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Determination of reference genes that are independent of feeding rhythms for circadian studies of mouse metabolic tissues

Reiko Nakao [a], Hiroki Okauchi [a,b], Chiaki Hashimoto [a,c], Naoyuki Wada [b,c], Katsutaka Oishi [a,c,d,*]

[a] Biological Clock Research Group, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan
[b] Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, Noda, Chiba, Japan
[c] Department of Applied Biological Science, Graduate School of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan
[d] Department of Computational and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwa-no-ha, Kashiwa, Chiba 277-8561, Japan

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ABSTRACT

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis is a popular method for the measurement of mRNA expression level and is a critical tool for basic research. The identification of suitable reference genes that are stable and not affected by experimental conditions is a critical step in the accurate normalization of RT-PCR. On the other hand, the levels of numerous transcripts exhibit circadian oscillation in various peripheral tissues and it is thought to be regulated by feeding rhythms in addition to the molecular circadian clock. Here, we investigated the effects of feeding schedule on the temporal expression profiles of 13 common housekeeping genes in metabolic tissues of mice fed during either the sleep or the active phase. The expression of most of these genes fluctuated dependently on feeding rhythms in the liver and WAT, but not in skeletal muscle. Two-way analyses of variance (ANOVA) identified 18S ribosomal RNA (*Rn18s*) as the only gene that was stably expressed throughout the day independently of feeding schedules in the liver and WAT, although RefFinder software showed that peptidylprolyl isomerase A (*Ppia*) was the most stably expressed housekeeping gene. Both ANOVA and RefFinder software determined that *Actb* was the preferred reference gene for skeletal muscle. Furthermore, NormFinder proposed that the optimal pairs of reference genes were beta-2 microglobulin (*B2m*)-*Ppia* in the liver, *Ppia*-TATA box binding protein (*Tbp*) in WAT, and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*Ywhaz*)-glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) in skeletal muscle, and that their stability value was better than that of a single stable gene. The appropriate reference gene pairs for normalizing genes of interest in mouse circadian studies are *B2m-Ppia* in the liver, *Ppia-Tbp* in WAT, and *Ywhaz-Gapdh* in skeletal muscle.

1. Introduction

An internal circadian system controls various physiological and behavioral rhythms such as sleep/wake cycles, body temperature, metabolism and hormone secretion. The master circadian clock is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. The molecular oscillator in the SCN consists of transcription/translation-based feedback loops that drive the 24 h expression of clock genes [1]. Studies of clock genes in mammals have revealed that molecular clock components function in various peripheral tissues such as the liver, heart, adipose tissue, kidneys and skeletal muscles, and that they are entrained to the SCN by systemic time cues such as neural, humoral and other signals including feeding and body temperature [1]. Transcriptome analyses have shown that hundreds of genes are rhythmically expressed in many tissues [2–4] and this process has been attributed mainly to the circadian clock. On the other hand, feeding status and temporal food intake can determine the circadian transcriptome independently of the molecular clock in the liver [5,6].

Gene expression analysis is important for biological research. Real-time reverse transcription-polymerase chain reaction (RT-PCR) is one of the most sensitive and reproducible means of quantifying mRNA expression [7]. The method requires the accurate normalization of genes of interest to a reference gene that is stable and unaffected by experimental conditions. Reference genes are generally selected from among housekeeping genes that are ubiquitously expressed in most tissues and not transcriptionally affected by experimental conditions. Among these, glyceraldehyde-3-

* Corresponding author at: Biological Clock Research Group, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology, Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan.
E-mail address: k-ooishi@aist.go.jp (K. Oishi).

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phosphate dehydrogenase (*Gapdh*), β-actin (*Actb*) and 18S ribosomal RNA (*Rn18s*) are the most popular. However, evidence suggests that the transcription levels of these genes vary considerably among developmental stages, cell types and experimental conditions [8–11].

We analyzed the circadian expression of housekeeping genes in metabolic tissues of mice fed during a reversed circadian schedule to determine those that are independent of circadian feeding schedules. The results showed that the mRNA expression levels of many popular housekeeping genes fluctuated dependently on the feeding cycle in the liver and WAT. The effects of feeding conditions and the time of day should be taken into consideration when evaluating mRNA expression in metabolic tissues.

2. Materials and methods

2.1. Animal care and study design

All animal experiments were performed according to the guidelines for animal experiments of the National Institute of Advanced Industrial Science and Technology (AIST). Our institutional Animal Care and Use Committee approved all experimental protocols (Permission #2016-020).

Six-week-old male C57BL/6J mice (Japan SLC Inc., Hamamatsu, Japan) were fed with a high-fat high-sucrose F2HFHSD diet (Oriental Yeast Co. Ltd., Tokyo, Japan) comprising 29.1% w/w (54.5% of total calories) fat, 20.7% w/w (17.2% of total calories) protein, 34.0% w/w (28.3% of total calories) carbohydrates (containing 20.0% w/w sucrose) *ad libitum* for two weeks under a 12-h light-12-h dark cycle (LD 12:12; lights on at Zeitgeber time (ZT) 0 and lights off at ZT12). The mice were individually housed in cages with running wheels and then separated into groups that were given food only during the sleep phase (ZT2-10) (Daytime feeding; DF) or only during the active phase (ZT14-22) (Nighttime feeding; NF) for one week. The mice were sacrificed at six-hour intervals and then the liver, epididymal white adipose tissue and gastrocnemius (skeletal) muscle were dissected and rapidly frozen in liquid nitrogen.

2.2. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from tissues using guanidinium thiocyanate followed by RNAiso (Takara Bio Inc., Otsu, Japan) according to manufacturer's instructions. The RNA concentration and absorbance at 260/280 nm were measured using a NanoDrop 2000 UV–Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Single-stranded cDNA was synthesized from 1.0 μg of total RNA using PrimeScript™ RT reagent kits with gDNA Eraser (Takara Bio Inc.). Real-time RT-PCR proceeded using SYBR® Premix Ex Taq™ II (Takara Bio Inc., Otsu, Japan) and a LightCycler™ (Roche Diagnostics, Mannheim, Germany). The reaction conditions for amplification were 95 °C for 10 s followed by 45 cycles of 95 °C for 5 s, 57 °C for 10 s and 72 °C for 10 s. The specificity of the PCR product was confirmed by melting curve analysis. Amplification efficiency was calculated from the slope of the standard curve as: $(10^{[-1/\text{slope}]} - 1) \times 100$ (Table 1). We detected the following common housekeeping genes using the Mouse Housekeeping Gene Primer Set (Takara Bio Inc., Otsu, Japan): ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1 (*Atp5f1*); β-2 microglobulin (*B2m*); hypoxanthine guanine phosphoribosyl transferase (*Hprt*); ribosomal protein, large, P1 (*Rplp1*); peptidylprolyl isomerase A (*Ppia*); ribosomal protein S18 (*Rps18*); phosphoglycerate kinase 1 (*Pgk1*); β-glucuronidase (*Gusb*); TATA box binding protein (*Tbp*); β-actin (*Actb*); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*Ywhaz*); glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), and 18S ribosomal RNA (*Rn18s*) using the primer sequences, 5′-GTAACCCGTTGAACCCCATT-3′ and 5′-CCATCCAATCGGTAGTAGCG-3′. Raw expression values were attributed to a standard curve generated using serially diluted cDNA.

Table 1
Real-time RT-PCR efficiency of evaluated genes.

	Gene symbol	Slope	R^2	Efficiency (%)
Liver	*Rn18s*	−3.532	1.00	91.9
	Ywhaz	−3.984	0.98	78.2
	B2m	−3.233	1.00	103.8
	Rps18	−3.534	1.00	91.9
	Rplp1	−3.262	1.00	102.6
	Hprt	−3.455	1.00	94.7
	Tbp	−3.684	0.99	86.8
	Actb	−3.963	0.96	78.8
	Ppia	−3.505	0.99	92.9
	Gusb	−3.512	1.00	92.6
	Atp5f1	−3.319	1.00	100.1
	Pgk1	−4.213	1.00	72.7
	Gapdh	−3.791	1.00	83.6
White adipose tissue	*Rn18s*	−3.488	1.00	93.5
	Ywhaz	−3.455	1.00	94.7
	B2m	−3.571	1.00	90.6
	Rps18	−3.299	1.00	101.0
	Rplp1	−3.318	1.00	100.2
	Hprt	−3.322	1.00	100.0
	Tbp	−4.515	0.99	66.53
	Actb	−3.283	1.00	101.6
	Ppia	−3.406	1.00	98.6
	Gusb	−3.368	1.00	98.1
	Atp5f1	−3.175	1.00	106.5
	Pgk1	−3.273	1.00	102.1
	Gapdh	−3.377	1.00	97.7
Skeletal muscle	*Rn18s*	−3.462	1.00	94.5
	Ywhaz	−3.777	1.00	84.0
	B2m	−3.349	1.00	93.4
	Rps18	−3.38	1.00	97.3
	Rplp1	−3.334	1.00	99.5
	Hprt	−2.962	1.00	117.6
	Tbp	−4.070	0.99	76.1
	Actb	−3.348	1.00	98.9
	Ppia	−3.477	1.00	93.9
	Gusb	−3.613	0.99	89.2
	Atp5f1	−3.391	1.00	97.2
	Pgk1	−3.545	1.00	91.5
	Gapdh	−3.591	1.00	89.9

2.3. Statistical analysis

Data are expressed as means ± standard error of the mean (SEM) and were evaluated by an analysis of variance (ANOVA) followed by the Tukey-Kramer post-hoc test using Excel-Toukei 2010 software (Social Survey Research Information Co. Ltd., Osaka, Japan). Differences were considered significant at $P < 0.05$. The stability of housekeeping gene expression was tested by RefFinder using a Ct value defined as the number of cycles required for the emitted fluorescence to reach a specific threshold level of detection that inversely correlated with the amount of template nucleic acid in the reaction mix. RefFinder is a web-based tool (http://fulxie.0fees.us/?type=reference#) that can select the most stably expressed housekeeping genes [12]. It integrates the statistical approaches of a comparative ΔCt method [7], BestKeeper [13], NormFinder [14] and geNorm [9] to rank candidate reference genes. The BestKeeper method estimates housekeeping gene stability based on an inspection of the calculated standard deviation (SD) of Ct values and any gene with a value >1 can be considered inconsistent [13]. The geNorm method selects applicable reference genes with a stability value that is below an arbitrary cut-off such as 0.5 [15]. NormFinder calculates gene stability values for either the most stable reference gene or the optimal combination of two genes. Threshold values in NormFinder