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Characterization of Expression in Mice of a Transgene Containing 3.3 kb of the Human Lactase-Phlorizin Hydrolase (LPH) 5' Flanking Sequence

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Abstract

Background and Aim—The regulation of human intestinal lactase-phlorizin hydrolase remains incompletely understood. One kb of pig and 2 kb of rat 5'-flanking sequence controls correct tissue, cell, topographic, and villus LCT expression. To gain insight into human LCT expression, transgenic mouse lines were generated from 3.3 kb of human LPH 5' flanking sequence from a lactase persistent individual fused to a human growth hormone (hGH) reporter bounded by an insulator.

Methods—Four lines were identified in which reporter expression was specifically detectable in the intestine and no other organ, two of which demonstrated hGH expression specific to small and large intestine. Quantitative RT-PCR was carried out on proximal to distal segments of small intestine at fetal days 16.5 and 18.5 and at birth, postnatal days 7 and 28 in line 22.

Results—In fetal intestine, hGH expression demonstrated a proximal to distal gradient similar to that in native intestine. There was no significant difference between hGH expression levels at 7 and 28 days in segment 3, the midpoint of the small intestine, where expression of endogenous lactase is maximal at 7 days and declines significantly by 28 days. Distal small intestine displayed high levels of hGH expression in enteroendocrine cells, which were shown to be a subset of the PYY cells.

Conclusions—Thus, a 3.3-kb LPH 5' flanking sequence construct from a lactase persistent individual is able to maintain postnatal expression in transgenic mice post weaning.

Keywords

Development; Gene expression; Enterocytes; Enteroendocrine cells; Gradients

Introduction

Lactase-phlorizin-hydrolase (LPH, lactase), a small intestinal enzyme, is responsible for the hydrolysis of lactose, the main carbohydrate in milk, into glucose and galactose. Lactase is specifically expressed by the absorptive enterocytes, the most abundant cell type in the small intestine, but by none of the other three intestinal cell types. Lactase messenger RNA (mRNA) and protein are only expressed by differentiated and non-proliferating cells on the villi of the small intestine [1].

In humans and all other mammals, lactase is expressed in the small intestine before birth and remains high during the nursing period. At weaning, the mammalian diet shifts from milk to solid food and lactase expression is reduced. Lactase is restricted to low or undetectable levels in the duodenum and distal ileum and reduced in the jejunum and proximal ileum [2,3]. Among most humans and all other mammals, the ability to digest lactose during adulthood is low or absent due to the reduced lactase levels. In humans, this condition is

known as lactase non-persistence or lactose intolerance and may cause diarrhea, flatulence, and abdominal pain in non-persistent individuals who consume dairy products. In contrast, a subpopulation of humans maintains high levels of lactase during adulthood, a condition known as lactase persistence.

Most human populations in the world are lactase nonpersistent. The lactase-persistent population is comprised largely of Northern Europeans and their descendants [4]. Although the mechanism of lactase persistence remains unknown, lactase persistence is inherited as an autosomal dominant gene [5]. No structural differences between the genes of persistent and non-persistent people have been identified, indicating that persistence arises from altered regulation of lactase gene expression [6]. Because the geographic distribution of lactase persistence matches the distribution of dairy farming, it has been hypothesized that dairy farming populations acquired an advantageous mutation in a lactase regulatory element which gave them the ability to use milk as food in adulthood [7]. Consistent with this hypothesis, genetic data indicate that persistence appeared about 10,000 years ago and has been strongly selected for [8].

Lactase expression is primarily regulated at the transcriptional level [2]. Approximately 100 bp of the proximal promoters of all mammals examined is highly homologous [9], suggesting that the causal variant of the unique human lactase persistence phenotype is not located in this region. Beyond the proximal promoter, the 5' flanking regions of different species diverge. In all mammals examined, the gene for MCM6 ends about 3 kb 5' to the transcriptional start site of lactase. The unique feature of the sequence between human lactase and MCM6 is that it contains five stretches of repetitive DNA, two Alu sequences consisting of 300 bp and three other short repetitive sequences [10]. It is unknown if these repetitive DNA sequences are involved in lactase persistence.

Previous transgenic studies indicate that all of the necessary regulatory information for accurate lactase expression is encompassed within 2 kb or less of the transcriptional start site of rat and pig LPH. A 1-kb pig lactase transgene was only expressed in the small intestine by absorptive enterocytes, indicating tissue and cell specificity [11]. This transgene also showed a post-weaning decline, but did not match the proximal-distal expression pattern of endogenous lactase. Expression of a 2-kb rat lactase construct was also tissue- and cell-specific, but did not follow endogenous lactase expression regionally or developmentally [12]. Lee et al. [13] analyzed three different-sized rat constructs of 0.8, 1.3, and 2 kb. The 0.8-kb transgene was expressed in multiple organs and showed low activity of the reporter. The 1.3-kb transgene was tissue specific, while the 2.0-kb transgene was tissue-specific, cell-specific, had a normal horizontal pattern, and demonstrated a maturational decline after weaning. These studies confirmed that the 2-kb rat 5' flanking region contains the regulatory elements necessary for tissue-, cell-, and developmental-specific expression and suggested that important elements were present distal to the proximal promoter. Several possible regulatory sites within this region of the rat gene had already been identified by Verhave et al. [14]. To date, however, comparison of distal 5' flanking sequences among different species has failed to find conserved regions that could represent critical transcription factor binding sites. Although SNPs in the human lactase 5' flanking sequence at -13.9 and -22 kb are associated with lactase phenotypes (reviewed in references [1] and [10]), the regulatory region for expression remains incompletely understood. The hypothesis of the current study is that critical regulatory elements for human LPH expression in the persistence phenotype lie within the 3.3-kb 5' flanking region between the end of the MCM6 gene and the LPH transcriptional start site.

Materials and Methods

Generation of Human LPH Constructs

By replacing the PUC12 backbone of POGH (Nichols Institute, San Juan Capistrano, CA) with a pBluescript II KS + (Stratagene) backbone, a promoterless human growth hormone vector (BSGH) was generated. The deletions of the human LPH promoter were constructed by PCR amplification, subcloning, and ligation reactions, resulting in human LPH constructs of various lengths with base pairs deleted. These constructs were used in transfection assays.

Transfection of LPH Constructs

To characterize the activity of the deletion constructs in vitro, transient transfection assays were carried out in Caco-2 cells just prior to confluence, using Lipofectamine-Plus reagent (Invitrogen). In order to use a constant amount of DNA in the transfections, pBluescript II KS + (Strata-gene) was added as a carrier. The Caco-2 cells used were verified to be mycoplasma-free, determined by the Mycoplasma detection kit (American Type Culture Collection, Manassas, VA).

The amount of hGH secreted into the media was used as a measure of transcriptional activity. The concentration of hGH was measured using an [¹²⁵I] immunoassay kit (Allegro hGH, Nichols Institute, San Juan Capistrano, CA). Human growth hormone activity of the promoterless BSGH vector was subtracted from all other constructs to correct for background. As a control for transfection efficiency, cells were co-transfected with eGFP. At the conclusion of the experiment, media were sampled for hGH assay and the cells harvested for FACS analysis of GFP expression. Calculated hGH levels were corrected for the percentage of cells transfected as measured by GFP expression.

Construction of the 3.3-kb hLPH hGH Transgene

The 3.3-kb 5' flanking region of the human lactase promoter was cloned from the DNA obtained from surgical specimen of a lactase persistent Caucasian male from the US. This cloned region spans the LPH 5' flanking region from the end of the MCM6 gene to the LPH transcriptional start site. The 3.3-kb construct was made by first amplifying the entire 3.3-kb region in two smaller pieces that overlap at a unique restriction site. The two pieces were digested with *Hin*DIII restriction enzyme. The restriction fragments from the two pieces were ligated. Next, the full 3.3-kb region was cloned upstream of a human growth hormone (hGH) reporter and chicken lysozyme 5' MAR insulator, which has proven useful in shielding constructs from positional effects in transgenic mice [16,17]. Since there was no significant difference in hGh expression among the top three hGH-expressing constructs (h600 bp, h2.3, and h3.3 kb (in Fig. 1), we focused on the h3.3-kb construct because it included a bigger fragment than the other two.

Generating Transgenic Mice

The complete 3.3-kb hLPH hGH transgene was linearized, purified, and microinjected into fertilized C57BL/6 mouse eggs. The eggs were re-implanted into the oviducts of pseudo-pregnant mice and developed to term. The mice were housed under standard conditions in the Animal Research at Children's Hospital (ARCH) facility and provided food and water ad libitum. Approval was obtained from the Institutional Animal Care Use Committee for all experiments involving mice.

Genotyping

To identify the transgenic mice, DNA from tail snips were analyzed for transgene incorporation by PCR with primers designed to amplify a 300-bp fragment of hGH sequence

(supplemental Fig. 1). Integrity of the tail DNA was verified by PCR for mouse glyceraldehyde-3-phosphate dehydrogenase (mGAPDH).

Tissue Collection

Tissue samples were taken from mice completely anesthetized with avertin (tribromoethanol). To determine tissue specificity of transgene expression, stomach, intestine, colon, liver, lung, heart, and kidney were collected on postnatal days (P) 7, 14, 21, and 28. The intestine was divided into five equidistant segments, where segment 1 is proximal duodenum and segment 5 is distal ileum. Samples were also collected from embryonic day 16.5, 18.0, and neonatal mice, and divided into proximal, middle, and distal thirds. Tissue samples were immediately processed for RNA isolation or frozen at -80°C .

RNA Isolation and cDNA Synthesis

RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Because it is critical that the RNA samples are DNA-free, all RNA samples were treated with DNase (DNA-freeTM, Ambion, Woodward) for 45 min at 37°C . RNA was quantified by optical density at 260 nm, integrity was confirmed by 1.2% agarose gel electrophoresis and cDNA was synthesized using iScript (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer's instructions.

Semi-Quantitative and Real-Time RT-PCR

Semi-quantitative and real-time reverse transcription-PCRs (RT-PCRs) were conducted as previously described [15]. Primer pairs were designed using Beacon Design software (PREMIER Biosoft International, Palo Alto, CA) and optimized as described previously [15]. Semi-quantitative RT-PCR was terminated in the linear range of amplification, and real-time RT-PCR was carried out using an iCycler and iQ SYBR Green Supermix (Bio-Rad) on four to ten animals in each group, corrected for the glyceraldehyde-3-phosphate dehydrogenase gene (*Gapdh*), and expressed relative to the calibrator, which was pooled adult mouse jejunal RNA.

All reactions were conducted in triplicate using 10 pmol of forward plus reverse primers at a denaturing temperature of 95°C , annealing temperature of 58°C and an extension temperature of 95°C . To calculate the efficiency of the qRT-PCR reaction, a standard curve was generated using threefold dilutions of mouse wild-type cDNA for both hGH and *Gapdh* primers. Transgene copy number in the mouse lines was determined by real-time PCR. The expression of hGH levels in each mouse line was expressed relative to normal human DNA, which has two copies of the GH gene.

Growth of Transgenic Mice

In order to determine if the hGH used as a reporter affected growth of the transgenic mice, a litter of transgenic and non-transgenic siblings were weighed weekly from 3–7 weeks after birth.

Measurement of Secreted hGH

Circulating levels of hGH in the blood of transfected mice were determined by radioimmunoassay using a commercially available kit (Allegro hGH, Nichols Institute), as previously described [12].

Immunohistochemistry

To investigate cell-specific transgene expression, immunohistochemistry for hGH (DakoCytomation, Carpinteria, CA USA,) was performed. Segments 1, 3, and 5 of the

intestine and colon were fixed overnight in 10% formalin, embedded in paraffin, and sectioned at 5 μ m. Tissue samples were deparaffinized, rehydrated, and then treated for 15 min with protease type XXIV (Sigma, St. Louis, MO; 0.03 mg/ml in PBS) for antigen retrieval. To block endogenous peroxidase activity, the sections were incubated with 3% hydrogen peroxide for 15 min, and blocked with a non-specific goat serum (Vectastain, ABC kit, Vector Laboratories) for 30 min at 37°C. Next, slides were incubated with an affinity-purified primary antibody, polyclonal rabbit anti-hGH (Dakocytomation, Carpinteria, CA) at a 1:400 dilution for 1 h at 37°C. Bound primary antibodies were visualized using a biotinylated secondary antibody and an avidin–biotin peroxidase conjugate (Vectastain ABC kit, Vector Labs). Signal was developed via incubation with 3, 3-diaminobenzide tetrahydrochloride (DAB) (Sigma, St. Louis, MO) substrate solution for 5 min. The tissue was lightly counterstained with methyl green and visualized by light microscopy. As a positive transgenic control, sections from the intestines of mice expressing the previously described rat LPH/hGH construct [12] were stained in parallel. Sections were also stained for mouse PYY (generous donation from A. B. Leiter, UMass Worcester).

Statistical Analysis

A parametric *t* test, non-parametric sign test, and signed rank test were used to compare hGH mRNA values between segments 1 and 3 of P7, segments 1 and 3 of P28, segments 3 and 5 of P7 and P28, respectively, and between the proximal and distal intestinal segments of 16.5-day embryos (E16.5) and neonatal (P0) mice. Significance was noted as *p* value 0.05, and is appropriately reported in figure legends.

It should be noted that we were only able to study the 3.3-kb 5' flanking sequence from a lactase persistent individual. The correlate from a non-persistent individual was not available.

Results

Verification of Construct Expression In Vitro

As shown in Fig. 1, constructs longer than 458 bp gave strong hGH expression when transfected into intestine-like Caco-2 cells. In comparison to shorter human LPH constructs, the h600-bp, h2.3-, and h3.3-kb constructs produced comparable levels of expression relative to the h118-bp construct. Furthermore, the h3.3-kb construct was more robust than the 2-kb rat construct previously shown to produce accurate expression in transgenic mice [12,13]. Therefore, it was hypothesized that this human construct would be appropriate for further study in transgenic mice. An insulator sequence was added to the construct to minimize transgene positional effects [16,17].

Expression and Transmission of the 3.3-kb hLPH hGH Transgene in Mice

Two rounds of pronuclear injections yielded 28 pups, eight (three females and five males) were positive for the transgene (Table 1). As determined by RT-PCR, offspring of one male expressed mRNA for hGH in the small and large intestine, but also in stomach, liver, lung, heart, and kidney. Adult offspring of the other four males specifically expressed the transgene only in the intestine.

Three female founders, F7, F19, and F23, were either infertile or did not transmit the transgene to any pups (Table 1). These founders were analyzed for expression of the transgene in stomach, intestine (divided into five equal-distant segments from proximal to distal intestine), colon, liver, lung, heart, and kidney (Table 2). Founder F7 showed hGH mRNA expression in multiple organs, while F19 showed expression in all the segments of

the small intestine and colon only. DNA analysis was positive for founder F23, but showed no detectable amount of hGH mRNA in the organs analyzed.

The male founders produced both male and female transgenic offspring, and positive female offspring were able to transmit the transgene, indicating that there was no sex-related transmission difference between the transgenic females and males. Lines 13, 20, and 22, were selected for further study (Fig. 2). In these lines, hGH expression was tissue-specific, being restricted to the small intestine and colon. Transgene copy numbers were 32, 35, and 58 in lines 13, 20, and 22, respectively. Further analysis was therefore focused on line 22, which had both strong expression of hGH in all small intestinal segments and the highest transgene copy number, by real-time RT-PCR.

Influence of hGH on the Growth of Mice

Mice from line 22 were weighed to investigate whether the expressed hGH influenced the growth of the transgenic mice. One litter was weighed from 3 to 7 weeks. The litter was composed of 11 mice, four females and seven males, of which three females and four males were positive for the transgene. The weights of the negative and positive mice were essentially the same over this period (Supplemental Fig. 2). Blood levels of hGH in transgenic line 22 mice had an average value of 5.1 ng/ml at 28 days.

Expression of the Transgene Along the Proximal–Distal Axis and During Development

Transgene hGH mRNA expression in fetal intestine at 16.5 days of gestation appeared to demonstrate a proximal to distal gradient by real-time RT-PCR. Maximal expression was in the proximal intestine and the lowest in the distal ileum (Fig. 3a) though this difference was not statistically significant. A similar pattern was seen on day 18.5 (data not shown). Immediately after birth, the transgene showed higher levels distally than proximally (Fig. 3b).

At 7 days, transgene hGH mRNA expression in the small intestine increased from segment 1 to 3 and was sustained in segments 4 and 5 (Fig. 4a). This pattern was also seen at 28 days, showing the highest median level in segment 3, and comparable levels in segment 5 (Fig. 4b). The expected mammalian developmental pattern of endogenous LPH mRNA expression is a characteristic decrease from the newborn to the post-weaning period (day P7 compared to day P28, Fig. 5). However, in our transgenic mice, hGH expression levels in segment 3 did not differ between P7 and P28 (Fig. 4). These data are consistent with persistence of human LPH transgene expression in this mouse model, and it supports the idea that the 3.3-kb 5' flanking region of human lactase, used in our transgenic construct, is sufficient to promote lactase persistence in mice. It is important to note that the 3.3-kb human LPH 5' flanking sequence was derived from a lactase-persistent individual, and thus behaves as expected. Unfortunately, we do not have parallel data from the 3.3-kb 5' flanking sequence of a lactase non-persistent individual. This would have enabled us to draw more definitive conclusions about the presence of lactase regulatory elements in this region.

As shown in Fig. 5, mouse LPH expression in our transgenic animals maintain the expected native pattern, consisting of a decline from the pre-weaning (P7) to the post-weaned period (P28). It shows that our transgene (reporting human LPH expression) does not interfere with normal mouse LPH expression.

Localization of hGH Expression

Cell and crypt/villus expression of the transgene was revealed using immunohistochemical or immunofluorescence techniques on intestinal segments 1, 3, and 5 from line 22 at 7 or 28 days of age (Figs. 6, 7). In some experiments, samples of intestine from previously

published 2-kb-rLPH-hGH transgenic mice [12] were stained as a positive control. As shown in Fig. 6a, patchy staining for hGH was identified in the supranuclear region of absorptive enterocytes on the upper villi, consistent with hGH cytolocalization. Careful examination of multiple sections revealed 5–10 hGH-positive absorptive enterocytes per villus. By comparison, the 2-kb-rLPH-hGH transgene achieved expression in a larger number of enterocytes in a comparable region (Fig. 6b). With the human transgene, we occasionally identified positive goblet or crypt cells, but expression in Paneth cells was not found (data not shown). Expression was identified, however, in a population of enteroendocrine cells. The tissue was first stained with an antibody against chromogranin A, considered a pan-enteroendocrine cell marker. Numerous positive cells were observed, but none was co-stained with hGH (data not shown). Subsequently, tissue samples were stained for PYY, known to be enriched in enteroendocrine cells in the ileum [19]. We identified a population of PYY-positive cells, a subset of which co-stained for hGH (Fig. 7). No other set of hGH-positive cells were identified.

Discussion

These studies were designed to test the hypothesis that the regulatory elements responsible for human lactase persistence are located in the 5' flanking region between the end of the MCM6 gene and the transcriptional start site of LPH. Expression of a human lactase transgene in mice has not been previously reported. During late fetal life, the 3.3-kb hLPH transgene was expressed in an expected proximal–distal pattern. Reporter expression at this time was highest in the proximal segment, decreasing distally, consistent with the pattern reported by Calvert et al. [18] for fetal development of lactase enzyme activity in native mouse intestine. During subsequent maturation, however, the proximal to distal gradient shifted such that hLPH mRNA expression was maintained at a high level from segment 3 to segment 5, and the expected decrease in LPH mRNA expression in these segments did not occur. This pattern suggests that sequences directing persistence of hLPH gene expression may be contained in the region of the 5'-flanking sequence used in this study.

In an effort to improve expression of the hLPH transgene, an insulator was added to our construct. The insulator is thought to shield constructs from positional effects in transgenic mice [16,17]. In previous studies, we showed that an LPH transgene could be incorporated into DNA without an insulator sequence, but frequently was not expressed or expressed inaccurately [12]. In an aforementioned study, only six founders (8%) were generated from 77 pups; only one out of the six showed specific expression of the lactase transgene. In contrast, from the 28 pups in the current study, eight pups (29%) positively expressed human LPH mRNA and transmission to offspring was seen in five male founders. In three of these, expression in offspring was intestine-specific. These data indicate that the use of the insulator was effective in increasing transgene expression in founders.

Previous studies of the LPH 5' flanking region in other transgenic models found that all of the necessary elements for cell- and developmental-specific expression were included within 1-kb (pig) or 2-kb (rat) of sequence 5' to the transcriptional start site [11,12,20]. Furthermore, elements required for correct crypt-villus and proximal–distal expression were also contained in the respective constructs. These data are in contrast to those in the present study in which tissue, cell, and crypt-villus expression were largely maintained, but developmental expression was more reflective of human LPH persistence than of native mouse LPH.

Finally, the proximal–distal gradient was altered with maintenance of high levels of expression distally in enteroendocrine cells. This suggests that critical elements for extinction of the transgene in enteroendocrine cells were missing from the construct studied

or that undetected similarities in the promoters of LPH and PYY accounted for the aberrant expression. Interestingly, all of the LPH proximal promoters studied to date are highly homologous in the first 100 bp, but diverge further 5' [1,10]. As shown by Lee et al. [13], rat transgenic constructs containing only 0.8 and 1.3 kb of 5'-flanking sequence failed to produce correct expression. Therefore, regions more distally 5' but within 2 kb are required for developmental regulation in the rat [12,13].

The 3.3-kb 5'-flanking region of the human LPH gene contains nearly 600 bp of repetitive DNA (Alu) sequences [10]. In contrast, the approximately 3-kb distance between the MCM6 and LPH genes in human and rodent (mouse and rat) genomes is essentially the same, suggesting that the repetitive DNA in humans has replaced non-critical sequence. The function of the Alu sequences remains unclear at present.

The expression of the hLPH transgene in PYY cells in this study recapitulates patterns previously described in investigations of human sucrase-isomaltase (SI) and rat liver fatty acid-binding protein (L-FABP) transgenes [21,22]. In these studies, as in the present one, expression of hGH in the PYY cells demonstrates that these cells contain the necessary machinery for expressing the cognate genes. Since LPH is not normally expressed in PYY cells, the data from our study suggest either that the hLPH transgene lacks elements that normally repress LPH gene expression in PYY cells, or that the promoter used in these studies contains sequences in common with PYY that permit expression. In studies of SI transgenic mice, Markowitz et al. examined human SI-hGH constructs with 200 bp, 3.3 and 8 kb of flanking sequence; All displayed hGH expression in enteroendocrine cells [21,22].

In contrast to the results presented here, Markowitz et al. did not detect expression of hGH in the duodenum, where native SI is faintly detectable, and very little in proximal jejunum, where native SI is strongly expressed. In distal jejunum, hGH was reported as about half the SI level, while in the ileum, hGH was about twice the SI level. Some of the high distal expression may have been due to the contribution of PYY cell expression. Consistent with our findings, Markowitz et al. reported that the intestine-specific expression pattern was observed in multiple lines and did not depend on copy number [21]. The striking similarity of the expression patterns of the human LPH described here and human SI in mice suggests that critical factors regulating both genes are different between mouse and human intestine.

In other studies, Simon et al. [23] examined expression patterns of fusion constructs of hGH and either a -596 bp or -4,000 bp of liver FABP 5' flanking sequence in different types of enteroendocrine cells of the intestine. They found that in comparison to native expression, patterns of transgene expression were similar in some cell types and altered in others. In particular, they found that the -596-bp hGH construct was expressed in PYY cells in the colon, but that expression of the -4,000-bp construct was completely suppressed, suggesting that PYY cells produce a strong inhibitor that binds to an element between -596 and -4,000 in the L-FABP gene. Since all of the enteroendocrine cells arise from the secretory lineage, which presumably originates from the same stem cell that generates all of the intestinal epithelial cells, the high expression we observe in the PYY cells is likely the result of a change specific to the PYY cells. The simplest mechanism would be a loss, or reduction, during cell differentiation, of an inhibitor that normally blocks LPH expression in PYY cells. In a preliminary search of the 400-bp immediately upstream of hSI, hLPH, and mPYY genes, we found similar transcription binding sites for GATA, HNF, PPAR, and several others, but not in a homologous order. An examination into these observations is, however, beyond the scope of this study.

A further point of interest is that fetal expression patterns of our transgene followed endogenous LPH exactly. Although enteroendocrine cells can be detected in developing

mouse intestine at E12.5 [24], and PYY cells are detectable and identifiable in the distal intestine late in gestation in rats [25], real-time RT-PCR analysis of PYY mRNA indicated that expression of the transgene in our study did not track native PYY expression, increasing distally (data not shown). Thus transgene expression in the intestinal absorptive cells is independent of PYY expression.

In contrast to the strong expression observed in the PYY cells, staining intensity of hGH in absorptive enterocytes was consistently patchy, indicating that the protein is present at low levels in some cells and barely detectable in others. Levels of endogenous LPH mRNA measured in the same samples are higher by fivefold or more, than hGH levels. The difference in level of expression compared to the endogenous gene appears to be unique to the lactase gene, as Markowitz et al. observed that a human SI trans-gene was expressed at levels comparable to that of endogenous mouse SI, although the comparison was based on band intensities from Northern blots [21]. In considering the possibility that key regulatory elements were missing from the transgene used in these studies, recent data on the distal promoter of the human LPH gene become relevant. Enattah et al. [26] identified two single-nucleotide polymorphisms (SNP) correlated with lactase persistence, a C → T SNP at -13,910 bp and a G → A SNP at -22,018 bp upstream of the lactase gene. That the two SNPs may be involved in transcriptional regulation has been suggested by cell transfection experiments using constructs containing the respective polymorphisms [1,20]. However, these constructs have not been examined in vivo.

In the present study, although the mouse line examined had a high copy number, hGH was not produced at a high enough level to affect the growth of the mice, consistent with previous studies. Krasinski et al. [12] showed that a low level of hGH from a single transgene was not sufficient to alter the growth of the mice. In contrast, Sweetser et al. [22] showed that transgenic mice producing high levels of hGH from high copy numbers of the I-FABP construct had increased growth rates compared to their wild-type litter-mates. Serum levels of hGH measured in the present study were found to be comparable to those reported in low copy number mice [12,22].

In summary, we have shown that a construct containing 3.3 kb of the human LPH 5' flanking sequence directs correct tissue, cell and crypt-villus expression of the hGH reporter. In contrast to previous reports of pig and rat LPH promoters expressed in transgenic mice [11,12], we found evidence for developmental persistence of hGH expression suggestive of the LPH persistence pattern seen in human intestine. However, additional studies with a construct containing the 5' flanking sequence from a lactase nonpersistent individual would have to be conducted to confirm our finding. We also observed robust expression of the transgene in a subset of PYY+ cells in the distal intestine, as reported previously for human SI and LFABP. Further studies are indicated to delineate the molecular basis of human LPH persistence and non-persistence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Relative expression of lactase promoter constructs in transfected CaCo-2 cells. The length of the human (*h*) or rat (*r*) lactase promoter in LPH- hGH transgene constructs seems to affect the levels of reporter expression in Caco-2 cells

Fig. 2.

Transgenic mouse lines 13, 20, and 22 gave intestine specific transgene expression. Intestine-specific hGH mRNA expression in adult mice from lines 13, 20, and 22 was analyzed by semi-quantitative RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase was used as a control for the input mRNA. The products were run on 2% agarose gel in Tris–Borate-EDTA buffer at 160 V for 25 min. A 100-bp ladder was loaded to show amplicon size (=300 bp)

Fig. 3.

Transgene hGH expression was detected in embryonic and newborn mice. Transgene hGH mRNA expression in fetal intestine at E16.5 showed a proximal to distal gradient of hGH mRNA levels, with maximal expression in the proximal segment and the lowest in the distal segment, parallel to that of endogenous LPH expression. Immediately after birth, the pattern of expression had reversed, with higher levels distally than proximally. Quantitative RT-PCR was used to assess relative mRNA expression, and $n = 3$ or 4 in each segment analyzed. All mRNA levels were computed relative to the same calibrator. The *solid black line* represents the medians

Fig. 4.

hGH transgene expression shows persistence through the post-weaning period. **a** t P7, transgene hGH mRNA expression in the small intestine increased from segment 1 to 3 (t test, sign test, and signed rank test, $p = 0.004$) and was sustained in segments 4 and 5. **b** The pattern seen at P7 was sustained at P28, also showing an increase from segment 1 to 3 (t test, sign test, and signed rank test; $p = 0.05, 0.04, 0.01$, respectively). Quantitative RT-PCR was used to assess mRNA expression $n = 9-10$ in each intestinal segment analyzed. All mRNA levels were computed relative to the same calibrator. The *solid black line* represents the medians

Fig. 5.

The transgenic mice maintain normal endogenous LPH expression. Intestinal segments 1, 3, and 5 were analyzed by real-time RT-PCR for native mouse lactase mRNA. The expected mammalian developmental pattern of endogenous LPH mRNA expression was observed with a significant decrease from the newborn (data not shown) to the post-weaning period. Student's *t* test performed on segment 3 in both day 7 and day 28 intestine yielded a *p* value = 0.01

Fig. 6. hGH protein is expressed in intestinal epithelial cells of line 22 p28 mice. The presence of hGH protein was detected by immunohistochemistry. **a** hGH staining (*black arrow*) in the supranuclear region of absorptive enterocytes on the upper villi. **b** Rat 2-kb LPH transgene Krasinski et al. [12] stained as a positive control. Staining is shown for segment 5 in line 22 at p28. Magnification $\times 40$

Fig. 7. hGH and PYY co-stain a subpopulation of enteroendocrine cells of line 22 p28 mice. Intestinal segment 5 sections were stained using immunofluorescence. **a** PYY cells (*red*); **b** hGH cells (*green*); and **c** co-expression of PYY and hGH (*yellow*). Of note is that not all PYY cells express hGH. Magnification $\times 40$

Table 1

Transmission of the 3.3-kb hLPH-hGH transgene from founder mice to offspring

Founder number	Transgenic pups/total pups	Transmission of transgene
7 (M)	0/37	No
13 (M)	5/14	Yes
15 (M)	24/44	Yes
19 (F)	0/0	No
20 (M)	5/14	Yes
22 (M)	18/47	Yes
23 (F)	0/13	No
28 (M)	18/35	Yes

Two litters produced 28 pups, only eight of which were positive for the transgene when DNA from tail snips was analyzed. *M* male, *F* female

Table 2

hGH-positive mice that did not transmit the transgene to their offspring

Founder	Stomach	Segment 1	Segment 2	Segment 3	Segment 4	Segment 5	Colon	Liver	Lung	Heart	Kidney
F7	+	+	+	+	+	+	+	-	+	-	+
F19	-	+	+	+	+	+	+	-	-	-	-
F23	-	-	-	-	-	-	-	-	-	-	-

hGH-positive mice who did not transmit the transgene to their offspring showed non-intestinal specific or no hGH mRNA expression. Messenger RNA expression in the founders (positive for hGH in their DNA) who did not transmit the hGH transgene to their offspring was analyzed by RT-PCR. Segment 1–5 refers to small intestine divided into five equal-distant segments from proximal to distal intestine. hGH expression (+) or absence (–)