Detection of differential gene expression in brown adipose tissue of hibernating arctic ground squirrels with mouse microarrays

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Yan, Jun, Adlai Burman, Calen Nichols, Linda Alila, Louise C. Showe, Michael K. Showe, Bert B. Boyer, Brian M. Barnes, and Thomas G. Marr. Detection of differential gene expression in brown adipose tissue of hibernating arctic ground squirrels with mouse microarrays. Physiol Genomics 25: 346–353, 2006. First published February 7, 2006; doi:10.1152/physiolgenomics.00260.2005.—Hibernation is an energy-saving strategy adopted by a wide range of mammals to survive highly seasonal or unpredictable environments. Arctic ground squirrels living in Alaska provide an extreme example, with 6- to 9-mo-long hibernation seasons when body temperature alternates between levels near 0°C during torpor and 37°C during arousal episodes. Heat production during hibernation is provided, in part, by nonshivering thermogenesis that occurs in large deposits of brown adipose tissue (BAT). BAT is active at tissue temperatures from 0 to 37°C during rewarming and continuously at near 0°C during torpor in subfreezing conditions. Despite its crucial role in hibernation, the global gene expression patterns in BAT during hibernation compared with the nonhibernating season remain largely unknown. We report a large-scale study of differential gene expression in BAT between winter hibernating and summer active arctic ground squirrels using mouse microarrays. Selected differentially expressed genes identified on the arrays were validated by quantitative real-time PCR using ground squirrel specific primers. Our results show that the mRNA levels of the genes involved in nearly every step of the biochemical pathway leading to nonshivering thermogenesis are significantly increased in BAT during hibernation, whereas those of genes involved in protein biosynthesis are significantly decreased compared with summer active animals in August. Surprisingly, the differentially expressed genes also include adipocyte differentiation-related protein or adipophilin (Adip), gap junction protein 1 (Gja1), and secreted protein acidic and cysteine-rich (Sparc), which may play a role in enhancing thermogenesis at low tissue temperatures in BAT.

As −2.9°C (3). Torpor is interrupted every 10–21 days throughout hibernation by arousal episodes, however, as arctic ground squirrels spontaneously rewarm to euthermic body temperature (36–37°C) and maintain that temperature for 15–24 h before slowly reentering torpor.

Once called the “hibernation gland,” brown adipose tissue (BAT) was originally observed in hibernators. In hibernating animals and most mammalian neonates, BAT is essential for heat production through nonshivering thermogenesis (NST). BAT can be found in body regions including interscapular, periaortic, perirenal, and intercostal regions (11). BAT mainly consists of brown adipocytes, endothelial cells, interstitial cells, and preadipocytes that can divide and differentiate to form new brown adipocytes. In contrast to white adipose tissue (WAT), BAT is richly vascularized and densely packed with mitochondria, which give rise to its brown color. Brown adipocytes in the BAT contain many small lipid droplets (multilocular), whereas white adipocytes in WAT contain one big lipid droplet (unilocular). In BAT, uncoupling protein 1 (UCP1), a BAT-specific protein, transports protons or proton equivalents across the mitochondria inner membrane without synthesizing ATP, thus resulting in part of the energy stored in the proton gradient being dissipated as heat.

BAT deposits persist in adult hibernators year round, although their mass, density of mitochondria, and UCP1 content increase two- to threefold in fall before hibernation (5, 24). Active NST in BAT at euthermic tissue temperatures during summer occurs under the circumstances of cold stress or diet-induced thermogenesis. NST in hibernation, however, occurs in BAT at tissue temperatures that can vary from 0 to 37°C as animals rewarm over 3–6 h during arousals and also continuously over weeks at tissue temperatures near 0°C in steady-state conditions during torpor in animals hibernating in subfreezing environmental temperatures (9). It is not known whether different gene products are required for active thermogenesis in tissue temperatures of 0 vs. 37°C.

The interspersed phylogenetic distribution of hibernating and nonhibernating species has led to the hypothesis that hibernation phenotype results from the differential expression of existing genes rather than the creation of novel genes (6, 12, 27). In support of this hypothesis, a number of differentially expressed genes of known function have been identified in tissues of different hibernating species at both mRNA and protein levels (6, 12). A switch from carbohydrate metabolism to fatty acid metabolism during hibernation has been demonstrated in various tissues (2, 4, 26, 32). A limited number of genes including muscle- and heart-type fatty acid binding protein, adipocyte-type fatty acid binding protein, cytochrome-c oxidase subunit 1, ATP synthase 6/8, and peroxisome prolif-
temperature change during a natural hibernation season of arctic ground squirrels, a small (10 g) temperature-sensitive data logger was implanted in the peritoneal cavity of a free-living adult male that was released at its home burrow and then recaptured a year later to retrieve the logger. Further details about the environment of the ground squirrel and the logger and surgery procedure were described previously (23). Animal protocols were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee.

Sample preparation. Total RNA was prepared from frozen tissue by homogenizing directly in Tri-reagent (Molecular Research Center), using a Polytron (Brinkman) with a 7-mm generator (Kinematica). RNA was processed according to the manufacturer’s directions, and RNA quality was assessed by 1% agarose gel electrophoresis using SYBR Green II poststaining (Cambrex). All RNA samples were subsequently linearly amplified with a modified T7 Eberwine procedure (30), and 2 μg of the amplified RNA was labeled with 60 μCi of [32P]dCTP as previously described (21).

Microarrays. Nylon microarrays (MA07, MA08, MA10, and MA11) printed with PCR-amplified mouse cDNAs were obtained from The Wistar Genomics Facility (http://www.wistar.upenn.edu/genomics). These arrays carry ~38,000 clones (9,600 on each array) including 4,392 sequence-verified clones (Research Genetics/In vitrogen mouse plates 1–17 and 51–79); 11,000 clones from the Mouse Brain Molecular Anatomy Project (“BMAP” clones, Research Genetics/In vitrogen); 15,000 sequence-verified clones from the National Institute of Aging (NIA Mouse 15K) mouse cDNA development libraries created from pre- and periimplantation embryos, embryonic day 12.5 female gonad/mesonephros, and newborn ovary; and 7,000 sequence-verified clones from the NIA 7.4K Mouse cDNA clone set with no redundancy within the set or with NIA Mouse 15K (http://lgsun1.grc.nia.nih.gov/cDNA/cDNA.html). The NIA clones are reported to be primarily full length. An additional 700 selected clones with functions related to the immune system were purchased from Research Genetics/In vitrogen. The gene lists, including GenBank accession numbers and Unigene build 134 cluster assignments, are available at http://showelab.wistar.upenn.edu/Wistar_Showe_Lab_Gene_lists.htm.

Hybridization and washing. Filters MA07, MA08, and MA11 were hybridized with the same labeled target in the same hybridization bottle with filters separated by nylon mesh. Filter MA10 was hybridized separately at an earlier date with the same aRNA preparation as used for the rest of the filters. The hybridization was carried out for 16 h at 42°C followed by 24 h at 35°C in 5 ml of MicroHyb buffer (Research Genetics). The lower temperatures and extended hybridization times were implemented to promote cross-species hybridization. Filters from both hybridizations were batch washed in a large container for consistency. Filters were rinsed at room temperature with 2× SSC-1% SDS to remove residual probe and MicroHyb solution and then transferred to preheated wash solutions in a temperature-controlled shaking water bath. One liter of solution was used for filters in the batch. Filters were washed twice for 30 min in 2× SSC-1% SDS at 50°C and then once for 30 min in 0.5× SSC-0.5% SDS at 55°C. Filters were then exposed to phosphorimager screens for 6 days and scanned at 50-μm resolution in a Storm Phosphorimager. The image analysis was performed with the ImaGene program (Biodiscov). 

Microarray data analysis. Only spots on the array satisfying flag = 0 (nonempty spot) and signal median > background median were included in the analysis. Local background median was subtracted from signal median as background-corrected signal. Background-corrected signals were divided by their median on the array as the normalized expression values. The normalized expression values of
technical replicates (samples split from the same total RNA extraction) were averaged as the final expression values and then again normalized. The final normalized expression values were log₂ transformed. Log fold change is defined as the difference between the means of log₂-transformed expression values in the winter hibernating group and the summer active group, i.e., $(\log_2(\text{expression value}_{\text{hibernating}}) - \log_2(\text{expression value}_{\text{active}}))$. The $P$ value of the two-tailed Student’s t-test was calculated by Excel (Microsoft). We used $P < 0.05$ and log fold change > 1 as the criteria for significant difference. GenBank accession numbers of all probes on the arrays were uploaded to Stanford Source (http://source.stanford.edu) to obtain the gene names and symbols. We combined multiple probes for the same gene and removed the unannotated (no gene symbols assigned) probes. When evaluating multiple probes corresponding to the same gene, we chose the probe with the lowest $P$ value. The significant genes were uploaded to GOMiner (http://discover.tau.ac.il/gominer/index.jsp) to identify the significant Gene Ontology (GO) categories. The enrichment in each category was calculated as the proportion of changed genes in the category relative to the expected proportion on the arrays: the ratio of changed genes in the category divided by the total number of genes in the category, divided by the same ratio for the genes on the entire arrays. The $P$ value of the significance of each GO category was calculated by one-sided Fishers exact test (33). All microarray data series were submitted to NCBI Gene Expression Omnibus (GEO) with accession number GSE4326.

### Primer design
The specificity of real-time PCR requires using exact or near-exact gene-specific primer sequences. Spermophilus lateralis and Spermophilus tridecemlineatus share on average 99% sequence identities with the arctic ground squirrel (Supplemental Table S1). We combined 81 arctic ground squirrel sequences (GenBank accession nos. DQ333962–DQ334051) sequenced from our pilot study with 8,816 S. lateralis sequences and 2,343 S. tridecemlineatus sequences downloaded from GenBank into a ground squirrel database. The mouse RefSeq sequences of the significant genes identified on the arrays were searched against the ground squirrel database with the BLAST program (1). Among the genes with identified orthologous sequences and the sources from which they are derived are listed in Supplemental Table S2. From the significant genes, we identified 668 probes that were significantly overexpressed during hibernation and 390 probes that were significantly downregulated.

### RESULTS
The body temperature of a typical free-living male arctic ground squirrel during hibernation is shown in Fig. 1. This 1-kg ground squirrel maintained core body temperature between 35 and 40°C during August and September before becoming sequestered in its burrow and beginning hibernation on October 1, 1998. For the duration of the 6-mo hibernation season, body temperature varied from 3°C to −2°C during torpor bouts that lasted 10–24 days between nine arousal episodes when body temperature returned to euthemic temperatures for 10–15 h.

Four Wistar mouse arrays (MA07, MA08, MA10, MA11) each carrying 9,600 probes were used to screen the differentially expressed genes between winter hibernating and summer active arctic ground squirrels. We used $P < 0.05$ and log fold change > 1 as the criteria for significant difference. Combining all four arrays, we identified 668 probes that were significantly overexpressed during hibernation and 390 probes that were significantly downregulated.
were significantly underexpressed during hibernation among the 38,400 probes on the arrays. When there were multiple probes corresponding to the same gene on the arrays, their results were often consistent. For example, five of eight probes distributed across four arrays corresponding to Succinate-CoA ligase, ADP-forming, β-subunit (Sucld2) showed significant overexpression in winter hibernating squirrels. This indicates that our array results are highly reproducible. After we removed the unannotated probes and combined multiple probes corresponding to the same gene on the arrays, we identified 408 genes overexpressed during hibernation and 217 genes underexpressed during hibernation among the 11,670 annotated genes on the arrays. The top five overexpressed genes during hibernation according to their log fold changes are Sucld2 (4.37), Gap junction protein α1 (Gja1, 3.70), Growth hormone-inducible transmembrane protein (Ghirm, 3.47), Progesterone receptor membrane component 1 (Pgrmc1, 3.39), and Dihydropyrimidinase dehydrogenase (Dld, 3.27), where the numbers in parentheses are log fold changes. The top five underexpressed genes during hibernation are Eukaryotic translation elongation factor 1γ (Eef1γ, −2.95), expressed sequence variant (Evesv, −2.52), Ribosomal protein S10 (RpS10, −2.36), Ribosomal protein L35 (RpL35, −2.36), and Syntaxin 6 (Stx6, −2.34).

The significant differentially expressed genes identified on the arrays were classified according to their GO categories. The significantly overrepresented GO categories and their gene members are listed in Tables 1 and 2. The important functional categories are summarized below.

**Metabolism.** The genes involved in fatty acid metabolism, the tricarboxylic acid (TCA) cycle, electron transport, and ATP synthesis were significantly overexpressed in hibernating animals compared with summer active animals. These include acetyl-CoA synthetase long-chain (Acsl1), acetyl-CoA dehydrogenase (Acadm, Acadl), and acetyl-CoA acetyltransferase (Acaac2) in fatty acid metabolism; succinate-CoA ligase (Sucld2, Sucld1), isocitrate dehydrogenase (Idh3a, Idh3b), and malate dehydrogenase (Mdh1) in the TCA cycle; cytochrome c somatic (Cycs), cytochrome b5 (Cyb5), NADH dehydrogenase (Ndufa8, Ndufb9), ubiquinol-cytochrome c reductase (Uqcrbc, Uqcrf1, Uqrh), and cytochrome-c oxidase (Coxbc, Cox7c, Cox17) in electron transport; and ATP synthase (Atp5a1, Atp5b, Atp5f1) in ATP synthesis. Lactate dehydrogenase (Ldh, Ldhb) involved in anaerobic metabolism was significantly overexpressed during hibernation. Methylmalonyl-CoA mutase (Mut) was also significantly overexpressed during hibernation.

**Protein biosynthesis.** The genes involved in protein biosynthesis were significantly underexpressed during hibernation, including Eukaryotic translation elongation factor 1α (Eef1d, Eef1g), translation initiation factor (Eif3s5), and a total of 23 ribosomal proteins.

**Transport.** Heart- and muscle-type fatty acid binding protein (Fabp3) responsible for intracellular transport of fatty acids was significantly overexpressed during hibernation. Solute carrier family 25 member 20 or carnitine/acyl-carnitine translocase (Slc25a20 or Cact) located at the mitochondrial inner membrane, where it transfers fatty acylcarnitines into mitochondria, was also significantly overexpressed during hibernation.

**Heat shock proteins.** There were 37 heat shock proteins present on the arrays. Heat shock 70-kDa protein 9A (Hspa9a) and DnaJ (Hsp40) homolog, subfamily B, member 9 (Dnajb9) were overexpressed during hibernation, whereas heat shock 10-kDa protein 1 (Hsp1) and heat shock 90-kDa protein 1α (Hspa1a) were underexpressed during hibernation.

**Antioxidant proteins.** Two isoforms of superoxide dismutase, Sod1 and Sod2, were both overexpressed during hibernation.

**BAT differentiation and remodeling.** Adipocyte differentiation-related protein or adipophilin (Adfp) and Gap junction protein 1 (Gja1) were overexpressed during hibernation. Secreted protein acidic and cysteine-rich (Sparc) and β-actin (Actb) were significantly underexpressed during hibernation.

**Others.** Asparagine synthetase (Asns) and Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation proteins (Ywhe, Ywhag, Ywhaz) were overexpressed during hibernation, whereas transforming growth factor β1-induced transcript 4 (Tgb14) and hemoglobin α1 (Hba1) were underexpressed during hibernation.

Quantitative real-time PCR was used to test 33 genes differentially expressed during hibernation, including 28 overexpressed and 5 underexpressed genes that were identified on the arrays. Ground squirrel specific primer pairs were used to guarantee the specificity of PCR reactions. The results are listed in Table 3. In the quantitative real-time PCR assay, all 33 genes showed changes of expression levels between hibernating animals and summer active animals in the same direction as the array results. Twenty-six genes (79%) showed significant differences ($P < 0.05$) in real-time PCR assay. Among the other seven genes, Atp5b ($P = 0.08$), Cox5c ($P = 0.06$), and Tgb14 ($P = 0.06$) showed less significant differences ($P < 0.1$), whereas the differences in Acaac2, Sucld1, Idh3a, and Sld were not significant ($P > 0.1$) in the real-time PCR assay. The log fold changes of 33 genes in the real-time PCR are plotted against those on the arrays in Fig. 2. The linear fit of the plot gives $y = 0.5885x + 0.0943$ ($R^2 = 0.4678$), where $y$ is the real-time PCR result and $x$ is the array result. Three additional genes that were absent on the arrays (Ucp1, Hsl, Fabp4) were also tested in the real-time PCR assay. The Ucp1 level was increased by 5.67-fold ($P < 0.001$), and the Hsl level was increased by 2.96-fold ($P = 0.03$), whereas the Fabp4 level was not significantly changed ($P = 0.805$) in hibernating animals compared with summer active animals.

**DISCUSSION**

Our results are consistent with the previous studies of differential gene expression in BAT during hibernation. The

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**Table 1. Significant GO categories classified by GOminer**

<table>
<thead>
<tr>
<th>GO Category</th>
<th>Total Genes on Arrays</th>
<th>Differentially Expressed Genes</th>
<th>Enrichment</th>
<th>Log2(ε(P))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA cycle</td>
<td>22</td>
<td>6</td>
<td>6.87</td>
<td>−3.79</td>
</tr>
<tr>
<td>Electron transport</td>
<td>177</td>
<td>17</td>
<td>2.42</td>
<td>−3.22</td>
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<tr>
<td>ATP synthesis</td>
<td>21</td>
<td>5</td>
<td>5.95</td>
<td>−2.93</td>
</tr>
<tr>
<td>Fatty acid metabolism</td>
<td>88</td>
<td>10</td>
<td>2.86</td>
<td>−2.62</td>
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<tr>
<td>Protein biosynthesis</td>
<td>357</td>
<td>27</td>
<td>3.45</td>
<td>−8.04</td>
</tr>
</tbody>
</table>

The enrichment in each category was calculated as the proportion of changed genes in the category relative to the expected proportion on the arrays. The $P$ value of the significance of each Gene Ontology (GO) category was calculated by 1-sided Fishers exact test (33). TCA, tricarboxylic acid.
Table 2. Genes in each significant GO category identified by GOminer

<table>
<thead>
<tr>
<th>GO Category</th>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Accession No.</th>
<th>Log Fold Change</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TCA cycle</strong></td>
<td>Dihydrolipoamide S-succinylltransferase</td>
<td>Dist</td>
<td>AI849904</td>
<td>2.30</td>
<td>0.002</td>
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<td></td>
<td>Isocitrate dehydrogenase 3 (NAD+) α</td>
<td>Idh3a</td>
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<td>Isocitrate dehydrogenase 3 (NAD+) β</td>
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<td>AU199946</td>
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<td>Malate dehydrogenase 1</td>
<td>Mdh1</td>
<td>BG064914</td>
<td>1.50</td>
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<td>Succinate-CoA ligase, ADP-forming, β-subunit</td>
<td>Sucl2a</td>
<td>AI835580</td>
<td>4.37</td>
<td>&lt;0.001</td>
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<td>Succinate-CoA ligase, GDP-forming, α-subunit</td>
<td>Suclgl1</td>
<td>BG071511</td>
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<td>0.03</td>
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<td><strong>Electron transport</strong></td>
<td>Cytochrome-c oxidase, subunit V1c</td>
<td>Cox5c</td>
<td>BG085306</td>
<td>2.21</td>
<td>&lt;0.001</td>
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<td>Cytochrome-c oxidase, subunit V1C</td>
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<td>Cytochrome b5</td>
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<td>Cytochrome c, somatic</td>
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<td>Dihydroxyacetone dehydrogenase</td>
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<td>Electron transferring flavoprotein, dehydrogenase</td>
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<td>NADH dehydrogenase (ubiquinone) β subcomplex, 9</td>
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<td>Thioredoxin 1</td>
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<td>Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1</td>
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<td><strong>Fatty acid metabolism</strong></td>
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<td>Cavelin, caveolae protein 1</td>
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<td>Sterol carrier protein 2, liver</td>
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<td><strong>Protein biosynthesis</strong></td>
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<td>Eukaryotic translation initiation factor 3, subunit 5</td>
<td>Eif3s5</td>
<td>AI836722</td>
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<td></td>
<td>Finkell-Biskis-Reilly murine sarcoma virus, ubiquitously expressed; ribosomal protein S30</td>
<td>Fau</td>
<td>BG087448</td>
<td>-1.76</td>
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<td>Laminin receptor 1 (ribosomal protein SA)</td>
<td>Lam1</td>
<td>BG072282</td>
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<td>Ribosomal protein L10A</td>
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<td>Ribosomal protein L18A</td>
<td>Rpl18a</td>
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<td>Ribosomal protein L17</td>
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<td>BG072985</td>
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<td>Rplp1</td>
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<td>Ribosomal protein S14</td>
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<td>Ribosomal protein S16</td>
<td>Rps16</td>
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<td>Ribosomal protein S5</td>
<td>Rps5</td>
<td>BI076433</td>
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<td>Ribosomal protein S8</td>
<td>Rps8</td>
<td>BG085869</td>
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<td>Ribosomal protein S9</td>
<td>Rps9</td>
<td>BG067495</td>
<td>-1.58</td>
<td>0.009</td>
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</table>

Genes in each significant GO category identified by GOminer and their log fold changes and P values in expression level differences on the arrays between hibernating animals and summer active animals are shown. Note that Acad, Acadm, and Cytb5 are involved in both electron transport and fatty acid metabolism.
overexpression of \textit{Fabp3} during hibernation on our arrays is consistent with References 13 and 19. The overexpression of cytochrome-c oxidase and ATP synthase on our arrays is consistent with Reference 20. However, as pointed out in References 20 and 28, the protein level of ATP synthase is low in BAT because the major role of BAT is heat production through futile proton cycling. This disagreement may be due to regulation at the posttranscriptional level. The overexpression of two isozymes of superoxide dismutase (\textit{Sod1} and \textit{Sod2}) during hibernation is consistent with Reference 10. Buzadzic et al. (10) showed that the activities of both \textit{Sod1} and \textit{Sod2} were increased in brown adipose tissue of European ground squirrels (\textit{Spermophilus citellus}) during hibernation. Superoxide dismutase acts as an antioxidant to destroy the harmful superoxide radicals in the body. The increased level of the antioxidant can protect the tissue from reactive oxygen species generated as a result of the intense metabolic activity sustained by this tissue during arousal.

![Log fold changes of 33 genes on the arrays vs. those in real-time PCR assay. The \(P\) values of their gene expression changes on the arrays and in real-time PCR assay are shown in Table 3.](http://physiolgenomics.physiology.org/)

**Table 3. Real-time PCR validation of 33 selected genes**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Name</th>
<th>Symbol</th>
<th>Array Experiment</th>
<th>Log fold change</th>
<th>(P) value</th>
<th>Real-Time PCR</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA synthetase long-chain family member 1</td>
<td>Acsvl</td>
<td>1.96</td>
<td>&lt;0.001</td>
<td>1.69</td>
<td>&lt;0.001</td>
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<tr>
<td>Acetyl-CoA dehydrogenase, median chain</td>
<td>Acadmn</td>
<td>2.29</td>
<td>&lt;0.001</td>
<td>1.28</td>
<td>&lt;0.001</td>
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<tr>
<td>Sterol carrier protein 2</td>
<td>Scp2</td>
<td>1.70</td>
<td>0.007</td>
<td>0.717</td>
<td>0.039</td>
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<tr>
<td>Acyl-CoA thioesterase 2</td>
<td>Acate2</td>
<td>1.04</td>
<td>0.003</td>
<td>1.88</td>
<td>0.008</td>
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<td>Acetyl-CoA acyltransferase 2</td>
<td>Acut2</td>
<td>1.23</td>
<td>0.007</td>
<td>0.38</td>
<td>0.20</td>
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<td>Succinate-CoA ligase, ADP-forming</td>
<td>Sacla2</td>
<td>4.37</td>
<td>&lt;0.001</td>
<td>2.47</td>
<td>&lt;0.001</td>
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<tr>
<td>Succinate-CoA ligase, GDP-forming, (\alpha)-subunit</td>
<td>Sacan1</td>
<td>1.11</td>
<td>0.03</td>
<td>0.10</td>
<td>0.65</td>
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<tr>
<td>Isocitrate dehydrogenase 3 (NAD(^{+})) (\alpha)</td>
<td>Idh3a</td>
<td>1.69</td>
<td>0.002</td>
<td>0.34</td>
<td>0.45</td>
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<tr>
<td>Malate dehydrogenase 1</td>
<td>Mdh1</td>
<td>1.50</td>
<td>0.004</td>
<td>1.05</td>
<td>0.037</td>
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<td>Cytochrome-c oxidase, subunit V1c</td>
<td>Cox6c</td>
<td>2.21</td>
<td>&lt;0.001</td>
<td>0.53</td>
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<td>Dihydrolipoamide dehydrogenase</td>
<td>Dld</td>
<td>3.27</td>
<td>0.001</td>
<td>0.44</td>
<td>0.18</td>
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<tr>
<td>Cytochrome (c), somatic</td>
<td>Ccys</td>
<td>3.25</td>
<td>&lt;0.001</td>
<td>0.72</td>
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<td>NADH dehydrogenase 1</td>
<td>Nda1a</td>
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<td>0.005</td>
<td>2.14</td>
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<td>Lactate dehydrogenase (\alpha)</td>
<td>Ldh(\alpha)</td>
<td>1.11</td>
<td>0.01</td>
<td>0.979</td>
<td>0.005</td>
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<tr>
<td>ATP synthase, (H^{+}) transporting mitochondrial F1 complex, (\beta)-subunit</td>
<td>Atp5b</td>
<td>2.29</td>
<td>&lt;0.001</td>
<td>1.51</td>
<td>0.08</td>
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<td>Fatty acid binding protein 3</td>
<td>Fabp3</td>
<td>2.54</td>
<td>0.004</td>
<td>4.26</td>
<td>&lt;0.001</td>
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<tr>
<td>Carnitine/acylcarnitine translocase</td>
<td>Cct</td>
<td>1.74</td>
<td>&lt;0.001</td>
<td>2.05</td>
<td>0.009</td>
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<td>Heat shock protein 9A</td>
<td>Hsp9a</td>
<td>2.62</td>
<td>&lt;0.001</td>
<td>0.76</td>
<td>0.006</td>
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<td>Superoxide dismutase 2</td>
<td>Sod2</td>
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<td>0.005</td>
<td>1.21</td>
<td>0.001</td>
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<td>Translation elongation factor 1 (\alpha)</td>
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<td>Ribosomal protein S16</td>
<td>Rps16</td>
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<td>0.005</td>
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<td>Ribosomal protein L3</td>
<td>Rpl3</td>
<td>-1.65</td>
<td>&lt;0.001</td>
<td>-0.67</td>
<td>0.023</td>
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<tr>
<td>Adipose differentiation-related protein</td>
<td>Adip1</td>
<td>0.833</td>
<td>0.01</td>
<td>3.26</td>
<td>&lt;0.001</td>
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<td>Growth hormone-inducible transmembrane protein</td>
<td>Ghitm</td>
<td>2.85</td>
<td>0.003</td>
<td>1.40</td>
<td>0.01</td>
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<td>Gap junction protein (\alpha)</td>
<td>Gja1</td>
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<td>0.001</td>
<td>2.17</td>
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<td>Secreted protein, acidic, cysteine rich</td>
<td>Sparc</td>
<td>-1.52</td>
<td>0.003</td>
<td>-2.14</td>
<td>&lt;0.001</td>
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<td>(\beta)-Actin</td>
<td>Actb</td>
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<td>Asparagine synthetase</td>
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<td>0.005</td>
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<td>Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, (\varepsilon) polypeptide</td>
<td>Ywhae</td>
<td>1.26</td>
<td>0.007</td>
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<td>Methylmalonyl-CoA mutase</td>
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<td>Protein tyrosine phosphatase 4a1</td>
<td>Ptp4a1</td>
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<td>Transforming growth factor (\beta1) induced transcript 4</td>
<td>Tgbf1i4</td>
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<td>RAB1, member RAS oncogene family</td>
<td>Rab1</td>
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</table>

Comparison between array experiment and real-time PCR assay for 33 selected genes is shown. Log fold change in real-time PCR was calculated from the difference in \(-C_T\) between hibernating animals and summer active animals, where \(C_T\) is critical threshold in real-time PCR. \(P\) value was calculated from 2-tailed Student’s \(t\)-test.
In BAT, NST is controlled by norepinephrine released by sympathetic nerves. Norepinephrine signaling through β-adrenoceptor activates hormone-sensitive lipase (Hsl) to cleave the triglyceride stored in the lipid droplet in brown adipocytes into free fatty acid and glycerol. In our real-time PCR assay, the Hsl level was significantly increased in hibernating animals compared with summer active animals. In cytosol, free fatty acid is bound with fatty acid binding proteins. Two types of fatty acid binding proteins exist in BAT: muscle and heart type (Fabp3) and adipose type (Fabp4). Hittel and Storey (19) showed that both types of fatty acid binding proteins are overexpressed during hibernation. We also showed that Fabp3 is overexpressed during hibernation on our arrays. Although Fabp4 was not present on our arrays, Fabp4 is not significantly overexpressed during hibernation in our real-time PCR assay. The fatty acid is activated to fatty acyl-CoA by acyl-CoA synthetases before entering mitochondria. On our arrays, acyl-CoA synthetase long-chain (Acsl1) is overexpressed during hibernation. Fatty acyl-CoA is transformed to fatty acyl-carnitine by carnitine palmitoyltransferase I and then transported into the mitochondria through carnitine/acyl-carnitine translocase (Cact) and recycled to fatty acyl-CoA by carnitine palmitoyltransferase II. This carnitine-mediated entry process is the rate-limiting step for fatty acid β-oxidation. On our arrays, Cact is significantly overexpressed during hibernation. After entering mitochondria, fatty acyl-CoA undergoes β-oxidation to generate acetyl-CoA. The enzymes involved in the first and last steps of the β-oxidation cycles, acyl-CoA dehydrogenase (Acadm, Acadl) and acyl-CoA acyltransferase (Acac2), are overexpressed during hibernation on our arrays. In mitochondria, acyl-CoA is further oxidized in the TCA cycle. Three of the eight enzymes in the TCA cycle, succinate-CoA ligase (Sucl2, Sucld1), isocitrate dehydrogenase (Idh3a, Idh3b), and malate dehydrogenase (Mdh1), are significantly overexpressed during hibernation on our arrays. Both fatty acid β-oxidation and the TCA cycle lead to the formation of the reduced electron carrier FADH and NADH, which are then oxidized by the electron transport chain. All three enzymes in the electron transport chain, NADH dehydrogenase (Ndufa8, Ndufb9), ubiquinol-cytochrome c reductase (Uqcrb, Uqcrcc2, Uqrcsf1, Uqcrh), and cytochrome-c oxidase (Cox6c, Cox7c, Cox17), are overexpressed during hibernation on our arrays. The respiration through the electron transport chain pumps protons out of the mitochondria matrix, leading to the formation of the proton gradient across the mitochondria membrane. As the protons or proton equivalents flow through Ucp1, the energy stored in the proton gradient is dissipated as heat. Although Ucp1 was not present on our arrays, we used real-time PCR to show that it was indeed overexpressed during hibernation. It is remarkable that nearly every step in the biochemical pathway leading to NST is elevated in BAT during hibernation. Under the above-freezing conditions of this experiment, thermogenic activity in the BAT of animals sampled during torpor should have been low and only activated during arousal. There is evidence that the translation of mRNA into proteins is inhibited during torpor (18, 29). The underexpression of a large number of genes involved in protein biosynthesis on our arrays also supports this view. The elevated mRNA levels of the genes involved in NST may be important for rapid translation after arousal to high body temperature (22). During hibernation NST has to work at tissue temperatures from 0 to 37°C in BAT, whereas the tissue temperature in BAT is always near 37°C in summer active animals. Among the differentially expressed genes identified on our arrays, some may potentially enhance the thermogenesis at low tissue temperature during hibernation. The overexpression of adfp gene during hibernation on our arrays indicates the enhanced brown adipocyte differentiation in BAT during hibernation. More mature adipocytes means higher thermogenic capacity in BAT. Gap junctions are intercellular channels for the diffusion of low-molecular-weight molecules between cells. Gja1 or connexin 43, a member of the gap junction proteins, has been shown to be overexpressed in the heart of hibernating hamsters, which may help hamsters avoid fibrillation during hibernation and arousal (25). The overexpressed Gja1 may enhance substrate transport through gap junctions in BAT and thus increase its thermogenic capacity during hibernation. Sparc is a matrix-associated protein that elicits changes in cell shape, inhibits cell cycle progression, and influences the synthesis of extracellular matrix. Sparc is underexpressed during hibernation on our arrays. Bradshaw et al. (7) showed that mice lacking Sparc exhibit an increase in the size and number of adipocytes. The underexpression of Sparc during hibernation may also play a role in the accumulation and differentiation of adipocytes in BAT. Actb, which is normally considered a housekeeping gene, is underexpressed during hibernation. This further suggests that BAT may have undergone structure remodeling to enable the differentiation and maturation of brown adipocytes to increase its thermogenic capacity at low tissue temperature during hibernation.

In conclusion, our microarray results not only agree with previous studies on BAT during hibernation but also have revealed the differential expression of a large number of genes not previously shown to be involved in hibernation. It is clear from this study that significant global changes occur at the mRNA level in BAT during hibernation. The fact that the genes involved in nearly every step of the biochemical pathway leading to NST are overexpressed in BAT during hibernation reflects the unique role of BAT as a “furnace” during hibernation. The differential expression of the genes involved in adipose differentiation, substrate transport, and structure remodeling may enhance the thermogenesis in BAT at low tissue temperature. Future studies including multiple stages throughout hibernation and concurring protein expression would give us a more complete understanding of the molecular mechanism underlying the hibernation phenotype.

ACKNOWLEDGMENTS

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GRANTS

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REFERENCES

Differential Gene Expression in Bat in Hibernation


