserves that would otherwise be used to maintain the steep thermal gradient between their body temperature (T_b) and that of the environment. The hypothalamic set point for T_b is adjusted downwards so that core T_b can fall to near ambient and metabolic rate is often reduced by >95%. By spending prolonged periods in torpor with only brief periodic arousals over the winter, small mammals can gain net energy savings of ~90% compared with the cost of remaining euthermic all winter (Storey, 2000). Although the torpid state is not intuitively the time for radical reorganization of metabolism, it is likely that a variety of other specific changes in gene expression also contribute to hibernation.

In an attempt to gain an overview of the key changes in gene expression that support entry into hibernation, we turned to DNA array technology. Atlas rat cDNA expression arrays (Clontech, Palo Alto, CA) were chosen; these feature 588 cDNAs representing proteins from a range of key biological processes including stress responses and signal transduction (Adryan et al., 1999). We chose to analyze changes in gene expression in brown adipose tissue (BAT) of euthermic versus hibernating thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*. BAT was chosen because of its critical role in hibernators in generating much of the heat that is used to warm the animal during arousal (Boyer and Barnes, 1999; Boyer et al., 1998; Nedergaard et al., 1989). The results revealed the prominent up-regulation of two genes for fatty acid binding proteins

(FABP), both the adipose- and the heart-type isoforms. FABPs play a key role in the intracellular transport of lipids and their strong up-regulation in BAT (and other organs) during hibernation may be crucial to the key role that lipid oxidation plays in supporting both basal metabolism during torpor and the extremely high rates of thermogenesis that return Tb to 37°C during arousal. The present chapter analyzes hibernation-induced and tissue-specific expression patterns of FABP transcripts and assesses key structural differences in the ground squirrel proteins that could aid their function at low temperature.

MATERIALS AND METHODS

Animals

Animals were captured, housed and organs harvested as described in chapter 2 and as described by Frerichs et al. (1998).

RNA Preparation and cDNA Library Construction

All materials and solutions used for RNA isolation were treated with 0.1% v/v diethylpyrocarbonate (DEPC) and autoclaved. Total RNA was isolated from tissues of hibernating and euthermic ground squirrels using Trizol solution (Gibco-BRL), following the manufacturer's protocol. Poly(A)⁺ RNA was then isolated using an oligo(dT)-cellulose column (Quiagen Inc, Valencia, CA) following the manufacturer's protocol; concentrations were determined spectrophotometrically at 260 nm. Equal amounts of poly(A)⁺ RNA from BAT of 3 hibernating individuals was used to construct a cDNA

library using cDNA synthesis and Uni-ZAP unidirectional cloning kits from Stratagene (San Diego CA).

Hybridization and Analysis of cDNA Arrays

Atlas rat cDNA expression arrays were purchased from Clontech. ³²P-Labeled cDNA probes were synthesized with mRNA purified from BAT of euthermic versus hibernating squirrels as per manufacturer's instructions. Arrays were soaked in deionized water immediately after opening, then pre-hybridized in 20 ml of ExpressHybe™ solution (Clontech) at 68°C for 15 minutes with continuous rotation. Aliquots of 1 x 10⁶ cpm of euthermic or hibernating BAT probe were then added to duplicate arrays and hybridization was carried out for 12 h at 68°C. Arrays were washed with a low stringency solution (2 x SSC, 1% SDS) and, if needed, a high stringency solution (0.1 x SSC, 0.5 % SDS) at 68°C until counts were reduced to 1000-5000 cpm as measured with a hand-held counter. Wet arrays were immediately covered in plastic wrap (to prevent drying) and exposed to a phosphorimaging (BioRad Laboratories) screen for 2-10 h to optimize exposure time. Array images generated from phosphorimager analysis were analyzed with the EST program (Adryan et al., 1999) which creates a composite image of two arrays (control and stress) and then transcript levels of each cDNA encoding 588 distinct genes were quantified relative to the intensities of 3 housekeeping genes (GAPDH, ubiquitin & tubulin) followed by comparison of relative levels in control versus stressed states.

RT-PCR Amplification and cDNA Retrieval from the BAT Library

A partial sequence (465 bp) of the H-FABP cDNA from 13-lined ground squirrel was retrieved by RT-PCR for use in another project (results not shown). First strand cDNA was synthesized from 0.5 µg of poly(A)⁺ RNA using Superscript II (Gibco-BRL) and an oligo-dT primer as described previously (Cai and Storey, 1996). PCR amplification was performed using Taq DNA polymerase (Perkin-Elmer, Wellesely, MA) and 1 µM forward and reverse primers. The forward 5'-GGTACCTGGAAGCTATG-3' and reverse 5'-CGCCTCCTTCTCGTAAGT-3' primers were synthesized by BioS&T (Lachine, PQ). Reaction mixtures were subjected to 30 cycles using a thermocycler (M.J. Research, Waltham, MA). A cDNA encoding rat A-FABP (IMAGE ID# 1771436) was purchased from Research Genetics (Huntsville, AL). DNA inserts isolated from a 0.7% TAE agarose gel were used to construct ³²P-labeled cDNA probes by the random primer procedure (Fahlman et al., 2000).

For primary screening approximately 35,000 plaques were grown on an agar plate. Two lifts were made from each plate using nylon membranes (Amersham). The membranes were alkali denatured, neutralized, UV-crosslinked, and allowed to air dry. The lifts were then hybridized with either A-FABP or H-FABP ³²P-labeled cDNA probes in a hybridization incubator using Denhardt's hybridization solution with 50% formamide. Plaques showing a strong hybridization signal were retrieved and subjected to two more rounds of screening to confirm the stronger signal and purify the clones. Purified phage were co-transfected into the XOLR *E. coli* strain with the Exassist helper phage to produce Bluescript plasmid vectors by *in vivo* excision. Purified plasmid DNA

was dot-blotted onto nylon membranes and tested with adipose or heart FABP probe to assess cross-reactivity and the presence of unique sequences.

DNA Sequencing and Protein Analysis

Isolated clones were sequenced by CMRS Inc. (Orleans, Ontario) using an automated DNA sequencing procedure and then the EditSeq translation program (DNASTAR Inc.) was applied. Nucleotide sequences were edited to remove any vector sequence, then loaded into the Blast program at the NCBI (Bethesda MD) for a similarity search in GenBank. Processed sequences were submitted to Genbank and accession numbers are given. Protein structure analysis was done using the “Molecules R Us utility” of the NCBI and the MolMol program (Version 2K.1) based on the known tertiary structures of human muscle FABP (MMDB Id: 3648). Deduced polypeptide sequences of ground squirrel FABPs were aligned with FABPs from other taxa using the algorithm of Feng and Doolittle in the DNAMAN program (Lynnon Biosoft).

Northern Hybridization Analysis

Total RNA was isolated from tissue samples, separated in formaldehyde agarose gels (16 µg of total RNA/lane) and then blotted onto a Nytran membrane by capillary action. The quality of total RNA was assessed by identification of the 18S and 28S ribosomal bands by ethidium bromide (EtBr) staining. DNA inserts were cut from the plasmid vector using *Bam*HI and *Xho*I and separated on a 1% agarose gel run in 40 mM Tris-acetate, 2 mM EDTA, pH 8.5. The inserts were purified and labeled with ³²P-dCTP using a random primer procedure. Northern blots were hybridized at 44°C, using labeled

probes (8×10^6 cpm/10 ml hybridization solution) and a sodium phosphate hybridization solution (Fahlman et al., 2000). Blots were then washed with increasing stringency and placed in autoradiography cassettes with phosphorimaging screens (Bio-Rad, Hercules CA). Transcript levels were quantified directly from the composite image generated by the Personal FX phosphorimager and the Quantity One program (Bio-Rad) and then normalized to the relative intensities of the EtBr stained 28S ribosomal band as described previously. RNA transcript sizes were estimated relative to the migration distance of RNA molecular weight markers (Gibco-BRL) in the formaldehyde gel.

RESULTS

Although the use of a rat array to analyze mRNA transcript levels in another species may have some drawbacks, for genes that have high nucleotide sequence identity the rat arrays can provide key initial evidence of the differential expression of genes in control versus stressed states. Indeed, we found that cross-species reactivity was high and ground squirrel probes cross-reacted with at least 90% of the cDNAs present on the array. Differential screening revealed two genes that were prominently up-regulated in BAT of hibernating ground squirrels, when compared with euthermic controls. These were the heart (H) and adipose (A) types of FABP (also known as FABP3 and FABP4, respectively; Vogel-Hertzel and Bernlohr, 2000) and their up-regulation during hibernation was subsequently confirmed via northern blotting. Overall, transcript levels of most of the 588 genes represented on the array did not change or significantly decrease during hibernation.

To obtain full length cDNAs, the BAT cDNA library was screened using a PCR product with the partial sequence of squirrel H-FABP cDNA and a plasmid containing rat A-FABP cDNA. Several candidate clones for each FABP were obtained and then subjected to a second round of screening. Cross hybridization of the excised clones revealed four that reacted with the H-FABP probe and three with the A-FABP probe. There was no cross-hybridization between the putative H-FABP and A-FABP clones. Restriction digests of the positive clones revealed size ranges of 400-700 bp and the largest were selected for sequencing. Clones pHFAB41 and pAFAB83 both contained full length open reading frames with start and stop codons, polyadenylation signals and polyA tails (Fig. 3.1). The processed cDNA sequences of the two clones were only 47% identical which may explain their lack of cross-hybridization. The open reading frame of pHFAB41 extended from 54 to 452 bp (Fig. 3.1A) and encoded a 133 amino acid protein that was identified from a Genbank search as mammalian heart-type FABP. The deduced amino acid sequence of ground squirrel H-FABP (GenBank accession no. AF327854) shared 91, 90.3 and 89.5 % amino acid sequence identity with rat, human and pig H-FABP, respectively (Fig. 3.2A). The predicted molecular mass of ground squirrel H-FABP was 14,818 Da. The open reading frame of pAFAB83 extended from 47 to 442 bp (Fig. 3.1B) and encoded a 132 amino acid protein with a predicted molecular mass of 14,730 Da. A search in Genbank identified the cDNA as encoding adipose-type FABP. The translated amino acid sequence of ground squirrel A-FABP (GenBank accession no. AF327855) shared 96.9% amino acid identity with human FABP, and 88.6% with both rat and pig proteins (Fig. 3.2B). The deduced polypeptide sequences of ground squirrel A-FABP and H-FABP were 64.6 % identical.

Figure 3.3 shows a northern blot analysis of the tissue distribution and relative transcript levels of H-FABP and A-FABP in euthermic versus hibernating squirrels. Of seven tissues tested, H-FABP mRNA was detected in BAT, heart, skeletal muscle and white adipose tissue (WAT) (Fig. 3.3A). During hibernation, H-FABP transcript levels rose in three of these: by 3.1-fold in BAT, 2.3-fold in skeletal muscle and 1.4-fold in heart (Fig. 3.4). A-FABP mRNA was detected only in BAT and WAT of euthermic animals (Fig. 3.3B) and transcript levels rose only in BAT during hibernation (by 2.3-fold) (Fig. 3.4). However, A-FABP, which was undetectable in euthermic heart, was detected in heart of hibernating animals (Fig. 3.3B, 3.3D) with an estimated 6.3-fold rise during hibernation (Fig. 3.4).

DISCUSSION

The use of cDNA array screening technology proved to be an effective way of searching for genes that were up-regulated by hibernation in ground squirrel BAT. A comparison with probe made from hibernating versus euthermic animals indicated that transcript levels of most genes did not change during hibernation but a small number (<1%) were up-regulated in the torpid state. This suggests that the transition to the hibernating state with its low metabolic rate and low Tb requires the up-regulation of very few genes. Other studies have also turned up only a few hibernation responsive genes. For example, screening of a cDNA library made from *S. lateralis* heart revealed only two genes that were up-regulated during hibernation (myosin light chain 1 and subunit 2 of NADH-ubiquinone oxidoreductase) (Fahlman et al., 2000) whereas two others were found using a PCR-based subtractive screen with *S. tridecemlineatus* heart

(pyruvate dehydrogenase kinase and pancreatic lipase) (Andrews et al., 1998). Limited gene up-regulation during hibernation is perhaps not surprising since, intuitively, the torpid state at low Tb is not the time for major biosynthesis of proteins. However, changes in the expression of a few specific genes is clearly important and each up-regulated gene confirmed to date has a defined role in supporting hibernation. The two FABPs identified in the present study also fit this pattern.

FABPs constitute a multi-gene family of small (~15 kDa) proteins that reversibly bind hydrophobic ligands such as long chain fatty acids, bile acids, prostaglandins and heme (Stewart, 2000; Veerkamp et al., 1999, Vogel-Hertzel and Bernlohr, 2000). *In vivo*, FABPs are believed to have a primary function in binding fatty acids and transporting them through the cytosol to various compartments within the cell. Nine types are known; these were first named for the organs in which they predominate but it is now known that some cell or tissue types express multiple forms (Stewart, 2000). H-FABP (or FABP3) is found in heart, skeletal muscle and mammary but FABP5 (the epidermal form) also occurs in heart and, as the present study shows, transcripts of A-FABP (FABP4) also appear in heart during hibernation (Fig. 3.3). We believe that this is the first report of A-FABP in a mammalian heart and its presence may serve a special need during hibernation. Notably, A-FABP has also been found in the hearts of Antarctic teleost fishes (Vayada et al., 1998) which further supports the idea that this functional isoform is important for low temperature function. BAT and WAT of ground squirrels contained both A-FABP and H-FABP transcripts. Both functional isoforms, as well as FABP5 have been reported in rat BAT (Vogel-Hertzel and Bernlohr, 2000) and expression of H-FABP increased in cold-exposed animals (Daikoku et al., 1997).

The importance of FABP expression to hibernation is supported by the results from Northern blot studies that show up-regulation of transcript levels for both H-FABP and A-FABP in BAT and heart during hibernation as well as H-FABP up-regulation in skeletal muscle. Notably, the two organs that contribute the most to thermogenesis during arousal - BAT which provides nonshivering thermogenesis to start the warm-up and skeletal muscle which initiates shivering thermogenesis once body temperature rises somewhat - both show elevated transcript levels for FABPs during hibernation. Heart is also a critical organ in the arousal process; it is rapidly warmed by the BAT surrounding it and a rapid rise in heart metabolic rate then allows a rapid distribution of warmed blood to all other organs. Although FABP protein levels were not measured in the present study, most genes in fatty acid metabolism are regulated at the transcriptional level (Veerkamp et al., 1999) so that an increase FABP transcripts would translate into an increase in protein product. Furthermore, experiments that both reduced (null mutations) or raised (transfections with FABP cDNA) FABP gene expression have been shown to cause significant changes in cellular fatty acid uptake, implying that changes in FABP protein correlated well with changes in gene expression.

Ground squirrels do not eat during the hibernating season and their winter metabolism is based almost exclusively on lipids, slowly consuming the huge reserves of body fat that are laid down during late summer and early autumn feeding (Boyer and Barnes, 1999). Hence, all components of lipid catabolism including intertissue and intracellular transport, storage and release mechanisms, and oxidative pathways need to be optimized for the challenges of hibernation. FABPs are one of these components. Although there are several suggestions for the *in vivo* function of FABPs (Vogel-Hertzel

and Bernlohr, 2000), it is generally agreed that they play a role in the intracellular trafficking of free fatty acids. Hence, the up-regulation of FABP genes during hibernation is probably effective in increasing intracellular supplies and movement of long chain fatty acids. This would help to support both basal lipid oxidation during torpor and, perhaps more importantly, the high rates of lipid catabolism that fuel thermogenesis during arousal.

Heat production during arousal is supplied first from non-shivering thermogenesis (NST) in BAT. BAT depots surround the major internal organs and arteries of hibernating species, particularly in the thoracic cavity (Wang and Lee, 1996). The mitochondria of BAT contain an uncoupling protein (UCP) which dissipates the proton gradient spanning the inner mitochondrial membrane and releases the energy that is normally captured in the synthesis of ATP as heat instead (Boyer et al., 1998, Nedergaard et al., 1989). Not surprisingly, the expression of UCP in BAT increases with cold exposure in rats (Daikoku et al., 1997) and seasonally in hibernators (Boyer et al., 1998; Nizielski et al., 1995). BAT relies on both its internal lipid stores and plasma triglycerides (derived from WAT) to fuel the fires of NST (Wilson et al., 1992). These two lipid sources may account for the presence of both H- and A- types of FABP in this adipose tissue. One of the unique features of the A type FABP is its ability to form a complex with hormone-sensitive lipase and this suggests that A-FABP functions to carry fatty acids away from intracellular lipid droplets after triglyceride hydrolysis (Veerkamp et al., 1999). In BAT, the primary destination of A-FABPs loaded with fatty acids would probably be the mitochondria where they would be used for oxidation and thermogenesis. This function for A-FABP may also be the reason for its induced presence in ground

squirrel heart during hibernation. Mammalian heart typically relies on fatty acids for about 70% of its fuel and during hibernation this may shift to almost 100% (Veerkamp et al., 1999). Typically, mammalian heart imports its fatty acids and it has been proposed that the role of H-FABP is to transport fatty acids from the sarcolemma to intracellular destinations; the up-regulation of H-FABP during hibernation in heart would increase this transport capacity and the delivery of fatty acids to the mitochondria whose capacity for electron transport is similarly enhanced during hibernation (Fahlman et al., 2000). Hearts of hibernating ground squirrels, however, also maintain substantial intracellular triglyceride lipid droplets (Burlington et al., 1972) which are probably needed because the demand for lipid fuels by heart during arousal could exceed the capacity for delivery of triglycerides by the blood. The induction of A-FABP in heart during hibernation would support the catabolism of intracellular lipid droplets and the transport of their released fatty acids by A-FABP to the mitochondria for oxidation. Our model, then, is one where A-FABP has a primary role in the transport of fatty acids that are released from intracellular lipid droplets via the action of hormone-sensitive lipase whereas H-FABP is involved in the importation of fatty acids into the cell (Fig. 3.5). An increase in the relative amounts of either protein would enhance the intracellular fatty acid (FA) transport capacity at low temperatures and thus the ability to transport or store FAs which are used exclusively to fuel all metabolic processes during hibernation. The up-regulation of both FABP isoforms in BAT and heart of hibernating animals serves the needs of both organs by giving them the means to exploit two sources of fatty acids from two different triglyceride pools - those in the blood and those in intracellular lipid droplets. The model would also fit the data for skeletal muscle which up-regulates the fatty acid importing

isoform, H-FABP, during hibernation to feed its dependence on blood-borne lipid delivery but does not have major intracellular lipid reserves.

Mammalian hibernators allow their core Tb to cool to close to 0°C, a value that is lethal to humans and most other mammals. Temperature change can have profound deleterious effects on proteins affecting, for example, their conformation, their kinetic/regulatory properties, their binding interactions with subcellular structures, and their integrated functioning within metabolic pathways. Indeed, the injurious effects of hypothermia on most mammals can be traced to differential effects of low temperature on the rates of ATP-producing and ATP-utilizing reactions (Storey, 2000). Clearly, however, the proteins of hibernators must remain functional over a wide temperature range and it is likely that selected structural differences in hibernator proteins, arising from discrete amino acid substitutions, may allow them to do so. The amino acid sequence alignments of ground squirrel FABPs with their homologues from human, rat and pig revealed a high degree of sequence similarity between species, about 90% in all cases (Fig. 3.3). Significantly, however, H-FABP showed three distinct amino acid substitutions as compared with the protein from the other species. These occurred at lysine 69, threonine 84, and asparagine 110 of the cDNA which correspond to residues 68, 83 and 109 of the mature protein after cleavage of the N-terminal methionine. Using the X-ray structure of the human heart/muscle FABP as a model (created with the use of RasMol v2.6 with data from the Brookhaven Protein DataBank, Id# 1 HMS), we determined that two of these substitutions occurred in the turns connecting beta-sheets in the protein, Lys 68 between beta-sheets D and E, and Asn 109 between beta-sheets H and I (Fig. 3.6). Both substitutions replace non-polar amino acids that are present in the other species. The

location of both turns covers a “gap” in the structure of all known FABPs which is believed to confer flexibility to the protein (Veerkamp et al., 1999; Vogel-Hertzel and Bernlohr, 2000). The third unique amino acid substitution in ground squirrel H-FABP, Thr 83, places a polar amino acid where hydrophobic amino acids were previously (Fig. 3.3A). Although this residue is not located in a turn region, it lies adjacent to Lys 68 in the tertiary structure. The side chains of all 3 of these amino acids project outwards from the plane of the secondary structure and may serve to stabilize the structure of the protein, allowing it to function effectively at low temperatures. An Asn 110 substitution has also been noted in the H-FABP from an Antarctic teleost fish (Vayada et al., 1998) which further supports the idea that the specific amino acid substitutions in ground squirrel FABP help to sustain protein function at low temperature. The presence of a lysine residue on the exposed surface of the protein may also enhance the interaction of the H-FABP with membranes at low temperatures where there is perhaps little collisional energy to transfer fatty acids from FABP to (mitochondrial) membranes (Bernlohr et al., 1997; Veerkamp et al., 1999). However, whereas H-FABP from hibernators showed these very distinct amino acid substitutions, the same could not be said for ground squirrel A-FABP. Two unique substitutions were noted: the lysine at position 53 was arginine in other species and the isoleucine at position 66 was lysine in the others. It is not currently known if these changes should significantly alter protein functionality, however, it is likely that A-FABP remains functional at low body temperatures.

Figure 3.1. cDNA sequences of hibernation-responsive clones pHFAB41 and pAFAB83 isolated from the ground squirrel brown adipose tissue cDNA library; clones encode H- and A-type FABP, respectively. Initiation codon (ATG), termination codon (TGA) and polyadenylation signal (AATAAA) are underlined in boldface type. Genbank accession numbers are AF327854 for H-FABP and AF327855 for A-FABP.

A. Clone pHFAB41

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gtacaagccttccatctctcgcagcttgcttctcagcctagcccagcttcacccATGGTGGACGCTTCGTGGGCACTGG 80
AAACTAGTGGACAGCAAGAATTTCGATGACTACATGAAGTCACTTGGTGTAGGCTTTGCTACGAGGCAAGTGGCCAGCAT 160
GACCAAGCCCACTACATCATCGAAAAGAATGGGGACACCATCATCTTAAAAACACAGAGTACCTTCAAGAACACGGAGA 240
TCAGTTTTCAGCTGGGAAGGAGTTTGATGAGACTACAGCAGATGACAGGAAGGTCAAGTCCACTGTGACGCTGGATGGA 320
GGAAAACCTGTCCACGTGCAGAAGTGGGACGGGCAAGAGACTACACTTGTGGGGAGCTAAATGATGGGAAACTCATCTT 400
GACACTCACCCATGGCAGTGTAGTGTGCACTGCACTTAAGAAAAAGAGGCATGAtcgaccagcactgtcactgactgtt 480
cctctgtcactggccaccctggactcagcaccacattgcctcattttttccctctggcattttgtataaacctacttt 560
gaggaaattcttctgggatcaggtgacaccagcctggatccccagtttccattgtgtatgtatgtgtttctttttttttt 640
taaaactgtatccaatatgctctgaagtATAAAAgcagagcagaggcc (A) n 688

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B. Clone pAFAB83

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agcctctttctcatcctaggaagavtaactccttgaaacctggcaaaATGGTGTGATGCTTTTGTAGGTACCTGGAAACTTG 80
TCTCCAGTGAATACTTTGATGATTACATGAAAGAAGTGGGAGTGGGCTTTGCCAOCGGAAGTGGCTGGCATGGCCAAA 160
CCCAACATGATCATCAGTGTGAATGGAGATGTGATCACCATTAGATCAGAAAGCACCTTAAAAACACTGAGATTTCTT 240
CAAACTGGGCCAGGAATTTGATGAAGTCACTGCAGATGACAGGAAAGTCAAGAGCATCATAACCTTAGATGGGGGTGTCC 320
TGGTTCAGGTGCAGAAGTGGGATGGCAAGTGCACCACATAAGAGAAAAAGAGAGGATGATAAGCTGGTGGTGGAAATGT 400
GTGATGAAAGGCGTCACTTCCACAGAGTTATGAGAGAGCATAAgccaaagagacacggcgacctggactgaagtttgtat 480
tcaaaactccacgacattctgttgatgaattgtcaaaaagatattatttttccataattaccaagcaactaatttt 560
ctcccgagctggattttgctaaatatggttggtttggttATAAAAactttttagattt (A) n 619

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Figure 3.2. Comparison of the deduced amino acid sequence of 13-lined ground squirrel H-FABP (A) and A-FABP (B) with the proteins from human, rat and pig. Shading shows those amino acids that differ from the ground squirrel sequence; unique amino acid substitutions in the ground squirrel sequences are underlined. Genbank accession numbers are 4096097, 6601400 and 2143386 for human, rat and pig H-FABP and 2144859, 5002228 and 4160392 for human, rat and pig A-FABP.

A. H-FABP

Squirrel MVDAFVGTWKLVD SKNFDDYMKSLGVGFATRQVASMTKPTTIIKNGDTIILKTQSTFKNTEISFQLGKE 70
 Human MVDAFVGTWKLVD SKNFDDYMKSLGVGFATRQVASMTKPTTIIKNGDTIILKTQSTFKNTEISFQLGVE 70
 Rat MVDAFVGTWKLVD SKNFDDYMKSLGVGFATRQVASMTKPTTIIKNGDTIILKTQSTFKNTEISFQLGVE 70
 Pig MVDAFVGTWKLVD SKNFDDYMKSLGVGFATRQVASMTKPTTIIKNGDTIILKTQSTFKNTEISFQLGVE 70

Squirrel FDETTADDRKVKSTITLDGGKLVHVQKWDGQETTLVRELNDGKLIL.TLTHGSVVCTRTRYEKEA 133
 Human FDETTADDRKVKSTITLDGGKLVHVQKWDGQETTLVRELNDGKLIL.TLTHGSVVCTRTRYEKEA 133
 Rat FDETTADDRKVKSTITLDGGKLVHVQKWDGQETTLVRELNDGKLILLTLTHGSVVCTRTRYEKEA 134
 Pig FDETTADDRKVKSTITLDGGKLVHVQKWDGQETTLVRELNDGKLIL.TLTHGSVVCTRTRYEKEA 133

B. A-FABP

Squirrel MCDAFVGTWKLVSSENFDDYMKVGVGFATRQVAGMAKPNMIISVNGDVITIKSESTFKNTEISFQLGOE 70
 Human MCDAFVGTWKLVSSENFDDYMKVGVGFATRQVAGMAKPNMIISVNGDVITIKSESTFKNTEISFQLGOE 70
 Rat MCDAFVGTWKLVSSENFDDYMKVGVGFATRQVAGMAKPNMIISVNGDVITIKSESTFKNTEISFQLGOE 70
 Pig MCDAFVGTWKLVSSENFDDYMKVGVGFATRQVAGMAKPNMIISVNGDVITIKSESTFKNTEISFQLGOE 70

Squirrel FDEVTADDRKVKSTITLDGGVLVHVQKWDGKSTTIKKRREDDKLVECVMKGVTSRTRYERA 132
 Human FDEVTADDRKVKSTITLDGGVLVHVQKWDGKSTTIKKRREDDKLVECVMKGVTSRTRYERA 132
 Rat FDEVTADDRKVKSTITLDGGVLVHVQKWDGKSTTIKKRREDDKLVECVMKGVTSRTRYERA 132
 Pig FDEVTADDRKVKSTITLDGGVLVHVQKWDGKSTTIKKRREDDKLVECVMKGVTSRTRYERA 132

Figure 3.3. Northern blot analysis of FABP transcript levels in tissues of euthermic and hibernating ground squirrels probed with ^{32}P -labeled cDNA for (A) H-FABP or (B) A-FABP. The 28S rRNA band of ethidium bromide stained nitrocellulose blots (C) demonstrates equal loading and high quality RNA in each lane. After hybridizing and washing, blots were exposed to imaging screens for 2 h. Hybridizing bands of about 850 nt (H-FABP) and 750 nt (A-FABP) are shown for brown adipose tissue (B), brain (Br), heart (H), kidney (K), liver (L), skeletal muscle (Sk) and white adipose tissue (W). An additional Northern blot (D) is shown to further demonstrate the presence of A-FABP in hibernating heart.

Figure 3.4. Histograms depicting the relative amounts of H-FABP (A) and A-FABP (B) mRNA transcripts in hibernating and euthermic ground squirrel tissues. Band intensities were quantified from phosphorimager analysis of northern blots, normalized to ethidium bromide-stained 28S rRNA band intensities, and ratios for hibernating versus euthermic samples were calculated. For A-FABP induction in heart, the fold increase during hibernation was calculated relative to the background intensity of controls which showed no A-FABP band; therefore, this represents a minimum fold increase. Data are means $\pm$ SEM, n=3 separate trials, each trial using a separate sample of total RNA isolated from pooled tissue of 3 animals.

A **B**

Figure 3.5. A model of intracellular fatty acid transport by adipose and heart-type fatty acid-binding proteins. While H-FABPs transport FFAs to the mitochondria from the plasma membrane, A-FABPs transport FFAs to and from triacylglycerol droplets. FFAs are either used to power the production of ATP by ATP synthase or heat by the uncoupling of the proton gradient spanning the inner mitochondrial membrane.

Figure 3.6. Three significant amino acid differences between 13-lined ground squirrel and human H-FABP. Charged or polar amino acid substitutions (bulls eyes) are superimposed on their respective secondary structural motifs from two (A or B) different perspectives. This figure was created with the use of RasMol v2.6 with data from the Brookhaven Protein DataBank, Id# 1 HMS.

A **B**

CHAPTER 4

THE TRANSLATION STATUS OF DIFFERENTIALLY EXPRESSED MRNAS IN THE HIBERNATING THIRTEEN-LINED GROUND SQUIRREL (*SPERMOPHILUS TRIDECEMPLINEATUS*)

INTRODUCTION

Hibernation is a state of profoundly depressed metabolic rate (often to <5% of euthermic resting rate) used by some small mammals to survive the cold winter months (Boyer and Barnes, 1999; Storey, 2000). By allowing their body temperature (T_b) to drop to near ambient, hibernators reduce the thermal gradient between themselves and the environment and greatly extend the time that their body fuel reserves can support survival.

Increasingly, an important role has been demonstrated for the differential expression of genes in the hibernation phenotype (Boyer and Barnes, 1999, Storey, 2000). At first glance however, this apparent up-regulation of genes seems incompatible with the suppression of transcription and translation which also occurs during hibernation (Frerichs et al., 1998; Knight et al., 2000; Martin et al., 2000; Zhegunov and Mikulinski, 1987). Little is known about the control of transcription during hibernation but the suppression of protein synthesis during hibernation has been linked with the phosphorylation of the α subunit of elongation initiation factor 2 (eIF2 α), which effectively blocks the delivery of the initiation Met-tRNA to the 43S pre-initiation complex by eIF2 (Frerichs et al., 1998). This paradox might be resolved, in part, by protein synthesis that is taking place during the periods of spontaneous arousal that are experienced by most hibernating mammals (Fig. 6.1) (Wang and Lee, 1996) or by differential translation of selected gene transcripts during torpor. Anecdotal evidence supports the latter option, a role for “interbout” arousals in replenishing essential gene products that are lost or damaged in the tissues and organs of hibernating animals (Barnes and Boyer, 1999, Knight et al., 2000; Martin et al., 2000).

The translation status of several mRNAs was analyzed by polysome profiling, Western blot analysis and *in vitro* protein synthesis, comparing the kidney and brown adipose tissues (BAT). The results suggest that suppression of protein synthesis occurs in the kidney but not in BAT during hibernation and that this regulatory dichotomy relates to the respective physiological roles of these tissues during hibernation. Furthermore, the data indicate that hibernators may uniquely up-regulate and sequester specific mRNAs as messenger ribonuclear protein (mRNP) “stress granules” (Kendersha et al., 1999) during torpor to enhance their stability and ensure their rapid translation upon return to euthermic body temperatures.

MATERIALS AND METHODS

Antibodies

Rabbit anti H-FABP antiserum was a gift of Dr. F. Spener, University of Munster, Germany. Rabbit anti rat OCT2 antiserum was a generously provided by Prof. Ken-ichi Inui, Kyoto University Hospital, Kyoto, Japan. The anti-PABP antibody 10E10 was generously provided by Dr. Gideon Dreyfuss (University of Pennsylvania, School of Medicine, Philadelphia, PA). Anti-TIA-1 antibody was obtained from Santa Cruz Biotechnology Ltd. (Santa Cruz, CA). Antibodies to eIF2 α and phosphorylated eIF2 α were purchased from Cell Signaling Laboratories (New England Biolabs), anti-COX4 monoclonal antibodies were purchased from Molecular Probes (Eugene, OR), and secondary HRP-conjugated antibodies were obtained from Santa Cruz Biotechnology Ltd.

Animals

Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) weighing 120 to 180 g were captured, housed and their organs harvested as described in chapter 2 and by Frerichs et al. (1998).

Hybridization and Analysis of cDNA Arrays

Atlas rat cDNA expression arrays were purchased from Clontech. ³²P-Labeled cDNA probes were synthesized with mRNA purified from BAT and kidney of euthermic versus hibernating squirrels as described in Chapter 3.

Polysome Profiles

Samples (500 mg) of frozen kidney or BAT were pulverized under liquid N₂ by mortar and pestle and then homogenized in 5 ml of polysome lysis buffer [25 mM Tris-HCl, pH 7.6, 25 mM NaCl, 10 mM MgCl₂, 250 mM sucrose, 100 µg/ml cyclohexamide, 5 U/ml RNase inhibitor (Promega)] using 10 strokes of a Douce homogenizer. The homogenate was centrifuged at 16,000 x g at 4 °C for 15 min. The supernatant was removed and mixed with 0.1 volume of detergent (5% w/v sodium deoxycholate, 5% v/v Triton X-100). Aliquots (1 ml) of the supernatant were layered on a 5 ml continuous sucrose gradient, 0.5 to 1.5 M, prepared in 300 mM NaCl, 10 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 100 µg/ml cyclohexamide, and 5 U/ml RNase inhibitor. The gradients were centrifuged at 4°C for 2 hours at 40,000 rpm in a SW41 rotor. Gradients were drained into 10 fractions of 500 µl each and immediately frozen at -70°C.

In Vitro Translational Assay

Kidney and BAT extracts were prepared as described previously (Knight et al., 2000; Martin et al., 2000; Zong et al., 1999). Protein content was adjusted to 10 mg/ml and the reaction started by the addition of 20 μ l of cytoplasmic extract to 80 μ l of assay mix (2 mM DTT, 0.4 mM ATP, 0.4 mM GTP, 0.4 mM creatine phosphate, 50 mg/ml creatine phosphokinase) plus 5 μ Ci/ml L-[3,4,5-³H(N)]-leucine (Amersham-Pharmacia). After incubation at 37°C for 30 minutes, reactions were stopped by the addition of 10% w/v trichloroacetic acid (TCA). Precipitated protein was pelleted by centrifugation, washed with 5% w/v TCA and resuspended in 1M NaOH for liquid scintillation counting. Background was defined as the signal present in the protein pellet when assays were run in the presence of 5 mM cyclohexamide, a protein synthesis inhibitor.

RNA Preparation and Northern Blot Analysis

All materials and solutions used for RNA isolation were treated with 0.1% v/v diethylpyrocarbonate (DEPC) and autoclaved. Total RNA was isolated from tissues or from fractions of the polysome gradients using Trizol solution (Gibco-BRL). RNA was separated on a 1% formaldehyde agarose gel and then blotted onto a Nytran membrane by capillary action. The quality of total RNA was assessed by the identification of well-defined 18S and 28S ribosomal bands after staining with ethidium bromide. Plasmid vectors containing cDNA inserts were digested with restriction endonucleases, size fractionated on a 1% agarose gel, purified and then labeled with ³²P using a random primer procedure. Northern blots were hybridized at 44°C for 12 hours, washed at

increasing stringency and then placed into autoradiography cassettes with X-ray film (X-Omat, AR, Kodak). Transcript levels were quantified by scanning the developed X-ray autoradiogram using a Scan Jet 3C scanner with DeskScan II V2.2 program (Hewlett Packard) and an Imagequant V5.0 program (Innovative Optical Systems Research). RNA transcript sizes were estimated from a plot of the molecular masses of RNA standards (Gibco-BRL) versus migration distance in the formaldehyde gel.

DNA Sequences

A plasmid containing the cDNA of mouse cytochrome c oxidase subunit IV (IMAGE ID# 535611) was purchased from Research Genetics (Huntsville, AL). A plasmid containing the cDNA of mouse organic cation transporter type 2 (IMAGE ID# 572016) was purchased from Research Genetics. A plasmid containing the entire H-FABP cDNA (GenBank accession no. AF327854) was retrieved from the BAT cDNA library (see Chapter 3).

Western blotting

Samples of frozen tissues from euthermic and hibernating ground squirrels were crushed under liquid nitrogen, weighed and then homogenized 1:3 w/v in Buffer P (1 % w/v SDS, 1% 2-mercaptoethanol, 50 mM Tris pH 6.8, 1 mM EDTA, 10 mM sucrose). Soluble protein content was measured using the Bio-Rad prepared reagent (Hercules, CA) with bovine serum albumin as the standard. An equal amount of protein from each sample was then boiled in 2 X SDS-PAGE loading buffer, centrifuged for 1 minute at 10,000 rpm and loaded onto two identical 15 % SDS-PAGE mini-gels run at 150 V for

45 minutes. Bio-Rad kaleidoscope pre-stained markers were used to track the progress of the proteins through the gel. Size fractionated protein samples were transferred to a polyvinylidene fluoride (PVDF) membrane by electroblotting. Membranes were blocked and incubated with a 1/1000 dilution of primary antibody. Blots were then washed and incubated with HRP-conjugated secondary antibodies and developed with colorimetric immunoblot-staining reagents (Bio-Rad).

RESULTS

Polysome profiles

The soluble extracts of kidney and brown adipose tissue (BAT) were subjected to sucrose gradient centrifugation (polysome profiling) to determine the effect of hibernation on the distribution of ribosomes and mRNAs between the translationally silent monosome/ mRNP fractions and actively translating polyribosome fractions (Zong et al., 1999). The position of ribosomes in the sucrose gradients was tracked by Northern blot analysis using a ^{32}P -labelled probe specific for the 18S ribosomal RNA (rRNA), the major RNA constituent of the 40S ribosomal subunit (Mikulits et al., 2000; Zong et al., 1999). Polyribosomes are typically present in heavier fractions (1-5) of the gradients whereas the proportion of monosomes becomes increasingly higher in the lighter fractions (Knight et al., 2000) (Fig. 4.1-4.3).

Figures 4.1 and 4.2 show the distribution of 18S ribosomal RNA as well as of the mRNA transcripts for two genes, cytochrome c oxidase subunit 4 (*Cox4*) and organic cation transporter type 2 (*Oct2*), in extracts from kidneys of euthermic versus hibernating squirrels. *Cox4* mRNA was used as a loading control because it was previously shown to

be unaffected by hibernation and highly expressed in both BAT and kidney (D. Hittel, unpublished observation). *Oct2* mRNA was identified as being upregulated during hibernation in kidney using cDNA array analysis. Fig. 4.1A and C show the distribution of 18S rRNA in euthermic versus hibernator extracts and show a clear shift in the proportion of 18S rRNA, and thus ribosomes, to higher fractions of lighter density during hibernation. This redistribution of ribosomes indicates a decrease in the number of active polyribosomes (present in the heavier fractions 1-5) and an increase in the numbers of translationally silent monosomes in hibernating kidney. This shift is particularly striking in fraction 6 which is essentially devoid of any RNA (message or ribosomal) in euthermic animals but contains significant signal in hibernating animals. This fraction was arbitrarily set as the dividing point between polysome and monosome fractions in the sucrose gradients (Knight et al., 2000). The *Cox4* cDNA probe bound strongly to an easily identifiable ~670 nucleotide band (Fig. 4.1B) which was identical in size to that reported for *Cox4* mRNA in most species that have been analyzed (Carter and Avadhani, 1991). The polysome profile of *Cox4* mRNA (Fig. 4.1B,D) followed roughly the trends described above for the 18S rRNA band. In euthermic kidney extracts there was a distinct distribution of *Cox4* mRNA between the heavy polyribosome and the monosome/mRNP fractions, again with little or no signal present in fraction 6. In hibernator extracts, the relative amount of *Cox4* mRNA transcripts associated with the heaviest polysome fractions (1-3) was distinctly reduced as compared with euthermic kidney whereas the mRNA associated with the monosome fraction was enhanced. The total amount of mRNA (as measured by radioactive signal) was roughly the same in the euthermic versus hibernator kidney profiles, so the change in the mRNA profile during hibernation was not

due to overall changes in *Cox4* mRNA abundance but instead to a translational repression that shifted a higher proportion of *Cox4* mRNA into the monosome fractions.

Comparison of commercial cDNA arrays hybridized with mRNA isolated from kidney of euthermic versus hibernating ground squirrels revealed genes that were putatively up-regulated during hibernation. One of these was the organic cation transporter type 2 (*Oct2*) whose mRNA abundance was 3.2-fold higher in kidney of hibernating, compared with euthermic, animals. Confirmation by Northern blot analysis indicated a mean 1.8-fold ($\pm$ 0.2 SEM) up-regulation of an ~2.2 kb band that hybridized with *Oct2* probe (Fig. 4.2A,C). Notably, studies have shown that cDNA arrays are more sensitive than Northern blots for quantifying changes in mRNA abundance (Zong et al, 1999). Figure 4.2B shows the polysome distribution of *Oct2* mRNA in kidney extracts from euthermic and hibernating animals. In euthermic extracts, a distinct partitioning of *Oct2* mRNA between the heaviest polysomes (fractions 1 and 2) and the unbound mRNA or mRNPs (fraction 9) was seen. During hibernation, however, the profile showed both a strong increase in the total amount of *Oct2* message (by 2.1 fold) (Fig. 4.2B,D) and surprisingly, that the vast majority of this mRNA was localized in fractions 6-9. Thus, although *Oct2* transcript levels clearly increased in hibernating kidney, most of these transcripts were sequestered to the translationally silent monosome and mRNP fractions with no detectable mRNA in the heaviest polysomes (fractions 1-2) despite the evidence that heavy polysomes are present in hibernating kidney (Fig 4.1A).

The gene for H-FABP was previously identified as being up-regulated in BAT of hibernating ground squirrels by cDNA array analysis (a 5.2 fold increase with subsequent confirmation by Northern blotting of a 3.1 fold $\pm$ 0.2 SEM increase (see chapter 3).

Probe bound strongly to an ~850 bp band that was previously identified as H-FABP mRNA after retrieval from a BAT cDNA library and sequencing (Chapter 3). Figure 4.3 shows the distribution of H-FABP mRNA, *Cox4* mRNA and 18S rRNA in the ribosomal gradient for extracts of BAT from euthermic versus hibernating animals. *Cox4* mRNA levels were again used as a control because they were unchanged in BAT of euthermic and hibernating animals. Unlike the situation in kidney, the 18S rRNA band migrated into higher density fractions of the sucrose gradient in the extracts from BAT of hibernating, compared with euthermic animals, indicating an increase in polyribosome density during torpor in this tissue (Fig. 4.3A). The polysome distribution of H-FABP mRNA in BAT extracts from euthermic animals showed a partitioning between the heaviest polysomes (fraction 1) and what is presumed to be the monosome and mRNP pool (fractions 7 & 8) (Fig. 4.3B,E). In BAT from hibernators a strong increase in overall H-FABP transcript abundance was seen as well as a proportional increase in the association of mRNA with polysome fractions 1-5. This indicates an increase in the translation status of H-FABP mRNA during hibernation (Zong et al., 1999). Interestingly, there was also an unexpected increase in the density of the monosome fractions containing H-FABP mRNA during hibernation (Fig. 4.3B,E). The polysome distribution of *Cox4* mRNA in euthermic BAT also showed a bimodal distribution and was present in both the heaviest polysome fraction 1 and in the monosome/mRNP fractions (Fig. 4.3C,F). During hibernation however, most of the *Cox4* mRNA was sequestered into the monosome and mRNP fractions, unlike the trends observed for 18S rRNA and H-FABP mRNA. This suggests that the heavy polyribosomes in hibernator BAT may disproportionately express those mRNAs that are crucial to the hibernation phenotype.

Unlike the situation for H-FABP, there was no significant change in the total amount of *Cox4* transcript in euthermic versus hibernating extracts, as judged from the sum of band intensities in the polysome profiles.

Protein synthesis

The *in vitro* translation rate and protein levels of OCT2, COX4 and H-FABP were investigated in the kidney and BAT of euthermic and hibernating animals. Figure 4.4 shows the results of studies aimed at assessing protein synthesis in kidney of hibernating squirrels. Measurements of global protein synthesis rates *in vitro* showed a strong depression of this metabolic function in kidney from hibernators. The rate of ^3H -leucine incorporation into protein in extracts from hibernating kidney was only $15\% \pm 3\%$ SEM of the corresponding value in extracts from euthermic kidney (Fig. 4.4A). Immunoblot analysis of OCT2 protein levels showed a significant decrease in the amounts of this protein in tissue from hibernating, versus euthermic, animals (Fig. 4.4B) which may be linked to the overall reduced rate of protein synthesis during hibernation. The amount of OCT2 protein dropped by $66\% \pm 22\%$ SEM (n=3 determinations on tissues from different individuals) whereas COX4 protein levels decreased only moderately (about 10-20%).

By contrast, the rate of *in vitro* translation (^3H -leucine incorporation into protein) did not change significantly in BAT from hibernators (Fig. 4.5A) and the amount of immunoreactive COX4 protein (Fig. 4.5B) remained constant despite the change in its translation status during hibernation (Fig. 4.3C,F). However, a significant increase in the amount of H-FABP protein was detected; H-FABP protein rose by 3.1 fold (± 0.4 SEM;

n=3 determinations on tissues from different individuals) in BAT from hibernating squirrels (Fig. 4.5B). This is consistent with the increase in both H-FABP transcript levels and their association with heavy polyribosomes (Fig. 4.3B,E).

eIF2 phosphorylation status

The phosphorylation status of eIF2 α in kidney and brown adipose tissue is linked to translation rate and polysome status during hibernation. Western blots quantifying the levels of the phosphorylated versus total amounts of eIF2 α revealed a striking increase in the amount of phosphorylated eIF2 α in the kidneys of hibernating ground squirrels, compared with euthermic controls whereas the total amount of eIF2 α did not change (Fig. 4.6). Although the exact ratios of phospho- versus dephospho- eIF2 α were not determined (Frerichs et al., 1998), previous studies have shown that even small amounts of phosphorylated eIF2 α significantly inhibit global protein synthesis by blocking the initiation of nascent polypeptides (Gray and Wickens, 1998; Kimball, 1999). A logical consequence of blocking translation initiation is the moderate to severe de-aggregation of polyribosomes (Frerichs et al., 1998; Knight et al., 2000; Martin et al., 2000) such as was observed in hibernating, but not euthermic kidney extracts. No phosphorylated eIF2 α was detected in BAT from either hibernating or euthermic animals although unphosphorylated eIF2 α was found in equal amounts under both conditions (data not shown).

Mammalian stress granules ?

The phosphorylation of eIF2 α has also been linked to the formation of cytoplasmic stress granules (SGs). These are mRNP structures that reversibly sequester

and protect untranslated mRNAs that accumulate during stress-induced translational arrest (Kedersha et al., 1999; Kedersha et al., 2000). Prominent protein constituents of SGs include two proteins: poly(A) binding protein I (PABP-1) and TIA-1, a self-aggregating, RNA-binding protein (Kedersha et al., 1999). The distribution of PABP-1 and TIA-1 in the polysome profiles of kidney extracts from euthermic and hibernating ground squirrels was investigated using Western blot analysis (Fig. 4.7). The initial prediction was that SGs would manifest themselves as medium density (hibernation-specific) particles containing both PABP-1 and TIA-1. PABP-1 was found most abundantly in fractions 8-10 in the profiles of both euthermic and hibernating kidney (Fig. 4.7A). However, in polysome profiles from hibernator kidney fractions 4, 5 and 7 also displayed prominent bands of PABP-I as well as two smaller cross-reacting bands that were not present in the profiles from euthermic kidney. This correlates roughly with the redistribution of *Cox4* and *Oct2* mRNAs in hibernating kidney polysome profiles (Figs. 4.1 & 4.2). In contrast to the results for PABP-1, TIA-1 did not change its distribution in the polysome profile of hibernator kidney (Fig. 4.7C). Both euthermic and hibernating polysome profiles showed near identical distribution of TIA-1 protein in fractions 8-10 (Fig. 4.7B).

DISCUSSION

The differential expression of genes is widely accepted as playing an important, if not essential, role in survival during mammalian hibernation (Srere et al., 1992; Storey, 2000). Among the many gene discovery techniques used, cDNA array screening technology has proven to be a relatively inexpensive and effective way of searching for

genes that are up-regulated during hibernation. However, there is an underlying paradox to gene up-regulation during hibernation. How is the expression of some genes increased at a time when the animal is in a state of deep torpor and when global metabolic rate, including overall rates of transcription and translation, are suppressed to very low values? This paradox may be resolved in part, by the existence of “interbout” arousals (IBAs) during which hibernators briefly re-warm from torpor to euthermic body temperatures before rapidly re-entering torpor (Knight et al., 2000). For ground squirrels, these IBAs occur frequently during the early and late parts of the hibernating season but only every 2-3 weeks during midwinter (Barnes and Boyer, 1999; Knight et al., 2000; Martin et al., 2000). During these energetically costly arousals, the rates of transcription and translation return to and even exceed the rates measured in summer active animals and this has led some researchers to speculate that the purpose of IBAs is to replenish specific gene products essential for survival during hibernation (Knight et al., 2000; Martin et al., 2000). We propose that the differential expression of hibernation-essential gene products may occur just prior to entry into the first major torpor bout during a time when the animals go through several “test-drops” - brief cycles of alternating torpor and euthermia with only moderate reductions in body temperature. These test-drops may pre-condition the animals by coordinating or shocking their bodies into both biochemical and physiological preparedness for an extended plunge into deep torpor. However, the evidence of the present study also indicates that expression of selected genes and synthesis of their gene products occurs during hibernation with differential controls applied so that only selected mRNA transcripts are translated by polyribosomes in the torpid state. To date, studies by our lab and others have identified only a small number of

genes that are up-regulated during hibernation (Barnes and Boyer, 1999; Fahlman et al., 2000; Storey, 2000). This suggests that the transition to the hibernating state, with its low metabolic rate and low Tb, requires relatively few but very specific changes to the expression of selected genes. The use of cDNA arrays that allow the simultaneous screening of hundreds of genes has been particularly instructive in this regard because these arrays have confirmed only a handful of putatively up-regulated genes during hibernation. In kidney, for example, screening revealed a very prominent response by *Oct2* when arrays were hybridized with mRNA from euthermic versus hibernating animals. The subsequent confirmation of *Oct2* mRNA up-regulation by Northern blots made the expression of this gene a good candidate for further study (Fig. 4.2A,C).

Organic cation transporters (OCT) are part of a larger family of transmembrane protein pumps that actively absorb and/or excrete endogenous and exogenous organic ions against their concentration gradients (Burckhardt, 2000). *In situ* hybridization evidence from rats indicates that OCT2 expression is localized in the basolateral membranes of cells lining the proximal tube (outer medulla) of the nephron. Not much is known about the regulation *Oct2* mRNA expression other than it is found predominantly in the kidney and that expression is regulated by sex-hormones such as testosterone whose levels fluctuate and influence hibernation (Urakami et al., 2000). Interestingly, *in vitro* evidence suggests that kidney proximal tubules are particularly sensitive to reactive oxygen species (ROS) generated during cold ischemia (Peters et al., 1998). A one-hundred fold decrease in heart rate reduces arterial pressure by 60% in hibernating ground squirrels and this dramatically reduces the rates of glomerular microfiltration and urine production by the kidneys (Anderson et al., 1990). Because of the greatly reduced

or “trickle” blood flow during hibernation, it is likely that the kidney and other organs become profoundly ischemic (Frerichs et al., 1998). It is not surprising, therefore, that global protein synthesis may be strongly suppressed with a selected elevation of expression of only selected genes, such as *Oct2*, which may be particularly sensitive to the cold and ischemia associated with hibernation.

The data show that eIF2 α is preferentially phosphorylated in the kidneys of hibernating animals (Fig. 4.6) and that this is linked to the de-aggregation of polyribosomes (Fig. 4.1). The phosphorylation of eIF2 α has also been linked to the suppression of protein synthesis in the brain of hibernating ground squirrels (Chen et al., 2001; Frerichs et al., 1998). In addition, mRNA transcripts for both a “control” gene (*Cox4*) that is not up-regulated during hibernation and for *Oct2* that is up-regulated in kidney are re-distributed to the translationally silent monosome and mRNA pools during hibernation (Figs. 4.1 and 4.2). This is in agreement with the general principle that hibernators depress all metabolic processes during torpor. Whereas the polysome profiles for *Cox4* mRNA showed roughly equal amounts of transcript in euthermic and hibernating animals, *Oct2* transcript levels were increased preferentially in hibernating animals (Fig. 4.2B,D). The purpose of increasing *Oct2* transcript levels while at the same time decreasing their translation status is further confused by the significant drop in OCT2 protein levels (Fig. 4.4B) in hibernating kidney. Because of the susceptibility of kidney proximal tubules in general, and membranes/membrane proteins (such as OCT2) specifically, to damage by ROS during hibernation, we propose that *Oct2* mRNA is up-regulated into a translationally silent pool in “anticipation” of the occurrence of damage during long term torpor such that OCT2 protein may be abundantly expressed during

IBAs when a rapid restoration of normal kidney function is required.

Storage of mRNAs as RNPs in untranslated pools during hibernation may be a mechanism by which animals can (a) save valuable mRNA transcripts over a 1-2 week torpor bout without damage or degradation and (b) rapidly re-initiate the synthesis of important protein products beginning either immediately when Tb begins to rise or during the short period of euthermic interbout arousal. Previous studies have linked the phosphorylation of eIF2 α to the assembly of mammalian stress granules (SGs), RNP structures that reversibly bind and protect mRNAs during a non-lethal disruption of cellular homeostasis. The present study shows that PABP-1, which is one of the proteins known to associate with these structures, redistributes in the polysome gradients from hibernating kidney, particularly into fractions 4, 5 and 7, but not 6 (Fig. 4.7A). These fractions also contain the majority of the *Oct2* and *Cox4* mRNAs (Fig. 4.1 and 4.2). In addition, the presence of ultrastructural changes in the nuclei of hibernating animals and indirect evidence for the binding of PABP to hibernator mRNAs (Knight et al., 2000) strengthens our argument for the presence of SGs during torpor. TIA-1, an RNA binding protein, has also been identified by *in situ* hybridization as an important constituent of mammalian SGs, yet our studies (Fig. 4.7B) did not demonstrate TIA-1 redistribution in the polysome gradient of hibernator kidney. Recent studies similarly demonstrate that TIA-1 does not redistribute in polysome gradients and that its association with SGs may not be strong enough to survive cell lysis and sucrose density ultra-centrifugation (Kedersha et al., 2000). To resolve this question further, *in situ* hybridization and fluorescence microscopy evidence must be collected on the cytoplasmic ultrastructure of cells from hibernating animals.

The gene for H-FABP was previously identified as being up-regulated during hibernation in ground squirrel BAT using cDNA arrays and Northern blot analysis (Chapter 3). *In vivo*, FABPs are believed to have a primary function in binding fatty acids and transporting them through the cytosol to various compartments within the cell (Veerkamp et al., 1999). Heat production during arousal from hibernation is supplied first from non-shivering thermogenesis (NST) in BAT. The mitochondria of BAT contain an uncoupling protein (UCP), which dissipates the proton gradient spanning the inner mitochondrial membrane and releases the energy that is normally captured in the synthesis of ATP as heat instead (Nedergaard et al., 1989). Not surprisingly, the expression of UCP in BAT increases with cold exposure in rats (Daikoku et al., 1997) and seasonally in hibernators (Boyer et al., 1998). Since BAT relies on both its internal lipid stores and plasma triglycerides to fuel NST, the up-regulation of H-FABP in BAT during hibernation could increase the transport capacity for delivery of fatty acids to the mitochondria for oxidation. The polysome distribution of H-FABP mRNA (Fig. 4.3B,D) along with the protein synthesis and Western blot data (Fig. 4.5) show that BAT responds very differently to hibernation than does kidney. The increased transcript levels and translation status of H-FABP mRNA resulted in a significant increase in H-FABP protein levels in BAT of hibernators. It is likely that this increase was first stimulated when the animals were placed in the hibernaculum as a response to cold exposure (Frerichs et al., 1998) but the high transcript levels and presence of H-FABP mRNA in polysomes of hibernating animals suggests that synthesis continues during torpor despite the low T_b and low metabolic rate. The lack of any detectable phosphorylation of eIF2 α and the unchanged rate of protein synthesis in BAT during hibernation (Fig. 4.5A) suggests that

this organ is regulated very differently from kidney during hibernation. This is probably due to the important physiological role that BAT plays in re-warming the organs of hibernating animals during arousal (Wang and Lee, 1996). Interestingly, whereas the numbers of heavy polyribosomes increased in hibernating BAT (Fig. 4.3A,D), *Cox4* mRNA re-distributed to the untranslated monosome/mRNP fractions (Fig. 4.3C,F). This surprising discovery may indicate that during hibernation only a specific subset of mRNAs, such as H-FABP, are translated in BAT. The protein products of these mRNAs likely have important physiological roles to play in BAT during hibernation. Future studies will focus on characterizing the entire mRNA complement of polysome and monosome fractions from euthermic and hibernating tissues using cDNA microarrays and/or differential screening techniques. This “ribonomics” approach will allow us to not only identify differentially expressed genes, but also those genes that are preferentially translated during hibernation (Zong et al., 1999).

Figure 4.1. Polysome analysis of soluble extracts of kidneys of euthermic (E) and hibernating (H) ground squirrels. RNA was extracted from 10 fractions of hibernating and euthermic samples (each 1-1.5 M sucrose gradient was 5ml), and blotted onto nylon membranes. Fraction numbers increase with decreasing density. Blots were hybridized with (A) [α - 32 P]dCTP-labeled 18S rRNA to track the position of the 40S ribosomal subunit and (B) a *Cox4* cDNA probe to show the polysome distribution of *Cox4* mRNA. Blots were scanned and mRNA intensity in each lane was expressed as a percentage of the total mRNA signal in the whole gradient. Panels A and B show representative blots; panels C and D show the corresponding mean signals in each fraction for n=3 trials using tissue from different animals. Triangles represent signal in euthermic kidney; squares represent signal in hibernating kidney.

Figure 4.2. Northern blot and polysome analysis of organic cation transporter 2 (*Oct2*) mRNA in the kidneys of euthermic and hibernating ground squirrels. (A) Representative northern blot using total RNA (15 μ g per lane) extracted from the kidneys of euthermic (lanes 1-3) and hibernating (lanes 4-6) squirrels and hybridized with [α - 32 P]dCTP-labeled *Oct2* cDNA probe. (B) Soluble tissue extracts from the kidneys of euthermic (E) and hibernating (H) ground squirrels were separated on 1-1.5 M sucrose gradients. Gradients were drained in 10 fractions and total RNA was extracted from each and blotted onto nylon membranes. Fraction numbers increase with decreasing density. Blots were subsequently hybridized with [α - 32 P]dCTP-labeled *Oct2* cDNA probe as in Fig.4.1. Triangles represent signal in euthermic kidney; squares represent signal in hibernating kidney.

A **C** **B** **D**

Figure 4.3. Polysome analysis of cytoplasmic extracts isolated from brown adipose tissue (BAT) of euthermic (E) and hibernating (H) ground squirrels. Total RNA was extracted from 10 fractions (each 1-1.5 M sucrose gradient was 5ml) 1-1.5 M sucrose gradients and blotted onto nylon membranes. Fraction numbers increase with decreasing density. Blots were hybridized with (A) [α - 32 P]dCTP-labeled 18S rRNA to track the position of the 40S ribosomal subunit, (B) H-FABP cDNA probe and (C) *Cox4* cDNA probe to show the distribution of these species between polysomes and monosomes. Blots were scanned and analyzed as described in Fig. 1. Representative blots are shown on the left and panels D-F show the corresponding mean signals for n=3 separate polysome separations. Triangles represent signal in euthermic BAT; squares represent signal in hibernating BAT.

Figure 4.4. (A) Rates of protein synthesis in vitro, measured by [^3H]-leucine incorporation into acid-precipitable protein, and (B) Western blot analysis of OCT2 and COX4 protein levels in kidneys of euthermic (E) and hibernating (H) ground squirrels. Protein synthesis data are means $\pm$ SEM, n=3 separately run trials each using tissue from different animals. Western blots are representative of n=3 trials.

A **B**

Figure 4.5. (A) Rates of protein synthesis in vitro, and (B) Western blot analysis of H-FABP and COX4 protein levels in brown adipose tissue from euthermic (E) and hibernating (H) ground squirrels. Data were analyzed as in Figure 4.4.

A **B**

Figure 4.6. Western blots of total (upper) and phosphorylated (lower) eIF2 α in kidney homogenates from euthermic (lanes 1-3) versus hibernating (lanes 4-6) ground squirrels.

Figure 4.7. Western blot analysis of polysome profiles from kidney of euthermic and hibernating ground squirrels using (A) anti-PABP-1 antibodies and (B) anti-TIA-1 antibodies. Samples from each fraction were mixed 1:1 v/v in SDS-PAGE sample buffer, boiled, size fractionated in duplicate on a 15 % acrylamide gel and then blotted onto PVDF membranes. Fraction numbers increase with decreasing sucrose density.

Appendix 4.1

In an effort to characterize the mRNA complement or translation status of polyribosomes from tissues of euthermic versus hibernating ground squirrels, cDNA microarrays containing 1700 human genes were used. Overall, only a low number of ground squirrel genes (< 50%), relative the numbers present on each glass slide, actually cross-reacted with the cyanine 3 or cyanine 5 probes, even when reciprocal labelling was conducted. This high number of false negatives may be attributed to the cross-species nature of these experiments and unfamiliarity with the technique.

Methods

The following microarray protocol was communicated by Bryant MacNile and by Qing Liu at the National Research Council (Ottawa, Ontario).

Total RNA was isolated from polysome fractions using Trizol reagent as described in the Chapter 4. The concentration and quality of purified RNA was determined by measuring the A_{260}/A_{280} ratio and by running an aliquot of each sample on a 0.7% formaldehyde gel. Total RNA was subsequently brought to a concentration of at least 1 $\mu\text{g}/\mu\text{l}$.

For 10-20 μg of purified total RNA, the reverse transcriptase (RT) reaction volume was 40 μl . On ice a standard first strand cDNA synthesis reaction was prepared as previously described for cDNA library screening (Chapter 2) except that 2 mM dCTP was added and 1mM of Cyanine 3 or Cyanine 5 (New England Nuclear) was used as probe. The control sample (euthermic) was labelled with a different dye (Cy3 or Cy5) than the experimental (hibernating) sample and then two additional samples were

reciprocally labeled to ensure that each dye was incorporated equally. The reactions were then incubated at 65°C for 5 minutes, then at 42°C for 5 minutes. Then 2 µl RT Superscript II (Life Technologies) and 1 µl Rnasin (optional, Promega) were added and incubated at 42°C for 2-3 hours in a covered water bath (dyes are light-sensitive). After a brief centrifugation, the mixture was placed on ice. Aliquots of 5 µl of 50 mM EDTA (pH 8) and 2 µl of 10N NaOH were added and incubated at 65°C for 20 minutes. The solution was then neutralized by addition of 4 µl of 5M acetic acid and the pH was checked. The two reactions (control and experimental, cy3 and cy5) were combined and 100 µl of isopropanol (50 µl per reaction volume) was added. DNA was precipitated on ice for 30 minutes followed by a 5 min centrifugation at 4°C. The isopropanol was poured off and the pellet was rinsed with ice cold 70% ethanol; after another brief centrifugation, any remaining alcohol was removed by pipetting. The DNA pellet was air dried under the cover of tin foil (although not completely) and then resuspended in 5µl of H₂O or TE. Some colour should be observed from the resuspended cDNA.

Labelled cDNA was used immediately. Human 1.7K slides (containing 1700 separate human cDNAs) were purchased from the microarray centre which is part of the Ontario cancer institute (OCI; <http://www.uhnres.utoronto.ca/services/microarray/>). To 100 µl of DIG Easy Hyb (Boehringer Mannheim) we added 5 µl of yeast tRNA (10 mg/ml) and 5 µl of sonicated salmon sperm single stranded DNA (10mg/ml). These mixtures were incubated at 65°C for 2 minutes and then cooled to room temperature. For each 1.7K human slide, 30 ul of hybe solution was combined with each pair of Cy5 and Cy3 labeled cDNA. These samples were then incubated at 65°C for 2 minutes, cooled to room temperature and then placed on a reliable surface. This hybe mixture was then

added to a 24 x 30 mm coverslip and gently lowered onto the slide. The slide(s) were then carefully placed into the hybridization chamber (we use a microscope slide box containing ~15ml of DIG Easy solution at the bottom). Clean microscope slides are placed at every second or third position in the box. These slides function as a platform onto which the hybridization arrays are placed. Typically, three hybridizations can be placed in a chamber. After the hybs were placed in the box, the lid was carefully placed over the slide box and the box was wrapped with plastic wrap. Slides were incubated on a flat surface in a 37°C incubator for 8-18 hours. After hybridization, arrays were removed from the box and dipped gently, but quickly in 1 X SSC (to let coverslip slide off). The arrays were then placed in a slide holder and staining dish (Diamed, cat# E/S258-4100-000) with fresh 1 X SSC. The arrays (with coverslips removed) were then washed 3 times for 10 minutes each at 50°C in clean slide staining boxes containing pre-warmed 1 X SSC, 0.1% SDS, with gentle occasional agitation, every 3-5 min. At the end of the three washes, the arrays were rinsed in 1 X SSC (plunging 4-6 times) and spin dried in a slide box with 3mm paper on the bottom with plate rotor in Sigma centrifuge at 500 rpm for 5 min. Arrays can be stored in the dark (for hours to days) until scanned. Arrays were scanned at the National Research Council (Ottawa, Ontario) using a Genefocus confocal scanning beam imaging system, the images were subsequently superimposed and quantified using the freeware microarray analysis program Scanalyze.

Overall, the results from this (Fig. 4.8) and other hybridizations (data not shown) indicate that the changes in the mRNA complement of kidney polyribosomes was semi quantitative; this means that there was a reduction in the numbers of active polyribosomes rather than a decrease in a specific subset of genes. However, there was

one gene (prothrombin) that was enriched in hibernating polyribosomes indicating perhaps, the selective translation of a few transcripts during hibernation. Prothrombin, other than its obvious role as a protein critical to the blood coagulation cascade has also been implicated in the formation of calcium oxalate crystals (kidney stones) which form spontaneously in urine (Ryall, 1999).

Figure 4.8. Preliminary analysis of human 1700 gene cDNA microarray hybridized to euthermic (Green) and hibernating (Red) probe from the polyribosome RNA isolated from the kidneys of ground squirrel. Shown is one section of the artificially coloured and superimposed images generated from the differential scanning of Cy3 and Cy5 dyes. Note high background levels and speckling effect.

In addition to kidney and BAT, I also collected polysome data from euthermic and hibernating ground squirrel brain (Fig. 4.9). Frerichs et al. (1998) have previously demonstrated that the phosphorylation of the alpha subunit of eIF2 in the brains of hibernating ground squirrels resulted in the moderate to severe disaggregation of polyribosomes. The distribution of the 18S ribosomal band (Fig. 4.9A) was shifted somewhat to the monosome fraction but the disaggregation was less severe than was previously described in hibernating kidney. However, as was seen in the hibernating kidney, the polysome distribution of Cox 4 mRNA was shifted to the translationally inactive monosome fractions during hibernation (Fig. 4.9A). This is interesting in light of the continued presence of heavy polyribosomes in hibernating animals and suggests perhaps, a mechanism for the exclusion of specific mRNAs from the heavy polyribosome fraction during hibernation.

During the polysome analysis experiments I developed an interesting technique for visualizing the RNA distribution in polysome gradients by adding ethidium bromide to the sucrose mixtures before making the gradient. Figure 4.9B shows that a high concentration (or band) of fluorescing RNA occurs in the hibernating profile of brain (see arrow) when compared with the euthermic profile, in which the RNA is distributed evenly throughout most of the gradient. In both gradients there is a highly fluorescent pellet containing the heaviest polyribosomes; this pellet was notably absent in the polysome gradient of hibernating kidneys (Fig 4.9C). This is agreement with my previous observations of the severe disaggregation of heavy polyribosomes in the cytoplasmic extracts of hibernating kidney.

Figure 4.9. (A) Polysome analysis of cytoplasmic extracts isolated from the brain of euthermic (E) and hibernating (H) 13-lined ground squirrels. Total RNA was extracted from 10 fractions of 1-1.5 M sucrose gradients (gradient was 5ml) and blotted onto nylon membranes. Fraction numbers increase with decreasing density. Blots were hybridized with (A) [α - 32 P]dCTP-labeled 18S rRNA to track the position of the 40S ribosomal subunit, and *Cox4* cDNA probe to show the distribution of these species between polysomes and monosomes. (B) Sucrose gradients, supplemented with ethidium bromide, of euthermic and hibernating brain cytoplasmic extracts showing the density distribution of RNA. (C) Ethidium bromide supplemented sucrose gradient of hibernating kidney extracts.

CHAPTER 5

HIBERNATION-RESPONSIVE GENE EXPRESSION ASSESSED BY cDNA ARRAY AND cDNA LIBRARY ANALYSIS

Entry into hibernation is associated with the up-regulation of a variety of genes that have specific functions to play in the torpid state. My studies with kidney and brown adipose tissue of ground squirrels have presented a detailed analysis of several of these including fatty acid binding proteins, cytochrome c oxidase and organic cation transporter (Chapters 2-4). Other authors have similarly reported selective gene up-regulation during hibernation in heart, skeletal muscle and a variety of other tissues in several species of hibernating ground squirrel (Storey, 2000). The gene screening techniques used in my studies (cDNA library screening, cDNA arrays) allow for a broad screening of potentially hundreds of genes to identify those that may be up-regulated during hibernation. However, both techniques revealed two general principles: (1) the comparison of gene expression in euthermic versus hibernating states showed that the major response to hibernation was either a reduction or no change in the levels of mRNA transcripts for most genes, and (2) only a selected few genes showed putative up-regulation during hibernation.

The present chapter summarizes additional results from differential screening techniques to describe a number of other genes that are putatively up-regulated during hibernation in ground squirrel kidney, brain or BAT. Although selected from differential screening, up-regulation of these genes remains unconfirmed by Northern blot or RT-PCR analysis, either because their transcripts were present at too low a copy number or hybridization/PCR conditions were not optimized. These genes provide a promising resource for future studies. What follows is a brief description of these hibernation-responsive clones, the techniques used to retrieve them and their possible significance for hibernation.

METHODS

A kidney cDNA library was constructed and screened as described in Chapter 2. Animals were obtained and organs harvested also as described in Chapter 2. Atlas 1200 gene cDNA arrays were purchased from Clontech (Palo Alto, CA), screened and analyzed as described in Chapter 3.

RESULTS and DISCUSSION

Differential screening of a kidney cDNA library

Screening of a hibernating kidney cDNA library produced relatively few genes that were (at first glance) hibernation-responsive. In total, 20 clones were pulled from the initial round of screening but this was subsequently reduced to 10 after secondary and tertiary screening. Cross hybridization studies revealed that two of these clones (Cox1 and ATPase 6/8) were present in duplicate, reducing the total number to 6 unique cDNA sequences. Of these, the largest (as determined by agarose gel electrophoresis) were chosen for sequencing and then putatively identified via homology searches in BLAST. Two of these clones, Cox1 and ATPase 6/8 were described in chapter 2. All remaining clones were positively identified from nucleotide similarities to full open reading frames (ORFs) or 3' untranslated regions (UTRs).

Clone 19.1

A homology search in BLAST revealed that clone 19.1 encoded the 3' sequence of a putative human RING finger protein (Fig. 5.1). The 82 % nucleotide sequence identity between the ground squirrel and human sequences suggests a high degree of

probability that they encode similar if not identical proteins in their respective species. The UTR contains no known secondary structural motifs, nor does it contain any of the protein coding sequence; it does however, contain a highly conserved polyadenylation sequence. The relatively high degree of sequence conservation may indicate an important regulatory role for this sequence, perhaps in mRNA stability or intracellular trafficking.

RING finger proteins have recently been shown to play a role in the ubiquitination pathway (Joazeiro and Weissman, 2000). Interestingly, many RING finger proteins possess ubiquitin-conjugating activity similar to the E3 protein (ubiquitin protein lyase) of the ubiquitin conjugating pathway. More significantly, RING finger proteins are believed to target the destruction of specific proteins and protein families (Joazeiro and Weissman, 2000). Selective removal or destruction of specific proteins in liver, skeletal muscle and kidney of hibernating ground squirrels has been reported (Martin et al., 2000). Hibernation-specific up-regulation of one or more RING finger proteins with ubiquitin-conjugating activity may provide the mechanism for targeting selected proteins for degradation during hibernation. Proteins targeted for destruction might include those that are cold/ischemia labile or that are non-essential for the survival during hibernation. The amino acids released from proteolysis of these proteins could contribute substrates for gluconeogenesis during torpor. Further characterization of this RING-finger protein may indicate which protein or suite of proteins are targeted for destruction during hibernation.

Figure 5.1. Alignment of kidney clone 19.1 with human hypothetical RING finger proteins hsrnf20 (Accession No. AF265230) and FLJ11189 (Accession No. AK002051) isolated from brain and testis.

```

19.1 .....A A T A A A 15
hsrnf20 TTGTGAATGTGGATTACAAAATGGTATAATTGGCTACTCTCCCTGGGTGGTTT 3551
FLJ11189 TTGTGAATGTGGATTACAAAATGGTATAATTGGCTACTCTCCCTGGGTGGTTT 1653
Consensus g a cc taatgaa
19.1 84
hsrnf20 3621
FLJ11189 1723
Consensus ttat tc aggat att agtc atttccatttact gca a taactt ttaag tcagctggcttct
19.1 153
hsrnf20 3691
FLJ11189 1793
Consensus tt taa gttatttaagttt atg tc ac ct aagcttttaatttctt ag c agataat
19.1 222
hsrnf20 3759
FLJ11189 1861
Consensus tt aa taatat c ttg ctt taatgaataaa atg aga g ga t actga tg
19.1 292
hsrnf20 3828
FLJ11189 1930
Consensus gt aaga a ctt tgggtg tctgg aa ttgatac gagag ttggtgagctatgaa ttactctc
19.1 362
hsrnf20 3898
FLJ11189 2000
Consensus a ctctt t a aggg t ttgtga cctt ttttcagaa attctgt aat tttctgtaggact
19.1 426
hsrnf20 3936
FLJ11189 2041
Consensus ttgt ctc acaagctt aattaagcaggattcagtt

```

Clone 28.1

A BLAST homology search of clone 28.1 revealed it to be the 3' UTR of lactoyl glutathione lyase or glyoxylase 1 (Fig. 5.2). The high degree of sequence identity between human and squirrel UTRs suggests not only that they likely encode the same proteins but that their respective UTRs possess an important regulatory function. Interestingly, the squirrel glyoxylase cDNA contains an extra sequence which is not present in the human sequence (Fig. 5.2), yet a putative polyadenylation sequence is still conserved near the poly(A) tail.

Glyoxylase 1 plays an important role in the detoxification of methyl glyoxyl and is a member of the metallothionein (GSH) transferase superfamily (Creighton and Hamilton, 2001). The increased expression of this protein during hibernation may help the kidney clear toxic organic species which accumulate during torpor or that are produced during the rapid reperfusion of the kidneys which occurs upon arousal from hibernation (Harlow and Braun, 1995).

Figure 5.2. Alignment of ground squirrel kidney clone 28.1 with the 3' UTR of human glyoxylase 1 (Accession No. XM_004478) cloned from human kidney.

[illegible]

Clone 24.4

Clone 24.4 was identified as the full length human cDNA encoding the protein CGI-97. Little is known about this protein other than it is highly conserved in several species, including *C. elegans* (Lai et al., 2000). This highly conserved protein of 250 amino acids is 98% identical with its human homologue. Structural analysis indicates that it is a highly alpha helical (48%) and soluble protein containing no membrane spanning or signal sequences. This protein has no assigned function, active domains, signal sequences or unique tissue distribution nor is its expression known to be altered by stress or disease states (Lai et al., 2000).

Figure 5.3. Shown are the full cDNA sequence of the kidney clone 24.4 (A) and the alignment of its putative amino acid sequence (B) with human CGI-97 protein (Accession no. AF151855). Start and stop codons as well as the polyadenylation signal in the cDNA sequence (A) are in bold and underlined.

A

GGCGCGCTC	TAGAACGAGT	GGATCCCCGG	GCTGCABGAA	TTCGGCACCA	GCGCCTGCGC	61
AGTGCCTCTC	CGAGGTGCGC	AGTGGGTCTG	CGAAGTGCGC	AGTGGCGGTC	GCGGAGGGCC	121
TAGCCCGTGT	CTGTGCTTAG	GGTTGCTGCG	GCCCTGGGGT	GTTGGAGTAC	GGCHCABATT	181
GCGCGGAGCG	CAGAGCCCTG	ATTCCCGCGC	CGCGATCTGT	GATGGGAGCT	<u>CATCTCTATC</u>	241
TTCACCCCCA	CCAACCAGAT	CCGCGTGACC	AACGTGGCGG	TGTTTCGCAT	GAAAGCGCGCA	301
GGCAAGCGCT	TGGAATACGC	CTGCTACAG	AACAAGGTGG	TGGGTGGCGG	CACGGCGTGG	361
AGGAAGACCT	GGAGCGAGTC	CTGCAGACT	ACTCCGTGTT	CTGTGAACGT	TCCAAAGGCC	421
AGGTCGCTAA	GAAGGAAGAC	CTCATCAGTG	CTTTCCGCAC	CGACGATCAA	ACAGAAATCT	481
GCAAGCAGAT	TTTGACTAAA	GGAGAGGTTG	AGGTATCTGA	TAAAGAAAGG	CACACAACAG	541
TGGAACAGAT	GTTTAGGGAC	ATTGCAACCA	TGTTGGCAGA	CAAGTGTGTC	AACCCGGAAA	601
CAAAGGAGAC	ATACACTGTT	ATCCTTATTG	AGAGAGCCAT	GAAAGGACAT	CACATATTAC	661
TCAAGCCCAA	TAAAGATACA	AAACAGCAGG	CTTTAGAAGT	GATAAGACAG	CTGAAGGAAA	721
AGATGAAGAT	TGAACGTGCT	CACATGAGGC	TCCGGTTTCT	TCTTCCAGTG	AATGAAGGGA	781
AGAAGCTCAA	GGACAGTGCT	AAGCCGTGTA	TCAAGGTTAT	AGAAGCGGAA	GACTATGGCC	841
AGCAGCTGTA	AATTGCTGTG	CTGATTTGAC	CAGGCTGCTT	CCGGGAAATT	GATGAGCTAA	901
TACAAAAGGA	AACAAAGGGC	AAAGGTTCTT	TGGAGTTCTT	CAATTGTAAT	GACGTGGAAG	961
AAGGAGATGA	GAAGTTGAAA	TGACATTCT	CAGTCTCTTC	AGCTATAAAA	CACATAAAAG	1021
AGATGTTTCT	GTTTCTGGTA	GCACGTGTCA	ATTTCTGTGA	CTTGGCCAAA	AAAGTGATCA	1081
ATTTGACATT	TCGTCTCTTG	GTTTCATGTG	TGCATAAAAG	ACTTCTTACA	TGAGGATAAT	1141
GTGGGAATGA	CTTTGTTTTA	ATTTTAAACA	GGGTGTTCAA	ACACAAATGA	AACCTAAAAG	1201
TACCAAGTCC	AGAGGGTCAT	TGTGCTGCTG	TCTCAGGCCT	TTATGGCACT	CCAAAATGAA	1261
AGCTTCCTGA	GACAACTCTT	GTGGCTAAAA	AGTTAAGTTT	TGTTATATTAT	ATAAGTGAGA	1321
TTATTGGGAA	ATATGCTCTG	ATGTTAAAAA	GGAGTATTTA	CTGGAAAAGA	CAACGTTTAA	1381
TACAGCTCTG	AAGGGTGTAT	ATCGTGTCAG	TGAAGCTTGG	ATGATAACGT	CTGTATTATC	1441
TGAAATGGGC	GTGTGGTGCG	TGTAGGACTG	AATGATGGTC	ATTGAATATG	CTGCCAAAAC	1501
TCAGATTGTC	CTTGCTAAAA	TATAAAGTCA	TTCGTTTCTA	ATTCTTAAAG	TTATATAACA	1561
TATATTAAAA	ACAGCTAAAA	TTATATGTGA	<u>TCAATAAACT</u>	ACDACTATTT	TTGTTAAAAA	1621
AAAAAAAAAA	AAA					

B

24.4tra		70
cgiprotein		70
Consensus	msiftptnqrltnvavvrakragkrfeiacynknkvgsrgvekdldvlgthsvfvnyskgqvakked	
24.4tra		140
cgiprotein		140
Consensus	lisafgtddgteickqiltkgvqvvdkerhtqlcqafrdiativadkcvnpethrpytvilieramkdi	
24.4tra		210
cgiprotein		210
Consensus	hysvk nkstkqqaalevikqlkeknkierahrllrfilpvnegkklk kklplikviesedyqqgleivc	
24.4tra		250
cgiprotein		250
Consensus	lidpgcfreideli ketkgkgslevlnlkdvcegdckfe	

Clone 3.3

Clone 3.3 was identified as the partial cDNA encoding the PPAR χ coactivator 2 (PGC-2) (Fig. 5.4A). This gene was only recently identified in brown adipose and a variety of other mouse tissues. Its physiological role is to enhance the transcriptional activity of genes activated by peroxisome-proliferator activated receptors (PPARs), possibly in response to cold and oxygen lack (Vega et al., 2000). Some of the genes activated by PPARs include FABPs, other fatty acid oxidation genes and mitochondrial encoded genes, all of which have been described in this and previous chapters.

Of the 29 amino acids encoded by clone 3.3, 28 were identical with the putative mouse protein (Fig. 5.4B), suggesting a highly conserved physiological function for PGC-2. Within this putative protein sequence there is a SCAN domain and leucine zipper motif which are characteristic of this class of PPAR coactivators (Vega et al., 2000).

Figure 5.4. (A) The sequence of kidney clone 3.3 with underlying translated protein sequence and (B) the carboxy terminal 29 amino acids of the putative amino acid sequence of ground squirrel clone 3.3 aligned with its mouse Pgc-2 homologue (Accession No. BC010724). Termination codon and polyadenylation signals (A) are underlined and in bold.

A

CTGGTGCAGGAGCAGCTACTGGCCATTCTACCTGAGGCAGCGCGGGCCCGGCGGCTCCGA
L V Q E Q L L A I L P E A A R A R R L R
CGCCGCGCGGACGTGCGCATCACCGGCTGAacggcgaggctgagcactttcttagactgg
R R A D V R I T G *
ggccatgatgcgtagggggtcggagcctggcacccccaccccatgttaatgaaaaacgagt
tttgacagctcctgtggccttagtggtgtctatatttggctccttcattgggtttatttc
acttgagtttatttcctacctcctttctgctattagagaaaggaagctggaagcccacga
gaataaagcctttgctgttcctgggc

B

PGC-2	MPAPFSCTPCSPAPFPCCEGAGDAPPSPSCSSPAPELPGAFAPINTAPYALAVLFPGASREGFETF	70
3.3		0
PGC-2	PCPFCFFYCDAGPFPAFFCLRELSQWLRFCLRTKEIVE	140
3.3		27
PGC-2		142
3.3		29

Clontech 588 and 1200 gene nylon cDNA array analysis

Brain

A significant number of genes were identified as hibernation-responsive by nylon cDNA array analysis. In two of the chapters presented in this thesis (Chapters 3 and 4) clones that were detected as hibernation-responsive using cDNA array analysis were confirmed and followed up. These encouraging results facilitated a shift in our laboratory towards cDNA array analysis as a tool for gene discovery in a wide variety of animal stresses.

Both brain and BAT of ground squirrels were assessed for hibernation-responsive genes using Atlas rat 1200 gene cDNA arrays. Representative arrays screened with radio-labelled cDNA extracted from brain of euthermic versus hibernating ground squirrels are shown in Figure 5.5. While several of the putative hibernation-responsive genes from array screening of BAT have been further studied (Chapters 3), many remain to be confirmed and analyzed. Table 5.1 shows ten unconfirmed hibernation-responsive clones identified from the array screening of ground squirrel brain. Several classes of genes/proteins are represented.

Perhaps the most interesting gene highlighted by the array screen for hibernation-responsive genes in brain was the cyclin-dependent kinase 2 (*cdc2*). This enzyme, in conjunction with a variety of cyclins, has been implicated in the regulation of transcription and translation by the phosphorylation of RNA Polymerase I and eIF-4E-Binding Protein 1, respectively (Heesom et al., 2001). The putative up-regulation of this gene during hibernation is interesting in light of the dramatic changes in transcription and translation rates that have been described in the brain and kidneys of hibernating animals

(Frerichs et al., 1998; Knight et al., 2000; Martin et al., 2000). This suggests that the activity of cdc2 kinase may be an important link in the chain of regulatory events that control the suppression of transcription and translation during hibernation.

The increased expression of protachykinin or substance P has been implicated in the generation and entrainment of circadian rhythmicity in the superchiasmatic nucleus (SCN) of the hypothalamus (Mikkelsen and Larsen, 1993). Interestingly, the SCN is the only part of the brain that remains translationally active during torpor and is believed to coordinate the entry into and exit from hibernation (O'Hara et al., 1999). The putative up-regulation of protachykinin in brain during hibernation may provide a key clue for future studies that address the timing of torpor cycles.

RalA and RalB are small GTPases that are implicated in critical cellular signaling pathways in the brain and other tissues. While not much is known about these proteins, they are believed to regulate the exocytosis of neuropeptide VI and regulate signal transduction by interaction with phospholipase D (Brymora et al., 2001). This may have important implications for the regulation of brain activity during torpor.

The putative up-regulation of Sug1, the 26S protease regulatory subunit 8 may have important implications in the regulation of protein degradation in the brain during hibernation. Interestingly, Sug1 has also been shown to play an important role in the regulation of transcript elongation by RNA polymerase II (Ferdous et al., 2001).

The extracellular calcium receptor and calretinin are both involved in calcium metabolism. They have been implicated in mechanisms of cell migration and outgrowth of neuronal processes in some types of neurons and may be involved in the signal

transduction mechanisms that mediate responses to cold and/or ischemia stresses associated with hibernation (Kakuta et al., 2001).

The up-regulation of the sodium potassium ATPase subunit B2 may be a proactive response to damage caused to this important plasma membrane pump during the cold, ischemic conditions associated with hibernation.

Finally, the increased expression of AP endonuclease and DNA topoisomerase suggests that the brain may be activating DNA repair pathways during hibernation. These might be involved either in damage prevention or repair in the cold, ischemic state or, alternatively, their up-regulation might be a preparatory measure to deal with potential damage due to oxidative stress during the arousal process, when oxygen free radical generation will sore.

Figure 5.5. Atlas rat 1200 gene cDNA arrays screened with mRNA from Euthermic (A) and Hibernating (B) ground squirrel brain. Arrays were exposed to X-ray film, scanned and quantified using the EST program to produce the data presented in Table 5.1.

A **B**

Table 5.1. Genes shown to be hibernation-responsive in the brain of the hibernating ground squirrel *S. tridecemlineatus*.

Gene	GenBank Accession Number	Fold increase in mRNA levels
Cell division control protein 2 homolog (CDC2)	Y00272	6.2
Protachykinin alpha precursor (alpha-PPT) substance P +	NM009312	4.2
RalB: GTP-binding protein	XM046784	4.2
Ral A; GTP-binding protein	XM035787	4.0
26S protease regulatory subunit 8; SUG1; p45/SUG; TAT-binding protein homologue 10 (TBP10); PSMC5	NM031149	4.0
Extracellular calcium-sensing receptor precursor (CASR); parathyroid cell calcium-sensing receptor:	XM033265	2.5
Calretinin	AI243411	2.3
Sodium/Potassium Transporting ATPase beta 2 subunit (ATP1B2)	XM042319	4.0
Apurinic/aprimidinic endonuclease (AP endonuclease; APEX; APEN)	NM024148	3.0
DNA topoisomerase II alpha (TOP2A):	XM008649	2.0

Brown Adipose Tissue

Hibernation-responsive genes in ground squirrel brown adipose tissue were assessed using Clontech Atlas 588 gene cDNA arrays. In addition to the heart and adipose type fatty acid-binding proteins that were discussed in Chapter 3, four other genes showed putative up-regulation in BAT during hibernation when euthermic versus hibernating samples were compared (Table 5.2). All of these could have potential roles in adjusting the metabolism of BAT during hibernation.

The gene for Janus kinase-1 (Jak-1) was up-regulated during hibernation. Jaks are tyrosine kinases that play important roles in the initial steps of cytokine receptor signalling (Hellgren et al., 2001). Jak-1, in particular, is enriched in adipose tissue, often representing up to one-third of all of the Janus kinase activity present in the tissue. Jak-1 is believed to react with the human growth hormone (HGH) receptor. It is possible that an increased expression of this gene during hibernation may activate signalling pathways that are physiologically relevant to the activity of BAT.

Increased expression of a putative high-density lipoprotein (HDL) receptor was also seen in hibernating BAT. Increased levels of this receptor protein could potentially enhance the uptake of blood-borne HDL particles into BAT during torpor and, thereby, enhance the lipid-fuelled thermogenic capacity of the tissue.

The physiological role of phospholipase A2 in brown fat is not known but the expression of this gene has been noted also in bovine adipose tissue after fattening. The enzyme may play a role in liberating free fatty acids (FFAs) from extensive triacylglycerol reserves so that they may be distributed to other tissues during hibernation.

The increased expression of the muscarinic acetylcholine receptor M2 was also noted in BAT from hibernating ground squirrels. Elevated receptor levels may increase the sensitivity of BAT to stimulation from the central nervous system so that it can respond (relatively) quickly to stimuli (i.e. during arousal), even at a low body temperature.

Table 5.2. Genes shown to be hibernation-responsive in brown adipose tissue from the hibernating ground squirrel *S. tridecemlineatus* as assessed using Clontech 588 gene Atlas nylon arrays: fold increase in mRNA levels during hibernation. Arrays were hybridized with probe from BAT of euthermic and hibernating animals, followed by scanning, quantification, normalization to the expression levels of 3 housekeeping genes (GAPDH, tubulin and ubiquitin), and then calculation of the ratio of mRNA levels in hibernation versus control.

Gene	GenBank Accession Number	Fold increase in mRNA levels
Janus (tyrosine) protein kinase-1	AJ000566	5.6
Putative high density lipoprotein receptor-1	U76204	3.4
Muscarinic acetylcholine Receptor M2	AB017655	3.1
Phospholipase A2	D00523	2.1

Liver and Pancreas

Hibernation-responsive genes in ground squirrel liver and pancreas were assessed using the Clontech 1200 gene Atlas nylon arrays. Table 5.3 lists three genes that were strongly up-regulated in liver during hibernation and two genes that were identified in pancreas. One of the genes that was hibernation-responsive in liver was the serine/threonine kinase, PCTAIR E2 (Mottram et al., 1993). This enzyme is highly similar to CDC2 kinase which was up-regulated in the brain of hibernators. PCTAIR E2 may have a similar function to CDC2 kinase with a possible role in transcriptional and translational control in the liver of hibernating ground squirrels.

Cytochrome P450 2C7 (CYPC27) was also up-regulated in liver of hibernating squirrels. The cytochrome P450 family is responsible for most of the drug and steroid metabolism in the liver. CYPC27 mRNA levels have been shown to be under the influence of growth hormone (Oinonen et al., 2000). This is interesting in light of the fact that Janus kinase activity (which is hibernation-responsive in BAT) has also been linked to the activated growth hormone receptor and this may indicate a regulatory role for this hormone in survival of hibernation.

Neurofibromin (NF-1) is a transcription factor that activates expression of the protein, p53, in the liver. P53 is responsive to a wide variety of cellular stresses and may be activated in the liver during hibernation; p53 has also been implicated as a transactivator or transrepressor of genes involved in the production and control of reactive oxygen species (Lee et al., 1999).

Two genes were hibernation-responsive in the pancreas. One was cytochrome P450 (B1). Transcript levels of this cytochrome P450 subtype have been shown to

increase strongly in the presence of dioxin in various tissue (Sutter et al., 1994). The apparent up-regulation of P450 (B1) in hibernating pancreas could play a role in detoxifying dangerous metabolites that accumulate during hibernation and arousal.

The gene for the neurokinin A receptor was also hibernation-responsive in pancreas. This receptor is thought to mediate the release of insulin and glucagon from the pancreas (Schmidt et al., 2000). This may be important in the regulation of blood glucose levels in hibernating animals.

Overall, then, the differential gene screening analyses presented in the current chapter have identified a wide variety of genes as putatively up-regulated in the tissues of thirteen-lined ground squirrels during hibernation. Each of these may have a key role to play in hibernation and future studies of these genes and the functions of the proteins that they encode may provide multiple new advances in our understanding of the metabolic regulation of hibernation.

Table 5.3. Hibernation-responsive genes in liver and pancreas from the hibernating ground squirrel *S. tridecemlineatus* as identified using the Clontech 1200 gene Atlas nylon arrays. The fold increase in expression in hibernating, compared with euthermic, liver is shown. Other details as in Table 5.2.

Gene	GenBank Accession Number	Fold increase in mRNA levels
LIVER		
Serine/threonine kinase PCTAIRE2 (PCTK2):	NM002595	4.0
Cytochrome P450 2C7 (CYP2C7); P450F; PTF1:	AW142236	3.1
Neurofibromin; neurofibromatosis protein type I (NF1); GTPase stimulatory protein:	NM012609	3.0
PANCREAS		
P450 IB1; C3H cytochrome P450; CYP1B1:	NM012940	4.3
Substance K receptor (SKR); neurokinin A receptor; NK-2 receptor	M57414	3.6

CHAPTER 6

GENERAL DISCUSSION: HIBERNATION PRECONDITIONING AND A ROLE FOR PPARs IN HIBERNATION-RESPONSIVE GENE EXPRESSION

Increasingly, an important role for differential gene expression has been acknowledged as an underlying mechanism in a wide variety of physiological adaptations. Changes in gene expression underlie animal adaptations to environmental stresses including cold, freezing and anoxia and, in our lab, have been identified as integral parts of mammalian hibernation, estivation, freeze tolerance and anaerobiosis (Storey, 2000). Each year new genes are discovered that contribute to the defences that allow animals to endure extreme environmental stresses. For example, adaptive changes in gene expression are known to include genes whose protein products enhance mitochondrial oxidative capacity (Cai and Storey, 1996; Fahlman et al., 2000), nucleate and control the size of ice crystals (Cai and Storey, 1997), and sequester potentially harmful metal ions that would otherwise react to form dangerous reactive oxygen species (ROS) (Larade and Storey, unpublished).

Overall, the work presented in this thesis demonstrates that differential gene expression may also play an important role in survival during mammalian hibernation. The goal of this research was to discover and characterise the expression of hibernation-responsive genes first in kidney, secondly in brown adipose tissue and also in other tissues using a variety of gene-discovery techniques. The mitochondrial-encoded core subunit of cytochrome c oxidase (Cox1) was shown to be up-regulated both at the mRNA and protein level in kidney and other tissues of the hibernating ground squirrel. It is believed that the increased expression of Cox1 specifically and the mitochondrial genome in general, may represent a proactive response to potential damage to the mitochondrial electron transport chain caused by cold and ischemia during hibernation. The increased expression of heart and adipose-type fatty acid binding proteins (FABPs) in brown

adipose, heart and skeletal muscle most likely facilitates the shift to low temperature lipid-fueled metabolism during hibernation. In addition, this study presented data that suggests that small amino acid differences in specific proteins of hibernating animals may explain the continued function of several hibernator enzymes at temperatures well outside their "normal" physiological parameters. Finally, I addressed an underlying paradox of investigating the up-regulation of genes at a time when the rates of both transcription and translation are generally thought to be significantly depressed. This investigation revealed that whereas the transcript levels of some genes increase during hibernation, the translation status or association of these mRNAs with actively translating polyribosomes is highly tissue dependent. Whereas transcript levels of the type-2 organic cation transporter (Oct2), which functions to excrete organic cations into the renal proximal tubules, are clearly up-regulated in the kidney of hibernating animals, the mRNA is sequestered into the cytoplasmic mRNA pool that is thought to be associated with RNA-binding proteins as mRNP particles. Interestingly, in brown adipose tissue, which remains physiologically "alert" during hibernation, there was no detectable suppression of protein synthesis or loss of polyribosomes. In fact, there was an increased presence of the previously described H-FABP on polyribosomes in BAT of hibernators as compared with euthermic polyribosomes. Overall, these data seem to indicate a tissue specific suppression of protein synthesis in hibernating animals, and that genes detected as up-regulated during hibernation may, in fact, be stored as cytoplasmic mRNP particles during torpor and held ready to be rapidly translated during arousal from hibernation. This could facilitate a rapid recovery from any damage caused by the cold and ischemia associated with hibernation. Overall, these changes in the expression of hibernation

responsive genes and their protein products contribute substantially to our understanding of the adaptations needed for hibernation and also suggest the involvement of a novel preconditioning mechanism for activating the unique stress responses that contribute the hibernation phenotype.

Hibernation is an intriguing research topic that has been largely overlooked, to date, from a molecular biology perspective. Whereas extensive physiological and biochemical data about hibernation has been gathered over the past 50 years, only recently has molecular biology been used to characterise changes in gene expression patterns associated with mammalian hibernation. Preliminary evidence suggests that the differential expression of known genes, rather than a subset of novel hibernation-specific genes, enables animals to express the hibernation phenotype (Srere et al., 1993; Storey, 2000). This is in keeping with the hypothesis that hibernation involves small changes at key metabolic regulatory points rather than a wholesale re-programming of gene expression networks. This also strengthens the hypothesis that hibernation is not a recently evolved phenomenon but the evolutionary extension of an ancient pattern of circadian heterothermy (Srere et al, 1992).

The expression of a pancreatic lipase in the hearts of hibernating ground squirrels provided the first indication that hibernation may governed in part, by the expression of genes outside of their normal expression profile (Andrews et al., 1998). My discovery of A-FABP expression in hibernating heart complements these findings and adds to the proposal that during hibernation, the tissue-specific expression patterns of certain genes are altered to adjust cell/tissue functionality to support their survival for extended periods of time under conditions (e.g. body temperature near 0°C, greatly reduced blood flow)

that are well outside the range of normal (euthermic) physiological parameters. Furthermore, recent evidence is beginning to suggest that hibernating animals utilise pre-existing stress response pathways in a pro-active manner to pre-condition themselves for the rigors of extended bouts of torpor. This hypothesis stems from the discovery that hibernation-responsive genes such as those encoding Cox and FABP proteins are also increasingly abundant in non-hibernator mammalian tissues subjected to ischemia and cold stress (Hittel and Storey, 2001; Daikoku et al., 1997; Van Itallie et al., 1993). This suggests that the hibernation phenotype may arise as a unique combination of a variety of stress responses. The physical manifestation of hibernation preconditioning process may be the “test drops” that ground squirrels experience in early autumn prior to entering an extended bout of torpor (Fig. 6.1) (Boyer and Barnes, 1999; Wang and Lee, 1996). During these short torpor bouts, the animals briefly suppress their metabolic rate (including body temperature and heart rate) for minutes to hours and then rapidly return to euthermic levels (Barnes and Boyer, 1999). The term hibernation preconditioning has been chosen to describe this process because of perceived similarities to the process of ischemic preconditioning (Cochrane et al., 1999). I propose that hibernators shock their organs with a combination of cold exposure and ischemia to activate endogenous stress response pathways. Specific expression of only a selected subset of genes during entry into torpor (or the early hours of torpor) also resolves the paradox of increased gene expression despite the known dramatic decreases in overall rates of transcription and translation that occur during torpor (Knight et al., 2000; Martin et al., 2000). By “shocking” the appropriate stress response pathways into readiness during a preconditioning phase, patterns of gene expression are induced prior to entry into an

extended bout of torpor. During arousal, these genes are then rapidly translated into protein products to either replace essential gene products, or produce proteins which repair or reduce the damage caused by cold and ischemia. These gene expression profiles may be patterned on the nucleus through the formation of stable promoter-transcription factor complexes or histone acetylation in promoter regions of hibernation-responsive genes (epigenetic memory). I have also discussed translation suppression and the apparent sequestering of functional mRNAs during hibernation. This is also a potential stress response activated by hibernation preconditioning where, in response to ischemia, cold, starvation or a combination of all three, translation initiation is blocked and mRNAs are sequestered into Poly(A) containing mRNP stress granules (Kedersha et al., 1999).

Figure 6.1. Body temperature of an Arctic ground squirrel (*Spermophilus paryii*) over the hibernation cycle. Telemetry data clearly shows the “test drop” phase (A) and inter-bout arousals (B) and periods of deep torpor (C). This figure was adapted from Boyer and Barnes (1999).

PPARs and Hibernation

Central, perhaps, to the transcriptional control of many hibernation-responsive genes are the peroxisome proliferator-activated receptors (PPARs). What follows is a brief overview of PPAR function and their possible role in the regulation of the gene expression responses described in this thesis.

PPARs belong to a family of orphan nuclear receptors whose endogenous ligands are not known. They are also members of the extended nuclear hormone receptor superfamily that also includes the vitamin D receptor, the retinoic acid receptor and the thyroid hormone receptor. PPARs are roughly divided into 3 subtypes (α , β and γ), each with its own unique tissue distribution and expression level, although some tissues express multiple isoforms. Overall, PPARs have been reported to play an important role in lipid homeostasis, metabolism and adaptive thermogenesis (Vega et al., 2000). PPAR α , the first PPAR discovered, has a role in regulating the transcription of enzymes involved in fatty acid oxidation, and is enriched in tissues with high oxidative energy demands such as BAT, heart and skeletal muscle (Vega et al., 2000). PPAR β has not been well characterised and, as such, will not be reviewed in this discussion. PPAR γ however, is thought to control the expression genes involved in lipogenesis or adipocyte differentiation and insulin sensitivity (Castillo et al., 1999). Because of its role in insulin sensitivity, PPAR γ has been targeted for the treatment of type II diabetes using the drug troglitazone (Rezulin TM), an exogenous ligand which strongly binds and activates this receptor. PPARs are of particular interest to the investigation of hibernation because of

their purported roles in both adaptive thermogenesis and lipid homeostasis, both of which can be linked to the activities of PPARs α and the PPAR coactivators PGC-1 and PGC-2.

PPAR Coactivators PGC-1 and PGC-2

PPARs act as trans-membrane platforms for the docking of large multi-protein complexes that, when stimulated by endogenous ligands, carry out the transcriptional activity initiated by the receptors (Fig. 6.2) (Castillo et al., 1999). These PPAR interacting proteins, also called coactivators, may possess histone acetyl transferase activity, and/or alter the conformation of the PPAR or other proteins to allow them to bind to specific regulatory sites upstream of target genes. Regardless of the mechanism, PPAR coactivators modify the activity of their respective PPAR, usually by enhancing the transcription of genes under their control in a ligand-dependent or independent fashion (Castillo et al., 1999). PGC-1 was first described as a coactivator of PPAR γ that is highly expressed in tissues such as BAT in response to cold (adaptive thermogenesis). Genes activated by the interaction of PGC-1 with PPAR γ include the nuclear respiratory factor 1 (NRF-1), a key transcriptional regulator of nuclear and mitochondrial encoded subunits of the mitochondrial electron transport chain. PGC-1 also stimulates the expression of uncoupling protein (UCP-1) in adipose tissue and UCP-2 in muscle (Castillo et al., 1999) yet surprisingly decreases their expression in the heart. This suggests that increased PGC-1 expression promotes energy production through coupled mitochondrial respiration in the heart while enhancing the thermogenic capacity (uncoupled respiration) of other tissues. Interestingly, PGC-1 has also been shown to interact with PPAR α , particularly in response to fasting to increase the expression of

mitochondrial fatty acid oxidation (FAO) genes (Lehman et al., 2000). Therefore, PGC-1 expression may link the increased expression of fatty acid oxidation genes (Andrews et al., 1998) with the increase in mitochondrial-encoded genes (Fahlman et al., 2000; Hittel and Storey, submitted manuscript) in the hearts of hibernating animals, possibly in response to a combination of cold exposure and fasting, both of which are known to increase PGC-1 expression (Lehman et al., 2000).

PGC-2 was recently identified by yeast two-hybrid screening as the second major coactivator of PPAR γ activity (Castillo et al., 1999). Unlike PGC-1 however, it does not interact with other PPARs or most other nuclear receptors. Induced expression of PGC-2 in cell lines that express endogenous PPAR γ results in the expression of several adipocyte markers such as A-FABP (Castillo et al., 1999). It is interesting, therefore, that PGC-2 was pulled from the screening of the kidney cDNA library with putatively increased expression during hibernation (Fig. 5.4). Unfortunately, because the sequence of mouse PGC-2 was only released to GenBank within the past few weeks, the identity of kidney clone 3.3 as the ground squirrel homologue of this coactivator came too late to allow a thorough investigation of the expression profile of PGC-2 to be compiled for inclusion in this thesis. The identification of PGC-2 in kidney of hibernating animals suggests that it may activate a limited adipogenic program in this organ. However, since PGC-1 expression elicited different effects in different tissues, the actual effects of enhanced PGC-2 expression in hibernator kidney must await experimental analysis. It would also be interesting to investigate the expression of PGC-2 in the heart of hibernating ground squirrels, since it controls the expression of A-FABP, which is normally expressed exclusively in adipose tissue. In fact, it would be prudent to conduct a

detailed study of PGC-1 and PGC-2 expression profiles, since these could represent the first fully elucidated hibernation-responsive transcriptional pathway. It would also be highly instructive to assess the responses of these activators to the test drops (Fig. 6.1) that precede full entry into extended periods of torpor. PPARs likely play an important role in lipid metabolism and adaptive thermogenesis. When their activities are modulated through interactions with coactivators such as PGC-1 and PGC-2, atypical gene expression patterns may allow the organs of hibernating animals to adapt to these extreme physiological challenges.

Figure 6.2 A model for the activity of the PPAR transcriptional regulatory complex. When activated by endogenous or exogenous ligands PPARs heterodimerize with the retinoid X receptor (RXR) and then bind to sequence specific target elements (PPREs) upstream of the PPAR target gene. Figure adapted from Barger and Kelly (2000).

Perspectives

A variety of techniques have been used successfully in this thesis to describe hibernation-responsive changes in gene expression. The protein products encoded by these genes likely alter the physiological parameters of the tissues and organs of hibernating animals so that they survive the reduction to a “trickle metabolism” caused by the reduced blood flow and low body temperatures during torpor.

In the future, all gene expression studies will include some form of DNA microarray technology. As the technology matures, the cost will drop substantially, a standardised platform will be adopted and the technique will become as routine as DNA sequencing is today. This will put the power of microarray technology in the hands of even the most modestly funded laboratory. No longer will single gene studies be sufficient, instead gene expression profiles will be captured and interpreted, leaving researchers with more time to characterize the protein products of the thousands of unknown genes still present in the genome. Microarrays will prove to be particularly useful in hibernation research because of their high degree of reproducibility and the capacity to test multiple time points in the hibernation cycle that their use will provide (Fig. 6.1).

For the purposes of cross-species hybridization, nylon cDNA arrays using ^{32}P as a probe produced the most consistent and reproducible data. Significantly, there was less problem with background noise using nylon arrays (Fig. 5.5), and a higher degree of cross-reactivity than was observed using cDNA glass microarrays (Fig. 4.8.). One drawback to using nylon arrays was the smaller number of available genes on the array compared with the thousands available using glass arrays; however, those genes chosen

for commercial nylon arrays tend to be fairly well characterised. The problem with cross-species hybridisation, in general, is that many genes have highly variable sequences, particularly across the species barrier and, therefore, only highly conserved genes tend to crossreact. This may also lead to false positives among closely related gene families. The only real way to eliminate this problem is to construct cDNA microarrays that are animal or tissue specific, such as by using low-redundancy ground squirrel cDNA libraries. This technique has been used successfully to describe the hypoxia-induced gene expression in the euryoxic fish, *Gillichthys mirabilis* (Gracey et al, 2001). Although this will require much initial work, the product is a highly ordered cDNA library which can be rapidly printed to multiple glass slides or nylon arrays and screened in much the same way as a cDNA library. This reduces the possibility of false positives, false negatives and increases the probability of finding small changes in low abundance and unknown genes. Studies of gene expression, polysome analysis (changes in translation status) and genes that specifically interact with poly(A)-binding protein, or other RNA binding proteins could also be rapidly investigated in multiple tissues (at multiple hibernation time points) of hibernating animals without worrying about missing genes due to low cross-reactivity.

Another interesting use for these arrays (and for all arrays in general) would be to treat them as large nuclear run-off experiments whereby the rates of transcription of thousands of genes could be monitored simultaneously. Using arrays to screen first for up-regulated genes, secondly for genes that are present in polysomes versus monosomes, and thirdly to run array run-off assays, will provide information about the transcript level, transcription, and translation status (3-dimensions of gene expression data) for hibernation-responsive genes.

The data on FABP presented in Chapter 3 raised the possibility of specific cold temperature adaptations of some hibernator proteins. Hibernators spend a significant portion of their lives at temperatures significantly lower than what is physiological for most mammals. This presents hibernators with a dilemma since they cannot adapt their metabolism to function primarily at low temperatures. Apart from the local fine tuning of metabolism by protein phosphorylation and differential gene expression, key enzymes may be cold-adapted enough so that they can maintain a relatively high proportion of their activity at low temperatures. Such adaptations have been inferred from studies of several hibernator proteins and enzymes such as FABP, glutamate dehydrogenase, and protein kinase A (Storey, 2000). It would be prudent therefore, to obtain and sequence the cDNAs for these genes in hibernating animals and compare them to non-hibernators. From the FABP data we have demonstrated that small changes in protein sequence (three residues in H-FABP) may lead to significant changes in protein functionality over a broad temperature range (Stewart et al., 1998).

In summary, this thesis presented several techniques for detecting and describing differential gene expression in hibernating thirteen-lined ground squirrels. I demonstrated that selected genes are differentially expressed during hibernation and that, in some cases, amino acid substitutions in the proteins that they encode (e.g. FABP) may support/enhance their function at low temperatures. I propose that hibernation-responsive gene expression patterns are established prior to entry into an extended bout of torpor through hibernation-preconditioning that activates pre-existing stress response pathways. Some of these pathways may be controlled by PPARs and their coactivators and the combinatorial nature of their *in vivo* expression patterns may lead to a unique

hibernation-specific expression of genes outside of their “normal” expression profile. The data in this thesis also demonstrated that hibernating animals may sequester most of their mRNA transcripts into mRNPs during torpor presumably to protect them from degradation during torpor. Overall, a variety of molecular events contribute to the expression and survival of the hibernation phenotype and with the advent of inexpensive genomic and proteomics technology, a full molecular explanation of mammalian hibernation is not only likely, but inevitable.

PUBLICATION LIST

RESEARCH PUBLICATIONS:

Peer-reviewed Articles:

Hittel DS, Voordouw G (2000) Overexpression, purification and immunodetection of DsrD from *Desulfovibrio vulgaris* Hildenborough. *Antonie van Leeuwenhoek*. 77: 271-280.

Hittel DS, Storey KB (2001) Differential expression of adipose and heart type fatty acid binding proteins in hibernating ground squirrels. *Biochim. Biophys. Acta* (in press).

Mizuno N, Hittel DS, Miki K, Voordouw G, Higuchi Y (2000) Preliminary X-ray crystallographic study of DsrD protein from the sulfate-reducing bacterium *Desulfovibrio vulgaris*. *Acta Crystallogr D Biol Crystallogr* 56:754-755

Hittel DS, Storey KB (2001) The Differential expression of a mitochondrial encoded subunit of cytochrome c oxidase in the kidney of a hibernating mammal. *J Exp Biology* (Submitted)

Hittel DS, Storey KB (2001) The translation status of differentially expressed mRNAs in the hibernating thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*). *Eur J Biochem* (Submitted)

Hittel DS, KB Storey (2001) The cloning and characterization of the PPARgamma coactivator 2 (Pgc2) from the hibernating 13-lined ground squirrel (*Spermophilus tridecemlineatus*) (in progress)

GENBANK SUBMISSIONS

Spermophilus tridecemlineatus ATP synthase 6 (ATP6) mRNA, complete cds; mitochondrial gene for mitochondrial product

gi|15421853|gb|AF411434.1|AF411434[15421853]

Spermophilus tridecemlineatus ATP synthase 8 (ATP8) mRNA, partial cds; mitochondrial gene for mitochondrial product

gi|15421786|gb|AF362073.1|AF362073[15421786]

Spermophilus tridecemlineatus cytochrome c oxidase subunit I (Cox1) mRNA, partial cds; mitochondrial gene for mitochondrial product

gi|12802821|gb|AF330007.1|AF330007[12802821]

Spermophilus tridecemlineatus adipose-type fatty acid-binding protein (FABP4) mRNA, complete cds

gi|12802819|gb|AF327855.1|AF327855[12802819]

Spermophilus tridecemlineatus heart-type fatty acid-binding protein (FABP3) mRNA, complete cds

gi|12802817|gb|AF327854.1|AF327854[12802817]

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