Housekeeping Gene Expression in Bovine Liver is Affected by Physiological State, Feed Intake, and Dietary Treatment¹

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ABSTRACT

Selection of appropriate housekeeping genes (HKG) for normalization of quantitative PCR data for genes of interest is critical for interpretation of results. Ideally, copy number of the chosen HKG mRNA will not vary with experimental treatments or physiological state in the tissue studied, which improves accuracy in detecting changes in genes of interest. Because of the liver's dynamic role in metabolism, physiological state or dietary treatments could alter mRNA expression of commonly used HKG. Therefore, the objective of this study was to evaluate stability of mRNA expression for a number of candidate HKG in bovine liver across different physiological and dietary experimental conditions during the periparturient period. A publicly available program (geNorm) was used to evaluate expression stability of 8 HKG (β -actin, glyceraldehyde 3-phosphate dehydrogenase, β -glucuronidase, peptidylprolyl isomerase A, polyubiquitin, ribosomal protein S9, ribosomal protein L32, and 18S ribosomal RNA) in 91 liver RNA samples. Screened samples included liver from cows in 3 groups: 1) cows receiving a dietary supplement pre- and postpartum ($n = 10$); 2) cows with clinical or subclinical ketosis ($n = 7$); and 3) cows consuming different amounts of energy prepartum ($n = 74$). In group 3, samples from d -65, -30, -14, 1, 14, 28, and 49 relative to parturition were included to enable characterization of HKG mRNA expression across different physiological states. Initial analyses indicated that mRNA for ribosomal protein S9 (*RPS9*) was one of the most stably expressed across different experiment types. To determine the best gene, 200 bootstrap repli-

cations of the original data set were performed to determine if the ranking of *RPS9* was superior to the other 7 genes evaluated. Average ranks and estimated standard errors for the top 3 genes were 1.64 ± 0.06 , 3.27 ± 0.10 , and 3.71 ± 0.12 for *RPS9*, *GAPDH*, and β -actin, respectively. Ribosomal protein S9 was ranked first 59% of the time and was never ranked lower than fifth. The lowest-ranked gene was polyubiquitin, ranked last 46.5% of the time (average rank = 6.85 ± 0.10). In this study, physiological state, amount of intake, or dietary treatment influenced the mRNA expression of commonly used HKG in bovine liver. Ideally, expression stability should be tested before collection of data in all experiments; however, we have shown that *RPS9* mRNA is stable across several physiological and diet-related experimental conditions for dairy cows, making it a good HKG in liver quantitative PCR experiments. **Key words:** liver, housekeeping gene, quantitative polymerase chain reaction

INTRODUCTION

Quantitative real-time PCR (qPCR) characterization of selected mRNA has become a popular method to study changes in relative copy number of mRNA in a variety of tissues and experimental settings. As summarized by Wong and Medrano (2005), qPCR is more sensitive than RNase protection assays, is less variable than band densitometry or probe hybridization as used with end-point assays, and can detect very small copy numbers for mRNA of interest. Because of its sensitivity, a stably expressed internal control gene, or housekeeping gene (HKG), is needed to accurately estimate copy number for mRNA. The expression of an "ideal" HKG will not vary with treatment or physiological state in the tissue studied (Thellin et al., 1999; Bustin, 2000, 2002). Expression of many of the common HKG currently used in experiments, however, varies with treatment or physiological state (Bustin, 2002; Wong and Medrano, 2005). As concerns about normalization to proper HKG have increased, methods to find HKG that are stably expressed under various experimental conditions and tissues of interest have received

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Table 1. Characteristics of liver RNA samples used for real-time quantitative PCR analysis of potential housekeeping genes

Sample set	Source of liver tissue	Physiological state	DRTP ¹	Dietary treatments ²	Samples, n
1	Dann et al. (2006) ³	Dry	-65	Limited energy intake prepartum	5
		Dry	-30	Ad libitum, limited, or restricted energy intake prepartum	13
		Dry	-14	Ad libitum, limited, or restricted energy intake prepartum	13
		Parturition/very early lactation	1	Ad libitum, limited, or restricted energy intake prepartum	13
		Lactating	14	Ad libitum, limited, or restricted energy intake prepartum	13
		Lactating	28	Ad libitum, limited, or restricted energy intake prepartum	13
		Lactating	49	Limited energy intake prepartum	5
2	Carlson (2006)	Parturition/very early lactation	2	Control or L-carnitine supplemented	5
		Lactating	10	Control or L-carnitine supplemented	5
3	Dann et al. (2005)	Ketotic, lactating	10 to 14	Ketosis induction, feed restriction	7
Total samples					92

¹DRTP = day relative to parturition.

²Dietary treatment descriptions: Dann et al. (2006) = 1 of 3 levels of energy intake fed prepartum, 150, 100, or 80% of NRC (2001) requirement for energy for mature cows in late gestation, common lactation diet provided 1.77 Mcal of NE_I/kg of DM; Carlson (2006) = either 0 (control) or 50 g of L-carnitine fed from d -14 to 21 relative to parturition, lactation diet provided 1.68 Mcal of NE_I/kg of DM; Dann et al. (2005) = at 5 DIM cows restricted to 50% of their ad libitum intake to induce ketosis.

³For description of RNA extraction methods for this experiment, see Loor et al. (2005, 2006).

more attention (Vandesompele et al., 2002; Pfaffl et al., 2004; Gabrielsson et al., 2005; Zhang et al., 2005).

The liver orchestrates the metabolic adaptations that are needed for lactation. As cows transition from pregnancy to lactation, the liver undergoes hypertrophy and gluconeogenesis increases (Bauman, 2000). Metabolic problems that occur during the transition period such as fatty liver can impair liver function, contribute to the development of ketosis (Bobe et al., 2004), and challenge the adaptive capability of the liver. The change in physiological state alone could alter expression of commonly used HKG because of the liver's dynamic role in metabolism. There is the potential for variation in expression of HKG mRNA in studies that evaluate changes in target mRNA copy number in the liver in both nonlactating and lactating cows, and this variation may increase when dietary treatments are imposed. Therefore, the objective of this study was to evaluate the suitability of a number of potential HKG for use in bovine liver studies that included different physiological states, different amounts of feed intake, and different dietary treatments.

MATERIALS AND METHODS

Samples of liver total RNA from previously conducted experiments (Dann et al., 2005, 2006; Loor et al., 2005, 2006; Carlson, 2006) were selected to create a set of samples (n = 92) that included various physiological states, amounts of feed intake, and dietary treatments for comparison of HKG mRNA stability (Table 1). A survey of the literature was conducted to determine

commonly used HKG, which included β -actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 18S ribosomal RNA (*18S*), and peptidylprolyl isomerase A (cyclophilin A; *PPIA*) as reported by Thellin et al. (1999), Zhong and Simons (1999), Suzuki et al. (2000), and Rhoads et al. (2003). Less commonly used HKG, including β -glucuronidase (*GUSB*), polyubiquitin (*UBC*), ribosomal protein S9 (*RPS9*), and ribosomal protein L32 (*RPL32*), were suggested by others using either qPCR (Blanquicett et al., 2002) or microarray studies (Zhang et al., 2005; Zhao et al., 2005).

Once candidate HKG were chosen, primers were designed to amplify specific cDNA sequences in each HKG using Primer Express Software v2.0 (Applied Biosystems, Foster City, CA). Sequences, product sizes, and accession numbers are summarized in Table 2. To avoid possible amplification of contaminating genomic DNA, all RNA samples were treated with DNase (DNase I, Qiagen Inc., Valencia, CA) before cDNA synthesis. Additionally, primers sets were designed to fall on or across exon-exon junctions.

Reverse transcription reactions were conducted using 100 ng of total RNA as a template with 50 ng of random hexamers, 50 μ M oligo(dT)₂₀, 10 mM dNTP, 40 U of RNasin (Promega, Madison, WI), and 200 U of SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) in a final volume of 20 μ L. To control for variability in cDNA synthesis, 2 separate pools of cDNA were made for each sample. Upon completion of reactions, each cDNA pool was diluted 1:4 using DNase- and RNase-free water.

Table 2. Primer sets used for the real-time quantitative PCR analysis

Gene ¹	Primer ²	Sequence ³	Length (bp)	Accession number ⁴
<i>ACTB</i>	ACTB.624F	5'-GCGTGGCTACAGCTTCACC	54	AY141970
	ACTB.677R	3'-TTGATGTCACGACGATTTC		
<i>GAPDH</i>	GAPDH.276F	5'-TGGAAAGGCCATCACCATCT	60	BC102589
	GAPDH.335R	3'-CCCACTTGATGTTGGCAG		
<i>GUSB</i>	GUSB.303F	5'-ATCGGCAGCGCCCACTACT	65	CV982833
	GUSB.367R	3'-CCCTCGTGCTCTAACACATGGAC		
<i>UBC</i>	PolyUB.3F	5'-TGGGTTCTCTGTGTCGTCTTG	76	AB099083
	PolyUB.78R	3'-TTTGACCTGTGAGTGAATGCGA		
<i>PPIA</i>	PPIA.204F	5'-CTTTCACAGAATAATTCGGGATT	65	BC105173
	PPIA.268R	3'-CAGTACCATTATGGCGTGTGAAG		
<i>RPS9</i>	RPS9.192F	5'-CCTCGACCAAGAGCTGAAG	62	DT860044
	RPS9.254R	3'-CCTCCAGACCTCACGTTTGTTC		
<i>RPL32</i>	RPL32.92F	5'-AAAGAGGACCAAGAAGTTCATTAGG	66	BC102748
	RPL32.157R	3'-CGCCAGTTC <u>CGCTT</u> GATTT		
<i>18S</i>	18S.1329F	5'-TGCATGGCCGTTCTTAGTTG	65	DQ222453
	18S.1393R	3'-AGAGTCTCGTTCGTTATCGGAATT		

¹*ACTB* = β -actin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *GUSB* = β -glucuronidase; *UBC* = polyubiquitin; *PPIA* = peptidylprolyl isomerase A (cyclophilin A); *RPS9* = ribosomal protein S9; *RPL32* = ribosomal protein L32; *18S* = 18S ribosomal RNA.

²Primer pairs for *ACTB* from Loo et al. (2005). Numbers after the gene abbreviation indicate bases in the single-stranded sequence used to design primers; F = forward, R = reverse.

³Exon-exon junction for RPL32.157R is underlined and bolded. All primer sequences are written 5' to 3'. The 3' notation indicates the starting point for the reverse primer in the single-stranded sequence used to design primers.

⁴GenBank.

Real-time PCR reactions were performed in triplicate in 384-well plates using SYBR Green I universal master mix (Applied Biosystems), 400 nM each of the forward and reverse primers (Integrated DNA Technologies, Coralville, IA), and 4 μ L of diluted cDNA in a final reaction volume of 10 μ L per well. All 92 samples were run on the same plate, with each of the 2 pools of cDNA occurring on different plates for the same gene. Amplification and data collection were carried out using an ABI Prism 7900 HT SDS instrument (Applied Biosystems) with reaction conditions of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C. In addition, dissociation curves were generated following the last cycle. To make standards, pooled liver RNA from several cows and time points was used to make cDNA as described for experimental samples. Serial dilutions were made from this cDNA stock to create a set of standards for the experiment. A standard curve was run for each gene on each plate to determine relative copy number of mRNA (Applied Biosystems, 2001). Data from each pool of cDNA were averaged for each sample within each gene. One sample did not amplify well for any of the 8 genes tested; therefore, its data were removed from the final data set before the HKG were ranked, leaving a final sample size of $n = 91$.

Following PCR data collection, a publicly available Visual Basic applet for Microsoft Excel, called geNorm

(Vandesompele et al., 2002), was used to rank the expression variability of HKG in all samples. The procedures outlined in the user's manual (http://medgen.ugent.be/~jvdesomp/genorm/geNorm_manual.pdf) were followed to calculate instability values for gene expression. Expression data calculated with standard curves were imported into geNorm and a measure of expression instability was calculated for all 8 genes. Briefly, geNorm uses an algorithm to calculate all possible pairwise combinations of expression ratios for each gene in each sample. The variation of these pairwise ratios for all samples in the data set is used to calculate a standard deviation, and the arithmetic mean of all pairwise variations for a gene is termed the gene instability value. Genes with stable expression have lower instability values and, therefore, the best rank. In a stepwise fashion, the gene with the largest value was excluded from the data set and the remaining genes were analyzed for a new gene instability measure. This process was repeated until only 2 genes remained.

Because the geNorm program cannot distinguish between the 2 top-ranked genes, bootstrap methodology (Efron, 1982; Boos, 2003; Gabrielsson et al., 2005) was used to determine if one of the top-ranked genes could be consistently ranked above the others. The ranking procedure (use of geNorm program for instability calculations) was bootstrapped by resampling from the origi-

Table 3. Descriptive statistics for ranks of potential housekeeping genes after 200 bootstrap replications

Gene ¹	Average rank	Standard deviation	Standard error
<i>RPS9</i>	1.64	0.909	0.064
<i>GAPDH</i>	3.27	1.409	0.099
<i>ACTB</i>	3.71	1.621	0.115
<i>GUSB</i>	4.18	1.528	0.108
<i>RPL32</i>	4.53	1.975	0.139
<i>18S</i>	5.35	2.236	0.158
<i>PPIA</i>	6.50	1.647	0.116
<i>UBC</i>	6.85	1.429	0.101

¹*ACTB* = β -actin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *GUSB* = β -glucuronidase; *UBC* = polyubiquitin; *PPIA* = peptidylprolyl isomerase A (cyclophilin A); *RPS9* = ribosomal protein S9; *RPL32* = ribosomal protein L32; *18S* = 18S ribosomal RNA.

nal set of 91 samples to make 200 new data sets. Using this methodology, for each new data set, expression data from a particular sample could have been included a minimum of 0 or maximum of 91 times. Each of these data sets was then imported into the geNorm program and ranked 1 through 8 as described above.

RESULTS AND DISCUSSION

Preliminary ranking analyses indicated that *RPS9*, *GAPDH*, and *ACTB* were the 3 most stably expressed genes across different sample types. Following 200 bootstrap replications, *RPS9* was ranked first 59% of the time, second 23.5% of the time, and third 14.5% of the time, making it the most stably expressed gene across samples. Additionally, *RPS9* was never ranked below fifth in any of the repetitions. Descriptive statistics for the overall ranks of all genes are summarized in Table 3. Although *GAPDH* is not usually a very stably expressed HKG (Bustin, 2000), it was the second-highest ranked gene in the group. Because of the clear separation of rankings for *RPS9* and other genes, however, we still would be hesitant to conclude that *GAPDH* is appropriate to use as the only HKG in an experiment. Polyubiquitin (*UBC*) was the least stably expressed gene throughout, never being ranked first, and ranked last 41.5% of the time. Densitometry analysis revealed that both *PPIA* and *GAPDH* varied widely in liver depending on physiological state and between well-fed or underfed cows (Rhoads et al., 2003). Our results tend to agree with the expression changes for *PPIA* but to a lesser extent with those for *GAPDH*; differences in methodologies make it impractical to further compare the 2 studies.

An alternative to using only 1 HKG is to use 2 or more. This methodology has been proposed by Vandesompele et al. (2002) and could offer more control of variability, provided that the HKG chosen are not in

pathways that are directly related. Tricarico et al. (2002) also expressed concern with using a single HKG in studies with normal and cancerous human liver and colon tissue. Data from the current study indicate that, for experiments in which samples are taken prepartum, postpartum, or both, and for experiments with dietary treatment groups, *RPS9* and *GAPDH* would be the best combination of HKG. The use of more than one HKG increases the accuracy of sample normalization but also increases the need for monetary and physical resources. For these reasons, we chose to find one stably expressed HKG across experimental types. Other researchers must consider what controls are best for each set of experimental conditions.

The main focus of this experiment was to find the most stably expressed HKG to use for normalization of other target genes. We also evaluated different combinations of genes across sample types to assess the variability in the expression ratios (Figure 1). Figure 1 shows expression ratios of the most stably ranked genes (*RPS9* and *GAPDH*; panel A) compared with the least stable genes (*UBC* and *PPIA*; panel B). Clearly, *UBC* and *PPIA* are not suitable for use as HKG across different physiological states or different amounts of feed intake because they vary widely between sample groups. Two other commonly used HKG, *18S* and *ACTB*, are shown in panel C. Although these plots show less variation than *UBC* and *PPIA*, they still vary across different categories of samples. Other problems exist with these 2 commonly used genes. High abundance of 18S rRNA in samples combined with the sensitivity of real-time qPCR make it difficult to use with less abundant mRNA (Wong and Medrano, 2005). Furthermore, rRNA can be lost during RNA purification steps (Bustin, 2000). The mRNA for *ACTB* varies in tissues with respect to disease state (Blanquicett et al., 2002; Soyoun and Taeuk, 2003) and fasting (Yamada et al., 1997). Finally, panel D of Figure 1 shows the reduction in variation across sample types when *RPS9* (the most stably expressed gene) was used with the least stable gene, *UBC*. Even though the plots are not as uniform as those for *RPS9* and *GAPDH*, the variability across sample types was greatly reduced because of the expression stability of *RPS9*.

To further illustrate temporal patterns of HKG expression, relative expression of all 8 HKG were plotted (Figure 2) for cows repeatedly sampled across the periparturient period (Lor et al., 2005, 2006; Dann et al., 2006). To eliminate the effects of disease state and because tissue samples were taken only once per cow, the ketotic cows (Dann et al., 2005) were removed from the data set before plotting. Samples from Carlson (2006) also were removed because these cows were not sampled before parturition. Although the overall pattern

Figure 1. Variation in gene expression ratios across liver samples in different categories when different pairs of housekeeping genes were used. The lower boundary of the box shows the 25th percentile, the line within the box represents the median, and the upper boundary of the box shows the 75th percentile. The error bars below and above the boxes represent the 10th and 95th percentiles, respectively; outliers are represented by dots. A) ribosomal protein S9 and GAPDH (*RPS9/GAPDH*); B) peptidylprolyl isomerase A and polyubiquitin (*PPIA/UBC*); C) 18S ribosomal RNA and β -actin (*18S/ACTB*); and D) ribosomal protein S9 and polyubiquitin (*RPS9/UBC*). Each panel shows gene expression ratios for the following categories: a) all data points, $n = 91$; b) dry cows, $n = 30$; c) parturition/very early lactation, $n = 18$; d) lactating cows, $n = 43$; e) dry or lactating cows fed for ad libitum intake, $n = 65$; f) dry or lactating cows fed for restricted intake, $n = 26$; g) cows with high energy intake prepartum, $n = 30$; h) cows with moderate energy intake prepartum, $n = 35$; i) cows with low energy intake prepartum, $n = 26$; j) cows fed a dietary supplement (L-carnitine), $n = 6$; and k) cows with clinical or subclinical ketosis, $n = 7$.

across time showed that most of the chosen HKG did not have large increases or decreases (Figure 2), the variation across samples for each gene contributed to the ranking and instability measures originally calculated by geNorm. As shown in Figure 2, the variability in relative expression level in all samples for each time point was smallest for *RPS9* and largest for *UBC*. This

again supports our findings that *RPS9* is a stable gene for use in experiments in which liver samples are taken over time from the same group of cows.

Measuring the stability of HKG mRNA selected for qPCR experiments is important for improving the accuracy of qPCR data. Variability in HKG expression that occurs between different physiological states and under

Figure 2. Temporal patterns of gene expression in liver RNA for 8 potential housekeeping genes evaluated using real-time quantitative PCR. Data in the figure are expression values relative to a control sample using a standard curve methodology. Samples were from cows repeatedly biopsied during the periparturient period (Dann et al., 2006; Loor et al., 2005, 2006). Gene names: *ACTB* = β -actin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *GUSB* = β -glucuronidase; *UBC* = polyubiquitin; *PPIA* = peptidylprolyl isomerase A (cyclophilin A); *RPS9* = ribosomal protein S9; *RPL32* = ribosomal protein L32; *18S* = 18S ribosomal RNA.

different dietary conditions was demonstrated in this study. We found that *RPS9* is a suitable HKG for studies of hepatic gene expression during the periparturient period. Because different tissue types may respond differently to physiological states and dietary conditions, the same set of HKG used in liver may not be stably expressed in other tissues. It is wise, therefore, to confirm expression stability for HKG before data collection begins.

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