Lactase persistence DNA variant enhances lactase promoter activity in vitro: functional role as a cis regulatory element

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Lactase persistence is a heritable, autosomal dominant, condition that results in a sustained ability to digest the milk sugar lactose throughout adulthood. The majority of the world’s human population experiences a decline in production of the digestive enzyme lactase-phlorizin hydrolase during maturation. However, individuals with lactase persistence continue to express high levels of the lactase gene into adulthood. Lactase persistence has been strongly correlated with single nucleotide genetic variants, C/T13910 and G/A22018, located 13.9 and 22 kb upstream from the lactase structural gene. We aimed to characterize a functional role for the polymorphisms in regulating lactase gene transcription. DNA in the region of the C/T13910 or G/A22018 human lactase variants was cloned upstream of the 3.0 kb rat lactase gene promoter in a luciferase reporter construct. Human intestinal Caco-2 cells were transfected with the lactase variant/promoter–reporter constructs and assayed for promoter activity. A 200 bp region surrounding the C/T13910 variant, associated with lactase non-persistence, results in a 2.2-fold increase in lactase promoter activity. The T/C13910 variant, associated with lactase persistence, results in an even greater 2.8-fold increase. The DNA sequence of the C/T13910 variants differentially interacts with intestinal cell nuclear proteins on EMSAs. AP2 co-transfection results in a similar repression of the C/T13910 variant/promoter–reporter constructs. The DNA region of the C/T13910 lactase persistence/non-persistence variant functions in vitro as a cis element capable of enhancing differential transcriptional activation of the lactase promoter. Such differential regulation by the C and T variants is consistent with a causative role in the mechanism specifying the lactase persistence/non-persistence phenotypes in humans.

INTRODUCTION

Intestinal lactase-phlorizin hydrolase (LPH, lactase) is the absorptive enterocyte membrane glycoprotein essential for digestive hydrolysis of lactose in milk. Lactase is present predominantly along the brush border membrane of differentiated enterocytes lining the villi of the small intestine. Expression of the lactase gene is temporally regulated during gut development and maturation (1,2). Lactase enzyme activity is maximal in the small intestine of pre-weaned mammals and declines markedly during maturation. In laboratory rodents, the maturational decline in lactase activity has been shown to occur at the time of weaning and contrasts with a maturational increase in enzymatic activity of other intestinal hydrolases essential for digestion of solid foods (comprehensively reviewed in 3). In humans, the activities of lactase and most of the other digestive hydrolases are maximal at birth. The age of onset of the post-weaning maturational decline of lactase activity in humans is variable, ranging from the toddler years to young adulthood.

Lactose is the predominant carbohydrate in breast milk. The lactose disaccharide consists of a glucose and a galactose molecule linked by a β(1–4) glycosidic bond. The digestive enzyme lactase catalyzes the hydrolysis of lactose to yield glucose and galactose that can then be absorbed across the intestinal mucosa membrane. The maturational decline in lactase activity renders most of the world’s adult human population intolerant to milk and other dairy products (4,5). Lactose present in dairy products cannot be digested in the small intestine, due to the reduced lactase level, and instead is fermented by bacteria in the distal ileum and colon. The fermentative products result in symptoms of diarrhea, gas bloat, flatulence and abdominal pain.

In a minority of adults, high levels of lactase activity persist in adulthood. This hereditary persistence of lactase is common

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primarily in people of northern European descent and is attributed to inheritance of an unidentified autosomal dominant mutation that prevents the normal maturation decline in lactase expression (6). Enattah et al. (7), using linkage disequilibrium and haplotype analysis in humans, have recently reported the identification of genetic variants located −13910 and −22134 bp upstream of the human lactase gene that are associated with hereditary lactase persistence/non-persistence in Finnish families (7). The −13910 bp C/T −13910 variant is located 5' to the lactase gene within intron 13 of the adjacent MCM6 gene (8) on chromosome 2. The intron 13 variant is a single nucleotide polymorphism, C to T. The authors report complete correlation between the lactase persistence/non-persistence phenotype and homozygosity for the C variant. Similarly, complete correlation between the lactase non-persistence phenotype and heterozygosity for the T variant allele. In the same study, a second variant (G/A 22018) was associated with the lactase persistence trait. Although not completely associated, this second variant may be involved in regulating the lactase activity. The C/T −13910 variant, however, is a strong candidate for the regulatory element involved in mediating lactase persistence. In a recent report, the lactase persistence allelic genotype T−13910/A−22018 has been shown to be associated with increased levels of lactase mRNA transcripts compared with the non-persistence allelic genotype C−13910/G−22018 (9). While strongly correlated with the lactase persistence/non-persistence phenotypes, the C/T −13910 variant has not yet been shown to be causative of the traits and a functional mechanism has not been defined (10). Enattah et al. (7) have postulated that the C/T −13910 variant affects an AP2 site, indicating that the C allele associated with lactase non-persistence is in the consensus binding motif whereas the T variant disrupts this motif. In the present paper, we report that the C/T −13910 lactase persistence/non-persistence variant functions as a cis element capable of directing differential transcriptional activation of the lactase promoter.

**RESULTS**

The lactase persistence C/T −13910 variant region enhances lactase promoter activity in intestinal cell culture

We have previously demonstrated that a 3.0 kb rat lactase promoter fragment (11) is capable of driving transcription in Caco-2 cells, a human adenocarcinoma-derived cell-line that mimics a small intestinal enterocyte phenotype with respect to expression of several digestive hydrolases including lactase (12). In order to determine whether the DNA sequence surrounding the C/T −13910 human lactase persistence variant is capable of regulating lactase promoter activity, Caco-2 cells were transfected with lactase promoter–reporter constructs harboring the C or T variant nucleotides. The constructs were generated by cloning the C/T −13910 region of the human lactase promoter upstream of the 3.0 kb rat lactase promoter in the luciferase reporter plasmid pGL3Basic. The C/T −13910 region (−14017 to −13800) was PCR-amplified from Caco-2 cell genomic DNA in order to generate the T variant reporter constructs p3kLac-T (forward orientation) and p3kLac-T' (reverse orientation). Similarly the C/T −13910 region was amplified from human RP11-34L23 BAC clone DNA to yield the C variant constructs p3kLac-C and p3kLac-C'. The C/T −13910 region in each construct was sequenced to confirm differences only at the −13910 single nucleotide variant. Transfected Caco-2 cell extracts were assayed for relative luciferase activity 48 h after transfection as shown in Figure 1.

Transfection with the lactase non-persistence associated C variant construct, p3kLac-C, results in a 2.2-fold induction of promoter activity driven by the 3 kb lactase promoter. Transfection with the lactase persistence-associated T variant construct, p3kLac-T, results in a 2.8-fold induction of promoter activity driven by the 3.0 kb lactase promoter. While both C/T −13910 regions are capable of enhancing promoter activity, the lactase persistence-associated T variant possesses a significantly greater activity compared to the non-persistence-associated C variant (P < 0.00001, p3kLac-T versus p3kLac-C).

Placement of the C/T −13910 variant region in both forward and reverse orientations results in comparable activation of the lactase promoter. Differential enhancement of lactase promoter activity mediated by the C/T −13910 variant region suggests a functional role for the DNA variant in specifying the lactase persistence/non-persistence phenotypes.

The G/A −22018 variant region directs minimal enhancement of lactase promoter activity in intestinal cell culture

A second genomic variant, G/A −22018, located upstream of the C/T −13910 variant region has also been associated with the lactase persistence/non-persistence traits. Although not completely associated, this second variant may also have a role in regulating lactase promoter activity. To determine whether the G/A −22018 variant region is involved in regulating the human lactase promoter, the G/A −22018 region (22 133–21 910) was cloned in both orientations upstream of the 3.0 kb rat lactase promoter to generate p3kLac-G, p3kLac-G', p3kLac-A and p3kLac-A'. Transfection with the lactase non-persistence-associated G variant constructs (p3kLac-G and p3kLac-G') or with the lactase persistence-associated A variant construct (p3kLac-A') results in a ~1.2- to 1.4-fold, induction of promoter activity (Fig. 2). The A variant region cloned in the reverse orientation (p3kLac-A') results in an ~0.8-fold reduction in promoter activity.

To determine whether the G/A −22018 variant region is capable of regulating the human lactase promoter in the context of the C/T −13910 variant, the G/A −22018 region was cloned upstream of the C/T −13910 region to generate p3kLac-GC and p3kLac-GT. The G −22018 variant and the C −13910 variant are both associated with lactase non-persistence. The addition of the G −22018 region upstream of the C −13910 region does not result in a significant change in lactase promoter activity (Fig. 3). The A −22018 variant and the T −13910 variant are both associated with lactase persistence. Addition of the A −22018 region upstream of the T −13910 Region results in a minimal, ~1.2-fold, increase in lactase promoter activity. Thus, the G/A −22018 variant region appears to direct minimal enhancement of the lactase promoter in context of the C/T −13910 variant region in the transfected reporter constructs.
AP2 overexpression represses lactase promoter activity in intestinal cell culture

It has been postulated that the C/T -13910 variant may affect an AP2 transcription factor binding site. The C allele, associated with lactase non-persistence, is in the consensus binding motif whereas the T variant disrupts this motif (7). In order to determine whether the transcription factor AP2 is involved in mediating the C/T -13910 region transcriptional activation of the lactase promoter, Caco-2 cells were co-transfected with the C/T -13910 variant/promoter constructs and AP2α and AP2γ expression constructs (Fig. 4). Co-transfection with the AP2α or AP2γ construct results in a 1.7-fold or greater repression in promoter activity driven by both C/T -13910 variant/promoter-reporter constructs as well as the 3.0 kb rat lactase promoter. This result suggests that AP2 regulatory cis element sequences may reside in the proximal rat lactase promoter but does not support a role for AP2 in mediating the lactase C/T -13910 variant effect.

Differential interaction between the C/T -13910 variant cis elements and intestinal cell nuclear proteins

In order to identify interaction between the C/T -13910 Variant cis element and intestinal cell nuclear proteins, we employed the electrophoretic mobility shift assay. C and T variant binding region oligonucleotide probes were incubated in the presence of Caco-2 cell (~90% confluent) nuclear extract and then migrated through a non-denaturing acrylamide gel. The EMSA in Figure 5 reveals the position of the rapidly migrating unbound probe at the base of the gel and DNA–protein complexes of slower mobility formed after incubation with Caco-2 nuclear extract in the absence of competition (lanes 4 and 5). A slow migrating DNA–protein complex, indicated by arrow, is more abundant in incubation with the T -13910 cis element probe (lane 4) compared with the C -13910 probe (lane 5). The T -13910 element DNA–protein complex is competed by 200-fold excess unlabeled T -13910 oligonucleotide (lane 3), but is not competed away by 200-fold excess of an unrelated Sp1 oligonucleotide (lane 2). The nuclear protein bound to the T -13910 cis element probe in Caco-2 cells therefore represents a specific DNA–protein interaction. In contrast, the C -13910 element DNA–protein complex in the same region is much less abundant and is not competed away by 200-fold excess C -13910 (lane 6) or Sp1 (lane 7) oligonucleotides. To confirm specificity of binding to the T -13910 cis element, EMSAs reactions were also performed with the T -13910 cis element probe in the presence of C -13910 cis element competitor DNA. The T -13910 cis element DNA–protein complex was not competed by 200-fold excess C -13910 cis element oligonucleotide (Fig. 6, lane 1). The nuclear protein interacting with the T -13910 cis element probe is thus a candidate factor that may be involved in differential transcriptional activation mediated by the C/T -13910 variant.
variant region. EMSA reactions performed with nuclear extract isolated from 7 day post-confluent Caco-2 cells result in a comparable T/C013910 cis element specific DNA–protein complex and AP2 antisera does not detect proteins interacting with the C/T/C013910 elements on gel supershifts (data not shown).

DISCUSSION

Hereditary persistence of lactase is common primarily in people of northern European descent and has been attributed to inheritance of a hitherto unidentified autosomal dominant mutation that prevents the normal maturational decline in lactase expression (6). The recent identification by Enattah et al. (7) of genetic variants associated with lactase persistence has prompted interest in determining whether the single nucleotide variants are causative. The intron 13 variant is a single nucleotide polymorphism (C to T) located at nucleotide 13,910 relative to the lactase start site and was completely correlated with lactase persistence/non-persistence. With respect to regulation of the lactase persistence/non-persistence phenotype, previous studies have suggested both transcriptional and post-transcriptional control mechanisms (6,13–18).

In the present study, we have focused on characterization of the role of the C/T−13910 and G/A−22018 regions in regulating the lactase gene promoter in intestinal cell culture. Specifically, the DNA surrounding the C/T−13910 and G/A−22018 polymorphisms was assayed for the ability to function as a transcriptional activator or repressor in transfected Caco-2 cells. With respect to the C/T−13910 region, DNA fragments harboring both the C and T variants are capable of enhancing lactase promoter (Fig. 1). Of note, however, the T variant results in greater promoter activation as compared to the C variant. Such an enhanced activity mediated by the T variant would be consistent with a functional role in the mechanism of lactase persistence. It is possible that a more dramatic effect was not seen due to the nature of transient transfection studies. Transfected Caco-2 cells contain multiple non-replicating episomal copies of the reporter constructs. Transiently transfected constructs are not always subject to regulatory mechanisms that involve complex chromatin structure, in contrast to the diploid chromosomal complement of the endogenous gene (19). In addition, the transfection constructs assayed likely lack other essential co-regulatory sequences located outside the 200 bp C/T−13910 region. Caco-2 cells, while a model of intestinal cell differentiation, are not identical to adult enterocytes in vivo and as such may also lack essential co-regulatory proteins needed to specify maximal phenotypic expression patterns. Thus, the C/T−13910 variants may exert a significantly greater differential regulatory effect in vivo in the context of the endogenous lactase gene. Compared with the C/T−13910 region, the G/A−22018 variant region results in minimal enhancement of the lactase promoter (Fig. 2). Unlike the C/T−13910 variant, the G/A−22018 variant was not completely correlated with the lactase...
persistence/non-persistence phenotype in the original report by Enattah et al. (7). Therefore, the G/A \(_{22018}\) variant may be associated with but not causative of the lactase phenotypes.

Enattah et al. (7) have postulated that the C/T \(_{13910}\) variant affects an AP2 binding site, noting that the C allele, associated with lactase non-persistence, is in the consensus binding motif, whereas the T variant disrupts this motif. To determine whether AP2 overexpression differentially regulates the C/T \(_{13910}\) variant element, we co-transfected Caco-2 cells with the variant–promoter constructs and with AP2\(\alpha\) and AP2\(\gamma\). Interestingly, AP2 over-expression results in a repression of the lactase promoter reporter constructs (Fig. 4). However, the fold repression is comparable for both the C and T allelic constructs. In addition, AP2 antisera do not detect proteins interacting with the C/T \(_{13910}\) element on gel supershifts (data not shown). These results do not support a direct role for AP2 binding.

The DNA sequence surrounding the C/T \(_{13910}\) variants, however, does interact differentially with intestinal cell nuclear proteins on gel shift assays. A slow migrating DNA–protein complex is more abundant in gel shift reactions performed with the T \(_{13910}\) \textit{cis} element probe than in reactions with the C \(_{13910}\) probe (Fig. 5, compare lanes 4 and 5). Such a differential gel shift binding pattern combined with the differential transactivation results suggests a possible mechanism to account for the lactase persistence/non-persistence phenotypes. Namely, the DNA sequence surrounding the T \(_{13910}\) variant may create a binding site for a transactivating protein that is capable of enhancing lactase transcription in adults with lactase persistence, thereby preventing the maturational decline. Such a mechanism would be consistent with a dominant mode of inheritance for lactase persistence associated with the T \(_{13910}\) allelic polymorphism. In individuals with lactase non-persistence, i.e. homozygous for the C \(_{13910}\) variant, the C nucleotide may disrupt the transactivating protein binding site and thus not allow for sustained lactase transcription into adulthood. There are likely to be additional regulatory control regions that specify the maturational decline in lactase in these individuals. Studies looking at the effect of the variants in stably transfected cell lines or in living animals will be necessary to confirm a role for the C/T \(_{13910}\) variant region in specifying lactase persistence or non-persistence. In addition, to characterize a potential regulatory mechanism directed by the C/T \(_{13910}\) region, further studies must be directed at identification of nuclear proteins interacting with this DNA region.

**MATERIALS AND METHODS**

**Materials and reagents**

Restriction endonucleases were purchased from Life Technologies (Rockville, MD, USA). Reagents for PCR were obtained from Qiagen (Valencia, CA, USA). Oligonucleotides used in restriction digestion reactions were purchased from Bioneer (Daejeon, Korea). The human lactase gene \(5\prime\)-flanking DNA sequence was amplified by PCR using primers \(5\prime\)-AGAAGTCACTCTCACGCCTCC-3\prime\) and \(5\prime\)-AGGATACCTCACTGCCATTTG-3\prime\). The amplified fragment was cloned in p3kLac-C. The T \(_{13910}\) and G \(_{22018}\) allelic promoter constructs were designed in p3kLac-C using oligonucleotides for the following restriction enzymes: A\(_{22018}\)-T\(_{13910}\), EcoRI and HindIII; G\(_{22018}\)-C\(_{13910}\), EcoRI and HindIII; C\(_{22018}\)-T\(_{13910}\), EcoRI and HindIII. The constructs were verified by sequencing using both strands.

Figure 3. Luciferase activity of intestinal cells transfected with G/A \(_{22018}\) and C/T \(_{13910}\) variant region lactase promoter–reporter constructs. Caco-2 cells were transfected with lactase promoter–reporter constructs and assayed for luciferase expression. p3kLac-C, human lactase gene C \(_{13910}\) variant region (open box). p3kLac-G-C, human lactase G \(_{22018}\) variant region (gray box) cloned in p3kLac-C. p3kLac-T, human lactase gene T \(_{13910}\) variant region (open box). p3kLac-A-T, human lactase A \(_{22018}\) variant region (gray box) cloned in p3kLac-T. Transfection efficiencies were normalized to renilla luciferase expression from a co-transfected pRL-CMV vector and expressed as relative luciferase activity (means±SD, \(n=3\)).
were synthesized by the protein and nucleic acid (PAN) facility of the Stanford University Medical Center.

**PCR amplification of the C/T\textsubscript{13910} and G/A\textsubscript{22018} regions of the human lactase gene locus**

A 218 bp fragment of the human lactase LCT gene C/T\textsubscript{13910} variant region was PCR-amplified using a forward oligonucleotide corresponding to nt $-14017$ to $-13994$, 5'-AGACGTAAGTTACATTTAATAC-3', and a reverse oligonucleotide corresponding to nt $-13800$ to $-13821$, 5'-CGTTAATACCTGACCTATC-3'. Both primers were synthesized with a 5' terminal MluI restriction site for subsequent cloning. The C/T\textsubscript{13910} region ($-14017$ to $-13800$) was PCR-amplified from Caco-2 cell genomic DNA to generate the T variant region DNA fragment. Similarly the C/T\textsubscript{13910} region was amplified from RP11-34L23, a human genomic DNA BAC clone (BACPAC Resources), to yield the C variant region DNA fragment. A 224 bp fragment of the lactase gene G/A\textsubscript{22018} variant region was PCR-amplified using a forward oligonucleotide corresponding to nt $-22133$ to $-22113$, 5'-TAAAGACATTTTACACTCTTC-3', and a reverse oligonucleotide corresponding to nt $-21910$ to $-21930$, 5'-AGAAAAATGGGTTTCCGCATG-3'. Both primers were synthesized with a 5' terminal KpnI restriction site. The G/A\textsubscript{22018} region ($-22133$ to $-21910$) was PCR-amplified from Caco-2 cell genomic DNA to generate the A variant region fragment. The G/A\textsubscript{22018} region was amplified from RP11-34L23 BAC clone DNA to yield the G variant region fragment.

**Subcloning of the lactase variant–promoter–reporter constructs**

C/T\textsubscript{13910} and G/A\textsubscript{22018} variant region promoter–reporter constructs were generated by cloning the PCR-amplified C/T\textsubscript{13910} or G/A\textsubscript{22018} variant region PCR products upstream of the lactase promoter in the previously described p3kLac construct (11). The p3kLac construct contains a 3.0 kb fragment of the rat lactase promoter cloned upstream of the luciferase cDNA in the reporter plasmid pGL3Basic (Promega). Specifically, the internal 218 bp MluI fragment of the C/T\textsubscript{13910} region PCR products was cloned into p3kLac to generate p3kLac-C and p3kLac-T (forward orientation) and p3kLac-C' and p3kLac-T' (reverse orientation). Similarly, the 224 bp KpnI fragment of the G/A\textsubscript{22018} region PCR products was cloned into p3kLac to generate p3kLac-G, p3kLac-G', p3kLac-A and p3kLac-A'. The combination G/A\textsubscript{22018} C/T\textsubscript{13910} variant region reporter constructs were generated by cloning the C/T\textsubscript{13910} region MluI fragments into p3kLac-G and p3kLac-A downstream of the G/A\textsubscript{22018} region to yield p3kLac-G-C and p3kLac-A-T. Incorporation of the C/T\textsubscript{13910} and G/A\textsubscript{22018} variant regions was confirmed by sequencing for all constructs generated.

**Transient transfection assays**

Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum. Forty-eight hours prior to transfection the cells were split and 35 mm dishes were seeded with $2 \times 10^5$ cells. For each reporter construct, a DNA transfection mixture was prepared consisting of 0.4 pmol of the reporter construct, 0.1 µg of pRL-CMV (Promega) as an internal control, and pBlueScript KS+ II to adjust to a final of 2.1 µg total DNA. The individual DNA mixtures were transfected in triplicate wells into cells (50–80% confluent) with Lipofectamine Reagent (BRL) according to the protocol of the manufacturer. For AP2 co-transfection experiments,
0.2 pmol of AP2x/pcDNA3.1+ or AP2γ/pcDNA3.1+ (gift of R. J. Weigel, Stanford University) (20) was transffected along with 0.4 pmol of the luciferase reporter constructs. Cells were harvested 48 h following transfection (70–90% confluent) and luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega) as described by the manufacturer, in a Monolight 3010 luminometer. Transfection with the dual reporters (firefly luciferase for the lactase promoter–reporter plasmids and renilla luciferase for the pRL-CMV control) allowed for simultaneous expression and measurement of both reporter enzymes. Experimental lactase promoter–reporter activities were normalized to the activity of the pRL-CMV control. Transient transfection efficiencies were controlled by co-transfection with the pRL-CMV plasmid.

**Electrophoretic mobility shift assays**

Nuclear proteins from Caco-2 cells harvested at 90% confluency and 7 days post confluency were extracted using the Nuclear Extract Kit from Active Motif (Carlsbad, CA, USA). Caco-2 cell nuclear extract purchased from Active Motif was also assayed. Protein was quantified with the protein assay from BioRad (Hercules, CA, USA). C/T-13910 variant region oligonucleotides (21mers) were synthesized with either the T or C variant centrally located and annealed to produce double-stranded probes (5’-GAT AATGTAGC/TCCCTGGCCTC-3’). DNA–protein interactions were detected using the LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL, USA) and 3’ end-biotinylated probes. Specifically, nuclear extracts (2 µg) were incubated with 20 fmol double-stranded, biotinylated DNA probes and 500 ng of poly(deoxyinosinate-deoxycytidylate) in a buffer containing 10 mM Tris, 50 mM KCl, 1 mM DTT, 0.05% NP-40 and 5 mM MgCl₂ in a total volume of 20 µl for 25 min at room temperature. Complexes were separated in a 5% native polyacrylamide gel in 0.5 x Tris-borate-EDTA buffer, transferred to a nylon membrane and cross-linked at 120 µl/cm². Detection of the complexes was performed using the LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL, USA) and 32P labeled fill-in oligonucleotides generated as previously described (11) and visualized by autoradiography.

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