J. Dairy Sci. 92:2007–2019
doi:10.3168/jds.2008-1655
© American Dairy Science Association, 2009.

Identification of internal control genes for quantitative polymerase chain reaction in mammary tissue of lactating cows receiving lipid supplements

A. K. G. Kadegowda,* M. Bionaz,†‡ B. Thering,†‡ L. S. Piperova,* R. A. Erdman,* and J. J. Loor†‡[1]
*Department of Animal and Avian Sciences, University of Maryland, College Park 20742
†Mammalian NutriPhysioGenomics, Department of Animal Sciences, and
‡Division of Nutritional Sciences, University of Illinois, Urbana 61801

ABSTRACT

Dietary lipid supplements affect mammary lipid metabolism partly through changes in lipogenic gene expression. Quantitative PCR (qPCR) is a sensitive, reliable, and accurate technique for gene expression analysis. However, variation introduced in qPCR data by analytical or technical errors needs to be accounted for via normalization using appropriate internal control genes (ICG). Objectives were to mine individual bovine mammary microarray data on >13,000 genes across 66 cows from 2 independent studies to identify the most suitable ICG for qPCR normalization. In addition to unsupplemented control diets, cows were fed saturated or unsaturated lipids for 21 d or were infused with supplements (butterfat, conjugated linoleic acid mixture, long-chain fatty acids) into the abomasum to modify milk fat synthesis and fatty acid profiles. We identified 49 genes that did not vary in expression across the 66 samples. Subsequent gene network analysis revealed that 22 of those genes were not co-regulated. Among those *COPS7A*, *CORO1B*, *DNAJC19*, *EIF3K*, *EMD*, *GOLGA5*, *MTG1*, *UXT*, *MRPL39*, *GPR175*, and *MARVELD1* (sample/reference expression ratio = 1 ± 0.1) were selected for PCR analysis upon verification of goodness of BLAT/BLAST sequence and primer design. Relative expression of *B2M*, *GAPDH*, and *ACTB*, previously used as ICG in bovine mammary tissue, was highly variable (0.9 ± 0.6) across studies. Gene stability analysis via geNorm software uncovered *MRPL39*, *GPR175*, *UXT*, and *EIF3K* as having the most stable expression ratio and, thus, suitable as ICG. Analysis also indicated that use of 3 ICG was most appropriate for calculating a normalization factor. Overall, the geometric average of *MRPL39*, *UXT*, and *EIF3K* is ideal for normalization of mammary qPCR data in studies involving lipid supplementation of dairy cows. These novel ICG could be used for normalization in similar studies as alternatives to the less-reliable *ACTB*, *GAPDH*, or *B2M*.
Key words: internal control gene, transcriptomics, milk fat

INTRODUCTION

Large-scale transcript analysis of lactating mouse mammary tissue has shown that regulation of mammary lipid synthesis occurs to a large extent at the level of mRNA expression (Rudolph et al., 2007). Although the molecular mechanisms by which dietary fatty acids regulate ruminant mammary lipogenic gene expression are not completely established, there is evidence of a role for transcriptional regulators and their target genes at least in bovine (Peterson et al., 2004; Harvatine and Bauman, 2006). The advent of bovine microarray (Loor et al., 2007) has enabled large-scale evaluation of mRNA expression in tissues due to nutrition or physiological state (Loor et al., 2005, 2006). Use of this technology promises to substantially increase our knowledge of transcriptional adaptations in bovine tissues.

Despite the advantages of microarrays, quantitative reverse transcription-PCR (**qPCR**) remains the method of choice for evaluation of mRNA expression as it is the most sensitive, reliable, and accurate technique available for gene expression analysis. Central to the applicability of qPCR is the fact that variation introduced because of analytical or technical errors during the procedure should be accounted for via normalization. Use of appropriate internal control genes (**ICG**) is the most reliable method currently used for data normalization (Vandesompele et al., 2002) because it can account for differences due to initial quantity of RNA, RNA handling, and variation in kinetics of the reverse transcription reaction. The selected ICG should not vary due to the type of cells or tissues or respond differently to treatments (Vandesompele et al., 2002). Thus, proper selection and evaluation of ICG is critical to avoid additional variation in the data.

There is no consensus on suitable ICG for studies of dietary lipid effects on ruminant mammary

Received August 26, 2008.
Accepted December 13, 2008.
[1]Corresponding author: jloor@uiuc.edu

transcriptomics. Most recent studies (via Northern blot or qPCR) have relied on individual ICG (commonly known as housekeeping genes) such as β-actin (*ACTB*; Peterson et al., 2003, 2004), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Baumgard et al., 2002; Sorensen et al., 2008), and cyclophilin (i.e., peptidylprolyl isomerase A to H, *PPIA-H*; Bernard et al., 2005) as well as ribosomal proteins (RPS4, RPS28; Piperova et al., 2000; Ahnadi et al., 2002). The geometric mean of *ACTB*, β-2-microglobulin (*B2M*), and 18S rRNA (Harvatine and Bauman, 2006) or that of *ACTB*, *GAPDH*, and ubiquitin have been used recently (Farke et al., 2008). However, *ACTB* and *GAPDH* were the least suitable or were highly variable ICG among several tested in bovine mammary tissue (Bionaz and Loor, 2007; Piantoni et al., 2008). Furthermore, the use of 18S rRNA for normalization is questionable because of its high abundance (Vandesompele et al., 2002).

The specific objective of this study was to identify more-robust ICG for normalization of qPCR through mining individual mammary tissue microarray data from 2 independent lactating dairy cow studies dealing with lipid supplementation. Pairwise analysis of mRNA expression ratios (Vandesompele et al., 2002) was used to identify potential ICG.

MATERIALS AND METHODS

RNA Extraction, PCR, and Primer Design and Testing

Mammary tissue samples (n = 66) from lactating dairy cows infused for 14 d with butterfat [a source of short- and long-chain fatty acids (**LCFA**)], LCFA, or a conjugated linoleic acid (**CLA**) mixture (Kadegowda et al., 2008), samples from cows fed saturated (EB100; Energy Booster 100, MSC, Carpentersville, IL) or unsaturated lipid supplements (fish oil) for 21 d (Thering et al., 2007), and unsupplemented controls were used in the study. Cows in the first study (Kadegowda et al., 2008) were in early lactation and cows in the second study were in mid lactation (Thering et al., 2007). Mammary tissue was collected via percutaneous biopsy as reported in Bionaz and Loor (2007), immediately frozen in liquid N_2, and preserved at −80°C for RNA extraction as described previously (Loor et al., 2005).

Total RNA was extracted from approximately 0.5 g of tissue using ice-cold Trizol reagent (Invitrogen, Carlsbad, CA). The RNA concentrations were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality was evaluated using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All samples had RNA integrity number >6.0. Genomic DNA was removed by DNase digestion and cleaned using RNeasy Mini Kit columns (Qiagen, Valencia, CA). A portion of the assessed RNA was diluted to 100 ng/μL using DNase-RNase-free water before cDNA synthesis by reverse transcriptase. Sufficient cDNA was prepared in a single run to perform PCR for all selected genes. Protocol for cDNA synthesis and qPCR are described elsewhere (Bionaz and Loor, 2007). Before starting the qPCR reaction, synthesized cDNA was diluted 1/4 (vol/vol) with free DNase-RNase-free water in experiment 1, whereas a dilution 1/3 (vol/vol) was used in experiment 2. For qPCR, 4 μL of diluted cDNA was combined with 6 μL of reaction mixture composed of 5 μL of 1× SYBR Green master mix (Applied Biosystems), 0.4 μL each of 10 μ*M* forward and reverse primers and 0.2 μL of DNase-RNase-free water in a MicroAmp Optical 384-well reaction plate (Applied Biosystems). For qPCR, each sample was run in triplicate and a 6-point relative standard curve plus the non-template control were used. The 4-fold-dilution standard curve was made using cDNA synthesized from a pooled RNA of all the samples. The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems) using the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. Despite different dilutions of cDNA, samples from both experiments were run in the same PCR plate.

The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 s plus 65°C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems). Primer Express 3.0 software (Applied Biosystems), optimized for use with Applied Biosystems PCR Systems, was used for primer design using default features, except for amplicon length, which was fixed, when possible, at a minimum of 100 bp. Primers were designed across exon junctions when feasible to avoid amplification of genomic DNA. The exon junctions were uncovered by blasting the sequence against the bovine genome (Genome Browser Gateway, 2008). Primers were aligned against publicly available sequences in National Center of Biotechnology Information (National Center of Biotechnology Information, 2008) and University of California Santa Cruz (USCS; Genome Browser Gateway, 2008). Primer features are reported in Table 1 and qPCR performance in Table 2. Before qPCR, primers were tested using the same protocol as for qPCR but without the dissociation step in a 20-μL reaction. Part of the qPCR product was run in a 2% agarose gel stained with ethidium bromide to assess the presence of the product to an expected size and presence of primer-dimer; the remainder was purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA) and sent to sequence at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology

Download English Version:

https://daneshyari.com/en/article/2439625

Download Persian Version:

https://daneshyari.com/article/2439625

Daneshyari.com