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Seasonally hibernating phenotype assessed through transcript screening

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Williams, Daryl R., L. Elaine Epperson, Weizhong Li, Margaret A. Hughes, Ruth Taylor, Jane Rogers, Sandra L. Martin, Andrew R. Cossins, and Andrew Y. Gracey. Seasonally hibernating phenotype assessed through transcript screening. *Physiol Genomics* 24: 13–22, 2005. First published October 25, 2005; doi:10.1152/physiolgenomics.00301.2004.—Hibernation is a seasonally entrained and profound phenotypic transition to conserve energy in winter. It involves significant biochemical reprogramming, although our understanding of the underpinning molecular events is fragmentary and selective. We have conducted a large-scale gene expression screen of the golden-mantled ground squirrel, *Spermophilus lateralis,* to identify transcriptional responses associated specifically with the summer-winter transition and the torpid-arousal transition in winter. We used 112 cDNA microarrays comprising 12,288 probes that cover at least 5,109 genes. In liver, the profiles of torpid and active states in the winter were almost identical, although we identified 102 cDNAs that were differentially expressed between winter and summer, 90% of which were downregulated in the winter states. By contrast, in cardiac tissue, 59 and 115 cDNAs were elevated in interbout arousal and torpor, respectively, relative to the summer active condition, but only 7 were common to both winter states, and during arousal none was downregulated. In brain, 78 cDNAs were found to change in winter, 44 of which were upregulated. Thus transcriptional changes associated with hibernation are qualitatively modest and, since these changes are generally less than twofold, also quantitatively modest. Unbiased Gene Ontology profiling of the transcripts suggests a winter switch to β-oxidation of lipids in liver and heart, a reduction in metabolism of toxic compounds and the urea cycle in liver, and downregulated electron transport in the brain. We identified just one strongly winter-induced transcript common to all tissues, namely an RNA-binding protein, RBM3. This analysis clearly differentiates responses of the principal tissues, identifies a large number of new genes undergoing regulation, and broadens our understanding of affected cellular processes that, in part, account for the winter-adaptive hibernating phenotype.

hibernation; torpor; microarray

HIBERNATION IS AN ADAPTIVE phenotype used by a wide range of mammalian species to conserve energy during the inclement months of winter. Seasonal hibernators, such as the golden-mantled ground squirrel, *Spermophilus lateralis*, cycle annually between a period of growth, fattening, and reproduction in summer and a period of minimal activity and fasting in winter. In summer, the animals are typical homeotherms, but during hibernation the animals become heterotherms, cycling between extended periods of torpor with low body temperatures (Tb) and brief arousal periods when Tb returns briefly to normal values (Fig. 1). This heterothermy requires that hibernating species exhibit a remarkable tolerance of core tissues to both profound cold and the damages associated with rapid warm reperfusion (reviewed in Ref. 6).

Hibernation is a tightly controlled and physiologically complex event. Thermal homeostasis is not simply abandoned, but instead the set point for thermoregulatory control is gradually lowered to a much-reduced Tb where it is maintained for the duration of the torpor bout. However, in contrast to poikilotherms, heat production can be spontaneously activated in a cooled, resting individual to arrest the decline in Tb or to maintain a constant, albeit lower, body temperature (5). In addition, endothermic heat production powers the spontaneous but brief arousals to the normothermic condition. By itself, reduced Tbs during hibernation should depress metabolism simply by virtue of the Arrhenius effect, but a controlled downregulation of metabolism occurs before the drop in Tb (20). In addition, there are major changes in the balance of intermediary metabolism with, for example, carbohydrate being replaced by lipid as the principal fuel. Furthermore, hibernation-induced bradycardia leads to reduced blood flow, and hibernating mammals have become important models of metabolic depression, ischemia, and various mechanisms accounting for hypometabolism and profound stress resistance of tissues (24).

Transitions between the summer-winter and torpid-aroused thermoregulatory states involve a complex physiological reorganization, all within strict circannual and circadian cycles. Changing expression of genes and their encoded products is likely to be a major part of this reorganization (36). A few attempts have been made to use unbiased screening methods at the mRNA level, such as differential or subtractive hybridization, although none of these studies has described the behavior of more than a handful of differentially expressed genes (recently reviewed in Ref. 6). Many of the identified genes appear to be involved in lipid metabolism and include the peroxisome proliferator-activated receptor proteins PPARγ and PGC-1α (13), pyruvate dehydrogenase kinase isoenzyme 4 and pancreatic lipase (1), apolipoprotein AI (15), and fatty acid-binding protein (22). For example, pancreatic lipase hydrolyzes triacylglycerides to liberate fatty acids for β-oxidation; this enzyme continues to show strong activity at cold temperatures, exhibiting >30% maximal activity at 0°C (1, 34).

While the expression responses of individual genes can offer specific insights, gene-by-gene investigations are unlikely to reveal the full scale and orchestration of events that underpin the complex physiological transition between summer and winter phenotypes, or between the torpid or aroused condition. However, contemporary functional genomic techniques do offer a viable means of generating a system-wide view of

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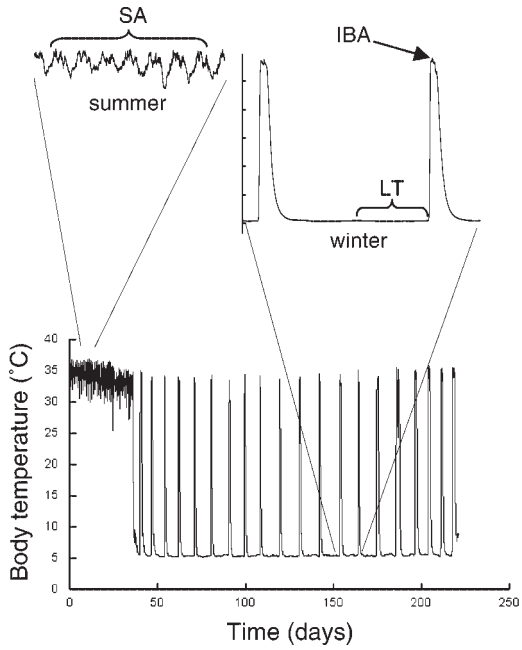


Fig. 1. Plot of body temperature (Tb) vs. time. Time points for RNA samples used in this study are indicated: SA, summer active; LT, late in a torpor bout; IBA, interbout aroused. Summer and winter *insets* show Tb vs. time for 8 and 16 days, respectively. Detailed information about each animal in the study is provided in Supplemental Table S1.

responses, since they quantify simultaneously the responses of many thousands of genes and their encoded products (17). Epperson et al. (14) undertook a protein screen of the golden-mantled ground squirrel, *S. lateralis*, using two-dimensional (2D) gels of liver tissue extracts and mass spectrometry to identify 69 proteins exhibiting changes in expression.

Here we have applied high-density microarray techniques using homologous *S. lateralis* cDNA probes in an open-ended and systematic search for genes in ground squirrels the expression of which changes with respect to season or hibernation stage. We have first constructed cDNA libraries from tissues of *S. lateralis* and selected ~10,000 clones for sequence characterization. Amplicons derived from these clones were used as homologous probes on a custom microarray that was used to compare expression profiles in brain, heart, and liver tissues isolated from ground squirrels at different points in their natural seasonal and hibernating cycles, focusing on changes between summer and winter animals and between animals in winter exhibiting deep torpor and those exhibiting arousal between torpor bouts. We have also compared responses between *S. lateralis* and the closely related 13-lined ground squirrel, *S. tridecemlineatus*.

METHODS

Animals and RNA sampling. All animal care and use procedures were approved by the University of Colorado Institutional Animal Care and Use Committee. *S. lateralis* were trapped in the field and maintained in the University of Colorado laboratory as described previously (26). Briefly, all animals were surgically implanted abdominally with radiotelemeters, and Tb was monitored for several months before death; the long-term history and thermoregulatory status at the death of each specimen were known in detail (a summary of this information is available in Supplemental Table S1; available at the *Physiological Genomics* web site).[1] Tissues from three main stages (torpid hibernators, interbout aroused hibernators, and summer active) of the circannual cycle of the squirrels were dissected out, rapidly frozen on liquid N_2, and then archived in a large tissue repository at −80°C. Liver tissue from telemetered 13-lined ground squirrels (*S. tridecemlineatus*) was generously provided by Dr. Hannah Carey (Univ. of Wisconsin, Madison) from a similar frozen tissue collection.

cDNA library and expressed sequence tag sequencing. Normalized cDNA libraries enriched for full-length clones were prepared from brain, heart, liver, testis, and embryo RNA samples. For each library except embryo (summer only), we pooled tissue samples isolated from animals killed at each stage of the circannual cycle. Total RNA was extracted from samples using Trizol (Invitrogen, San Diego, CA), and poly(A)+ RNA was purified over oligo(dT) resin (Ambion). During first-strand synthesis, adaptors containing the rare asymmetrical restriction sites for *Sfi*I were incorporated into the cDNA using a template-switching mechanism at the 5′-end of the RNA transcript (45). All libraries were normalized using the same pool of poly(A)+ RNA that was used for first-strand synthesis essentially as described (8). Second-strand synthesis of the normalized cDNA was performed using long-distance PCR (Advantage 2 polymerase, Clontech) using limited cycles (5–8 cycles). The double-stranded cDNA was digested with *Sfi*I, size fractionated over Sephacryl-500 (Amersham), and directionally cloned into the *Sfi*I sites of either λ-FLC (7) (heart, embryo, and testis) or λTriplEx2 (Clontech), and plasmids were released by mass excision in BM25.8 cre-recombinase-expressing *Escherichia coli*.

Additional cDNA libraries were prepared using suppression subtractive hybridization (12) to generate populations of cDNA fragments that were deliberately enriched for hibernation-regulated genes. RNA from summer active animals was subtracted against interbout aroused or torpid samples. The resulting cDNA fragments were either directly cloned in a T-vector (pGEMT-easy, Promega) or biotinylated, and their full-length hybrids were isolated by hybridization to first-strand cDNA populations as described (19). Libraries were propagated in DH10B *E. coli* (Invitrogen), and random colonies were picked into 384-well microtiter plates containing Luria-Bertani (LB) supplemented with 10% glycerol and antibiotic. In total, 32 plates (numbered 01–32) of cDNA clones were picked. Full details of the cDNA libraries constructed for this study can be found at **http://legr.liv.ac.uk/squirrelbase/library.htm**.

From each library, expressed sequence tag (EST) sequences were generated from three to five 384-well microtiter plates. The sequences were either 5′-orientated for the directionally cloned libraries or were sequenced in one direction using a T7 oligonucleotide for the T-cloned cDNAs. In total, 9,475 high-quality sequences were assembled into 4,998 unique sequences and annotated on the basis of the results of basic local alignment search tool (BLAST) homology and Conserved Domain Database (CDD) protein domain searches using "EST-ferret" (**http://legr.liv.ac.uk/EST-ferret**), a custom analysis pipeline that assembles and annotates cDNA sequences using a series of PERL scripts (W. Li, unpublished observations). We were able to annotate 3,082 of these putative genes based on their homology with previously characterized genes. The EST sequences generated during this project have been deposited in GenBank under accession numbers CO732290–CO741092.

Microarray construction and hybridization. The *S. lateralis* microarray was constructed using 12,288 PCR-amplified cDNA clones that were contact printed onto polyacrylic acid-coated glass slides (J. Lee, unpublished data) using standard protocols (**http://derisilab.ucsf.edu/microarray/index.html**). For each tissue sample, total RNA was extracted from tissues using Trizol (Invitrogen). Fluorescently labeled

[1] The Supplemental Material for this article (Supplemental Tables S1 and S2 and Supplemental Figs. S1 and S2) is available online at **http://physiolgenomics.physiology.org/cgi/content/full/00301.2004/DC1**.

cDNA was synthesized using amino-allyl adducts coupled to cysteine-3 (Cy3) and Cy5 fluors (**http://derisilab.ucsf.edu/microarray/index.html**) and compared with a reference RNA by hybridization to two arrays with reversal of the labeled fluorophores. The reference RNA pool was prepared for each tissue by pooling RNA samples isolated from animals sampled under all conditions. Microarrays were hybridized overnight at 65°C, washed, and scanned and the images scanned and quantified (GenePix 4000A, Axon Instruments). The microarray data were submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (GEO accession no. GSE2033).

Data analysis. For each array, spatial- and intensity-based trends in the data were removed by Lowess normalization (GeneSpring, Agilent). Data for each pair of fluor-reversed slides were combined by averaging the ratios derived from the two measurements. The expression of each cDNA was then normalized to the median ratio observed for that transcript in the summer animals. The expression data for redundant cDNAs that aligned in the same sequence contig and were thus represented by replica spots on array were averaged to yield the dereplicated data set that was employed in our analysis. Statistical tests were performed to identify genes that exhibited significant differences between the different conditions, using significance analysis of microarrays (SAM)(39). To estimate the percentage of genes identified by chance in these gene lists, 500 permutations of the measurements were tested, and the false discovery rate was adjusted so that 2 or fewer genes per list would be expected to be false positives. Genes that exhibited a $<$1.3-fold change in expression between summer active and the winter states were then removed from the lists of significant genes. To assess the enrichment of particular classes of genes in each list, each list was divided into 25 Gene Ontology (GO) (3) categories of the "biological process" domain, and the significance of their over- or underrepresentation relative to all identified genes with a GO classification on the array was estimated using a Fisher exact test (44) with a multiple-testing correction (4).

Table 1. *GO categories of genes in SquirrelBASE*

Categories	
Behavior	10
Cell communication	390
Cell adhesion	63
Cell recognition	4
Cell-cell signaling	34
Response to external stimulus	139
Signal transduction	212
Cell growth and/or maintenance	1308
Cell cycle	128
Cell growth	11
Cell motility	26
Cell organization and biogenesis	80
Cell proliferation	38
Homeostasis	10
Stress response	77
Transport	292
Metabolism	973
Amino acid and derivative metabolism	4
Biosynthesis	146
Carbohydrate metabolism	56
Catabolism	137
Coenzymes and prosthetic group metabolism	18
Electron transport	58
Energy pathways	38
Lipid metabolism	102
Nucleobase, nucleoside, nucleotide, and nucleic acid metabolism	396
Oxygen and reactive oxygen species metabolism	7
Protein metabolism	12
Regulation of metabolism	8
Xenobiotic metabolism	13

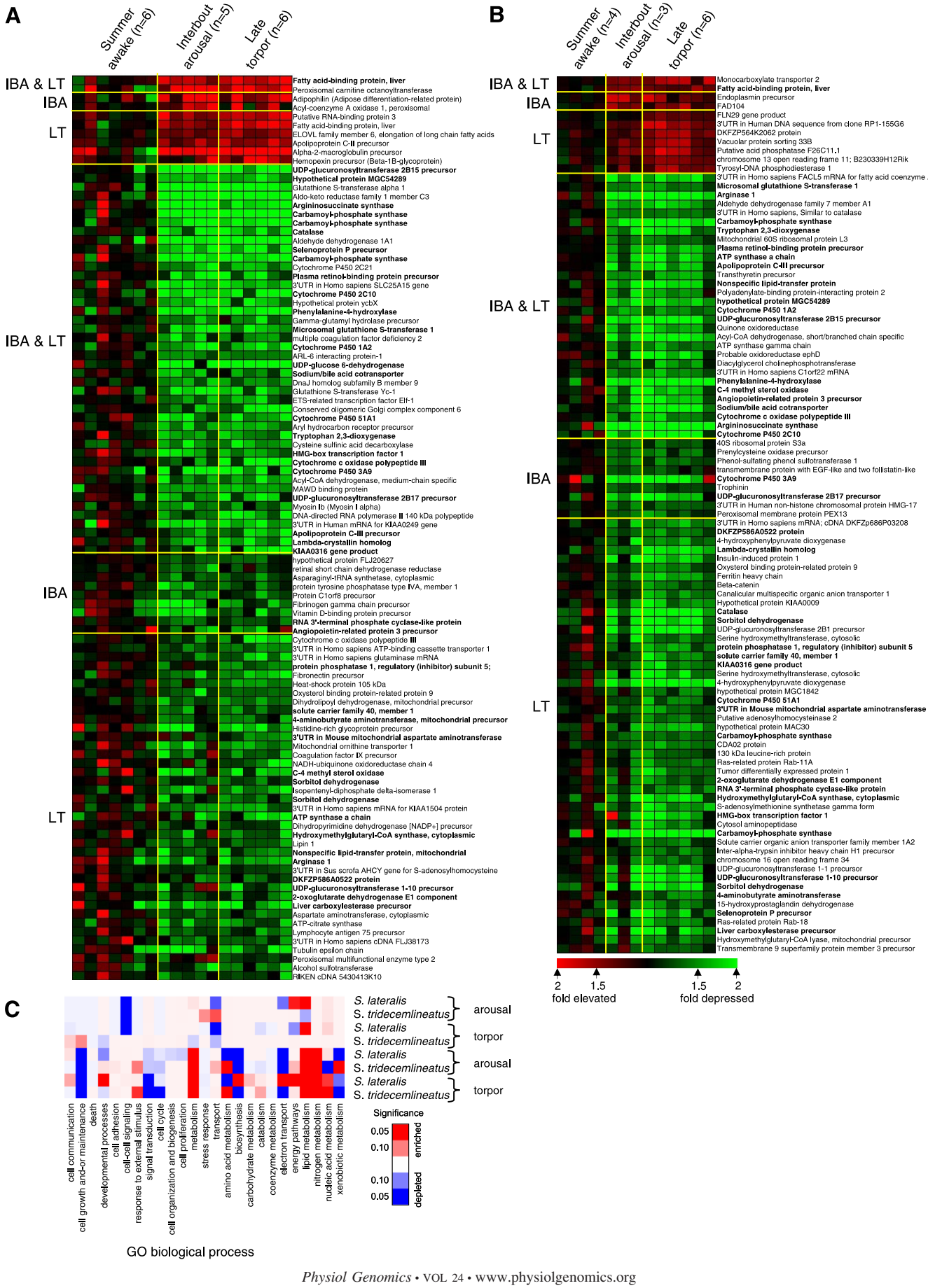
Full list of clones, their assembly into 3,263 singletons and 1,835 contigs, and their Gene Ontology (GO) categories can be viewed at **http://legr.liv.ac.uk/squirrelbase/squirrelbase_3_0/index.htm.**

RESULTS

The first step in establishing a transcriptional profile of the hibernating phenotype was to develop a high-quality resource of cDNA clones that would serve as microarray probes. We used two approaches to create this cDNA resource. First, we prepared normalized cDNA libraries from brain, liver, and cardiac muscle that were largely nonredundant and contained a high proportion of full-length genes. The source RNA for the libraries was isolated from animals killed throughout the year to capture the diversity of transcripts that might be expressed in different seasons. We also prepared cDNA libraries from testis and whole embryo, since these offer a high diversity of expressed transcripts (9). Second, we prepared subtracted cDNA libraries that were purposely enriched for genes that were regulated during the circannual cycle. cDNAs from these libraries were then isolated and used to construct a 12,288-spot *S. lateralis* microarray. EST analysis of 5′-sequence reads of a random selection of 8,874 clones revealed that as many as 5,109 putatively unique cDNAs were represented on the array. Of these, 3,082 have been furnished with putative identities from sequence alignment, and 1,798 of these have GO annotation terms including 109 from lipid metabolism and 396 from nucleic acid metabolism categories (Table 1). This provides broad representation from metabolic pathways; for example, the ground squirrel collection contained 68% of the genes identified from the mouse genome that are involved in fatty acid metabolism ("enzyme commission" category), and the corresponding figure for prostaglandin and leukotriene metabolism was 42%.

In total, 56 RNA samples from liver, brain, and cardiac muscle from animals with a precisely defined thermal history were hybridized to 112 microarrays. Each sample was assessed with reversal of the fluors used to label RNA for each replicate slide, since this greatly improves the accuracy of the resulting expression data (43). For each tissue, we created a balanced distribution of sample types by comparing five or more RNA samples from summer animals with a similar number of either interbout aroused or torpid animals (Fig. 1). The data presented in this study are confined to the 3,082 putatively nonredundant cDNAs with a putative identity. To identify genes that exhibited a significant difference in expression between summer active and the winter states, we employed a published signal-to-noise statistic that takes into account the multiple comparisons implicit in large-scale microarray studies (39) (Supplemental Table S2).

Liver responses. Liver was selected for analysis because of its central role in metabolic homeostasis and the processing of dietary nutrients. Figure 2*A* shows the relative expression of the cDNAs identified as differentially expressed between summer active and either interbout aroused or torpid animals, or both. The predominant feature of the transcriptional response of hibernators was a reduction in transcript level of a large number of genes, with 44 cDNAs exhibiting decreased transcript levels in both interbout aroused and late torpid animals, and 9 and 39 cDNAs reporting decreased transcript levels in either interbout aroused or torpid individuals, respectively (Fig. 2*A*). In contrast, just 10 cDNAs were elevated in the winter



animals, 2 of which were elevated in both winter conditions, while others were elevated specifically in interbout aroused (2 cDNAs) or torpid animals (6 cDNAs). Overall, there was a striking similarity in the transcript profiles of liver in the two hibernating conditions, with many genes exhibiting a similar trend toward either down- or upregulation in both winter states, although falling short of being significantly different from summer themselves. Indeed, just two cDNAs exhibited statistically significant differences in expression between the interbout aroused and late torpid groups, one an unknown EST and the other the anti-proliferation gene transducer of erb-B2 (*TOB1*)(Supplemental Table S2), and both genes were <1.4-fold elevated in late torpor.

To identify biological themes in this large-scale gene expression study, we profiled the distribution of genes in each list across 25 categories of the biological process domain as defined by the GO annotations (3) (**http://www.geneontology.org**). This procedure yielded a heuristic measure of the likelihood that a particular category was over- or underrepresented in each gene list compared with that expected by random selection from the entire list of annotated squirrel cDNAs present on the microarray. The resulting probability values are presented in Fig. 2*C* as a heat map, or GO-Matrix (refer to Ref. 18), to highlight the most distinctive functional themes of the liver response.

Hibernating mammals switch from carbohydrate to lipid as their principal fuel during the winter hibernating season (6). Consistent with this, 7 of the 10 transcripts that were identified as elevated in the hibernators were involved in lipid metabolism (Fig. 2*A*). Indeed, the GO-Matrix for *S. lateralis* (Fig. 2*C*) also revealed a statistically significant overrepresentation of genes in the GO category "lipid metabolism" in the list of elevated genes for both winter states ($P \leq 0.05$). The highest ranked gene that was elevated in both aroused and torpid animals was liver fatty acid-binding protein (*FABPL*; 1.5- and 1.9-fold induced in aroused and torpor, respectively). In ground squirrels, *FABPL* exhibits a temperature-independent binding capacity that serves to maintain lipid transport in the cold (37) and has been shown to be upregulated at the protein level in *S. lateralis* liver (14). Also induced was adipophilin, an adipose differentiation-related protein (*ADFP*) that localizes to the surface of intracellular lipid droplets and whose mRNA expression has previously been correlated with the accumulation of lipid droplets (21), as well as a long-chain fatty acyl elongase (*ELOVL6*) that is normally highly expressed in adipose tissue (28). We also detected the elevated expression of two peroxisomal genes, carnitine *O*-octanoyltransferase (*CROT*) and acyl-CoA oxidase 1 (*ACOX1*), that participate in the β-oxidation of fatty acids. Our data also revealed the differential regulation of two members of the apolipoprotein family with the transcript for CII (*APOC2*) being elevated during torpor, whereas that of CIII (*APOC3*) was decreased. This is pertinent to the metabolism of hibernating mammals, since *APOC2* stimulates lipoprotein lipase to hydrolyze triglycerides and thus provide free fatty acids for cells, whereas in contrast, *APOC3* inhibits lipase activity (32). α-2-Macroglobulin (*A2M*) transcript levels were also elevated in the liver, consistent with previous reports of the elevated expression of this gene at both the transcript and protein level in hibernating squirrels (15, 36). The expression of *A2M* may prevent blood clotting (35).

A major function of the liver is the breakdown of endogenous waste products, and genes involved in detoxification pathways dominated the lists of repressed genes in both winter states. For example, we detected a significant repression of five cytochrome P450 genes (of 10 P450 genes present on the array) and two UDP-glucuronosyltransferases (*UGT2B15, UGT2B17*), all of which participate in the metabolism and subsequent elimination of potentially toxic xenobiotics and endogenous compounds. Consistent with this biological theme, we also detected decreased transcript levels for genes involved in the reduction of electrophilic compounds including catalase (*CAT*), three glutathione-*S*-transferase genes (*GSTA1, GSTA3, MGST1*), aldehyde dehydrogenase (*1A1*), and aldo-ketoreductase (*AKR1C3*). These latter two genes are depressed also at the protein level during hibernation (15).

The liver is also responsible for the disposal of amino groups derived from the metabolism of amino acids in the form of urea. Our data revealed that the expression of almost every gene involved in the urea cycle is reduced in the liver of the torpid squirrels (Fig. 2*A*). Indeed, every gene printed on the array whose GO annotation was associated with the urea cycle was discovered in the list of repressed transcripts. The GO-Matrix confirmed these findings and revealed a highly significant enrichment of genes in the GO category "nitrogen metabolism" ($P \leq 0.0001$). Thus we detected reduced transcript levels for genes that supply ammonia and nitrogen for the synthesis of urea, glutaminase (*GLS2*), and aspartate aminotransferase (*GOT2*) as well as genes that participate in the urea cycle itself, argininosuccinate synthase (*ASS*), arginase (*ARG1*), ornithine transporter (*SLC25A15*), and carbamoyl phosphate synthase (*CPS1*). The frequency of genes in the GO category "metabolism" was also enriched in the lists of downregulated genes in both winter states ($P \leq 0.01$).

Comparison with hepatic responses in 13-lined ground squirrel. To determine whether the differential expression of these genes is conserved in other hibernating squirrels, we undertook a further expression analysis by hybridizing hepatic RNA from summer and hibernating 13-lined ground squirrels, *S. tridecemlineatus*, to the *S. lateralis* microarray. We compared the expression of four summer active animals with that

Fig. 2. Analysis of circannual gene expression in liver from 2 species of hibernating ground squirrel, *Spermophilus lateralis* (*A*) and *S. tridecemlineatus* (*B*). The expression of each cDNA is presented as the ratio of transcript abundance in each individual animal relative to its median abundance in the control summer active group of animals. Each row represents a different cDNA, and each column represents the expression of the corresponding transcript in an individual animal from each of the three groups. Red indicates a relative increase in transcript abundance in the hibernators, and green represents a decrease, with saturated color indicating a 2-fold or greater change in expression. The cDNAs exhibiting statistically significant up- or downregulation relative to the summer active animals have been grouped according to the direction of change (up- or downpointing arrows) and whether it applies to one, the other, or both winter groups. Within each of these 6 groups, genes are listed in decreasing order of statistical significance. Compared with summer animals, the average fold increase and fold decrease of these genes in the *S. lateralis* winter animals were 1.85 and 1.67, respectively, and in the *S. tridecemlineatus* 1.44 and 1.66, respectively. Genes in bold type were found to be significantly differentially expressed in the liver of both species. *C*: GO-Matrix, a pseudo-color map of the significant over- or underrepresentation (red or blue, respectively) of Gene Ontology (GO) categories within each list of statistically significant genes shown in *A* and *B*. Saturated colors represent *P* values <0.05.

of three interbout aroused and six late torpid *S. tridecemlineatus*. Using available sequence data for the two species, we estimate that the nucleotide sequence similarity of the coding regions of orthologous genes is >85%, indicating that *S. tridecemlineatus* mRNA is expected to hybridize with the arrayed *S. lateralis* probes. Indeed, a similar number of spots on the array were detected by hybridization of cDNA from either species (data not shown). The relative proportion of up- and downregulated genes was similar to that observed in *S. lateralis*, with the expression of just 11 transcripts increasing in the winter animals, while 88 were reduced (Fig. 2*B*, Supplemental Table S2). A search for mRNAs that were differentially regulated in both species of squirrel during hibernation revealed 41 that were differentially expressed in at least one winter condition in either species (genes in bold type; Fig. 2, *A* and *B*). Only a single gene, *FABPL*, showed a small but significant increase in expression in both species, 1.4- and 1.7-fold in *S. tridecemlineatus* and *S. lateralis,* respectively. In contrast, there was considerable overlap between the lists of downregulated genes, with 40 genes being significantly repressed in both species. Notably, this group of common repressed genes included four of the same cytochrome P450 genes, both UDP-glucuronosyltransferases, and, with the exception of *GLS2* and *SLC25A15*, all the genes involved in urea metabolism. The GO-Matrix provided further evidence that the response of the two species was functionally similar, since the distribution of GO terms in *S. tridecemlineatus* closely matched that observed for *S. lateralis* (Fig. 2*C*).

Cardiac responses. Hibernating mammals exhibit profound changes in heart rate during the hibernating season, with heart rate decreasing from 200–300 to 3–5 beats/min during torpor but returning to normal rates during each interbout arousal (27). The transcriptional profile of cardiac muscle revealed that the heart undergoes greater transcriptional changes between torpor and interbout aroused states than was detected in liver. Figure 3*A* shows that 59 and 115 cDNAs were elevated specifically during interbout arousal and torpor, respectively, but that only 7 cDNAs were elevated in both conditions (Supplemental Fig. S1 and Supplemental Table S2). Again, the GO-Matrix analysis was used to guide the interpretation of the predominant changes in gene expression (Fig. 3*C*). Genes induced in both winter states compared with summer included peroxisomal 2,3-*trans*-enoyl-CoA isomerase (*PECI*) and peroxisomal multifunctional enzyme 2 (*HSD17B4*), consistent with a switch to fatty acid β-oxidation during hibernation. Indeed, genes involved in lipid metabolism were generally enriched in the lists of genes elevated during arousal ($P = 0.01$) and torpor ($P = 0.005$). During arousal, these included a subunit of the mitochondrial trifunctional enzyme (*HADHA*), adipophilin (*ADFP*), and two acyl-CoA dehydrogenases (*ACADVL, ACADM*), and in torpor we detected elevated levels of *FABPL* and *APOC2*. Elevated expression of these latter two genes was also a marker of torpor in liver. The transcriptional profile of the heart during arousal was also enriched for genes involved in "energy pathways" ($P = 0.002$). Notable as induced during arousal were isocitrate dehydrogenase (*IDH3A*) and succinyl-CoA ligase [*SUCLA2*; also upregulated at the protein level (14)], which may indicate modulation of the citric acid cycle and oxidative phosphorylation pathways in the aroused phase of hibernation.

A diverse set of 123 genes exhibited significantly depressed transcript levels in the heart of torpid animals, but, in contrast to the liver, no genes exhibited reduced transcript levels in the hearts of interbout aroused animals (Fig. 3*A*). Although we were unable to detect a coherent functional theme using the GO-Matrix, the expression signature did include some specific genes whose function could be linked to the depressed metabolism of the torpid heart. For example, the list included peroxisome proliferator-activated receptor-γ coactivator (*PPARGC1*), which has been shown to regulate the level of oxidative phosphorylation in muscle tissue (29). Also depressed were two ribonuclease P subunits (*RPP14, RPP30*) that are involved in synthesis of tRNA molecules, an observation that is consistent with prior studies showing that translation is arrested during torpor (41). We also discovered a number of cell cycle control genes in the list of torpor repressed genes, yet cell cycle genes were absent in the list of transcripts elevated in torpor. These included CHK2 checkpoint homolog (*CHEK2*), cyclin L1 (*CCNL1*), CDK regulatory subunit associate protein 1 (*CDK5RAP1*), and p53-binding protein (*MDM2*). A depletion of cell cycle transcripts in the heart of torpid animals may be linked to a decrease in cell cycle progression, as might be predicted in a bradycardiac heart.

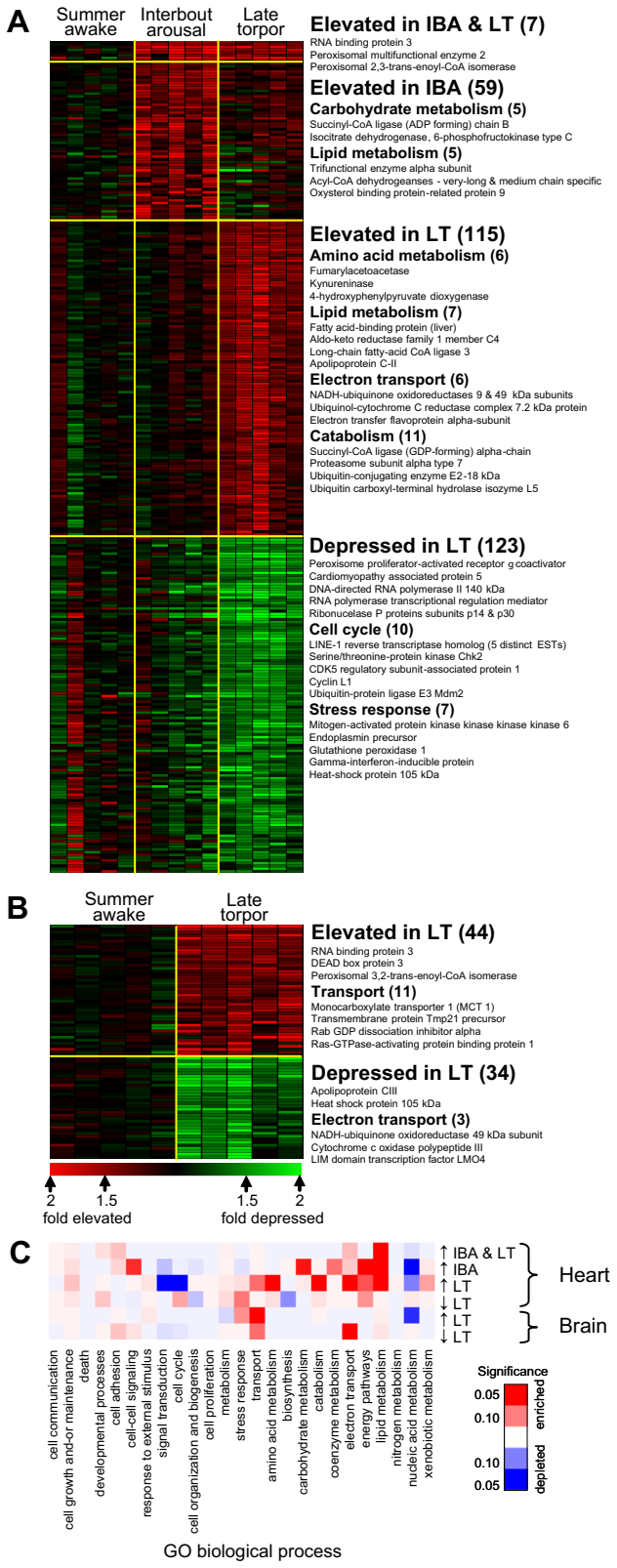
Brain responses. In brain, the function of the transcriptional changes associated with hibernation remains cryptic. We detected just 44 transcripts that were elevated in the brain of torpid animals and 34 transcripts that were depressed (Fig. 3*B*, Supplemental Fig. S2, and Supplemental Table S2), and all but one of these genes (*RBM3*) exhibited less than twofold changes in transcript abundance between the summer active and torpid states. The GO-Matrix (Fig. 3*C*) revealed that genes participating in "transport" processes were enriched in both lists of genes, and that the list of repressed genes was especially enriched for genes involved in "electron transport" ($P = 0.04$). The brain of torpid animals did express elevated transcript levels of *PECI* as did cardiac muscle during arousal and torpor, suggesting that *PECI* expression may be associated with torpor in more than one tissue type.

Common tissue responses. Our analysis has revealed that the expression of just one gene (cDNA clone 21h19) was elevated in all three tissues of *S. lateralis* at late torpor (1.6, 1.6, and 2.3-fold elevated in liver, heart, and brain tissue, respectively; Supplemental Table S2). The EST sequence of this cDNA contained a complete open reading frame that shared 96% identity with mouse RNA-binding protein 3 (*RBM3*) and 64% identity with mouse cold-inducible RNA-binding protein (*CIRBP*). We also identified two genes that were repressed in all three tissues of *S. lateralis* during torpor, but their functional significance to the hibernating phenotype is unclear. One of these was a conserved oligomeric Golgi complex component 6 (*COG6*), which plays a role in normal Golgi function of protein transport. The other gene was KIAA0316 (or focal adhesion kinase 1) (*PDZH10*), a nonreceptor-associated protein tyrosine kinase that has been implicated in diverse signaling pathways.

DISCUSSION

In addition to the specific gene expression changes in the liver, heart, and brain of hibernators as described in the RESULTS above, at least three major patterns are apparent in these data.

These are seen by comparing torpid with interbout aroused hibernators (comparisons within the torpor cycle), summer with winter animals (comparisons over the seasonal cycle), and 13-lined with golden-mantled ground squirrels (comparisons between ground squirrel species).



Comparisons within the torpor cycle. We show that transcript levels in winter remain remarkably stable regardless of whether the animal was torpid or aroused; thus transitions in body temperature from 37 down to 4°C apparently do not involve a large-scale restructuring of the transcriptome. The small differences in gene expression between the two winter states were evident in all three tissues (summarized in Table 2), and are put into sharp relief when compared with responses of a cold-tolerant poikilotherm, the common carp, exposed to an imposed chronic cooling regime from 30 down to 10°C. Thus, in liver of *S. lateralis*, we found that just two genes were different between torpid (4°C) and aroused (37°C) winter animals. This can be compared with ~1,700 genes responding to cooling across seven tissues in the carp. Indeed, it has been estimated that as much as one-third of the transcriptome undergoes differential expression in carp (18), and these showed much greater fold changes than the less than twofold changes observed in the hibernating species. This dramatic response underpins the cold compensation strategy of many poikilotherms when metabolism and most other aspects of cellular performance are stepped up to offset the rate-depressing effects of cold and to maintain activity at all levels of organization. The lack of direct thermal influence on the winter transcriptome during hibernation is thus more consistent with a noncompensatory strategy of response, one in which activity is suppressed in the cold leading to greater energetic savings. Another argument for avoiding a massive reprogramming of gene expression immediately before the onset of torpor is that the transcriptome is preset for the rapid resumption of protein synthesis during the frequent but brief arousals without the need for additional transcriptional regulation. Our data also suggest that transcript turnover is low during torpor, as we saw no evidence that mRNA levels were decreased in total RNA amounts extracted from the tissues of torpid animals, consistent with earlier observations (15, 23, 31). Of course, these general conclusions are limited to those genes represented on our microarray, and at this stage we cannot exclude important roles for the regulation of other unrepresented transcripts. Nor does it exclude other regulatory events during the torpor cycle, such as changes in protein expression or in posttranslational modification, both of which are likely to be regulated during hibernation (16, 38, 42).

A distinctive feature of the transcriptional signature of the heart of hibernators was that a diverse set of genes exhibited depressed transcript levels during torpor but not during the

Fig. 3. Differential gene expression in the heart (*A*) and brain (*B*) of *S. lateralis*. The expression of each cDNA is presented as the ratio of transcript abundance in each individual animal (5 animals per condition) relative to its median abundance in the control summer active animals. Expression data for the brain of interbout aroused animals could not be collected. Genes are listed in decreasing order of statistical significance. Red indicates a relative increase in transcript abundance in the hibernators, and green represents a decrease, with saturated color indicating a 2-fold or greater change in expression. Examples of major functional groups enriched in each list, the number of corresponding genes, and the names of representative or particularly significant genes are indicated. In heart, the average induction in both IBA and LT animals was 1.54-fold, in IBA 1.41-fold, and in LT 1.39-fold. The average repression of genes in LT heart was 1.45-fold. In brain, the average fold induction and fold repression of genes in the hibernators was 1.4 and 1.54, respectively. *C*: GO-Matrix, a pseudo-color map of the significant over- or underrepresentation (red or blue, respectively) of GO categories within each list of statistically significant genes. Saturated colors represent P values <0.05.

Table 2. *Summary of genes in liver, cardiac, and brain tissues that differ in transcript expression between different stages of the seasonal and hibernating cycles in the golden-mantled ground squirrel, S. lateralis*

Comparison	Downregulated Genes	Upregulated Genes
	Liver	
SA/IBA	9	2
SA/LT	39	6
SA with both	44	2
IBA/LT	0	2
	Cardiac	
SA/IBA	0	59
SA/LT	123	115
SA with both	0	7
IBA/LT	0	0
	Brain	
SA/LT	34	44

Values are nos. of genes deemed as significantly different between the indicated stages according to the statistical tests described in METHODS. SA, summer active; LT, torpor; IBA, interbout aroused.

interbout aroused state. These particular mRNAs might be more sensitive to degradation and have shorter half-lives during torpor but are restored to normal levels during interbout arousal, as was observed previously with *A2M* (15). In this scenario, no specific regulatory mechanism would be responsible for their reduced levels during torpor, a finding consistent with observations that RNA polymerase II-driven transcription is quelled during torpor but restored during interbout arousal (40). Likewise, the transcripts that are elevated during torpor may be a consequence of enhanced resistance to degradation rather than new transcription. Although there is evidence supporting the existence of a global mechanism to protect mRNAs from degradation during torpor (15), the elevated levels of specific transcripts in the heart may indicate that some mRNAs, for example those of lipid genes, are specifically protected or inherently more stable than others.

Comparisons over the seasonal cycle. We found larger differences when comparing summer and winter animals irrespective of hibernation stage; thus in liver we found 45–55 genes displaying summer-winter differences in abundance, and in cardiac tissue we found 120–175 genes. In liver we showed that, of the genes displaying regulation, most exhibited decreased transcript levels in the winter compared with the summer, the difference between up- and downregulated genes being ~10-fold. In contrast, the heart showed a more balanced proportion of up- and downregulated genes, although as already indicated we observed no significantly downregulated genes in the aroused animals in winter.

Thus the winter transcript pattern is broadly related not so much to excursions in body temperature during torpor:arousal cycles but more to the production of a characteristic winter-specific transcriptome for the entire hibernation season. It is worth emphasizing that the screening technique used here allows comparison between replicate individuals within each of the three treatment groups. This has allowed us to observe significantly greater variation between individuals in the summer active group compared with the two winter groups, which might have its basis in nutritional, hormonal, social status, or gender-based variation between energetically active summer individuals. This may have reduced the number of significant gene expression changes recovered in this study, and larger numbers of replicate animals or more closely controlled conditions or treatment of these animals might well reveal more genes displaying the seasonal response pattern. Nonetheless, these data strongly support a conclusion that more adjustments to the transcript pool are made on a seasonal basis to prepare the animals for hibernation than those required for the torpor bout itself.

Comparisons between ground squirrel species. In liver we find a degree of consistency in gene regulation responses between the two hibernating ground squirrel species. This relates not so much to specific genes but to classes of genes or to biological processes in which they are involved, which supports the idea that modulations of these specific processes are important and conserved components of the hibernating phenotype. The discrepancy in the overlap of the sets of differentially expressed genes in the two hibernating species may be attributed to difficulties in the statistical detection of the modest transcriptional changes between the summer and winter animals, changes that may be masked due to interindividual variation and the difference in the number of individuals analyzed for each species (13 individuals of *S. tridecemlineatus* vs. 18 used for *S. lateralis*). Alternatively, differences in the transcriptional response may well be linked to some more subtle interspecific differences in the hibernating phenotype of the two species.

Molecular phenotype. The GO-Matrix method allows the interpretation of gene responses using strict, statistically based, and unbiased inferences rather than the more subjective selection of genes with known regulatory properties. This has revealed some consistent patterns in gene responses related particularly to the regulation of lipid biosynthesis and metabolism, and to the elimination of nitrogen waste products. While the former is well known, the involvement of so many genes has not to date been recorded as being involved in hibernation, but clearly it involves the coordinated regulation of a large set of genes. The latter group of pathways has not to our knowledge been subjected to any scrutiny, despite urea metabolism being assessed as part of estimates of protein turnover. Here we show a highly consistent reduction in multiple genes involved in the urea cycle and the breakdown of waste products, which together comprise 19 of the 39 downregulated genes in the liver of torpid animals, relative to summer active animals. These new data provide supporting evidence that both the excretory and biosynthetic functions of the liver are depressed during hibernation, responses that are consistent with prolonged fasting and metabolic changes (25).

A notable observation is the upregulation in all tissues examined of a single gene, *RBM3,* which is homologous to *CIRBP*. Both genes are highly conserved mammalian cold shock proteins with an RNA-binding capacity (11, 30). *CIRBP* is also cold responsive in amphibians (33), and we have recently described the upregulated expression of a similar gene in seven different tissues of a cooled teleost fish (18). Thus the induction of these closely related RNA-binding proteins points to a central role for them during cold exposure in vertebrates. Although the exact role of these proteins in the cold response is unclear, evidence from *Xenopus* suggests that *CIRBP*, through interactions with another RNA-binding protein, *HuR*,

binds to specific mRNAs and regulates the deadenylation of their poly(A) tail (2). Furthermore, in mammals, *RBM3* mRNA is translated even under mild hypothermic conditions due to the presence of an internal RNA entry site (IRES) (10). Because RNA molecules are expected to exhibit greater secondary structure as temperature decreases, we hypothesize that *RBM3* and other cold-inducible RNA-binding proteins may function as RNA chaperones to facilitate the processing of RNA molecules in the cold. Future studies to determine the functional significance of RNA-binding proteins on both the behavior of RNA molecules and the physiology of cooled animals may aid in the understanding of this complex phenotype.

Our results present a snapshot of the transcriptional differences that exist between three broad phenotypic states: summer active animals, torpid animals, and animals aroused from torpor. However, these conditions may not fully represent all of the changes that must occur, since hibernation involves not only the ability to enter and reverse torpor but the entire process of converting the summer homeotherm to a winter heterotherm. Thus the torpor stage of hibernation is just one component of a circannual cycle of physiological events that prepare the animal for this dramatic transition, and profiling of the physiological status of animals throughout the year will increase our understanding of the hibernating phenotype. In particular, animals preparing for hibernation in the fall may exhibit intermediate phenotypes that are informative of the mechanisms underpinning this transition. This period is characterized by increased food consumption accompanied by significant weight gain and relaxed body temperature control. In contrast, animals collected in summer present a phenotype from the most disparate time of the circannual cycle with respect to torpor. In the present study, we chose to compare these most contrasting times of the year, summer vs. winter, yet our results showed that the transcriptional differences between these two conditions were still modest. Future studies will need to profile more individual animals sampled across all seasons and physiological states with larger and more complete probe sets to thoroughly identify with statistical confidence the transcriptional changes that orchestrate the hibernating phenotype.

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