Gene 545 (2014) 56–60

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Single-nucleotide polymorphisms and activity analysis of the promoter and enhancer of the pig lactase gene

Hai-ting Du [a,1], Hong-yan Zhu [b], Jia-mei Wang [a], Wei Zhao [a], Xiao-li Tao [b], Cai-feng Ba [a], Yu-min Tian [b], Yu-hong Su [b,*]

[a] Basic Medical College of Liaoning Medical University, Liaoning, China
[b] Animal Science and Veterinary Medicine College of Liaoning Medical University, Liaoning, China

ARTICLE INFO

Article history:
Received 11 November 2013
Received in revised form 1 May 2014
Accepted 2 May 2014
Available online 6 May 2014

Keywords:
Pig
Lactase
SNP
Luciferase reporter gene
Promoter

ABSTRACT

Lactose intolerance in northern Europeans is strongly associated with a single-nucleotide polymorphism (SNP) located 14 kb upstream of the human lactase gene: −13,910*C/T. We examined whether SNPs in the 5′ flanking region of the pig lactase gene are similar to those in the human gene and whether these polymorphisms play a functional role in regulating pig lactase gene expression. The 5′ flanking region of the lactase gene from several different breeds of pigs was cloned and analyzed for gene regulatory activity of a luciferase reporter gene. One SNP was found in the enhancer region (−797*G/A) and two were found in the promoter region (−308*G/C and −301*A/G). The promoter C_{-308},G_{-301}(Pro-CG) strongly promotes the expression of the lactase gene, but the promoter G_{-308},A_{-301}(Pro-GA) does not. The enhancer A_{-797}(Enh-A) genotype for Pro-GA can significantly enhance promoter activity, but has an inhibitory effect on Pro-CG. The Enhancer G_{-797}(Enh-G) has a significant inhibitory effect on both promoters. In conclusion, the order of effectiveness on the pig lactase gene is Enh-A + Pro-GA > Enh-A/G + Pro-CG > Enh-G + Pro-GA.

1. Introduction

Lactase (LCT), also known as lactase-phlorizin hydrolase, is present on the apical membrane of intestinal epithelial cells of nearly all mammals, and is necessary for hydrolyzing the disaccharide lactose in milk to yield the monosaccharides glucose and galactose for mucosal absorption. Lactose intolerance is a heritable, autosomal dominant, condition that results in a persistent inability to digest lactose throughout adulthood. The pig *LCT* gene is located on chromosome 15q13. Expression of *LCT* is restricted to the proximal and middle portions of the small intestine. The 1-kb sequence of the upstream fragment of *LCT*, located at the positions −997 to +10 in the pig *LCT* gene, has been shown by genetic association to control small intestine-specific expression and the post-weaning decline of LCT in transgenic mice (Troelsen et al., 1994; Wang et al., 2006). Temporally, lactase enzyme activity is maximal in newborn mammals and declines dramatically during maturation. The data demonstrate that the post-weaning decline of LCT is mainly due to a transcriptional down-regulation. The region from −17 to −994 in the pig *LCT* carries *cis*-elements that direct small intestine-specific expression and a post-weaning decline of a linked rabbit β-globin gene (Buller and Grand, 1990; Freund et al., 1990; Lee et al., 2002a, 2002b; Thomsen et al., 1995). The "*LCT* enhancer," located at positions −894 to −798 in the pig *LCT* gene, is necessary for high differentiation-dependent LCT expression in intestinal cells. The promoter, located at positions −313 to +5, was cloned and shown to be functional in the human intestinal cell line Caco-2.

The proximal promoter of the *LPH* gene alone is not able to initiate a high level of transcription in differentiated intestinal cell lines. Upstream sequences located between −894 and −798 play an essential role in initiating higher levels of expression in differentiated intestinal cell lines. This region contains at least three *cis*-elements (at −894 to −880, −880 to −875, and −833 to −814) with functional importance for *LCT* enhancer activity (Troelsen et al., 2003a, 2003b). The pig *LCT* enhancer was studied by mutation analysis, transfection experiments, and electrophoretic mobility shift assays. The data show that the sequences CE-LPH2a (*cis*-element lactase phlorizin hydrolase 2a) and CE-LPH 2b (*cis*-element lactase phlorizin hydrolase 2b) bind nuclear factors from intestinal cell nuclear extracts (Spodsberg et al., 1999; Troelsen et al., 1992; Troelsen et al., 1994). The enhancer region is not conserved, in contrast to the proximal promoter region. Furthermore, the enhancer is active in intestinal cells (Caco-2), but not in non-intestinal cells

Abbreviations: LCT, lactase; LPH, lactase-phlorizin hydrolase; SNP, single-nucleotide polymorphism; LP, lactase persistent; LNP, lactase non-persistent; PCR, polymerase chain reaction; DMEM, Dulbecco's Modified Eagle's Medium.

* Corresponding author at: No. 40 Songpo Road, Section 3, Linghe District, Jin Zhou City, Liao Ning Province, China.
E-mail addresses: haoyunhaiting@gmail.com (H. Du), haoyunyuhongsu@gmail.com (Y. Su).
[1] Postal address: No. 40 Songpo Road, Section 3, Linghe District, Jin Zhou City, Liao Ning Province, China.

http://dx.doi.org/10.1016/j.gene.2014.05.007
0378-1119/

(HeLa). The pig *LCT* enhancer is only able to enhance expression when it is located upstream of an intestinal-specific promoter such as the *LPH* promoter or the sucrase-isomaltase (SI) promoter. When located upstream of a SV40-derived promoter the *LCT* enhancer has no stimulatory effect. In addition to the lack of promoter promiscuity, the pig *LCT* enhancer is not a classical enhancer in the sense that it is promoter-specific and it is orientation- and location-dependent (Troelsen et al., 2003a, 2003b).

In field tests, we found that non-pathological diarrhea of pigs that was not treatable with anti-bacterial and anti-viral therapies could be alleviated by adding lactase to the diet. This phenomenon raised the question as to whether pigs have lactose intolerance similar to humans. As a first step, the SNPs of the 5′ flanking region of the pig *LCT* gene have been examined and their influence on *LCT* gene expression studied. This study has important practical implications that will aid in the dissection of the molecular mechanisms underlying the lactose intolerance observed in pigs and in humans.

2. Materials and methods

2.1. Subcloning of the lactase 5′ flanking region of the pig LCT gene

The 5′ flanking region was amplified from genomic DNA from several foreign (Large white, Landrace and Duroc) and domestic breeds (Hebao pigs and Min pigs) to detect the polymorphic site. DNA fragments corresponding to the 5′ flanking region of the pig *LCT* gene (−997 to +10) were generated by polymerase chain reaction (PCR) amplification using the following primers:

Forward: 5′-TTCCTGAGTTCCAAAGAGTG-3′;

Reverse: 5′-CTAGGAACTGTTAGGAGGTATG-3′.

PCR amplification was carried out in LA PCR buffer (Mg^{2+} Plus) containing 0.25 U of TaKaRa LA Taq, 20 μM primers, and 0.4 mM dNTPs in a final volume of 25 μL. Thirty-five cycles of PCR (94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min) was carried out. The DNA sequence of the 5′ flanking region of pig *LCT* gene was inserted into the plasmid pMD 18-T vector by the TA cloning technique. Sequencing results were analyzed using DNAMan software.

2.2. Subcloning of the lactase SNP region promoter–reporter and enhancer–promoter–reporter constructs

DNA fragments corresponding to the nucleotide sequence surrounding the enhancer −797*G/A, and −308C/G SNP regions of the pig lactase gene were generated by PCR amplification. A 145-bp fragment of the pig lactase gene −797*G/A SNP region was amplified using a forward oligonucleotide corresponding to nt −930 to −910, 5′-TCGG*GGTACC*GATATGCAGAAATAAAGGTAG-3′, and a reverse oligonucleotide corresponding to nt −785 to −805, 5′-TCGC*GAGCTC*TTTCAG TATCTGCAAAACAGT-3′. Primers were synthesized with a 5′-terminal *Kpn*I site and a 3′-terminal *Sac*I site for subsequent cloning.

Similarly, a 317-bp fragment of the *LCT* G/A_{-301} and C/G_{-308} variant region was amplified using a forward oligonucleotide corresponding to nt −313 to −294, 5′-CTA*GCTAGC*AAAAAGTTTGGTAAGGACCT-3′, and a reverse oligonucleotide corresponding to nt +5 to −16, 5′-CGG*AAGC TT*GGAACTGTTAGGAGGTATGTG-3′. Primers were synthesized using a 5′-terminal *Nhe*I restriction site and a 3′-terminal *Hin*dIII restriction site for subsequent cloning.

PCR fragments covering the region from −313 to +5 of the pig *LCT* promoter were cloned into the reporter plasmid pGL3-basic (Promega) upstream of the luciferase cDNA to generate the reporter plasmids pGL3-ProGA and pGL3-ProCG. The internal 146 bp *Kpn*I fragment of the −797*G and −797*A SNP region PCR products was cloned separately upstream of the pGL3-ProGA and pGL3-ProCG sequences to generate pGL3-ProGA-enhA, pGL3-ProGA-enhG, pGL3-ProCG-enhA and pGL3-ProCG-enhG. Proper incorporation of the promoter and enhancer SNP regions was confirmed by sequencing.

2.3. Cell culture and transfections

Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum. Forty-eight hours prior to transfection, the cells were split and 96-well tissue culture plates were seeded at 10^4 cells per well. For each reporter construct, a DNA transfection mixture was prepared consisting of 1 μg of the reporter construct, 0.01 μg of pRL-TK (Promega) as an internal control, and pBluescript KS + II added to obtain a final amount of 1.1 μg total DNA. The individual DNA mixtures were transfected in quadruplicate wells into Caco-2 cells at 70–80% confluency using Lipofectamine reagent (BRL) according to the manufacturer's protocol. Cells were harvested 48 h following transfection (90% confluent) and luciferase activity was measured using the Dual-Glo® Luciferase Assay System (Promega) in a total waveband luminometer. Transfection with the dual reporters (firefly luciferase for the lactase promoter–reporter plasmids and *Renilla* luciferase for the pRL-TK control) allowed for simultaneous expression and measurement of both reporter enzymes. Experimental lactase promoter–reporter and enhancer–promoter–reporter activities were normalized to the activity of the pRL-TK internal control and expressed as relative luciferase activity (mean ± SD). Statistical significance was determined using Student's unpaired t test.

3. Results

3.1. Polymorphism analysis of the 5′ flanking region of the pig LCT gene

The number of each breed of pigs used is shown in Table 1. The results show one SNP in the enhancer region: −797*G/A, and two in the promoter region: −308*G/C and −301*A/G. Interestingly, these SNPs were associated as follows: −308G with −301A and −308C with −301G. Fig. 1 shows the DNA wave crest figure of the polymorphic sites: −797G/A, −301G/A, and −308C/G.

3.2. Activity analysis of the promoter and enhancer of the pig LCT gene

To investigate the role of the −308*G/C and −301*A/G in regulating the promoter of the pig *LCT* gene, we initially generated DNA constructs composed of a pGL3 basic promoterless luciferase reporter construct fused to a 317-bp fragment of the pig *LCT* promoter gene corresponding to the −308*G/C and −301*A/G SNPs, cloned upstream of a luciferase reporter gene (Fig. 2), which we named pGL3-ProGA and pGL3-ProCG. Intestinal Caco-2 cells were transfected with the *LCT* promoter-luciferase constructs and assayed 48 h after transfection. Transfection with pGL3-ProGA resulted in a 2.1-fold induction of promoter activity. Transfection with pGL3-ProCG resulted in a 7-fold induction of promoter activity. The difference between Pro-GA and Pro-CG was highly significant ($P < 0.001$), showing that the Pro-CG is a strong promoter of the luciferase gene.

To investigate the role of −797*G/A on the enhancer of pig *LCT*, a 146-bp fragment of pig lactase enhancer gene corresponding to the −797*G/A SNP was cloned upstream of the pGL3-ProGA and pGL3-ProCG constructs, yielding four plasmids that we named pGL3-ProGA-

Table 1
Sequencing result of 5′ flanking region polymorphic site of different breeds of pig *LCT*.

Breed	Num	−301*G	−301*A	−308*G	−308*C	−797*G	−797*A
Min pig	14	12	2	2	12	1	13
Hebao pig	18	16	2	2	16	17	1
Landrace	14	14	0	0	14	0	14
Large white	16	16	0	0	16	0	16
Duroc	16	16	0	0	16	0	16
Total	78	74	4	4	74	18	60