Propionate induces the bovine cytosolic phosphoenolpyruvate carboxykinase promoter activity

Qian Zhang, Stephanie L. Koser, and Shawn S. Donkin¹

Department of Animal Sciences, Purdue University, West Lafayette, IN 47907

ABSTRACT

Cytosolic phosphoenolpyruvate carboxykinase (*PCK1*) is a critical enzyme within the metabolic networks for gluconeogenesis, hepatic energy metabolism, and tricarboxylic acid cycle function, and is controlled by several transcription factors including hepatic nuclear factor 4 α (HNF4 α). The primary objective of the present study was to determine whether propionate regulates bovine *PCK1* transcription. The second objective was to determine the action of cyclic AMP (cAMP), glucocorticoids, and insulin, hormonal cues known to modulate glucose metabolism, on bovine *PCK1* transcriptional activity. The proximal promoter of the bovine *PCK1* gene was ligated to a Firefly luciferase reporter and transfected into H4IIE hepatoma cells. Cells were exposed to treatments for 23 h and luciferase activity was determined in cell lysates. Activity of the *PCK1* promoter was linearly induced by propionate, and maximally increased 7-fold with 2.5 mM propionate, which was not muted by 100 nM insulin. Activity of the *PCK1* promoter was increased 1-fold by either 1.0 mM cAMP or 5.0 μ M dexamethasone, and 2.2-fold by their combination. Induction by cAMP and dexamethasone was repressed 50% by 100 nM insulin. Propionate, cAMP, and dexamethasone acted synergistically to induce the *PCK1* promoter activity. Propionate-responsive regions, identified by 5' deletion analysis, were located between –1,238 and –409 bp and between –85 and +221 bp. Deletions of the core sequences of the 2 putative HNF4 α sites decreased the responsiveness to propionate by approximately 40%. These data indicate that propionate regulates its own metabolism through transcriptional stimulation of the bovine *PCK1* gene. This induction is mediated, in part, by the 2 putative HNF4 α binding sites in the bovine *PCK1* promoter.

Key words: hormone, short-chain fatty acid, *PCK1*, promoter

INTRODUCTION

Phosphoenolpyruvate carboxykinase (**PCK**; EC 4.1.1.32), a key enzyme of gluconeogenesis, tricarboxylic acid (TCA) cycle function, and several metabolic networks that affect energy metabolism in the liver and kidney, catalyzes the irreversible formation of phosphoenolpyruvate from oxaloacetate (Rognstad, 1979; She et al., 2000; Burgess et al., 2007). It is well documented in nonruminant species that the gene for the cytosolic form of PCK (*PCK1*) is actively regulated by hormones and nutrients at the transcriptional level, most notably through induction by glucagon and glucocorticoids, effects that are repressed by insulin action (Hanson and Reshef, 1997). Accordingly, expression of *PCK1* is induced by starvation and reduced during feeding (Hanson and Reshef, 1997). However, in ruminants, the expression of *PCK1* is not altered during feed restriction (Velez and Donkin, 2005), but is induced in response to increased feed intake (Greenfield et al., 2000) and monensin feeding (Karcher et al., 2007), conditions that are linked to increased ruminal propionate production. Expression of *PCK1* mRNA is increased in neonatal calves and at least maintained with postruminal propionate infusions in lactating dairy cows despite elevated insulin concentrations (Zhang et al., 2015), a hormone that potently and rapidly represses *PCK1* mRNA expression (Granner et al., 1983; Chakravarty and Hanson, 2007).

Studies using primary rat hepatocytes and H4IIE rat hepatoma cells indicate that short-chain fatty acids (**SCFA**), including propionate and butyrate, modulate expression of both *PCK1* mRNA and *G6PC* mRNA (Massillon et al., 2003). Binding of hepatic nuclear factor 4 α (**HNF4 α**) to mouse *G6PC* promoter is required for stimulation of transcription by propionate and butyrate (Massillon et al., 2003). Hepatic nuclear factor 4 α is a member of the nuclear receptor superfamily abundantly expressed in the liver and required for liver-specific gene expression in response to hormones and nutrients (Sladek et al., 1990; Babeu and Boudreau, 2014). Upon binding to its recognition sites in a gene promoter, HNF4 α is able to recruit coactivators of transcription factors, such as peroxisome proliferator-

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¹Corresponding author: sdonkin@purdue.edu

activated receptor γ coactivator-1 α , to stimulate transcriptional activity (Gonzalez, 2008). The critical role of HNF4 α in activating transcription of the *PCK1* and *G6PC* gene has also been confirmed using hepatocytes from mice lacking hepatic HNF4 α (Rhee et al., 2003).

These observations led us to question if the induction of *PCK1* mRNA expression by propionate in liver of dairy cattle might be due to direct activation of the bovine *PCK1* promoter. We were likewise interested in determining the relationship between propionate, glucagon, glucocorticoids, and insulin on bovine *PCK1* gene transcription. In addition, the bovine *PCK1* promoter was analyzed to reveal putative transcription factor binding sites and the functionality of the putative HNF4 α binding sites in mediating responses to propionate and butyrate were tested. Here, we show that propionate has an inductive effect on the transcriptional activity of the bovine *PCK1* promoter, which is dominant to the repressive effect of insulin and synergistic to the inductive effect of the combination of cyclic AMP (cAMP) and dexamethasone. We also demonstrate that 2 putative HNF4 α -binding sites within the bovine *PCK1* promoter are responsible, in part, for propionate-induced *PCK1* transcription.

MATERIALS AND METHODS

Promoter-Luciferase Reporter Constructs

The bovine *PCK1* proximal promoter sequence was obtained from National Center for Biotechnology Information database (<https://www.ncbi.nlm.nih.gov/>). The DNA sequence containing the proximal promoter region from -1,238 through +221 bp relative to the transcription start site (TSS) of the bovine *PCK1* gene (NCBI Gene ID: 282855) was linked to a Firefly luciferase reporter. The promoter region corresponds to bases 59,151,952 through 59,150,494 of the bovine chromosome 13. To determine the propionate-responsive

elements within the bovine *PCK1* promoter, 5' nested deletions of the proximal promoter region were individually linked to the Firefly luciferase reporter. The target promoter sequences were amplified from bovine genomic DNA by PCR using the primers presented in Table 1. The amplified PCR products were purified using agarose gel electrophoresis and the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and each was ligated into the pDrive cloning vector (Qiagen), which was used to transform Qiagen EZ Competent Cells (Qiagen). Positive clones were grown individually overnight for plasmid DNA isolation using the Wizard Plus SV Miniprep kit (Promega, Madison, WI). The cloned sequences were then excised from pDrive and ligated separately into the pGL3-Basic vector (Promega). Each *PCK1* promoter-luciferase plasmid was used to transform competent JM109 *Escherichia coli*, and positive clones were grown individually overnight for plasmid DNA isolation using the Wizard Plus SV Miniprep kit (Promega). Insert sequence and orientation of the promoter regions were verified by sequencing at the DNA Sequencing Low Throughput Laboratory of Purdue Genomics Core Facility (Purdue University, West Lafayette, IN) using the ABI 3700 sequencer (Amersham Biosciences, Piscataway, NJ).

In Silico Promoter Sequence Analysis

The bovine *PCK1* proximal promoter sequence (-1,238/+221) was analyzed using the TRANSFAC transcription factor analysis tool (Biobase, Beverly, MA). The search parameters were limited to liver-specific factors, high-quality matrices, and set to minimize false positives.

Site-Directed Mutagenesis

The essentiality of the putative HNF4 α binding sites within the bovine *PCK1* gene located at +68 through

Table 1. Primer sequences used to amplify the promoter regions of the bovine *PCK1* gene and for the site-directed mutagenesis

Promoter construct	Primer sequence (5'-3')
<i>PCK1</i> promoter (-1,238/+221)	Forward: GGTCTCATTGCTCAAGTGTAATCG
<i>PCK1</i> promoter (-815/+221)	Forward: AATCCACAAGGCAGGTGTGACTGA
<i>PCK1</i> promoter (-409/+221)	Forward: ACCACTGCTCTATTCTGGCAACCA
<i>PCK1</i> promoter (-251/+221)	Forward: CAGAAGTTGTGTAAAGGCCTGCCA
<i>PCK1</i> promoter (-85/+221)	Forward: AGTCGAGCCTCTCTGGGTGTG
All promoter regions of <i>PCK1</i>	Reverse: AGAGTTGAGGGTGTCCATGGTTGT
Mutant HNF4 α (+68_+72)	Forward: AAGGGACCCTTTGGCTGACCTGATCGTCCA
	Reverse: TGGACGATCAGGTCAGCCAAAGGGTCCCTT
Mutant HNF4 α (-1,078_-1,074)	Forward: AGGTTCCAGGAAGGGGTCCCTTACAAGAGG
	Reverse: CCTCTTGTAAGGGACCCCTTCCTGGAACCT
Double mutant HNF4 α (+68_+72)/HNF4 α (-1,078_-1,074)	Forward: AAGGGACCCTTTGGCTGACCTGATCGTCCA
	Reverse: TGGACGATCAGGTCAGCCAAAGGGTCCCTT

+72 bp and -1,078 through -1,074 bp relative to TSS for the bovine *PCK1* promoter responsiveness to the tested treatments were determined using site-directed mutagenesis. Mutations of the putative HNF4 α binding sites were generated using the GENEART Site-Directed Mutagenesis System kit (Life Technologies, Grand Island, NY) following the manufacturer protocols. The primers used for the mutagenesis are shown in Table 1. Three mutant promoters were tested relative to the wild-type promoter [*PCK1*_(WT)] in these experiments. The mutant HNF4 α _(+68_+72)⁻ was created by deleting the core sequence of the putative HNF4 α binding site at +68 through +72 bp (5'-CTCTG-3') using the *PCK1*_(WT) as template. The mutant HNF4 α _(-1,078_-1,074)⁻ was created by deleting the core sequence of the putative HNF4 α site at -1,078 through -1,074 bp (5'-CAAAG-3') using the *PCK1*_(WT) as template. The double mutant HNF4 α _(+68_+72)⁻/HNF4 α _(-1,078_-1,074)⁻ was created by deleting the core sequences of the putative HNF4 α site at +68 through +72 bp (5'-CTCTG-3') and at -1,078 through -1,074 bp (5'-CAAAG-3') using the mutant HNF4 α _(-1,078_-1,074)⁻ as template. All promoter-luciferase reporter plasmids were sequenced at the DNA Sequencing Low Throughput Laboratory of Purdue Genomics Core Facility to verify the target mutations.

Cell Culture

Rat hepatoma H4IIE cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 4.5 mM glucose supplemented with 10% fetal bovine serum (Sigma-Aldrich) and antibiotics (10 mg/L piperacillin and 10 mg/L ciprofloxacin; Sigma-Aldrich) at 37°C, 95% relative humidity, and 5% CO₂. Cells were seeded in 24-well Corning Costar tissue culture plates (Corning Inc., Tewksbury, MA) at a target density of 75,000 cells per cm² and grown for 24 h to achieve approximately 80% confluence.

Test of Promoter Function

Transient transfections in H4IIE cells were performed using Lipofectamine 2000 (Invitrogen Inc., Waltham, MA) according to the manufacturer's instruction. For transfection, cell culture medium was DMEM supplemented with 1% BSA (Merck Millipore, Billerica, MA) but without antibiotics. A volume of 100 μ L of the lipofectamine-plasmid mixture containing 98 μ L of Opti-MEM I Reduced Serum Medium (Life Technologies Inc.), 2 μ L of lipofectamine, 0.8 μ g of each tested plasmid, and 0.008 μ g of a Renilla pRL-CMV luciferase

plasmid (Promega) was added to each well. Cotransfection of Renilla pRL-CMV luciferase plasmid was used to normalize transfection efficiency.

Five hours after transfection, medium was removed by aspiration and replaced with the treatment additions to DMEM containing 1% BSA and antibiotics (10 mg/L of piperacillin and 10 mg/L of ciprofloxacin). Cells were exposed to treatments for 23 h. Basal promoter activity and responsiveness to propionate and hormones were tested in a series of complementary experiments. Briefly, DMEM supplemented with 1% BSA and antibiotics (10 mg/L of piperacillin and 10 mg/L of ciprofloxacin) was used to determine the basal promoter activity. Responsiveness of the *PCK1* promoter was determined in the presence of 0, 0.25, 0.5, 1.0, or 2.5 mM propionate. The effects of SCFA and glycerol on the *PCK1* promoter were tested in DMEM containing 1% BSA and antibiotics and either 2.5 mM acetate, 2.5 mM propionate, 2.5 mM butyrate, or 2.5 mM glycerol. Medium without SCFA or glycerol served as the control. The relationship between propionate and hormone additions was determined in the presence of either 2.5 mM propionate, 100 nM insulin, 1.0 mM 8-Br-cAMP, 5.0 μ M dexamethasone, or their combinations. To determine the location of DNA regulatory elements required for propionate action, 5' nested deletions of *PCK1* proximal promoter ligated to luciferase reporter were individually transfected into H4IIE cells and exposed to either 0 or 2.5 mM propionate. The role of HNF4 α in *PCK1* promoter response to propionate and butyrate was determined using the following mutants: HNF4 α _(+68_+72)⁻, HNF4 α _(-1,078_-1,074)⁻, and the double mutant HNF4 α _(+68_+72)⁻/HNF4 α _(-1,078_-1,074)⁻. Each mutant plasmid was individually transfected into H4IIE cells, and cells were exposed to either no addition, 2.5 mM propionate, or 2.5 mM butyrate.

Luciferase Activity

Following 23 h of exposure to treatments, medium was removed by aspiration and cells were washed with 250 μ L of ice-cold 1 $\times$ PBS and harvested in 80 μ L of 1 $\times$ passive lysis buffer (Promega). Firefly luciferase and Renilla luciferase were quantified with the Dual-Luciferase Reporter Assay system and the Stop & Glo Reagent kit according to the manufacturer's instruction (Promega). Fluorescence was determined with Magellan 5.0 software (Tecan, Research Triangle Park, NC) using a Tecan GENios Pro spectrofluorometer. Promoter activity was expressed as the ratio of Firefly luciferase to Renilla luciferase, and treatment effect was expressed as the fold change relative to the no-addition controls.

Statistical Analysis

All experiments were conducted in at least 3 separate cell preparations using 3 replicates per treatment. Data were analyzed for normality using the Univariate procedure of SAS 9.2 (SAS Institute Inc., Cary, NC), and any heteroscedastic data were adjusted to achieve normality using a natural logarithm transformation. Analyses of variance were performed using the Proc Mixed procedure of SAS 9.2. Data from multiple promoter luciferase reporter constructs were evaluated using a model that accounted for the fixed effects of construct and cell preparation. Data from the full length *PCK1* promoter (−1,238/+221) were evaluated using a model that accounted for the fixed effects of treatment and cell preparation. Contrast statement was included to test linearity for the dose-response experiment. A linear regression trend line with coefficient of determination values was generated using an Excel spreadsheet (Microsoft Corp., Redmond, WA). For the data that were not adjusted using logarithm transformation, least squares means and standard errors were reported. For the data that were adjusted using logarithm transformation, least squares means and 95% confidence intervals were reported using the original scale. Tukey-Kramer studentized adjustments

were used to separate treatment means when main effects were significant. Means were considered different when $P < 0.05$.

RESULTS

Basal Promoter Activity of the Bovine *PCK1* Gene

The pGL3-luciferase family of constructs served as controls in this experiment. The promoterless parent vector, pGL3-Basic, served as a negative control, and pGL3-SV40 Promoter containing a SV40 promoter element served as a positive control. Activity of the positive control pGL3-SV40 Promoter was significantly greater than the negative control pGL3-Basic ($P < 0.05$; Figure 1). The ability of the bovine *PCK1* promoter (−1,238/+221) to drive luciferase expression was greater ($P < 0.05$) than the pGL3-Basic negative control and similar to the pGL3-SV40 Promoter positive control (Figure 1). Compared with the full-length *PCK1* promoter (−1,238/+221), the ability to drive luciferase expression was increased ($P < 0.05$) for the *PCK1* promoter (−409/+221) and promoter (−251/+221; Figure 1). The activity of the minimum *PCK1* promoter (−85/+221) was still greater than the pGL3-Basic negative control ($P < 0.05$; Figure 1).

Figure 1. Basal activity of the *PCK1* promoter reporter constructs in rat hepatoma H4IIE cells. Cells were transfected with each individual promoter reporter construct and incubated in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO) containing 1% bovine serum albumin (Merck Millipore, Billerica, MA) and antibiotics for 23 h. The pGL3-Basic (Promega, Madison, WI) was promoterless and used as a negative control, and the pGL3-SV40 Promoter served as positive control. Values are LSM and 95% CI, $n = 3$. Labeled means without a common letter (a–c) differ at $P < 0.05$.

Figure 2. Effect of propionate on the activity of the bovine *PCK1* promoter in rat hepatoma H4IIE cells. Cells were transfected with the *PCK1* promoter (−1,238/+221) reporter construct and exposed to 0, 0.25, 0.5, 1.0, or 2.5 mM of propionate for 23 h. Treatment effects are expressed as the fold change relative to the no-addition control. Values are LSM and 95% CI, $n = 3$. Labeled means without a common letter (a–d) differ at $P < 0.05$. Induction of the *PCK1* promoter activity shows a linear regression with increasing propionate concentrations.

Dose-Dependent Induction of Bovine *PCK1* Transcription by Propionate

Propionate at 0.25, 0.5, 1.0, and 2.5 mM linearly increased ($P < 0.05$) the activity of the bovine *PCK1* promoter (−1,238/+221; Figure 2). Compared with 0 mM propionate, 0.25 mM propionate induced ($P < 0.05$) the *PCK1* promoter activity 0.5-fold, and 2.5 mM propionate increased ($P < 0.05$) the promoter activity approximately 7-fold (Figure 2).

Responsiveness of Bovine *PCK1* Transcription to SCFA and Glycerol

Activity of the bovine *PCK1* promoter (−1,238/+221) was induced ($P < 0.05$) by 2.5 mM propionate and 2.5 mM butyrate, but not by 2.5 mM acetate ($P \geq 0.05$; Figure 3). The induction by butyrate was greater ($P < 0.05$) than propionate (Figure 3). Glycerol, another gluconeogenic substrate, did not affect ($P \geq 0.05$) the *PCK1* promoter activity, indicating a lack of general effect of gluconeogenic precursors on *PCK1* transcription (Figure 3).

Responsiveness of Bovine *PCK1* Transcription to Propionate and Hormonal Cues

Dexamethasone and cAMP, when present alone, induced ($P < 0.05$) the bovine *PCK1* promoter

(−1,238/+221) by approximately 1-fold compared with the no-addition control (Figure 4). The combination of cAMP and dexamethasone increased ($P < 0.05$) the *PCK1* promoter activity by 2.2-fold, which was repressed 50% by insulin (Figure 4). Propionate alone induced ($P < 0.05$) the promoter activity by approximately 7-fold (Figure 4). When propionate was combined with cAMP or dexamethasone, the inductive effects were magnified ($P < 0.05$) 11- and 9-fold, respectively, and the combination of propionate, cAMP, and dexamethasone synergistically increased ($P < 0.05$) the promoter activity about 23-fold, which was greater ($P < 0.05$) than the induction by propionate alone (Figure 4). In presence of propionate, insulin was without effect ($P \geq 0.05$) on the bovine *PCK1* promoter activity (Figure 4).

Propionate-Responsive Regions Within the Bovine *PCK1* Promoter

Compared with the *PCK1* promoter (−1,238/+221), deletion from −1,238 to −409 bp resulted in increased basal promoter activity but caused a significant loss of propionate inducibility ($P < 0.05$; Figure 5). Further deletion from −409 to −85 bp did not alter the responsiveness to propionate (Figure 5). The minimum promoter construct (−85/+221) was sufficient to mediate responsiveness to propionate compared with the negative control pGL3-Basic ($P < 0.05$; Figure 5). Thus, the 2 regions from −1,238 to −409 bp and from −85

Figure 3. Effects of short-chain fatty acids and glycerol on the activity of the bovine *PCK1* promoter in rat hepatoma H4IIE cells. Cell were transfected with the *PCK1* promoter (−1,238/+221) reporter construct and exposed to 0 or 2.5 mM acetate, 2.5 mM propionate, 2.5 mM butyrate, or 2.5 mM glycerol for 23 h. Treatment effects are expressed as the fold change relative to the no-addition control. Values are LSM and 95% CI, n = 3. Labeled means without a common letter (a–c) differ at $P < 0.05$.

to +221 bp relative to TSS accounted for the robust induction of the bovine *PCK1* promoter by propionate.

Role of the HNF4 α Binding Sites in Propionate-Induced Bovine *PCK1* Transcription

In silico analysis of the bovine *PCK1* promoter sequence using TRANSFAC revealed 2 putative HNF4 α binding sites at +68 to +72 and −1,078 to −1,074 bp relative to TSS, respectively (Figure 6). The role of HNF4 α binding sites in mediating propionate and butyrate regulation of bovine *PCK1* transcription was examined by deleting the core sequences of HNF4 α binding site at +68 to +72 bp [mutant HNF4 $\alpha_{(+68-72)}$], at −1,078 to −1,074 bp [mutant HNF4 $\alpha_{(-1,078-1,074)}$], or both sites [double mutant HNF4 $\alpha_{(+68-72)}/$ HNF4 $\alpha_{(-1,078-1,074)}$]. Compared with the *PCK1*_(WT), the mutant HNF4 $\alpha_{(+68-72)}$, mutant HNF4 $\alpha_{(-1,078-1,074)}$, and double mutant HNF4 $\alpha_{(+68-72)}/$ HNF4 $\alpha_{(-1,078-1,074)}$ had increased basal promoter activity, but responsiveness to propionate or butyrate was depressed by about 40% ($P < 0.05$; Figure 7). The basal activity and responsiveness to propionate or butyrate were not different among the 3 mutants. These results indicate the putative HNF4 α binding sites are responsible, at least in part, for propionate- and butyrate-mediated induction of bovine *PCK1* transcription.

DISCUSSION

Feeding strategies that increase ruminal propionate production are often used to meet increased glucose demand and reduce the effect of fatty liver and ketosis during early lactation in dairy cows (Baird, 1982; Duffield et al., 1998; Aschenbach et al., 2010). The gluconeogenic effect of propionate appears to be mediated by elevated expression of *PCK1* mRNA (Zhang et al., 2015). Induction of *PCK1* mRNA expression in primary rat hepatocytes and H4IIE cells in response to propionate is due to transcriptional activation (Massillon et al., 2003). Available data indicate that transcription of *PCK1* in liver requires an important transcription factor HNF4 α (Puigserver, 2005; Gonzalez, 2008). In this study, we demonstrated that propionate, a major gluconeogenic precursor in ruminants, directly regulates bovine *PCK1* transcription.

The increase in transcription of bovine *PCK1* in response to propionate and butyrate appears to be a specific effect of these SCFA, as acetate was without effect. Likewise, the effects on *PCK1* do not appear to be mediated by the availability of gluconeogenic precursors, as glycerol was also without effect. Short-chain fatty acids have been recently identified as ligands for G-protein-coupled receptors, GPR41 and GPR43, also known as free fatty acid receptor (FFAR) 3 and

FFAR2, respectively (Brown et al., 2003; Layden et al., 2013). These 2 receptor types show ubiquitous expression throughout the human body (Tang et al., 2012) and bovine tissues (Wang et al., 2009; Yonezawa et al., 2009). Acetate and butyrate preferentially bind to FFAR2 and FFAR3, respectively, whereas propionate binds to both receptors. The receptor preferences may have provided some possible explanations for the different responsiveness of bovine *PCK1* transcription to SCFA.

Two propionate-responsive regions were identified by 5' deletion analysis, and promoter analysis revealed 2 putative STAT3 binding sites as well as 2 putative HNF4α binding sites within these 2 regions (Figure 6). In the present study, we did not test the functionality of STAT3 in mediating responses of bovine *PCK1* to propionate and butyrate. It has been reported that STAT3 mediates negative regulation of transcription of gluconeogenic genes, including *PCK1* and *G6PC* in mouse liver and HepG2 cells (Ramadoss et al., 2009), which makes it less likely to be involved in the positive

regulation of bovine *PCK1* transcription by propionate and butyrate. The 2 putative HNF4α binding sites are involved but not completely responsible for the induction, as deletions of the core sequences of these 2 sites decreased the responsiveness to propionate by approximately 40%. It has been reported that HNF4α plays an important role in regulation of *PCK1* and *G6PC* gene expression by both hormonal and nutritional cues (Gonzalez, 2008). Binding sites of HNF4α have been identified within the promoter of the rat *PCK1* and *G6PC* (Hanson and Reshef, 1997; Massillon et al., 2003; Chakravarty et al., 2005). The induction of mouse *G6PC* transcription by propionate and butyrate requires binding of HNF4α to the *G6PC* promoter (Massillon et al., 2003). Future studies are needed to confirm the binding of HNF4α to these sites as well as other *cis*-elements within the bovine *PCK1* promoter to mediate the full responsiveness to SCFA.

Although *PCK1* is a critical enzyme for gluconeogenesis, it also plays an important cataplerotic role in oxaloacetate (OAA) metabolism. Sufficient OAA in

Figure 4. Effects of propionate and hormonal cues on the activity of the bovine *PCK1* promoter in rat hepatoma H4IIE cells. Cell were transfected with the *PCK1* promoter (−1,238/+221) reporter construct and exposed to 2.5 mM propionate, 100 nM insulin, 1.0 mM cyclic AMP (cAMP), 5.0 μM dexamethasone, or the combinations indicated for 23 h. Treatment effects are expressed as the fold change relative to the no-addition control. Values are LSM and 95% CI, n = 3. Labeled means without a common letter (a–d) differ at *P* < 0.05.

Figure 5. Identification of propionate-responsive regions within the bovine *PCK1* promoter. Rat H4IIE cells were transfected with the *PCK1* promoter (−1,238/+221) and its nested 5′ deletions separately and exposed to either 0 or 2.5 mM of propionate for 23 h. Propionate induction for each promoter reporter is expressed as fold change relative to the no-addition control. Values are LSM and 95% CI, n = 3. Labeled means without a common letter (a–c) differ at $P < 0.05$.

the cell is necessary to enable to capacity for the complete oxidation of acetyl-CoA that is generated from fatty acids through β -oxidation during negative energy balance in dairy cattle (Baird et al., 1968; Li et al.,

2012). Increased *PCK1* activity that is unmatched by change in PCK activity may lead to diminution of OAA (Jitrapakdee et al., 2008). Propionate can replenish the OAA pool through propionyl CoA and methylmalonyl-

Figure 6. A diagram of the proximal promoter of the bovine *PCK1* gene. Transcription start site is indicated as TSS. Putative transcription factor binding sites within the promoter (−1,238 through +221) was identified using the TRANSFAC transcription factor analysis tool (Biobase, Beverly, MA). The search parameters were limited to liver-specific factors, high-quality matrices, and were set to minimize the number of false positives. Abbreviations for common transcription factors: C/EBP = CCAAT/enhancer binding protein, NF-1A = nuclear factor 1A, STAT3 = signal transducer and activator of transcription 3, HNF4 α = hepatic nuclear factor 4 α , SREBP = sterol regulatory element-binding protein, Sp1 = specificity protein 1, USF = upstream stimulatory factor, HNF-1 β = hepatic nuclear factor 1 β , CREB = cyclic AMP response element binding protein.

CoA metabolism (Aschenbach et al., 2010). In contrast, butyrate cannot be converted to OAA. The present data indicate butyrate is a potent inducer of bovine *PCK1* transcription and exhibits roughly double the effect of a similar concentration of propionate. Because butyrate carbon does not enter the OAA pool, the potential exists to reduce the oxidative capacity of the tricarboxylic acid cycle by increased *PCK1* activity that is not matched by metabolism that replenishes OAA. Induction of *PCK1* by butyrate without a matched increase in propionate supply may lead to a pattern of metabolism marked by a reduction of OAA pool, and therefore compromised capacity for acetyl-CoA oxidation. A resulting buildup of the intracellular acetyl CoA may potentiate hepatic ketogenesis in an attempt to shuttle energy to peripheral tissues. Increased butyrate to propionate ratio in the rumen by dietary supplementation of α -amylase is reported to be accompanied with elevated blood concentrations of BHB, one of the ketone bodies in dairy cattle (DeFrain et al., 2005; Tricarico et al., 2005, 2008). Increased ketogenesis in response to increased ruminal supply of butyrate (Huhtanen et al., 1993) supports a role of butyrate in altering hepatic energy metabolism. Our data have suggested an underlying mechanism for these observations. The regulation of bovine *PCK1* transcription and effect of propionate and butyrate proportions in conjunction with effects on cellular capacity for gluconeogenesis and fatty acid

oxidation were not investigated in the present study and warrant further investigation.

In addition to regulation by propionate and butyrate, the bovine *PCK1* promoter activity was also regulated by cAMP, dexamethasone, and insulin. The pattern of regulation by these hormones in bovine is similar to rat in that *PCK1* is stimulated by glucagon (via cAMP) and glucocorticoids and repressed by insulin (Hanson and Patel, 1994; Hanson and Reshef, 1997). These similarities are not surprising because the promoter (–1,000 bp to the TSS) of the rat and bovine *PCK1* gene have several high conserved regions that correspond to several transcription factor binding sites, such as the cAMP response element (Yang et al., 2009), which is involved in the full induction of rat *PCK1* promoter by both cAMP (Roesler, 2000) and glucocorticoids (Imai et al., 1990; Scott et al., 1998). A putative cAMP response element was also identified within the bovine *PCK1* promoter located at –94 bp (Figure 6) and may be the key regulatory element mediating the responsiveness of the bovine *PCK1* promoter to cAMP and dexamethasone.

Even though the regulation of *PCK1* transcription by cAMP, dexamethasone, insulin, and propionate has been reported in rodents (Hanson and Reshef, 1997; Massillon et al., 2003), to our knowledge this is the first study to investigate the relationship between propionate and these hormonal cues in regulation of *PCK1*

Figure 7. The role of the putative HNF4 α binding sites within the bovine *PCK1* promoter in mediating the responsiveness to propionate or butyrate. Rat H4IIE cells were transfected with the *PCK1*_(WT) and the mutant HNF4 α _{(+68_+72)⁻}, mutant HNF4 α _{(-1078_-1074)⁻}, and double mutant HNF4 α _{(+68_+72)⁻}/HNF4 α _{(-1078_-1074)⁻} separately and exposed to either 0 or 2.5 mM of propionate or 2.5 mM butyrate for 23 h. Inductions by propionate or butyrate for each promoter reporter are expressed as fold change relative to the no-addition control. Values are LSM $\pm$ SEM, n = 3. Labeled means without a common letter (a–c) differ at *P* < 0.05.

transcription in any species. The extent of induction of bovine *PCK1* transcription in response to propionate far exceeded the induction by cAMP and dexamethasone, and this effect is not repressed by insulin, indicating propionate is a primary regulator for bovine *PCK1* expression. The dominant induction of *PCK1* transcription by propionate over the repressive effect of insulin is consistent with the unique characteristic of ruminants that gluconeogenic rate is increased after feeding despite increased insulin concentration in blood (Aschenbach et al., 2010). Additionally, stimulation of *PCK1* transcription by hormonal signals, such as cAMP and dexamethasone, is likely to be more important during feed restriction. Otherwise, a decrease in expression of *PCK1* mRNA would be an anticipated outcome of reduced propionate supply; but clearly this is not the case in vivo, as *PCK1* mRNA in liver is not altered during feed restriction over a 5-d period in dairy cows (Velez and Donkin, 2005).

CONCLUSIONS

These data demonstrate hormonal and nutritional regulation of *PCK1* transcription in bovine. The promoter activity of bovine *PCK1* is induced by cAMP, dexamethasone, propionate, and butyrate. Insulin exhibits a dominant inhibition on the cAMP- and dexamethasone-induced *PCK1* transcription, but fails to repress propionate-induced *PCK1* transcription. Propionate, as a major gluconeogenic precursor in ruminants, controls its own metabolism in liver via inducing *PCK1* transcription.

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