Preference of IRES-mediated initiation of translation during hibernation in golden-mantled ground squirrels, *Spermophilus lateralis*

Peipei Pan and Frank van Breukelen

*School of Life Sciences, University of Nevada, Las Vegas, Las Vegas, Nevada*

Submitted 15 November 2010; accepted in final form 25 May 2011

Pan P, van Breukelen F. Preference of IRES-mediated initiation of translation during hibernation in golden-mantled ground squirrels, *Spermophilus lateralis*. Am J Physiol Regul Integr Comp Physiol 301: R370–R377, 2011. First published May 25, 2011; doi:10.1152/ajpregu.00748.2010.—Mammalian hibernation involves virtual cessation of energetically consumptive processes normally vital to homeostasis, including gene transcription and protein synthesis. As animals enter torpor, the bulk of initiation of translation is blocked at a body temperature of 18°C in golden-mantled ground squirrels (*Spermophilus* (*Callospermophilus*) *lateralis*). Previous data demonstrated regulation of cap-dependent initiation of translation during hibernation. We asked what happens to cap-independent, specifically, internal ribosome entry site (IRES)-mediated initiation of translation during hibernation. We analyzed polysome fractions for mRNAs that are known to contain or not to contain IRES elements. Here, we show that mRNAs harboring IRES elements preferentially associate with ribosomes as a torpor bout progresses. Squirrels allowed to naturally complete a torpor cycle have a higher IRES preference index than those animals that are prematurely aroused from torpor. Data indicate that this change in preference is not associated with gene expression, i.e., change is due to change in mRNA association with ribosomes as opposed to mRNA abundance. Thus, although processes like transcription and translation are virtually arrested during torpor, ribosomes are preferentially loaded with IRES-containing transcripts when squirrels arouse from torpor and translation resumes. Differential translation of preexisting mRNAs may allow for the preferential production of key stress proteins critical for survival of physiological insults that are lethal to other mammals.

cap-dependent initiation of translation; polyribosomes (polysomes); ribosome; internal ribosome entry site

Although normally vital to homeostasis, protein synthesis represents ∼20–30% of standard metabolic rate (42). This energetic outlay is incompatible with the metabolic rates of mammalian hibernation; translation is severely reduced to near-negligible levels during torpor (0.13–0.5% of euthermic values) and is fully restored during the interbout arousal (55, 66). When mRNAs are actively translated, they will associate with multiple ribosomes, forming polyribosomes or polysomes. Polysome analyses revealed that initiation of translation is markedly reduced during the entrance into torpor when \( T_b \) reaches 18°C (55). However, elongation of nascent peptides continues slowly throughout the torpor bout. At the end of the torpor bout, few polysomes remain. As \( T_b \) begins to rise, some new initiation occurs, but translational initiation and elongation are only fully recoupled when \( T_b \) reaches 18°C. A survey of major regulatory initiation factors that could be involved in the active suppression of initiation of translation revealed one regulatory locus in livers of hibernating ground squirrels (57). Eukaryotic initiation factor 4E (eIF4E), the cap-binding protein, was regulated during torpor. Regulation of eIF4E, and thus the regulation of cap-dependent initiation of translation, is known to occur by at least two distinct mechanisms: 1) direct phosphorylation of eIF4E and 2) interaction of eIF4E with binding proteins, e.g., eIF4E-binding protein 1 (4E-BP1). 4E-BP1 was absent in summer but present in winter in golden-mantled ground squirrels (57). In summer, squirrels apparently control initiation of translation through reversible phosphorylation of eIF4E. In winter, squirrels appear to control initiation of translation through reversible binding to 4E-BP1. 4E-BP1 is differentially phosphorylated between the torpid and active states (57). Given the role of eIF4E in regulating initiation, these data suggest that squirrels actively downregulate cap-dependent initiation of translation during hibernation.

We asked what happens to cap-independent initiation during hibernation when cap-dependent translation is inhibited. More specifically, what is the role for internal ribosome entry site (IRES)-mediated initiation of translation (IRESmt), which allows for translational initiation to occur independent of the commonly used 5'-mRNA cap structure (19)? IRESmt is a well-documented and -validated cap-independent translation mechanism (30). In IRES use, a subset of mRNAs contain higher-ordered RNA structures in the 5′-untranslated region. Theses structures allow for the recruitment of eukaryotic initiation factors (eIF) independent of the 5′,7-methyl guanosine cap of mRNA and eIF4E function (52). IRESmt is important during physiological stresses, including mild hypothermia, hypoxia, and oxidative stress (6, 7, 18, 19, 22, 49). The utilization of IRESmt promotes differential protein expression geared toward enhancing survivorship (29). Similarly, if hibernators used IRESmt, one might expect the translation of stress proteins that could enable the squirrels to withstand the hard-
ships of hibernation. To determine the role of IRESmt in hibernation, we asked if IRES-containing transcripts are preferentially associated with ribosomes during a torpor bout.

MATERIALS AND METHODS

Animals and tissue collection. Adult golden-mantled ground squirrels (Spermophilus (Callospergophilus) lateralis) were captured in July and early August from southern Nevada and California. Some animals were killed immediately as a seasonal control [summer active (SA), \( T_b = \sim 37^\circ C \)]. The remaining squirrels were maintained in a diet of rat chow supplemented with sunflower seeds. These squirrels were implanted in October with temperature-sensitive radiotelemeters (Minimitter, Sun River, OR) that allowed for the precise determination of pulsed feedings.

Animals spontaneously stopped feeding, and were housed in an environmental chamber at 4°C and (Minimitter, Sun River, OR) that allowed for the precise determina-

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Correlation coefficients \((r)\) for the standard curve were \(>0.98\). Reaction efficiency \((E)\) of the reactions was calculated by the equation: 

\[
E = 10^{\frac{1}{\text{slope}} - 1}
\]

Polysomes were isolated using a sucrose density gradient with an ISCO flow system as previously described [45]. The gradients were recovered in 12 1-nil fractions. The early fractions contain monosomes and oligosomes, whereas polysomes are restricted to later fractions. We pooled the fractions into light (early fractions 2–5) and heavy (later fractions 7–12) pools to test if there were differences in oligosomes vs. heavy polysomes.

Quantitative real-time PCR. Polysome RNA was isolated from the pooled fractions with TRIzol Reagent (Invitrogen, Carlsbad, CA). Polysome RNA for each sample was used in a reverse transcription reaction to synthesize single-stranded cDNA (SuperScript III Reverse Transcriptase; Invitrogen). A total RNA sample was isolated from a single SA animal and used as a standard reference to avoid issues with differential efficiencies of quantitative real-time PCR (qRT-PCR) plates (Fig. 1). A reference standard curve was constructed for each assayed transcript from this serially diluted and quantified total RNA

Table 1. Oligonucleotide primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>GenBank Accession No.</th>
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<tbody>
<tr>
<td>β-Actin</td>
<td>5’-TGGCATATGATGACTGGC-3’</td>
<td></td>
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<tr>
<td>GAPDH</td>
<td>5’-AGAAGCTGGAGTAACGAT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BiP/GAPRT78</td>
<td>5’-CATCCTCTGGGAAAGA-3’</td>
<td></td>
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<tr>
<td>c-Myc</td>
<td>5’-GACACCTCTTGTGAAAGA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaM kinase II</td>
<td>5’-GAGGATAGAGATGGAAAAGC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28S</td>
<td>5’-CGAAGATCTCTTCAAACCA-3’</td>
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Known non-ribosomal entry site (IRES)-containing transcripts include actin and GAPDH, whereas BiP, c-myc, and calmodulin kinase (CaM kinase) II transcripts are known to harbor IRES elements. 28S rRNA was used to normalize for the amount of ribosomes. Amplified fragments were sequenced, and sequences have been deposited in GenBank.
(dilution factors of 1×, 5×, 25×, 125×, and 625×). qRT-PCR was used to investigate the association of both IRES (BiP, c-myc, and CaM kinase II)- and non-IRES (GAPDH and β-actin)-containing transcripts with ribosomes in various stages of a torpor bout from golden-mantled ground squirrels with corresponding specific primers (Table 1). qRT-PCR was performed on both the polysome fractions and for standard curve construction using the SYBR Green I kit with ROX (Bio-Rad, Hercules, CA). Reactions (1 reaction/animal for both the transcript of interest and 28S rRNA along with the standard curve) were performed on a single plate to reduce interplate variability. Preliminary experiments revealed little difference between replicate plates provided the reaction efficiencies were high. All PCR reactions were run with the following program: 3 min at 95°C, 15 s at 95°C, 30 s at 52°C, and 30 s at 72°C (40 cycles) with a mixture containing 1 µl of cDNA template, 12.5 µl qPCR Supermix, and 200 nM of each primer in a total volume of 25 µl (Bio-Rad). Melt curves to ascertain that real specific products were synthesized were obtained using the following program: 1 min at 95°C, 1 min at 55°C, and 80 cycles of 10 s at 55°C with a step of 0.5°C every cycle.

Construction of IRES preference, abundance indexes, and statistical analyses. Results from qRT-PCR were compared against a reference standard curve made from total RNA of a single SA animal (Fig. 1). The raw critical threshold (Ct) values were then normalized for the amount of starting material and expressed as the concentration of transcript of interest-to-28S rRNA ratio (62). From earlier work (55 and references therein), we know that, during torpor, there is a dramatic reduction in the quantity of protein synthesis (and therefore heavy polysomes). The normalization to ribosomal RNA removes quantitative changes in translation to the analysis (see DISCUSSION). The ratios were log-transformed, and the IRES preference index was obtained by summing the log-transformed values from the known IRES-containing transcripts and then subtracting the log-transformed values from the known non-IRES-containing transcripts [IRES preference index = ∑log (normalized IRES transcript concentration) − ∑log (normalized non-IRES transcript concentration)]. To make the data on IRES preference more accessible as a single value for comparison, we converted the average values for all animals at a given torpor stage into an RGB color scale. Values are on a scale of 0–255, in which 0 is magenta (lowest IRES preference index value as a % of maximum IRES preference index value) and 255 is green (maximum IRES preference index value). IRES preference indexes were analyzed by average linkage cluster analyses (uncentered correlation) for all individual animals to test if there was a switch in the preference as a function of torpor. Data for both heavy and light fractions on IRES preference index were subjected to paired t-test to determine if there was an effect of fraction pool. Analyses demonstrated no effect of fraction pool, e.g., light vs. heavy fractions on IRES preference (paired t-test, P > 0.05), and values for both pools were summed in all graphical representations.

The IRES abundance index was determined for these mRNAs in a similar fashion to the IRES preference index except qRT-PCR was performed on total RNA as opposed to polysome RNA from these same animals. The results from transcript abundance were subjected to ANOVA with Tukey adjustment for multiple comparisons and differences where P < 0.05 were considered significant (26).

**EDTA treatment control.** Lysates from SA and early arousal (Tb ~10°C) animals were loaded on a sucrose gradient, and polysomes were isolated as previously described (45; same methodology as used above). Parallel lysates were pretreated with 30 mM EDTA before loading. All experiments were performed as above except total RNA was subjected to analysis by the Bio-Rad Experion system (indicates integrity and concentration; analyses as per the manufacturer’s directions) to show disruption of RNA from heavy material by EDTA.

**RESULTS**

We exploited qRT-PCR to determine the quantity of specific mRNAs that associated with ribosomes during torpor. To avoid issues with differential efficiencies of qRT-PCR plates and changes in the quantity of protein synthesis as a function of torpor state, the raw Ct values were compared against a reference standard curve made from total RNA of a single animal, transformed to the amount of starting material and expressed as the concentration of the transcript of interest-to-28S rRNA ratio. Our reactions had reaction efficiencies of 92.84 ± 2.51% (Fig. 1).

**Fig. 2.** EDTA treatment disrupts polysome association with mRNA measured by qRT-PCR. A lysate from a summer active (SA) animal was loaded on a sucrose gradient as indicated in the text (control). A parallel lysate was treated with 30 mM EDTA before loading (+ EDTA). The abundance of specific RNA species was measured by qRT-PCR. 28S rRNA was used as a loading control for the amount of ribosome. β-Actin is encoded by a noninternal ribosome entry site (IRES) containing transcript. BiP’s transcript harbors an IRES element. Values are indicated as the percentage of the maximum value in the gradient for each RNA species. Note that EDTA treatment shifts the RNA distribution. [RNA], RNA concentration.
RNA association with proteins is disrupted by pretreatment with EDTA (3a). EDTA treatment disrupted the polysome profile in our experiments (Figs. S2, 2, and 3). We demonstrate that EDTA treatment shifted polysome profiles toward monosome- and oligosome-bearing fractions for total RNA (Fig. S2) as well as for specific messages amplified by qRT-PCR (Fig. 2). We note that, as a result of EDTA treatment, shifts in 28S rRNA coincide with shifts in transcript levels (Fig. 2). We show sample profiles for early arousing (T$_b$ $\sim$10°C) and SA ground squirrels to demonstrate the differences in RNA distributions as a function of state (Fig. 3). Such differences were used as a basis for the calculated IRES preference index.

As a torpor bout progresses, mRNAs that harbor IRES sequences preferentially associate with ribosomes (Fig. 4). Squirrels in the summer experience an IRES index that is biased toward cap-dependent initiation of translation. During winter, squirrels entering torpor have similar indexes as summer squirrels, indicating preference for cap-dependent initiation of translation. However, as a torpor bout progresses, IRESmt becomes more and more dominant until the highest IRES indexes are obtained during the arousal process. This result suggests that, as squirrels arouse from torpor and translation resumes, their ribosomes are preferentially loaded with mRNAs containing IRES elements. To ensure that these changes were bona fide, we performed average linkage cluster analyses (uncentered correlation) on our obtained IRES preference indexes and found two groups (Fig. 5) demonstrating a shift in the preference of transcripts to associate with the ribosomes as a function of torpor.

To detect if the observed changes in IRES preference were the result of a bias of association of IRES participants with ribosomes as opposed to differential expression of mRNA, a similar index was performed using qRT-PCR from total RNA (as opposed to polysome fractions) from these same animals. An abundance index was determined for these mRNAs in a similar fashion to the IRES preference index (Fig. 6). No significant differences were found in the transcript abundance (ANOVA with Tukey adjustment for multiple comparisons, $P > 0.05$ for all comparisons), demonstrating that the observed effect of association of IRES-containing transcripts to ribosomes was not the result of changes in concentration of mRNAs but rather changes in association.

Normally, golden-mantled ground squirrels spontaneously arouse from torpor in a very predictable fashion. However, squirrels may also be induced to arouse prematurely before the completion of the torpor bout through various disturbances such as shaking, loud noises, or sudden shifts in ambient temperature (unpublished observations). We alarm-aroused squirrels by gentle shaking before the completion of the torpor bout. Premature arousal from torpor affects the IRES preference index (Fig. 7). These data indicate that there is a shift during the torpor bout such that animals that spontaneously arouse are poised to experience greater IRESmt (ANOVA, $P < 0.05$). Although animals who had a T$_b$ of 10°C show a

![Fig. 3. IRES-containing mRNAs are relatively more abundant on heavy polysomes in early arousing squirrels. Lysates from SA (left) and early arousing (ARO; body temperature $\sim$10°C; right) animals were load on a sucrose gradient as indicated in the text. The abundance of specific RNA species was measured by qRT-PCR. Note that, although most of the 28S and non-IRES-containing GAPDH RNA is found in the lighter fractions in the ARO animal, the IRES-containing BiP is found most frequently in a heavy polysome-containing fraction.](http://ajpregu.physiology.org/)

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significantly reduced IRES preference when alarm aroused, IRESmt may be exploited by the time animals reach T_b of 20°C during the arousal process (Fig. 7).

**DISCUSSION**

To balance energetic outlays with available resources, mammalian hibernators must arrest most processes normally vital to homeostasis. Accordingly, ground squirrels downregulate processes of transcription and translation (55, 56). Of note is that, while much of this downregulation is accomplished through passive mechanisms, there is a role for active suppression of cap-dependent initiation of translation (57). In this study, we investigated a cap-independent process of initiation, e.g., IRESmt. We analyzed polysome fractions for mRNAs that are known to contain or not to contain IRES elements.

As a torpor bout progresses, mRNAs that harbor IRES sequences preferentially associate with ribosomes (Fig. 4). As translation resumes when the squirrels’ T_b is increased during arousal, the availability of IRES-containing transcripts may have tremendous physiological consequences. One possible explanation for increased association of IRES participants to ribosomes may be differential gene expression, i.e., if there was an increase in mRNA abundance for these IRES-containing transcripts then there may simply be increased association of these transcripts through mass action reaction rates. Although the severely restricted transcription available during torpor would make this possibility unlikely (56), we determined if there were any changes in mRNA abundance. Our analyses demonstrate that the IRES-harboring transcripts that we examined were not differentially expressed (Fig. 6). Rather, the squirrels exploit an available mechanism of IRESmt to gain differential translation of preexisting transcripts as the squirrels begin to arouse from torpor and translation resumes.

The use of IRESmt has been demonstrated in other systems in response to physiological insults and preferentially encodes for stress proteins (3, 16, 63). Indeed, glucose-induced starvation in yeast resulted in IRESmt, which increased survivability (16). We contend that the use of IRESmt in hibernators may help provide a protective phenotype by allowing increased expression of stress proteins as squirrels arouse. For instance, during entrance to hibernation, heart rate drops precipitously before any observed change in T_b (31). This reduced blood supply may provide an ischemic insult, and indicators of oxidative stress such as conjugated dienes and ubiquitylated protein concentrations increase during entrance into torpor (reviewed in Ref. 4). The induction of stress proteins may help to serve to mitigate this damage. A number of known IRES participants increase during hibernation. These include BiP/GRP78 (12, 28), connexin 43 (24), hypoxia-inducible factor (HIF-1; see Ref. 32), and c-Jun (34). BiP is an endoplasmic reticulum-resident chaperone that may facilitate proper folding of proteins and function during torpor (12, 28). Connexin 43 is associated with increased conductivity in the heart, and it may play a role in maintaining cardiac rhythms during torpor (14, 43, 44). HIF-1 is a transcription factor and may serve to enhance hypoxia tolerance (32). c-Jun is a transcription factor critical for efficient axonal regeneration (38). Recent data suggest a need for substantial axonal regeneration during arousal from hibernation (58).

One might expect enormous changes in transcript abundance as a result of the tremendous physiological changes inherent to hibernation. However, numerous investigations of differential gene expression examining total mRNA have found relatively few differences in transcript abundance as a function of torpor (e.g., see Refs. 61 and 65). This seemingly surprising result...
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We suspect that IRES-containing transcripts may represent only a small fraction of total transcript associated with ribosomes but that this fraction is increased during a torpor bout.

We asked if squirrels might prepare for arousal, i.e., must squirrels load up ribosomes with IRES-containing transcripts to arouse from torpor? We investigated this hypothesis by alarm-arousing ground squirrels prematurely from their torpor bouts. During a natural, spontaneous arousal, IRES preference index is relatively high and remains constant throughout the arousal process (Fig. 7). In contrast, alarm-aroused squirrels at 10°C demonstrate IRES preference indexes that are similar to animals obtained earlier in the torpor bout, e.g., early torpor or late torpor. However, by the time the squirrels have reached a Tb of 20°C, IRES preference index is indistinguishable from naturally aroused squirrels. Thus, it appears that squirrels are normally prepared for an arousal.

Although hibernation is assumed to be a very effective survival strategy, as many as 40–70% of ground squirrels may die in a given year during the winter (47). Any mechanism that might ameliorate the damage associated with the hardships of torpor should be under significant evolutionary selective pressure. Therefore, it follows that a mechanism that allows for the differential translation of preexisting messages that preferentially encode for stress proteins during the torpor cycle is of great importance. Arousing with a new set of proteins may be the critical adaptation that allows a hibernator to survive physiological insults that are lethal to most other mammals.

Fig. 6. IRES-harboring transcript abundance was not changed as a function of torpor bout. IRES abundance index demonstrates that there is no preferential expression of IRES genes during the torpor bout. An abundance index was calculated similarly to the IRES preference index used in Figs. 4, 5, and 7 except the values were obtained using total RNA and not ribosome-associated RNA. Values represent means ± SE, n = 3 animals for each state. There are no significant differences between the various states (P > 0.05, ANOVA with Tukey correction for multiple comparisons). These data indicate that the observed changes in IRES preference are the result of preferential translation and not differential gene expression.

Fig. 7. Premature arousal from torpor affects the IRES preference index. Animals (n = 3 for each group) were allowed to spontaneously arouse from torpor as a normal function of the torpor cycle (natural arousal) or were forced to arouse prematurely (alarm arousal). The abscissa represents the Tb of the squirrel when sampled. Data from alarm-aroused squirrels at Tb ≤ 10°C are significantly different from all other values (ANOVA, P = 0.045). No other differences were found. Some data points overlap.
REFERENCES

DISCLOSURES

ACKNOWLEDGMENTS

REFERENCES


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