Available online at www.sciencedirect.com
ScienceDirect
Cryobiology 53 (2006) 310–318
CRYOBIOLOGY
www.elsevier.com/locate/ycryo

Evidence for a reduced transcriptional state during hibernation in ground squirrels ☆

Pier Jr Morin*, Kenneth B. Storey

Institute of Biochemistry and Department of Chemistry, Carleton University, 1125 Colonel By Drive, Ottawa, Ont., Canada K1S 5B6

Received 14 March 2006; accepted 4 August 2006
Available online 18 September 2006

Abstract

During mammalian hibernation, metabolic rate can be reduced to <5% of the euthermic rate as a result of coordinated suppression of multiple energy expensive metabolic processes. Gene transcription is one of these and the present study examines mechanisms of transcriptional control that could contribute to lowering the rate of gene expression in torpor. Histone deacetylases (HDAC) have been linked to gene silencing and measured HDAC activity was 1.82-fold higher in skeletal muscle of hibernating thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*, compared with euthermic controls. Western blotting also showed that HDAC1 and HDAC4 protein levels were 1.21-and 1.48-fold higher, respectively, in muscle from torpid animals. Histone H3 was also evaluated by Western blotting. Total histone H3 was unchanged but two forms of covalently modified histone H3 that are associated with active transcription (phosphorylated Ser 10 and acetylated Lys 23) were significantly reduced by 38–39% in muscle during hibernation. Finally, RNA polymerase II activity was measured using a PCR-based approach; activity in muscle from hibernating squirrels was only 57% of the euthermic value. These data support an overall decrease in transcriptional activity in skeletal muscle of hibernating animals that is accomplished by multiple molecular mechanisms.

Keywords: Transcription; RNA polymerase II; Histone deacetylase; Metabolic rate depression; *Spermophilus tridecemlineatus*

To survive winter, various small mammals enter hibernation. During hibernation, metabolic rate is often reduced to just 1–5 % of the normal euthermic rate and body temperature falls to near ambient. This allows hibernators to save up to 90% of the energy they would normally consume if they were to remain euthermic during the winter months [31]. Heartbeat and breathing rate are profoundly depressed during hibernation and organ perfusion rates can drop to ~10% of normal [11]. Strong metabolic rate depression is achieved by a coordinated suppression of the rates of energetically costly cell functions such as transmembrane ion pumping and protein synthesis [29]. Rates of translation are well known to be reduced in mammals in the face of stresses such as starvation or hypoxia [5,8] and in hibernation the rate of protein synthesis can be reduced to extremely low levels by combining

☆ The research was supported by a discovery grant from the Natural Sciences and Engineering Council of Canada (OPG 6793) to K.B.S. and by an Ontario Graduate Scholarship to P.J.M. K.B.S. holds the Canada Research Chair in Molecular Physiology.
* Corresponding author. Fax: +1 613 520 2569.
E-mail address: pier.morin@gmail.com (P. Morin).

0011-2240/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.cryobiol.2006.08.002

reversible phosphorylation controls on the translation apparatus with the rate effects of a >30 °C decrease in body temperature. Indeed, the rate of radiolabelled leucine (^{14}C) incorporation in brain of torpid 13-lined ground squirrels, *Spermophilus tridecemlineatus*, was only 0.04% of the value measured in euthermic brain [12]. Similar findings were reported for *S. tridecemlineatus* kidney [14].

Gene transcription is another main energy consuming processes in cells, typically requiring ~1–10% of the overall cell energy budget [26]. Given the strong suppression of translation in hibernation, it is reasonable to propose that the overall rate of transcription would also be reduced. Indeed, studies with several different animal systems suggest that transcriptional suppression is a general component of hypometabolism (reviewed in [29]). Data for hibernating mammals is limited to date but tends to concur. For example, the rate of incorporation of [^{3}H]uridine into RNA in brain was only ~12% in torpor of the rate in euthermic ground squirrels [3]. The same principle was documented by [^{3}H]uridine incorporation into RNA in multiple organs of hamsters [24] and from nuclear run-on assays performed on liver extracts of *S. lateralis* [30].

Multiple regulatory controls could be involved in reducing the rate of gene transcription during hibernation. It is well known that histone acetylation and deacetylation are linked to transcriptional activity and that histone deacetylases (HDACs) are associated with gene silencing. HDAC activity can be affected by changes in oxygen levels and is known to be induced under hypoxic conditions [17] and reduced under oxidative stress [1]. Transcriptional controls could also be applied in other ways such as by regulation of key enzymes including RNA polymerase II.

The present study looks at the transcriptional state of skeletal muscle in euthermic versus hibernating *S. tridecemlineatus* and assesses multiple parameters that could be involved in regulating the overall rate of transcription including analysis of RNA polymerase II, HDAC, and histone H3. The information gathered here provides strong evidence of an overall suppression of gene transcription in ground squirrel muscle during hibernation.

Materials and methods

Animals

Thirteen lined ground squirrels, *S. tridecemlineatus* (130–180 g), were captured by a licensed trapper (TLS Research, Michigan) and transported to the Animal Hibernation Facility at the National Institute of Neurological Disorders and Stroke (NIH, Bethesda, MD). Hibernation experiments were conducted by the laboratory of Dr. J.M. Hallenbeck using their standard protocols [6]. Animals were kept on a fall day/night light cycle in shoebox cages maintained at 21 °C and fed *ad libitum* until they entered and finished the pre-hibernation phase of hyperphagia that maximizes lipid stores. A sensor chip was introduced under the squirrel skin and the body temperature of each animal was monitored electronically. When squirrels had reached a plateau weight gain of 220–240 g, they were placed in their cages containing wood shavings into a cold chamber at 4 °C and 60% humidity. The hibernaculum was kept in constant darkness, except for a photographic red safe light (3–5 lux), and could be entered only through a darkened anteroom. Noise within the chamber was kept to a minimum. Individuals settled into hibernation after different lengths of time but all were sampled on the same day after each individual had been hibernating for 2–5 days (as indicated by continuous body temperature readings of ~6 °C). Animals that had not entered torpor after at least three days in the cold room and that showed continuous high body temperatures (36–38 °C) were sampled as controls. All animals were sacrificed by decapitation and tissues were excised, frozen immediately in liquid nitrogen and then transported to Ottawa on dry ice where they were then placed at −80 °C until use.

Western blotting

Frozen skeletal muscle (mixed hind leg thigh muscle) samples (~500 mg) from euthermic and hibernating ground squirrels were crushed under liquid nitrogen, weighed, and then homogenized with a Polytron homogenizer in 2 mL of buffer containing 100 mM Mops, 25 mM Hepes, 25 mM β-glycerophosphate, 5 mM EDTA, 1 mM EGTA and 250 μM Na_3VO_4, adjusted at pH 7.4, with 1 mM phenylmethylsulphonyl fluoride (PMSF) added immediately before homogenization. After centrifugation at 10,000*g* for 10 min at 4 °C, supernatants were collected and soluble protein concentrations were determined using the Coomassie blue dye-binding method and the Bio-Rad prepared reagent (Bio-Rad, Hercules, CA). SDS–polyacrylamide gel electrophoresis and blotting to polyvinylidene difluoride membranes was carried out essentially as

in Morin and Storey [20] with 10% gels (5% stacking gel), 20 μg of protein per well, and electrophoresis at 200 V for 45 min. Wet transfer of proteins onto membranes used a transfer buffer solution containing 25 mM Tris (pH 8.5), 192 mM glycine, and 10% v/v methanol at 4 °C for 1.5 h at 0.3 mA. Following transfer, the PVDF membrane was blocked for 1 h in TBST (50 mM Tris–HCl, pH 6.8, 150 mM NaCl, 0.05% v/v Tween 20) with 2.5% w/v powdered skim milk. This was decanted and then membranes were incubated overnight at 4 °C with the different antibodies at a 1:1000 v:v dilution in 5 mL of TBST. The RNA polymerase antibodies were purchased from Covance Research (Berkeley, USA); total polymerase protein was quantified using monoclonal antibody 8WG16 that recognizes the C-terminal heptapeptide repeat present on the largest subunit of pol II whereas phosphorylated polymerase was assessed with monoclonal antibody H14 recognizing phosphorylated Ser 5 in the heptapeptide repeat YSPTSPS in the C-terminal domain. Polyclonal antibodies recognizing histone deacetylase and histone H3 were from Cell Signaling (Boston, USA). Subsequently, the membrane was incubated with HRP-linked anti-rabbit or anti-mouse secondary antibody (1:2000 v:v dilution) in TBST for 1 h and then blots were developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's protocol. The data were obtained from the same set of replicates (euthermic and hibernating samples prepared from different individual animals), but for each protein analyzed, these replicates were run on separate gels and transferred to different membranes that were then each probed with one of the antibodies. Bands were visualized using a Syngene (Bio-Rad, Hercules, CA) and band intensities were quantified using the Gene Tools program. After quantifying the immune bands, the blots were restained using Coomassie blue to demonstrate equal protein loading between samples. Three Coomassie blue stained bands that did not change in intensity between samples were chosen and their combined intensity was quantified in each lane. The band intensity of immunoreactive material in each lane was then normalized against the combined intensity of these three Coomassie blue stained bands. Mean normalized band densities ± SEM were then calculated for samples from hibernating versus euthermic animals and significant differences between the groups were tested using the Student's *t*-test. The ratio hibernating:euthermic band intensity was calculated and plotted; error bars on the final histograms are the sum of SEM values for hibernating and euthermic trials.

RNA polymerase II assay

RNA polymerase II activity was assayed using a PCR-based approach. Nuclear extracts were prepared from thigh muscle of euthermic and hibernating ground squirrels using a slight modification of the method described by Dignam et al. [9]. Briefly, tissue samples were disrupted using a Dounce homogenizer in homogenization buffer (10 mM Hepes, 10 mM KCl, 10 mM EDTA, and 1 mM DTT, pH 7.9) with 1 mM PMSF added prior to homogenization. Samples were centrifuged at 10,000*g* for 10 min at 4 °C and then supernatants were discarded. Pellets were resuspended in extraction buffer (20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 50% v/v glycerol, and 1 mM DTT, pH 7.9) and then placed horizontally on ice and rocked on a rocking platform for 1 h. Samples were then centrifuged at 10,000*g* for 10 min at 4 °C. Supernatants containing nuclear extracts were collected and protein concentration was measured with the Bio-Rad assay. A series of samples were made up containing a volume of supernatant containing 100 μg of nuclear protein combined with 100 μL of transcription buffer (100 mM Hepes, 400 mM KCl, 25 mM $MgCl_2$, 5 mM EDTA, and 10% v/v glycerol), 5 μL of 0.1 M DTT, 3 μL each of 100 mM NTPs (ATP, CTP, GTP, UTP) (Fermentas), and 1 μg of a Promega PGL3-Promoter Vector containing the gene for EGFP (enhanced green fluorescent protein) with a SV40 promoter inserted in the multiple cloning site, kindly provided by Dr. W. Willmore, Carleton University. Initial trials were run in the presence of 0.1 mg of α-amanitin, a RNA polymerase II inhibitor, to confirm that the activity detected was specific to RNA polymerase II action. Samples were adjusted to a final volume of 500 μL with distilled water and then reactions were incubated at 37 °C. Individual samples were removed at 1, 5, 10, 25, and 45 min and total RNA was isolated immediately using Trizol reagent (Gibco-BRL, Bethesda, MD). Each sample was mixed in 1 mL Trizol followed by the addition of 200 μL of chloroform and mixing. Samples were centrifuged at 10,000 rpm at 4 °C for 15 min, and then the top layer was removed (~600 μL) and transferred into another tube. A 500 μL aliquot of isopropanol was added, followed by incubation at 21 °C for 10 min to precipitate the RNA. Samples

were centrifuged at 10,000 rpm for 10 min at 4 °C to pellet the RNA; the supernatant was discarded and the RNA pellet was washed with 70% ethanol and then resuspended in DEPC-treated water. A 30 μg aliquot of this total RNA was used for first strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) and following the manufacturer's protocol. Primers for the EGFP gene were designed. The forward primer was 5′-GGCCACA AGTTCAGCGTGTC-3′ and the reverse was 5′-TGCCGTCCTCGATGTTGTGG-3′. PCR was conducted as in Eddy et al. [10] using the basic protocol recommended with the Invitrogen reagents. Briefly, the reaction mixture consisted of 5 μL of cDNA, 1.25 μL of primer mixture (0.5 μM forward and 0.5 μM reverse), 15 μL of sterile water, 2.5 μL of 10 times PCR buffer (Invitrogen), 1.25 μL of 5 times $MgCl_2$, 0.5 μL of 10 mM dNTPs, and 0.125 μL of *Taq* Polymerase (Invitrogen) in a total volume of 25 μL. The cycles performed for amplification consisted of an initial step of 2 min at 94 °C, followed by amplification steps of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min repeated 37 times; the final step was at 72 °C for 2 min. PCR products were separated on a 1.0% agarose gel. Bands were visualized with ethidium bromide on a UV box. A product of ~420 bp corresponding to EGFP was obtained. Band intensity for bands in euthermic and hibernating samples at each time point were determined. A standard curve of band intensity versus ng of DNA was constructed from a low mass DNA ladder (Invitrogen) loaded on the same gel as the sample and used to determine the ng DNA produced at each time point. Data were plotted (ng DNA vs time), three initial rates were calculated for euthermic and hibernating samples (ng DNA produced per minute per μg nuclear protein) and mean rate ± SEM was reported. The ratio of hibernating to euthermic initial rates was determined.

Histone deacetylase assay

Histone deacetylase (HDAC) was assayed using the HDAC assay kit from Cayman Chemicals (Ann Arbor, USA), according to manufacturer's instructions. Briefly, ~100 mg of muscle was homogenized in 50 mM Tris buffer, pH 7.4, followed by centrifugation at 10,000*g* for 10 min at 4 °C and collection of the supernatant. A 15 μL aliquot of sample was added to 10 μL of HDAC assay buffer and equilibrated at room temperature for 10 min before addition of 15 μL of HDAC assay substrate and incubation for 1 h at 30 °C. Then 20 μL of the diluted activator solution was added and the mixture was incubated at room temperature for 10 min. Fluorescence of samples was then measured in a microplate reader (excitation = 350–380 nm, emission = 440–460 nm).

Results

Histone deacetylase protein levels

HDAC protein was assessed via Western blotting in skeletal muscle from euthermic and hibernating *S. tridecemlineatus* using polyclonal antibodies recognizing HDAC1 and HDAC4 (Fig. 1). Each antibody crossreacted with a single protein band on the blot at 62 and 140 kDa, respectively, the known molecular weights of these two isozymes. The histogram in Fig. 1B shows the ratio of HDAC1 and HDAC4 protein levels in hibernating versus euthermic muscle. The amounts of HDAC1 and HDAC4 protein were significantly higher in muscle from hibernating squirrels; levels were 1.21- and 1.48-fold higher, respectively, compared with euthermic controls ($P < 0.05$).

Histone deacetylase activity in 13-lined ground squirrel muscle

Histone deacetylase (HDAC) activity has been associated with the silencing of genes [21]. Total HDAC activity was assayed using a kit from Cayman Chemicals following the manufacturer's protocol. Mean HDAC activity in muscle from euthermic ground squirrels was 31,878 ± 2135 relative fluorescence units per gram fresh weight (RFU/gfw) and significantly higher, 57,972 ± 7471 RFU/gfw, in hibernating squirrels, a 1.82-fold increase (data are means ± SEM, $n = 3$ samples from individual animals; $P < 0.05$, as assessed by the Student's *t*-test).

Histone H3 protein levels in S. tridecemlineatus muscle

Histone H3 seems to play a role in the silencing of genes just as HDAC does. Polyclonal antibodies for total histone H3, phosphorylated histone H3 (Ser 10) and acetylated histone H3 (Lys 23) were used to quantify histone H3 expression during hibernation (Fig. 2). Total histone H3 protein did not change in muscle of hibernating animals compared with euthermic samples. However, both the amount of phosphorylated histone H3 and the amount of acetylated

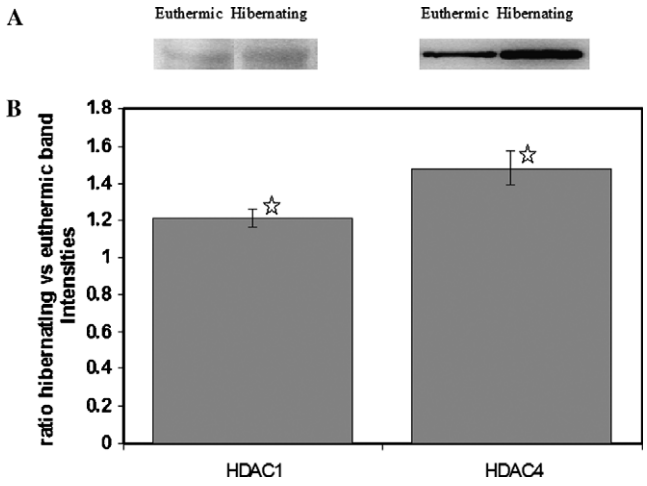

Fig. 1. (A) Western blots showing HDAC1 and HDAC4 protein expression in skeletal muscle of euthermic and hibernating *S. tridecemlineatus.* (B) Histogram showing relative protein expression levels in hibernation vs euthermia; data are means ± SEM for $n = 4$ independent trials. ☆ Values are significantly different ($P < 0.05$) from the corresponding euthermic control value.

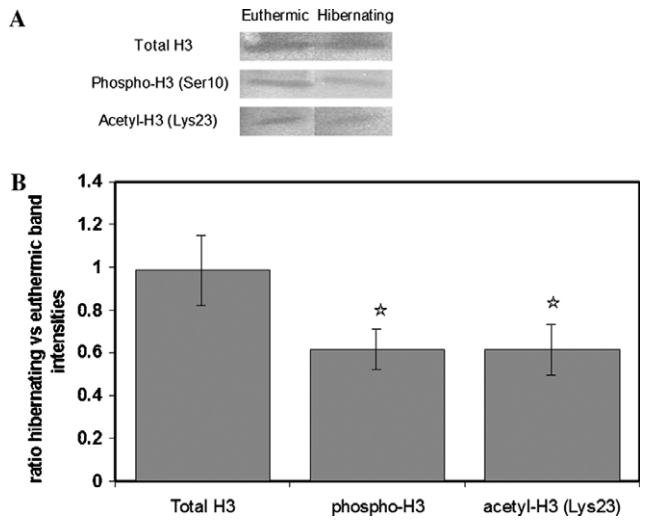

Fig. 2. (A) Total histone H3, phosphorylated histone H3 (Ser 10), and acetylated histone H3 (Lys 23) protein expression in skeletal muscle of euthermic and hibernating *S. tridecemlineatus.* (B) Histogram showing relative expression levels of histone H3 in hibernating squirrels; data are means ± SEM for $n = 3$ independent trials. ☆ Values are significantly different ($P < 0.05$) from the corresponding euthermic control value.

protein decreased significantly in muscle during hibernation to values that were 61% and 62% of the comparable values in euthermic muscle ($P < 0.05$).

RNA Polymerase II protein expression in S. tridecemlineatus

RNA polymerase II protein levels were measured in muscle of *S. tridecemlineatus*. Both total protein and the amount of phosphorylated RNA polymerase II were measured using Western blotting (Fig. 3). Fig. 3B shows the ratio of RNA polymerase protein levels in muscle from hibernating versus euthermic animals. Total protein content of RNA polymerase II remained unchanged during hibernation whereas the amount of phosphorylated enzyme (phospho-Ser 5 in the heptapeptide repeat YSPTSPS in the C-terminal domain) increased significantly by 1.79-fold ($P < 0.05$) in torpid animals.

RNA polymerase II activity in S. tridecemlineatus

RNA polymerase II activity was measured by its ability to transcribe the EGFP gene with the mRNA

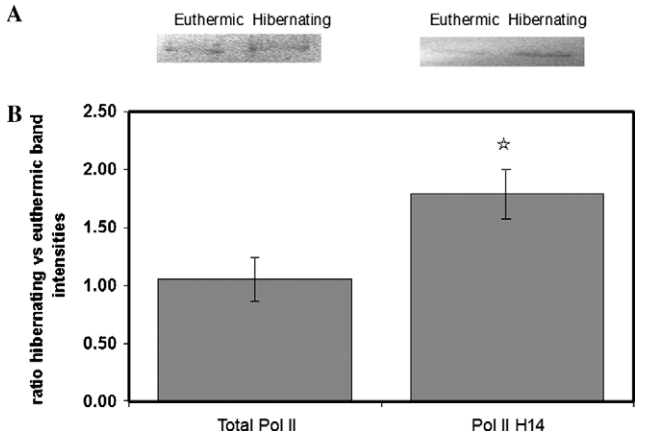

Fig. 3. (A) Total RNA polymerase II protein and the amount of phosphorylated RNA polymerase II (the antibody recognizes phospho-Ser 5 in the heptapeptide repeat YSPTSPS in the C-terminal domain) in skeletal muscle of euthermic and hibernating *S. tridecemlineatus*. (B) Histogram showing relative protein expression in hibernating vs euthermic squirrels; data are means ± SEM for $n = 3$ independent trials. ☆ Values are significantly different ($P < 0.05$) from the corresponding euthermic control value.

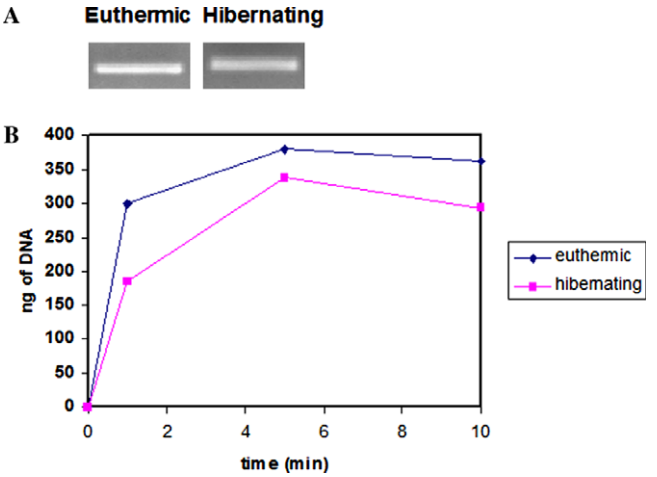

Fig. 4. (A) EGFP amplification by RNA polymerase II in samples of skeletal muscle from euthermic and hibernating ground squirrels after 5 min incubation at 37 °C. (B) Graphical representation of a typical result obtained for a RNA polymerase II assay; ng DNA produced are plotted versus time. EGFP was amplified for up to 45 min but reached a plateau after 5 min.

product then amplified by PCR before visualization on ethidium bromide stained agarose gels. The purpose of this assay was to compare the state of RNA polymerase II activity in euthermic and hibernating muscle samples; all assays were conducted at a constant 37 °C. Typical assay results are shown in Fig. 4. Initial trials used a series of incubation times (1, 5, 10, 25, and 45 min at 37 °C) but Fig. 4B shows that DNA production reached a plateau after 5 min and a 5 min incubation was used in all subsequent analyses and for calculations of enzyme activity. Calculated RNA polymerase II activity in nuclear extracts from euthermic muscle was 0.63 ± 0.03 ng DNA produced/min/μg nuclear protein. The value in muscle from hibernating squirrels was significantly lower, 0.36 ± 0.01 ng DNA produced/min/μg nuclear protein, or 57% of the euthermic value (data are means ± SEM, $n = 3$ samples from individual animals; $P < 0.05$).

Discussion

While many recent studies have shown that rates of protein translation are strongly suppressed during hibernation, much less is known about transcription during torpor. Selected genes are clearly up-regulated (as is the synthesis of selected proteins) [28,29] and several studies indicate that the overall

rate of transcription is reduced in torpor [3,24,30]. However, the mechanisms of transcriptional control in hibernation have not previously been explored. Our results begin to examine transcriptional regulation in hibernating *S. tridecemlineatus* and identify several mechanisms that could contribute to global transcriptional suppression in torpor.

Histone acetylation has been linked with transcriptionally active chromatin [13]. The principle behind this is that acetylation of histones can make chromatin more accessible to the transcriptional machinery [19] due to modification of nucleosomal conformation [22]. Phosphorylation of histone H3 at serine residue 10 has also been linked with transcriptional activation [7]. Examination of both of these modifications of histone H3 in ground squirrel muscle are consistent with a state of reduced transcriptional activity in the torpid state. Hence, although histone H3 total protein levels remained constant between euthermic and hibernating states, both the amount of phosphorylated histone H3 (Ser 10) and the amount of acetylated histone H3 (Lys 23) were reduced by 38–39% in hibernation as compared with euthermia. These findings suggest that at least two forms of posttranslational modification contribute to regulating histones during hibernation and that the actions of both of these mechanisms on histone H3 are consistent with an overall decrease in transcriptional activity in skeletal muscle during hibernation. Histones H2A/H2B and H4 are also subject to acetylation that regulates their activity [23,25] and these may also be modified in a parallel way during hibernation.

To further analyze transcriptional control in hibernators, histone deacetylase activity and protein levels were measured. Since histone acetylation is associated with active transcription, an increase in histone deacetylase activity would predictably result in a slow down of transcriptional activity. Three major classes of mammalian histone deacetylases are known. Class I consists of HDAC 1, HDAC2, HDAC3, and HDAC8 and these are known to be recruited by DNA binding factors and to act as transcriptional repressors [18]. Class II histone deacetylases consist of HDAC4, HDAC5, HDAC6, and HDAC7 and function as transcriptional corepressors [2]. A third class of HDAC consists of enzymes that are analogous to the yeast Sir2 protein which is also involved in transcriptional silencing [15]. Total HDAC activity was 1.82-fold higher in skeletal muscle of hibernating squirrels versus euthermic controls. Furthermore, both HDAC1 and HDAC4 protein levels were elevated in hibernation (by 1.21- and 1.48-fold, respectively); other Class I and Class II HDACs may similarly increase in torpor. Both the increase in HDAC activity and in HDAC protein levels point to a regulated reduction in transcriptional activity in muscle of thirteen-lined ground squirrels during hibernation.

RNA polymerase II activity was also measured in muscle of euthermic and hibernating ground squirrels. Previous data gathered from nuclear run-on assays in golden-mantled ground squirrels or from rates of [^{3}H]uridine incorporation into RNA in hamsters indicated that the overall rate of transcription was reduced in organs of torpid animals [3,24,30]. Direct analysis of RNA polymerase II in the present study showed a substantial reduction in enzyme activity in muscle from torpid animals; the activity in nuclear extracts from hibernator muscle was only 57% of the value in euthermic muscle. Note that these values are for polymerase activity measured in 37 °C incubations whereas polymerase activity *in vivo* during hibernation would be very much lower due to the effects of a body temperature that could be as much as 35 °C lower than euthermic values. The present data showing a change in polymerase activity between the euthermic and torpid states (when measured at a constant assay temperature) indicates that transcriptional repression during hibernation occurs as a result of a stable modification(s) of the protein. Total polymerase protein did not change in hibernation so this argues that the change in activity is the result of a modification of the enzyme protein. RNA polymerase II is known to be covalently modified by phosphorylation at Ser 2 and Ser 5 of a peptide sequence (YSPTSPS) that is repeated multiple times in the C-terminal domain (CTD) and phosphorylation at these sites has been linked with transcriptional control. The Pol II H14 Ab used in the present study recognizes enzyme that is phosphorylated on the 5th serine residue in this sequence. The data show that the amount of phosphorylated (Ser5) RNA polymerase II protein rose by 1.79-fold during hibernation suggesting that transcriptional control during hibernation may also be applied by modifying the phosphorylation state of RNA polymerase II. This specific form of RNA polymerase II is interesting. It was initially thought that this phosphorylated form of the enzyme was associated with active transcription; if this were so, the change in phosphorylation state measured in hibernation would contradict our other findings. However, subsequent studies have monitored RNA

polymerase II phosphorylation states at both transcriptionally active and inactive stages of the cell cycle and showed that hyperphosphorylation of the RNA polymerase II CTD could not be used as an indicator of transcriptional activity [16]. While it seems that a hyperphosphorylated CTD correlates with polymerase being located at the transcription initiation position of genes, phosphorylation of Ser 2 and Ser 5 is not a prerequisite for transcription. In fact, it has been shown that inhibiting the kinases responsible for phosphorylating the CTD did not lead to transcription inhibition [27] and that CTD-less RNA polymerase II can still transcribe genes [4]. The increase in phosphorylated RNA polymerase content measured here may just mean that RNA polymerase II in hibernating muscle is positioned at the initiation position, waiting to transcribe selected genes, but without necessarily being transcriptionally active.

Overall, then, the results presented here point towards a decrease in transcriptional activity in skeletal muscle of hibernating *S. tridecemlineatus*. This supports the idea that metabolic rate depression during hibernation involves a coordinated and regulated suppression of the rates of multiple energy-expensive cellular activities including the overall rate of gene transcription.

Acknowledgments

We thank Dr. J.M. Hallenbeck, National Institute of Neurological Disorders and Stroke, Bethesda, MD for providing ground squirrel tissues, Dr. W.G. Willmore, Carleton University for the EGFP containing vector, and J.M. Storey for editorial commentary on the manuscript.

References

[1] I.M. Adcock, B. Cosio, L. Tsaprouni, P.J. Barnes, K. Ito, Redox regulation of histone deacetylases and glucocorticoid-mediated inhibition of the inflammatory response, Antioxid. Redox. Signal. 7 (2005) 144–152.

[2] N.R. Bertos, A.H. Wang, X.J. Yang, Class II histone deacetylases: structure, function, and regulation, Biochem. Cell. Biol. 79 (2001) 243–252.

[3] L.S. Bocharova, R.Ya. Gordon, V.I. Arkhipov, Uridine uptake and RNA synthesis in the brain of torpid and awakened ground squirrels, Comp. Biochem. Physiol. B. 101 (1992) 189–192.

[4] S. Buratowski, P.A. Sharp, Transcription initiation complexes and upstream activation with RNA polymerase II lacking the C-terminal domain of the largest subunit, Mol. Cell. Biol. 10 (1990) 5562–5564.

[5] T.M. Casey, J.L. Pakay, M. Guppy, P.G. Arthur, Hypoxia causes downregulation of protein and RNA synthesis in noncontracting Mammalian cardiomyocytes, Circ. Res. 90 (2002) 777–783.

[6] Y. Chen, M. Matsuhita, A.C. Nairn, Z. Damuni, D. Cai, K.U. Frerichs, J.M. Hallenbeck, Mechanisms for increased levels of phosphorylation of elongation factor-2 during hibernation in ground squirrels, Biochemistry 40 (2001) 11565–11570.

[7] P. Cheung, C.D. Allis, P. Sassone-Corsi, Signaling to chromatin through histone modifications, Cell 103 (2000) 263–271.

[8] D.J. DeGracia, R. Kumar, C.R. Owen, G.S. Krause, B.C. White, Molecular pathways of protein synthesis inhibition during brain reperfusion: implications for neuronal survival or death, J. Cereb. Blood Flow Metab. 22 (2002) 127–141.

[9] J.D. Dignam, R.M. Lebovitz, R.G. Roeder, Accurate transcription initiation by RNA Polymerase II in a soluble extract from isolated mammalian nuclei, Nucleic Acids Res. 11 (1983) 1475–1489.

[10] S.F. Eddy, P. Morin Jr., K.B. Storey, Cloning and expression of PPAR-gamma and PGC-1alpha from the hibernating ground squirrel, *Spermophilus tridecemlineatus*, Mol. Cell Biochem. 269 (2005) 175–182.

[11] K.U. Frerichs, C. Kennedy, L. Sokoloff, J.M. Hallenbeck, Local cerebral blood flow during hibernation, a model of natural tolerance to "cerebral ischemia, J. Cereb. Blood Flow Metab. 14 (1994) 193–205.

[12] K.U. Frerichs, C.B. Smith, M. Brenner, D.J. DeGracia, G.S. Krause, L. Marrone, T.E. Dever, J.M. Hallenbeck, Suppression of protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation, Proc. Natl. Acad. Sci. USA 95 (1998) 14511–14516.

[13] T.R. Hebbes, A.W. Thorne, C. Crane-Robinson, A direct link between core histone acetylation and transcriptionally active chromatin, EMBO J. 7 (1988) 1395–1402.

[14] D. Hittel, K.B. Storey, The translation state of differentially expressed mRNAs in the hibernating 13-lined ground squirrel (*Spermophilus tridecemlineatus*), Arch. Biochem. Biophys. 401 (2002) 244–254.

[15] S. Imai, C.M. Armstrong, M. Kaeberlein, L. Guarente, Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase, Nature 403 (2000) 795–800.

[16] E. Kim, L. Du, D.B. Bregman, S.L. Warren, Splicing factors associate with hyperphosphorylated RNA polymerase II in the absence of pre-mRNA, J. Cell Biol. 136 (1997) 19–28.

[17] M.S. Kim, H.J. Kwon, Y.M. Lee, J.H. Baek, J.E. Jang, S.W. Lee, E.J. Moon, H.S. Kim, S.K. Lee, H.Y. Chung, C.W. Kim, K.W. Kim, Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes, Nat. Med. 7 (2001) 437–443.

[18] P.S. Knoepfler, R.N. Eisenman, Sin meets NuRD and other tails of repression, Cell 99 (1999) 447–450.

[19] D.Y. Lee, J.J. Hayes, D. Pruss, A.P. Wolffe, A positive role for histone acetylation in transcription factor access to nucleosomal DNA, Cell 72 (1993) 73–84.

[20] P. Morin Jr., K.B. Storey, Cloning and expression of hypoxia-inducible factor 1alpha from the hibernating ground squirrel, *Spermophilus tridecemlineatus*, Biochim. Biophys. Acta 1729 (2005) 32–40.

[21] H.H. Ng, A. Bird, Histone deacetylases: silencers for hire, Trends Biochem. Sci. 25 (2000) 121–126.

[22] V.G. Norton, B.S. Imai, P. Yau, E.M. Bradbury, Histone acetylation reduces nucleosome core particle linking number change, Cell 57 (1989) 449–457.
[23] L.P. O'Neill, B.M. Turner, Histone H4 acetylation distinguishes coding regions of the human genome from heterochromatin in a differentiation-dependent but transcription-independent manner, EMBO J. 14 (1995) 3946–3957.
[24] P.G. Osborne, B. Gao, M. Hashimoto, Determination in vivo of newly synthesized gene expression in hamsters during phases of the hibernation cycle, Jpn. J. Physiol. 54 (2004) 295–305.
[25] C. Puerta, F. Hernandez, L. Lopez-Alarcon, E. Palacian, Acetylation of histone H2A.H2B dimers facilitates transcription, Biochem. Biophys. Res. Commun. 210 (1995) 409–416.
[26] D.F. Rolfe, G.C. Brown, Cellular energy utilization and molecular origin of standard metabolic rate in mammals, Physiol. Rev. 77 (1997) 731–758.
[27] H. Serizawa, J.W. Conaway, R.C. Conaway, Phosphorylation of C-terminal domain of RNA polymerase II is not required in basal transcription, Nature (Lond.). 363 (1993) 371–374.
[28] K.B. Storey, Mammalian hibernation. Transcriptional and translational controls, Adv. Exp. Med. Biol. 543 (2003) 21–38.
[29] K.B. Storey, J.M. Storey, Metabolic rate depression in animals: transcriptional and translational controls, Biol. Rev. Camb. Philos. Soc. 79 (2004) 207–233.
[30] F. van Breukelen, S.L. Martin, Reversible depression of transcription during hibernation, J. Comp. Physiol. [B]. 172 (2002) 355–361.
[31] L.C.H. Wang, T.F. Lee, in: M.J. Fregley, C.M. Blatteis (Eds.), Handbook of Physiology: Environmental Physiology, Torpor and Hibernation in Mammals: Metabolic, Physiological, and Biochemical Adaptations, Oxford University Press, New York, 1996, pp. 507–532.