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# Epigenetic regulation of hibernation-associated *HP-20* and *HP-27* gene transcription in chipmunk liver

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ABSTRACT

The chipmunk hibernation-related proteins (HPs) HP-20 and HP-27 are components of a 140-kDa complex that dramatically decreases in the blood during hibernation. The *HP-20* and *HP-27* genes are expressed specifically in the liver and are downregulated in hibernating chipmunks. Hibernation-associated physiological changes are assumed to be under genetic control. Therefore, to elucidate the molecular mechanisms of hibernation, here we examined the mechanisms behind the altered *HP-20* and *HP-27* gene expression in nonhibernating versus hibernating chipmunks. Chromatin immunoprecipitation (ChIP) analyses revealed that histone H3 on the *HP-20* and *HP-27* gene promoters was highly acetylated at lysine (K) 9 and K14 and highly trimethylated at K4 in the liver of nonhibernating chipmunks, while these active histone modifications were nearly absent in hibernating chipmunks. Furthermore, histone acetyltransferases and a histone methyltransferase were associated with the *HP-20* and *HP-27* gene promoters primarily in nonhibernating chipmunks. Consistent with a previous finding that HNF-1 and USF can activate *HP-20* and *HP-27* gene transcription by binding to the proximal promoter region, ChIP-quantitative PCR (qPCR) analyses revealed that significantly less HNF-1 and USF were bound to these gene promoters in hibernating than in nonhibernating chipmunks. These findings collectively indicated that the hibernation-associated *HP-20* and *HP-27* gene expression is epigenetically regulated at the transcriptional level by the binding of HNF-1 and USF to their proximal promoters, and that histone modification has a key role in hibernation-associated transcriptional regulation.



## 1. Introduction

Mammalian hibernation is a unique physiological adaptation that allows life to be sustained at very low body temperatures (Tb), which can fall below 5°C. During hibernation, the heart and breathing rates drop, and the metabolic rate falls to only a few percent of the euthermic level, enabling the animal to conserve a considerable amount of energy [1]. The hibernation-associated physiological changes are assumed to be under genetic control [2,3]. In the chipmunk (*Tamias asiaticus*), hibernation-related proteins (HPs), HP-20, HP-25, HP-27, and HP-55, were found as components of a 140-kDa complex that decreases dramatically in the blood during hibernation [4]. The 140-kDa complex begins to decrease before hibernation onset, remains at a low level during hibernation, and then starts to increase before hibernation ends [4]. Kondo et al. showed that hibernation onset is marked by an increase in HP-20c, a complex consisting of HP-20, HP-25, and HP-27, in the brain [5]. This increase, like the decrease in the 140-kDa complex level in the blood, is independent of Tb changes, supporting the idea that hibernation is regulated by a circannually controlled molecular network.

HP-20, HP-25, and HP-27 are highly homologous to each other [4,6], and belong to the C1q and tumor necrosis factor superfamily [7]. The *HP-20*, *HP-25*, and *HP-27* genes are expressed specifically in the liver and are downregulated in hibernating chipmunks and ground squirrels [6]; these downregulations are responsible for the reduced 140-kDa HP complex level in the blood. These three HP genes are located close to each other on the genome in ground squirrel [8]. We previously revealed that *HP-20*, *HP-25*, and *HP-27*

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gene transcription can be activated *in vitro* by HNF-1, HNF-4 and USF, and HNF-1 and USF, respectively [9–13]. Since the fluctuation of the 140-kDa complex level was independent of Tb changes [5], the mRNA expression of these *HP*s in the liver is thought to be controlled by endogenous rhythms, rather than by the Tb or by metabolic inhibition at low Tb.

We recently revealed that the differential *HP-25* gene transcription in the liver between nonhibernating and hibernating chipmunks is epigenetically regulated [11]. The acetylation and methylation of certain histone residues generally have roles in gene activation, whereas a lack of histone acetylation is a marker of gene repression [14–16]. Another group also showed that histone modifications in the skeletal muscle of the ground squirrel are responsive to torpor-arousal cycles [17]. These findings together suggest that the epigenetic regulation of gene expression involving reversible histone modifications has key roles in mammalian hibernation. In the case of the *HP-25* gene, the differential histone modification between nonhibernating and hibernating chipmunks is regulated by the binding of HNF-4 to the gene promoter, and this binding is modulated by small heterodimer partner (SHP) [11]. Although the transcript levels of the *HP-20*, *HP-25*, and *HP-27* genes fluctuate similarly in concert with hibernation [6], these genes are activated by different transcription factors [9–13]. Thus, in this study, we sought to reveal the molecular mechanisms behind the hibernation-associated changes in *HP-20* and *HP-27* gene expression. Our results indicated that the hibernation-associated transcriptional regulation of the *HP-20* and *HP-27* genes in the liver is accomplished by histone modification, similar to the *HP-25* gene, and that the differential histone modification between nonhibernating and hibernating chipmunks is regulated by the binding of HNF-1 and USF to the proximal promoter region.

## 2. Materials and methods

### 2.1. Animals

Male chipmunks (*Tamias asiaticus*) were used as previously described [11]. All of the protocols were in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kitasato University, and all experimental procedures were approved by the same committee.

### 2.2. RT-qPCR of primary transcripts and mRNA

Total RNA prepared from the liver using ISOGENII (Nippon Gene) was treated with DNaseI. To amplify the primary transcript or mRNA of the *HP-20*, *HP-27*, and *albumin* genes, quantitative PCR was performed with more than five samples in technical triplicate. The primers used were listed in Supplementary Table 1. *Beta-actin* was used as an endogenous control for normalizing gene expression. For further details please refer to the supplemental information.

### 2.3. Chromatin immunoprecipitation (ChIP) analysis

ChIP was carried out as described previously [12]. All of the ChIP experiments were performed three times with independent chromatin preparations. The antibodies used for ChIP were described previously [11]. The ChIP-qPCR data were normalized using the percent input method and were shown as the value in the liver of hibernating chipmunks relative to that for nonhibernating chipmunks. For further details please refer to the supplemental information.

### 2.4. Immunoblotting

Immunoblotting was performed using the same nuclear extracts as described previously [11].

### 2.5. Bisulfite sequencing

Bisulfite sequencing was performed as described previously [13].

### 2.6. Transfection and luciferase assays

The promoter-reporter plasmid constructions, transfection, and Luciferase assays were carried out as described previously [9,11,13].

### 2.7. Statistical analysis

Student's t-test (2-tailed 2, type 2) and the Tukey-Kramer Multiple Comparison test were used to determine the difference from a control group. Two-way ANOVA was used to analyze the interaction between treatments, and post hoc multiple comparison using the Tukey-Kramer test was performed when the interaction was significant with $p < .001$.

## 3. Results

### 3.1. HP-20 and HP-27 genes are regulated at the transcriptional level in concert with hibernation

We previously showed by northern analyses that the *HP-20* and *HP-27* genes are downregulated in the liver of hibernating chipmunks [6]. However, northern analysis only indirectly indicates gene transcription, since the mRNA levels represent the sum of transcriptional and post-transcriptional events. Here, we first confirmed our initial finding by quantitatively comparing the primary transcript levels of the *HP-20* and *HP-27* genes between nonhibernating and hibernating chipmunks. This approach can be used to determine a gene's transcriptional state as an alternative to the nuclear run-on assay [18]. To compare the levels of *HP-20* and *HP-27* primary transcripts in the liver of nonhibernating versus hibernating chipmunks, we performed RT-qPCR using a pair of primers, one located in exon 1 and one in intron 1 (Fig. 1). We also analyzed the mRNA levels of these genes by RT-qPCR. Since the *albumin* mRNA and primary transcript levels do not change with hibernation [6,11], we used the *albumin* gene as a control. The *HP-20* and *HP-27* mRNA levels were significantly decreased in the liver of hibernating chipmunks (Fig. 1B), consistent with the northern analysis results [6]. Furthermore, the *HP-20* and *HP-27* primary transcripts were severely reduced in the liver of hibernating compared with nonhibernating chipmunks, while the *albumin* primary transcript was not significantly different (Fig. 1A). These results indicated that the *HP-20* and *HP-27* genes in the liver of nonhibernating chipmunks change from a transcriptionally active state to a transcriptionally repressed one in association with hibernation.

### 3.2. Active histone marks on the HP-20 and HP-27 gene promoters occur during the nonhibernation state

We recently revealed that the chipmunk *HP-25* gene is epigenetically regulated in concert with hibernation [11]. Other studies have also shown that post-translational modifications on histone proteins in the skeletal muscle of ground squirrels are responsive to hibernation cycles [17,19]. Since the total amounts of histone H3 acetylated at lysine (K) 9 and K14 and trimethylated at K4, which are hallmarks of gene activation [14,15], in the liver are similar

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