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Gene Up-Regulation in Heart during Mammalian Hibernation

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A cDNA library prepared from heart of hibernating golden-mantled ground squirrels, *Spermophilus lateralis,* was differentially screened to clone genes that were up-regulated during hibernation. Two differentially expressed clones were found after three rounds of screening and were confirmed as up-regulated by Northern blotting. Clone Ang6 encoded a polypeptide with 116 amino acids that was identified as the ventricular isoform of myosin light chain 1 ($MLC1_v$). Clone Ang19 coded for 274 amino acid residues of the mitochondrially encoded protein subunit 2 of NADH–ubiquinone oxidoreductase (ND2). Both proteins showed high amino acid sequence identity with their human counterparts, 97.5% for $MLC1_v$ and 66% for ND2. Northern blot hybridization revealed differential expression of these genes in multiple organs during hibernation. Transcript levels of both were approximately twofold higher in heart and three- to fourfold higher in skeletal muscle of hibernating, versus euthermic, animals. ND2 was also up-regulated in hibernator liver. Hibernation-induced up-regulation of $MLC1_v$ suggests that a restructuring of myosin subunit composition could contribute to changes in muscle contractility needed for hypothermic function, whereas changes in ND subunit composition may affect the function of the electron transport chain during hibernation. © 2000 Academic Press

Key Words: Spermophilus lateralis; NADH–ubiquinone oxidoreductase; myosin light chain subunit 1; cDNA library screening.

Hibernation is the key to winter survival for many small mammals living in seasonally cold environments. Through a combination of regulated metabolic rate depression, a resetting of the hypothalmic set point for body temperature, and the consequent steep reduction in body temperature (T_b) to near ambient, many small mammals can lower their metabolic rate during hibernation to <2% of the corresponding euthermic rate. As a result, the net energy savings during the winter season (including the cost of periodic arousals) can be as much as 88%, compared with the costs of remaining euthermic over the same time (46). The induction and maintenance of the hibernating state require a variety of metabolic adjustments, including those that regulate metabolic rate suppression, readjust metabolism for function under extreme hypothermia, change patterns of fuel use to a primary reliance on lipids, and put in place mechanisms for rapid rewarming of body tissues upon arousal. For example, mammalian organs (including those of hibernating species in the summer months) sustain significant, even lethal, metabolic injuries due to hypothermia if core T_b drops by more than just a few degrees (20), yet hibernating mammals may routinely suppress core T_b by over 30°C and spend 2–3 weeks at time in a torpid state before returning uninjured to euthermia. The mechanisms of hibernation regulation include both seasonal changes and hibernation-induced adjustments to a variety of metabolic functions (39, 40, 46). These can involve changes to the types or amounts of selected proteins and enzymes in hibernator cells, such as elevated amounts of uncoupling protein (UCP) in brown adipose tissue that support thermogenesis during arousal (9, 31).

With the advent of good techniques for evaluating changes in gene expression, a number of recent studies have begun to identify organ-specific changes in gene and protein expression that contribute to hibernation. Whereas mRNA transcript and/or protein levels of many genes

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appear to be unchanged during hibernation (13, 26, 33) and a few are specifically down-regulated (37), selected genes are now known that are specifically up-regulated during hibernation. These include the genes for α_2-macroglobulin in liver (38), moesin in intestine (18), pyruvate dehydrogenase kinase isozyme 4 and pancreatic lipase in heart (2), and UCP isoforms in multiple tissues (9). Furthermore, in ground squirrel brain, a 98-kDa protein with a phosphotyrosine moiety is present in membrane fractions throughout a hibernation bout but disappears within 1 h of arousal (33), whereas increased expression of "intermediate-early" genes for selected transcription factors (c-fos, junB, c-Jun) occurs during late torpor and peaks during arousal (32).

Our current interest is in heart. This organ plays a vital role in hibernation, for it must continue to circulate blood throughout the entire hibernation course, although operating at a much lower body temperature and higher peripheral resistance than during euthermia (46). Indeed, whereas heart rate during hibernation may be only 1/30 or less of the euthermic value, the force of myocardial contraction is actually increased during torpor. Furthermore, although skeletal muscles showed some disuse atrophy during hibernation, cardiac tissue mass actually increased by 21% and so did heart oxidative capacity, as assessed by citrate synthase activities (48). Hence, some reorganization of gene expression to benefit heart function during hibernation should be expected. Changes to heart protein products could define the difference between the ready endurance of deep hypothermia by hibernating mammals and the lethal consequences that equivalent hypothermia exposure would have for most mammals, including man.

In the present study the role of differential gene expression in supporting heart function during hibernation was examined in golden-mantled ground squirrels, *Spermophilus lateralis*. This medium-sized species (250–300 g when fully fattened) is native to the mountains of western North America. The hibernation season runs from about October to May, depending on latitude and elevation, with torpor bouts that can last 1–3 weeks interspersed with brief arousals. Using differential screening of a cDNA library made from heart of hibernating squirrels, we found two genes that were up-regulated during hibernation. These were identified as the mitochondrially encoded gene for NADH–ubiquinone oxidoreductase subunit 2 (*Nad2*) and the nuclear-encoded gene for ventricular myosin light chain 1 ($MLC1_v$). Each may have a significant function in sustaining and adjusting heart function during hibernation.

MATERIALS AND METHODS

Animals

Golden-mantled ground squirrels (*S. lateralis*) were captured in the Crooked Creek area of the White Mountains of California during the summer and were treated essentially as described by Frank *et al.* (16). Briefly, animals were housed individually in rat cages at the animal care facility of the University of California, Irvine and were maintained at 22°C on a fall (10L:14D) photoperiod. The animals were maintained on water and a semisynthetic diet that was a modified version of Purina 5001 rodent diet produced by the Test Diet Division of Purina Mills, Inc. (Richmond, IN, U.S.A.) (16). Each animal was given 150 g of diet every 5 days and the animals were allowed to feed and fatten for 8 weeks. At the end of this time (September), control animals were sacrificed and tissues were rapidly excised and flash-frozen in liquid nitrogen. Remaining animals were moved to a cold room at 4°C and induced to hibernate. After 30 days of hibernation (October), the animals were sacrificed while torpid; all animals had been torpid for at least 2 days in their current hibernation bout and had body temperatures within ~1°C of ambient (16). Tissue samples on dry ice were air-freighted to Ottawa 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 diethylpy-

rocarbonate (DEPC) and subsequently autoclaved. Total RNA was isolated from hearts of hibernating and euthermic ground squirrels using Trizol solution (BRL), following the manufacturer's protocol. Poly(A)$^+$ RNA was isolated from total RNA using an oligo(dT)–cellulose column (New England Biolabs) with 0.25 g oligo(dT) and 1 mg of total RNA, following the protocol of Cai and Storey (12). The concentration of the poly(A)$^+$ RNA was determined spectrophotometrically at 260 nm. Poly(A)$^+$ RNA from the hearts of hibernating animals was used to construct a cDNA library using a cDNA library and Uni-ZAP unidirectional cloning kits from Stratagene (San Diego, CA, U.S.A.), following the manufacturer's instructions. Double-stranded cDNA was fractionated using the Sephacryl S-500 column supplied with the kit; cDNA samples in fractions 1–4 were mixed and used for ligation to the Uni-ZAP cosmid vectors.

Differential Screening of the cDNA Library

^{32}P-Labeled single-stranded cDNA probes were synthesized from poly(A)$^+$ RNA isolated from hearts of hibernating and euthermic animals. Into an autoclaved, DEPC-treated, 1.5-ml microfuge tube, 2 μl poly(A)$^+$ RNA template (about 1 μg) and 6 μl DEPC ddH$_2$O were added. The mixture was heated at 65°C for 5 min and then 5 μl of 5× first-strand buffer (BRL), 1.5 μl dNTPs without dCTP (5 mM for each nucleotide), 1 μl oligo(dT) primer (200 ng/μl, NEB), 1 μl RNasin (5 U/μl, Promega), and 2.5 μl dithiothreitol (0.1 M, 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 μl M-MLV reverse transcriptase (200 U/μl, BRL) and 5 μl [^{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 μl EDTA (0.5 M), 1 μl sodium dodecyl sulfate (10% w/v), and 3 μl 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 μl Tris–HCl (1 M, pH 7.4) and 3 μl 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 μl with TE buffer; a 2-μl aliquot was removed for scintillation counting. Approximately one million cpm of probe/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 heart of either hibernating or euthermic animals in a hybridization incubator (LAB-Line Instruments) using Denhardt's hybridization solution with 50% formamide (4). Plaques showing a stronger signal with the probe from hibernator heart than with the probe from euthermic heart were retrieved and subjected to two more rounds of screening to confirm the stronger signal and to 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 heart of hibernating ground squirrels and to determine whether the same genes were also up-regulated in other organs (kidney, liver, skeletal muscle). Total RNA was isolated from tissue samples using Trizol, separated in a formaldehyde agarose gel using 16 μg of total RNA/lane, and then blotted onto a Nytran membrane (Schleicher & Schull) using a standard procedure (34). The quality of total RNA was determined by identification of the 18S and 28S ribosomal bands. The DNA inserts from the tertiary screened clones were cut from the plasmid vector using *Bam*H I 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 using a Geneclean III kit (Bio 101,

Vista, CA, U.S.A.) and were labeled with ^{32}P using a random primer procedure (34). Northern blots were hybridized at 42°C, using labeled probes (8×10^6 cpm/10 ml hybridization solution) and a sodium phosphate hybridization solution (11). 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 ddH_2O to visualize ribosomal RNA bands and destained in ddH_20 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. Furthermore, the staining of the blot confirmed that the transfer from the gel to the Nytran membrane had been successful and that there was no major degradation of RNA in any lane. RNA transcript sizes were estimated from a plot of RNA molecular mass (BRL standards) versus migration distance in the formaldehyde gel.

DNA Sequencing

Isolated clones were sequenced by Bio S&T Inc. (Lachine, Quebec, Canada) 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 (ORF) for each clone were loaded into a BLAST program at NCBI (U.S.A.) for a similarity search in GenBank.

RESULTS

Identification and Isolation of Up-Regulated Clones during Hibernation

A cDNA library was successfully constructed with the poly(A)$^+$ RNA isolated from heart of hibernating ground squirrels. The titer was $\sim 5 \times 10^5$ plaque-forming units/ml. About

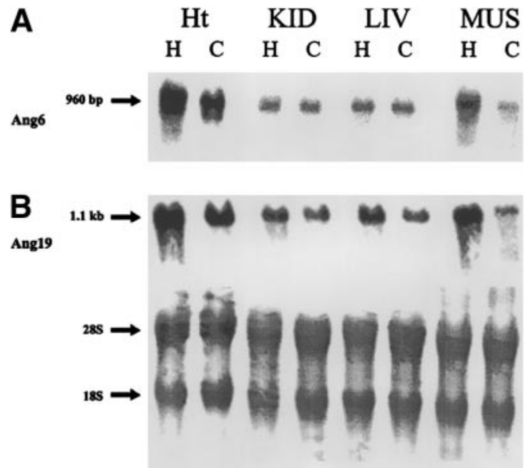

FIG. 1. Northern blot analysis of total RNA from heart of euthermic and hibernating golden-mantled ground squirrels, *S. lateralis,* probed with the hibernation-responsive clones isolated through differential screening of a cDNA library constructed from hearts of hibernating ground squirrels. (A) Blot probed with Ang6. (B) Blot probed with Ang19. Also shown are the methylene blue-stained 28S and 18S ribosomal RNA bands; all blots were stained before probing to verify that RNA was not degraded and that there was equal loading in the lanes. Tissues are heart (Ht), kidney (KID), liver (LIV), and leg skeletal muscle (MUS) from hibernating (H) versus euthermic control (C) animals. A representative blot of three trials is shown.

350,000 plaques from the cDNA library were differentially screened using ^{32}P-labeled single-stranded cDNA probes made from poly(A)$^+$ RNA from heart of hibernating versus euthermic animals. A total of eight clones showing stronger signal in heart of hibernators were isolated after the third round of differential screening. Northern blot analysis using the ^{32}P-labeled cDNA inserts from isolated clones as probes confirmed a positive up-regulation during hibernation in heart for two clones: Ang6 and Ang19 (Fig. 1). The Ang6 probe hybridized with a single band on the Northern blot with a size of ~960 nucleotides, whereas hybridization with the Ang19 probe showed a single band with a size of ~1100 nucleotides.

DNA Sequence Analysis of Ang6 and Ang19

Ang6 and Ang19 were subjected to automated sequencing. The results for Ang6 showed

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CGGGCGCTGGGCCAGAACCCCACACAAGCCGAGGTGCTCCGTGTCCTGGGGAAGCCAAAA   60  NUCLEOTIDE
 R  A  L  G  Q  N  P  T  Q  A  E  V  L  R  V  L  G  K  P  K   20  AMINO ACID

CAAGAAGAACTTAATAACAAGATGATGGATTTTGAAACGTTCCTGCCCATGCTCCAGCAC  120
 Q  E  E  L  N  N  K  M  M  D  F  E  T  F  L  P  M  L  Q  H   40

ATATCCAAGAATAAGGACACGGGCACATATGAGGACTTCGTGGAGGGGCTGCGGGTCTTC  180
 I  S  K  N  K  D  T  G  T  Y  E  D  F  V  E  G  L  R  V  F   60

GACAAGGAGGGCAACGGCACTGTCATGGGTGCCGAGCTCCGCCATGTGCTGGCCACACTG  240
 D  K  E  G  N  G  T  V  M  G  A  E  L  R  H  V  L  A  T  L   80

GGGGAGAGGCTGACAGAGGATGAGGTGGAGAAATTGATGGCTGGGCAAGAGGACTCCAAT  300
 G  E  R  L  T  E  D  E  V  E  K  L  M  A  G  Q  E  D  S  N  100

GGCTGCATCAACTATGAAGCATTTGTGAAGCATATCATGGCCAGCTGAgcctcccacagg  360
 G  C  I  N  Y  E  A  F  V  K  H  I  M  A  S  *               115

gagcccagggagggcgagctggggatgtctcatctcccatcatgatgctgacaccagtgg  420
cctggagctgtgggaaggaggggagtggaccaaggctccggcacaagccctcagagctct  480
ctgcacggttgtcatctctgcctggggctgtgctcattgtgaatgcattctgtctccatc  540
cagagccttataaataaatgacttcctcctttcaaaaaaaaaaaaaaaa             590
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FIG. 2. cDNA sequence and putative amino acid sequence (in boldface) of hibernation-responsive clone Ang6 identified as encoding the ventricular isoform of myosin light chain 1. The stop codon is indicated by an asterisk and the polyadenylation tract is underlined. Accession No. in GenBank is AF170306.

a 590-bp cDNA insert that included a polyadenylation signal beginning at nucleotide 553 and a poly(A) tail starting at nucleotide 574 (Fig. 2; GenBank Accession No. AF170306). The sequence contained a single open reading frame, potentially encoding a polypeptide of 115 amino acids. No start codon was found but the C terminus was complete, terminating with a TGA stop codon at position 346–348. Data from a similarity search in GenBank showed that the clone had high similarity to the DNA sequence of mammalian genes coding for the ventricular isoform of myosin light chain subunit 1. A comparison of the deduced amino acid sequence with that of the protein from other mammalian and avian sources suggested that, although the C terminus was complete, nucleotides encoding 79–85 amino acid residues were missing from the N terminus. Very high sequence identity was found when the 115-amino acid sequence of ground squirrel $MLC1_v$ was compared with the corresponding portion of $MLC1_v$ from other species. Ground squirrel and human $MLC1_v$ (19, 23) showed 97.5% of residues identical. Compared with the sequence shown in Fig. 2, human $MLC1_v$ showed only 3 amino acid differences, substitutions of R, T, and S at positions 20, 26, and 114, respectively. The rat sequence also showed 97.5% identity (27) with 90% identity for chicken (30) and 80% for mouse $MLC1_v$ (7). Clone Ang6 had a long untranslated region after the C terminus (Fig. 2) but a similarity search using this untranslated region in the BLAST program in GenBank showed no relationship of this region to any known genes.

The DNA sequence of clone Ang19 contained 852 bp including the poly$(A)^+$ tail (Fig. 3; GenBank Accession No. AF169642). The result of a similarity search with this sequence indicated a high similarity to the mitochondrial gene (*Nad2*) encoding subunit 2 of NADH–ubiquinone oxidoreductase (ND2). Therefore, the cDNA sequence was translated using the genetic code for vertebrate mitochondria. A single ORF was found, starting at nucleotides 3–5 and ending at residues 825–827 with the stop codon TAA (Fig. 3). A comparison of the putative amino acid sequence of the ground squirrel clone with that of ND2 from other mammalian species revealed a complete C terminus but suggested that 73 amino acids were missing from the N-terminal portion of the ground squirrel clone. However, alignment of the 274 amino acid residues of the ground squirrel sequence with the equivalent C-terminal portion of the sequences from other mammals showed a high percentage of identical residues throughout. Sixty-six percent of the residues in the ground squirrel protein were identical with the human

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gcACAATAATTAACTTCATAAACTTAGGTCAATGAACCTTATCCAACCCACACAATCAAATTTCATCATTCATATTTACAATCGCACTCTCA   92   NUCLEOTIDE
   T  M  I  N  F  M  N  L  G  Q  W  T  L  S  N  P  H  N  Q  I  S  S  F  M  F  T  I  A  L  S      30   AMINO ACID
   I  L  F  *  N  *  L  S  *  *  *  *  M  T  *  T  T  *  *  Y  *  *  L  *  I  M  M  *  M  A           HUMAN

  ATAAAGATAGGACTAGCCCCATTTCACCTGTGAGTACCAGAAGTAACCCAAGGAATCCCACTTAAATCAGGCCTAATCATGCTAACATGA  182
   M  K  M  G  L  A  P  F  H  L  W  V  P  E  V  T  Q  G  I  P  L  K  S  G  L  I  M  L  T  W      60
   *  *  L  *  M  *  *  *  *  F  *  *  *  *  *  *  *  *  T  *  *  T  *  *  *  L  L  *  *  *

  CAAAAAATCGCTCCAATCTCCATCATTTACCAAATCGCTTATTCCATAAACTCTACCCTCATACTATTTATAGGAACCCTGTCAATTATA  272
   Q  K  I  A  P  I  S  I  I  Y  Q  I  A  Y  S  M  N  S  T  L  M  L  F  M  G  T  L  S  I  M      90
   *  *  L  *  *  *  *  *  M  *  *  *  S  P  *  L  *  V  S  *  L  *  T  L  S  I  *  *  *  *

  CTAGGAGGCTGAGGAGGACTTAACCAAACCCAACTACGAAAAATCTTAGCATATTCATCAATCGCCCATATAGGATGAATAATAGTAATC  362
   L  G  G  W  G  G  L  N  Q  T  Q  L  R  K  I  L  A  Y  S  S  I  A  H  M  G  W  M  M  V  I     120
   A  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  T  *  *  *  *  *  *  A  V

  GTTACATATAACCCAACCTTAACAACATTTAACCTAATCATCTATATCCTACTAACCTTCAACATATTTATACTTCTCTATCATTATAAA  452
   V  T  Y  N  P  T  L  T  T  F  N  L  I  I  Y  I  L  L  T  F  N  M  F  M  L  L  Y  H  Y  K     150
   L  P  *  *  *  N  M  *  I  L  *  *  T  *  *  *  I  *  *  T  T  A  *  L  *  *  N  L  N  S

  AATTCTACTACTACTTCCCTATCAAATCTATGAAATAAGTTTCCCCTTTTAGCTTCCATAATTTTAATTGTATTAATATCGCTAGGAGGA  542
   N  S  T  T  T  S  L  S  N  L  W  N  K  F  P  L  L  A  S  M  I  L  I  V  L  M  S  L  G  G     180
   S  T  *  *  L  L  *  *  R  T  *  *  *  L  T  W  *  T  P  L  *  P  S  T  *  L  *  *  *  *

  CTACCCCCCCTAACAGGATTTGCACCAAAATGAATTATTCTCAAAGAACTTATCTCAAACAACAATATTATTCTTTCCACATTAATAGCA  632
   L  P  P  L  T  G  F  A  P  K  W  I  I  L  K  E  L  I  S  N  N  N  I  I  L  S  T  L  M  A     210
   *  *  *  *  *  *  *  L  *  *  *  A  *  I  E  *  F  T  K  *  *  S  L  *  I  P  *  I  *  *

  ATATTAGCACTCCTAAATTTATACTTCTACACACGACTTATTTATTCAACATCCCTAACCCTATTTCCATCATTTAACAACGCAAAAATA  722
   M  L  A  L  L  N  L  Y  F  Y  T  R  L  I  Y  S  T  S  L  T  L  F  P  S  F  N  N  A  K  M     240
   T  I  T  *  *  *  *  *  *  *  L  *  *  *  *  *  *  *  I  *  *  L  *  M  S  *  *  V  *  *

  AAATGACAATTCGAAAACACAAAACCCATACCTCTCCTACCAAACTTTATCATCACTTCCACCCTTTCCCTTCCATTAATGCCCCTCCTC  812
   K  W  Q  F  E  N  T  K  P  M  P  L  L  P  N  F  I  I  T  S  T  L  S  L  P  L  M  P  L  L     270
   *  *  *  *  *  H  *  *  *  T  *  F  *  *  T  L  *  A  L  T  *  *  L  *  *  I  S  *  F  M

  ACACTACTAAACTAAaaaaaaaaaaaaaaaaaaaaaaaaa                                                    852
   T  L  L  N  *                                                                               274
   L  M  I  L
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FIG. 3. cDNA sequence and putative amino acid sequence (in boldface) of hibernation-responsive clone Ang19 isolated from the ground squirrel heart cDNA library and identified as encoding subunit 2 of NADH–ubiquinone oxidoreductase (ND2). Also shown for comparison is the C-terminal amino acid sequence of human ND2 (73 amino acids are missing at the N terminus). Nucleotide sequence was translated based on the genetic code for vertebrate mitochondrial DNA. The stop codon is indicated by an asterisk. Accession No. in GenBank is AF169642.

protein (Fig. 3) (1), with 63% for harbor seal (3), 58% for mouse (8), and 54% for wallaroo (22).

Tissue-Specific Expression

Tissue-specific levels of *MLC1*$_v$ and *Nad2* transcripts were assessed via Northern hybridization with Ang6 and Ang19 as probes and using total RNA samples from heart, kidney, liver, and hind leg skeletal muscle of hibernating and euthermic ground squirrels (Fig. 1). A comparison of transcript amounts in different organs of control, euthermic animals showed, not unexpectedly, that transcript levels for the ventricular isoform of *MLC1* were highest in heart with quite low levels of expression in control kidney, liver, and muscle. *Nad2* transcript levels in euthermic animals were also highest in heart but substantial expression was also seen in the other three organs.

To quantify changes in transcript levels between euthermic and hibernating states, band intensities were first normalized relative to the quantified density of their corresponding 28S ribosomal band to compensate for any unequal loading of RNA onto the formaldehyde gel or unequal transfer of RNA from the gel to the Nytran membrane. Normalized transcript levels in euthermic and hibernating states were then used to determine the ratio of transcript levels in hibernating versus control samples for four organs (Fig. 4). Hybridization with the Ang6 probe showed that transcript levels for *MLC1*$_v$ were much higher in heart and skeletal muscle

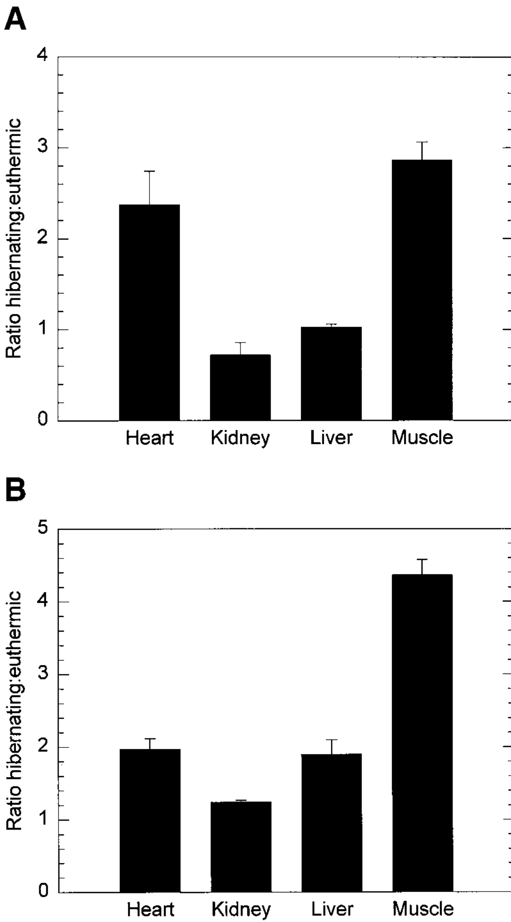

FIG. 4. Histograms showing the ratio of relative amounts of mRNA transcripts in organs of hibernating versus euthermic ground squirrels as determined by hybridizing with (A) probe Ang6 to detect *MLC1*$_v$ transcripts and (B) probe Ang19 to detect *Nad2* transcripts. Northern blots of total RNA (as per Fig. 1) were scanned, band intensities were quantified using Imagequant, and then band intensities were normalized relative to their corresponding 28S ribosomal bands. The ratios of normalized band intensities for hibernating versus control samples were then calculated. Data are means ± SE, $n = 3$ (except for $n = 2$ for kidney and liver for Ang19) separately run Northern blots; each trial contained an RNA sample prepared from a separate pool of tissue, each pool containing material from five animals.

of hibernating animals than in euthermic controls (Fig. 4A). The ratio of transcript levels in hibernator versus control tissues was 2.37 ± 0.64 (mean ± SE, $n = 3$) in heart and 2.87 ± 0.34 in muscle. Barton *et al.* (7) reported that the ventricular isoform of MLC1 and the slow skeletal muscle isoform were indistinguishable by size and sequence homology in the mouse; hence, it is not surprising that the Ang6 probe detected high transcript levels in skeletal muscle. In contrast, *MLC1*$_v$ transcript levels were unchanged in kidney and liver of hibernators, with ratios of transcript levels (hibernator/control) being 0.72 ± 0.24 and 1.02 ± 0.06, respectively.

Hybridization with the Ang19 probe showed higher transcript levels for *Nad2* in heart, liver, and skeletal muscle from hibernating animals, compared with euthermic controls (Fig. 4B). The ratio of transcript levels in hibernator versus control tissues was 1.97 ± 0.25 in heart, 1.90 ± 0.50 in liver, and 4.36 ± 0.37 in muscle. However, transcript levels were unchanged in kidney, the ratio being 1.24 ± 0.05.

DISCUSSION

Metabolic rate of hibernating golden-mantled ground squirrels can be reduced to as little as 4% of the basal metabolic rate of the euthermic animal (36). With such low rates of energy expenditure, the torpid state is clearly not a time to expect a major remodeling of cellular protein types and amounts. Indeed, a number of studies have now suggested that only modest restructuring occurs without large changes in mRNA or protein levels (13, 26, 32). Instead, the rates of numerous cellular processes are strongly suppressed in a coordinated manner primarily using reversible regulatory mechanisms. Thus, the rate of protein synthesis was reduced to only 0.04% of the mean euthermic rate in brain of *Spermophilus tridecemlineatus* (17) and activities of key metabolic enzymes fall sharply, including pyruvate dehydrogenase (which gates carbohydrate entry into the tricarboxylic acid cycle) activity, which was reduced to just 4% of the euthermic value in heart of hibernating *S. lateralis* (10), and Na^+K^+ATPase, which was lowered to 40–60% of euthermic values in hibernator organs (25). Hibernation-induced activity changes of both enzymes were due to reversible phosphorylation control, which also

allows for a rapid reversal and reactivation of these key metabolic loci upon arousal.

The present study shows, however, that up-regulation of selected genes, with probable enhanced production of their protein products, also contributes to adaptation during hibernation. Our results for differential screening of a cDNA library made from mRNA from heart of hibernating *S. lateralis* isolated two clones that were up-regulated during hibernation and identified as encoding the ventricular isoform of myosin light chain 1, a nuclear-encoded gene, and subunit 2 of NADH–ubiquinone oxidoreductase, a mitochondrially encoded gene. Transcript levels of both genes were increased by about twofold in heart of hibernating animals, compared with euthermic controls, and both genes were also strongly up-regulated by three- to fourfold in skeletal muscle. This suggests that the roles that both gene products play in hibernation may be generally applicable to all muscle types. A similar analysis of differential gene expression in heart of another ground squirrel, *S. tridecemlineatus,* identified two other genes that were up-regulated during hibernation: pyruvate dehydrogenase kinase isozyme 4 and pancreatic lipase (2). The former enzyme inactivates pyruvate dehydrogenase via protein phosphorylation and its up-regulation correlates well with the strong suppression of pyruvate dehydrogenase activity in hibernator heart (10), whereas expression of the lipase during hibernation is believed to aid triglyceride hydrolysis at low body temperatures.

The clones isolated from the *S. lateralis* heart cDNA library contained nucleotide sequences encoding the complete C-terminal end of their respective proteins but the N terminus was missing in both cases. However, the 115 amino acids of ground squirrel $MLC1_v$ and the 274 residues of ND2 shared a high percentage of identical residues with the respective proteins from other mammalian sources. Indeed, identity was 97.5% between the ground squirrel and the human or the rat $MLC1_v$ and 54–66% for ground squirrel ND2 compared with other mammals.

Muscle contraction is dependent on the sliding action of actin and myosin, powered by ATP energy. The myosin molecule is composed of two heavy chains (MHC) and four light chains (MLC), and the light chains are further classified as regulatory light chains (MLC2), which can be phosphorylated, or alkali light chains (MLC1 and MLC3; also called essential light chains) (24). Myosin alkali light chain 1 shows atrial ($MLC1_a$) and ventricular ($MLC1_v$) isoforms. The role of the alkali light chains is not clear (5, 6), but the isomers are differentially expressed in various muscle types and in non-muscle tissues (30), and it has been suggested that the isoforms have different contractile properties (6). The light chains are not essential for enzymatic activity, but their addition increases the velocity of muscle contraction (41). The alkali light chains could be involved in generating the force of contraction (42) and altered proportions of MHC and MLC could determine the contractile velocities of muscle (35, 44).

During hibernation, body temperature (T_b) falls by as much as 35°C and the hibernator heart must continue to function at a T_b of 0–5°C for several weeks at a time, a low temperature that would cause severe hypothermia damage to heart of most mammals. Furthermore, regulation of cardiac function must be maintained in hibernators to master the increased peripheral resistance at low T_b, and, thus, the cardiac contraction force must be maintained (47). Indeed, the force of contraction by ground squirrel heart papillary muscle increases two- to fivefold during the hibernation season, especially at low temperatures (46). Wang and Lee (47) suggested that the increase in sarcoplasmic reticulum (SR) Ca^{+2} storage and release observed in hibernating cardiac muscle could explain this enhanced contractility and this was supported by studies showing a decreased contractility at lower temperatures when SR function was impaired with caffeine (45).

However, the present study and that of Morano *et al.* (28) suggest that another contribution to increased contractility of heart during hibernation may come from a change in the composition of muscle proteins. Hearts of hibernating

ground squirrels had higher levels of mRNA transcripts for *MLC1*$_v$ than did euthermic animals and increased abundance of mRNA transcripts typically correlates with enhanced synthesis and increased levels of the protein product concerned. This implies that levels of the MLC1$_v$ subunit increase in heart during hibernation. This could change the ratio of alkali light chain subunit types within the myosin molecule, which could, in turn, alter the contractile properties of myosin at low body temperatures. Additional evidence of myosin restructuring during hibernation comes from a study of European hamsters (*Cricetus cricetus*), which showed that the relative abundance of myosin heavy chain isoforms, α and β, in heart changed with metabolic state (28). The β isozyme dominated in summer animals (79% of total) and in winter-active hamsters kept at 22°C, whereas during hibernation the activity of the α isozyme rose to 53% of total. Changes in the regulatory myosin light chains also occurred (28). Thus, both our results and those of Morano *et al.* (28) suggest that substantial myosin restructuring is a feature of hibernation.

Mitochondria contain their own DNA, and the mitochondrial genome of vertebrates consists of a double-stranded, circular DNA molecule (49), approximately 16 kb in length (14). The mtDNA of most vertebrates encodes 22 tRNAs, 2 rRNAs, and 13 proteins and contains only one major noncoding region (49). The encoded proteins are subunits of respiratory chain enzymes, including seven of the subunits of NADH–ubiquinone oxidoreductase, the first complex of the electron transport chain (29). ND2 is one of these. The entire complex in vertebrate tissues contains as many as 40 subunits, as well as nine iron–sulfur centers, two bound quinones, and one flavin mononucleotide bound as a prosthetic group (29).

Changes in the levels of mitochondrial transcripts for NADH–ubiquinone oxidoreductase and other electron transport chain proteins have been observed as responses to environmental stresses in various animals and cell lines, and these plus the evidence of *Nad2* up-regulation in hibernator heart, skeletal muscle, and liver suggest that changes in subunit availability or subunit composition of these complexes may serve different functional needs. A down-regulation of mitochondrial transcripts, but not cytoplasmic transcripts, was seen during oxidative stress in mammalian fibroblasts (15), and ischemia suppressed the expression of mitochondrial, but not nuclear, genes encoding subunits of cytochrome *b* in rat kidney (43). Exposure to 4°C differentially increased mitochondrial transcript levels in the liver of rats (21). In anoxia-tolerant turtles, mitochondrial transcripts encoding subunit 5 of ND as well as subunit 1 of cytochrome oxidase were up-regulated in liver during anoxic submergence (12), as were ND subunit 4 and cytochrome *b* in turtle brain (W. G. Willmore and K. B. Storey, unpublished). Thus, it appears that increased transcription of mitochondrial genes can be a response to both hypothermia and hypoxia and one or both might be the stimulus for *Nad2* up-regulation in *S. lateralis* heart, for, in addition to the hypothermic conditions of hibernation, hypoxic episodes could also arise due to apnoic breathing patterns in the torpid state.

In summary, this study identified two genes that are up-regulated in heart and other organs of hibernating golden-mantled ground squirrels; these encode the ventricular isoform of myosin light chain 1 (MLC1$_v$) and subunit 2 of NADH–ubiquinone oxidoreductase (ND2). Transcripts of both were approximately twofold higher in heart and three- to fourfold higher in skeletal muscle during hibernation versus euthermia. Elevated MLC1$_v$ transcript levels could result in changes to the myosin subunit composition during hibernation, which might alter muscle contractility to improve hypothermic function. Differential expression of the ND2 gene suggests that changes in subunit composition may also affect the function of the electron transport chain during hibernation. Not only do these data indicate that adaptive changes to selected cellular functions (contraction, electron transport chain) are needed for successful hibernation but they also suggest that these same functions may be impaired during hypotherma in nonhibernating species, providing targets for further re-

search on hypothermia survival and cold preservation of organs.

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