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The translation state of differentially expressed mRNAs in the hibernating 13-lined ground squirrel (*Spermophilus tridecemlineatus*)

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Abstract

The translation state of differentially expressed mRNAs were compared in kidney and brown adipose tissue of the hibernating ground squirrel, *Spermophilus tridecemlineatus*. Polysome analysis revealed a striking disaggregation of polyribosomes during hibernation and the redistribution of *Cox4* (cytochrome *c* oxidase subunit 4) and *Oct2* (organic cation transporter type 2) transcripts into monosome and mRNP fractions of kidney cytoplasmic extracts. Additionally, OCT2 protein levels decreased in kidney of hibernating animals in line with a strong decrease (85%) in translation rate compared with euthermic kidney. There was no translational depression in brown adipose tissue during hibernation and the H isoform of fatty-acid-binding protein (H-FABP), that is up-regulated during hibernation, was increasingly abundant in the heavy polyribosome fraction isolated from the brown adipose of hibernators. This may indicate the existence of a tissue-specific mechanism for the translational control of a subset of genes that are physiologically relevant to the survival of hibernation.

Keywords: *Spermophilus tridecemlineatus*; Polysome; Kidney; Brown adipose tissue; cDNA array; Mammalian hibernation

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 [1,2]. By allowing their body temperature (Tb)[1] 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 [1,2].

Reversible protein phosphorylation is an important mechanism by which hibernating animals make "acute" adjustments to the activities of various metabolic functions during entry into or arousal from hibernation [2,3]. Many enzymes in catabolic and anabolic pathways as well as key functional proteins (such as ion-motive ATPases) are reversibly inhibited by the actions of protein kinases or protein phosphatases that alter the phosphorylation state of these enzymes/proteins during torpor [1,2]. Increasingly, however, an important role has been demonstrated for the differential expression of genes in the hibernation phenotype [1,4]. Several genes have been identified that are preferentially expressed in the tissues of hibernating mammals. These include α2-macroglobulin in the liver, pyruvate dehydrogenase kinase isozyme 4, pancreatic lipase, and mitochondrial-encoded genes in the heart [4–6], and a variety of genes in other tissues [1].

At first glance this apparent up-regulation of genes seems incompatible with the suppression of transcription and translation which also occurs during hibernation [3,7,8]. Little is known about the control of

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[1] *Abbreviations used:* *Cox4* and COX4, respectively, the mRNA-encoding cytochrome c oxidase subunit 4 and its protein product; *Oct2* and OCT2, respectively, the mRNA encoding the organic cation transporter type 2 and its protein product; BAT, brown adipose tissue; H-FABP, heart-type fatty-acid-binding protein; PABP, poly(A)-binding protein 1; TIA-1, T-cell intracellular antigen-1; eIF2α, the α subunit of elongation initiation factor 2; Tb, body temperature; mRNP, messenger ribonuclear protein; HRP, horseradish peroxidase; SSC, sodium saline citrate; TCA, trichloroacetic acid; PVDF, polyvinylidine fluoride; rRNA, ribosomal RNA; SGs, stress granules; IBA, "interbout" arousals; ROS, reactive oxygen species; NST, nonshivering thermogenesis; UCP, uncoupling protein.

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transcription during hibernation; however, the suppression of protein synthesis during hibernation has been linked to the phosphorylation of the α subunit of elongation initiation factor 2 (eIF2α), which effectively blocks the delivery of the initiation Met-tRNA to the 43S preinitiation complex by eIF2 [3]. 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 [1] or by differential translation of selected gene transcripts during torpor. Anecdotal evidence supports the former option, a role for "interbout" arousals in replenishing essential gene products that are lost or damaged in the tissues and organs of hibernating animals [7–9].

The present study focuses on the latter option, the idea that differential translation of selected gene products could occur during torpor to provide posttranscriptional regulation in the expression of genes whose transcript levels are elevated during hibernation. We describe the translation state of these mRNAs by polysome profiling, Western blot analysis, and in vitro protein synthesis, comparing the kidney and brown adipose tissue (BAT). Our results suggest that suppression of protein synthesis occurs in the kidney but not in BAT during hibernation and that this regulatory dichotomy relates to their respective physiological roles during hibernation. Furthermore, the data indicate that hibernators may uniquely up-regulate and sequester specific mRNAs as messenger ribonuclear protein (mRNP) "stress granules" [10] 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 Professor Ken-ichi Inui, Kyoto University Hospital, Kyoto, Japan. The anti-PABP antibody 10E10 was generously provided by Dr. Gideon Dreyfuss (School of Medicine, University of Pennsylvania, Philadelphia, PA). Anti-TIA-1 antibody was obtained from Santa Cruz Biotechnology Ltd. (Santa Cruz, CA). Antibodies to eIF2α and phosphorylated eIF2α 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 in Illinois during August by a trapper licensed by the U.S. Department of Agriculture (TLC Research, Batlett, IL) and shipped by air to the animal housing facility of the National Institutes of Health campus (Bethesda, MD) [3]. Animals were housed in a holding room in individual shoebox cages maintained at 22–24 °C, with 60% relative humidity and 12/12-h light/dark cycle; free access was given to food and water. Individuals were weighed weekly to determine when they entered and finished the prehibernation phase of hyperphagia that maximizes body lipid reserves prior to hibernation. When animals had exhibited a rapid increase in body weight (by 220–240 g), most were placed in a dark chamber at 5–6 °C and 60% humidity to facilitate hibernation. Other animals were sacrificed as euthermic controls. After placing them in the hibernation chamber, most animals entered a state of deep torpor within 3 to 8 days. Animals were sampled as hibernators after 1 to 7 days of continuous torpor. For sampling, all animals were euthanized by intracardiac injection of pentobarbital sodium, and tissues were rapidly excised, flash-frozen in liquid nitrogen, and transported to Ottawa on dry ice where the remaining experiments were performed.

Hybridization and analysis of cDNA arrays. Atlas rat cDNA expression arrays were purchased from Clontech. ^{32}P-labeled cDNA probes were synthesized with mRNA purified from BAT and kidney of euthermic versus hibernating squirrels as per the manufacturer's instructions. Arrays were soaked in deionized water immediately after opening, then prehybridized in 20 ml of ExpressHybe solution (Clontech) at 68 °C for 15 min with continuous rotation. Aliquots of 1×10^6 cpm of euthermic or hibernating probe made from BAT or kidney 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 × SSC, 1% SDS) and, if needed, a high-stringency solution (0.1 × 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 and exposed to a phosphorimaging screen (Bio-Rad Laboratories) for 2–10 h to optimize exposure time. Array images generated from phosphorimager analysis were analyzed with the EST program [11] 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 followed by comparison of relative levels in control versus stressed states.

Polysome profiles. Samples (500 mg) of frozen kidney or BAT were pulverized under liquid N_2 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 $MgCl_2$, 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*g* at 4 °C for 15 min. The supernatant was

removed and was mixed with 0.1 vol 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 $MgCl_2$, 100 μg/ml cyclohexamide, and 5 U/ml RNase inhibitor. The gradients were centrifuged at 4 °C for 2 h 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 [3]. Protein content was adjusted to 10 mg/ml and the reaction started by the addition of 20 μl of cytoplasmic extract to 80 μl of assay mix plus 5 μCi/ml L-(3,4,5-^{3}H(N))-leucine (Amersham-Pharmacia) [3]. 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 1 M 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 cylcohexamide, 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 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 ^{32}P using a random primer procedure. Northern blots were hybridized at 44 °C for 12 h, 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.

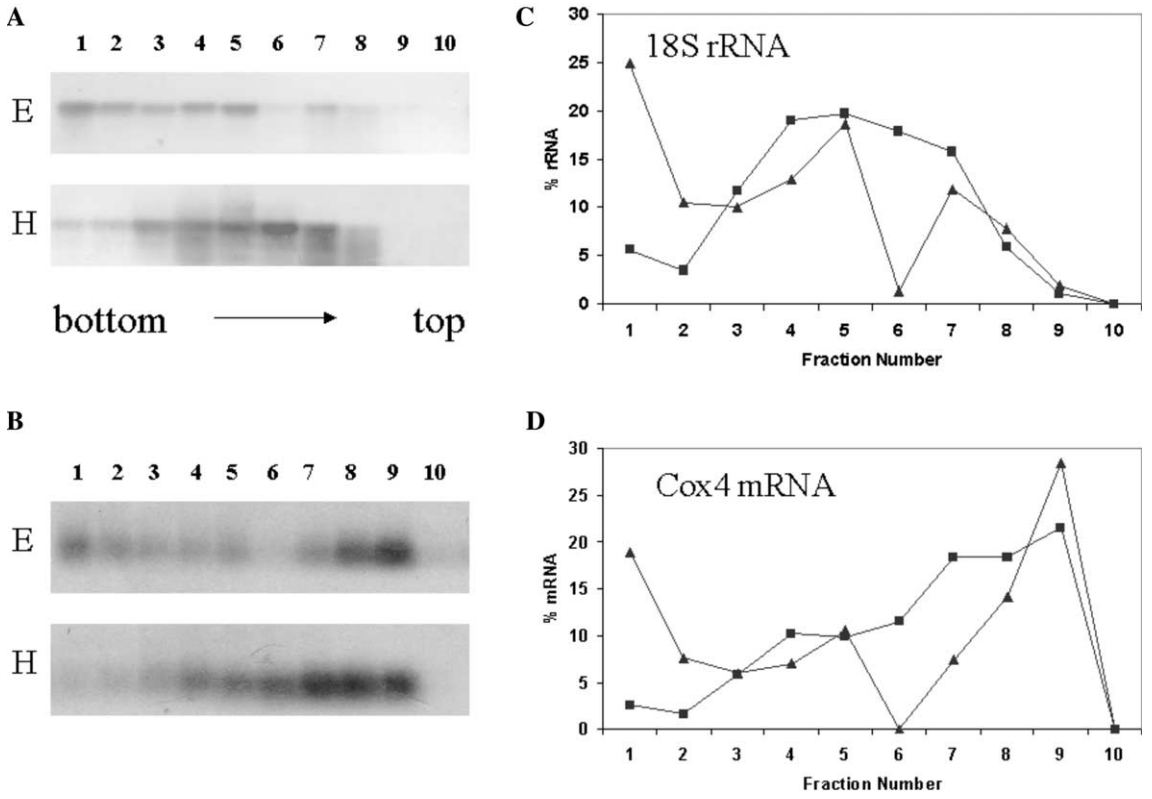

Fig. 1. Polysome analysis of soluble extracts of kidneys of euthermic (E) and hibernating (H) ground squirrels. After separation of extracts on a 0.5–1.5 M sucrose gradient, RNA was recovered from each of 10 fractions and blotted onto nylon membranes. Fraction numbers increase with decreasing density. 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. A and B, representative blots; C and D, the mean signal in each fraction for $n = 3$ trials using tissue from different animals. Triangles represent signal in euthermic kidney; squares represent signal in hibernating kidney.

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 previously retrieved from a cDNA library made from BAT of hibernating *S. tridecemlineatus* [12].

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 × SDS–PAGE loading buffer, centrifuged for 1 min at 10,000 rpm, and loaded onto two identical 15% SDS–PAGE minigels run at 150 V for 45 min. Bio-Rad kaleidoscope prestained markers were used to track the progress of the proteins through the gel. Size fractionated protein samples were transferred to a polyvinylidine 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 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 and actively translating polyribosome fractions [9]. The position of ribosomes in sucrose gradients was tracked by Northern blot analysis using a ^{32}P-labeled probe specific for the 18S ribosomal RNA (rRNA), the major RNA

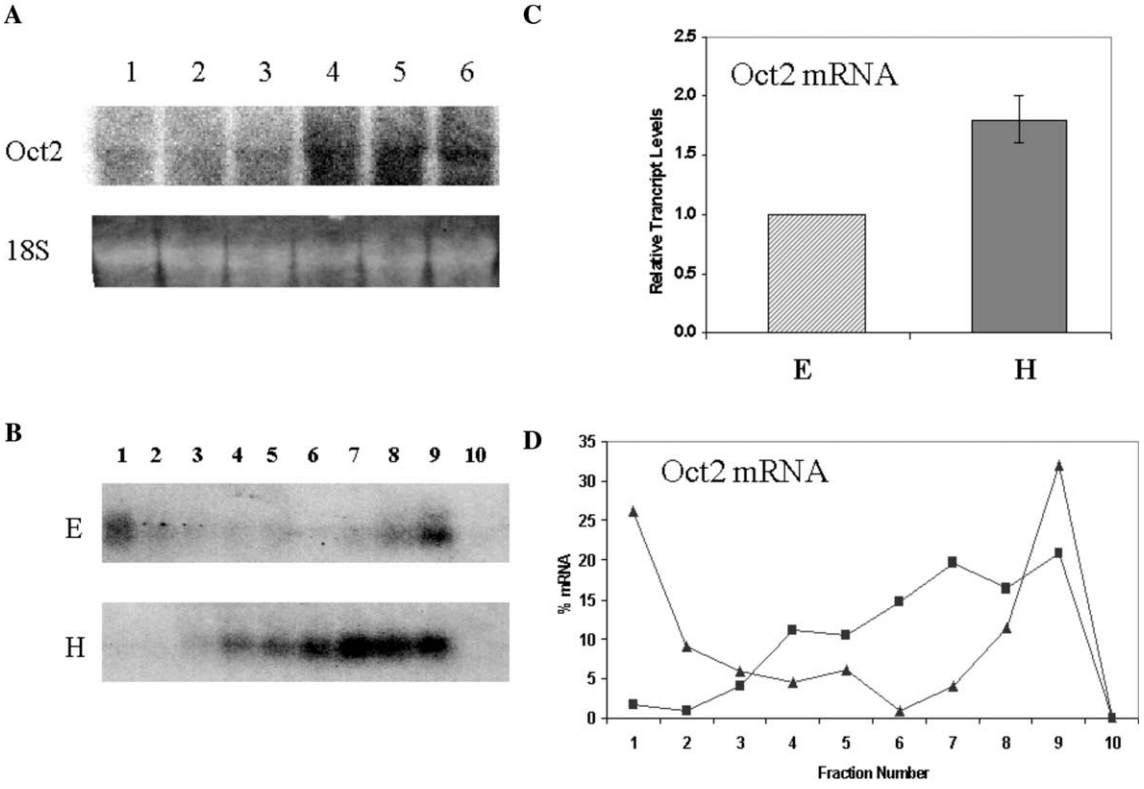

Fig. 2. Northern blot and polysome analysis of organic cation transporter 2 (*Oct2*) mRNA in the kidneys of euthermic and hibernating ground squirrels. (A) Representative Northern blot using total RNA (15 μg per lane) extracted from the kidneys of euthermic (lanes 1–3) and hibernating (lanes 4–6) squirrels 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 0.5–1.5 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 and analyzed relative to the intensity of the 18S rRNA band (A, C) or as in Fig. 1 (D). Triangles represent signal in euthermic kidney; squares represent signal in hibernating kidney.

constituent of the 40S ribosomal subunit [13,14]. Polyribosomes are typically present in heavier fractions [1–5] of the gradients whereas the proportion of monosomes becomes increasingly higher in the lighter fractions [9,13,14] (Figs. 1–3).

Figs. 1 and 2 show the distribution of 18S ribosomal RNA as well as of the mRNA transcripts for two genes,*Cox4* and *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 [15]. *Oct2* mRNA was identified as being up-regulated during hibernation in kidney using cDNA array analysis. Figs. 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 [3,9,13]. 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 [9,13,14]. The *Cox4* cDNA probe bound strongly to an easily identifiable ~670-nucleotide band (Fig. 1B) which was identical in size to that reported for *Cox4* mRNA in most species that have been analyzed [15]. The polysome profile of *Cox4* mRNA (Figs. 1B and 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

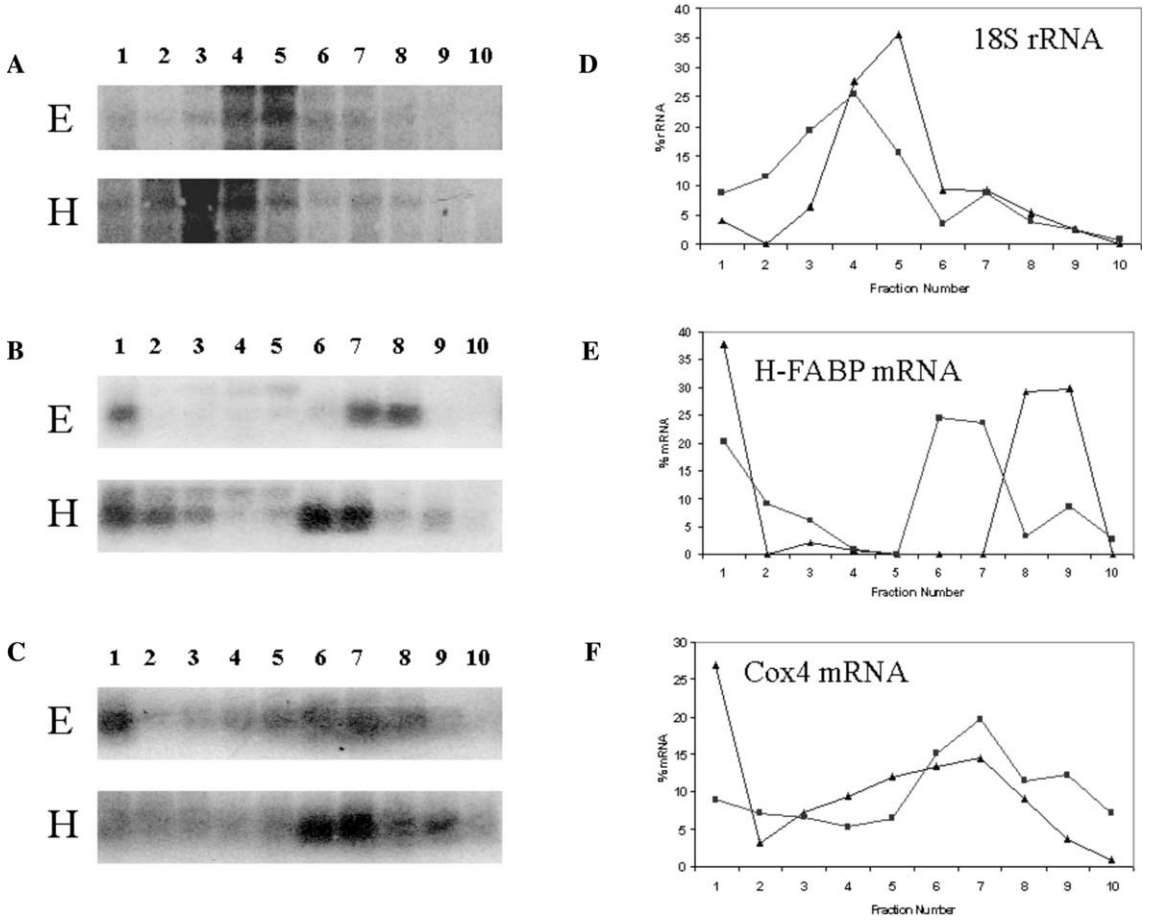

Fig. 3. Polysome analysis of cytoplasmic extracts isolated from brown adipose tissue (BAT) of euthermic (E) and hibernating (H) ground squirrels. Total RNA was extracted from 10 fractions of 5-ml sucrose gradients (each 0.5–1.5 M) and blotted onto nylon membranes. Fraction numbers increase with decreasing 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 D–F show the corresponding mean values for $n = 3$ trials. Triangles represent signal in euthermic BAT; squares represent signal in hibernating BAT.

relative amount of *Cox4* mRNA transcripts associated with the heaviest polysome fractions (1–3) was distinctly reduced 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 [9,13,14].

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 (±0.2SE) up-regulation of an ~2.2-kb band that hybridized with *Oct2* probe (Figs. 2A and C). Notably, studies have shown that cDNA arrays are more sensitive than Northern blots for quantifying changes in mRNA abundance [13]. Fig. 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) (Figs. 2B and 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. 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 ± 0.2-fold (SE) increase [12]. 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. Fig. 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. As mentioned previously, *Cox4* mRNA levels were used as a control because they were unchanged in 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. 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 and 8) (Figs. 3B and 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 state of H-FABP mRNA during hibernation [13,14]. Interestingly, there was also an unexpected increase in the density of the monosome fractions containing H-FABP mRNA during hibernation (Figs. 3B and 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 the monosome/mRNP fractions (Figs. 3C and 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.

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. Fig. 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 [^{3}H] leucine incorporation into protein in extracts from hibernating kidney was only $15 \pm 3\%$ (SE) of the corresponding value in extracts from euthermic kidney (Fig. 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. 4B) which may be linked to the overall reduced rate of protein synthesis during hibernation. The amount of OCT2 protein dropped by $66 \pm 22\%$ (SE) ($n = 3$ determinations on tissues from different individuals) whereas COX4 protein levels decreased only moderately (about 10–20%).

By contrast, the rate of in vitro translation ([^{3}H] leucine incorporation into protein) did not change significantly in BAT from hibernators (Fig. 5A) and the amount of immunoreactive COX4 protein (Fig. 5B) remained constant despite the change in its translation

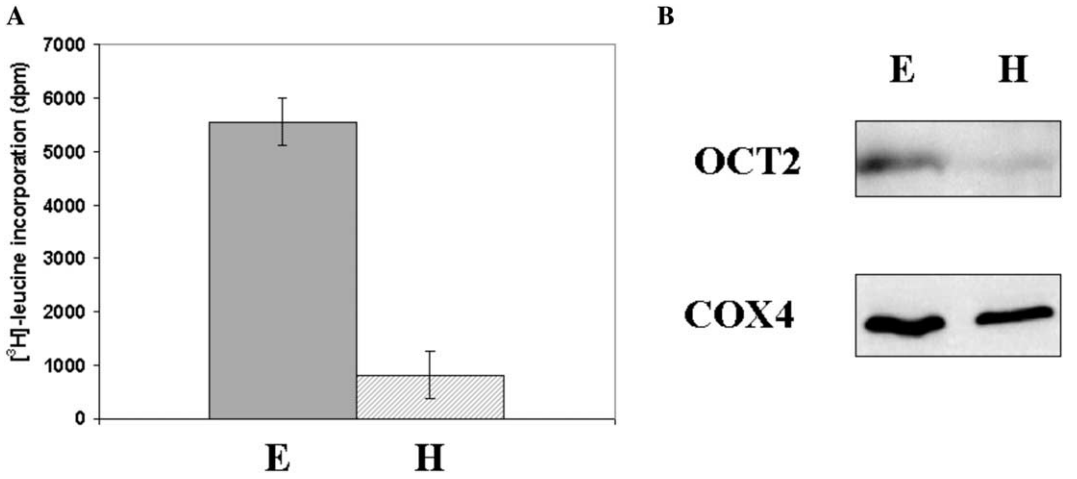

Fig. 4. Rates of protein synthesis in vitro, measured by [^{3}H]leucine incorporation into acid-precipitable protein (A) and Western blot analysis of OCT2 and COX4 protein levels in kidneys of euthermic (E) and hibernating (H) ground squirrels (B). Protein synthesis data are means ± SE; $n = 3$ separately run trials each using tissue from different animals. Western blots are representative of $n = 3$ trials.

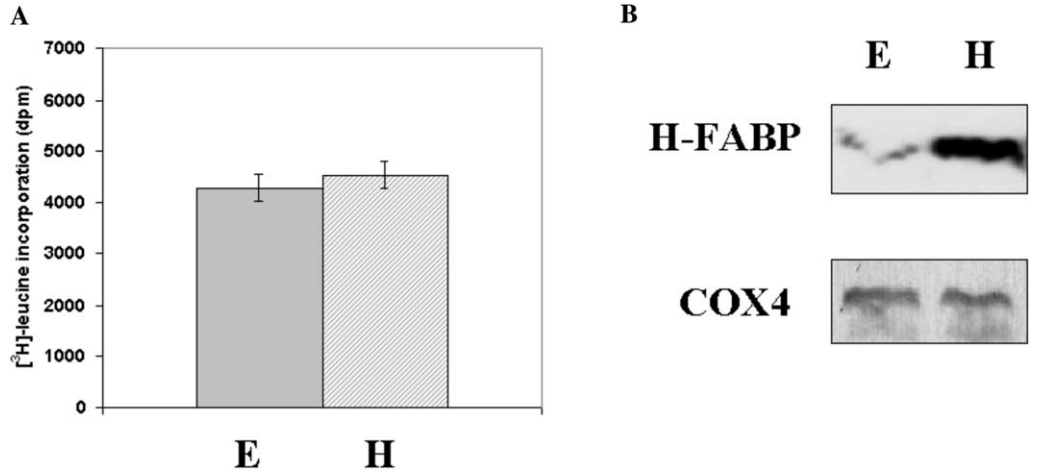

Fig. 5. Rates of protein synthesis in vitro (A) and Western blot analysis of H-FABP and COX4 protein levels in brown adipose tissue from euthermic (E) and hibernating (H) ground squirrels (B). Data were analyzed as in Fig. 4.

state during hibernation (Figs. 3C and F). However, a significant increase in the amount of H-FABP protein was detected (3.1 ± 0.4-fold (SE)) ($n = 3$ determinations on tissues from different individuals) in BAT from hibernating squirrels (Fig. 5B). This is consistent with the increase in both H-FABP transcript levels and their association with heavy polyribosomes (Figs. 3B and E).

The phosphorylation status of eIF2α in the 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 eIF2α revealed a striking increase in the amount of phosphorylated eIF2α in the kidneys of hibernating ground squirrels, compared with euthermic controls whereas the total amount of eIF2α did not change (Fig. 6). Though the exact ratios of phospho- to dephospho-eIF2α were not determined [3], previous studies have shown that even small amounts of phosphorylated eIF2α significantly inhibit global protein synthesis by blocking the initiation of nascent polypep-

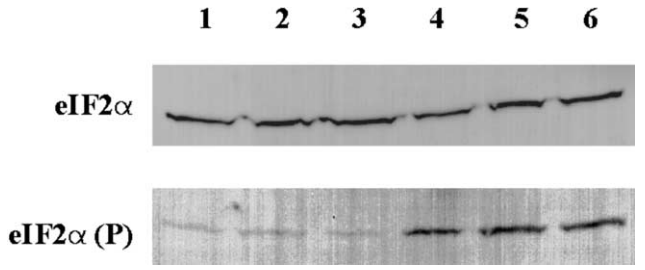

Fig. 6. Western blots of total (top) and phosphorylated (bottom) eIF2α in kidney homogenates from euthermic (lanes 1–3) versus hibernating (lanes 4–6) ground squirrels.

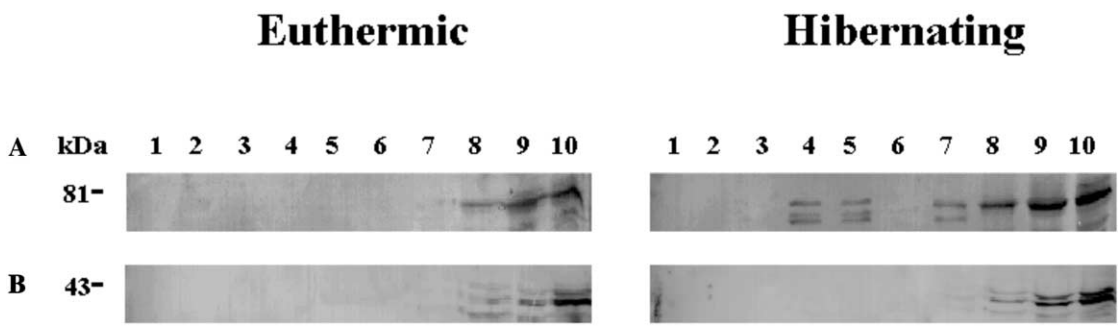

Fig. 7. Western blot analysis of polysome profiles from kidney of euthermic and hibernating ground squirrels using (A) anti-PABP-1 antibodies and (B) anti-TIA-1 antibodies. Samples from each fraction were mixed 1:1 (v/v) in SDS–PAGE sample buffer, boiled, size fractionated in duplicate on a 15% acrylamide gel, and then blotted onto PVDF membranes. Fraction numbers increase with decreasing sucrose density.

tides [14,17]. A logical consequence of blocking translation initiation is the moderate to severe disaggregation of polyribosomes which was observed in hibernating, but not euthermic, kidney (this study), brain, and liver extracts [3,7,9,18]. No phosphorylated eIF2α was detected in BAT from either hibernating or euthermic animals although unphosphorylated eIF2α was found in equal amounts under both conditions (data not shown).

The phosphorylation of eIF2α 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 [10,18]. Prominent protein constituents of SGs include two proteins, poly(A)-binding protein I (PABP-1) and TIA-1, a self-aggregating, RNA-binding protein [10]. We investigated the distribution of PABP-1 and TIA-1 in the polysome profiles of kidney extracts from euthermic and hibernating ground squirrels using Western blot analysis (Fig. 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. 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. 1 and 2). Contrary to the results for PABP-1, TIA-1 did not change its distribute in the polysome profile of hibernator kidney (Fig. 7C). Both euthermic and hibernating polysome profiles showed near identical distribution of TIA-1 protein in fractions 8–10 (Fig. 7B).

Discussion

The differential expression of genes is widely accepted as playing an important, perhaps essential, role in survival during mammalian hibernation [1,4]. 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 rewarm from torpor to euthermic body temperatures before rapidly reentering torpor [1,7,9]. 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 [7,9]. 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 [7–9]. 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 euthermy with only moderate reductions in body temperature. These test drops may precondition the animals by coordinating or shocking their bodies into both biochemical and physiological preparedness for an extended plunge into deep torpor [1,19]. 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 [1,4–6]. 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 over 500 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 (Figs. 2A and C).

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 [20]. 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 [20,21]. Not much is known about the regulation *Oct2* mRNA expression other than that it is found predominantly in the kidney and that expression is regulated by sex hormones such as testosterone whose levels fluctuate and influence hibernation [21,22]. Interestingly, in vitro evidence suggests that kidney proximal tubules are particularly sensitive to reactive oxygen species (ROS) generated during cold ischemia [23–26]. A 100-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 [22]. Because of the greatly reduced or "trickle" blood flow during hibernation, it is likely that the kidney and other organs become profoundly ischemic [3]. It is not surprising, therefore, that protein synthesis in general may be strongly suppressed with a elevation of expression of only selected genes, such as *Oct2*, that may be particularly sensitive to the cold and ischemia and are targeted for destruction during hibernation.

Our data shows that eIF2α is preferentially phosphorylated in the kidneys of hibernating animals (Fig. 6). Previous studies have linked the phosphorylation of eIF2α with the suppression of protein synthesis and disaggregation of polyribosomes in the brain of hibernating ground squirrels [3,7]. We have shown that 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 redistributed into the translationally silent monosome and mRNA pools during hibernation (Figs. 1 and 2). This is in agreement with the general principle that hibernators depress all metabolic processes during torpor [1,2]. 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 (Figs. 2B and D). The purpose of increasing *Oct2* transcript levels while at the same time decreasing their translation state is further confused by the significant drop in OCT2 protein levels (Fig. 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 [25,26], 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 need to be abundantly expressed during IBAs when a rapid restoration of normal kidney function is required. The physiological basis of this anticipatory response may be the test drops that hibernating mammals experience prior to entering their first bout of torpor [19]. We propose that these test drops are a hibernation preconditioning process in which the organs of animals are shocked to activate preexisting stress response pathways which subsequently protect the animal from the rigors of cold torpor [15].

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- to 2-week torpor bout without damage or degradation and (b) rapidly reinitiate the synthesis of important protein products either beginning immediately when Tb begins to rise or during the short period of euthermic interbout arousal. Previous studies have linked the phosphorylation of eIF2α to the assembly of mammalian SGs, RNP structures that reversibly bind and protect mRNAs during a nonlethal disruption of cellular homeostasis [10]. 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. 7A). These fractions also contain the majority of the *Oct2* and *Cox4* mRNAs (Figs. 1 and 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 [9,18] 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 [10], yet our studies (Fig. 7B) did not find TIA-1 redistribution in the polysome gradient of hibernator kidney. Recent studies [18] 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 ultracentrifugation. To resolve this question, 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 [12]. 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 [27]. Heat production during arousal from hibernation is supplied first from nonshivering 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 [19]. Not surprisingly, the expression of UCP in BAT increases with cold exposure in rats [28] and seasonally in hibernators [1]. Since BAT relies on both its internal lipid stores and its 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 (Figs. 3B and D) along with the protein synthesis and Western blot data (Fig. 5) show that BAT responds very differently to hibernation than does kidney. The increased transcript levels and translation state 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 [28] 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 Tb and low metabolic rate. The lack of any detectable phosphorylation of eIF2α and the unchanged rate of protein synthesis in BAT during hibernation (Fig. 5A) suggests that this organ is regulated very differently from kidney during hibernation [29]. This is probably due to the important physiological role that BAT plays in rewarming the organs of hibernating animals during arousal [1,28]. Interestingly, whereas the number of heavy polyribosomes increased in hibernating BAT (Figs. 3A and D), *Cox4* mRNA redistributed to the untranslated monosome/mRNP fractions (Figs. 3C and 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 identify not only differentially expressed genes, but also those genes that are preferentially translated during hibernation [13,14].

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