Complex Interactions among Tissue Restricted Transcription Factors and Cofactors are Critical for Intestine Specific Gene Expression

Herbert van Wering

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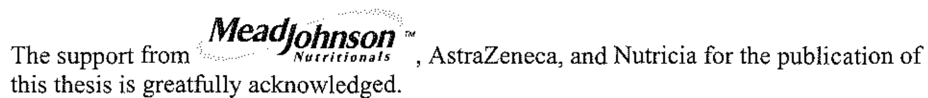
Herbert M. van Wering

On the cover: The John Hancock building and Prudential tower in Boston, Massachusetts. This oil painting by Stephen D. Krasinski was painted from a picture taking while having diner on a fishing boat on the Charles river. Present on this trip were Herbert, Naomi, Maartje, Evelien, Steve, and Joanna. This view represents one of the most beautiful views of the backbay during a late summer sunset.



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Complex Interactions among Tissue Restricted Transcription Factors and Cofactors are Critical for Intestine Specific Gene Expression

Complexe interacties tussen weefsel gebonden transcriptie factoren zijn belangrijk in de expressie van intestinale genen

Proefschrift

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Het kleinste bezit het grootste,
dat geldt ook andersom.
Een prikkelende gedachte
is de vraag 'waarom'?
Waarom kan het grote niet zonder al het klein?
En waarom is er niets kleins als er geen groot kan zijn?
Een evenwicht tussen beiden, schept een brede visie.
En geeft de mens met deze blik een gave: precisie.
Precisie in het kleine bouwt een brug naar al het groot
en stelt deze visie op het leven aan het bewustzijn bloot.

Marieke Gielen, Boston 2002.

"Pappie, dat met al die gekleurde lijntjes op die stroken papier, dat deed ik ook op de kleuterschool, waarom doe jij dat nu nog…."?

Proefschrift J.H. van Wering, Amsterdam 1981

Nu begrijp ik pas dat je eerst moet hebben geleefd om dat soort lijntjes te kunnen trekken……….
Mijn dank is groter dan is te beschrijven in woorden. Mooi dat je me deze kans gaf, dank je Pappie!

Proefschrift H.M. van Wering, Boston 2002

Preface

This study was a joint effort between the Division of Pediatric Gastroenterology and Nutrition, Department of Pediatrics, Erasmus MC/Sophia Kinderziekenhuis, Rotterdam, The Netherlands, and the Division of Pediatric Gastroenterology and Nutrition, The Floating Hospital for Children, Tufts University School of Medicine, Boston, Massachusetts, USA, which later became Division of Gastroenterology and Nutrition, Department of medicine, Children's Hospital Boston, Boston MA, 02115; Department of Pediatrics, Harvard Medical School, USA.

Research described was supported by a American Digestive Health Foundation Student Research Fellowship Award and research Fellowship Stipend from the American Gastroenterology Association (AGA), Bethesda, Maryland USA; by Nutricia Research Foundation Stipend Zoetermeer, The Netherlands; Foundation "De Drie Lichten", Hilversum, The Netherlands; NIH Research Grant # R01-DK-32658-19; by the Silvio O. Conte Digestive Disease Core Center Grant P30-DK-34928.

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Chapter 1

Introduction

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1 Background

1.1 Intestinal epithelial cell differentiation and development is a dynamic, continuous process

During ontogenesis and throughout the lifespan of mammals, the small intestine undergoes an integrated pattern of differentiation (128,141). The complex structure of the mature epithelium occurs as a result of a series of developmental transitions beginning in the embryo and terminating after weaning (95,128,141). Beginning on embryonic day 8 (E8) in mice, interactions between visceral endoderm and mesoderm produce an undifferentiated stratified epithelium and, by E9, a primitive gut tube is formed. The cells undergo rapid cytodifferentiation beginning on E15, and by E19, an epithelial monolayer overlies nascent villi. It is during this important transition that the expression of certain intestine-specific genes is first detected (96,97). Villi are separated at their bases by a proliferative compartment which is converted to a mature crypt of Lieberkühn during the first two weeks of postnatal life. The intestine undergoes final differentiation at weaning whereby the panel of genes expressed establishes the protein patterns that are responsible for digestion and absorption of nutrients. The mechanisms underlying intestinal development are unknown, but an understanding of the developmental history of transcriptional events of specific genes will provide insight into the combined effects of temporally regulated transcription factors.

The columnar intestinal epithelium of the mature intestine is a highly dynamic system in which the cells are continuously renewed by a process involving cell generation and migration from the stem cell population located near the base of the crypt to differentiated cells of the villus (56,79,141). From stem cells, four major terminally differentiated cell types arise: absorptive enterocytes, goblet cells, enteroendocrine cells and Paneth cells (41). Absorptive enterocytes, comprising approximately 95% of epithelial cells on villi, are polarized cells with an apical brush border that contains transporters, receptors, and membrane-anchored hydrolases, including lactase-phlorizin hydrolase (LPH) and sucrase-isomaltase (SI). Goblet cells synthesize mucins, which protect the epithelium and produce a medium in which terminal digestion and absorption can occur optimally (25). Enteroendocrine cells produce a variety of hormones necessary for regulation of intestinal processes (116). Paneth cells produce a number of proteins including those of the innate immune system such as, lysozyme and defensins (115). An additional cell type that is derived form the enterocyte lineage are the M-cells, which are also produced in crypts adjacent to the Peyer's patches (95,155). Precursor cells undergo several rounds of cell division while they reside in the crypt compartment and, with the exception of Paneth cells which migrate to the base of crypts, migrate from crypts toward the tip of the villi. As the precursor cells approach the crypt-villus junction, the proliferating phase ends and the cells then acquire a functionally differentiated phenotype. The cells continue to migrate up the villi, undergo apoptosis (95), and are shed (83) into the intestinal lumen three to five days (depending on species) after their initial appearance on villi (17,40). This continuous process is a model for cellular differentiation. In addition to differentiation-specific regulation along the crypt/villus (vertical) axis, intestinal gene expression is also controlled along the proximal-to-distal (horizontal) axis resulting in distinct functions in different regions of intestine as well as by the developmental gradient. The molecular mechanisms underlying the dynamic processes of intestine-specific gene expression, cell fate determination, cellular differentiation, and intestinal development are poorly understood at present.

The critical mechanisms for the determination of specific cell lineages remains unclear, but the influence of specific early transcription factors are beginning to be discovered. Recent investigations in intestinal stem cells by Yang et. al. (161) have shown that *Math1*, a basic helix-loop-helix transcription factor, is required for secretory cell lineage commitment in the mouse

intestine. Loss of *Math1* results in depletion of goblet, enteroendocrine, and Paneth cells without affecting absorptive enterocytes. These results suggest that the secretory cells (goblet, enteroendocrine, and Paneth cells) arise from a common progenitor that expresses *Math1*, whereas absorptive cells (enterocytes) arise from a progenitor that is *Math1*-independent. These discoveries are relevant because the downstream signaling from these early specification genes dictates the eventual hierarchy of differentiative transcription factors present in specified intestinal cells. By delineating the overlapping expression of transcription factors and target genes during fetal and postnatal development, along vertical axis, and along the horizontal gradient, the critical modulators that regulate the transcription of specific intestinal genes can be defined.

1.2 Transcriptional regulation and RNA processing:

Transcriptional control in the context of cellular differentiation involves a network of interactions between *cis*-acting regulatory elements in the DNA of a particular gene and *trans*-acting factors (transcription factors) that bind to *cis*-acting elements. Most of the transcriptional regulatory DNA sequences are in the 5′-flanking region of genes, although intronic (14) and 3′ control (87) also occur. The rate of transcription of target genes is mainly regulated by the rate of the formation of the transcription initiation complex (45), which binds to TATA-box and start site of target genes (Fig. 1). Promoters can include a TATA-box, a start site or both of these control elements. The formation of the transcriptional initiation complex starts with the initial binding of TATA-box binding protein (TBP) to the TATA-box. Hereafter, the transcription factors that bind to their *cis*-acting elements upstream form the TATA-box facilitate the forming of the transcriptional initiation complex. Specific transcription factors manipulate the formation of transcriptional initiation complex by their presence, abundance, affinities for their *cis*-acting elements, and physical interactions with other proteins. Transcription factors are cell-type and tissues specific and have expression patterns that are divers during the life of the cell. Presence of a certain transcriptional repressor in tissue specific cells will repress the transcriptional activation in this cell type, but not in other cell type that do not produce this repressor. Abundance of transcription factors can favor the transcriptional activation in a certain direction. When, a transcriptional activator is in higher abundance than a transcriptional repressor, the transcription will be overall directed towards the activation state, depending on affinity differences as well. The affinity of specific transcription factors for their cognate binding sites are defined by the specific sequences contained within the binding site and the configuration of the DNA-binding domains in transcription factors. The physical protein-protein interactions among transcription factors and other transcriptional modulators such as co-activators or co-repressors (72) provide an additional mechanism for control of transcription. The *cis/trans*-acting elements regulate the formation rate of the transcription initiation complex, which is required for the transcriptional activity of specific genes. These data show that transcriptional activation occurs through complex mechanisms whereby a large number of proteins each play a role in the transcription process. The transcriptional process is the rate limiting step in the presence and abundance of proteins.

The binding of transcription factors at *cis*-acting elements of the 5′-flanking regions of target genes, modulate the assembly rate of the transcriptional initiation complex (45). The effect of these transcription factors result in either higher assembly rate of transcriptional initiation complex, which results in higher transcriptional activity (activated transcription) or lower assembly rate of the transcriptional initiation complex, which results in a lower transcriptional activity (repressed transcription) (158).

Gene transcription/RNA processing

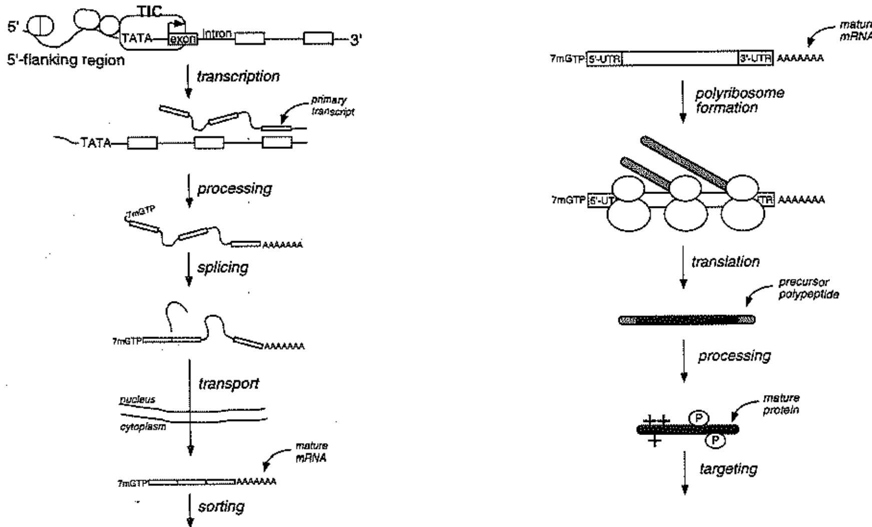


Figure 1: Schematic representation of the process of gene transcription and processing of RNA. Transcriptional initiation complex (TIC) is necessary for the basal transcription of genes. The circles represent transcription factors and co-factors that form protein complexes that regulate transcription. The interaction of proteins found in nucleus with each other and the DNA and their function in the transcription process is the main scoop of this thesis work. Courtesy of S.D. Krasinski.

The transcriptional activators once bound to their *cis*-elements facilitate assembly of the transcriptional initiation complex, either by direct contact with proteins that are part of the transcription machinery, or indirectly through the recruitment of coactivators that may facilitates strand separation and open complex formation (45). Transcriptional repressors once bound to their *cis*-elements can repress formation of the transcriptional initiation complex by several mechanisms, including direct interference with binding of transcriptional activators, or by direct interaction with the transcriptional initiation complex, analogous to how activators activate gene transcription. Also, transcriptional repressors cause repression by indirect mechanisms, like recruitment of corepressors that may cause strand tightening complex formation (45). In contrast to transcription factors that bind directly to their consensus binding sites at the DNA (*cis*-element), co-factors do not bind directly to the DNA, but generally form protein-protein complexes with *cis*-acting transcription factors. Cooperative regulation by multiple transcription factors that results in a transcription rate that is greater than the sum of the activation of each transcription factor individually is referred to as synergy. Cooperative regulation by multiple transcription factors that results in a transcription rate that is lower than the sum of the activation of each transcription factor individually is referred to as antagonism. Interactions between transcription factors and co-factors in their role in the regulation of gene transcription for specific intestinal genes will be the main focus of this thesis.

Although the abundance of proteins available in the cell is regulated mainly by the transcription process, other processes subsequent to transcription play a role in establishing the biologically active protein products. Newly transcribed RNA, named pre-mRNA, is processed and spliced into mature mRNA, which is released into the cytoplasm and is sorted to specific regions within the cell (Fig. 1). These processes include capping of the 5′ end of the pre-mRNA

with 7methylGTP (3) and addition of a poly A tail (24) to the 3′ end, both of which protect the mRNA in the cytoplasm. Splicing processes involve cutting the intronic regions and precisely fusing the exons in-frame (Fig. 1). The mature mRNA consists of three specific regions. The 5′-untranslated region (5′-UTR) has been suggested to play a role in the regulation of the rate of translation (78). The open reading frame is the translated portion of the mRNA. The 3′-UTR has been suggested to contain localizing (60,61) and stabilizing signals (78). The mRNA in the cytoplasm is than translated into precursor polypeptides by the ribosomes. Post-translational processing allow the cell to react to a large variety of extracellular stimuli and manipulate its gene expression repertoire accordingly (13). These post-translational processes include phosphorylation and glycosylation. Protein phosphorylation is the mechanism of choice for situations where rapid changes in transcriptional activity is required (18). Glycosylation is important in sorting and transport as well as peptide folding.

The final biologically active proteins and their role in the cell are determined by the abundance of their mRNAs and post-transcriptional processes. The pathway from transcription of target genes to the final processes that establish the biologically active proteins is shown in Fig. 1. The transcriptional process is the rate limiting step that regulates the abundance of proteins in the cell. It is this process that is the main focus of this thesis and is discussed below for intestine-specific genes.

1.3.1 Lactase-phlorizin hydrolase and sucrase-isomaltese.

Lactase-phlorizin hydrolase (LPH) and sucrase-isomaltese (SI) are both trans-membrane glycoproteins that protrude from the apical membrane of the absorptive enterocyte into the intestinal lumen. LPH is a beta-galactosidase responsible for the cleavage of the disaccharide lactose, which is the principle carbohydrate of mammalian milk, into its absorbable monosaccharides, glucose and galactose. LPH also shows specificity for phlorizin, a naturally occurring glucoside found in some mushrooms, bark and apple tree leaves (15). SI contains isomaltase and sucrase activities, and plays a key role in the final degradation of glycogen and starch (51).

LPH and SI are both expressed in absorptive enterocytes on villi demonstrating tissue-, cell-type, and differentiation-specific expression (64,142,157). The cells in the proliferative crypt compartment do not express either LPH (121) or SI (140). However, as the cells emerge from the crypts, and differentiate, the cells start to express LPH and SI. In adult mice, high levels of LPH and SI mRNA are expressed in the lower half of the villus with decreasing levels towards the tip of villi, in contrast to the respective proteins which are expressed throughout the entire villi (63,121,131). The hypothesis is that the cell since LPH and SI are stable and anchored at the cell membrane, it can "turn off" the transcription of LPH and SI genes. Although the expression patterns of LPH and SI show similarities along the crypt-villus axis, these proteins demonstrate differences along the horizontal and developmental gradients. This is exemplified in all mammals by the lactase specific activity which is high at birth and declines during weaning, whereas the sucrase specific activity is low at birth and increases rapidly to adult levels during weaning (63). These enzymatic changes coincide with the transition from a milk-based diet, in which the primary carbohydrate is lactose, to a diet of solid foods that contain α-dissaccharidases. The mechanism underlying this differential pattern of regulation is unknown.

1.3.2 Intestine-specific gene expression for lactase-phlorizin hydrolase (LPH) and sucrase-isomaltase (SI) coincides with critical transitions in intestinal differentiation and development.

LPH is expressed at the time of villus formation in fetal mice whereas SI expression is detectable but low at this time. At E18, the proximal intestine is characterized by the presence of primitive villi lined by an epithelial monolayer of columnar cells, whereas the distal intestine continues to exhibit a stratified multi-layered epithelium. LPH mRNA is first detected, at this age and is found in all of the epithelial cells lining the primitive villi (96,120) but cannot be detected at this age in the stratified multi-layered epithelium of the distal intestine. In contrast to the increasing expression of LPH, SI expression remains detectable but low at this age (96,120). Since LPH is first expressed at the time of villus formation in the developing gut, it is a good marker for this event during the intestinal development.

In contrast to the uniform expression of LPH mRNA in fetal intestine, expression in postnatal intestine undergoes a topographic redistribution. At E22 (day of birth) and immediately after birth, LPH mRNA becomes restricted to the lower half of the villi, whereas LPH proteins remain detectable along the length of the villi (Fig. 2) (120).

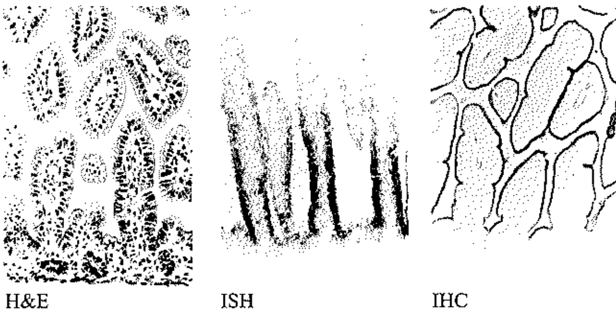


Fig. 2. LPH mRNA and immunoreactive protein expression in the jejunum of 7-day-old rats. H&E staining (left panel) shows the structure of the crypts and villi. In situ hybridization assays (ISH) demonstrate that LPH mRNA is expressed at the lower half of villi (middle panel). LPH protein, revealed by immunohistochemistry (ICH) is present, along the length of the villi (right panel). Courtesy of Edmond Rings.

This pattern does not appear to be a result of suckling, since prevention of suckling does not influence the distribution of LPH mRNA (120). The restriction to the lower half of the villi remains essentially unchanged at all subsequent ages, including adult animals. Neither LPH mRNA nor protein is detectable in crypts (120). Therefore, LPH is an excellent marker for intestinal development around birth and during suckling.

During the suckling/weaning transition, LPH and SI expression undergo redistribution that parallels changes in the dietary nutrients.

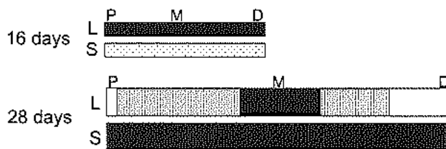


Fig. 3. Distribution of LPH(L) and SI (S) protein along the horizontal axis of small intestine in 16-, and 28-days-old rats (121). P, M, and D indicate proximal, middle, and distal small intestine. Bars represent intestines at relative length and growth. White segments represent no immunodetection. Light shaded segment represents low expression. Middle dark shaded segments represent patchy expression (approximately half of the cells express proteins) and dark shaded areas represent expression in all enterocytes.

The pre-weaning (16 days) expression of LPH is high throughout the small intestine, in contrast to the low expression of SI. As is shown in Fig. 3, during weaning and after weaning (28 old days), LPH expression per enterocyte is reduced and is also restricted to the jejunum and

proximal ileum (M) (63) but during weaning SI expression rapidly reaches adult levels throughout the entire small intestine (77,121). Also, SI mRNA is mainly found from crypt/villus junction to the mid of villus and lower expression from mid villus to tip of the villus in adult rat jejunum (140). These data demonstrate that the changes of LPH and SI expression to adult levels coincide with the final transition of intestine development during weaning. Therefore LPH and SI are excellent markers for final intestinal developmental differentiation process.

1.3.3. Intestine-specific gene expression for lactase-phlorizin hydrolase (LPH) and sucrase-isomaltase (SI) along the horizontal and developmental gradient coincides with the abundance of its respective mRNA and pre-RNA.

Lactase and sucrase specific enzyme activities correlate with their protein expression patterns and abundance of their mRNAs along the horizontal and developmental gradients suggesting fundamental control at the level of gene transcription. This lab was the first to show that the developmental pattern of lactase and sucrase activity in rats (16) and the genetic lactase pattern in humans (30) is coordinated with the abundance of its mRNAs (Fig.4), findings subsequently confirmed by this laboratory (31,63) and others (32,47,58,68,80). As shown in Fig. 4, the specific activity of lactase is high in the pre-weaning period (7 to 14 days) and restricted to the middle segment of small intestine after weaning (21 and 28 days), whereas sucrase activity is low before weaning (7 to 14 days), and increases just prior to weaning. In the adult, sucrase activity is isolated to the proximal and middle segments of the mouse small intestine.

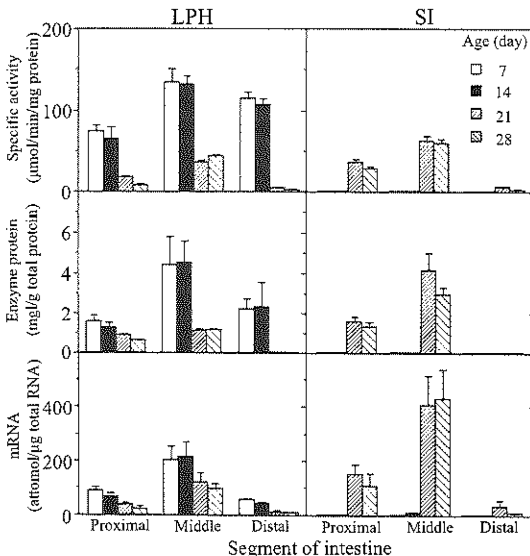


Fig.4. Quantitative determination of LPH and SI specific activity, protein, and mRNA in different intestinal segments during postnatal development (63). Proximal intestinal segments were taken from the proximal 1- to 2-cm of small intestine adjacent to the pylorus; middle segments were taken from the geometric center of the small intestine; and distal segments were taken from the distal 1- to 2-cm of small intestine adjacent to the ileocecal junction. Fragments were taken from 7, 14, 21, and 28 days old mice, respectively. Specific activity, protein and mRNA of LPH and SI determinations were carried out using activity tests, rocket immunoelectrophoresis, and RNase protection assays, respectively. Courtesy of S.D. Krasinski

These patterns of lactase and sucrase specific activity closely parallel the levels of LPH and SI and mRNA. In addition, Krasinski et. al. (63) demonstrated that the mRNA levels of LPH and SI are also closely correlated with the abundance of the pre-mRNAs. Pre-mRNA is the direct product of transcription of the LPH gene (Fig.1) and can be used as an indicator of transcription rate. These data demonstrate that the levels of protein correlate well with the abundance of their

respective mRNAs and pre-mRNAs demonstrating that the abundance of LPH and SI is regulated in gene transcription.

Developmental pattern of LPH during weaning in human demonstrates differential patterns in geographical different populations. Additionally in Fig. 4 is confirmed the horizontal and developmental expression of LPH and SI as previously described. The developmental decline of LPH at weaning in all mammals and the majority of humans is not regulated by the presence of lactose substrate, but rather is genetically "hard-wired" (94). The LPH decline occurs in humans at around age 5, although a minority of humans, mainly those from or related to societies of northern and western Europe (a traditional milk drinking population), continue to express LPH throughout adulthood. Since LPH and SI are expressed in tissue-, cell type-, and differentiation-specific manner and their expression along the horizontal and developmental gradient coincide with specific transitions in the intestinal development, LPH and SI genes are well established markers for these cell differentiation and intestine developmental processes.

1.3.4. The 5′-flanking regions contain important information for tissue-, cell type-, and differentiation-specific as well as the horizontal and developmental expression patterns.

The 5′-flanking region of the LPH gene regulates tissue-, cell type-, and differentiation-specific expression patterns as well as the horizontal and developmental expression patterns of LPH. Transgenic studies show that 2038 base pairs (bp) of 5′-flanking region of the rat LPH promoter directs reporter (human growth hormone) expression to absorptive enterocytes on small intestinal villi (64). Transgene expression was not detected in crypts, enteroendocriene (Fig 5), and goblet cells. These data demonstrate that information for enterocyte- and tissue-specific LPH gene expression is contained in the 2038 bp 5′-flanking region. Other transgenic studies using the pig and rat LPH 5′-flanking sequence (76,145) are consistent with these findings.

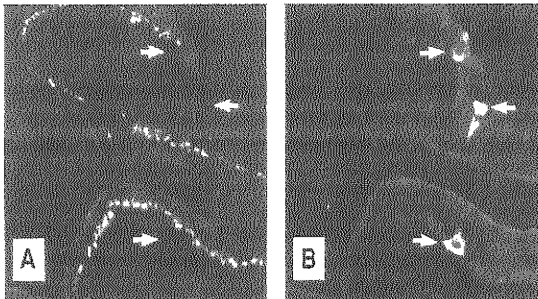


Fig 5. Cellular distribution of human growth hormone (hGH) in proximal segment of transgenic mouse small intestine. Dark-field exposure immunofluorescence microscopy revealing that hGH (A), visualized with FITC, is not present in (B) enteroendocrine cells (detected with Texas red). *Arrows* show serotonin-containing enteroendocrine cells. These data demonstrate that the transgene is not expressed in endocrine cells.

The transgene expression pattern along the horizontal axis was shifted proximal than that of the endogenous LPH expression. The developmental expression of the transgene was also ectopic. However, a report by Lee et. al. demonstrated in transgenic mice, that a 2.0 kb of the rat 5′-flanking sequence directed appropriate horizontal and developmental expression of the transgenic (76). In addition, a report by Troelsen et. al. (145) described that a 1038 bp region of the pig LPH 5′-flanking region directed correct horizontal and developmental LPH expression.

Transgene expression pattern along the horizontal and developmental gradient is also regulated by the SI 5′-flanking sequence. A construct containing bases -3424 to +54 of the human SI gene directed the appropriate intestine-specific transcription and differential expression along the crypt-villus axis (82). The horizontal expression pattern is regulated by additional DNA regulatory regions in the human SI gene. In addition, an evolutionarily

conserved region between human and mouse (nucleotides -210 to +54 of the mouse SI gene) directed the appropriate transcription to enterocytes in developmental and differentiation-dependent patterns that was parallel to that of the endogenous gene (152).

Together these data demonstrate that the LPH and SI (63,76,82,131,145) tissue-, cell type-, and differentiation-specific expression patterns as well as the horizontal and developmental expression patterns of LPH are regulated by their 5′-flanking region.

1.3.5. The proximal LPH and SI promoters both contain binding sites for intestinal transcription factors.

The effects of intestinal transcription factors on the regulation of LPH and SI gene expression will provide insights in differentiation process of intestinal enterocytes. Homology comparisons of the 5′-flanking sequence among the human, pig, mouse and rat LPH genes and the 5′-flanking sequence of human and mouse SI genes reveal conserved regions at putative binding sites for a number of intestinal transcription factors, including Cdx-2, GATA, and hepatocyte nuclear factor-1 (HNF-1) transcription factor families (Fig. 6).

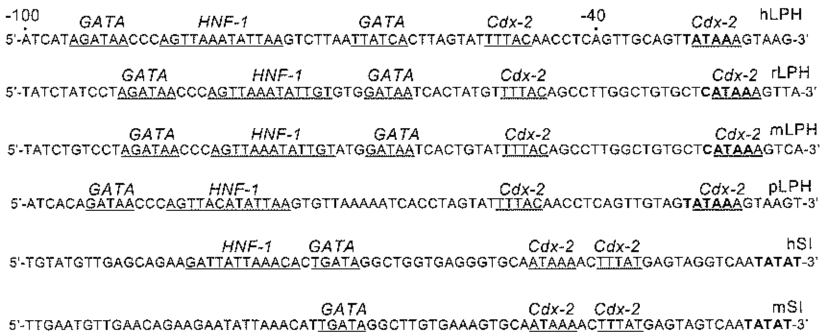


Fig. 6. The -100 to -20 bp region of the human (h), rat (r), mouse (m), pig (p) LPH and mouse and human SI 5′-flanking region sequences. These promoters contain conserved binding sites for GATA (A/TGATAA/G), HNF-1 (GTTAATNATTAAC), and Cdx-2 (TTTAT/C) (underlined). TATA-boxes are in *boldface* type. The mouse sequences were obtained from the genetic data base of the Celera Discovery system (Rockville, MD) (courtesy Dr. M. Fleming).

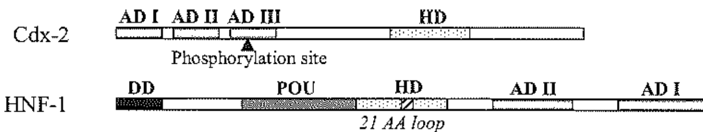
Since the cognate binding sites of these transcription factors are in close proximity to each other and to the TATA-box in the LPH and SI promoters, we hypothesize that these factors together might interact and play an important role in the regulation of LPH and SI gene expression. In addition, since the number and arrangement of these binding sites is specific to each gene (e.g. one of each Cdx-2 and HNF-1 binding sites in LPH, and two of each in the SI), it is further hypothesized that each of these factors individually as well as in cooperativity will demonstrate differential effects on each of the promoters.

1.4 Intestinal-specific transcription factors

1.4.1 Transcription factors Cdx-2, GATA-4, -5, and -6 and HNF-1α are only expressed together in the small intestinal epithelium.

Expression of numerous transcription factors identified in other tissue has been shown in the gastrointestinal tract. Binding sites for Cdx-2, HNF-1, and GATA factors were identified in the 5'-flanking region of rat and human LPH and human SI genes. Cdx-2, HNF-1 and GATA factors (Fig. 7) are expressed contiguously together only in intestinal epithelium. The intestinal expression of Friend of GATA (FOG) cofactors in unclear. These transcription factor families have all been shown to influence the regulation of cellular differentiation in intestinal as well as non-intestinal systems. These factors and cofactors have been separated in two groups, including members of homeodomain containing transcription factor family and members of transcription factor family containing zinc fingers. The role these factors play in the intestine-specific cellular development and gene expression is only beginning to be revealed.

Homeodomain proteins



Zinc finger proteins

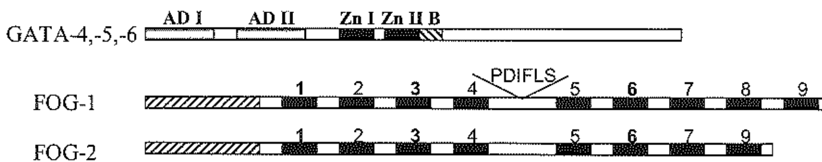


Figure 7. Schematic representation of the three major transcription factors and FOG-1and FOG-2 co-factor family used in this thesis report. Cdx-2 contains three N-terminal activation domains (AD) of which the third one at amino acid serine position 60 is a phosphorylation site, and a homeodomain (HD). HNF-1α contains a dimerazation domain (DD), POU-domain POU, homeodomain (HD) with an extra 21-AA loop, and two C-terminal activation domains, and the GATA-4, -5, and -6 subfamily of zinc finger proteins contain two N-terminal activation domains (AD) and two zinc fingers (Zn) and a basic region (B) near the C-terminus. FOG-1 contains an N-terminal active site, 9 zinc fingers, and a PDIFLS where FOG-2 only contains 8 zinc fingers.

1.4.2 Homeobox protein families.

The homeobox gene family is important for developmental pattern formation and organogenesis in multiple species (28). These proteins were also shown to bind to specific DNA-binding sequences within the regulatory regions of specific target genes and regulate their rate of transcription. The homeodomain contains three well-defined α-helices of which helices two and three form a helix-turn-helix motif that binds to the DNA of specific target genes. Walters et al. (153) showed that many homeobox genes are expressed in the adult human small intestinal mucosa. These data suggest that homeobox transcription factors may play a role in the regulation of intestinal development and transcription of specific genes. Two examples of homeodomain proteins are Cdx-2 and HNF-1α and are described further.

1.4.2.1 Cdx-2 transcription factor

Cdx-2 (Fig. 7), a member of the homeobox gene family related to *Drosophila* caudal, is necessary for embryonic development, and may play an important role in intestinal differentiation. Mice homozygous for the Cdx-2 null mutation fail to implant, and die between E 3.5 and E 5.5, whereas heterozygous knockout mice develop adenocarcinoma of the small intestine and colon (20). Cdx-2 is first detected at E 3.5 in extra-embryonic tissue (20). Within the embryo, Cdx-2 expression first occurs at E 8.5 in the posterior gut, tailbud, posterior region of the neural tube, and unsegmented paraxial mesoderm before the development of somites (20). In adult mammals, Cdx-2 is highly expressed in small intestine and colon (39,54,55,132) and is also detected in pancreas (127). In adult mouse small intestine, Cdx-2 is expressed in all epithelial cells in crypts and on villi, but only at low levels in Paneth cells at the bases of crypts (53,127). Stable transfection of Cdx-2 into IEC-6 cells, which are undifferentiated crypt-like cells that do not express Cdx-2, result in inhibition of cell growth, induction of marked morphological differentiation, and activation of intestine-specific gene expression (132). Together, these data suggest that Cdx-2 is important for implantation during early embryogenesis as well as development and maintenance of a differentiated intestinal epithelium.

As described above, Cdx-2 can modulate epithelial cell phenotype, the regulation of cellular growth as well as transcription of multiple genes expressed in the intestinal epithelium. The gene expression repertoire of cells is constantly modulated by a variety of extra-cellular stimuli. Protein phosphorylation is one of the posttranslational modifications of choice for situations where rapid change of transcriptional activity is required (119). Rings et al. (119) shown that the Cdx-2 activation domain is phosphorylated at position serine 60 (S60) via a mitogen-activated protein kinase (MAPK) pathway. S60-phophorylated and S60-non-phosphorylated Cdx-2 have different transactivating capacities and show different spatial expression patterns along the crypt-villus axis, suggesting distinct functions for these two forms as they relate to intestinal differentiation.

Originally shown to bind a conserved region in the sucrase-isomaltase (SI) gene called SI footprint 1 (SIF1) (131,157), Cdx-2 has now been demonstrated to bind and regulate the activity of the promoters of several intestinal genes, including SI (131,138), lactase-phlorizin hydrolase (LPH) (34,48,107,145,146), clusterin (133), calbindin-D9K (CaBP9K) (5,69), carbonic anhydrase 1 (CA1) (26,27), HOX C8 (138), the vitamin D receptor (160), guanylyl cyclase C (GC-C) (111), and human apolipoprotein B (73). Cdx-2 has also been shown to activate the glucagon promoter in a pancreatic islet cell line (70). Most of these genes contain the Cdx-2 binding consensus, TTTAY (Y is C or T), in a conserved position within the first 100 bp of the transcriptional initiation site in the 5´-flanking sequence. Localization to a position adjacent to the core promoter (i.e., TATA-box and transcriptional start site) suggests a critical role for Cdx-2 in the transcriptional regulation of gastrointestinal genes.

Recently, it has been suggested that Cdx-2 may modulate the activity of the promoters of certain intestinal genes through interactions at their TATA-boxes. Rat clusterin, a glycoprotein that has been associated with apoptosis, and rat and human CaBP9K, a mammalian intestinal vitamin-D-dependent Ca^{2+}-binding protein, both have been shown to bind Cdx-2 at their TATA-boxes using supershift and competition EMSAs (5,69,133). The rat clusterin and the rat and human CaBP9K promoters contain a Cdx-2 binding consensus sequence at their TATA-boxes. Cdx-2 may modulate transcription through this site as observed in transient co-transfection assays using intestinal and non-intestinal cell lines (5,69,133). Xu et al. (159) have demonstrated that Cdx-2 is capable of binding to its own TATA-box and repressing Cdx-2 expression. Thus, these studies suggest an alternative mechanism for Cdx-2 regulation of intestinal gene expression by interaction at the core promoter. Since mutations that disrupt Cdx-2 binding were not

introduced into this site, mechanism by which Cdx-2 regulates gene transcription through this site remains unclear.

The genes for rat and human LPH contain a well characterized Cdx-2 binding site located within a region called cis-element (CE)-LPH-1a (-54 to -40 bp) (12,34,146,147). Sequence analysis (Fig. 5) of the rat and human LPH genes also reveal Cdx-2 binding sites overlying the rat and human LPH TATA-boxes. Whether this binding site actually binds Cdx-2 or plays a role in the transcription of the LPH gene has yet not been determined but will be discussed in thesis.

Although it is widely known that Cdx-2 binds to CE-LPH1a (34,65,146,148), several reports have suggested that non-Cdx-2-proteins also bind to CE-LPH1a (12,48,88,108,146,147). Using EMSAs, several studies revealed the presence of non-Cdx-2 complexes interacting at CE-LPH1a, but did not report a function associated with this binding activity (12,34,48,108). Mitchelmore et. al. (88) suggested that a homeodomain protein, HOXC11, interacts at CE-LPH1a and potentiates HNF-1α-dependent transcription of the LPH promoter, but did not report direct protein binding to this site. Troelsen et al. (146) have shown that a specific mutation outside of the Cdx-2 consensus but within CE-LPH1a demonstrated a higher transcriptional activity than that of the wild type promoter suggesting the presence of a silencing element within CE-LPH1a. However, an alternative complex forming at CE-LPH1a by EMSAs was not reported. A focus of this thesis is linking binding of a non-Cdx-2-complex to CE-LPH1a with a function in the regulation of the human LPH promoter.

1.4.2.2 HNF-1 homeodomain transcription factor family

HNF-1 transcription factors (Fig. 7) contain a novel DNA-binding homeodomain distantly related to the POU domain and bind as dimers to the consensus sequence GTTAATNATTAAC (85,149). The homeodomain of HNF-1 is novel since it contains an insertion of 21 amino acids between two of the homeobox helices (28). Dimerization is stabilized by a cofactor of HNF-1 (DCoH) (46,86). Two members of the HNF-1 family, HNF-1α and HNF-1β, share highly homologous DNA binding domains but have divergent activation domains at the C-terminus (28) suggesting diverse regulation of target genes.

Two reports of mice lacking HNF-1α gave variable results (75,114), including decreased growth rate, sterility, liver enlargement, renal dysfunction, and non-insulin-dependent diabetes mellitus. These mice also exhibit a reduction in the expression of certain liver genes, such as albumin, α1-antitrypsin, phenylalanine hydroxylase, and liver fatty acid binding protein (L-FABP) (1,75,114). Indeed, oligonucleotide microchip expression analysis revealed alterations in the expression over 800 liver genes in HNF-1α knockout mice (126). The enlarged liver in these animals has been attributed to an accumulation of lipid due to decreased expression of the *l-fabp* gene (1). Despite alterations in other tissue, gastrointestinal structure was normal in these animals, although expression of the ileal sodium-dependent bile transporter was reduced (126). HNF-1β knockout mice have a disorganized visceral endoderm and die at E7.5 (4,21) and therefore do not provide any information about their role in intestinal differentiation.

Originally thought to be liver-specific, HNF-1α and HNF-1β are both expressed also in kidney, stomach, and small and large intestine (6,66,85). HNF-1β is also expressed in ovary and lung (141). In mice, HNF-1α mRNA is uniformly distributed in the stratified epithelium at E10.5 and in the epithelium of primitive gut tube at E15.5 suggesting a role in early intestinal morphogenesis (10). In mouse small intestine, HNF-1α and HNF-1β mRNAs are expressed at high levels in crypt cells, and expression is gradually reduced until there are low levels at the villus tips (125). Expression of HNF-1α mRNA in the crypt and lower villus is consistent with a role in the induction of cellular differentiation and intestinal gene expression along the vertical

axis. Although the pattern of HNF-1α mRNA has been well characterized, little is known regarding the pattern of expression of the HNF-1α protein and is discussed in this thesis.

Originally thought to be specific for the activation of liver genes (85), HNF-1α has now been shown to activate the promoter of several genes expressed in the intestine, including guanylin (49), aminopeptidase N (106), phosphoenolpyruvate carboxykinase (7), aldolase B (42), the ileal sodium-dependent bile acid transporter (126) the sodium-glucose cotransporter (SGLT1) (126), LPH (130), and SI (156). The pig LPH promoter is activated by HNF-1α through binding to an HNF-1 site (called CE-LPH2c) (130), which is conserved in position in the human (-90 to -70 bp) and rat (-86 to – 66 bp) LPH genes (Fig. 5). The human SI promoter has two HNF-1 binding sites, sucrase-isomaltase footprint 2 (SIF2) (-90 to -70) and SIF3 (-176 to -159 bp), that interact with both HNF-1α and HNF-1β (157). These two family members, which can form homodimers as well as heterodimers with each other, differentially activate both LPH and SI promoters (11,130,157). In addition, Mitchelmore et al. (89) demonstrated that HNF-1α physically associates with Cdx-2 to cooperatively activate the pig LPH promoter. HNF-1α also cooperates with SP1 to activate the SGLT1 promoter (84). Two recent reports have indicated that the homeobox transcription factor, HNF-1α, activates certain liver-specific genes by recruitment of coactivator proteins with intrinsic histone acetylase activity (112,129). Soutoglou et al (129) found that HNF-1α physically associates with the histone acetyltransferases (HATs) CREB-binding protein and p300/CBP-associated factor, and that the HNF-1α-recruited HAT activity was important for the transcriptional activation of a genome-integrated promoter containing HNF-1 binding sites. Using *hnf-1α* null mice, Parrizas et al (112) demonstrated that recruitment of HAT activity by HNF-1α is necessary for the activation of certain genes in vivo. They showed that although HNF-1α may occupy HNF-1 binding sites on promoters, recruitment of HAT activity is necessary for transcription. The results of these studies indicate that HNF-1α occupies target gene promoters in diverse tissues but plays an obligate role in transcriptional activation only in cellular- and promoter-specific contexts in which it is required to recruit HAT activity. The effect of HNF-1α on the human and rat LPH and human SI promoters in combination with other intestine-specific transcription factors has not been described thus far and is a focus of this thesis.

1.4.3. Zinc finger transcription factor and cofactor families

The zinc finger gene family is divers and have been described to be important for developmental of multiple organs in different species, including heart, muscle, gonads, and lung. Zinc finger regions are evolutionarily highly conserved domains and form a loop structure that can mediate binding to specific *cis*-acting elements and/or physical interaction with other proteins. Two zinc finger families are described below that are found to be expressed in the intestine and suggested to play a role in the intestinal development. Two groups are described further, GATA zinc finger family are transcription factors bind directly to DNA, whereas Friend of GATA (FOG) is a multiple zinc finger family of cofactors that mediate through protein-protein interaction.

1.4.3.1. GATA factors

The GATA family of transcription factors has been implicated in cell lineage differentiation, organ development, and cell-specific gene expression during vertebrate development (67,90,93,103,110,113,128,154). Six GATA transcription factors have been

described in vertebrates, each of which is defined by two evolutionarily conserved zinc fingers of the motif Cys-X_2-Cys-X_{17}-Cys-X_2-Cys (90)(Fig. 7) that mediate binding to the nucleotide sequence element (A/T)GATA(A/G). The GATA family is generally categorized into two subfamilies, GATA-1, -2, -3 and GATA-4, -5, -6 based on expression patterns and amino acid homologies. GATA-1, -2, -3 are expressed in developing blood cells and are critical for hematopoiesis (109), whereas GATA -4, -5, -6 have a more diverse pattern of expression and are found in small intestine, heart, liver, lung, and gonad where they play critical roles in regulating tissue-specific gene expression (2,59,71,101,102,134). Comparison of the deduced amino acid sequence of GATA-4, -5, -6 reveals a low level of identity with that of the GATA-1, -2, -3 subfamily (90). However, within the GATA-4, -5, -6 subfamily, a high level of sequence homology is present across the two zinc fingers and the adjacent C-terminal basic region (85-95%) (90).

Mice homozygous for the GATA-4 null allele die by E9.5 and lack both a primitive heart tube and foregut (67,91). GATA-5 knockout mice survive and reproduce despite pronounced genitourinary abnormalities in females (93), but show no gross abnormalities in intestinal structure or histology (intestinal gene expression was not reported). Interestingly, a GATA-5 null mutation in zebrafish resulted in embryonic lethality with an identical phenotype to that observed in GATA-4 null mice, suggesting a similar role for GATA-5 in zebrafish to that of GATA-4 in mice (117). GATA-6 null mice die before gastrulation (62,103) and therefore provide little information regarding intestinal development. Interestingly, in GATA-4 null mice, GATA-6 was up-regulated whereas in GATA-6 null mice, GATA-4 was down regulated, suggesting that expression of GATA-4 and -6 is dependent on each other (67,91,103). In *Drosophila* and *C. elegans*, GATA homologs have been implicated in gut development (37,118,128). These studies, however, demonstrate independent functions for individual GATAs, although functional redundancy among GATA subfamily members has been reported (105,143).

GATA-4, -5, -6 are expressed together only in cardiac tissue and small intestine, but individually, or in overlapping patterns in stomach, liver, lungs, spleen, ovary, testis, and bladder (2,59,71,101,102,134). Expression of all three intestinal GATA mRNAs is detected as early as E9.5 in the primitive gut and continues to be expressed in intestinal epithelium throughout embryogenesis (2,38,71,101,102). In both mouse (2) and chicken (38) intestine, GATA-4 transcripts are distributed in epithelial cells all along the villus, with increasing levels accumulating toward the villus tips. In chicken intestine (38), GATA-5 mRNA is localized to differentiated cells of the villi, whereas GATA-6 transcripts are localized to cells of the upper crypt and lower villus. The similarities and differences in the pattern of GATA-4, -5, -6 expression patterns suggest common and independent regulatory pathways for the control of intestine and cardiac gene expression.

GATA-4 regulates, independently and in cooperation with other proteins, multiple cardiac-restricted promoters including those from the cardiac troponin C (cTnC) (52), atrial natriuretic factor (ANF) (29,74,124), brain natriuretic peptide (BNP) (43), myosin heavy chain (MHC) (100), the rat troponin I promoter (TnI) (104), and carnitine palmitoyltransferase I β (CPT-Iβ) (98) genes as well as genes from nervous system A1 adenosine receptors (A1ARs) (122). Characterization of the cTnC promoter revealed that the C-terminal zinc finger and adjacent basic region of GATA-4 are necessary for DNA binding, and that the N-terminus contains two independent activation domains (92). Several transcription factors including Krüppel-like factor, SP1, Nkx2.5(29,74,124), nuclear factor of activated T cells 3 (NAFT3)(92), serum response element (SRE) (8) and a multifunctional zinc finger transcription factor (YY1) (9) can associate specifically with the C-terminal zinc fingers of GATA proteins resulting in modulation of transcriptional activation. GATA-4 and GATA-6 also are capable of physically interacting with each other in cardiac myocytes (19). GATA-4 is also involved in the regulation

of the Mullerian-inhibiting substance (MIS) gene (144). GATA-4 physically associates with nuclear receptor SF-1 which results in the synergistic activation of MIS promoter. GATA-5 has been suggested to be a potential transcriptional activator of the β-myosin heavy chain promoter (99) and to regulate the expression of Nkx2.5 which is expressed early in cardiac tissue. GATA-6 has been shown to play a specific role in gene expression in lung tissue (90). Interestingly, GATA-5, but not GATA-6 also physically associates with Nkx2.5 and together cooperatively activates the ANF promoter (29). These data demonstrate that GATA transcription factors physically interact with a number of other transcription factors, which result in modulation of the transcriptional activity of tissue-specific target genes.

Although the function of GATA-4, -5, -6 in cardiogenesis has been under intense investigation, only recently has the role of these factors in the control of intestinal gene expression been studied. Gao. et al. (38) showed that GATA-4, -5, and -6 can each activate the promoter for the Xenopus *i-fabp* gene. Fitzgerald et. al. (35) demonstrated a 15-fold induction of the human LPH promoter using a chicken GATA-6 expression vector. A report by Fang et al. (33) showed that all three GATA subfamily members are capable of activating the rat LPH promoter. The SI promoter has not been reported to be regulated by GATA factors, nor has interaction between GATA factors and other transcriptional regulators been defined.

1.4.3.2. Friend of GATA-1, and -2 (FOG-1,and -2) cofactors

Friend of GATA (FOG) is a recently described family of evolutionarily conserved multi-zinc finger transcriptional co-factors that bind to GATA factors and modulate GATA-specific activation of target genes during cellular differentiation. Two FOG cofactors have been described FOG-1 and FOG-2, based on their protein structure and expression patterns (137,139). FOG-2 contains eight zinc fingers (Fig. 7), of which four are of the C2H2 type and four of the C2HC type. By comparison, FOG-1 contains nine zinc fingers, with finger eight missing in FOG-2. This additional zinc finger is largely responsible for the difference in size between FOG-1 and FOG-2. The zinc fingers are the most conserved domains of FOG-1 and FOG-2. Like FOG-1, FOG-2 shares homology with the Drosophila zinc finger protein, U-shaped, which serves as a co-factor for the GATA factor, Pannier, (81) suggesting evolutionar conservation of a critical structure/function relationship.

FOG-1 is highly expressed in parallel with GATA-1 in erythroid and megakaryocytic cell lines and in spleen, liver and intestine (151). As with GATA-1, FOG-1 plays a critical roll in hematopoiesis. The targeted disruption of FOG-1 in mice leads to embryonic lethality, with mice dying of severe anemia between E11 and E12 (113,150). FOG-1 can either further activate (synergy) or repress the GATA-activation of target genes. FOG-1 also interacts with the transcriptional co-repressor C-terminal binding protein-2 (CtBP2), which may be recruited by FOG-1 to repress gene expression (50). However, FOG-1 can also have an effect on GATA-1 activation without CtBP2 (57). FOG-1 cofactors are mainly expressed and interact with GATA-1 transcription factors.

FOG-2 is more widely expressed then FOG-1, generally paralleling that of GATA-4. In mice, FOG-2 mRNA is first detected in developing heart and septum transversum at E8.5 followed by expression in neural epithelium and the urogenital ridge at E11.5 (81,137,139). At E15.5, expression is detected in the developing lung, kidney, and gut (139). In adult mice, FOG-2 is expressed highly in heart, brain, and testis, and at lower levels in lung, liver, and kidney (139). FOG-2 expression in adult small intestine has not been reported.

As with GATA-4, FOG-2 plays a critical role in cardiogenesis. Mice harboring a targeted mutation of FOG-2 die of congestive heart failure at E13 with a syndrome of tricuspid atresia.

These mice also demonstrate an absent tricuspid valve, a large atrial septum defect (ASD), a vertical septum defect (VSD), an elongated left ventricular outflow tract, rightward displacement of the aortic valve, and pulmonic stenosis. The molecular mechanism by which FOG-2 deficiency leads to endocardial defects is unclear (136).

FOG-2 can cooperate with GATA-4 to activate the MHC promoter in Cos-7 cells, whereas FOG-2 inhibits the GATA-4 activation of this promoter using other cell lines. The repressor mechanism is also shown in primary rat cardiomyocytes for the promoters of the ANF, BNP and cTnC genes (81,137,139). Interestingly, expression of FOG-2 repressed the synergistic activation of the ANF promoter by GATA-4 and Nkx2.5, reducing the level of activation to that of Nkx2.5 alone. These data demonstrate that FOG-2 represses the GATA-4-specific as well as cooperative activation of cardiac gene promoters.

FOG-2 function requires, in part, physical association with GATA-4 as well as specific structures that are independent of the protein-protein interaction domains. As with GATA-1/FOG-1 interactions (151), the N-terminal zinc-finger of GATA-4 is required for physical association with FOG-2 (137). Indeed, disruption of GATA-4/FOG-2 interactions through a targeted mutation in the N-terminal zinc finger of GATA-4 not only interferes with physical association and FOG-2-mediated repression in vitro, but also results in tricuspid atresia in vivo (22), demonstrating the importance of this physical interaction for cardiogenesis. Indeed, the n-terminal zinc finger of mammalian GATA factors is highly related to the N-terminal zinc finger of Drosophila GATA factor, Pannier, which has been shown to interact physically with a FOG-like protein, U-shaped (23,44), suggesting that GATA-FOG interactions have been conserved throughout evolution. Although FOG-2 consists of eight zinc-fingers, zinc fingers 1 and 6 are independently capable of physically associating with GATA-4 (135). As with FOG-1, FOG-2 also contains the conserved CtBP2 terminal domain, and is both necessary and sufficient to repress GATA-4-dependent transcription by FOG-2 (135). Although studies on the critical importance of FOG-2 for cardiogenesis are beginning to emerge, there are as yet no reports on the role of FOG-2 in intestine. The mechanisms by which FOG co-factors regulate the LPH gene expression will be revealed in this thesis.

1.5 Protein-protein association for the regulation of gene expression.

Protein-protein association mediated through homeodomain and zinc finger regions might be a mechanism to regulate the tissue and cell specific level of abundant gene expression. Synergy of a specific gene promoter is defined by a higher transcriptional activation by a combination of two or more transcription factors than the additive transcriptional activation of these transcription factors individually. $A = [F1 + F2]/([F1] + [F2])$ where A = total activation, [F1] = activation from transcription factor 1, [F2] = activation from transcription factor 2, and [F1 + F2] = activation from transcription factor 1 & 2 together. The ratio of the effects of both factors together over the additive effect of individual factors will predict interactions. If $A = 1$, then there is an additive effect; $A > 1$, synergistic; $A < 1$, antagonistic. The families of transcription factors are members of homeodomain and zinc finger families. Members of homeodomain and zinc finger families have both been evolutionarily conserved. Moreover, in *Drosophila*, interactions between the zinc finger of Ftz-F1 a member of the nuclear receptor super family and the homeodomain of the *fushi tarazu* (FTz) protein result in the cooperative activation of the *engrailed* gene (36,162). Therefore these data suggest that protein-protein interaction between zinc-fingers and homeodomains is an evolutionary conserved mechanism of gene regulation.

The physical interaction between HNF-1α and other proteins enables these proteins to form stable DNA/protein complexes which can facilitate gene transcription. HNF-1α has been reported to be necessary for claudin-2 expression in mice and was able to enhance claudin-2 promoter activity only in the presence of Cdx-2 (123). In addition as mentioned above, Cdx-2 and HNF-1α physically associate and cooperatively activate the pig LPH promoter (89). The cooperative activity between Cdx-2 and HNF-1α requires an HNF-1 binding site, but not Cdx-2 binding sites (89). Also, HNF-1α is capable of physically interacting with histone acetyltransferases (HATs), CREB-binding protein (CBP), p300/CBP-associated factor (P/CAF), Src-1, and RAC3 (129). HNF-1α may recruit multiple co-activators which activate transcription by coupling nucleosome modification and recruitment of the general transcription machinery. HNF-1α may therefore be capable mediating chromatin destabilization, which opens the DNA strang of target genes in order to enable the transcription machinery to transcribe the codons in this open-reading frame. Together, these data suggest that HNF-1α has the ability to physically associate with diverse proteins, which support nucleosome modification, necessary for gene transcription.

The GATA-4/Nkx2.5 interaction can cause cooperative activation at promoters of specific genes, whereas other promoters might be down-regulated by that complex. GATA-4 and Nkx2.5 physically associate through the C-terminal zinc finger and basic region of GATA-4 and homeodomain of Nkx2.5 causing a cooperative activation of atrium natriuretic factor (ANF) (29,74,124) and cardiac α-actin (αCA) promoters (124). Their models for cooperative activation of these promoters suggest that the physical interaction between GATA-4 and Nkx2.5 causes a conformational change in Nkx2.5 which opens its activation domains and DNA binding domain. These data show a functional co-localization of protein-protein and DNA-binding domains in the GATA-4 and Nkx2.5 transcription factors, suggesting a relative equal importance. Although, both transcription factors must bind to the DNA for cooperative activation of ANF promoter, the cooperative activation of αCA promoter requires only Nkx2.5 binding. However, in contrast to the cooperative activation of the ANF and αCA promoters, the combination of GATA-4 and Nkx2.5 causes a down regulation of the carnitine palmitoyltransferase-Iβ (CPT-Iβ) cardiac promoter (98). These data demonstrate that the same GATA-4 and Nkx2.5 complexes synergistically activate the ANF promoter but down regulate the CPT-Iβ activation, suggesting that the cooperative regulation of protein-protein complexes is regulated by promoter specific elements.

The Cdx-2, HNF-1 and GATA-4, -5, and -6 transcription factor families are only expressed together in the intestine and have individually demonstrated roles in the regulation of intestinal genes, including LPH and SI, transcription. These transcription factors might cooperatively regulate these intestinal genes. One report (89) describes the synergy between Cdx-2 and HNF-1α at the pig LPH promoter. Whether these three transcription factors act in concert to regulate the human LPH and SI genes, and the role of FOG-2 cofactor is unknown, and is a focus of this thesis.

1.6. SPECIFIC AIMS OF THE STUDY

As might be concluded from the general introduction about gene regulation, our understanding of how transcription factors regulate transcription of LPH and SI is extensive. Both LPH and SI are enterocyte specific membrane anchored enzymes that are necessary for the digestion of nutrients present in the specific diets of mammals according to their age before and after weaning. Previous work by Grand et. al. has demonstrated that LPH and SI are expressed in complex patterns along the vertical, horizontal, and developmental gradient of the small intestine. LPH and SI expression patterns coincide with important transitions during small intestine development. Furthermore, Krasinski et. al. demonstrated that specific regions in the LPH promoter contain the information necessary for the specific patterns of expression. Krasinski et. al also demonstrated that the vertical, horizontal, and developmental LPH and SI expression patterns are regulated at the level of transcription. Therefore, LPH and SI genes are perfect markers for cell differentiation of small intestine.

Seqeunce analysis of the 5´-flanking regions of LPH and SI genes revealed consensus binding sites for Cdx-2, HNF-1, and GATA transcription factors. The families of these transcription factors have all been indicated to play a role in cell differentiation and morphogenesis. These transcription factors are turned on in early stages of intestinal development, are only expressed together in the small intestine, and their binding sites are in close proximity to each other and the TATA-box regions suggesting that they play an important role in cell differentiation and intestine specific gene regulation. The close proximity of these binding sites to each other and the fact that these transcription factors have been shown individually to cooperate with comparable proteins for the regulation of gene expression, it is hypothesized that these factors act in concert to regulate LPH and SI transcription.

Therefore, the aims of this study were:

- To define the independent and cooperate function among Cdx-2, HNF-1, and GATA transcription factors in the regulation of LPH and SI gene expression.

- To define the importance of Cdx-2 and its binding sites in the LPH 5´-flanking region.
  A. Effect of a Cdx-2 consensus binding site overlapping the TATA-box of LPH gene.
  B. Effect of non-Cdx-2 protein binding at CE-LPH1a on the LPH gene expression.

- To define the mechanism of GATA-5 and HNF-1α functional synergy at the LPH promoter in vivo.

- To determine the mechanism of differential control of LPH transcription by individual members of the GATA transcription factor family

- To define the function of FOG-1 and 2 in regulating the GATA-mediated expression of the LPH gene in the absence and presence of HNF-1α.

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Chapter 2

Differential activation of intestinal gene promoters: Demonstration of functional interactions between GATA-5 and HNF-1α

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ABSTRACT

The effects of GATA-4, -5, and -6, HNF-1α and β, and Cdx-2 on the rat and human lactase-phlorizin hydrolase (LPH) and human sucrase-isomaltase (SI) promoters were studied using transient transfection assays in Caco-2 cells. GATA factors and HNF-1α were strong activators of the LPH promoters, whereas HNF-1α and Cdx-2 were strong activators of the SI promoter, although GATA factors were also necessary for maximal activation of the SI gene. Co-transfection of GATA-5 and HNF-1α together resulted in a higher activation of all three promoters than the sum of the activation by either factor alone, demonstrating functional co-operativity. In the human LPH promoter, an intact HNF-1 binding site was required for functional synergy. This report is the first to (a) demonstrate differential activations of the LPH and SI promoters by multiple transcription factors co-transfected singly and in combination, and (b) show that GATA and HNF-1 transcription factors co-operatively activate intestinal gene promoters. Synergistic activation is a mechanism by which higher levels of tissue-specific expression might be attained by overlapping expression of specific transcription factors.

INTRODUCTION

Absorptive enterocytes originate from undifferentiated, multipotent stem cells located near the base of small intestinal crypts (20). Through a continuous process of cell division, migration, differentiation, apoptosis, and exfoliation, these cells are renewed every 3-5 days. Absorptive enterocytes comprise over 95% of mucosal cells on small intestinal villi and are responsible for the terminal digestion and absorption of nutrients. To carry out these specialized functions, absorptive enterocytes acquire a differentiated phenotype characterized by the expression of functionally relevant proteins such as digestive enzymes, receptors, transporters, and cytoplasmic carriers. Differences in the panel of genes expressed along the proximal-to-distal (horizontal) axis result in regional differences in intestinal function. The mechanisms that underlie the process of intestinal differentiation are poorly understood.

Analysis of the effects of specific transcription factors in the regulation of absorptive enterocyte-specific differentiation can be studied by the use of marker genes whose expression parallels that of key events in the differentiation process. Lactase-phlorizin hydrolase (LPH) and sucrase-isomaltase (SI) are intestinal disaccharidases that are expressed in villus enterocytes and display a differentiation-specific pattern of expression. The LPH and SI genes are similarly expressed in jejunum and proximal ileum, but demonstrate different patterns during development. LPH gene expression is highest in newborn mammals and declines during weaning, whereas SI gene expression is low or undetectable at birth and increases to adult levels during weaning. In most humans, LPH expression declines, similar to that in other mammals, but in a subpopulation of humans, LPH expression remains high throughout adult life. Previous work in our laboratory have shown that the developmental pattern of lactase activity in rats (8) and the genetic pattern in humans (14) is correlated with the abundance of its mRNA, and also that the horizontal and developmental patterns of LPH expression in rats are transcriptionally regulated (32). It is now widely accepted that LPH expression is controlled mainly by the rate of gene transcription.

Studies in transgenic mice indicate that the 5'-flanking regions of both the LPH and SI genes direct transgene expression to villus epithelium (33, 40, 41, 64, 68). Analysis of published sequence reveals that the LPH (6, 71) and SI (9, 63, 73) genes contain GATA, HNF-1, and Cdx-2 consensus binding sites in their 5'-flanking regions (Fig. 1). These transcription factor families have all been shown to influence the regulation of cellular differentiation. Since GATA, HNF-1, and Cdx-2 factors are expressed contiguously only in intestinal epithelium, and since their cognate binding sites in the LPH and SI genes are adjacent to the TATA-box and to each other, we hypothesized that these transcription factors together are important regulators of LPH and SI gene expression.

The GATA family of zinc finger proteins, defined by a highly conserved DNA-binding domain that interacts specifically with DNA elements containing a consensus WGATAR sequence (W = A or T; R = A or G), has been implicated in cell lineage differentiation during vertebrate development. GATA-1, -2, and -3 are expressed in developing blood cells during hematopoiesis, whereas GATA-4, -5, and -6 are expressed together only in cardiac tissue and small intestine, but individually, or in overlapping patterns in stomach, liver, lungs, spleen, ovary, testis, and bladder (3, 29, 36, 50, 51). In both mouse (3) and chicken (18) intestine, GATA-4 transcripts are distributed all along the villus, with increasing levels accumulating toward the villus tips.

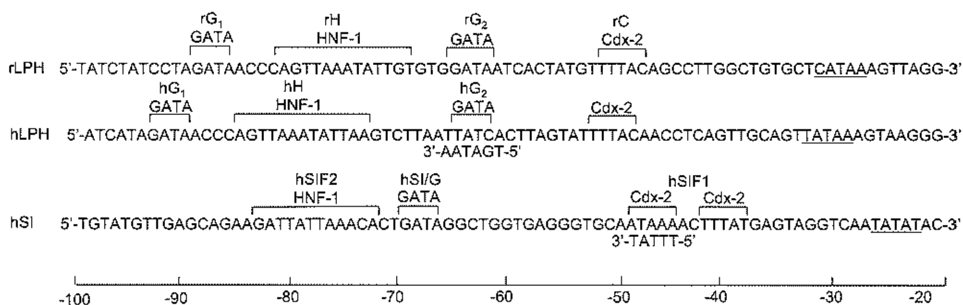

Fig. 1. The -100 to -20 bp regions of the rat LPH (rLPH), human LPH (hLPH), and human SI (hSI) 5'-flanking sequence contain binding sites for GATA (WGATAR; W = A or T, R = A or G), HNF-1 (GTTAATNATTAAC; N = A, C, G, or T), and Cdx-2 (TTTAY; Y = A or T) transcription factors (indicated in italics above the DNA sequence). In the human and rat LPH genes, rG_1 and hG_1 are the 5' GATA sites whereas rG_2 and hG_2 are the 3' GATA sites. The 3' GATA site in rLPH is indicated with a question mark (?) because it is not a true 6-base consensus. The hG_1 site is the lactase upstream element (LUE) characterized previously using GATA-6 (17). hSI/G refers to the GATA site in the human SI gene. rH and hH are the HNF-1 consensus sites that correspond to CE-LPH2c in the pig LPH gene (59). rC and hC are the CE-LPH1a sites that bind Cdx-2 (7, 16, 22, 53, 65, 66). The HNF-1 (hSIF2 & hSIF3) and Cdx-2 (hSIF1) binding sites in the human SI gene have been previously characterized (60, 72). The TATA-boxes are underlined.

In chicken intestine (18), GATA-5 mRNA is localized to differentiated cells of the villi, whereas GATA-6 transcripts are localized to cells of the upper crypt and lower villus. Mice homozygous for the GATA-4 (35, 48) or GATA-6 (31, 52) null allele die in utero and therefore provide little information regarding maintenance of intestinal differentiation in adults. GATA-5 knockout mice survive and reproduce despite pronounced genitourinary abnormalities in females (49), but show no gross abnormalities in intestinal structure or histology (intestinal gene expression was not reported). Recent studies have suggested that GATA-4, -5, and -6 may modulate the expression of intestinal genes (15, 17, 18). Gao et. al. (18) showed that GATA-4, -5, and -6 can stimulate directly the activity of the promoter for the Xenopus intestinal fatty acid binding protein (I-FABP) gene. Fitzgerald et. al. (17) demonstrated a 15-fold induction of a human LPH promoter-reporter construct using a chicken GATA-6 expression vector. Fang et. al. (15) showed that all three members of this subfamily are capable of activating the rat LPH promoter. Two potential GATA-binding sites are present in the rat and human LPH genes (Fig. 1), although the 3' site in the rat LPH gene contains only 5 of the 6-bp consensus. The human SI gene contains a single putative GATA motif within 100 bp 5' to the transcriptional start site, the characterization of which has not yet been reported.

HNF-1 transcription factors contain a novel DNA-binding homeodomain distantly related to the POU domain and bind as dimers to the consensus sequence, GTTAATNATTAAC (42, 67). Two members of this family, HNF-1α and HNF-1β, have been described and share highly homologous DNA binding domains, but divergent activation domains (43, 55). Originally thought to be liver-specific, these two proteins are also expressed in kidney, stomach, and small and large intestine (5, 34, 43). HNF-1β is also expressed in lung and ovary (43). In mouse small intestine, HNF-1α and HNF-1β mRNAs are expressed at high levels in crypt cells and expression is gradually reduced until there are low levels at the villus tips (58). Reports of mice lacking HNF-1α gave variable results (1, 38, 54) including sterility, liver enlargement, renal dysfunction, non-insulin-dependent diabetes mellitus, and reduction in the expression of certain liver genes such as albumin, α1-antitrypsin, phenylalanine hydroxylase, and liver fatty acid binding protein (L-FABP). The effect of HNF-1α gene disruption on gastrointestinal function

has not been described. HNF-1β knockout mice have a disorganized visceral endoderm and die at embryonic day 7.5 (4, 11). In addition to regulating the expression of liver genes, HNF-1 factors also modulate genes that are expressed only in small and/or large intestine, including LPH (59), SI (72), and guanylin (23). In the pig LPH gene, mutational analysis in cotransfection experiments localized an HNF-1α-mediated response to an HNF-1 binding site within 227 bp of the transcriptional start site (called CE-LPH2c) (59), which is conserved in position in the human (-90 to –70 bp) and rat (-86 to –66 bp) LPH genes (Fig. 1). The human SI promoter has two HNF-1 binding sites, SIF2 (-90 to -70 bp) and SIF3 (-176 to -159 bp), that interact with both HNF-1α and HNF-1β (72). These two family members, which can form homodimers as well as heterodimers, differentially activate both the LPH and SI promoters (59, 72) suggesting that the relative abundance of HNF-1 isoforms may be important for the regulation of genes with HNF-1 binding sites.

Cdx-2, a homeodomain-containing transcription factor that binds to the consensus, TTTAY (Y = A or C), is highly expressed in adult mammalian small intestine and colon, and is also detectable in pancreas (19, 25, 26, 60). In mouse small intestine, Cdx-2 is expressed in all epithelial cells in both crypts and on villi, with the exception of Paneth cells at the base of crypts (24). Mice heterozygous for a Cdx-2 null mutation develop adenocarcinoma of the small intestine and colon, whereas homozygote null mutant embryos die between 3.5 and 5.5 days post coitum (10). Cdx-2 has been shown to induce differentiation in IEC-6 cells, suggesting a role in cellular differentiation (61). Cdx-2 has also been shown to bind and activate the LPH (7, 16, 22, 53, 65, 66) and SI (60) genes via conserved cis-elements called CE-LPH1a and SIF1, respectively, both of which are adjacent to their TATA boxes (Fig. 1). CE-LPH1a contains a single binding site whereas SIF1 contains two adjacent sites on opposite strands. Additional Cdx-2 binding sites, CE-LPH1b (59) and CE-LPH-1c (47), have been identified in the pig LPH gene.

At present, the relative importance of GATA-4, -5, and -6, HNF-1α and β, and Cdx-2 on LPH and SI gene expression, and possible species differences between rat and human LPH gene expression have not been characterized together under the same experimental conditions. Further, possible interactions among these transcription factors have only recently been examined. Michelmore et al. (47) have demonstrated that HNF-1α and Cdx-2 physically interact and co-operatively activate the pig LPH promoter. In the present study, we demonstrate for the first time that GATA, HNF-1, and Cdx-2 transcription factors regulate the LPH and SI promoters differently, and that GATA-5 and HNF-1α synergistically activate these promoters.

MATERIAL AND METHODS

Chemicals and reagents. All chemicals were purchased from Sigma Chemical Company (St Louis, MO), Gibco-BRL (Gaithersburg, MD) or Fischer Scientific (Fair Lawn, NJ) unless indicated otherwise. Enzymes were purchased from Gibco-BRL, Promega Biotec (Madison, WI) or Pharmacia Biotech (Piscataway, NJ) unless indicated otherwise. Radioactive nucleotides and ^{14}C chloramphenicol were purchased from DuPont-New England Nuclear (Boston, MA). ^{35}S methionine (Redivue™) was purchased from Amersham Life Science (Arlington Heights, IL).

Plasmids. The human growth hormone gene was used as a reporter in transfection assays. As described previously (33), the human growth hormone gene was subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA). This construct, referred to as bluescript growth hormone

(BSGH), served as a promoterless control for background and the basic construct into which all promoter sequences were subcloned. Sequence from the rat LPH (rLPH) gene (-2038 to +15 bp) was isolated (71) and subcloned into BSGH 5' to the human growth hormone reporter as previously described (33). A segment of the human LPH (hLPH) gene (-1025 to +11 bp) was amplified by PCR from human DNA isolated from discarded jejunum of patients undergoing elective gastric bypass surgery. The 5' (5'-ATGGGTACCAAGATACTTATTATAGGAAGAGGA-3') and 3' (5'-ATCTAAGCTTTTTCTAGGAACTGTTAGGAGG-3') primers, designed from published sequence (6), contain *Kpn* I and *Hind* III sites, respectively (underlined), to facilitate subcloning into BSGH. A segment of the human SI (hSI) gene (-183 to +54 bp, gift of Dr. P. Traber, U. Penn.) (73) was also amplified (5'-ATGGGTACCTGACAGTACAATTACTAATTAAC-3 and 5'-ACGTAAGCTTAGCCTGTTCTCTTTGCTATG-3) and subcloned into BSGH. Deletions and mutations of all other rat LPH, human LPH, and human SI promoter-reporter plasmids were constructed by PCR using the same *Kpn* I-*Hind* III strategy. Mutations were introduced into primer and template oligonucleotides. All constructs were confirmed by sequencing.

The Rous sarcoma virus (RSV) promoter and the enhancer sequence within its long terminal repeat were fused to the chloramphenicol acetyltransferase (CAT) gene (called RSVCAT) and used to control for transfection efficiency (21). The RSV promoter without the enhancer sequence (21) was fused to the human growth hormone gene (called RSVdE) and used as a control promoter-reporter construct in co-transfection experiments. This promoter does not contain GATA, HNF-1, or Cdx-2 binding sites.

Previously characterized expression vectors for mouse GATA-4 (35), GATA-5 (51), and GATA-6 (50) (gifts of M. Parmacek, U. Penn.), mouse HNF-1α (34) and HNF-1β (43) (gifts of G. Crabtree, Stanford), and mouse Cdx-2 (60) (gift of P. Traber, U. Penn.) were employed in the present study. The GATA and Cdx-2 expression vectors were constructed from pcDNA3 (Invitrogen, Carlsbad, CA) and pRC-CMV (Invitrogen), respectively, both of which contain cytomegalovirus (CMV) promoters. The HNF-1 expression vectors were constructed from pBJ5, which contains the SRα (human T cell lymphotropic virus type 1) promoter. pRC-CMV (Invitrogen) served as a negative control expression vector for all co-transfection experiments. All expression vectors were authenticated by supershift EMSAs using extracts from transfected cells, as described below.

Cell culture and transfections. The Caco-2 cell line was the principal cell line used in the present study. Originally derived from a human colon adenocarcinoma, Caco-2 cells differentiate upon confluence exhibiting characteristics of small intestinal absorptive enterocytes, including a microvillus membrane and expression of small intestinal genes. The Caco-2 cells used in the present study have been previously characterized with respect to LPH and SI expression (69).

All cells were grown in Dulbecco's modified Eagle's medium (Bio Whittaker, Walkersville, MD) supplemented with 5 μg of penicillin-streptomycin per ml and containing 10% fetal calf serum. Cells at 80-95% confluence were collected by trypsinization, and aliquots were transfected using 10 μg of the promoter-reporter constructs, and, when indicated, 8.0 μg of expression vector and 1.0 μg of RSVCAT as a control for transfection efficiency. pBluescript II KS+ (Stratagene) was added as a carrier (total DNA/transfection=35 μg). Transfections were carried out by electroporation (Pulse Controller II, Bio-Rad, Hercules, CA) at 300 volts and 950 μF in cytomix buffer (240 mM KCl, 0.3 mM $CaCl_2$, 20 mM HPO_4/KH_2PO_4 pH 7.6, 50 mM HEPES pH 7.6, 4 mM EGTA pH 7.6, 10 mM $MgCl_2$) supplemented with fresh glutathione (3.08 mg/ml). After transfection, cells were plated at 75-90% confluence in 6-well plates. Media were

replaced after 24 h, and cells were harvested after 48 h (i.e., 24 h after the last media change). All plates were confluent at the time of harvest.

The amount of human growth hormone secreted into the media over a 24 h period was used as an indicator of transcriptional activity. The concentration of human growth hormone was measured using an ^{125}I immunoassay kit (Allegro hGH, Nichols Institute, San Juan Capistrano, CA). To analyze CAT activity, cell lysates were prepared by freeze-thaw cycling, and the protein concentration of the supernatant was determined by a Coomassie protein assay (Pierce, Rockford, IL). CAT activity was measured in cell lysates as described (56). Transcriptional activity was expressed as total human growth hormone secreted into the media over 24 h relative to total CAT activity in cell lysates (expressed as mg hGH/Unit CAT activity). The transcriptional activity of BSGH was subtracted from all other constructs to correct for background.

Nuclear extracts. Nuclear extracts were isolated from confluent cells grown on 10-cm plates directly (untransfected), or after transfection of specific expression vectors. For transfected cells, 8 μg of expression vector were transfected as described above, and two transfection reactions were combined on 10-cm dishes. Nuclear extracts were prepared by a modification of published methods (2). Cells were harvested by scraping, and resuspended in ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), incubated on ice for 10 min, and lysed by vortexing for 10 s. Nuclei were pelleted in a microcentrifuge for 10 s at full speed and extracted in 100 μl of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) for 20 min on ice. The resulting lysate was cleared of cellular debris by centrifugation for 15 min, and stored as a nuclear extract at -80°C. The protein concentration was determined as described by Kalb and Bernlohr (28)

In vitro transcription-translation. Labeled (^{35}S methionine) and unlabeled proteins were synthesized using a reticulocyte lysate transcription-translation system (TNT, Promega) according to the manufacturers instructions. Since all expression vectors contain a T7 DNA-dependent, RNA polymerase site between the viral promoter and the cDNA insert, a T7-based TNT system was employed. However, since in vitro transcription of the GATA-5 expression vector using a T7 polymerase transcription-translation system was inefficient, and since the GATA-5 coding region could not be amplified by PCR, a 1.5 kb *Sma* I-*Eco* RV fragment containing the GATA-5 coding region was subcloned into the *Sma* I site of pGEM-7Zf(-), and plasmids whose orientation enabled correct transcription from the SP6 polymerase site were isolated (screened by restriction digests, confirmed by sequencing). GATA-5 was efficiently synthesized from this plasmid using an SP6 TNT system.

EMSAs. Oligonucleotides used as probes and competitors in EMSAs are shown in Fig. 2. Probes were made by annealing a 10-fold molar excess of 10 or 11 base reverse strand oligonucleotide to the forward strand by boiling for 2 min in annealing buffer (100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA), then slow cooling for 2 h (< 32°C). The annealed oligonucleotides were extended with the large fragment of DNA polymerase I (Klenow) in the presence of ^{32}P dATP in extension buffer (50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT) for 1 h at 37°C.

Name	Sequence	Localization (bp)
Oligonucleotides containing GATA binding sites:		
xIFABP/G	5'-GGAGATCCCTGTAC**AGATAT**GGGGAGAC-3'	xIFABP, -57 to -30 (18)
rG_1	5'-TCTATCCT**AGATAA**CCCAGTTAAA-3'	rLPH, -98 to -75
rG_2	5'-TATTGTGT**GGATAA**TCACTATGTT-3'	rLPH, -74 to -51
hG_1	5'-GTGATCAT**AGATAA**CCCAGTTAAA-3'	hLPH, -103 to -80
hG_1m	5'-GTGATCAgctcgAACCCAGTTAAA-3'	---
hG_2	5'-ATACTAAG**TGATAA**TTAAGACTTA-3'	hLPH, -53 to -76
hG_2m	5'-ATACTAAGTGAgAATTAAGACTTA-3'	---
hSI/G	5'-TTAAACAC**TGATAG**GCTGGTGAGG-3'	hSI, -79 to -56
Oligonucleotides containing HNF-1 binding sites:		
rβ-Fib/H	5'-CAAACT**GTCAAATATTAAC**TAAAGGGAG-3'	rβ-Fib, -102 to -75 (12)
rH	5'-AACCCA**GTTAAATATTGTG**TG-3'	rLPH, -86 to -66
hH	5'-AACCCA**GTTAAATATTAAG**TC-3'	hLPH, -90 to -70
hHm	5'-AACCCAGgTAAATAgTAAGTC-3'	---
hSIF2	5'-GCAGAAG**ATTATTAAACACT**G-3'	hSI, -90 to -70
hSIF3	5'-AGTACA**ATTACTAATTAAC**TT-3'	hSI, -179 to -159
Oligonucleotides containing Cdx-2 binding sites:		
rC	5'-ATGT**TTTAC**AGCCTTGGCTGT-3'	rLPH, -55 to -35
hC	5'-TAT**TTTAC**AACCTCAGTTGC-3'	hLPH, -55 to -36
hSIF1	5'-GGGTGCA**ATAAA**AC**TTTAT**GAGTA-3'	hSI, -57 to -34
Oligonucleotide containing multiple binding sites:		
hG_1H	5'-GTGATCAT**AGATAA**CCCA**GTTAAATATTAAG**TC-3'	hLPH, -103 to -70

Fig. 2. Oligonucleotides used as probes and competitors in EMSAs. The names used in the present study, specific sequences, and localization in their respective genes are indicated. Binding sites are in bold. An "m" indicates a mutation introduced into the binding site and the mutation itself is shown in lower case letters. xIFABP/G and rβ-Fib/H are previously characterized probes for binding of GATA (18) and HNF-1 (12) factors, respectively.

After removing an aliquot for specific activity determinations by TCA precipitation, the labeled probe was separated from the short antisense oligonucleotides using a spin column with a 12 base cutoff (STE Select-D G-25, 5 Prime-3 Prime, Inc, Boulder CO). Purified probes were stored at –20°C. The specific activity of all probes exceeded 10^7 cpm/pmol. Competitors were synthesized by annealing equimolar amounts of full-length sense and antisense oligonucleotides as described above.

EMSAs were carried out using 10,000 cpm of probe, 5 μg of protein from nuclear extracts, 1.0 μg poly dI:dC-poly dI:dC, and 2 μg bovine serum albumin in EMSA reaction buffer (25 mM Tris, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 8% glycerol) unless indicated otherwise. These conditions resulted in maximal specific binding and minimal non-specific binding as determined by competition EMSAs and supershifts. After a 20 min incubation at room temperature, the reaction was run on a 5% non-denaturing polyacrylamide gel at constant voltage for 1-2 h. The gel was dried and exposed to film. Competitors and antibodies were preincubated with the nuclear extract for 10 min prior to the addition of the probe. The Cdx-2 antibody was a gift from Dr. D. Silberg (University of Pennsylvania) and was previously

characterized (60). All other antibodies were purchased from Santa Cruz Biochemicals (Santa Cruz, CA) as the concentrated gel shift stock.

RT-PCR. RNA was isolated from Caco-2 cells grown on 6-well plates using TRIzol Reagent (Gibco-BRL) according to the manufacturers instructions. To synthesize first strand cDNA, purified RNA was annealed to oligo dT and extended with Superscript II (Gibco-BRL) according to the manufacturers instructions. PCR was carried out on the first strand cDNA using the following oligonucleotides: human LPH (5'-GTCCCACTACCGTTTTTCCA-3', 5'-CGTCTGTGGTAGGTCCCAGT-3'), human SI (5'-CTCTCCATCGGTCTTTCCAA-3', 5'-AGAAGGCTCTGGGAGGTGTT-3'), human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5'-GGGTCATCATCTCTGCCCCCTCTG-3', 5'-CCATCCACAGTCTTCTGGTGGCA-3'), and mouse GATA-5 (5'-GCCTCTTCTCCCACTCTCCT-3', 5'-GTAGGACCCCACTGAGACCA-3'). After an initial melting step at 95°C for 2 min, the oligonucleotides were annealed to the cDNA template at 55°C for 40 s, extended at 72°C for 60 s, and melted at 95°C for 20 s. After 30 cycles, the reaction was extended for 10 min at 72°C, then cooled to 4°C. The amplification products were analyzed on 1.8% agarose gels stained with ethidium bromide.

Statistics. The t test or the one-way analysis of variance (ANOVA) was employed in all statistical analyses using InStat software (Graphpad Software, Inc., San Diego, CA). The Dunnett's or Tukey multiple comparison test was employed for all statistically significant ANOVA analyses.

RESULTS

Characterization of individual effects of transcription factors on the LPH and SI promoters. The individual effects of multiple transcription factors on the LPH and SI promoters have not been compared under the same experimental conditions. Thus, transient co-transfection assays in Caco-2 cells were carried out using individual expression vectors for each of the transcription factors under study. As shown in Fig. 3A, GATA factors are capable of activating the LPH and SI promoters. Transcriptional activities of promoter-reporter constructs were increased when co-transfected with expression vectors for GATA-4, GATA-5, or GATA-6 as compared to that with pRC-CMV, a plasmid containing only the CMV promoter. GATA-stimulated transcriptional activities were higher for constructs that contain consensus GATA binding motifs in their promoters (i.e., rLPH108, rLPH2038, hLPH118, hLPH1025, and hSI183). Activation was generally strongest with the GATA-5 expression vector demonstrating statistically significant increases from pRC-CMV controls for rLPH108, rLPH2038, hLPH118, and hSI183 (see Fig. 3A for P-values). Transcriptional activities of the basal promoter constructs, rLPH37 and hLPH37, and the negative control promoter-reporter construct, RSVdE, co-transfected with the GATA expression vectors revealed minimal responses, although all were significantly increased over the pRC-CMV controls with GATA-5 co-transfection ($P < 0.01$ for each). These data demonstrate that GATA-family transcription factors activate the LPH and SI promoters, consistent with previous reports for LPH (15, 17). Activation of the SI promoter by GATA-5 has not been previously reported.

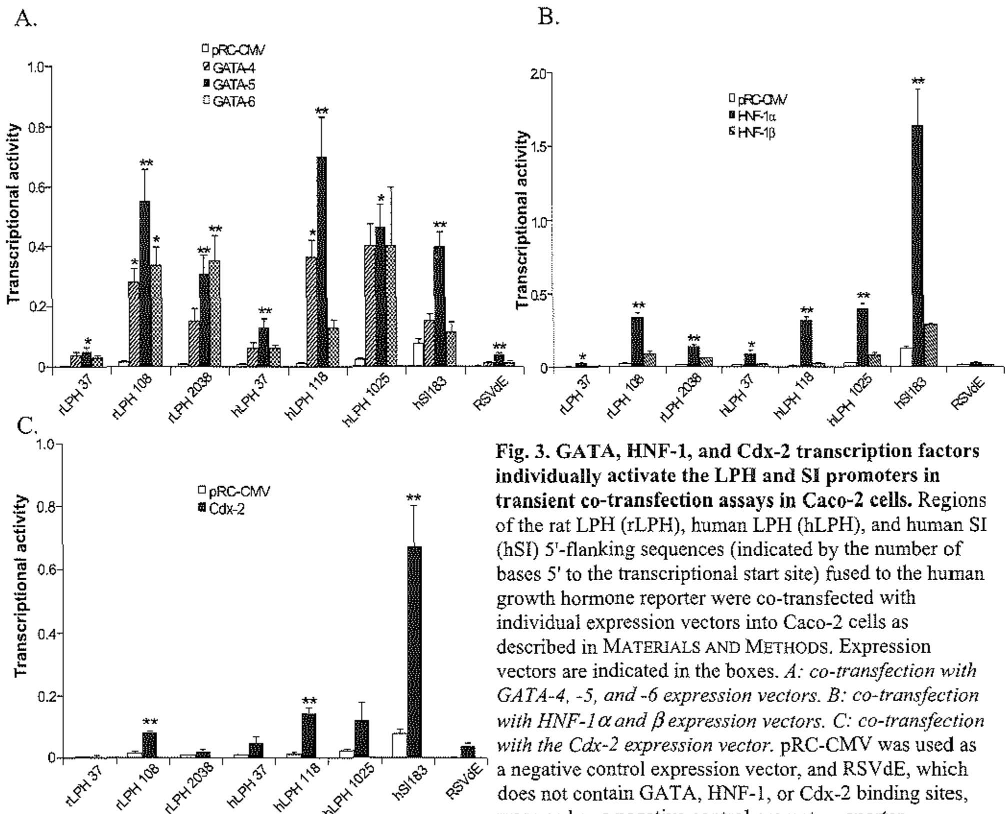

Fig. 3. GATA, HNF-1, and Cdx-2 transcription factors individually activate the LPH and SI promoters in transient co-transfection assays in Caco-2 cells. Regions of the rat LPH (rLPH), human LPH (hLPH), and human SI (hSI) 5'-flanking sequences (indicated by the number of bases 5' to the transcriptional start site) fused to the human growth hormone reporter were co-transfected with individual expression vectors into Caco-2 cells as described in MATERIALS AND METHODS. Expression vectors are indicated in the boxes. *A: co-transfection with GATA-4, -5, and -6 expression vectors. B: co-transfection with HNF-1α and β expression vectors. C: co-transfection with the Cdx-2 expression vector.* pRC-CMV was used as a negative control expression vector, and RSVdE, which does not contain GATA, HNF-1, or Cdx-2 binding sites, was used as a negative control promoter-reporter construct. Transcriptional activity (mean ± SEM, n = 4) is expressed as total human growth hormone secreted into the media over 24 h relative to total CAT activity in cell lysates per well (mg hGH/Unit CAT activity). Significant differences from the pRC-CMV controls are indicated: *P < 0.05, **P < 0.01.

HNF-1α was also capable of activating the LPH and SI promoters (Fig. 3B). For all constructs that contain HNF-1 binding motifs in their promoters (i.e., hLPH108, rLPH2038, hLPH118, hLPH1025 and hSI183), mean transcriptional activities with HNF-1α co-transfection were significantly greater than that with pRC-CMV (P < 0.01 for each). The hLPH37 and rLPH37 constructs were also significantly increased with HNF-1α co-transfection (P < 0.05). The transcriptional activities after HNF-1β co-transfection were not significantly greater than the unstimulated controls, although mean values were always higher. These findings are consistent with previous reports that demonstrate activation of both the LPH and SI promoters by HNF-1α (46, 59, 72)

Cdx-2 was also capable of activating the LPH and SI promoters (Fig. 3C). The transcriptional activities of the rLPH108 (P < 0.001), hLPH118 (P < 0.001), and hSI183 (P < 0.01) constructs, all of which contain Cdx-2 binding sites, were significantly increased from the pRC-CMV controls when the Cdx-2 expression vector was co-transfected. Activation of the LPH and SI promoters by Cdx-2 has been well characterized (16, 60, 65).

Differences among the rat and human LPH and human SI promoters were determined by comparing the transcriptional responses (fold change over baseline) for rLPH108, hLPH118, and hSI183 (Table 1).

Expression vector	rLPH108	hLPH118	hSI183
GATA-4	18.6 ± 3.1*	28 ± 4.4‡	2 ± 0.3*‡
GATA-5	36.5 ± 7.1*	53.7 ± 10.2†	5.3 ± 0.7*†
GATA-6	22.4 ± 4.2*†	10 ± 2*	1.6 ± 0.5†
HNF-1α	16 ± 1.3*	31.4 ± 2.6*†	13.5 ± 2†
HNF-1β	3.2 ± 0.8	2.4 ± 0.6	2.4 ± 0.1
Cdx-2	5.3 ± 0.5	10.9 ± 1.4	9 ± 1.7

Table 1. Transcriptional responses of LPH and SI promoter-reporter constructs co-transfected with expression vectors in Caco-2 cells. Values are mean (± SEM) transcriptional response (n = 4), expressed as fold change over baseline (transcriptional activity$_{expression\ vector}$/transcriptional activity$_{pRC\text{-}CMV}$). Transcriptional responses of rLPH108, hLPH118, and hSI183 to each of the expression vectors were compared.

Values with the same symbol in each row are significantly different; *P<0.05, †P<0.01, ‡P<0.001.

These constructs were chosen because they contain all of the GATA, HNF-1, and Cdx-2 binding sites under study, but little additional sequence. With GATA-4 and GATA-5 co-transfection, the transcriptional responses of the rLPH108 and hLPH118 were each significantly greater than that of hSI183 (see Table 1 for P values). With GATA-6 co-transfection, the transcriptional response for rLPH108 was significantly greater than either hLPH118 ($P < 0.05$) or hSI183 ($P < 0.01$). With HNF-1α co-transfection, the transcriptional response for hLPH118 was significantly greater than either rLPH108 ($P < 0.05$) or hSI183 ($P < 0.01$). Data from this table demonstrate that the three promoters respond differently to individual transcription factors.

To characterize binding site affinities, competition EMSAs were carried out using test probes for GATA (xIFABP/G), HNF-1 (rβ-Fib/H), and Cdx-2 (hSIF1), and titrations of specific competitors for each of the binding sites under study (Fig. 4). Competition for GATA-5 (Fig. 4A) was apparent and similar for the 5' GATA sites in the rat and human LPH gene (rG_1 and hG_1, respectively), the 3' GATA site in the human LPH gene (hG_2), and the GATA site in the human SI promoter (hSI/G). The 3' GATA site in the rat LPH gene (rG_2), which contains only 5 of the 6-base consensus, demonstrated minimal competition (lanes 7-9) similar to that of the non-specific control (lanes 22-24). The specific competitor (lanes 19-21), xIFABP/G, showed the most efficient competition, even though this sequence also contains only 5 of the 6-base consensus (although different from that of rG_2).

Competition for HNF-1α (Fig. 4B) and Cdx-2 (Fig. 4C) transcription factors were highest using binding sites from the human SI promoter (hSIF3 and hSIF1, respectively) as compared to the HNF-1 (rH and hH) and Cdx-2 (rC and hC) binding sites in the LPH promoters. Comparison of the two HNF-1 binding sites in the SI promoter (hSIF2 and hSIF3) demonstrated that HNF-1 has a higher affinity for hSIF3 than hSIF2, as previously reported (72).

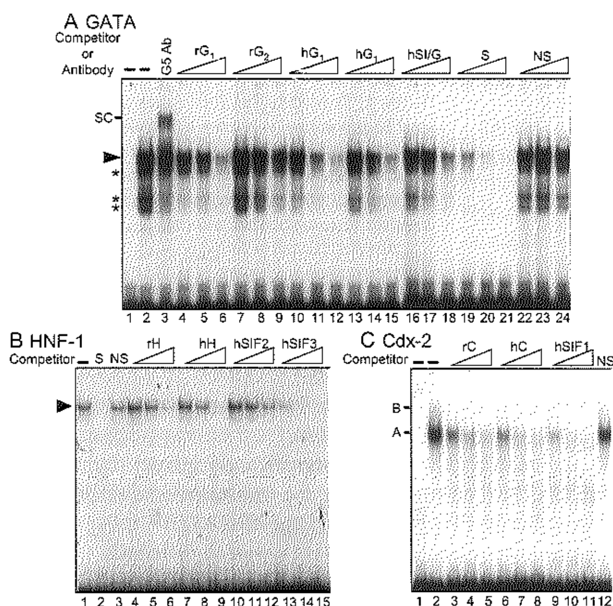

Fig. 4. GATA, HNF-1, and Cdx-2 binding sites in the rat LPH, human LPH, and human SI genes demonstrate different affinities in protein-DNA interactions. *A:* using xIFABP/G as a test probe for GATA binding (lane 1) and reticulocyte lysates (5 μl) enriched in mouse GATA-5 (TNT, Promega) (lanes 2-24), competition EMSAs were carried out using 20-, 50-, and 200-fold molar excess of double-stranded oligonucleotides containing the putative GATA sites under study (lanes 4-18). Specific (S) and non-specific (NS) titrations were carried out with xIFABP/G (lanes 19-21) and hSIF2 (lanes 22-24), respectively, for comparison. A supershiftcomplex (SC) with a GATA-5 antibody (lane 3) demonstrated that the main complex (arrowhead) is authentic GATA-5. Other complexes, which also specifically competed and supershifted, are indicated with an asterisk (*). *B:* using the rβ-Fib/H oligonucleotide as a test probe for HNF-1 binding and nuclear extracts (5 μg) from Hep-G2 cells, which are enriched in HNF-1, competition of the HNF-1 complex (arrowhead, lane 1) was carried out using 10-, 50-, and 200-fold molar excess of oligonucleotides containing the HNF-1 binding sites under study (lanes 4-15). The specific (S) and non-specific (NS) competitors (rβ-Fib/H and hSIF1, respectively) were added at 200-fold molar excess. *C:* using the hSIF1 site as a test probe (lane 1) and nuclear extracts from Colo-DM cells (lanes 2-12), which are enriched in Cdx-2, competition of the Cdx-2 complexes A and B (lane 2) were carried out using 20-, 100-, and 500-fold molar excess of competitors (lanes 3-11). The non-specific competitor (NS) was hSIF3 and was added at a 500-fold molar excess (lane 12).

Combined effects of transcription factors on LPH and SI expression. To characterize the combined effects of multiple transcription factors, and to identify possible functional interactions among transcription factor families, co-transfection experiments were carried out using all possible combinations of GATA-5, HNF-1α, and Cdx-2. These transcription factors were chosen because they were either strong activators (GATA-5 and HNF-1α) or the only member (Cdx-2) of the transcription factor families under study. Further, all are expressed on villi coincident with LPH and SI expression. As shown in Fig. 5A, the individual effects of HNF-1α and Cdx-2 produced significantly greater transcriptional activities for hSI183 than for rLPH108 and hLPH118. The combination of GATA-5 plus HNF-1α resulted in mean transcriptional activities that were similar for all three promoter constructs. The combinations of GATA-5 plus Cdx-2, HNF-1α plus Cdx-2, and all three transcription factors together resulted in mean transcriptional activities that were significantly greater for hSI183 than for either of the LPH promoter-reporter constructs ($P < 0.05$ for each). GATA-5 plus HNF-1α produced the highest activation of all combinations for rLPH108 and hLPH118, whereas all three expression vectors together produced the greatest response for hSI183.

Additive, synergistic, or antagonistic interactions among transcription factors were identified by comparing the effect of multiple expression vectors co-transfected together to the additive effect of each of the expression vectors co-transfected singly. This relationship, termed interaction response, is depicted as the logarithm of the ratio of the combined over the added

individual effects. Values of -0.1 to 0.1 were defined as additive effects, greater than 0.1, synergistic effects, and less than -0.1, antagonistic effects. As shown in Fig. 5B, the combination of GATA-5 plus HNF-1α demonstrated synergistic responses for all three promoters with hLPH118 having the highest mean interaction response (interaction response of 0.67, which corresponds to a 5-fold increase of combination over added individual activities).

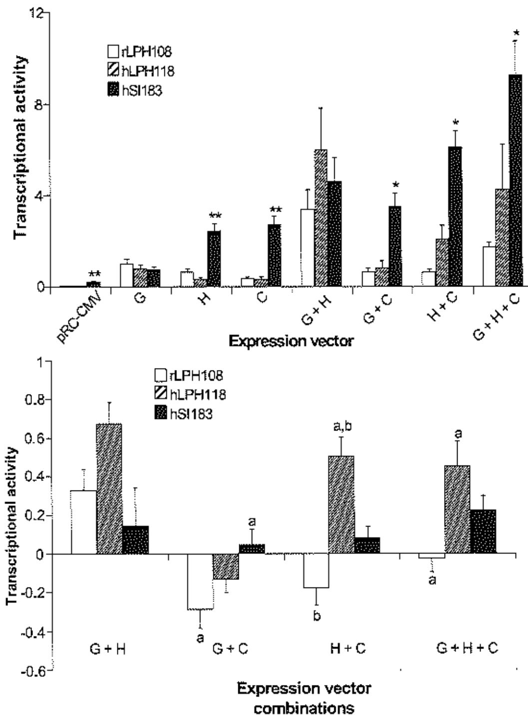

Fig. 5. Combinations of expression vectors reveal differential activation of promoters and functional interactions among transcription factors. *A:* transcriptional activities (mean ± SEM, n = 5) after co-transfection of all possible combinations of GATA-5 (G), HNF-1α (H), and Cdx-2 (C) expression vectors were compared among rLPH108, hLPH118, and hSI183. Unstimulated expression (pRC-CMV) is indicated as a control. Significant differences from rLPH108 and hLPH118 are indicated: *$P < 0.05$, **$P < 0.01$. *B:* interactions among transcription factors were characterized by comparing the activity of multiple expression vectors co-transfected together to that of individual expression vectors co-transfected singly and added together. This relationship, termed interaction response, is depicted as the mean (± SEM, n = 5) logarithm of the ratio of the combined over the added individual effects (e. g., log [(GATA-5 + HNF-1α)/((GATA-5) + (HNF-1α))]). Values of -0.1 to 0.1 were defined as additive effects, greater than 0.1 synergistic effects, and less than -0.1 antagonistic effects. Interaction responses for each combination of expression vectors were compared among rLPH108, hLPH118, and hSI183. Bars with the same letter within each expression vector treatment group were significantly different: [a]$P < 0.05$, [b]$P < 0.001$.

The combination of GATA-5 plus Cdx-2 was antagonistic for the LPH promoters and additive for the hSI183 construct, the difference between rLPH108 and hSI183 being statistically significant ($P < 0.05$). The rat and human LPH promoters responded differently to the combination of HNF-1α plus Cdx-2 as well as the combination of all three expression vectors together. The rLPH108 construct demonstrated additive or antagonistic responses, whereas the hLPH118 construct demonstrated synergistic responses (see Fig. 5B for P values). The combination of HNF-1α plus Cdx-2 was additive for hSI183 whereas the combination of all three transcription factors together demonstrated synergistic interactions on this promoter. These data demonstrate that interactions among transcription factors are determined, in part, by the configuration of the binding sites in the promoters under study.

The effect of combinations of transcription factors on endogenous expression of the LPH and SI genes were characterized in Caco-2 cells using RT-PCR (Fig. 6).

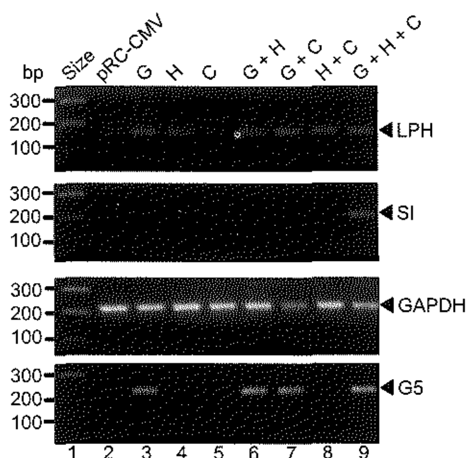

Fig. 6. GATA-5, HNF-1α, and Cdx-2 activate endogenous LPH and SI gene expression in Caco-2 cells. RT-PCR was carried out on RNA isolated from Caco-2 cells transfected with all combinations of the GATA-5 (G), HNF-1α (H), and Cdx-2 (C) expression vectors as described in MATERIALS AND METHODS. Amplification of endogenous human LPH and SI mRNAs are indicated by 166 and 201 bp fragments, respectively. Amplification of GAPDH mRNA (208 bp fragment) was used as a positive control for all RNAs and that of mouse GATA-5 mRNA (G5, 226 bp fragment) was used as positive control for transcription of the GATA-5 expression vector. A size marker (lane1) was included. pRC-CMV was used as the negative control expression vector (lane 2).

An amplified product corresponding to LPH mRNA was identified in all lanes. The intensity of this product on agarose gels was similar between baseline (pRC-CMV-transfected, lane 2) and the Cdx-2-transfected cells (lane 5), and increased when either GATA-5 or HNF-1α expression vectors were transfected (lanes 3, 4, 6-9). However, there was no evidence for synergistic enhancement of LPH mRNA abundance by any combination of transcription factors. An amplified product reflecting SI mRNA abundance was identified only in the lane corresponding to expression of all three transcription factors (lane 9). These data demonstrate that GATA, HNF-1, and Cdx-2 transcription factors differentially modulate the endogenous expression of the LPH and SI genes.

Cell-specific activation of the human LPH promoter by GATA-5. To determine if the GATA-5 response is cell-specific, co-transfection experiments with the GATA-5 expression vector and the hLPH118 construct were carried out in different cell lines. Table 2 shows a wide range of transcriptional responses (fold change over baseline) in both intestinal (T-84, Caco-2, HT-29, Colo-DM) and non-intestinal (Hep-G2, MDCK, HeLa, 3T3) cell lines. The highest responses were found in T-84, Caco-2, (both colonic adenocarcinoma), Hep-G2 (hepatocellular carcinoma), and MDCK (kidney epithelial) cells, whereas the lowest responses were observed in HeLa (cervical carcinoma), Colo-DM (adenocarcinoma of the colon), and 3T3 (fibroblast) cells. Parallel results were found for rLPH108 and hSI183 (not shown). EMSAs using a probe specific for HNF-1 or Cdx-2 binding (Fig. 7) revealed a close correlation between the presence of HNF-1 binding activity and GATA-5

Cell line	Transcriptional response
Intestinal cells	
T84	109 ± 7
Caco-2	84 ± 20
HT-29	19 ± 6
COLO-DM	5 ± 2
Non-intestinal cells	
HepG2	95 ± 26
MDCK	62 ± 13
HeLa	9 ± 6
NIH/3T3	0

Table 2. Transcriptional responses of hLPH118 to GATA-5 co-transfection in different cell lines. Values are mean (± SEM, n = 3) transcriptional responses (fold increase over baseline) with co-transfection of the GATA-5 expression vector.

activation of hLPH118 (Table 2). Those cell lines that had abundant HNF-1 binding activity (Fig. 7A) also displayed high levels of GATA-5 activation responses (Table 2), whereas those

cell lines with no evidence of HNF-1 binding activity demonstrated limited GATA-5 responses. There was no correlation between the presence of Cdx-2 binding activity (Fig. 7B) and GATA-5 activation.

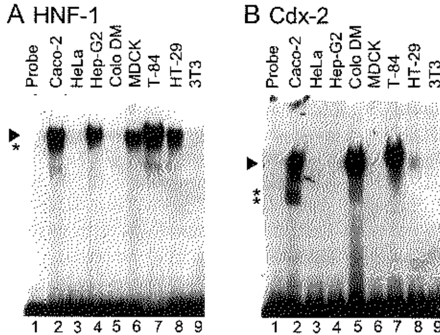

Fig. 7. HNF-1 and Cdx-2 binding activity is different in different cell lines as determined by EMSAs. *A:* using the hH (human HNF-1 binding site) probe, EMSAs were carried out using 10 μg of nuclear extract from different cell lines. The main complex (arrowhead) and secondary complex (*) competed specifically and supershifted with HNF-1α and/or HNF-1β antibodies (not shown). *B:* using the hC (human Cdx-2 binding site) probe, EMSAs were carried out using 10 μg of nuclear extract from different cell lines. The main complex (arrowhead) and secondary complexes (*) competed specifically and supershifted with the Cdx-2 antibody (not shown). The secondary complexes become more intense with repeated thawing and refreezing of nuclear extracts (not shown), suggesting that these complexes represent degradation products of Cdx-2.

To determine if the introduction of HNF-1 into HeLa cells, which do not synthesize endogenous HNF-1 (Fig. 7A), results in GATA-5 activation, GATA-5 and HNF-1α expression vectors were co-transfected (individually and in combination) with hLPH118, and the transcriptional activity was determined. Parallel experiments were carried out in Caco-2 cells as a control. As shown in Fig. 8, GATA-5 demonstrated minimal activation of hLPH118 in HeLa cells as compared to that in Caco-2 cells ($P < 0.001$).

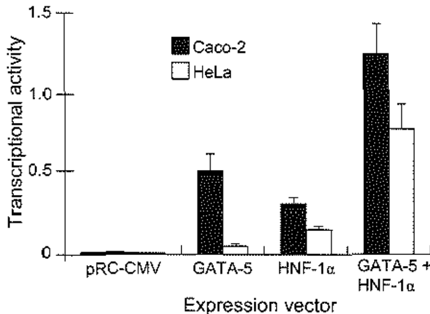

Fig. 8. GATA-5 activates hLPH118 in HeLa cells when HNF-1α is co-transfected. Transient co-transfection assays (mean ± SEM, n = 6) were carried out in HeLa and Caco-2 cells using hLPH118 and expression vectors for GATA-5 and HNF-1α, singly, and in combination. Significant differences from HeLa cells within each expression vector treatment group are indicated: $^{**}P < 0.01$.

In contrast, significant differences by cell line could not be found for co-transfection with HNF-1α alone or with HNF-1α plus GATA-5. Co-transfection of both GATA-5 and HNF-1α resulted in a synergistic activation of hLPH118 in both cell lines. Transfection of GATA-5 or HNF-1α into HeLa cells resulted in specific protein levels similar to that in transfected Caco-2 cells, as determined by supershift EMSAs (not shown), demonstrating that HeLa cells are synthesizing the transcription factors from the transfected expression vectors. These data suggest that GATA-5 activation of the human LPH promoter is dependent on the presence of HNF-1 transcription factors.

Mutational analysis of the GATA and HNF-1 binding sites in the human LPH promoter. To characterize the effects of disruptions in protein-DNA interactions during individual and combined GATA-5 and HNF-1α co-transfections, mutations were introduced into the GATA and HNF-1 binding sites of the human LPH promoter. To first demonstrate that the introduced

mutations disrupt specific protein-DNA interactions, EMSAs were carried out using nuclear extracts from Caco-2 cells transfected with the GATA-5 expression vector and a probe that spans the 5' GATA and the HNF-1 site of the human LPH gene (called hG_1H). As shown in Fig. 9, two main complexes were identified (arrowheads). The faster mobility complex was competed with

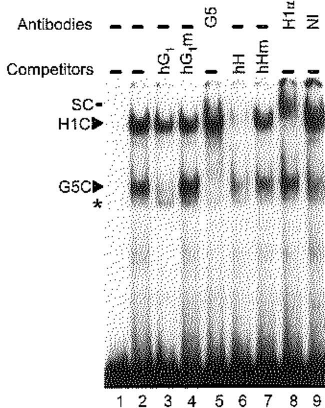

Fig. 9. Specific mutations disrupt specific protein-DNA interactions. EMSAs were carried out using a probe that spans the 5' GATA and the HNF-1 sites of the human LPH gene (hG_1H, lanes 1-9) and nuclear extracts from Caco-2 cells transfected with the GATA-5 expression vector (lanes 2-9). Competitions (200-fold molar excess) were carried out using oligonucleotides spanning only the GATA (hG_1) or HNF-1 (hH) sites as well as oligonucleotides spanning both of these sites, but with specific mutations introduced into the GATA or HNF-1 binding motifs (hG_1m and hHm, respectively). Supershifts were carried out using antibodies specific for GATA-5 (G5) or HNF-1α (H1α). Nonimmune (NI) serum was used as a negative control. GATA-5-specific complex (G5C), HNF-1-specific complex (H1C), and supershift complexes (SC) are indicated. The asterisk (*) indicates a non-specific complex that neither competes nor supershifts.

an oligonucleotide containing the wild-type GATA site (hG_1, lane 3), but not with an oligonucleotide containing the specific mutation in the GATA motif (hG_1m, lane 4). This complex also supershifted with a GATA-5 antibody (lane 5) and was therefore indicated as GATA-5 complex (G5C). The slower mobility complex competed specifically with an oligonucleotide containing an intact human HNF-1 site (hH, lane 6), but not with an oligonucleotide containing a mutated site (hHm, lane 7), and supershifted specifically with an HNF-1α antibody (lane 8). This complex was indicated as HNF-1 complex (H1C). Noteworthy is that specific competition with hH revealed partial competition of the GATA-5 complex. An oligonucleotide probe containing the 3' GATA site (hG_2) also binds GATA factors, but a mutation introduced into this site (hG_2m) does not compete (not shown). These data demonstrate that mutations introduced into the GATA and HNF-1 sites of the human LPH promoter disrupt specific protein-DNA interactions at these sites.

To test the importance of the GATA and the HNF-1 binding sites in the human LPH promoter, transient co-transfections using all combinations of wild-type and mutated sites in the hLPH118 construct and the GATA-5 and HNF-1α expression vectors were carried out in Caco-2 cells. As shown for GATA-5 activation (Fig. 10, top panel), a promoter-reporter construct containing 55 bp of 5'-flanking sequence of the human LPH gene (h55wt), which includes the Cdx-2 binding site, but not the GATA or HNF-1 sites, demonstrated a significantly lower transcriptional activity than that of the wild-type hLPH118 (wt) construct ($P < 0.01$). Mutations introduced into either GATA site alone (G_1m or G_2m) had no effect on GATA-5-stimulated activity, but mutations introduced into both GATA sites (G_1mG_2m) resulted in a significantly reduced GATA-5 response ($P < 0.01$). Interestingly, all constructs containing mutations in the HNF-1 site had mean GATA-5-stimulated activities that were less than 70% of that of h118wt; significantly lower activities were found for G_2mHm and G_1mHmG_2m ($P < 0.01$ for each). These data demonstrate that GATA-5 is capable of activating the human LPH promoter through either of the two GATA motifs, and that full GATA activation is dependent on an intact HNF-1 binding site. In HNF-1α co-transfection experiments (Fig. 10, middle panel), all constructs containing a mutation in the HNF-1 binding site (Hm) had transcriptional activities that were less than 40% of that of h118wt, although none were statistically significant.

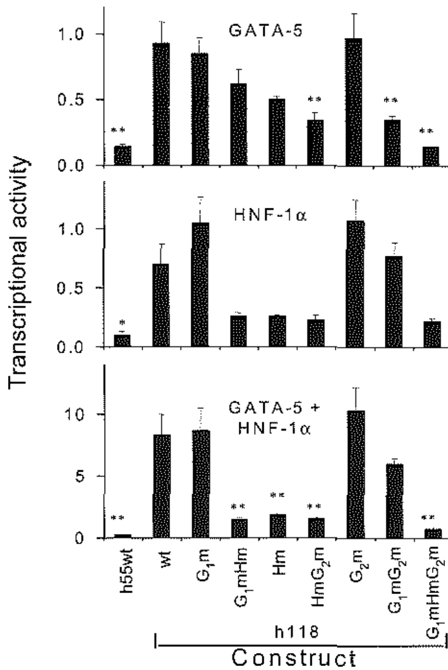

Fig. 10. Functional co-operativity between GATA-5 and HNF-1α is dependent on an intact HNF-1 binding site. Transient co-transfections using all combinations of wild-type and mutated sites in the hLPH118 (h118) construct and the GATA-5 and HNF-1α expression vectors were carried out in Caco-2 cells. h55wt is a promoter-reporter construct containing 55 bp of 5'-flanking sequence of the human LPH gene. Specific mutations were introduced according to the gel shift probes depicted in Fig. 2 (e. g., the h118G_1m promoter-reporter construct contains the hG_1m mutation). Transcriptional activities (mean ± SEM, n = 4) were determined as described in MATERIALS AND METHODS. The co-transfected expression vectors are indicated in the upper center of each panel. Those means that were significantly different from h118wt (Dunnett's test) are indicated: *$P < 0.05$, **$P < 0.01$.

HNF-1α-stimulated activities were not affected by constructs that contain only GATA mutations. These data suggest that the HNF-1 binding site is critical for HNF-1α-stimulated activation.

Co-transfection of both the GATA-5 and HNF-1α expression vectors demonstrated significantly lower transcriptional activities from h118wt for all constructs containing HNF-1 mutations (Fig. 10, lower panel). Individual GATA mutations alone had no effect on transcriptional activity, although mutations in both GATA sites resulted in a mean transcriptional activity that was 72% of that of h118wt ($P > 0.05$, not significant). These data suggest that the HNF-1 binding site is critical for combined activation by GATA-5 and HNF-1α.

DISCUSSION

This is the first report to extensively compare under the same experimental conditions the effect of specific transcription factors on multiple intestinal gene promoters and to identify functional co-operativity between two major classes of transcription factors, the zinc-finger GATA proteins and the homeobox HNF-1 family. Studies to date have generally examined regulation of individual intestinal gene promoters, and only recently have the effect of multiple transcription factors been examined (46, 47, 72), but only on single promoters. In the present report, we found that GATA, HNF-1, and Cdx-2 transcription factors regulate the rat LPH, human LPH, and human SI promoters differently, and that GATA-5 and HNF-1α co-operatively activate these promoters. Together, these data suggest that the specific expression of intestinal genes is modulated by complex interactions involving multiple transcription factors.

Our data are in agreement with previous reports demonstrating individually that members of the GATA-4, -5, and -6 subfamily are capable of activating the human (17) and rat (15) LPH promoters, that HNF-1α is capable of activating the pig LPH (59) and human SI (72) promoters, and that Cdx-2 is capable of activating the pig (65) and rat (16) LPH and human SI (60)

promoters. Our data also parallel a recent report by Mitchelmore et. al. (47), who showed that HNF-1α and Cdx-2 co-operatively activate the pig LPH promoter, a finding we have also demonstrated for the human LPH promoter. Thus, our data are in general agreement with previous reports.

GATA and HNF-1 transcription factors together are key activators of LPH gene expression, whereas Cdx-2 has a comparatively weak effect on the LPH promoters. Although GATA-4, -5, and -6, HNF-1α, and Cdx-2 are all capable of activating the LPH promoters individually in co-transfection assays, comparative analyses reveal that the rat and human LPH promoters generally demonstrate greater transcriptional responses to the GATA factors and HNF-1α than to Cdx-2 (Fig. 3). Similarly, activation of endogenous LPH transcription in Caco-2 cells occurred with over-expression of GATA-5 or HNF-1α, but not with that of Cdx-2 (Fig. 6). In contrast to our data, in which Cdx-2 showed no effect on LPH mRNA abundance, Fang et. al. (16) reported a modest 2-fold increase in LPH mRNA abundance in Cdx-2-transfected Caco-2 cells, which could be due to a more sensitive quantification technique (densitometry of the negative image of ethidium bromide-stained bands in agarose gels) employed in their study. Our results depict the comparative effects of single and multiple transcription factors, and thus, relative to GATA-5 or HNF-1α co-transfection, Cdx-2 appears to be a comparatively weak activator of endogenous LPH gene transcription in Caco-2 cells. Finally, the combination of GATA-5 and HNF-1α produces the strongest activation of the LPH promoters demonstrating functional co-operativity between these two transcription factor families (discussed below). Together, these data suggest that members of the GATA and HNF-1 transcription factor families are significant activators of LPH gene expression, whereas Cdx-2, although perhaps necessary for LPH expression in vivo, has a comparatively lesser role in modulating LPH gene expression.

Although some experiments argue that HNF-1 and Cdx-2 have a greater role than GATA factors in the modulation of the SI promoter, other experiments suggest that all three transcription factor families are important in the activation of SI gene expression. As shown in Table 1, transcriptional responses for the human SI promoter were strongest for HNF-1α and Cdx-2 as compared to that for the GATA factors. Further, greater basal expression of the human SI promoter as compared to the LPH promoters could be due to the relative abundance of endogenous transcription factors synthesized in Caco-2 cells. Based on amount of extract used, intensity of complexes on autoradiographic film, and length of exposure time, data from EMSAs suggest that Caco-2 cells synthesize abundant amounts of HNF-1 and Cdx-2 proteins, but limited amounts of GATA proteins (not shown). Thus, it could be argued that HNF-1 and Cdx-2 have a greater role than GATA factors in the activation of the SI promoter (in contrast to the LPH promoters) resulting in a greater basal (unstimulated) expression. HNF-1 and Cdx-2 have greater affinities for SIF3 and SIF1, respectively, as compared to their cis-element counterparts in the LPH genes (Fig. 4), which could explain the greater basal transcriptional activities of the SI vs. the LPH promoters. Noteworthy is that hSIF1 contains two Cdx-2 motifs whereas rC and hC (i.e., CE-LPH1a) has a single binding motif which likely accounts for the differences in affinity between the two elements.

Co-transfection experiments using combinations of expression vectors, however, suggest that all three transcription factor families co-operatively activate the human SI promoter. For hSI183, co-transfection of the combination of GATA-5, HNF-1α, and Cdx-2 expression vectors produced the highest transcriptional activity of any combination of transcription factors (Fig. 5A), and demonstrated a strong synergistic interaction response (Fig. 5B). Further, all three transcription factors were also important for endogenous SI gene expression, since an amplified product corresponding to human SI mRNA was detected only in the RNA isolated from Caco-2 cells transfected with the GATA-5, HNF-1α, and Cdx-2 expression vectors (Fig. 6). Activation

of SI individually by HNF-1 and Cdx-2 transcription factors has previously been demonstrated (60, 72), but the present study is the first to show activation of the SI promoter by the GATA family, and co-operative activation of the SI promoter by members of the three different transcription factor families.

The rat and human LPH promoters, with some exceptions, demonstrated similar responses to the transcription factors under study. Both promoters were significantly activated by GATA, HNF-1, and Cdx-2 transcription factors (Fig. 3), and their patterns with co-transfection of multiple expression vectors were similar to each other (Fig. 5A). A significant difference between the two promoters, however, was revealed by co-transfection of the GATA-6 expression vector, in which the rat LPH promoter demonstrated a greater transcriptional response than the human LPH promoter (Table 1). This suggests a preferential activation of the rat promoter by GATA-6, and may indicate differential affinities of the GATA factors for specific sequence in the binding sites, although this has not been specifically tested. A second difference was found with HNF-1α co-transfection, in which the human LPH promoter demonstrated a significantly greater activation response than the rat LPH promoter (Table 1), suggesting a preferential activation of the human LPH promoter by HNF-1α (although no difference in HNF-1 binding affinity was noted between the rat and human HNF-1 sites; Fig. 4B). Finally, the combination of HNF-1α plus Cdx-2 as well as the combination of all three expression vectors demonstrated antagonistic or additive responses for rLPH108, but a strong synergistic response for hLPH118 (Fig. 5B, discussed further below). Together, these data suggest that the position, configuration, and/or specific sequence within binding motifs of the LPH promoter may influence the magnitude and direction (activation vs. repression) of responses to specific combinations of transcription factors. Implications for the differential regulation of LPH gene expression between humans and other mammals are unknown at this time.

The basal promoter constructs (i.e., rLPH37 and hLPH37), with varying degrees of statistical significance, were activated by many of the transcription factors tested (Fig. 3). It is possible that sequence in the human growth hormone reporter gene may activate reporter expression in response to specific expression vectors. However, the activity of BSGH after specific transcription factor stimulation was subtracted from that of all other constructs stimulated with the same transcription factor thus eliminating activity due to the reporter itself. In a preliminary report (70), we showed that Cdx-2 is capable of binding and activating the LPH and SI promoters at their TATA-box regions. Thus, Cdx-2 may directly activate these promoters at their TATA-boxes, whereas GATA and HNF-1 factors, which do not bind to the -37 to -1 bp region (not shown), may activate transcription of a basal promoter indirectly (in contrast to direct protein-DNA interactions), or by direct interactions with the transcriptional initiation complex.

In the human LPH gene, we confirm that the two GATA motifs bind GATA-5 with similar affinities (Fig. 4A), and demonstrate that both sites play a role in the activation of the human LPH promoter (Fig. 10). Mutations introduced into either one of the sites had little effect on GATA-stimulated activity, but mutations introduced into both sites resulted in a significant reduction in GATA-5-stimulated transcriptional activity (Fig. 10). In a previous report, Fitzgerald et. al. (17) demonstrated that a mutation in the 5' GATA site of the human LPH promoter resulted in a decrease in GATA-6 activation from the wild-type control. The reason for the apparent difference between our study and that of Fitzgerald et. al. is uncertain, but could be due to differences in the ability of chicken GATA-6 as compared to mouse GATA-5 to activate the human LPH promoter. In the human LPH gene, the 5' GATA site (hG_1) is on the forward strand whereas the 3' GATA site (hG_2) is on the reverse strand, which could result in possible differences in the ability of different GATA proteins to activate the LPH gene through the hG_2 site. We conclude from our data that GATA-5 is capable of activating the human LPH promoter

through either GATA motif, and that the two sites together do not activate the LPH gene any more than either of the sites individually.

Binding site affinity experiments suggest that GATA-5 prefers to bind WGATAT. Unbiased analysis of random GATA motifs revealed the consensus for the GATA-1, -2, and -3 subfamily is WGATAR, where W is A or T and R is A or G, but clear preferences in core and adjacent sequences were noted (30, 45). A similar characterization of the GATA-4, -5, and -6 subfamily has not been reported. Comparison of the relative affinity of GATA-5 for each of the GATA motifs (Fig. 4A), as summarized in Fig. 11, reveals that GATA-5 binds equivalently if an "A" or "T" (but not a "G") is in the 5' position, and prefers a "T" in the 3' position, although it continues to bind with less affinity if an "A" or "G" are present in this position. We therefore propose that the GATA-5 consensus is WGATD, where D is A, G, or T.

Consensus	**A/TGATAA/G**	Affinity for GATA-5
xIFABP/G	5'-TAC**AGATAT**GGG-3'	++
rG1	CCT**AGATAA**CCC	+
rG2	TGT**GGATAA**TCA	+/-
hG1	CAT**AGATAA**CCC	+
hG2	AAG**TGATAA**TTA	+
hSI/G	CAC**TGATAG**GCT	+

Fig. 11. Sequence analysis demonstrates that GATA-5 preferentially binds a AGATAT motif. Based on affinity experiments shown in Fig. 4A, the relative affinities of GATA-5 for each of the GATA motifs were assigned either "++" for strong competition, "+" for intermediate competition, or "+/-" for weak or lack of competition. The core motif (bold) and adjacent sequence are shown.

In co-transfection experiments, the combination of HNF-1α and Cdx-2 demonstrated synergistic activation of the human LPH promoter (hLPH118), but antagonistic interactions on the rat LPH promoter (Fig. 5B). Our data on the human LPH promoter are in agreement with a previous report by Mitchelmore et. al. (47), who demonstrated that HNF-1α and Cdx-2 functionally interact resulting in a synergistic activation of the pig LPH promoter. Although the reason for the apparent species difference in response to HNF-1α and Cdx-2 is not readily known, structural similarities and differences among the rat, human, and pig LPH promoters were noted. There is greater conservation of sequence identity in the first 100 bp of 5'-flanking region between the human and pig LPH (93 %) genes than between the human and rat LPH (78 %) genes. This includes the HNF-1 binding motif, in which the 3' half is completely conserved between the human and pig sequence and more closely correlates with the HNF-1 consensus than that of the rat LPH HNF-1 (rH) site. Noteworthy, however, is that rH and hH demonstrate similar affinities for HNF-1 binding in competition EMSAs (Fig. 4). Sequence differences also include the TATA boxes, in which the human and pig LPH genes contain a true "TATA" motif, whereas the rat LPH gene contains a "CATA" motif, although the effect of such a difference on transcriptional regulation is unknown. The recent demonstration of a second Cdx-2 binding site (called CE-LPH1c) 5' to the HNF-1 site in the pig LPH gene (47) is unlikely to explain the species differences in response to these transcription factors because CE-LPH1c is not present in the human LPH gene, but is present in the rat LPH gene (-102 to -97 bp). Since rLPH108 does not demonstrate synergistic activation by HNF-1α and Cdx-2, it is likely that the presence or absence of this site does not play a direct role in the co-operative activation by these two transcription factors. Finally, with regard to binding site position, the distance between the 5' base of the HNF-1 motif and that of the Cdx-2 binding site at CE-LPH1a is 33 bp for the human and pig promoters and 28 bp for the rat LPH promoter, which represents a difference of approximately one-half turn of the helix which could affect the ability of HNF-1α and Cdx-2 to interact. At present, the process underlying the co-operative interactions between HNF-1α and Cdx-2 is unknown, but the findings in the present study imply that the mechanism is more

complex than simple protein-protein interactions and the presence of HNF-1 and Cdx-2 binding sites.

In the present report, we demonstrate for the first time functional co-operativity between GATA-5 and HNF-1α. This co-operativity was strongest for the human LPH promoter, but was present for the rat LPH and human SI promoters (Fig. 5B) as well. Functional co-operativity between zinc-finger GATA proteins and homeobox transcription factors has been previously demonstrated for GATA-4 and Nkx-2.5, a cardiac-restricted homeobox protein (13, 37, 57). GATA-4 was shown to physically associate with Nkx-2.5 and co-operatively activate cardiac-specific promoters. Further, mutational analysis of each transcription factor indicated that physical interaction was required for functional synergy. Deletional analysis in GST pull-down assays revealed that the carboxy-terminal zinc finger of GATA-4 was required to interact with Nkx-2.5, and helix III of the homeodomain of Nkx-2.5 was necessary to associate with GATA-4. Both regions are known to bind their cognate DNA binding sites, demonstrating a close association between protein-protein and protein-DNA interactions. Indeed, the homeodomain of HNF-1α was shown to be necessary for interactions with Cdx-2 (47). Based on these models, we hypothesize that the C-terminal zinc finger of GATA-5 and the homeodomain of HNF-1α physically interact, and that this interaction is necessary for synergistic activation of the human LPH promoter.

Based on the findings in the present study, we hypothesize that the patterns of expression of certain intestinal genes are due, at least in part, to the simultaneous presence of GATA, HNF-1, and Cdx-2 transcription factors. Members of all three transcription factor families are present in the upper crypt and lower villus, which corresponds to the region that LPH and SI transcription is activated during intestinal differentiation. However, the net effect on intestinal gene expression during differentiation is far more complex than the simple presence of these transcription factors and their cognate binding sites. Data in the present study suggest that the number of binding sites and their relative positions on the promoter is important. Further, as shown by transcription from longer promoter constructs (Fig. 3) or from the endogenous genes (Fig. 6), which do not always parallel expression of shorter promoter constructs, other interactions involving sequence further upstream may also influence transcriptional activation. Finally, interaction with other transcription factors, such as HOXC11 (46), as well as with specific cofactors, including friend of GATA-2 (FOG-2) (39, 62), dimerization co-factor for HNF-1 (DCoH) (44), and p300 (27) must be considered.

We conclude that the complex hierarchies of control that govern gene expression during intestinal differentiation and development are determined by the stoichiometry of transcription factors and cofactors within intestinal cells at any given time, and the type, number, and arrangement of cis-acting elements in the regulatory regions of intestinal genes. LPH and SI, markers of intestine-specific gene expression and intestinal differentiation, are regulated by complex interactions involving multiple transcription factors. Synergistic activation by combinations of transcriptions factors is likely a mechanism by which specific patterns of tissue-specific expression might be attained by the overlapping expression of specific transcription factors. Characterizing specific interactions will provide insight into the regulatory mechanisms that control the pathways of intestinal differentiation, and is presently under study in our laboratory.

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Chapter 3

Hepatocyte-nuclear-factor-1alpha (HNF-1α), GATA-4 and caudal-related homeodomain protein Cdx2 functionally interact to modulate intestinal gene transcription: Implication for the developmental regulation of the Sucrase-Isomaltase gene.

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ABSTRACT

Sucrase-isomaltase, an intestinal specific gene, is strongly induced in the differentiated small intestinal villous epithelium during the suckling-weaning transition in mice. We have previously identified cis-acting elements within a short evolutionarily conserved SI promoter. However, the nature and profile of expression of the interacting proteins have not been fully characterized during this developmental transition. Herein, we show that HNF-1α, GATA-4, Cdx2 and Cdx1 are the primary transcription factors from the adult mouse intestinal epithelium to interact with the SIF3, GATA and SIF1 elements of the SI promoter. We then aimed to study whether HNF-1α, GATA-4 and Cdx2 can cooperate in the regulation of SI gene expression. Immunolocalization experiments revealed that HNF-1α is detected in rare epithelial cells of suckling mice and become progressively expressed in the villous epithelial cells during the suckling-weaning transition. GATA-4 protein is exclusively expressed in villous differentiated epithelial cells of the proximal small intestine, decrease in expression in the ileum and become undetectable in the colon. HNF-1α, GATA-4 and Cdx2 can interact *in vitro* and *in vivo*. In addition, these factors activate SI promoter activity in co-transfection experiments where GATA-4 requires the presence of both HNF-1α and Cdx2 to influence this activity. These findings imply a combinatory role of HNF-1α, Cdx2 and GATA-4 for the temporal and positional-dependent regulation of SI transcription during intestinal development.

INTRODUCTION

The maturation of the intestinal epithelium involves several steps that are initiated early during embryogenesis and completed at the reach of adulthood (1,2). In mice, important changes in the acquisition of mature enterocyte functions are observed during the third postnatal week that corresponds to the suckling-weaning transition. Among these changes are the activation of transcription of distinct genes related to specific functions of the intestinal epithelium (3). Sucrase-isomaltase (SI)[1], an enzyme expressed in the brush border of mature enterocytes, represents one of the most extensively studied genes activated during this developmental transition (4). SI is first detectable in mouse embryonic intestine and remains stable through the first two weeks of life after birth. At the time of the suckling-weaning transition, a dramatic induction of SI mRNA and protein is observed. A proximal to distal gradient of SI expression and activity is then established with highest level in the jejunum and no detectable level in the colon (3,5,6). A vertical gradient of expression is also observed along the crypt-villus axis with a maximal level in the lower two-third part of the villi and no expression in the crypts (3,7,8). Although the distribution of SI expression is well documented during intestinal development, the exact developmental mechanisms that govern SI and intestinal gene expression are still poorly defined.

In the past, we have identified a number of SI promoter elements important to support expression in intestinal epithelial cells (9). Furthermore, a short evolutionarily conserved SI gene promoter was shown to recapitulate the developmental expression of SI in transgenic mice (6). This indicated that all the elements required for the developmental regulation of the SI gene are comprised within this short region. This short promoter contains three identified regulatory sites, SIF1 (Sucrase Isomaltase Footprint 1), SIF3 and GATA.

The SIF1 element interacts with Cdx transcription factors (10,11) that belong to mammalian homeobox gene family related to the *Drosophila melanogaster* gene *caudal* (12). The expression of Cdx1 and Cdx2 is restricted to the intestinal epithelium (13). Cdx1 protein is mainly found in the crypt compartment whereas the Cdx2 protein is detected in both the crypt and the villus compartments (13-15). Cdx2 stimulates differentiation and expression of SI in intestinal epithelial cells (16). However, the specific role of the SIF1 element in the regulation of SI promoter activity *in vivo* is unclear.

The SIF3 element interacts with the transcription factors HNF-1α and HNF-1β that bind to DNA as either homodimers or heterodimers. They are expressed in a variety of tissues, including liver, kidney and intestine and have been implicated in the regulation of multiple genes (17). HNF-1α stimulates SI promoter activity via the SIF3 element whereas HNF-1β down-regulates this effect (18). Although HNF-1α mRNA has been localized in the crypts and bottom of the villi in the small intestine (19), the exact pattern of HNF-1α protein distribution during intestinal development is unknown.

More recently, we have identified a GATA element localized in the proximal region of the SI promoter (20,21). GATA-4, 5, and 6 are expressed in the intestine as well as various mesoderm- and endoderm-derived tissues such as heart, liver, lung and gonad where they play critical roles in regulating tissue-specific gene expression (22). GATA-4 transcripts are detected in villi of the small intestine both in mice (23) and chicken (24) whereas the GATA-6 mRNA is predominantly localized at the crypt-villus junction (24). However, the exact distribution of GATA proteins in the intestine has not been addressed. GATA-4 and GATA-6 play critical roles in the definitive formation of the endoderm early during embryonic life (25-28). Their involvement in the maintenance of the intestinal epithelium remains speculative.

In this study, we investigated whether HNF-1α, GATA-4 and Cdx2 could activate the developmental regulated SI gene through combinatorial interactions. We report that HNF-1α and

GATA-4 protein expression is temporally and regionally modulated in a way that correlates well with the activation of SI expression during the suckling-weaning transition in the intestine. We show that these proteins physically interact with each others and functionally cooperate in the activation of the SI gene promoter. These findings imply a combinatory role of HNF-1α, Cdx2 and GATA-4 for the maturation of the small intestinal mucosa.

EXPERIMENTAL PROCEDURES

Plasmid construction, mutagenesis and transgenic mice—The -201 to +54mSI-luciferase and –134 to + 40 IPAL-luciferase constructs were described elsewhere (18,29). Point mutagenesis of the SI promoter was performed using a PCR-based mutagenesis approach (30). The following oligonucleotides were designed to create point mutations (underlined) of the specific elements: SIF1m (sense) 5'GTGAAAGTGCAAGGCAAC GGGATGAGTAGTCAA-3' and SIF1m (antisense) 5'-TTGACTACTCATCCCGTT GCCTTGCACTTTCAC-3'; GATAm (sense) 5'-TAAACATTGATCGGCTTGTG AAAG -3' and GATAm (antisense) 5'-CTTTCACAAGCCGATCAATGTTTA-3'. Integrity of the mutant constructs was confirmed by sequence analysis. The -8.5 to +54 SIF1mutantSI-hGH plasmid was constructed and injected to produce transgenic mice as described previously (18). The presence of the transgene in mouse genomic DNA was determined as described previously (18,31,32). Transgene founders of the BGSJL/F1 strain (Jackson Laboratory, Bar Harbor, ME) were bred with normal CD1 mice (Charles River) and offspring were analysed for the transgene by PCR.

Cell culture and transfections—HEK293T, COS-7 and Caco-2 cells were maintained in DMEM supplemented with 4.5 g/liter D-glucose, 25 mM HEPES, 10 % fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml of streptomycin in 5 % CO_2. For luciferase assays, transfections were performed using Lipofectamine (Life Technologies Inc., CA) according to manufacturer's recommendations. Cells at 30-40% confluence were incubated with 1.2 µg of total DNA and 2.5 µl of lipofectamine /ml of OPTI-MEM for 18 h. The media was then changed to DMEM complete medium containing 10% FBS. The luciferase and renilla activities were determined 48 h after the transfection using the dual luciferase assay kit (Promega Biotech, Madison, WI). Each experiment was repeated at least three times in triplicate. For stable transfections, Caco-2/15 cells were trypsinized 48 h following transfection and maintained in selection medium containing 1 mg per ml of G418.

RNA analysis—RNA was extracted from multiple tissues or from cultured cells and Ribonuclease (RNase) protection assays were performed as previously described (18). Riboprobes for the detection of mSI, hSI, hGH, h36B4 and m36B4 mRNA were prepared as previously described (18).

Immunohistochemistry—Immunohistochemistry was performed exactly as described previously (13,15). After blocking with protein-blocking agent (Coulter-Immunotech, Miami, FL), the slides were incubated overnight at 4°C with affinity-purified antibodies. The following antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) were used: anti-goat GATA-4 (SC-1237) at a concentration of 0.2 µg/ml; anti-rabbit GATA-4 (H-112) at a concentration of 0.2 µg/ml; anti-goat HNF-1α (SC-6547) at a concentration of 0.067 µg/ml. Cdx2 protein was detected with the affinity purified CNL antibody (1:1000) (15). All primary antibodies were visualized with a biotinylated secondary antibody and alkaline phosphatase detection system. The slides were developed with nitroblue tetrazolium chloride (NBT) and 5-Bromo-4-chloro-3-

indolyl-phosphate (BCIP; Roche Molecular Biochemicals, Indianapolis, IN). The tissue was lightly counterstained with 2% neutral red and mounted with Permount (Fisher Scientific). Immunohistochemistry was repeated at least 3 times for each antibody with sections obtained each time from different mice. Each animal was routinely sacrificed between 10-12 AM to prevent any modification of expression related to the circadian rhythm as it has been reported for HNF-1proteins (33). Specificity of the signal for each antibody was confirmed by blocking experiments as previously described (13).

Isolation of nuclear proteins from adult intestinal epithelium—Nuclear protein was isolated from the intestinal epithelium of adult mice by an adaptation of a previously described method that used human intestine (34). Briefly, mice were sacrificed and the intestine was separated in sections of jejunum, ileum and proximal colon. Each section was open longitudinally and rinsed with cold PBS. The sections were further cut in 5mm pieces and incubated in 5 ml of cold MatriSperse (Becton Dickinson, Franklin Lakes, NJ) in 15ml tubes at 4°C for 18 to 24 hr. The epithelial layer was dissociated by gentle manual shaking. The epithelial suspension was collected, centrifuged and washed with cold PBS. Nuclear proteins were then isolated from the epithelial cell pellet as described previously (9).

Western Blot analysis—20 μg of nuclear protein extracts was analyzed by a 4-12% Bis-Tris NuPAGE (Invitrogen, Carlsbad, CA) electrophoresis and transferred to an Immobilon-P membrane. Western blot was then performed exactly as described previously (18). The following antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) were used: HNF-1α affinity-purified goat polyclonal antibody raised against a peptide mapping to the carboxy terminus of human HNF-1α (SC-6545); GATA-4 affinity-purified goat polyclonal antibody raised against a peptide mapping to the carboxy terminus of mouse GATA-4 (SC-1237); HDAC-2 affinity-purified rabbit polyclonal antibody raised against a peptide mapping to amino acids 435-488 of human HDAC-2 (SC-7899). A Cdx2 affinity-purified rabbit polyclonal antibody raised against a peptide mapping to the N-terminus of murine Cdx2 (CNL) was used for Cdx2 detection (15).

Electrophoretic Mobility Shift Assays (EMSA)—EMSA was performed essentially as described previously (10). The reactions were performed in 20 μl of binding buffer (10) containing 5 μg of nuclear extracts, 1 μg of poly dIdC and 20,000 cpm of ^{32}P-labeled DNA probe during 30 min. Complexes were then separated on a 4% polyacrylamide gel at room temperature (SIF1 and SIF3) or 4°C (GATA), dried and exposed on a phosphoimager screen. The following double stranded oligonucleotides were used as DNA probes: GATA: 5'-AAACATTGATAGGCTTGTGA-3'; MUT-GATA: 5'-AAACATTGATGGGCTTGTG A-3'; α1-globin GATA: 5'-GATCTCCGGCAAC TGATAAGGATTCCCTG-3'. The SIF1 and SIF3 DNA probes have been described previously (9). When indicated, antibodies were included in the binding reaction for supershift analysis.

GST-pulldown—GST-protein interaction assays were performed essentially as described elsewhere (35). Briefly, bacterial expression of GST fusion proteins was induced in medium containing 0.1μM isopropyl-1-thio-D-galactopyranoside for 3 h. The bacterial pellet was resuspended in PBS containing proteinase inhibitors and sonicated to disrupt the bacteria. The bacterial residues were removed by centrifugation, and the supernatant was incubated with GST beads (Amersham Pharmacia Biotech) on a rotating wheel for 30 min. The beads were washed 6 times with PBS and the GST fusion proteins eluted with 10 mM reduced glutathione according to the manufacturer's instructions. Reduced glutathione was removed by dialysis in dialysis buffer (0.1 M NaCl, 1 mM EDTA, 20 mM Tris pH 8, 0.5% NP-40 and 5% glycerol). HNF-1α,

GATA-4 and Cdx2 proteins were labeled with [^{35}S] methionine by coupled in vitro transcription and translation using the TNT reticulocyte lysate system (Promega) according to the manufacturer's instructions. GST fusion proteins (20μg) were incubated with labeled proteins and pre-washed GST beads (10μl) in 200 μl of HND buffer (10 mg/ml bovine serum albumin, 20 mM Hepes, 50 mM NaCl, 0.1% Nonidet P-40, 5 mM dithiothreitol) at 4 °C for 1 hr. The beads were then washed 4 times with MTPBS (PBS, 0.1% Nonidet P-40) buffer and proteins eluted from the beads in SDS sample buffer. The eluted proteins were separated on a 4-12% NuPAGE electrophoresis gel.

Immunoprecipitation (IP)-EMSA analysis—IP-EMSA was performed essentially as described elsewhere (36). Transiently transfected HEK293T cells were washed three times in PBS and lysed with lysis buffer A (150 mM NaCl, 40 mM Tris-HCl at pH 7.6, 10% glycerol, 0.3% NP-40) containing protease and phosphatase inhibitors (15) for 20 min and pelleted. Supernatants containing 150 μg of total protein were allowed to bind anti-FLAG (Sigma, St.Louis, MO) agarose beads 5 hrs at 4°C. Pellets were washed three times in lysis buffer A and three times in EMSA binding buffer. Immunoprecipitated proteins were dissociated with deoxycholate for 10 min on ice, neutralized with 1% of NP-40 and analyzed by EMSA.

Results

HNF-1α, GATA-4, Cdx2 and Cdx1 interact with the SI promoter in adult mouse enterocytes—The SI gene promoter contains several cis-acting elements important for transcriptional regulation (9) (Fig. 1A). The identity of proteins able to interact with these elements has been previously investigated with the use of intestinal cell lines. We wanted to confirm the nature of proteins of the mouse intestinal epithelium susceptible to interact with the SIF3, GATA and SIF1 elements. Since the SI gene is highly transcriptionally active in the adult small intestinal epithelium, nuclear extracts were prepared from adult mouse isolated enterocytes, incubated with a SIF3, GATA or SIF1 ^{32}P- labeled probe and migrated on a polyacrylamide gel. As previously reported, the SIF3 probe produced a single complex that was supershifted when an HNF-1α polyclonal antibody was included in the binding reaction (Fig. 1B) (18).

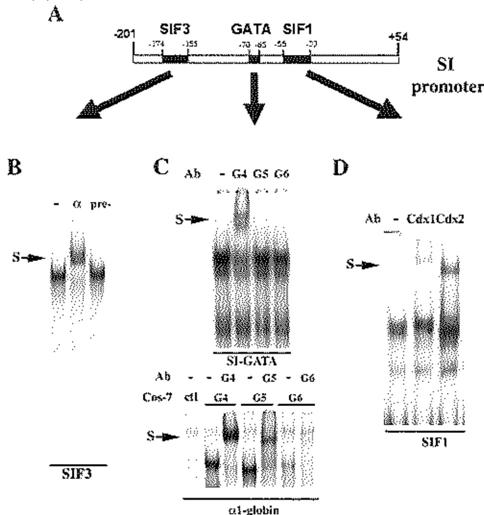

Fig. 1. Interaction of nuclear extracts of mouse adult intestinal epithelial cells with the mouse SI promoter. *(A),* Diagram of the short evolutionarily conserved SI promoter with previously characterized SIF3, GATA and SIF1 elements. ^{32}P-labeled SIF3 (*B*), SI-GATA (*C*), α1-globin-GATA (*C*), and SIF1 (*D*) oligonucleotides were used for EMSA as previously described *(9)*. 5 μg of nuclear extract of intestinal epithelial cells isolated from adult mice was used for each binding reaction. When indicated, specific antibodies (Ab) were included in the binding reaction for supershift (S) analysis. The following antibodies were used: α (HNF1α); pre- (pre-immune serum); G4 (GATA-4), G5 (GATA-5); G6 (GATA-6); Cdx1; Cdx2. Ctl: control.

The GATA probe produced an intense complex that was mostly supershifted with the addition of a GATA-4 polyclonal antibody (Fig. 1C, upper panel). The addition of GATA-5 or GATA-6 polyclonal antibodies did not supershift this complex (Fig. 1C, upper panel). Control experiments performed with nuclear extracts prepared from GATA-5 and GATA-6 transfected COS-7 cells confirmed the ability of both antibodies to supershift a GATA specific complex (Fig. 1C, lower panel). This suggested that GATA-4 was the main protein in adult intestinal epithelium to interact with the SI-GATA site. Incubation of intestinal nuclear extracts with the SIF1 probe produced two very close migrating complexes (Fig. 1D). Supershift analysis showed that both Cdx1 and Cdx2 complexes were present and separable by size (Fig. 1D).

Mutagenesis of the SIF1 element abolishes SI promoter activity in vivo—The role of SIF3 and GATA elements in the regulation of SI promoter activity in vivo has been previously characterized (18,20). The SIF1 element is crucial for high level of SI promoter activity in vitro (9,10). To evaluate the importance of the SIF1 element in the regulation of SI gene in the intact mouse intestinal epithelium, we designed a transgenic construct harboring point mutations within this element and linked nucleotides -8.5kb to +54 of the mSI gene to the hGH reporter gene (Fig 2A). The wild-type construct supports hGH expression in enterocytes of transgenic mice (18,32) and the SIF1 mutation abolishes in vitro interaction of Cdx proteins (10).

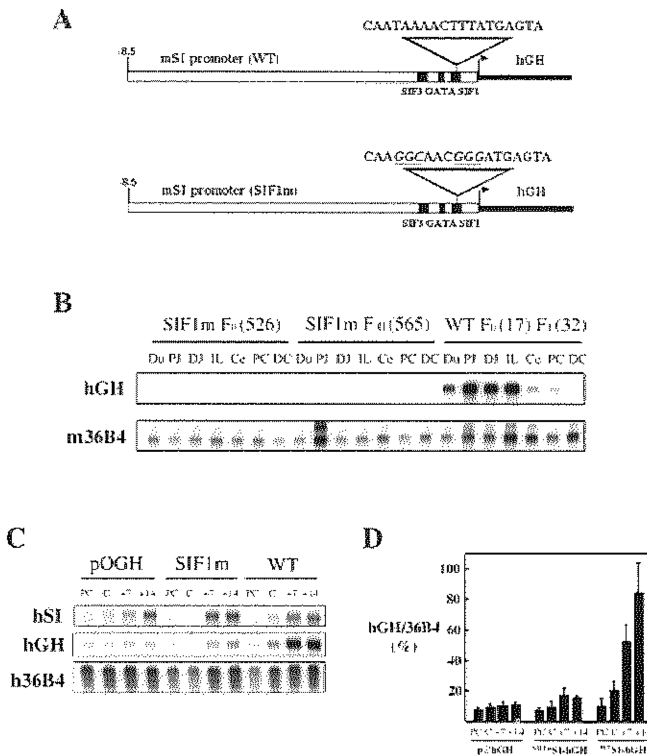

Fig. 2. Effect of the SIF1 mutation on SI promoter activity in transgenic mice and Caco-2/15 cells. *A*, Diagram of the SI reporter constructs used to generate transgenic mice and stable Caco-2/15 cell lines. Mutations within the SIF1 element are indicated in italic and underlined. *B*, Total RNA was isolated from intestinal tissues of 2 different adult founder lines obtained with the –8.5 to +54 SIF1mSI-hGH construct and analyzed by an RNase protection assay for the simultaneous detection of hGH and 36B4 mRNA. Total RNA isolated from founder 17 with the genomic integrated -8.5 to +54 WTSI-hGH construct was used as a reference control. Du, duodenum; PJ, proximal jejunum; DJ, distal jejunum; IL, ileum; Ce, cecum; PC, proximal colon; DC, distal colon. C, Caco-2/15 cells were stably transfected with the neomycin resistance pRC/CMV vector in combination with either the empty hGH reporter vector (p∅hGH), the WTSI-hGH construct (WT) or the SIF1mSI-hGH construct (SIF1m). Total RNA was extracted from G418 resistant cell populations at different days of cell confluency (PC, pre-confluent; *C*, confluent; +7, 7 days after confluence; +14, 14 days after confluence). RNase protection assay was performed using hSI, hGH and h36B4 riboprobes. D, Intensity of the signals was measured from 3 to 4 independent experiments by densitometric analysis using a phosphoimager (Molecular Dynamics). Expression level of hGH mRNA was reported to the h36B4 signal and represented graphically (mean ± SD).

The effect of the SIF1 mutation on hGH expression in two transgenic lines was compared to the previously characterized WT $F_0(17)F_1(32)$ transgenic line (32). Total RNA isolated from different portions of the intestine was analyzed by an RNase protection assay. Each RNA sample was incubated with probes for hGH and an internal control (36B4) in the same hybridization solution in order to correlate hGH mRNA levels among these different lines. The WT transgenic line showed high level expression of hGH in the small intestine (Fig. 2B), as previously reported (32). In contrast, no detectable level for hGH mRNA was found in the intestine of the SIF1 mutant transgenic lines (Fig. 2B). To further analyze the importance of the SIF1 element to regulate SI promoter activity in vivo, we utilized the Caco-2/15 cell line that spontaneously differentiates in post-confluence with concomitant high induction of SI expression. The WT and the SIF1 mutant constructs were co-transfected with a neomycin resistance expression vector (pRC/CMV) to stably transfect Caco-2/15 cells. As a control, Caco-2/15 cells were co-transfected with the empty hGH reporter and pRC/CMV. Expression of hSI, hGH and h36B4 was determined in RNA samples isolated from neomycin resistant population of clones at different times of cell confluence. Expression of the WT construct increased in a similar manner to endogenous human SI mRNA during cellular differentiation (Fig. 2C and D). In contrast, the SIF1 mutant construct showed stable level of hGH expression during differentiation (Fig. 2C and D) and was comparable to the level detected in Caco-2/15 stably co-transfected with the empty vector (Fig. 2C and D). These results indicate that the SIF1 element is essential to activate the SI promoter in vivo.

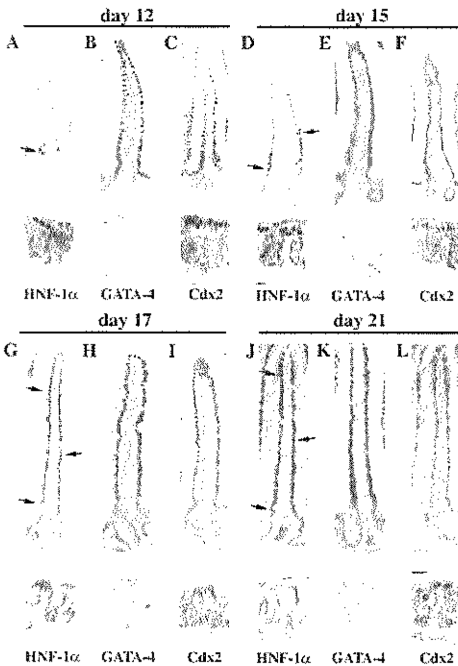

Fig. 3. Distribution of HNF-1α, GATA-4 and Cdx2 proteins in the mouse intestine during the suckling-weaning transition.
Immunohistochemistry was performed to detect HNF-1α (*A*, *D*, *G* and *J*), GATA-4 (*B*, *E*, *H* and *K*) and Cdx2 (*C*, *F*, *I* and *L*) in the jejunum (upper panels) and the colon (lower panels) at post-natal day 12 (*A*, *B* and *C*), 15 (*D*, *E* and *F*), 17 (*G*, *H* and *I*) and 21 (*J*, *K* and *L*). The antibodies were developed with NBT/BCIP (purple nuclei) and the tissue sections were lightly stained with neutral red. The arrows indicate positive HNF-1α labeled cells. Original magnification: 200 X.

The combined temporal and spatial distribution of HNF-1α, GATA-4 and Cdx2 correlates well with the induction of SI expression during the suckling-weaning transition—Although our results demonstrate that the SIF1 element is essential for SI gene transcription in enterocytes, it is unlikely that Cdx proteins alone are responsible for the strong induction of SI expression during the suckling-weaning transition. Indeed, the pattern of Cdx2 protein expression remains primary unchanged throughout post-natal development and the Cdx1 protein is mainly found in the crypt compartment where SI is not expressed (13). Since HNF-1α and GATA-4 interact with the SI promoter (Fig. 1), we investigated the expression profile of these proteins by immunohistochemistry from post-natal day 12 to post-natal day 21. This transition period reflects the major changes observed for SI gene expression (6). The Cdx2 protein distribution was monitored as a reference control.

At post-natal day 12, a period of time when SI is undetectable in the small intestine, the HNF-1α protein was detected in the nucleus of very rare enterocytes of the jejunum (Fig. 3A, upper panel). This pattern was observed throughout the entire small intestinal tract (data not shown). In contrast, HNF-1α was detected in the nucleus of mostly all epithelial cells of the proximal colon (Fig. 3A, lower panel). The GATA-4 protein was mainly detected in the nucleus of enterocytes localized in villi of the jejunum (Fig. 3B, upper panel) whereas it was undetectable in the ileum (data not shown) as well as the colon (Fig. 3B, lower panel). Cdx2 protein was mainly detected in villus-enterocytes of the small intestine as well as in the surface colonocytes of the proximal colon with the use of the CNL antibody that recognizes the unphosphorylated form of the serine 60 residue of Cdx2 (15)(Fig. 3C). At post-natal day 15, HNF-1α became detected in the nucleus of enterocytes localized mainly at the crypt-villus junction in the jejunum while the pattern in the proximal colon remained the same as compared to post-natal day 12 (Fig. 3D). GATA-4 was restricted to the nucleus of villus enterocytes in the jejunum (Fig. 3E, upper panel) and remained undetectable in the colon (Fig. 3E, lower panel). Cdx2 remained unchanged compared to day 12 (Fig. 3F). At post-natal day 17, HNF-1α and GATA-4 were primarily detected in all enterocytes of the villus compartment in the jejunum (Fig. 3G and H) and the ileum (not shown) while the Cdx2 pattern remained unchanged as compared to previous time points (Fig. 3I). GATA-4 remained undetectable in the colon (Fig. 3H, lower panel). From post-natal day 21 to adulthood, HNF1α was detected along the entire axis of the small intestine in abundance in the nucleus of villous enterocytes with only rare positively stained nuclei in crypt epithelial cells (Fig. 3J). GATA-4 protein was detected in the nucleus of villous epithelial cells of the entire small intestine (Fig. 3K, upper panel) with no detectable signal in the colon (Fig. 3K, lower panel). Cdx2 localization was comparable to the earlier time points during development (Fig. 3L).

To confirm the changes observed in HNF-1α and GATA-4 expression, western blots were performed with nuclear extracts obtained from isolated enterocytes at different time during post-natal development. A profound increase in HNF1α protein level was observed during the suckling-weaning transition, while the level of HDAC-2, a protein used as a control, remained relatively unchanged (Fig. 4A). GATA-4 protein level was decreased toward the distal part of the intestine at both days 17 and 22 with no detectable level in the colon (Fig. 4B). While the level of Cdx2 protein increased toward the distal part of the intestine, both HNF-1α and HDAC-2 proteins remained relatively unchanged (Fig. 4B). Thus, HNF-1α and GATA-4 are subjected to a complex regulation during post-natal development that parallels the restricted induction of SI gene transcription in the differentiated epithelium of the small intestine (5,6,31,32).

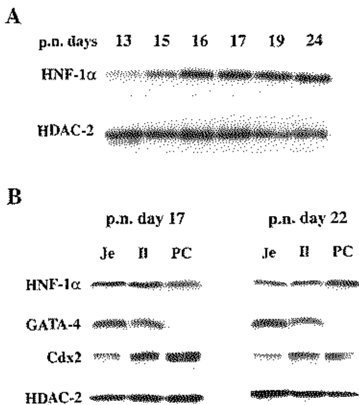

Fig. 4. Western analysis of HNF-1α, GATA-4 and Cdx2 in the mouse intestinal epithelium during the suckling-weaning transition. *A*, Nuclear extracts were prepared from isolated epithelial cells of the jejunum from post-natal (p.n.) day 13 to day 24 and analyzed by western with the use of an HNF-1α polyclonal antibody. The blot was striped and incubated with an HDAC-2 polyclonal antibody to monitor protein integrity. *B*, Nuclear extracts were prepared from isolated epithelial cells of the jejunum (Je), ileum (Il) and proximal colon (PC) at post-natal day 17 and 22 and analyzed by western with the use of HNF-1α, GATA-4, Cdx2 and HDAC-2 polyclonal antibodies.

HNF-1α, GATA-4 and Cdx2 interact in vitro and in vivo—We next explored whether HNF-1α, GATA-4 and Cdx2 could physically interact. An *in vitro* GST pull-down assay was performed with the use of recombinant GST-GATA-4 and GST-HNF-1α proteins and *in vitro*-translated [^{35}S] methionine-labeled HNF-1α, GATA-4 and Cdx2 proteins (Fig. 5A). The GST-GATA-4 recombinant protein was able to pull-down Cdx2 protein as opposed to the GST protein or beads alone (Fig. 5A, left panel). Furthermore, GST-HNF-1α was able to interact with GATA-4 and Cdx2 proteins (Fig. 5A, right panel). The interaction still occurred when both GATA-4 and Cdx2 labeled proteins were simultaneously included with GST-HNF-1α (Fig. 5A). To test whether HNF-1α, GATA-4 and Cdx2 could interact natively, we conducted an IP-EMSA experiment with the use of protein extracts from HEK293T cells co-transfected with FLAG-Cdx2 or empty FLAG (control), both in the presence of HNF-1α and GATA-4 expression vectors. Immunoprecipitated FLAG-Cdx2 (F-Cdx2) extract interacted with the SIF1 element whereas the immunoprecipitated FLAG (F) did not produce any specific band (Fig. 5B, lanes 1 and 2). The IP-F-Cdx2 produced specific complexes when incubated with the SIF3 or GATA labeled oligonucleotides in contrast to the IP-F control extract (Fig. 5B, compare lanes 4 and 7 to lanes 3 and 6). The addition of HNF-1α or GATA-4 polyclonal antibodies resulted in the supershift of SIF3 and GATA upper complexes respectively, confirming the presence of these proteins in the IP-F-Cdx2 complex (Fig. 5B, lane 5 and 8). These results suggest that native HNF-1α, GATA-4 and Cdx2 proteins co-exist in a same complex.

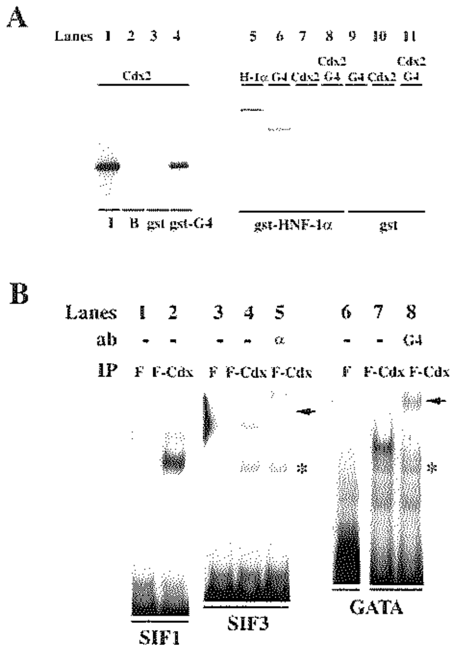

Fig. 5. HNF-1α, GATA-4 and Cdx2 physically interact. *A,* A GST pull-down assay was performed with GST-GATA-4 (gst-G4 left panel), GST-HNF-1α (right panel) or GST (as a control of specificity) attached to Sepharose beads and incubated with *in vitro* translated [^{35}S] methionine-labeled HNF-1α (H-1α, lane 5), GATA-4 (G4, lanes 6, 8, 9 and 11) and Cdx2 (lanes 2, 3, 4, 7, 8, 10, 11) proteins. After repeated washing procedures, the labeled proteins were subjected to a SDS-PAGE and detected by autoradiography. B, beads; I, 20% input. *B,* An IP-EMSA was performed with the use of total extracts from HEK293T co-transfected with empty-FLAG, HNF-1α and GATA-4 expression vectors (IP-F) or with FLAG-Cdx2, HNF-1α and GATA-4 (IP-F-Cdx2). Following the IP, proteins attached to the beads were dissociated by deoxycholate treatment and subjected to an EMSA with the use of SIF1-labeled oligonucleotides (lanes 1 and 2), SIF3-labeled oligonucleotides (lanes 3 to 5) or GATA-α1-globin-labeled oligonucleotides (lanes 6 to 8). When indicated, specific antibodies (ab) for HNF-1α (α) and GATA-4 (G4) were included in the binding reaction for supershift analysis (arrows). Uncharacterized complexes are indicated by asterisks.

HNF-1α, GATA-4 and Cdx2 cooperate to activate intestinal promoter activity—To further characterize the functional significance of the protein-protein interaction among HNF-1α, GATA-4 and Cdx2 on SI gene transcription, we performed co-transfection experiments in Caco-2 cells with the use of the SI promoter linked to a luciferase gene reporter. Similar constructs harboring mutations within HNF-1, GATA and Cdx interacting sites were also used to monitor

the specificity of these interactions (Fig. 6A). These mutations have been previously characterized by EMSA (10,37). Co-transfection of either Cdx2 or HNF-1α expression vector with the SI promoter construct increased the transcriptional activity whereas co-transfection of the GATA-4 expression vector alone did not significantly influence SI activity in Caco-2 cells (Fig. 6B).

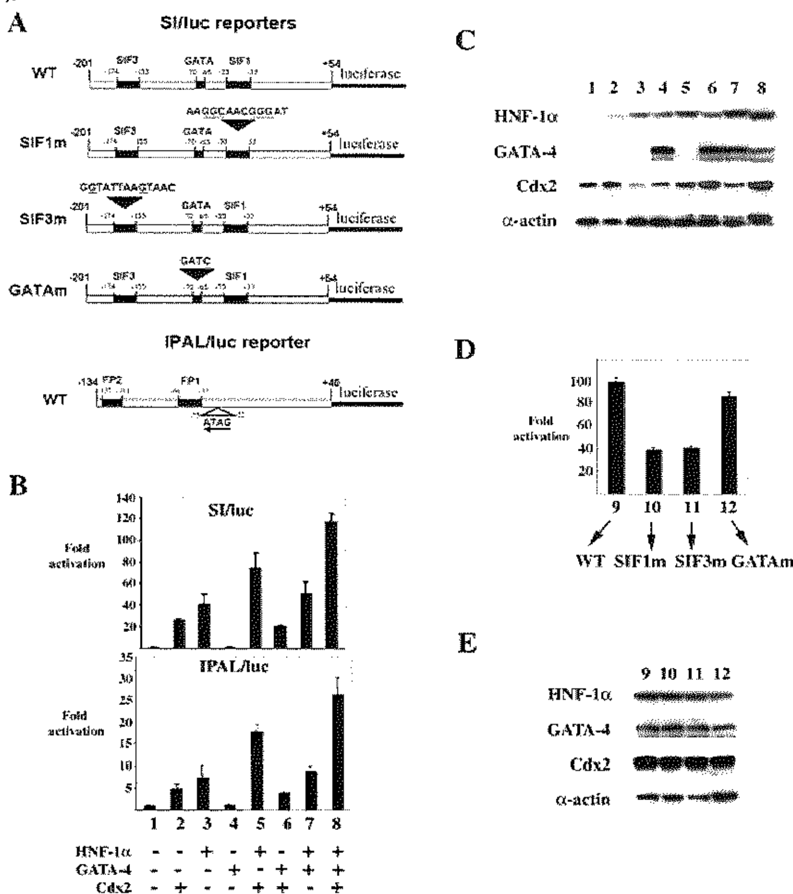

Fig. 6. HNF-1, GATA-4 and Cdx2 synergistically activate the intestinal SI promoter in Caco-2 cells. *A*, Diagram of the SI and IPAL constructs used in co-transfections. The different mutations within the SIF3, GATA and SIF1 elements are underlined. *B*, Caco-2 cells were co-transfected using LipofectAMINE with 300 ng of SI-luciferase or IPAL-luciferase reporter vectors, 2 ng of CMV-renilla, and different combinations of Cdx2 (50 ng), HNF-1α (150 ng) and GATA-4 (200 ng) expression vectors. The pRC/CMV plasmid was used as an empty vector to calibrate the various amounts of expression vectors used in each condition. Results obtained in triplicate were reported as fold difference (mean ± SD) from transfections with the reporter construct alone and are representative of three independent experiments. *C*, Western blot analysis was performed with the use of HNF-1α, GATA-4, Cdx2 and actin antibodies on protein extracts from Caco-2 cells co-transfected as described in B.*D*, Caco-2 cells were co-transfected with Cdx2, HNF-1α and GATA-4 expression vectors in combination with wild-type or mutant SI-luciferase constructs. *E*, Western blot analysis was performed on protein extracts from Caco-2 cells co-transfected as described in D.

Although some minor synergy in the transcriptional activation of SI was observed when both Cdx2 and HNF-1α were combined, the addition of GATA-4 with both Cdx2 and HNF-1α enhanced the synergistic activation of the SI promoter (Fig. 6B). This effect was observed only when Cdx2 and HNF-1α were simultaneously co-transfected with GATA-4 (Fig. 6B). Similar results were obtained when an IPAL (intestinal phospholipase A/lysophospholipase) promoter-luciferase construct was used in the co-transfection assay (Fig. 6B), suggesting that the interaction among HNF-1α, GATA-4 and Cdx2 could be important for the regulation of other intestinal genes. A Western blot was performed in parallel to confirm the production of HNF-1α, GATA-4 and Cdx2 proteins in these co-transfection experiments (Fig. 6C). Individual mutations within the HNF-1 and Cdx sites of the SI promoter resulted in a reduction of the synergistic effect of HNF-1α, GATA-4 and Cdx2 on SI gene promoter activity, while a mutation in the GATA site did not affect the combined effect of these factors on SI transcriptional activity (Fig. 6D). A western blot confirmed that similar levels of HNF-1α, GATA-4 and Cdx2 proteins were produced in these co-transfection experiments (Fig. 6E). These results confirmed that GATA-4 synergies with HNF-1α and Cdx2 in the transcriptional activation of the SI promoter and that a functional GATA-interacting site is not required for this effect.

Discussion

The understanding of molecular mechanisms that govern tissue-specific gene expression often lead to the identification of transcription factors responsible for tissue specialization and maturation. In the past and herein, we used the SI gene as a model to study the molecular basis of the establishment and maintenance of the intestinal phenotype. A short evolutionarily conserved SI promoter contains cis-acting elements that interact with HNF-1α, GATA-4 and Cdx proteins. Interestingly, similar elements are present in a number of intestinal specific gene promoters such as IPAL (29), lactase-phlorizin hydrolase (21,38,39), and iFABP (24,40). We have recently described the importance of the HNF-1 and GATA elements in the regulation of SI promoter activity *in* vivo (18,20). In the current study, we showed that the Cdx element is essential for SI gene transcription. Our findings also indicated that HNF-1α protein expression was induced in the villous epithelium during the suckling-weaning transition. In addition, GATA-4 protein was restricted to the villous epithelium with a decreasing gradient of expression along the horizontal axis of the intestine. We demonstrate that HNF-1α, GATA-4 and Cdx2 interact and cooperate for the transcriptional activation of intestinal specific genes. Our results imply that the Cdx element is required for SI gene transcription but that combinatorial interactions of HNF-1α, GATA-4 and Cdx2 are important for the temporal and spatial regulation of SI gene expression during post-natal development.

The HNF-1α protein is detected in only few epithelial cells in suckling animals to become progressively expressed in differentiated enterocytes during the suckling-weaning transition. In adult mice, HNF-1α mRNA has been found to be highest in crypts of the small and large intestine with a decreasing gradient along the vertical axis (19). Our analysis shows that HNF-1α protein is abundantly detected in the villous enterocytes with only minor expression in the crypt compartment. The molecular network involved in the regulation of HNF-1α has been partly characterized in embryoid bodies (41) but remains largely unknown in the intestinal epithelium context. GATA-4 protein expression is also restricted to villous epithelial cells but is not expressed in the most distal part of the intestine. GATA-4 transcripts were originally reported to be present in the mouse small intestinal villus region and undetectable in the colon (23). Other studies have reported that the GATA-4 mRNA was localized in the entire tract of the

intestinal epithelium late in mouse embryonic development (42) and up-regulated along the crypt-villus axis in chicken (24). Our findings demonstrate that the GATA-4 protein is restricted to the small intestinal villus region and absent in the colon, a pattern that is established early during embryonic development[2]. The molecular mechanisms that control GATA-4 expression are still to be determined. Our findings coupled to others suggest that regulation of HNF-1α and GATA-4 rely on transcriptional and post-transcriptional mechanisms that promote the regionalization of gene expression along both the horizontal and vertical axis during intestinal development.

The overall distribution of HNF-1α, GATA-4 and Cdx2 proteins during the suckling-weaning transition corresponds well with the induction of SI (6) and IPAL (43) mRNA expression. Nevertheless, it is likely that post-translational modifications may play a role in the functional interaction of these factors to regulate intestinal gene transcription. We have previously reported that phosphorylation of the serine-60 residue within the Cdx2 protein that occurs in the crypt compartment reduces its transactivation capacity (15). The phosphorylation of GATA-4 by certain activated-kinase pathways influences both binding and transactivation potency (44,45). Two different GATA family members (GATA-1 and GATA-3) are direct targets of CREB-binding protein (CBP) histone acetyltransferase activity (HAT) and the acetylation of family-conserved GATA-specific domains results in the modification of their activation capacity (46-48). In addition, other transcription factors and co-factors are likely to be involved in the modulation of SI gene expression. For example, Cux/CDP interacts with and represses SI promoter activity via an element that encloses the GATA site (20). Furthermore, Cdx2 and GATA-4 interact with the cofactor p300 (49,50) while HNF-1α interacts with both CBP and CBP-associated factor (PCAF) (51). Whether these post-translational and physical interactions occur along the vertical and horizontal axis of the intestine during development remains to be determined.

The Cdx2 and HNF-1α proteins can interact via their respective homeodomain region *in vitro* (52). Our results suggest that Cdx2, HNF-1α and GATA-4 all interact with each other and are part of a same complex. However, detailed mechanisms and nature of the domains involved for the formation of a stable complex with transcriptionally active intestinal genes *in vivo* will need further studies. It has been suggested that GATA proteins can influence the activity of certain gene promoters without physically interacting with DNA but through physical interactions between other proteins binding to regulatory elements of the promoter (53). GATA-5 and HNF-1α cooperatively activate the lactase gene promoter, an effect that is independent of intact GATA interacting site (21). Our results suggest that the transcriptional activation of the SI promoter by GATA-4 does not require an intact GATA site but depends on Cdx2 and HNF-1α. Therefore, it is possible that other family members such as GATA-5 and GATA-6 could influence SI promoter activity via similar indirect interactions.

The importance of Cdx2 in the maintenance of the intestinal phenotype has been demonstrated in knockout experiments (54,55). Ectopic expression of Cdx2 within the stomach epithelium results in the formation of intestinal metaplasia (56). The GATA-4 protein plays a role in the specification of the mouse gastric epithelium as demonstrated with the use of chimeric mice (57) and stimulates the differentiation of embryonic cells into extraembryonic endoderm (58). The possible role of HNF-1α in the formation and maintenance of the gastrointestinal tract has not been yet characterized carefully (59,60). The future challenge will be to investigate the combinatory roles of HNF-1α, GATA-4 and Cdx2 in the morphogenesis and differentiation of the intestinal epithelium during development.

FOOTNOTES

[1] The abbreviations used are: SI, Sucrase-isomaltase; SIF, Sucrase Isomaltase Footprint; Cdx, Caudal-related homeodomain protein; HNF-1, Hepatocyte Nuclear Factor-1; IPAL, intestinal phospholipase A/lysophospholipase; PCR, polymerase chain reaction; hGH, human Growth Hormone; bp, base pairs; RNase, ribonuclease; DMEM, Dulbecco's modified Eagle's medium; NBT, nitroblue tetrazolium chloride; BCIP, 5-Bromo-4-chloro-3-indolyl-phosphate; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; HDAC, histone deacetylase; CBP, CREB-binding protein; HAT, histone acetyltransferase; PCAF, CBP-associated factor.
[2] *F. Boudreau et al., unpublished observations*

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Chapter 4

Cdx-2 activation of the lactase-phlorizin hydrolase promoter: Identification of a non-Cdx-2 repressor complex at CE-LPH1a

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ABSTRACT

Cdx-2 is an intestine-specific homeodomain-containing transcription factor that activates the promoters of intestinal genes through specific interactions with the consensus sequence, TTTAT/C. Here, we demonstrate that Cdx-2 interacts with the lactase-phlorizin hydrolase (LPH) promoter at cis-element (CE)-LPH1a (-54 to -40 bp) as well as the LPH TATA-box. Introduction of a mutation (TATAtA) into the LPH TATA-box that disrupts Cdx-2 binding, but not binding of the TATA-binding protein, does not reduce Cdx-2-stimulated activation, suggesting that Cdx-2 does not activate the LPH promoter through interactions at the TATA-box. Characterization of CE-LPH1a using EMSAs revealed binding of a novel, non-Cdx-2 complex in multiple cell lines that is independent of the Cdx-2 binding site. Mutational analysis in transient transfection assays revealed a repressor function for this protein, and thus it was designated as nuclear factor-LPH1/repressor (NF-LPH1/R). These data are consistent with the hypothesis that NF-LPH1/R represses LPH gene expression in non-Cdx-2-producing cells, and that this repression is released in cells that synthesize Cdx-2, such as those in the intestinal epithelium.

Abbreviation: Lactase-phlorizin hydrolase, LPH; sucrase-isomaltase, SI; cis-element-LPH1a, CE-LPH1a; nuclear factor-LPH1/repressor, NF-LPH1/R; SI footprint1, SIF1; TATA-box binding protein, TBP.

INTRODUCTION

Cdx-2, a member of the homeobox gene family related to *Drosophila* caudal, is necessary for embryonic development, and may play an important role in intestinal differentiation. Mice homozygous for the Cdx-2 null mutation fail to implant, and die between embryonic day (E) 3.5 and 5.5, whereas heterozygous mice develop adenocarcinoma of the small intestine and colon [1]. Cdx-2 is first detected at E3.5 in extra-embryonic tissue [1]. Within the embryo, Cdx-2 expression first occurs at E8.5 in the posterior gut, tailbud, posterior region of the neural tube, and unsegmented paraxial mesoderm before the development of somites [1]. In adult mammals, Cdx-2 is highly expressed in small intestine and colon [2-5] and is also detected in pancreas [6]. In small intestine of adult mice, Cdx-2 is expressed in all epithelial cells in crypts and on villi, but only at low levels in Paneth cells at the bases of crypts [7, 6]. Stable transfection of Cdx-2 into IEC-6 cells results in inhibition of cell growth and induction of marked morphological differentiation suggesting a role for Cdx-2 in cellular differentiation [8]. Together, these data suggest that Cdx-2 is important for development and maintenance of a differentiated intestinal epithelium.

Originally shown to bind a conserved region in the sucrase-isomaltase (SI) gene called SI footprint 1 (SIF1) [5, 9, 10], Cdx-2 has now been demonstrated to bind to the consensus TTTAY (Y is C or T) and regulate the activity of the promoters of several other intestinal genes, including lactase-phlorizin hydrolase (LPH) [11-16], clusterin [17], calbindin-D9K (CaBP9K) [18, 19], carbonic anhydrase 1 [20, 21], HOX C8 [9], the vitamin D receptor [22], guanylyl cyclase C [23], and human apolipoprotein B [24]. Cdx-2 has also been shown to activate the glucagon promoter in a pancreatic islet cell line [25]. In the human LPH gene, a Cdx-2 binding site, TTTAC, localized in a region called cis element (CE)-LPH1a at -54 to -40 bp, has been well characterized [26, 11-16]. Additional Cdx-2 binding sites, CE-LPH1b [27] and CE-LPH1c [28], have been identified in the pig LPH gene.

Recently, it has been suggested that Cdx-2 may modulate the activity of the promoters of certain intestinal genes through interactions at their TATA-boxes. Rat clusterin, a glycoprotein that has been associated with apoptosis, and rat and human CaBP9K, a mammalian intestinal vitamin-D-dependent Ca^{2+}-binding protein, both have been shown to bind Cdx-2 at their TATA-boxes using supershift and competition EMSAs [18, 19, 17]. These studies have suggested that Cdx-2 may activate or repress transcription at these sites. In addition, Xu et al. [29] have recently demonstrated that Cdx-2 binds to its own TATA-box and represses Cdx-2 expression. Sequence analysis revealed that a Cdx-2 binding site is also present overlapping the rat and human LPH TATA-boxes. Thus, we hypothesize that in addition to Cdx-2 regulation at CE-LPH1a, Cdx-2 may also modulate the activity of the rat and human LPH promoters by interaction at their TATA-boxes. Furthermore, although it is widely known that Cdx-2 binds to CE-LPH1a [11, 13, 15, 30], several reports have suggested that non-Cdx-2-proteins also bind to CE-LPH1a [26, 12, 28, 14-16]. In this report, we demonstrate that a non-Cdx-2-complex binds at CE-LPH1a to a site that is independent of the Cdx-2 binding site. This complex is expressed in intestinal as well as non-intestinal cell lines, and may repress LPH gene transcription in the absence of Cdx-2.

EXPERIMENTAL

Electrophoretic mobility shift assays (EMSAs)

To characterize specific protein/DNA interactions, EMSAs were carried out using probes and competitors as shown in Fig. 1.

Name	Sequence	Location (bp)
r37/1	5'-TGTGCTC**ATAAA**GTTAGGATCCTTCCACATGCTTCT-3'	rLPH, -37 to -1
h37/1	5'-GCAGTT**ATAAA**GTAAGGGTTCCACATACCTCCTAACA-3'	hLPH, -37 to -1
h37/1m1	5'-GCAGggcTAAAGTAAGGGTTCCACATACCTCCTAACA-3'	-----------
h37/1m2	5'-GCAGTTAgccAGTAAGGGTTCCACATACCTCCTAACA-3'	-----------
h37/1m3	5'-GCAGTTATAgAGTAAGGGTTCCACATACCTCCTAACA-3'	-----------
h26/1	5'-GTAAGGGTTCCACATACCTCCTAACA-3'	hLPH, -26 to -1
r55/35	5'-ATGT**TTTAC**AGCCTTGGCTGT-3'	rLPH, -55 to -35
h55/37	5'-TAT**TTTAC**AACCTCAGTTG-3'	hLPH, -55 to -37
h59/37	5'-TACCTAT**TTTAC**AACCTCAGTTG-3'	hLPH, -59 to -37
h59/37m1	5'-TACCTAT**TTT**gcAACCTCAGTTG-3'	-----------
hSIF1	5'-GGGTGCA**ATAAA**AC**TTTAT**GAGTA-3'	hSI, -57 to -34

Fig. 1:Oligonucleotides used as probes and competitors in EMSAs. The names used in the present study, specific sequences (forward strand), and localization in their respective genes are indicated. Cdx-2 binding sites are in bold and TATA-boxes are underlined. An 'm' indicates a mutation introduced into the oligonucleotide and the mutation itself is shown in lower case letters. The 'r' and 'h' indicate rat and human sequence, respectively.

Probes were made by annealing a 10-fold molar excess of 10 base reverse strand oligonucleotide to the forward strand, and extending using the large fragment of DNA polymerase I (Klenow, Gibco-BRL, Gaithersburg, MD) and [^{32}P] dATP (DuPont-New England Nuclear, Boston, MA) as previously described [13]. The specific activity of all probes exceeded 10^6 cpm/pmol. EMSAs were carried out using 10,000 cpm of probe and 5 µg of protein from nuclear extracts or in vitro transcribed and translated protein, labeled with [^{35}S] methionine (Redivue™, Amersham-Pharmacia Life Science, Arlington Heights, IL). Nuclear extracts were prepared as previously described [13] and labeled proteins were synthesized using a reticulocyte lysate transcription-translation system (TNT, Promega Biotech, Madison, WI) according to the manufacturer's instructions. In competition or supershift EMSAs, competitors (200-fold molar excess unless otherwise indicated) or antibodies (1 µl, undiluted), respectively, were preincubated with the nuclear extract for 10 minutes prior to the addition of the probe. The Cdx-2 antibody used in supershift assays was a gift from Dr. D. Silberg (University of Pennsylvania) and was previously characterized [5].

Plasmids

The human growth hormone (hGH) gene was used as a reporter in co-transfection assays. Human LPH promoter-reporter constructs included h37wt, which contains the TATA-box, and h59wt, which contains the TATA-box and CE-LPH1a (-54 to -40 bp). Specific mutations were also introduced into the human LPH promoter sequence of these two constructs. All plasmids, including those with introduced mutations, were constructed by PCR using a *Kpn*I-*Hind*III strategy, as previously described [13]. Heterologous promoter constructs were synthesized by inserting single (TK 1x) and multiple (TK 4x) copies of wild type -59 to -37 bp sequence of the human LPH promoter upstream of the thymidine kinase (TK) promoter by PCR. All constructs were confirmed by sequencing. A previously described expression vector for mouse Cdx-2 [5] (gift of P. Traber, U. Penn.) was employed in the present study. pRC-CMV (Invitrogen) served as a negative control expression vector for all co-transfection experiments.

Cell culture and transient co-transfection assays

Cells were maintained in DMEM (BioWhittaker, Walkersville, MD) supplemented with 5 µg/ml penicillin-streptomycin and containing 10% FCS. Transient co-transfection assays were

carried out by electroporation as previously described [13]. The amount of hGH secreted into the media over 24 h was measured using an [^{125}I] radio-immunoassay kit (Allegro hGH, Nichols Institute, San Juan Capistrano, CA). To control for transfection efficiency, all transcriptional activities were expressed relative to pXGH5, a constitutively active metallothionine-I promoter.

Statistics

The one-way analysis of variance (ANOVA) and Tukey-Kramer or Dunnett multiple comparisons tests were employed in all statistical analyses using InStat software (Graphpad Software, Inc., San Diego, CA).

RESULTS

A Cdx-2 binding site (TTTAT) was identified on the reverse strand overlapping the TATA-box of the rat and human LPH genes. To determine if Cdx-2 binds to sequence containing these sites, competition and supershift EMSAs were carried out using the -37 to -1 bp regions as probes. As shown in Fig. 2, a major complex with a similar mobility was identified with each probe using nuclear extracts from Colo-DM cells, which are enriched in Cdx-2 [5].

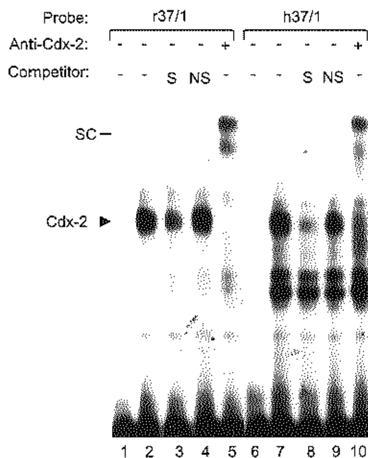

Fig. 2: Cdx-2 binds to the TATA-box regions of the rat and human LPH genes. EMSAs using r37/1 and h37/1 as probes (indicated across the top of the figure) demonstrated a major protein-DNA complex (arrowhead) using 5 µg of Colo-DM nuclear extracts. This complex was partially competed using a specific oligonucleotide (lanes 3 & 8), but was not competed using a non-specific (NS) oligonucleotide (lanes 4 & 9), and also formed a supershift complex (SC) using a mouse Cdx-2 antibody (lanes 5 & 10).

These complexes competed specifically and were supershifted with a Cdx-2 antibody demonstrating that they contain Cdx-2. Thus, in addition to the known Cdx-2 binding site in CE-LPH1a [26, 11, 13, 15, 30], Cdx-2 is also capable of binding to sequence near the LPH TATA-box.

To characterize affinities of the different binding sites for Cdx-2, competition EMSAs were carried out using hSIF1 as a probe (Fig. 3), which is a previously described test probe for Cdx-2 binding [13, 5]. Owing to the two Cdx-2 binding sites in this probe, two complexes, A and B, were observed, as previously described [5]. Using titrations of oligonucleotides containing each binding site under study, both r37/1 and h37/1 revealed weaker affinities for Cdx-2 than the previously characterized upstream rat and human binding sites at CE-LPH1a (r55/35 and h55/37) and hSIF1, with hSIF1 having the highest affinity for Cdx-2, as previously shown [13, 14].

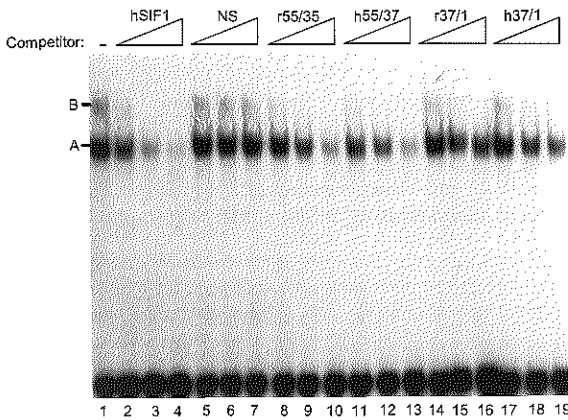

Fig. 3: Cdx-2 has a lower affinity for binding sites in the LPH TATA-box regions than those in SIF1 and CE-LPH1a. EMSAs using hSIF1 as a test probe and Colo-DM nuclear extracts (5 µg) revealed two major complexes (A and B, lane 1), as previously described [5]. Competition EMSAs using a 20-, 50-, and 200-fold molar excess of Cdx-2 binding sites LPH promoters (lanes 8-19) as well as a non-specific competitor (NS) (lanes 5-7) revealed that r37/1 and h37/1 (lanes 14-16 & 17-19, respectively) do not compete for Cdx-2 as well as rat and human CE-LPH1a (r55/35 and h55/37, lanes 8-10 & 11-13, respectively) or hSIF1 (lanes 2-4)

To further localize Cdx-2 binding in the TATA-box region, competition EMSAs were carried out using the following probes: h37/1, which includes the human LPH TATA-box; and h26/1, which does not contain the TATA-box, but includes the first 26 bp of 5′-flanking region (Fig. 4A).

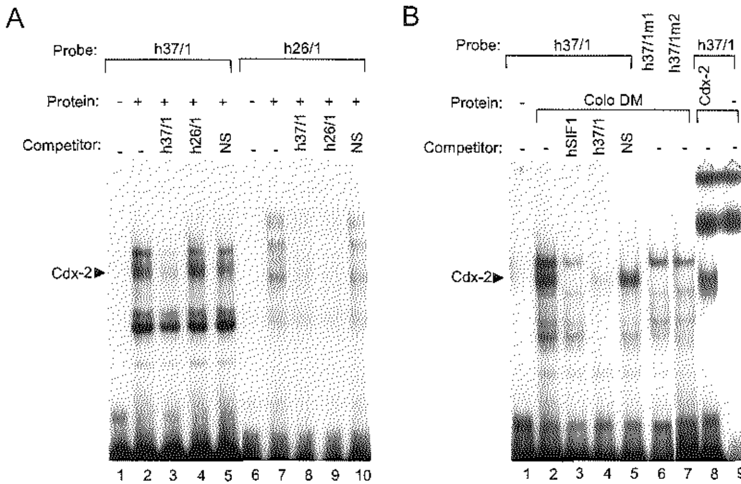

Fig. 4: Cdx-2 binds to the Cdx-2 consensus site overlapping the TATA-box of the human LPH gene. A: Cdx-2 binding is localized to -37 to -26 bp. EMSAs were carried out using h37/1 (lanes 1-5), which includes the human LPH TATA-box, and h26/1 (lanes 6-10), which does not include the TATA-box, as probes, and Colo-DM nuclear extracts (5 µg). The h37/1, h26/1, and non specific (NS) oligonucleotides were used as competitors as indicated across the top of the figure. Protein-DNA complexes formed with the h26/1 probe (lane 7) do not coincide with the mobility of the Cdx-2-specific complex (arrowhead) indicating that this probe does not bind Cdx-2. B: Cdx-2 binds to the consensus sequence overlapping the TATA-box. EMSAs were carried out using h37/1 (lane 1-5, 8 & 9), h37/1m1 (lane 6), and h37/1m2 (lane 7) as probes and Colo-DM nuclear extracts (5 µg) or in vitro synthesized proteins (lanes 8 & 9). The Cdx-2 complex (arrowhead) formed with labeled h37/1 (lane 2) was competed using hSIF1 (lane 3) and h37/1 (lane 4), but not with a non-specific (NS) oligonucleotide. Probes with mutations introduced into the putative Cdx-2 binding site overlapping the TATA-box do not bind Cdx-2 (lanes 6 & 7). In vitro-synthesized, radiolabeled Cdx-2 binds h37/1 (lane 8), further demonstrating the mobility of the Cdx-2/DNA complex using a semi-purified protein rather than a nuclear extract. A negative control labeling reaction (ie, TNT reaction without template) demonstrates a non-specific signal near the top of the gel (lane 9), but not a Cdx-2-specific complex.

Complexes that formed with the h37/1 probe (lane 2) were competed specifically using excess, unlabeled h37/1 oligonucleotide (lane 3), whereas none of the complexes were competed using h26/1 or a non-specific oligonucleotide (lanes 4 and 5, respectively). In contrast, complexes that formed with the h26/1 probe (lane 7) were competed specifically with a molar excess of both h37/1 and h26/1 oligonucleotides (lane 8 and 9, respectively). Protein-DNA complexes observed with the h26/1 probe have different mobilities than those present using the h37/1 probe (lanes 7 and 2) and none form a supershift complex with a Cdx-2-specific antibody (not shown). These data demonstrate that Cdx-2 does not bind to the first 26 bp of the human LPH 5′-flanking region, but does bind to a sequence that includes an 11 bp region between –37 and –26 bp, which contains the human LPH TATA-box and the putative Cdx-2 binding site.

To localize Cdx-2 binding to the consensus sequence overlapping the TATA-box, additional EMSAs were carried out using probes with mutations introduced into the consensus sequence (Fig. 4B). The h37/1 probe forms multiple complexes, only one of which is competed by hSIF1 (lane 3), suggesting that this protein-DNA complex (arrowhead) contains Cdx-2. The specific oligonucleotide, h37/1, competed all bands (lane 4). Probes containing mutations introduced into both the 3′ and 5′ regions of the Cdx-2 site overlapping the TATA-box (h37/1m1 and h37/1m2, respectively) were unable to bind Cdx-2 (lane 6 and 7). In addition, in vitro-synthesized, radiolabeled Cdx-2 demonstrated a single, specific Cdx-2 complex using h37/1 as a probe (lane 8). These data show that Cdx-2 binds to the consensus sequence overlapping the TATA-box in the human LPH promoter.

Cdx-2 is known to bind and activate the LPH promoter at CE-LPH1a[11-16], but the interaction of Cdx-2 with the LPH TATA-box is a novel finding. To characterize the function of the Cdx-2 binding sites at CE-LPH1a and overlapping the TATA-box, transient co-transfection assays were carried out in Caco-2 cells using wild type (h37wt and h59wt) and mutated human LPH promoter-reporter constructs and the pRC-CMV and Cdx-2 expression vectors. The h37m3 construct has a mutation in the Cdx-2 binding site overlapping the TATA-box that disrupts Cdx-2 interactions as confirmed by competition EMSAs (not shown), but does not disrupt TBP-TATA interactions [31]. The h59m1 construct has a mutation in CE-LPH1a that is known to disrupt Cdx-2 binding [15]. As shown in Fig. 5, h37wt, which does not contain CE-LPH1a, was activated 8-fold over baseline by Cdx-2, suggesting that Cdx-2 may activate this construct through interactions at other sites, perhaps though the site overlapping the TATA-box.

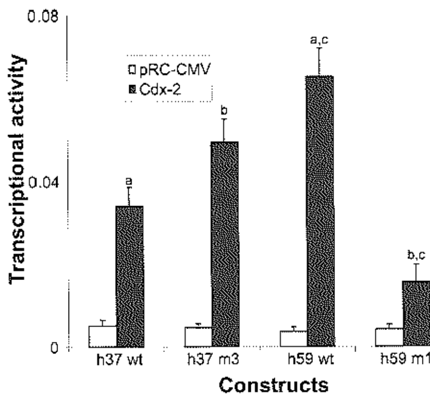

Fig. 5: Identification of Cdx-2 response elements in the human LPH promoter. Transient co-transfection assays were carried out using wild-type and mutated human LPH promoter sequence fused to the human growth hormone reporter, as described in Experimental. Transcriptional activities are expressed relative to that of the active metallothionine promoter. Values are mean ± SEM of n = 4. Values with the same letter are significantly different: a, b, $P < 0.01$; c, $P < 0.001$, using the Tukey-Kramer test.

However, introduction of a mutation into this site, as revealed by h37m3, did not reduce the transcriptional response to Cdx-2, suggesting that an intact Cdx-2 binding site overlapping the TATA box does not mediate Cdx-2 activation. The h59wt construct was activated 24-fold by Cdx-2. The Cdx-2-stimulated transcriptional activity of h59wt was significantly greater than that

of h37wt ($p < 0.01$), suggesting that the addition of CE-LPH-1a results in a greater activation of the LPH promoter by Cdx-2. Introduction of a mutation into CE-LPH 1a (h59m1) that is known to disrupt Cdx-2 binding to DNA [15] resulted in a significantly reduced Cdx-2-mediated activation as compared to the wild type h59wt construct ($p < 0.001$). This demonstrates that the presence of an intact Cdx-2 binding site at CE-LPH1a mediates Cdx-2 activation. The Cdx-2-stimulated transcriptional activity of h59m1 was also lower than that of h37wt and h37m3 ($p < 0.01$) suggesting the presence of a silencer between -59 and -37 bp of the human LPH promoter. Taken together, these data suggest that Cdx-2 activates the LPH promoter through interactions at CE-LPH1a, but not through interactions with the Cdx-2 binding site overlapping the TATA-box, and that a silencer may be present within human CE-LPH1a.

Although it is widely known that Cdx-2 binds to a in CE-LPH1a [11-16], several reports have suggested that non-Cdx-2-proteins also bind to CE-LPH1a [28, 15, 16]. To characterize such proteins, EMSAs were carried out using CE-LPH1a (h59/37) as a probe and nuclear extracts from different cell lines (Fig. 6A). Nuclear extracts from Caco-2 cells form a previously characterized protein-DNA complex (lane 2, arrowhead) [26, 11, 13, 15, 30]. Nuclear extracts from non-intestinal cells, including HeLa, Hep-G2, MDCK, and NIH/3T3 cells, each form a protein-DNA complex with a slower mobility (lanes 3-6) than that of Cdx-2.

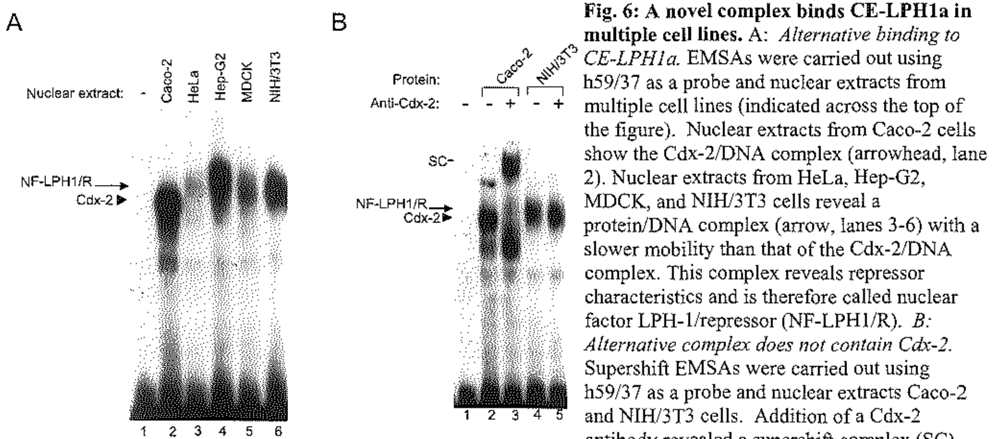

Fig. 6: A novel complex binds CE-LPH1a in multiple cell lines. A: *Alternative binding to CE-LPH1a.* EMSAs were carried out using h59/37 as a probe and nuclear extracts from multiple cell lines (indicated across the top of the figure). Nuclear extracts from Caco-2 cells show the Cdx-2/DNA complex (arrowhead, lane 2). Nuclear extracts from HeLa, Hep-G2, MDCK, and NIH/3T3 cells reveal a protein/DNA complex (arrow, lanes 3-6) with a slower mobility than that of the Cdx-2/DNA complex. This complex reveals repressor characteristics and is therefore called nuclear factor LPH-1/repressor (NF-LPH1/R). *B: Alternative complex does not contain Cdx-2.* Supershift EMSAs were carried out using h59/37 as a probe and nuclear extracts Caco-2 and NIH/3T3 cells. Addition of a Cdx-2 antibody revealed a supershift complex (SC) with Caco-2 nuclear extracts (lane 3), but not with NIH/3T3 nuclear extracts (lane 5) indicating that the alternative complex does not contain Cdx-2.

This complex does not contain Cdx-2, as revealed by supershift EMSAs using NIH/3T3 nuclear extracts and a Cdx-2 antibody (Fig. 6B). These data suggest an alternative binding activity occurring at CE-LPH1a in multiple cell lines that is distinct from Cdx-2. As shown below, this complex reveals repressor characteristics and is therefore called nuclear factor LPH-1/repressor (NF-LPH1/R).

To characterize the DNA binding characteristics of NF-LPH1/R, competition EMSAs were carried out using Caco-2 and NIH/3T3 nuclear extracts (Fig. 7). Caco-2 cells were used as a source of Cdx-2 in order to define the mobility of the Cdx-2 complex, and NIH/3T3 cells were chosen because these cells demonstrate abundant NF-LPH1/R and do not synthesize Cdx-2 (Fig. 6).

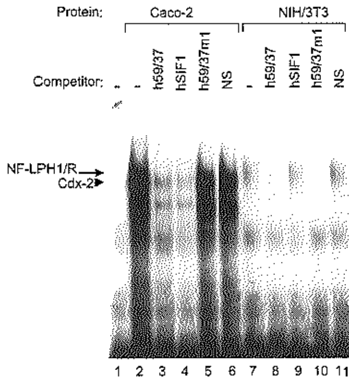

Fig. 7: NF-LPH1/R does not bind to the consensus Cdx-2 site. EMSAs were carried out using h59/37 as a probe and nuclear extracts from Caco-2 or NIH/3T3 cells. Nuclear extracts from Caco-2 cells (lane 2) revealed binding of Cdx-2 (arrowhead, lane 2). The h59/37 and hSIF1 oligonucleotides competed for Cdx-2, but h59/37m1 and a non-specific (NS) oligonucleotide did not compete for Cdx-2 (lanes 3-6, respectively). Nuclear extracts from NIH/3T3 cells (lane 7) revealed binding of NF-LPH1/R (arrow). The oligonucleotides h59/37 (lane 8) and h59/37m1 (lane 10) competed for NF-LPH1/R, but the hSIF1 (lane 9) and NS (lane 11) oligonucleotides did not compete for NF-LPH1/R.

Nuclear extracts from Caco-2 cells demonstrated a Cdx-2 complex that was competed by h59/37 and hSIF1, but not by h59/37m1, which does not bind Cdx-2 [15], or a non-specific oligonucleotide (NS). In contrast, NF-LPH1/R was competed away by h59/37 and h59/37m1, but not by hSIF1 or a non-specific competitor. These data demonstrate that NF-LPH1/R binds to a different sequence than that of Cdx-2.

Data from co-transfection assays described in Fig. 5 are consistent with silencer activity associated with CE-LPH1a. To determine if NF-LPH1/R is a candidate repressor that binds CE-LPH1a, the -59 to -37 bp region was transferred upstream of the active thymidine kinase (TK) promoter, and transient transfection assays were carried out in NIH/3T3 cells, which synthesize NF-LPH1/R, but not Cdx-2. As shown in Fig. 8, CE-LPH1a was transferred in single (TK1x) and multiple (TK4x) copies.

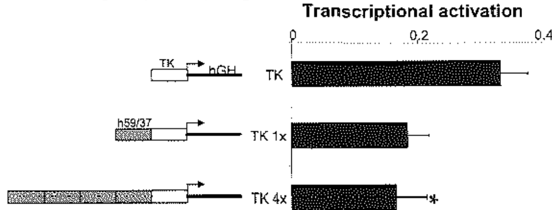

Fig. 8: NF-LPH1/R has repressor activity that is transferable to a heterologous promoter. Transient transfection assays were carried out using NIH/3T3 cells, which synthesize NF-LPH1/R, but not Cdx-2. A schematic representation of the promoter-reporter constructs is shown on the left. An open box indicates the TK promoter; a grey box indicates the h59/37 region; and the solid line indicates the human growth hormone (hGH) reporter. Transcriptional activity is expressed relative to pXGH-5 as described in Experimental. Data are mean ± SEM of n = 4. * $p<0.05$ compared to TK, using the Dunnett test.

TK1x demonstrated a consistently lower transcriptional activity than that of the TK promoter alone, but failed to demonstrate statistical significance. However, TK4x, which contains four copies of the -59 to -37 bp region, was significantly lower than that of TK alone ($p < 0.05$) demonstrating an approximate 50 % reduction. These data revealed that in the absence of Cdx-2, but in the presence of NF-LPH1/R, CE-LPH1a mediates silencer activity. These data are consistent with the hypothesis that NF-LPH1/R represses the LPH promoter in cells that do not produce Cdx-2.

DISCUSSION

Cdx-2 is an intestine-specific homeodomain transcription factor that regulates the promoters of intestinal genes through specific interactions with the consensus sequence TTTAY. Here, we show that Cdx-2 not only activates the promoter of the intestinal LPH gene through interactions with the consensus site within CE-LPH1a at -54 to -40 bp, as previously demonstrated by us [13] and others [11-16], but also that Cdx-2 associates with a binding site overlapping the LPH TATA-box. We further show that a non-Cdx-2 protein also interacts with CE-LPH1a. Our data are consistent with the hypothesis that this protein, termed NF-LPH1/R, which is expressed in multiple cell lines, represses the human LPH promoter.

Our data demonstrate that Cdx-2 is capable of binding to the TATA-boxes of both the rat and human LPH genes. This finding is consistent with previous reports that have shown that Cdx-2 binds also to Cdx-2 sites overlapping the TATA-boxes of other genes, including those of rat clusterin [17] and rat and human CaBP9K [18, 19]. For the rat clusterin gene, Cdx-2 activates a promoter-reporter construct containing rat clusterin 5′-flanking and untranslated sequence in which a single Cdx-2 binding site overlapping the TATA-box is present [17]. For the CaBP9K genes, which also contain Cdx-2 binding sites overlapping their TATA-boxes, Cdx-2 both activates and represses a reporter fused to the CaBP9K promoter depending on the promoter (rat vs. human) and cell line used for transfection [18, 19]. Cdx-2 may repress the Cdx-2 promoter at its own TATA-box [29]. However, none of these studies [18, 19, 17] introduced a mutation into the Cdx-2 consensus binding site overlapping their respective TATA-boxes, and thus the importance of this site for Cdx-2 regulation is unknown.

In the present study, we introduced a novel mutation into the human LPH TATA-box that disrupts Cdx-2 binding, but not TBP binding. This mutation is based on a study by Wu et. al., [31] which showed that the TATA-box (TATAAA) of the adenovirus major late promoter (AdMLP) containing a mutation (TATAtA) binds TBP at a higher affinity than that of the wild type sequence, but demonstrates a lower transcriptional activation of the AdMLP promoter. Interestingly, a parallel mutation introduced into the TATA-box of the human LPH gene, which disrupts Cdx-2 binding (confirmed by EMSAs), was activated by Cdx-2 to a mean value that was not decreased from that of the wild type promoter. Since no other Cdx-2 binding sites are present within the first 37 bp of the LPH 5′-flanking sequence, Cdx-2 activation of the human LPH construct containing only 37 bp of 5′-flanking region is most likely due to direct protein-protein interaction with the TBP complex. Similar mechanisms have been reported for the transcriptional activation of this construct by GATA and HNF-1 transcription factors [13]. Our data are consistent with the hypothesis that Cdx-2 is capable of binding to the Cdx-2 consensus site overlapping the LPH TATA-box, and may even compete with TBP for binding to this site. Support for this mechanism is given by a report that suggested competition by Cdx-2 with TBP at the TATA-box of its own promoter [29], and a report that demonstrated competition by PRH, a homeodomain protein, with TBP for binding at the TATA-box of genes expressed in hematopoietic cells [32]. The importance of Cdx-2/TATA interactions in vivo is unknown at this time.

Although it is widely known that Cdx-2 binds to CE-LPH1a [11-16], several reports have suggested that non-Cdx-2-proteins also bind to CE-LPH1a [28, 15, 16]. Using EMSAs, several studies reveal the presence of non-Cdx-2 complexes interacting at CE-LPH1a, but none report a function associated with this binding activity [26, 11, 12, 14]. Mitchelmore et. al. [28] suggested that a homeodomain protein, HOXC11, interacts at CE-LPH1a and potentiates HNF-1α-dependent transcription of the LPH promoter, but did not report direct protein binding to this site. Further, Troelsen et al. [15] reported that a specific mutation outside of the Cdx-2 binding site at CE-LPH1a demonstrated a higher transcriptional activity than that of the wild type

promoter suggesting the presence of a silencing element within CE-LPH1a, but did not report an alternative complex forming at CE-LPH1a by EMSAs. The present report links for the first time binding of a non-Cdx-2-complex (NF-LPH1/R) to CE-LPH1a with repression of the human LPH promoter. NF-LPH1/R binds to a site different from that of Cdx-2, and is produced in multiple cell lines. Binding of NF-LPH1/R to CE-LPH1a was consistently associated with a decrease in transcriptional activity, both in the wild type context (Fig. 5) and as a heterologous promoter (Fig. 8), supporting a repressor role for this protein complex.

The close proximity of Cdx-2 and NF-LPH1/R binding within CE-LPH1a suggests that the binding of these proteins is mutually exclusive. The complex that binds to CE-LPH1a (Cdx-2 and NF-LPH1/R) would therefore depend on the relative abundance and affinity of each protein for their respective consensus binding sites. Accordingly, NF-LPH1/R could act as a general repressor of LPH gene transcription in multiple tissues, but in the presence of Cdx-2, such as in intestinal epithelium, Cdx-2 could preferentially bind CE-LPH1a and activate the LPH promoter in a tissue-specific manner. We have previously shown that GATA and HNF-1 transcription factors synergistically activate an LPH promoter-reporter construct containing 118 bp of 5′-flanking sequence, which includes GATA and HNF-1 binding sites [13, 33]. This and the present study support a model in which NF-LPH1/R acts as a repressor of LPH transcription in non-Cdx-2 producing cells (i.e., non-intestinal cells), that this repression is released by Cdx-2, and that overlapping expression of GATA factors with HNF-1α results in high levels of intestine-specific LPH gene expression.

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Chapter 5

Physical interaction between GATA-5 and HNF-1α results in synergistic activation of the human lactase-phlorizin hydrolase promoter

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ABSTRACT

GATA-4, -5, and -6 zinc finger and HNF-1α homeodomain transcription factors are expressed in the intestinal epithelium and synergistically activate the promoter of intestinal genes. Here, we demonstrate that GATA-5 and HNF-1α physically associate both in vivo and in vitro, and that this interaction is necessary for cooperative activation of the lactase-phlorizin hydrolase (LPH) promoter. Further, physical association is mediated by the C-terminal zinc finger of GATA factors and the homeodomain of HNF-1α. Deletion of HNF-1α activation domains or interruption of HNF-1 binding sites in the LPH promoter results in a complete loss of cooperativity, whereas deletion of GATA-5 activation domains or interruption of GATA binding sites results in a reduction, but not an elimination of cooperativity. We hypothesize that GATA/HNF-1α-cooperativity is mediated by HNF-1α through its activation domains which are oriented for high levels of activation through binding to DNA and physical association with GATA factors. These data suggest a paradigm whereby intestine-specific gene expression is regulated by unique interactions among tissue-restricted transcription factors co-expressed in the intestine. Parallel mechanisms in other tissue as well as in *Drosophila* suggest that zinc finger/homeodomain interactions are an efficient pathway of cooperative activation of gene transcription that has been conserved throughout evolution.

INTRODUCTION

The intestinal epithelium is a dynamic structure that undergoes a highly regulated process of cell division, migration, cell fate determination, and differentiation (1-3). During intestinal development, interactions between visceral endoderm and mesoderm at E8 in mice result in the formation of a primitive foregut that rapidly undergoes cytodifferentiation so that by E19, an epithelial monolayer overlies nascent villi. During the first two weeks of postnatal life, a proliferating compartment develops into the crypts of Lieberkühn. Stem cells located near the base of crypts rapidly divide and give rise to four terminally differentiated cell types, which migrate both basally and apically. Cells migrating to the base of crypts become Paneth cells, whereas those migrating up the crypt toward villi become absorptive enterocytes, goblet cells, and enteroendocrine cells. At the crypt-villus junction, the proliferative phase ends and cells acquire a differentiated phenotype characterized by the synthesis of functionally relevant proteins. The cells continue to migrate up the villi, enter an apoptotic cycle, and are shed into the intestinal lumen approximately three days after their initial appearance on villi. The molecular mechanisms underlying the dynamic processes of intestine-specific gene expression and cellular differentiation during development are poorly understood.

Absorptive enterocytes comprise ~95% of epithelial cells on villi and are the cells responsible for the terminal digestion and absorption of nutrients. Lactase-phlorizin hydrolase (LPH), the enzyme critical for the digestion of milk lactose, is an absorptive enterocyte-specific protein that serves as a marker for intestine-specific gene expression and intestinal differentiation (4,5). In rats, LPH mRNA is detected as early as E18 in the proximal intestine when primitive villi are formed (6). LPH expression is highest at birth and continues to be highly expressed throughout the suckling period. After weaning, LPH expression per enterocyte is reduced and is also restricted to the jejunum and proximal ileum (4,5). This developmental decline also occurs in humans at around age 5, although a subset of the human population continues to synthesize high levels of LPH throughout adulthood (7,8). The close correlation between the lactase activity and its mRNA in rats (4,9), and humans (10,11), and transcription rate experiments (4) indicate that LPH is regulated mainly by gene transcription.

Transgenic studies indicate that information for enterocyte-specific LPH gene expression in vivo is contained in the 5′-flanking region (12-14). Identification of specific binding sites within the first 100 bp of 5′-flanking region in the LPH genes of several species has led to the demonstration that GATA, hepatocyte nuclear factor-1α (HNF-1α), and Cdx-2 transcription factors are activators of the LPH promoter (15-21). Due to the close proximity of GATA and HNF-1 sites on the LPH promoter, and the near-exclusive co-expression of these two families of transcription factors in intestinal epithelium (22-29), we hypothesized that members of the GATA and HNF-1 families of transcription factors interact to modulate LPH gene expression.

The GATA family of transcription factors has been implicated in cell lineage differentiation during vertebrate development. Defined by two evolutionarily conserved zinc fingers of the motif Cys-X_2-Cys-X_{17}-Cys-X_2-Cys that mediate binding to the consensus DNA sequence WGATAR (W = A or T, R = A or G) , the GATA family is generally categorized into two classes based on expression patterns and amino acid homologies. GATA-1, -2, and -3 are expressed in developing bone marrow cells and are critical for hematopoiesis (30), whereas GATA-4, -5, and -6 have a more diverse pattern of expression that includes small intestine, heart, liver, lung, and gonad (22-24,31-33). The GATA-4, -5, -6 subfamily has been shown to modulate promoter function of intestinal genes including the rat and human LPH (16,17,21), human sucrase-isomaltase (SI) (21), and *Xenopus* intestinal fatty acid binding protein (I-FABP) (25) genes. Although the wide-ranging expression patterns of GATA-4, -5, and -6 argue against these proteins as being master regulators of tissue- or cell-type-specific gene expression, there is

increasing evidence that this subfamily might be critical in regulating cell-specific gene expression through unique interactions with other semi-restricted transcription factors and co-factors (34).

HNF-1α is a member of a transcription factor family that contains a modified homeodomain and binds as dimers to the consensus sequence, GTTAATNATTAAC (35,36). Originally thought to be liver-specific, HNF-1α is expressed in the intestinal epithelium (26-29,37), and has been shown to modulate the promoter of many genes expressed in the intestine (18,20,21,38-45). We have recently shown that GATA subfamily members and HNF-1α synergistically activate the LPH and SI promoters (21), suggesting that members of these two transcription factor families interact to produce high levels of enterocyte-specific gene expression.

The goal of the present experiments was to characterize the role that GATA and HNF-1α transcription factors play in regulating intestine-specific gene expression by defining the mechanism by which these two transcription factors function to synergistically activate the human LPH promoter. In these studies, the importance of critical structures in GATA factors and HNF-1α, including domains responsible for protein-protein interaction, DNA-binding domains, and activation domains, for cooperative activation was determined. Using GATA-5 as a model, the results of this study reveal that physical association between GATA-5 and HNF-1α is required for the synergistic activation of the human LPH promoter, and that this interaction occurs through the C-terminal zinc finger and basic region of GATA-5 and the homeodomain of HNF-1α. Identification of parallel mechanisms of protein-protein interaction between zinc finger and homeodomain proteins in other vertebrate tissue (46-48) as well as in *Drosophila* (49,50) suggest that zinc finger/homeodomain interactions are an efficient mechanism for synergistic activation of gene transcription that has been conserved throughout evolution.

MATERIALS AND METHODS

Plasmids–Previously characterized expression vectors for mouse GATA-4 (G4-CMV), (51), GATA-5 (G5-CMV), (24) (gifts of M. Parmacek, University of Pennsylvania) and HNF-1α (27) (gift of G. Crabtree, Stanford University) were obtained for these studies. Since the original HNF-1α expression vector replicates inefficiently during bacterial amplification, the HNF-1α coding region was PCR-amplified (5′-ATACGGATCCATGGTTTCTAAGCTGAGCCAGCTG-3′ and 5′-GTATGAATTCCTTACTGGGAAGAGGAGGCCATCTG-3′) and subcloned into the *BamHI* and *EcoRI* sites of pcDNA1 (called H1α-CMV). This plasmid was amplified efficiently in E. coli DH5α cells.

For in vivo protein-protein interaction studies, an expression vector for FLAG-tagged GATA-5 (G5-FLAG) was constructed by fusing the GATA-5 coding region to that of FLAG in the pFLAG-CMV™-2 expression vector (Sigma). This was done using site-directed mutagenesis (52) by inserting a second *EcoRI* site at the 5′ side of the GATA-5 coding region (mutagenic oligonucleotide, 5′-CTTTGGTACATGGAATTCGAGAGCTCCCAAC-3′) resulting in *EcoRI* sites flanking the open reading frame. The GATA-5 coding region was then subcloned in-frame into the *EcoRI* site of pFLAG-CMV™-2, oriented by restriction digests, and confirmed by sequencing.

For in vitro protein-protein interaction studies, glutathione S-transferase (GST) fusion vectors were constructed for GATA-5 (GST-G5) and HNF-1α (GST-H1α). GST-G5 and GST-H1α were made by site-directed mutagenesis and PCR amplification, respectively, by

introducing *BamHI* and *EcoRI* sites adjacent to the start and stop codons: GATA-5, 5′-CAAGCTTTGGTACATGGATCCTCCCCGCGCGA-3′, 5′-GGTGACAGTTTCCGAATTCCCTAGGCCAAG-3′; HNF-1α, 5′-ATACGGATCCATGGTTTCTAAGCTGAGCCAGCTG-3′, 5′-GTATGAATTCCTTACTGGGAAGAGGAGGCCATCTG-3′. Both constructs were subsequently subcloned in-frame into the *BamHI* and *EcoRI* sites of pGEX-2TK (Promega). The constructs were confirmed by sequencing.

For transfection studies, the human LPH promoter containing 118 bp of 5′-flanking region was fused 5′ to the human growth hormone reporter (called h118wt), as previously described (21). This region contains two GATA sites and an HNF-1 site as previously described (21). Transcription factor-DNA interactions were characterized, in part, using human promoter-reporter constructs containing mutations in the two GATA sites (h118mG1G2), in the HNF-1 site (h118mH), and in all three sites together (h118mG1G2H). Mutations introduced into these sites, have been previously shown to disrupt specific protein-DNA interactions (21). pRC-CMV (Invitrogen) served as a negative control expression vector for all co-transfection experiments.

Immunoprecipitation of FLAG™-tagged protein–In vivo GATA-5/HNF-1α interactions were determined by supershift EMSA detection of HNF-1α in proteins from Cos-7 cells co-transfected with G5-FLAG and H1α-CMV and immunoprecipitated with anti-FLAG antibodies. Cos-7 cells were co-transfected at 70% confluence with G5-FLAG and H1α-CMV using Effectene™ reagent (Qiagen) according to the manufacturer's protocol. After two days, confluent 100 mm plates were washed using 10 ml of cold PBS and scraped in 1 ml PBS. Cells were then pelleted in a microfuge at 4° C and supernatants were discarded. The pelleted cells were resuspended in 150 μl of lysis buffer (10 mM HEPES pH 7.0, 1 mM EDTA, 60 mM KCl, 0.5% Nonidet P-40, 1 mM DTT, and 1% proteinase inhibitor [Sigma]), incubated on ice for 5 min, and centrifuged for 5 min at 4° C. The supernatants were discarded and the pelleted nuclei were resuspended in 650 μl of Buffer A (150 mM NaCl, 40 mM Tris pH 8, 10% glycerol, 0.3% Nonidet P-40, and 1% proteinase inhibitor) and incubated on ice for 20 min. After centrifugation for 10 minutes at 4° C, the supernatants were transferred to a new tube and incubated for 1 hour with protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) to clear non-specific proteins. Beads were pelleted and supernatants were transferred to a fresh tube. Anti-FLAG™ M2 affinity gel (Sigma), which is a purified murine anti-FLAG IgG_1 monoclonal antibody attached to agarose, was gently mixed with nuclear extracts for 5 hours at 4° C. The agarose was then washed 3 times in Buffer A followed by 3 washes in 1x EMSA buffer (20 mM HEPES pH 7.5, 60 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 12.5% glycerol). GATA-5-FLAG/HNF-1α complexes were than released by incubating the agarose in 40 μl 0.8% deoxycholic acid (Sigma) on ice for 15 min. After adding 10% Nonidet P-40 to final ~1%, the agarose was pelleted and 12 μl were analyzed by EMSAs.

Electrophoretic mobility shift assays (EMSAs)–EMSAs were carried out as previously described (21) using previously characterized GATA and HNF-1 binding sites as probes and competitors. These sites included the GATA site from the Xenopus intestinal-fatty acid binding protein gene (xIFABP) (25) and the HNF-1 site in the rat beta-fibinogen gene (rβ-Fib) (53). Probes were made by annealing single-stranded oligonucleotides and extending with [^{32}P] dATP (DuPont-New England Nuclear) using the large fragment of DNA polymerase I (Klenow, Gibco-BRL) as previously described (21). The specific activity of all probes exceeded 10^6 cpm/pmol. Proteins were incubated with 10,000 cpm of probe for 20 min at RT and separated in 8% non-denaturing polyacrylamide gels. For competition or supershift EMSAs, competitors (200-fold molar excess) or antibodies (1 μl, undiluted), respectively, were pre-incubated with the nuclear

extract for 10 minutes prior to the addition of the probe. All antibodies were purchased from Santa Cruz Biotechnology.

Site-directed mutagenesis–Site-directed mutagenesis was carried out using the method of Kunkel et. al. (52). To delete or disrupt specific structures within GATA-5 and HNF-1α, mutations were introduced into G5-pGEM, which contains the GATA-5 coding region oriented for efficient transcription from the SP6 RNA polymerase site in pGEM-7Zf(-) (21), and HNF-1α-CMV. The G5-pGEM vector was used because mutagenesis with G5-CMV was not achieved. DNA containing GATA-5 mutants synthesized from the G5-pGEM uracil template were re-inserted into the pcDNA-3 expression vector for transient transfection studies using a *BamHI*/*EcoR*I subcloning strategy. Fig. 1 shows the mutagenic oligonucleotides used in this study. The oligonucleotides were phosphorylated using T4 polynucleotide kinase and ATP, and annealed to the uracil templates by incubation for 2 minutes at 70°C, followed by slow cooling to 40° C. Uracil templates for both G5-pGEM and H1α-CMV required the use of reverse strand oligonucleotides. After annealing, a double-stranded hybrid was synthesized using T7 DNA polymerase and T4 DNA ligase, and used to transform competent E. coli cells. After an overnight incubation at 37°C, plasmids were isolated using a mini-prep kit (Qiagen) and tested with *BamH*I or *EcoR*I digest. The identities of the mutant clones were confirmed by DNA sequencing.

Name	Oligonucleotide	Mutation	Effect
GATA mutations			
mut 1	5'-GGTGTTTGCTGT*GAATTC***TTA**GGAGCCCTTGAT-3'	**Stop**, *EcoRI*	Deletion of C-terminal domain
mut 2	5'-TGTCTGGATACTCT*GAATTC***ACA**TTGCCAGTGGC-3'	**Stop**, *EcoRI*	Deletion of C-terminal domain, basic region
mut 3	5'-GAAGGCTCCGGA**CAT***GGATCC*AGGGGCTACGTC-3'	*BamHI*, **Start**	Deletion of activation domains
mut 4	5'-TTGACCCCGTT**CAT***GGATCC*ATAGAGGCCGCAG-3'	*BamHI*, **Start**	Deletion of activation domains, Zn finger I
mut 5	5'-GCCACAGGCGT**TAGA**CACTGGTTCGCC-3'	**C270S**	Cysteine to serine substitution in Zn finger II
HNF-1α mutations			
mut 1	5'-GGTGGAGGTCC*GAATTC***TCA**GTCCATGGCCAAC-3'	**Stop**, *EcoRI*	Deletion of activation domains
mut 2	5'-GTAGACACGCAC*GAATTC***TCA**AAGGTTGGAGCC-3'	**Stop**, *EcoRI*	Deletion of activation domains and partial deletion of the homeodomain

Fig. 1: Oligonucleotides used for the introduction of mutations in GATA-5 and HNF-1α. The name, oligonucleotide sequence, type of mutation, and effect of the mutation are indicated. Uracil templates for both G5-pGEM and H1α-CMV required the use of reverse strand oligonucleotides.

In vitro transcription and translation–Unlabeled and labeled wild-type and mutant GATA-5 and HNF-1α proteins were synthesized using the TNT™ transcription/translation kit (Promega) according to the manufacturers instructions. G5-pGEM and H1α-CMV utilized SP6 and T7 RNA polymerases, respectively. Labeled proteins were synthesized using [^{35}S]-methionine (Redivue, Amersham-Pharmacia Biotech).

GST pull-down assays–To characterize in vitro protein-protein interactions, GST pull-down assays were carried out. GST fusion proteins were expressed in E. coli DH5α and purified using GSH-Sepharose (Amersham Pharmacia Biotech) beads according to standard protocols

(54). Beads coated with glutathione were incubated in 160 μl of binding buffer (20 mM HEPES pH 7.5, 100 mM KCl, 5 mM EDTA, 5 mM EGTA, 0.5% BSA) and mixed for 2 hours at 4°C. The beads were then washed 5x with PBS and bound proteins were released by boiling in SDS-sample buffer and resolved by SDS/PAGE. The gels (10% polyacrylamide) were run at constant voltage (150V) for 40 minutes, dried, and exposed to film.

Cell culture and transient co-transfection assays–HeLa cells were used for transient co-transfection assays because GATA-specific and GATA/HNF-1α cooperative activation can be distinguished in cells that do not synthesize endogenous HNF-1α. Transient co-transfection assays were carried out by electroporation as previously described (21). The amount of human growth hormone reporter secreted into the media over 24 h was measured using an [^{125}I] radioimmunoassay kit (Allegro hGH, Nichols Institute). To control for transfection efficiency, all transcriptional activities were expressed relative to pXGH5, a constitutively active metallothionine-I promoter fused to the human growth hormone gene.

Statistics–The t test or the one-way analysis of variance (ANOVA) was employed in all statistical analyses using InStat software (Graphpad Software, Inc.). Multiple comparisons were carried out by the Dunnett multiple comparison test.

RESULTS

GATA-5 and HNF-1α synergistically activate the human LPH promoter (21) suggesting that these two transcription factors physically associate. To test the hypothesis that GATA-5 and HNF-1α physically associate in vivo, Cos-7 cells were co-transfected with G5-FLAG and H1α-CMV, and FLAG-associated proteins were immunoprecipitated from nuclear extracts using beads coated with anti-FLAG antibodies. Detection of HNF-1α in the G5-FLAG immunoprecipitate indicates GATA-5/HNF-1α interactions.
As shown by EMSAs using an HNF-1 binding site as a probe (Fig. 2), a protein/DNA complex was identified that supershifts with an HNF-1α antibody demonstrating that HNF-1α was present in the anti-FLAG immunoprecipitate from G5-FLAG-transfected cells.

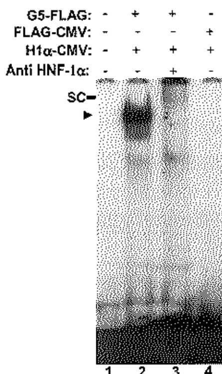


Fig. 2: GATA-5 and HNF-1α physically associate in vivo. EMSAs using the rβ-Fib probe (53) were carried out on nuclear extracts from Cos-7 cells that were co-transfected with the G5-FLAG and H1α, and immunoprecipitated using beads coated with anti-FLAG antibodies. A major protein/DNA complex (arrowhead) was detected (lane 2) that forms a supershift complex (SC) using an HNF-1α antibody (lane 3). Nuclear extracts from Cos-7 cells co-transfected with FLAG-CMV and H1α were used as a negative control (lane 4).

HNF-1α was not detected in nuclear extracts from Cos-7 cells co-transfected with FLAG-CMV and H1α-CMV indicating that HNF-1α does not bind to FLAG alone. These data demonstrate that HNF-1α physically associates with GATA-5 in vivo.

To characterize critical domains in GATA-5 necessary for physical association and functional cooperativity with HNF-1α, mutations were introduced into GATA-5 to delete or disrupt specific structures (Fig. 3A). The capacity of wild type and mutant GATA-5 proteins to

associate physically with HNF-1α in vitro was then tested by GST pull-down assays using GST fused to HNF-1α. As indicated by the presence of a labeled protein on SDS-polyacrylamide gels (Fig. 3B, upper panel), GATA-5 proteins that have intact C-terminal zinc finger and basic regions (GATA-5 wild type, mut 1, mut 3, and mut 4) are pulled down by GST-H1α. In contrast, GATA-5 mut 2, in which the basic region and C-terminal domain are deleted, and GATA-5 mut 5, in which the structure of the C-terminal zinc finger is disrupted, are not pulled down by GST-H1α.

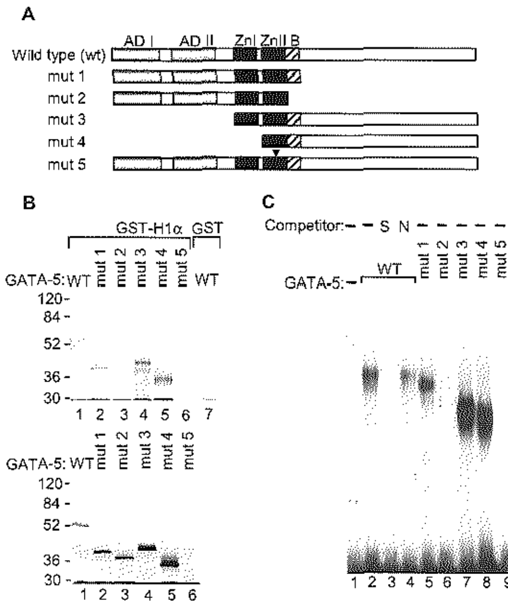


Fig. 3: C-terminal zinc finger and basic regions of GATA-5 are necessary for physical association with HNF-1α and binding to DNA. *A: Schematic representation of wild type and mutant GATA-5 proteins.* GATA-5 contains two N-terminal activation domains (AD I and AD II), two zinc fingers (Zn I, N-terminal zinc finger; Zn II, C-terminal zinc finger), and a basic region (B) near the C-terminus (24). The C-terminal domain is deleted in mut 1, whereas the C-terminal domain and basic region are deleted in mut 2. Both activation domains are deleted in mut 3, whereas both activation domains and the N-terminal zinc finger are deleted in mut 4. GATA-5 mut 5 is a cysteine-to-serine substitution in the C-terminal zinc finger at amino acid 270. *B. The C-terminal zinc finger and basic region of GATA-5 are necessary for physical association with HNF-1α.* GST pull-down assays (upper panel) were carried out using GST-H1α incubated with labeled in vitro transcribed and translated wild type (lane 1) and mutant GATA-5 (lanes 2-6). GATA-5 wild type, mut 1, mut 3, and mut 4, were pulled down by GST-H1α whereas GATA-5 mut 2 and mut 5 were not. GATA-5 was not pulled down by GST alone (lane 7). All proteins used in GST pull-down assays were synthesized as shown by direct loading of TNT products (lower panel). *C. The C-terminal zinc finger and basic region are critical for binding to DNA.* EMSAs were carried out using the xIFABP probe (25) and wild type and mutant GATA-5 proteins as indicated across the top of the figure (lanes 2-8). GATA-5 wild type forms a complex with (lane 2) that is competed with a specific oligonucleotide (S, lane 3) but not with a non-specific oligonucleotide (N, lane 4). GATA-5 wild type, mut 1, mut 3, and mut 4 all bind to DNA, whereas GATA-5 mut 2 and mut 5 do not bind to DNA.

GST alone does not pull down wild type GATA-5 indicating that GST does not interact with GATA-5. Direct loading of labeled TNT products (Fig. 3B, lower panel) indicates that proteins of predicted sizes were synthesized. These data demonstrate that the C-terminal zinc finger and basic regions of GATA-5 are required for physical association with HNF-1α.

To map DNA binding domains in GATA-5, EMSAs were carried out using a GATA binding site as a probe and in-vitro synthesized wild type and mutant GATA-5 proteins (Fig. 3C). A protein/DNA complex is formed with wild type GATA-5 that is competed away by a specific oligonucleotide but not by a non-specific oligonucleotide, indicating that the GATA-5 binds the DNA specifically. GATA-5 mut 1, mut 3, and mut 4 bind DNA but GATA-5 mut 2 and mut 5 do not. These data are parallel to the GST pull-down experiments demonstrating that the domains critical for GATA-5/DNA interaction also map to the C-terminal zinc finger and basic region. Thus, regions in GATA-5 that mediate protein-protein interaction with HNF-1α and DNA binding are co-localized to the C-terminal zinc finger and basic regions.

The functional importance of specific structures in GATA-5 for synergistic GATA-5/HNF-1α activation of the human LPH promoter was tested by transient co-transfection assays

using wild type or mutant GATA-5, and wild type HNF-1α expression vectors (Fig. 4). To test specific effects of transfected wild type and mutant GATA-5 and HNF-1α, a model system utilizing cells that do not synthesize appreciable amounts of these factors was necessary. HeLa cells provided such a model system (15,16,25,55) and were used throughout the remainder of this study. pRC-CMV was used as a negative control. The human LPH promoter (h118wt) was independently activated by HNF-1α, as previously described in Caco-2 cells (21), whereas GATA-5 alone only minimally activated this promoter. The human LPH promoter was synergistically activated by the combination of GATA-5 plus HNF-1α together, as indicated by a

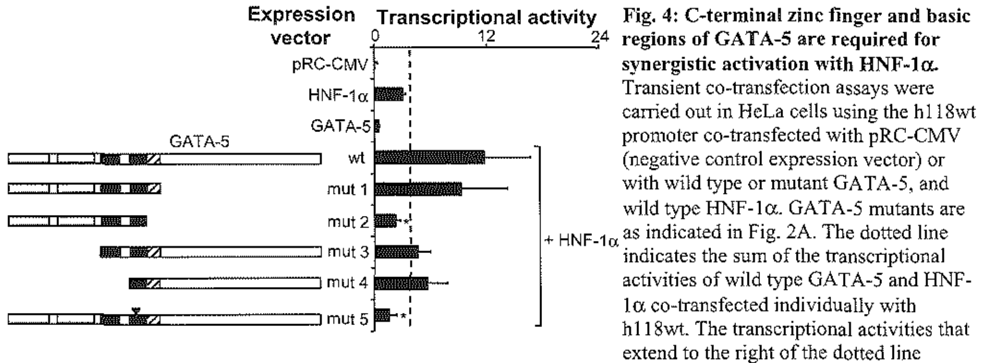


Fig. 4: C-terminal zinc finger and basic regions of GATA-5 are required for synergistic activation with HNF-1α. Transient co-transfection assays were carried out in HeLa cells using the h118wt promoter co-transfected with pRC-CMV (negative control expression vector) or with wild type or mutant GATA-5, and wild type HNF-1α. GATA-5 mutants are as indicated in Fig. 2A. The dotted line indicates the sum of the transcriptional activities of wild type GATA-5 and HNF-1α co-transfected individually with h118wt. The transcriptional activities that extend to the right of the dotted line indicate synergistic activation. Data are mean ± SEM of n = 5. * $p<0.05$ compared to h118wt co-transfected with GATA-5 and HNF-1α.

transcriptional activity that was ~3 fold greater than the sum of the individual transcriptional activities of GATA-5 and HNF-1α alone (indicated by the dotted line). GATA-5 mut 1, mut 3, and mut 4 all demonstrate synergistic activation of the human LPH promoter when co-transfected with HNF-1α as indicated by a mean activation that extends to the right of the dotted line. Noteworthy, however, is that synergy, although present, is greatly reduced with muts 3 and 4, which do not contain GATA-5 activation domains. GATA-5 mut 2 and mut 5, which do not physically associate with HNF-1α or bind to DNA, fail to show synergistic activation of the human LPH promoter. These data suggest that the C-terminal zinc-finger and basic region of GATA-5 are required for synergistic activation of the human LPH promoter.

To characterize functional domains in HNF-1α, mutations were introduced into wild type HNF-1α that result in C-terminal deletions (Fig. 5A). The capacity of these proteins to physically associate with GATA-5 in vitro was assessed by GST pull-down assays using GST linked to GATA-5 (Fig. 5B, upper panel). As indicated by the presence of labeled protein on SDS polyacrylamide gels (Fig. 5B, upper panel), wild type HNF-1α (filled arrowhead) and HNF-1α mut 1 (open arrowhead), in which the activation domains are deleted, physically associate with GST-G5 (lanes 1 and 2, respectively). However, HNF-1α mut 2, in which the activation domains plus a segment of the C-terminal side of the homeodomain are deleted, does not physically associate with GST-G5. All proteins were synthesized as shown by direct loading of the TNT reactions (Fig. 5B, lower panel).

DNA binding domains in HNF-1α were mapped by EMSAs using wild type and mutant HNF-1α synthesized in vitro (Fig. 5C). Wild type HNF-1α forms a protein/DNA complex which supershifts using an HNF-1α antibody, indicating that the complex contains HNF-1α. HNF-1α mut 1 binds DNA (lane 4), but HNF-1α mut 2 does not bind DNA (lane 5). These data demonstrate that an intact homeodomain in HNF-1α is critical for HNF-1α/DNA binding to

DNA. Thus, regions in HNF-1α that mediate protein-protein interaction with GATA-5 and DNA binding are co-localized to the homeodomain.

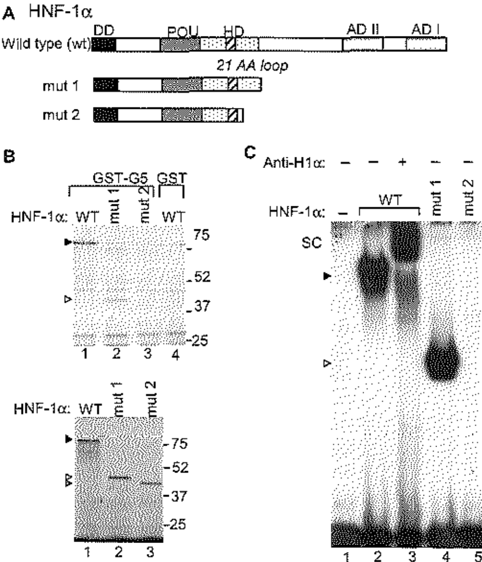


Fig. 5: An intact homeodomain in HNF-1α is necessary for physical association with GATA-5 and binding to DNA. *A. Schematic representation of wild type and mutant HNF-1α proteins.* HNF-1α contains an N-terminal dimerization domain (DD), a POU domain (POU), a homeodomain (HD) that has a unique 21-amino acid loop (21 AA loop), and two C-terminal activation domains (AD I and AD II). The activation domains are deleted in HNF-1α mut 1, and the activation domains plus a segment of the C-terminal side of the homeodomain are deleted in HNF-1α mut 2. *B. An intact homeodomain of HNF-1α is required for physicalassociation with GATA-5.* GST pull-down assays were carried out using GST-G5 incubated with in vitro-labeled wild type and mutant HNF-1α proteins (upper panel). HNF-1α wild type (filled arrowhead, lane 1) and HNF-1α mut 1 (open arrowhead, lane 2) physically associate with GATA-5, whereas HNF-1α mut 2 does not (lane 3). HNF-1α does not interact with GST alone (lane 4). All wild type and mutant HNF-1α proteins were synthesized as indicated by direct loading of labeled products (lower panel). HNF-1α mut 2 is indicated by the shaded arrowhead. *C. An intact homeodomain is required for HNF-1α binding to DNA.* EMSAs were carried out using the rβ-Fib probe (53) incubated with wild type and mutant HNF-1α proteins. Probe alone is indicated in lane 1. Wild type HNF-1α forms a protein/DNA complex (filled arrowhead, lane 2) that is supershifted (SC) using an HNF-1α antibody (lane 3). HNF-1α mut 1 (open arrowhead, lane 4) is capable of forming a complex with DNA with faster mobility than that of wild type HNF-1α. HNF-1α mut 2 does not form a protein/DNA complex (lane 5).

The role of HNF-1α activation domains in the cooperative activation of the human LPH promoter were characterized by transient co-transfection assays using hl18wt and wild type and mutant HNF-1α (mut 1). For comparison, wild type GATA-5 and GATA-5 mut 3, which contains a deletion of the GATA-5 activation domains, were also used. This allowed comparative analyses of the activation domains of GATA-5 and HNF-1α together (Fig. 6).

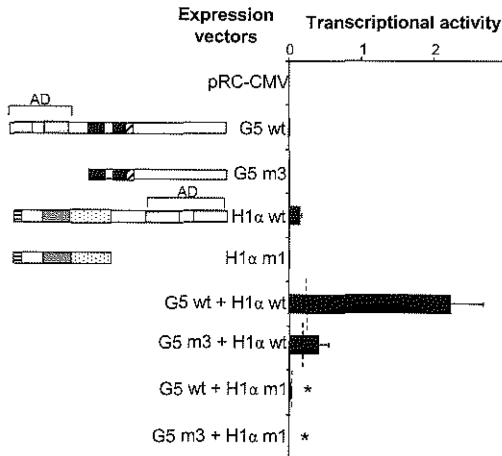


Fig. 6: Activation domains of HNF-1α are required for synergistic activation of the human LPH promoter. Transient co-transfection assays were carried out in HeLa cells using hl18wt, and wild type and mutant expression vectors for GATA-5 and HNF-1α. pRC-CMV was used as a negative control. Wild type and mutant GATA-5 and HNF-1α proteins are as indicated in Fig. 2A and 5A, respectively. The dotted lines indicate the sum of the transcriptional activities of wild type and mutant GATA-5 and HNF-1α expression vectors individually co-transfected with hl18wt for that specific combination of expression vectors. Transcriptional activities that extend to the right of the dotted lines indicate synergistic activation of the hl18wt promoter. Data are means ± SEM of n = 4. * $p<0.05$ compared to hl18wt co-transfected with wild type GATA-5 and HNF-1α.

Consistent with previous data (Fig. 4), deletion of GATA-5 activation domains reduces, but does not eliminate functional synergy. In contrast, deletion of the HNF-1α activation domains eliminates independent as well as cooperative activation of the human LPH promoter. Lack of activation by HNF-1α mut 1 is not due to the inability to associate with GATA-5 or to inefficient DNA binding because these functions remain intact (Fig. 5).

Since domains in GATA-5 and HNF-1α that confer physical association are co-localized with DNA binding domains, it is not possible by mutagenesis of GATA-5 or HNF-1α to differentiate the importance of GATA/HNF-1α interaction from DNA binding. However, it is possible to independently determine the importance of protein-DNA interaction by introducing mutations that disrupt DNA interaction into the binding sites in the human LPH promoter. As shown in Fig. 7, transient co-transfection assays were carried out using wild type and mutant h118 promoter-reporter constructs that have the GATA and HNF-1 binding sites either intact or mutated.

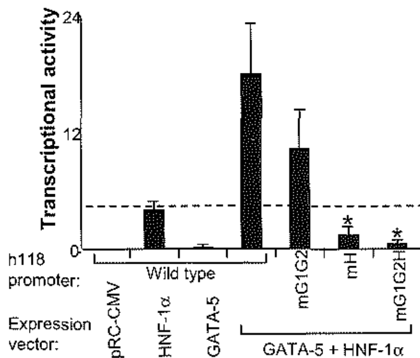


Fig. 7: GATA/HNF-1α synergistic activation of the human LPH promoter requires intact HNF-1 binding sites on the DNA. Transient co-transfection assays were carried out in HeLa cells using wild type GATA-5 and HNF-1α expression vectors co-transfected with wild type and mutant promoter-reporter constructs. The h118mG1G2 promoter-reporter construct contains mutations in both GATA sites, whereas h118mH contains a mutation in the HNF-1 binding site. The h118mG1G2H construct contains mutations in all three binding sites. The dotted line indicates the sum of transcriptional activities of GATA-5 and HNF-1α expression vectors co-transfected individually with h118wt. Transcriptional activities that extend above the dotted line indicate synergistic activation. Data are mean ± SEM of n = 5. * $p<0.05$ compared to h118wt co-transfected with GATA-5 and HNF-1α.

As shown by the middle bar, the wild type human LPH promoter co-transfected with GATA-5 and HNF-1α demonstrates synergistic activation. GATA-5 and HNF-1α co-transfected with h118mG1G2 demonstrate synergistic activation, although the transcriptional activity is reduced from that of the wild type promoter. However, constructs containing mutations in the HNF-1 binding site alone (h118mH) or together with the mutated GATA binding sites (h118mG1G2H), do not show synergistic activation and are significantly lower than that of the wild type promoter ($p < 0.05$). These data demonstrate that HNF-1 binding sites in the promoter are necessary for GATA-5/HNF-1α cooperative activation.

Sequence alignment of GATA-4 and GATA-5 reveals 85% homology in the C-terminal zinc finger and basic regions (24), which are the domains responsible for interaction with HNF-1α. To test the hypothesis that GATA-4 and HNF-1α are also capable of physically associating and cooperatively activating the human LPH promoter, GST pull-down and transient co-transfection assays were carried out. As indicated by the presence of a labeled protein in SDS-polyacrylamide gels (Fig. 8A, upper panel), GATA-4 is pulled down with GST-H1α similar to that of GATA-5.

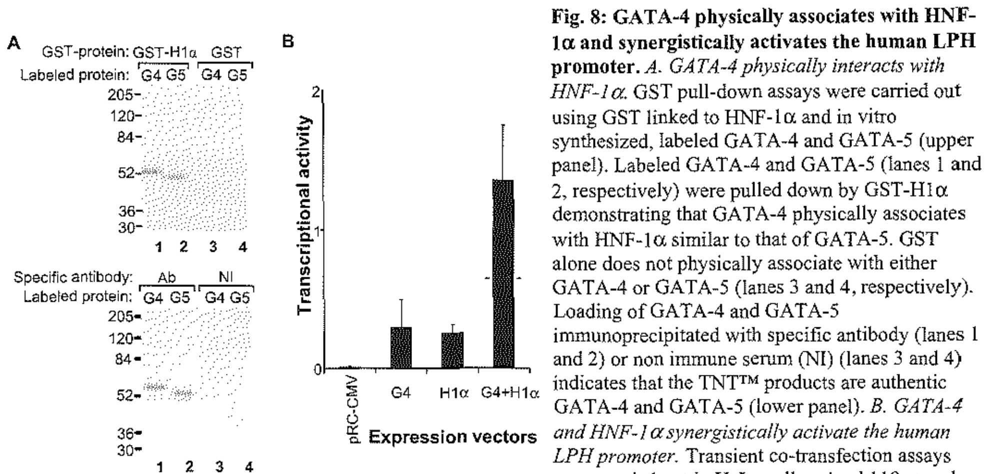


Fig. 8: GATA-4 physically associates with HNF-1α and synergistically activates the human LPH promoter. *A. GATA-4 physically interacts with HNF-1α.* GST pull-down assays were carried out using GST linked to HNF-1α and in vitro synthesized, labeled GATA-4 and GATA-5 (upper panel). Labeled GATA-4 and GATA-5 (lanes 1 and 2, respectively) were pulled down by GST-H1α demonstrating that GATA-4 physically associates with HNF-1α similar to that of GATA-5. GST alone does not physically associate with either GATA-4 or GATA-5 (lanes 3 and 4, respectively). Loading of GATA-4 and GATA-5 immunoprecipitated with specific antibody (lanes 1 and 2) or non immune serum (NI) (lanes 3 and 4) indicates that the TNT™ products are authentic GATA-4 and GATA-5 (lower panel). *B. GATA-4 and HNF-1α synergistically activate the human LPH promoter.* Transient co-transfection assays were carried out in HeLa cells using hl18wt and GATA-4 and HNF-1α co-transfected separately or together. pRC-CMV is used as a negative control. Transcriptional activities above the dotted line indicate synergistic activation. Data are means ± SEM of n = 5.

Immunoprecipitations were carried out using antibodies for GATA-4 and GATA-5 demonstrating that the TNT reactions resulted in the synthesis of authentic GATA-4 and GATA-5 proteins (Fig. 8A, lower panel). These data suggest that GATA-4, like GATA-5, physically associates with HNF-1α. Transient co-transfection assays carried out in Hela cells (Fig. 8B) demonstrate that GATA-4 and HNF-1α independently and synergistically activate the human LPH promoter. These data suggest that GATA-4 is capable of interacting with HNF-1α by mechanisms similar to those described for GATA-5.

DISCUSSION

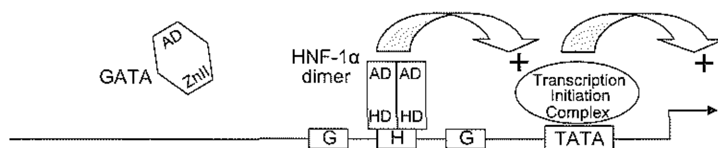
The GATA zinc finger and HNF-1 homeodomain families have been implicated as regulators of tissue-specific gene expression (34,37). The mRNAs of members of both of these transcription factor families are detected in the foregut as early as E9.5 for GATA factors and E10.5 for HNF-1α. Both are expressed in the intestinal epithelium throughout adulthood, suggesting a critical role for these two families of transcription factors in intestinal function (29,34,56). This report shows for the first time that physical association between members of each of these transcription factor families, namely GATA-4 or GATA-5 and HNF-1α, results in the cooperative activation of the promoter of an intestine-specific gene, LPH, suggesting functional convergence of two critical intestinal transcriptional regulatory pathways to maintain high levels of intestine-specific gene expression.

We have previously shown that GATA-5 and HNF-1α synergistically activate the human LPH promoter in the Caco-2 intestinal cell line (21). In the present report, we demonstrate that this synergistic activation requires physical association between GATA-5 and HNF-1α, and that this interaction is mediated by the C-terminal zinc finger and basic region of GATA-5 and homeodomain of HNF-1α. Our data further demonstrate that HNF-1α activation domains are necessary not only for independent HNF-1α activation, but also for synergistic activation with GATA-5. Deletion of GATA-5 activation domains however, results in a reduction, but not an elimination of synergy, suggesting that the GATA-5 activation domains are not necessary, but

serve as additional activators for maximal synergy. Mutational analysis of the human LPH promoter further revealed that intact HNF-1 binding sites, but not GATA binding sites, are necessary for synergistic activation (Fig. 7), suggesting that binding of HNF-1α to the LPH promoter is a critical component of synergy. Although GATA-5 binding sites may not be directly necessary for cooperative activation, the presence of GATA binding sites adjacent to the HNF-1 site might recruit GATA factors in close proximity for HNF-1α interaction resulting in maximal levels of synergistic activation.

The requirement for physical association between GATA-5 and HNF-1α is demonstrated by mutational analysis of GATA-5 (Fig. 3 and 4) and of the human LPH promoter (Fig. 7). As shown in Fig. 3 and 4, GATA-5 mut 5 (C270S) fails to associate with HNF-1α, bind DNA, and synergistically activate hl18wt. However, promoter mutation experiments (Fig. 7) reveal that GATA binding to DNA is not necessary for functional synergy. Thus, failure to synergistically activate the LPH promoter by GATA-5 mut 5 must be due to its inability to physically associate with HNF-1α, rather than its inability to bind DNA. Taken together, these data demonstrate that physical interaction is required for functional synergy. Based on these data, we hypothesize that GATA/HNF-1α synergy is mediated by HNF-1α through its activation domains which are oriented for high levels of activation through a combination of binding to DNA and physical association with GATA factors (Fig. 9). GATA/HNF-1α interactions might unmask the HNF-1α activation domains either by a conformational change or by recruitment of additional proteins that modulate transcriptional activity. Interaction between zinc fingers and homeodomains may be a critical biological mechanism for gene regulation.

HNF-1α-independent activation



GATA/HNF-1α cooperative activation

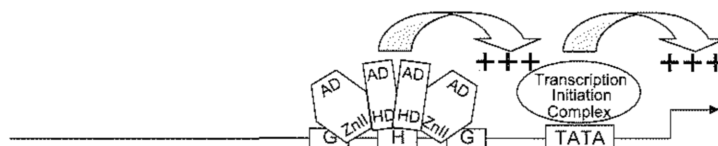


Fig. 9. Schematic representation of a model for GATA/HNF-1α cooperative activation of the human LPH promoter. AD, activation domains; HD, homeodomains; ZnII, C-terminal zinc finger of GATA factors; G, GATA binding site at the LPH promoter; H, HNF-1 binding site; TATA, is the TATA-box binding site for TBP.

For example, similar to the model presented here is the well-characterized model for the synergistic activation of cardiac promoters by GATA-4 and Nkx2.5 (47,48,57-60), a homeodomain-containing transcription factor like HNF-1α. In this model, the C-terminal zinc finger and basic region of GATA-4 and the homeodomain of Nkx2.5 are required for physical association which, in turn, is necessary for synergistic activation of several genes (47,48,57). Furthermore, intact Nkx2.5 binding sites on cardiac promoters are required for synergistic activation (47,48,57). HNF-1α cooperatively activates the sodium-glucose transporter (SGLT1)

with SP-1, a zinc finger-containing protein (44). In *Drosophila*, interactions between the zinc finger of Ftz-F1, a member of the nuclear receptor superfamily, and the homeodomain of the *fushi tarazu* FTz protein result in the cooperative activation of the *engrailed* gene (49,50). These findings are parallel to the GATA-5/HNF-1α model proposed here, and suggest an evolutionarily conserved structure/function relationship that preserves a mechanism of cooperative activation of multiple genes in diverse tissues.

HNF-1α has been shown to interact through its homeodomain with other transcription factors in the intestine. A report by Sakaguchi et al. (45) suggested that HNF-1α was able to enhance claudin-2 promoter activity only in the presence of another member of the homeodomain transcription factor family, Cdx-2. In addition, Michelmoore et. al. (20) demonstrated that HNF-1α physically interacts with Cdx-2, to cooperatively activate the pig LPH promoter. Interestingly, similar to the GATA-5/HNF-1α model presented in this study, HNF-1α, but not Cdx-2, must bind to the DNA for cooperative activation. Since HNF-1α binds to the DNA as a dimer (35,36), it remains possible that for the specific regulation of the LPH gene in vivo, all three factors form a tri-molecular complex whereby the HNF-1α dimer binds GATA factors, Cdx-2, or a combination of both. This hypothesis is consistent with our previous studies (21), which show that the human LPH and SI promoters demonstrate synergistic activation when co-transfected with GATA-5, HNF-1α, and Cdx-2 together. A similar mechanism has also been reported for the cardiac α-actin (αCA) promoter, which is up-regulated through combinatorial interactions of at least three cardiac tissue-enriched transcription factors, GATA-4, Nkx2.5, (which also forms dimers, like HNF-1α), and serum response factor (SRF) (48). HNF-1α may also recruit co-activators such as CREB-binding protein and p300/CBP-associated factor, forming a tri-molecular complex that activates transcription by coupling nucleosome modification with recruitment of proteins for the general transcription machinery (61). Together, these data suggest that HNF-1α has the ability to physically associate with diverse proteins, and act as a linker to the DNA in order to synergistically activate intestinal gene promoters. The magnitude of cooperative activation may be altered by the abundance of different transcription factors and their affinity for HNF-1α.

Physical association between GATA family members and HNF-1α is necessary for the synergistic activation of the human LPH promoter providing a mechanism by which tissue-restricted transcription factors can interact to attain high levels of tissue-specific gene expression. However, although GATA (22,24) and HNF-1α (29) transcription factors are co-localized in the intestinal epithelial where LPH expression is high (5,6), they are also co-expressed in primitive intestinal epithelial cells prior to the onset of LPH gene expression (23,24,32,37). We therefore hypothesize that co-expression of GATA factors and HNF-1α is necessary but not sufficient for high levels of cell-specific LPH gene expression. Interaction with other tissue-restricted transcription factors and co-factors, such as SP-1 (44), FOG-2 (62,63), Cdx-2 (20) and dimerization co-factor of HNF-1α (64) as well as other information further upstream in the LPH promoter (18,65) must be considered when characterizing specific expression of the LPH gene in vivo.

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Chapter 6

DUAL MECHANISMS FOR THE GATA-4 ACTIVATION OF THE HUMAN LACTASE-PHLORIZIN HYDROLASE PROMOTER

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ABSTRACT

In the recent study, the importance of GATA elements in the LPH promoter, the critical structures in GATA factors, including domains responsible for DNA-binding, protein-protein interaction, and activation domains, for GATA-4-specific as well as GATA-4/HNF-1α cooperative activation were determined. Using wild type and mutated human LPH constructs and wild type and mutated GATA-4 proteins, the results of this study revealed that the two GATA elements in the human LPH promoter and the GATA-4 activation domains are critical for the GATA-4 independent activation, but not for GATA-4/HNF-1α synergy. The physical interaction between GATA-4 and HNF-1α is mediated through the C-terminal zinc finger of GATA-4 and is important for the GATA-4/HNF-1α cooperative activation of human LPH gene. The GATA elements in the human LPH promoter and the GATA-4 activation domains are not important for the GATA-4/HNF-1α cooperative activation of human LPH gene. As is described above GATA-4 and GATA-5 demonstrated distinct as well as redundant mechanisms for the regulation of human LPH promoter (1), we used GATA-4 and GATA-5 as models in the comparisons for DNA binding affinities among the GATA elements of the human LPH promoter. Therefore, this report suggests that the human LPH promoter is activated by GATA-4 using a dual mechanism defined by GATA-4-specific as well as GATA-4/HNF-1α cooperative activation. Also the distinct GATA-4-specific activation over that from GATA-5 is due, in part, to a higher affinity of GATA-4 for a GATA element in the human LPH promoter. Since the GATA-4 horizontal expression of GATA-4 coincides with that of LPH in intestine of adult mice, it is hypothesized that GATA-4 together with HNF-1α is important for adult levels of LPH expression.

INTRODUCTION

The GATA family of transcription factors is a group of evolutionarily conserved transcriptional regulator that are defined by two zinc fingers of the motif Cys-X_2-Cys-X_{17}-Cys-X_2-Cys that mediate binding to the consensus DNA sequence WGATAR (W = A or T, R = A or G). The GATA family is generally categorized into two classes based on expression patterns and amino acid homologies. GATA-1, -2, and -3 are expressed in developing bone marrow cells and are critical for hematopoiesis (2), whereas GATA-4, -5, -6 are expressed in individually, or in overlapping patterns in cardiac tissue, small intestine, stomach, liver, lungs, spleen, ovary, testis, and bladder (3-8). Expression of all three intestinal GATA mRNAs is detected as early as E9.5 in the primitive gut and continues to be expressed in intestinal epithelium throughout embryogenesis (3,5-7,9). In both mouse (3) and chicken (9) intestine, GATA-4 transcripts are distributed in epithelial cells all along the villus, with increasing levels accumulating toward the villus tips. In chicken intestine (9), GATA-5 mRNA is localized to differentiated cells of the villi, whereas GATA-6 transcripts are localized to cells of the upper crypt and lower villus. Although the small intestine of adult mice is the only tissue in which all three GATA-4, -5, and -6 mRNAs are expressed (10), independent and overlapping functions of this GATA subfamily are unknown.

Targeted disruption of the GATA-4, -5, -6 genes demonstrate phenotypes that are consistent with their individual expression patterns. Mice homozygous for the GATA-4 null allele die by E9.5 and lack both a primitive heart tube and foregut (11,12). GATA-5 knockout mice survive and reproduce despite pronounced genitourinary abnormalities in females (13), but show no gross abnormalities in intestinal structure or histology (intestinal gene expression was not reported). GATA-6 null mice die before gastrulation (14,15) and therefore provide little information regarding intestinal development. Interestingly, a GATA-5 null mutation in zebrafish resulted in embryonic lethality with an identical phenotype to that observed in GATA-4 null mice, suggesting a similar role for GATA-5 in zebrafish to that of GATA-4 in mice (16). In *Drosophila* and *C. elegans*, GATA homologs have been implicated in gut development (17-19). These studies, however, demonstrate independent functions for individual GATA's, although functional redundancy among GATA subfamily members has been reported (20,21).

The GATA-4, -5, -6 subfamily has been shown to modulate promoter function of intestinal genes including the rat and human lactase phlorizin hydrolase (LPH) (1,22-24), human and mouse sucrase-isomaltase (SI) (24,25), and *Xenopus* intestinal fatty acid binding protein (I-FABP) (9) genes. We have recently shown that GATA-4 or -5 together with hepatic nuclear factor-1α (HNF-1α) synergistically activate the LPH and SI promoters (1,24,25). Similar to these data, Durocher et. al. have shown that GATA-4 or GATA-5 together with Nkx-2.5 synergistically activate the atrial natriuretic factor (ANF) promoter (26) demonstrating that GATA factors display redundant roles, suggesting fundamentally important pathways in the regulation of gene transcription. Identification of parallel mechanisms of protein-protein interaction between zinc finger and homeodomain proteins in other vertebrate tissue (26-28) as well as in *Drosophila* (29,30) suggest that zinc finger/homeodomain interactions are an efficient mechanism for synergistic activation of gene transcription that has been conserved throughout evolution.

As we have recently reported (1), that GATA-4, in contrast to GATA-5, is capable of activating the human LPH promoter independently of HNF-1α. The present report defines the dual mechanisms of GATA-4 activation, characterized by GATA-4 specific and GATA-4/HNF-1α cooperative activation, for the transcriptional regulation of the human LPH promoter. These mechanisms are characterized by: (1) HNF-1α-independent activation (in contrast to GATA-5), in which the DNA binding and activation domains are required, and (2) HNF-1α-dependent

cooperative activation (similar to GATA-5), in which physical association is required but not the GATA-4 activation domains or GATA-4/DNA binding. This study is the first to demonstrate distinct regulatory mechanisms for activation of the human LPH promoter by two conserved GATA factors.

MATERIALS AND METHODS

Plasmids– To construct plasmids templates for the synthesis of RNA probes for RNase protection assays, mouse cDNA sequence (GATA-4, bp 456 to 736; GATA-5, bp 253 to 707; HNF-1α, bp 2936 to 3174) was amplified and subcloned into pBluescript II KS(+). Templates for antisense and sense probes were linearized and transcribed accordingly: GATA-4 antisense, *EcoR*I, T3; GATA-5 antisense, *Aat*II, T7; HNF-1α antisense, *Kpn*I, T7; HNF-1α sense, *HinD*III, T3. A previously described template was used to synthesize a probe for the identification of mouse LPH mRNA (31).

Previously characterized expression vectors for mouse GATA-4 (G4-CMV), (11), GATA-5 (G5-CMV), (7) (gifts of M. Parmacek, University of Pennsylvania) and HNF-1α (32) (gift of G. Crabtree, Stanford University) were obtained for these studies. Since the original HNF-1α expression vector replicates inefficiently during bacterial amplification, the HNF-1α coding region was PCR amplified and subcloned into the *BamH*I and *EcoR*I sites of pcDNA1 (called H1α-CMV), as previously described (1). This plasmid was amplified efficiently in E.coli DH5α cells.

For transfection studies, the human LPH promoter containing 118 bp of 5′-flanking region was fused 5′ to the human growth hormone reporter (called h118wt) (24). This region contains two GATA sites, an HNF-1 site, and a Cdx-2 site as previously described (24). Transcription factor-DNA interactions were characterized, in part, using human promoter-reporter constructs containing mutations in the two GATA sites (h118mG1G2), in the HNF-1 site (h118mH), and in all three sites together (h118mG1G2H). Mutations introduced into these sites, have been previously shown to disrupt specific protein-DNA interactions (24). pRC-CMV (Invitrogen) served as a negative control expression vector for all co-transfection experiments.

For in vitro protein-protein interaction studies, a glutathione S-transferase (GST) fusion vector was constructed for HNF-1α (GST-H1α) as previously described (1).

RNase protection assays-The quantitative pattern of GATA and HNF-1α expression was compared to that of LPH using RNase protection assays as previously described (31,33). Total RNA was isolated from mouse tissues using Trizol Reagent™ (Invitrogen), quantified by optical density at A260 nm, and checked on an agarose gel. [^{32}P]-labeled probes were hybridized at 68°C in 50% formamide overnight, digested with RNase A and T1, and the protected fragments were separated on 6% denaturing polyacrylamide gels and revealed by autoradiography.

Site-directed mutagenesis–To delete or disrupt specific structures within GATA-4 and GATA-5, site-directed mutagenesis (34) was carried out on the GATA-4 and GATA-5 coding region using mutagenic oligonucleotides. The oligonucleotides were phosphorylated using T4 polynucleotide kinase and ATP, and annealed to the uracil templates by incubation for 2 minutes at 70°C, followed 20 minutes incubation at 40° C. The protocol was continued according to previously described method (1). All uracil templates required the use of reverse strand oligonucleotides. GATA-4 mut1 was constructed using the oligonucleotide 5'-GAGGCGGCAGCCATGGATCCCCAGGATGCTC-3' which introduced a *BamH*I (underlined)

and a start codon (bold), and resulted in a deletion of the GATA-4 activation domains. GATA-4 mut2 was constructed using the oligonucleotide 5'-GCCGCAGGCATT**AGA**TACAGGCTCACC-3', the mutation is indicated in bold, which resulted in a cysteine-to-serine substitution in the C-terminal zinc finger at amino acid position 290. The identities of the mutant clones were confirmed by DNA sequencing.

In vitro transcription and translation–Unlabeled and labeled wild-type and mutant GATA-4 and GATA-5 proteins were synthesized as previously described (1) using the TNT™ transcription/translation system (Promega). Labeled proteins were synthesized using [^{35}S]-methionine (Redivue, Amersham-Pharmacia Biotech).

GST pull-down assays–To characterize in vitro protein-protein interactions, GST pull-down assays were carried out as previously described (1). GST fusion proteins were expressed in E. coli DH5α and purified using GSH-Sepharose (Amersham Pharmacia Biotech) beads according to standard protocols (35).

Electrophoretic mobility shift assays (EMSAs)– To define the capability of wild type and mutated proteins to bind DNA, EMSAs were carried out as previously described (24). A test oligonucleotide that contains a GATA site from Xenopus intestinal-fatty acid binding protein gene (xIFABP) was used as a probe (9). Specific and non-specific oligonucleotides, and the two GATA sites located in the human LPH promoter (24) were used as competitors. The oligonucleotides are listed below, Specific competitor (S), 5'-GGAGATCCCTGTACAGATATGGGGAGAC-'3, non-specific (SIF1), 5'-GGGTGCACTAAAACTTTATGAGTA-'3, GATA element (hG1, -95 10 -88 bp), 5'-GTGATCATAGATAACCCAGTTAAA-'3, and GATA element (hG2, -67 to -61 bp) 5'-ATACTAAGTGATAATTAAGACTTA-'3 as previously characterized (24). For competition or supershift EMSAs, competitors (200-fold molar excess) or antibodies (1 µl, undiluted), respectively, were pre-incubated with the nuclear extract for 10 minutes prior to the addition of the probe. All antibodies were purchased from Santa Cruz Biotechnology.

Cell culture and transient co-transfection assays–To characterize the functional role of GATA on the LPH promoter, transient co-transfection assays were carried out using HeLa cells because GATA-specific and GATA/HNF-1α cooperative activation can be distinguished in cells that do not synthesize endogenous HNF-1α as previously described (1). Cells were transfected using Effectene™ reagent (Qiagen) according to the protocol provided by the manufacturer. Optimal conditions for both cell lines were: 1 µg total DNA (0.4 µg of promoter/reporter construct and 0.2 µg of expression vector) and 4 µl of Effectene™ reagent. Media were replaced after 24 h, and the experiment was concluded after an additional 24 h, as previously described (24). The amount of human growth hormone reporter secreted into the media over 24 h was measured using an [^{125}I] radioimmunoassay kit (Allegro hGH, Nichols Institute). To control for transfection efficiency, all transcriptional activities were expressed relative to pXGH5, a constitutively active metallothionine-I promoter fused to the human growth hormone gene.

Densitometry quantification-The films of EMSAs were scanned under epi-white light using the Gel Doc 2000™ (Bio-Rad, Hercules, CA). The relative density of bands form film were determined by densitometry using the Quantity One software (Bio Rad, Hercules, CA).

Statistics–The t test or the one-way analysis of variance (ANOVA) was employed in all statistical analyses using InStat software (Graphpad Software, Inc.). Multiple comparisons were carried out by the Dunnett test.

RESULTS

To define the regulation of LPH gene expression in adult mice expression patterns along the horizontal gradient of GATA factors in small intestine, RNase protection assays were carried out (Fig. 1).

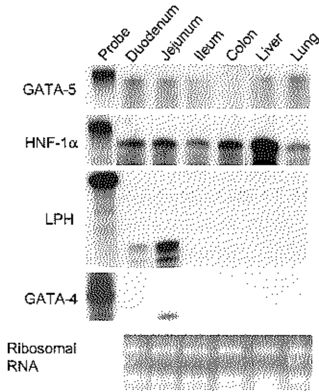

Fig. 1: The horizontal patterns of expression of GATA-4 mRNA in adult mice coincides with that of LPH. RNase protection assays were carried out using probes for GATA-4, GATA-5, HNF-1α, and LPH (lane 1) incubated with RNA isolated from duodenum, jejunum, ileum, colon, heart and lung (lanes 2-7, respectively). The quality of RNA is demonstrated by intact ribosomal bands in an agarose gel (bottom panel).

RNA was isolated from duodenum, mid-jejunum, ileum and colon as well as liver and lung of adult mice. GATA-4 mRNA was detected in mid-jejunum, whereas GATA-5 mRNA was detected in duodenum, mid-jejunum, and ileum, but was not detected in colon. Both liver and lung express GATA-5 mRNA, as previously shown (10). HNF-1α mRNA was detected in every tissue with liver being the most abundant, as previously shown (32). LPH mRNA was detected in small intestine with highest levels in mid-jejunum, as previously shown (31). The quality and quantity of RNA is demonstrated by distinct ribosomal RNA bands on agarose gel (bottom panel). These data indicate that the horizontal expression of GATA-4 mRNA parallels that of LPH mRNA in adult mice suggesting that GATA-4 is an activator of LPH along the horizontal gradient in vivo.

To define the potential importance of GATA-4 and HNF-1α in the regulation of LPH gene expression along the vertical gradient in vivo, immunohistochemistry was carried out using serial sections of mid-jejunum from adult mice and mouse GATA-4 and HNF-1α antibodies (Fig. 2). GATA-4 and HNF-1α are both expressed on villi and are not detected in goblets (arrows) or crypts. The vertical expression patterns of GATA-4 and HNF-1α coincide with the villus-specific expression of LPH, suggesting that GATA-4 and HNF-1α might act together to cooperatively activate the human LPH promoter.

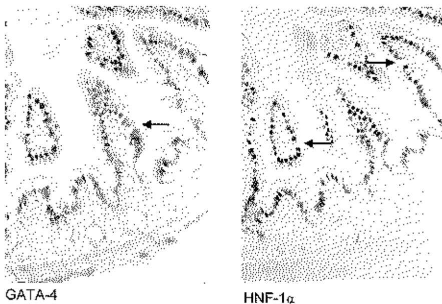

Fig. 2: GATA-4 and HNF-1α are both expressed in epithelial cells on villi of adult mice. Immunohistochemistry was carried out on serial sections of mid jejunum of adult mice using mouse antibodies for GATA-4 and HNF-1α. Goblet cells (arrows) do not demonstrate staining for either GATA-4 or HNF-1α.

To define potential similarities and differences in the function of intestinal GATA factors, transient co-transfection assays were carried out using the wild type human LPH promoter (hl18wt) and GATA-4, GATA-5, and HNF-1α expression vectors co-transfected singly and in combination (Fig. 3). HeLa cells were

used since these cells do not synthesize endogenous HNF-1α (24). An empty expression vector (pRC-CMV) was used as a control for background activation of the human LPH promoter. As recently reported (1), GATA-4 independently activated the human LPH promoter as compared to GATA-5 which is known not to activate the human LPH promoter in the absence of HNF-1α (24).

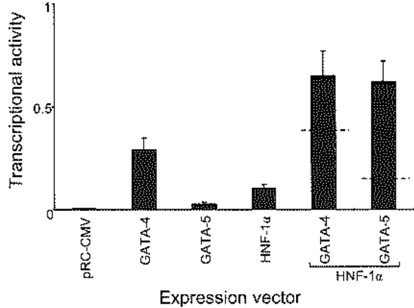

Fig. 3: GATA-4 activates the human LPH promoter by distinct mechanisms that are independent and dependent on HNF-1α. Transient co-transfection assays were carried out in HeLa cells using the h118wt promoter co-transfected with pRC-CMV (negative control expression vector) or wild type GATA-4, GATA-5, and HNF-1α, singly and in combination. The dotted line indicates the sum of the transcriptional activities of wild type GATA and HNF-1α co-transfected individually with h118wt. The transcriptional activities above the dotted line indicate synergistic activation. Data are mean ± SEM of n = 5.

HNF-1α activated the human LPH promoter independently, as previously described (1,24). Both GATA-4 and GATA-5 synergistically activated the human LPH promoter with HNF-1α, as previously shown (1). These data suggest that GATA-4 activates the human LPH promoter by dual mechanisms that are both independent and dependent on HNF-1α.

To determine the importance of the binding sites in the proximal LPH promoter for the dual mechanisms of GATA-4 activation, transient co-transfection assays were carried out in Hela cells using wild type and mutated h118 constructs (Fig. 4A), and pRC-CMV and GATA-4 expression vectors (Fig. 4B top panel).

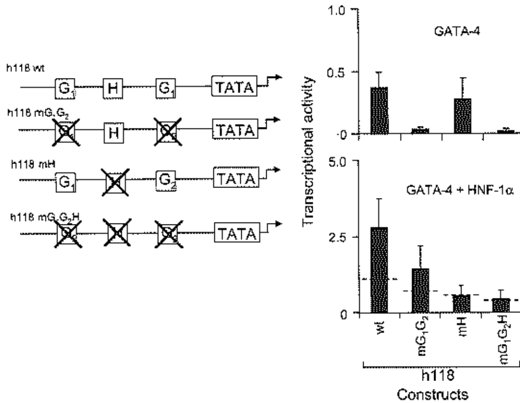

Fig. 4: Different binding sites in the human LPH promoter are necessary for GATA-4-specific and GATA-4/HNF-1α cooperative activation. A schematic representation of wild type and mutant h118 LPH constructs. Wild type (h118 wt) construct contains the first 118 bp of human LPH 5'-flanking sequence. The mutated h118mG_1G_2, h118mH, and h118 mG_1G_2 constructs contain mutations introduced into both GATA-binding sites, the HNF-1 site, and in all three sites together, respectively. B. GATA-4-specific activation requires intact GATA binding sites in the human LPH promoter (top panel), whereas GATA-4/HNF-1α cooperative activation requires an intact HNF-1 site and not intact GATA sites (bottom panel). Transient co-transfection assays were carried out in HeLa cells using wild type and mutated h118 constructs and the GATA-4 expression vector. pRC-CMV was used as a negative control. Data are means ± SEM of n = 5.

The wild type h118 construct and the h118mH construct, which contains a mutation in the HNF-1 binding site, were similarly activated by GATA-4. However, constructs containing mutations introduced into both GATA binding sites (h118mG_1G_2) or into both GATA and the HNF-1 binding sites (h118mG_1G_2H) were not activated by GATA-4. These data demonstrate that the GATA binding sites are critical for the GATA-4-specific activation of the human LPH promoter.

To test the importance of GATA and HNF-1 binding sites in the human LPH promoter for the GATA-4/HNF-1α cooperative activation, transient co-transfection assays in HeLa cells were carried out using wild type and mutated h118 constructs co-transfected with GATA-4 and HNF-1α expression vectors together (Fig. 4B bottom panel). The h118wt construct was

synergistically activated by a combination of GATA-4 and HNF-1α, as also shown in Fig. 3, and as previously reported (1). The $h118mG_1G_2$ construct remained synergistically activated by GATA-4 and HNF-1α together although this construct was activated less than that of h118wt. However, introduction of a mutation into the HNF-1 binding site alone or in combination with both GATA sites, resulted in a complete loss of synergistic activation by GATA-4 and HNF-1α. These data demonstrate that cooperative GATA-4/HNF-1α activation of the human LPH promoter requires HNF-1 binding sites, similar to that previously shown for GATA-5/HNF-1α cooperativity (1).

To characterize critical domains in GATA-4 necessary for the GATA-4-specific and the GATA-4/HNF-1α cooperative activation, mutations were introduced into GATA-4 that delete or disrupt specific structures (Fig. 5A). To test the capacity of wild type and mutated GATA-4 proteins to bind DNA which is necessary for GATA-4-specific activation, EMSAs were carried out using in vitro synthesized proteins and a previously described probe that binds GATA factors (24). As shown in Fig. 5B, wild type GATA-4 forms a protein/DNA complex (lane 2) which, in turn, forms a supershift complex (SC) using a mouse antibody for GATA-4 (lane 3). GATA-4 mut 1, which contains a deletion of the activation domains, binds DNA (lane 4), whereas, GATA-4 mut 2, which contains a mutation in the C-terminal zinc finger that disrupts the structure of this zinc finger (lane 5), is incapable of binding DNA as previously shown (36). These data confirm that the C-terminal zinc finger of GATA-4 is critical for DNA binding.

To test the importance of specific domains in GATA-4 for the physical interaction with HNF-1α, GST pull-down assays (Fig. 5C, top panel) were carried out using in vitro labeled wild type and mutated GATA-4 proteins. Wild type GATA-4 and GATA-4 mut 1 physically interact with HNF-1α, but GATA-4 mut 2 does not (lanes 1-3, respectively). Wild type and mutated GATA-4 proteins were synthesized as shown by the direct loading of in vitro labeled proteins (Fig. 5C, bottom panel). These data demonstrate that the C-terminal zinc finger is critical not only for DNA binding, but also for direct protein-protein interaction with HNF-1α, as previously shown for GATA-5 (1).

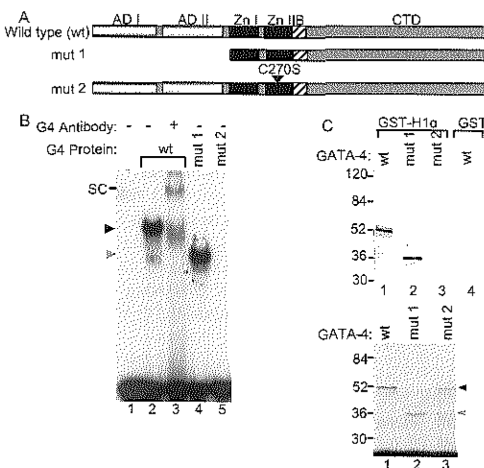

Fig. 5: The C-terminal zinc finger of GATA-4 mediates DNA binding and physical interaction with HNF-1α. A. Schematic representation of wild type and mutant GATA-4 proteins. GATA-4 contains two N-terminal activation domains (AD I and AD II), two zinc fingers (Zn I, N-terminal zinc finger; Zn II, C-terminal zinc finger), and a basic region (B) near the C-terminus (36). Both activation domains are deleted in mut 1. GATA-4 mut 2 is a cysteine-to-serine substitution in the C-terminal zinc finger at amino acid 290. B. The GATA-4 C-terminal zinc finger mediates DNA binding. EMSAs were carried out using the previously described GATA binding site as a probe (1) and wild type and mutant GATA-4 proteins as indicated across the top of the figure (lanes 2-5). A protein-DNA complex (black arrowhead, lane 2), forms a supershift complex (SC) with a mouse GATA-4 antibody (lane 3). GATA-4 mut 1 (gray arrowhead) binds to DNA, whereas GATA-4 mut 2 does not bind to DNA. C. The GATA-4 C-terminal zinc finger is required for the physical interaction with HNF-1α. GST pull-down assays (upper panel) were carried out using GST-H1α incubated with labeled in vitro transcribed and translated wild type (lane 1) and mutant GATA-4 (lanes 2 and 3). GATA-4 wild type (black arrowhead) and mut 1 (gray arrowhead) were pulled down by GST-H1α whereas GATA-4 mut 2 was not. GATA-4 was not pulled down by GST alone (lane 4). All proteins used in GST pull-down assays were synthesized as shown by direct loading of TNT products (lower panel).

To test the importance of GATA-4 activation domains and the C-terminal zinc finger for the GATA-4-specific and GATA-4/HNF-1α synergistic activation, transient co-transfection assays in HeLa cells (Fig. 6) were conducted using GATA-4 mutants. To test the importance of these domains for the GATA-4 specific activation, GATA-4 wild type and mut 1 were co-transfected independently of HNF-1α (Fig. 6, top panel). Wild type GATA-4 was capable of activating h118wt, whereas GATA-4 mut 1, which binds DNA, activates h118wt 60% lower than that of GATA-4 wild type. GATA-4 mut 2, which contains a mutation that disrupts the C-terminal zinc finger, binds DNA, but does not activate h118wt. These data demonstrate that the GATA-4-specific activation requires GATA-4 activation domains and an intact C-terminal zinc finger.

To determine whether the activation domains and the C-terminal zinc finger are important for the GATA-4/HNF-1α synergistic activation of the human LPH promoter, transient co-transfection assays were carried out using HeLa cells. The h118wt was co-transfected with wild type and mutated GATA-4 and HNF-1α expression vectors (Fig. 6, bottom panel). The h118wt construct was synergistically activated by the combination of GATA-4 plus HNF-1α together, as indicated by a transcriptional activity that was ~2 fold greater than the sum of the individual transcriptional activities of GATA-4 and HNF-1α alone (indicated by the dotted line). GATA-4 mut 1 demonstrates synergistic activation of h118wt when co-transfected with HNF-1α, although reduced as compared for that of h118wt co-transfected with GATA-4 and HNF-1α together. However, GATA-4 mut 2, which does not physically interact with HNF-1α or bind with DNA, does not synergistically activate h118wt construct with HNF-1α. However, promoter mutation experiments (Fig. 4) reveal that GATA binding to DNA is not necessary for functional synergy.

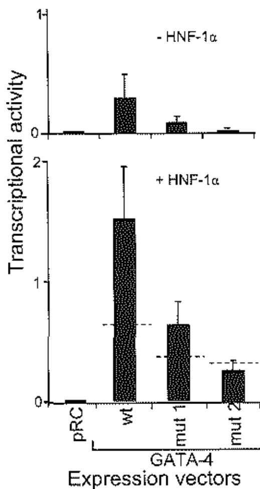

Fig. 6: The GATA-4-specific activation requires GATA-4 activation domains but GATA-4/HNF-1α cooperative activation does not. Transient co-transfection assays were carried out in HeLa cells using h118wt, and wild type and mutant expression vectors for GATA-4 singly (top panel) as well as together with the HNF-1α expression vector (bottom panel). pRC-CMV was used as a negative control. The dotted lines indicate the sum of the transcriptional activities of wild type or mutant GATA-4 and wild type HNF-1α (data not shown) expression vectors individually co-transfected with h118wt for that specific combination of expression vectors. Transcriptional activities that follow above the dotted lines indicate synergistic activation of the h118wt promoter. Data are means ± SEM of n = 5.

Thus, failure to synergistically activate the LPH promoter by GATA-4 mut 2 must be due to its inability to physically associate with HNF-1α, rather than its inability to bind DNA. These data demonstrate that the mechanism of GATA-4/HNF-1α synergy requires the physical interaction with HNF-1α mediated through an intact C-terminal zinc finger. Synergistic activation does not require GATA-4 activation domains, similarly to the previously described GATA-5/HNF-1α synergistic activation of h118wt (1).

To test whether distinct functions among the GATA factors are mediated through binding affinity differences of GATA-4 and GATA-5 to GATA elements in the human LPH promoter, competition EMSAs were carried out (Fig. 7A). Specific (S), non-specific (NS) oligonucleotides, as well as oligonucleotides containing each of the GATA binding sites of the human LPH

promoter were used as competitors to characterize binding affinities among GATA binding sites for GATA-4 and GATA-5. Since GATA-4 and GATA-5 proteins are of different sizes, it was possible to assay these GATA proteins together and directly compare the differences in GATA binding sites. Supershift analyses demonstrate that the top band is GATA-4 and the bottom band is GATA-5. EMSAs using the unlabeled oligonucleotide version of the probe was used a specific competitor, and both unlabeled hG_1 and hG_2 oligonucleotides demonstrated that the specific oligonucleotide competed stronger for GATA proteins than either hG_1 and hG_2 as previously shown (1), where the hG_1 oligonucleotide competed GATA-4 and -5 stronger than the hG_2 oligonucleotide. These data suggest that the two GATA binding elements of the human LPH promoter between -95 and -88 bp (hG_1) and between -67 and -61 bp (hG_2) have a differential affinities for GATA proteins.

To characterize different binding affinities between hG_1 and hG_2 for GATA-4 and GATA-5, densitometry quantification of each band was carried out (Fig. 7A.) A premixed amount of GATA-4 and GATA-5 was used for all binding assays (lanes 2-14). The band density in all lanes was used to calculate the ratio between amount of GATA-4 or GATA-5 that was bound to the probe.

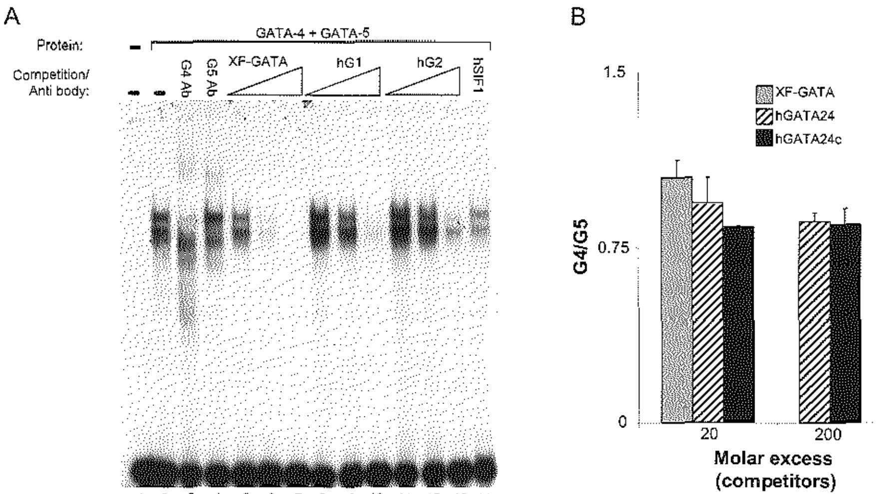

Fig. 7: GATA binding site in the human LPH promoter have distinct affinities for GATA proteins. A. GATA binding element between -95 and -88 bp demonstrated higher affinity for GATA factors than the GATA binding element between -67 and -61 bp of human LPH 5'flanking sequence. EMSAs were carried out using the previous xFABP described GATA probe (Fig. 5B) and a standardized premix of wild type GATA-4 and GATA-5 proteins (lanes 2-14). GATA-4 (filled arrowhead) and GATA-5 (open arrowhead) bind to DNA, are of different sizes (lane 2), and form a supershift complex (SC) with GATA-4 (G4 Ab) and GATA-5 (G5 Ab) mouse antibodies, respectively (lanes 3 and 4). Competition EMSAs were carried out using unlabeled oligonucleotides from xIFABP (lanes 5-7), both GATA binding site in the 5'-flanking region of the human LPH gene (lanes 8-13), and a non-specific (lane 14) as competitors (indicated on the top of the gel). 2x, 20x, and 200x molar excess were used for each competitor. B. Densitometry among GATA-4 and GATA-5 band density for both GATA binding elements revealed higher affinity for GATA-4 over GATA-5.

The GATA-4 and GATA-5 incubated with probe and no competitor (lane 2) was used to standardize loading differences between GATA-4 and GATA-5. The 200 molar excess of unlabeled oligonucelotides competed the GATA proteins completely and were not considered for densitometry. Specific competitor (unlabeled probe) showed, as expected, that each band was

competed equally. However competition with the hG_1 and hG_2 oligonucleotides revealed differential competition demonstrated by a greater reduction in GATA-4 binding to the probe (Fig, 7B). These data demonstrate that the GATA binding elements in the human LPH promoter have a preference for GATA-4 compared to GATA-5, suggesting that the higher affinity of GATA binding elements for GATA-4 might mediate, in part, the GATA-4-specific activation of transcription of LPH in vivo.

DISCUSSION

The GATA-4, -5 , and -6 subfamily of transcription factors are expressed together in the mammalian small intestine as early as E9.5 (10) and activate promoters of intestinal genes (1,9,22-24), but distinct functions among these factors in the regulation of intestinal gene expression have not been previously reported. In the present study, we show for the first time distinct functions among individual GATA factors in their ability to activate the human LPH promoter. GATA-5 activates the LPH promoter in the presence of HNF-1α but is incapable of activating this promoter in the absence of HNF-1α (24). In contrast, GATA-4, in addition to synergistic activation with HNF-1α, is capable of activating the LPH promoter independently of HNF-1α. The GATA-4-specific activation requires GATA-4 binding to DNA and the GATA-4 activation domains. In the present report we have shown that the mechanism of GATA-4/HNF-1α cooperativity requires the physical interaction between GATA-4 and HNF-1α and that this interaction is mediated through the C-terminal zinc finger of GATA-4. This GATA-4/HNF-1α mechanism is similar to the previously shown mechanism of the GATA-5/HNF-1α cooperativity which is mediated through the C-terminal and basic region of GATA-5 and the homeodomain of HNF-1α. Further, both GATA-4/HNF-1α and GATA-5/HNF-1α cooperativity require HNF-1α binding to DNA (1) and the HNF-1α activation domains. These findings are in agreement with our previously reported model which states that the GATA/HNF-1α synergy is mediated by HNF-1α through its activation domains which are oriented for high levels of activation through a combination of binding to DNA and physical association with GATA factors (1). Although dual mechanisms for GATA-4 have been reported in the regulation of the Müllerian inhibiting substance gene (MIS) (21), this study is the first to demonstrate distinct regulatory mechanisms for activation of the human LPH promoter by two conserved GATA factors, suggesting that these factors play a critical role in the transcriptional regulation of intestinal genes in vivo.

Zinc finger-homeodomain interactions may be an important mechanism for tissue-specific gene regulation. We have previously shown that GATA factors physically interact with a member of the homeodomain transcription factor family, HNF-1α, in vivo, which is required for the cooperative activation of intestine-specific genes, like LPH (1) and SI (25). The zinc finger/homeodomian interaction mechanism is also demonstrated for other tissue-specific genes (10,37) as well as in *Drosophila* (29,30). The redundant function of GATA factors in the zinc finger-homeodomain synergy mechanism and the fact that this mechanism is found in other vertebrate tissue as well as in *Drosophila* suggest that zinc finger-homeodomain interactions are an efficient mechanism for synergistic activation of gene transcription that has been conserved throughout evolution in which conserved GATA factors play an important role.

This study demonstrates for the first time different binding affinities of GATA elements in the promoter of an intestinal gene. We have demonstrated by competition EMSAs that the hG_1 (-95 to -88 bp) has a higher affinity for GATA proteins than the hG_2 (-67 to -61 bp) (Fig 7A). Additionally both GATA elements preferentially bind GATA-4 over GATA-5 (Fig. 7B). The alignment of the 5'-flanking sequence of the human, rat, mouse, and pig LPH gene, revealed that hG_1 (-95 to -88 bp) is evolutionarily conserved, suggesting that is element is important for GATA regulation of transcription. However, hG_2 (-67 to -61 bp) revealed differences in

sequence among mammals and may mediate species-specific GATA regulation at LPH promoter, as previously described (24).

Distinct functions among the GATA-4, -5, and -6 transcription factor family have not been reported for the regulation of intestinal-specific gene transcription and may, in part, be explained by the differential binding affinities between these GATA factors for the *cis*-acting elements in promoters of target genes. We have demonstrated by densitometry that the human GATA elements preferentially bind GATA-4 over GATA-5 in vitro (Fig. 7B) which may, in part, explain the GATA-4-specific activation of the human LPH promoter. Consistent with these data are the affinity differences of GATA factors for the GATA element in the promoters of cardiac genes. GATA-4 has a higher affinity for the GATA element in the α- and β-myosin heavy-chain (α- and β-MHC) promoters than GATA-6, which explained, the higher transcriptional activation of these promoters by GATA-4 (38). Additionally, in the same report, it is suggested that the differential activation of the atrial natriuretic factor promoter by GATA-4 and GATA-6 correlates well with their affinity differences for the GATA sites in this promoter. Further, in another report, it was demonstrated that GATA-5, and -6 activate the cardiac troponin I promoter better than GATA-4, which was suggested to be regulated by the *cis*-acting elements of the promoter (39). Finally, in a previous report, it is demonstrated that GATA-6 activates the mitogen-responsive phosphoprotein Dab2 promoter, but GATA-4 does not (40). In contrast to the recent and previous reports, Morrisey et al suggested that the GATA-6-specific activation is not regulated through the zinc finger regions, but rather through N-terminal or C-terminal domains. However, our recent data clearly demonstrates that the distinct functions between GATA-4 and GATA-5 correlates with the distinct affinities for the GATA elements. These data suggest that the GATA elements in intestine-specific promoters have different affinities for members of the GATA-4, -5, and -6 subfamily, which may explain, in part the distinct functions among these GATA factors for the transcriptional regulation of intestine-specific genes.

Although the wide-ranging expression patterns of GATA-4, -5, and -6 argue against these proteins as being master regulators of tissue- or cell-type-specific gene expression, the phenotypes of mice which contain targeted disruption of GATA-4, -5 , and -6 suggest distinct functions of these factors. In addition, the expression pattern of GATA-4 mRNA along the horizontal gradient coincides with that of LPH mRNA in adult small intestine, but not the GATA-5 mRNA expression pattern, suggesting that GATA-4 is a critical component for the transcription of LPH along the horizontal gradient. Since Cdx-2, a caudel related homeodomian transcription factor, also physically interacts with GATA factors (25)and HNF-1α (41) and we have previously shown that LPH is also synergistically activated by a combination of GATA, HNF-1α, and Cdx-2 (24), it remains possible that these transcription factors bind and act in concert to regulate LPH gene transcription. Finally, both GATA-4 and GATA-5 with HNF-1α are capable of cooperatively activating the human LPH promoter and GATA-5 is expressed in enterocytes of the intestine where LPH is not expressed, it is reasonable to hypothesize that these factors are important but not sufficient for the complex expression patterns of LPH. Interaction with other tissue-restricted transcription factors and co-factors, such as SP-1 (37), FOG-2 (42,43), Cdx-2 (41) and dimerization co-factor of HNF-1α (44) as well as other information further upstream in the LPH promoter (45,46) must be considered when characterizing specific expression of the LPH gene in vivo.

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Chapter 7

Friend of GATA (FOG) cofactors mediate repression of the GATA activation of the lactase-phlorizin hydrolase gene promoter

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ABSTRACT

Friend of GATA (FOG) is a family of multi-zinc finger cofactors that modulate the GATA activation of target genes. Two members have been identified thus far, FOG-1 and FOG-2. We have previously shown that the lactase-phlorizin hydrolase (LPH) promoter is activated by GATA and HNF-1α transcription factors, both individually and cooperatively (*Am. J. Physiol.* 281:G69-G84, 2001). The aim of this study was to define the role of FOG cofactors on the GATA-specific and GATA/HNF-1α cooperative activation of the LPH promoter. Both FOG-1 and FOG-2 mRNAs were detected in RNA isolated from adult mouse jejunum by RT-PCR demonstrating that these two cofactors are expressed in mammalian small intestine. Both FOG-1 and FOG-2 repressed the GATA-4-specific activation of the human LPH promoter to less than 70% (FOG-1) or 10% (FOG-2) of its activated value. FOG-1 and FOG-2 had no effect on the HNF-1α-stimulated activation, demonstrating that FOG repression is GATA-specific. FOG cofactors also repressed the GATA/HNF-1α cooperative activation of the LPH promoter. Introduction of critical amino acid substitutions in the N-terminal zinc finger of GATA factors, which have been shown to disrupt physical interactions with FOG, result in the reduction of FOG-specific repression of the GATA/HNF-1α cooperative activation, but not the GATA-4-specific activation. These data suggest that FOG cofactors expressed in the intestine modulate LPH gene expression by repressing the GATA-specific and GATA/HNF-1α cooperative activation of the LPH promoter. This repression likely occurs via physical associations between GATA and FOG proteins that involve, in part, the N-terminal zinc fingers of GATA factors.

INTRODUCTION

Friend of GATA (FOG) is a family of multi-zinc finger, transcriptional co-factors that modulate GATA-specific activation of target genes during cellular differentiation. FOG-1 is highly expressed in parallel with GATA-1 in erythroid cell lines and in spleen, liver, and testis (25). As with GATA-1, FOG-1 plays a critical role in hematopoiesis. The targeted disruption of *fog-1* in mice leads to embryonic lethality, with mice dying of severe anemia between embryonic day 11 and 12 (24), similar to that found in *gata-1* null mice (8). FOG-2 is more widely expressed than FOG-1, generally paralleling that of the GATA-4, -5, -6 subfamily. In mice, FOG-2 mRNA is first detected in developing heart and septum transversum (E8.5) followed by expression in neural epithelium and the urogenital ridge (E11.5) (21, 22). At E15.5, expression is detected in the developing lung, kidney, and gut (22). In adult mice, FOG-2 is expressed highly in heart, brain, and testis, and at lower levels in lung, liver, and kidney (22). As with GATA-4, FOG-2 plays a critical role in cardiogenesis. Mice harboring a targeted mutation of *fog-2* die of congestive heart failure at E13 characterized by a thin ventricular myocardium, tricuspid atresia, and a defective vasculature (20, 23), similar to that found in *gata-4* null mice (15, 17). Indeed, knock-in of a GATA-4 substitution that is incapable of physically associating with FOG-2 exhibits a remarkably similar constellation of defects (3) suggesting that interactions between GATA-4 and FOG-2 are essential for normal heart development. FOG-1 and FOG-2 are both expressed in fetal intestine (3, 16, 22, 25), but the expression and function of these two proteins in adult intestine have not been reported.

FOG proteins have been shown to modulate the transcriptional activities of the GATA proteins through interactions with the N-terminal zinc finger of GATA factors (GATA factors have a pair of zinc fingers). Depending on the context, FOG can act to further activate (synergize) or repress the GATA-activation of target genes (10, 16, 24, 25). For example, the N-terminal zinc finger of GATA-1 associates with FOG-1 resulting in synergistic activation of the erythroid-specific NF-E2 promoter (24, 25). However, FOG-1 represses the GATA-1-activation of the M1α promoter (10). Similarly, the α-myosin heavy chain (α-MHC) promoter is activated by FOG-2 in COS cells, but repressed by FOG-2 in neonatal rat cardiomyocytes (16). FOG-1 interacts with the transcriptional co-repressor CtBP2, which may be recruited by FOG-1 to repress gene expression (10), although knock-in of a FOG-1 substitution that does not interact with CtBP2 exhibits no phenotypic abnormalities (11). Although FOG-2 also contains the conserved CtBP2 binding site, PIDLS, the GATA-4-specific repression of cardiac promoters was recently localized to a novel N-terminal domain (aa 1-247) of FOG-2 (19). This domain, which is also conserved in FOG-1, is both necessary and sufficient to repress GATA-4-dependent transcription. These data demonstrate a multifunctionality of FOG proteins that is dependent on the configuration of target promoters as well as the expression of other components within cells.

GATA-4, -5, and -6 subfamily expressed in intestinal epithelium (1, 9), and activate the promoters of intestinal genes, including those of the intestinal fatty acid binding protein (9), sucrase-isomaltase (12), and lactase-phlorizin hydrolase (LPH) (5, 6, 12). We have further shown that GATA factors physically associate with the homeobox transcription factor, hepatocyte nuclear factor-1α (HNF-1α), and synergistically activate the LPH promoter (27). Finally, GATA factors demonstrate independent functions characterized by the ability of GATA-4, and inability of GATA-5, to activate the LPH promoter in the absence of HNF-1α (12, 26, 27). In the present study, we demonstrate that FOG-1 and FOG-2 are expressed in the adult mouse small intestine and repress the GATA-4-specific and GATA/HNF-1α cooperative activation by a mechanism that requires at least one intact GATA zinc finger.

METHODS

RT-PCR-To characterize FOG expression in adult mouse small intestine, RT-PCR was conducted using RNA isolated from tissue of adult mice. RNA was isolated using Trizol Reagent™ (Gibco-BRL) according to the manufacturers instructions. After annealing the RNA to oligo dT, first strand cDNA was synthesized using Superscript II (Gibco-BRL) according to the manufacturers instructions. PCR was then conducted using a thermocycler (MJ Research) by melting at 94°C (30 sec), annealing at 55°C (30 sec) and extending at 72°C (1 min) for a total of 30 cycles. The following oligonucleotides were used: FOG-1, 5′-TGGTGCAGGGTTACCAAAGT-3′, 5′-TTGATGACTGCGGTAGCAAG-3′; FOG-2, 5′-AGTCTTCAGCGAAGCAGCTC-3′, 5′-TGACCATGCCTCTGTTTTTG-3′; SI, 5′-ACACGTTGCTGCCCTATTTC-3′, 5′-CATAAAAGCTGGACCCACA-3′. Expected sizes of amplification products were: FOG-1, 194 bp; FOG-2, 299 bp; SI, 146 bp.

Plasmids– Previously characterized expression vectors for mouse FOG-1 (FOG-1-CMV) (25) (gift of S. Orkin) and FOG-2 (FOG-2-CMV) (21) (gift of J. Leiden) were obtained for these studies. Previously characterized expression vectors for mouse GATA-4 (G4-CMV) (15), GATA-5 (G5-CMV) (18) (gifts of M. Parmacek, University of Pennsylvania) and HNF-1α (14) (gift of G. Crabtree, Stanford University) were also obtained for these studies. Since the original HNF-1α expression vector replicates inefficiently during bacterial amplification, the HNF-1α coding region was PCR amplified and subcloned into the *BamHI* and *EcoRI* sites of pcDNA1 (called H1α-CMV), as previously described (27). This plasmid was amplified efficiently in E.coli DH5α cells.

To introduce a mutation into the N-terminal zinc finger of GATA-4 that has been previously shown to disrupt FOG-2 interactions (19), site-directed mutagenesis was carried out using the method of Kunkel et al (13) as previously described (27). This mutation, a glutamic acid-to-lysine substitution at amino acid 215 (E215K, mut1), was introduced using the mutagenic oligonucleotide, 5′-CACAATTGACAC<u>ACT</u>TTCTGCCTTCTGAG-3' (mutation underlined).

For transfection studies, the human LPH promoter containing 118 bp of 5′-flanking region was fused 5′ to the human growth hormone reporter (called h118wt) as previously described (12). This region contains previously characterized GATA and HNF-1 binding sites. An h118 construct in which the GATA sites were mutated (called h118mG1G2) was also used in these studies. Mutations introduced into these sites have been previously shown to disrupt specific protein-DNA interactions (12). pRC-CMV (Invitrogen) served as a negative control expression vector for all co-transfection experiments.

Cell culture and transient co-transfection assays–All cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 5 μg of penicillin-streptomycin per ml and containing 10% fetal calf serum. Transient co-transfection assays were carried out in Caco-2 and HeLa cells. Caco-2 cells were used to define the function of FOG cofactors in an intestinal cell line. HeLa cells were used because GATA-specific and GATA/HNF-1α cooperative activation can only be distinguished in cells that do not synthesize endogenous HNF-1α, exempting Caco-2 cells because they make abundant amounts of HNF-1α. Cells at 80-95% confluence were collected by trypsinization and plated onto 6-well plates the day before transfection. Cells were transfected using Effectene™ reagent (Qiagen) according to the protocol provided by the manufacturer. Optimal conditions for both cell lines were: 1 μg total DNA (0.4 μg of promoter/reporter construct and 0.2 μg of expression vector) and 4 μl of

additional 24 h, as previously described (27). All plates were confluent at the time of harvest. The amount of human growth hormone reporter secreted into the media over 24 h was measured using an [^{125}I] radio/immunoassay kit (Allegro hGH, Nichols Institute). To control for transfection efficiency, all transcriptional activities were expressed relative to pXGH5, a constitutively active metallothionine-I promoter fused to the human growth hormone gene.

Statistics–The one-way analysis of variance was employed in all statistical analyses using InStat software (GraphPAD Software, Inc.). Multiple comparisons were carried out by the Tukey multiple comparison test.

Results and Discussion

To determine if FOG cofactors are expressed in the adult mammalian small intestine, RT-PCR was carried out on RNA isolated from the mid jejunum of a 10 week old mouse. As shown in Fig. 1, an amplification product of predicted size for FOG-1 was clearly identified in adult mouse jejunum as well as in spleen and liver, as previously shown (25).

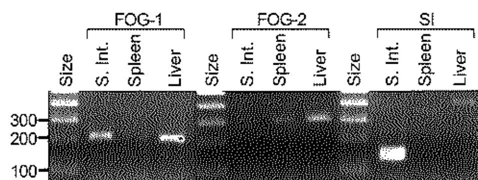

Fig. 1. FOG-1 and FOG-2 are expressed in adult mouse small intestine. RT-PCR was carried out on RNA isolated from the mid jejunum of a 10 week old mouse using oligonucleotides specific for mouse FOG-1, FOG-2, and sucrase-isomaltase (SI). Size markers are indicated on the left in bp. Expected sizes of amplified products: FOG-1, 194 bp; FOG-2, 299 bp; SI, 146 bp.

A feint, but detectable amplification product of predicted size for FOG-2 was also identified in mouse jejunum. A FOG-2 amplification product was also detected in spleen and liver, as previously published for liver (22). On the right, is a positive sucrase-isomaltase control for intestinal RNA. This experiment demonstrates that FOG cofactors are expressed in adult mouse jejunum.

GATA factors have been shown to activate certain intestinal gene promoters, including those of the intestinal fatty acid binding protein (9), sucrase-isomaltase (2, 12), and LPH (5, 6, 12) genes. In non-intestinal systems, FOG cofactors have been shown to modulate the GATA activation of target genes (4, 7, 16, 19, 21, 25). To define the role of FOG cofactors on the GATA activation of intestinal genes, the effect of FOG cofactors on the GATA activation of the LPH promoter was determined using transient co-transfection assays in the Caco-2 intestinal cell line. As shown in Fig. 2, both FOG-1 and FOG-2 repressed the GATA-4 and GATA-5 activation of the LPH promoter, and FOG-2 repressed the GATA-6 activation of this promoter.

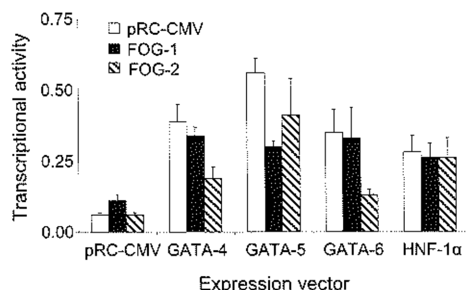

Fig. 2. FOG-1 and FOG-2 repress the GATA-activation of the human LPH promoter in the Caco-2 intestinal cell line. Transient co-transfection assays were carried out using h118 and expression vectors for pRC-CMV (empty vector control), GATA-4, GATA-5, GATA-6, or HNF-1α independently (open box) or along with expression vectors for FOG-1 (filled box) or FOG-2 (hashed box).

This repression was GATA-specific because neither FOG-1 nor FOG-2 repressed the HNF-1α activation. This study demonstrates that FOG cofactors are capable of modulating the GATA activation intestinal gene promoters in an absorptive enterocyte-like cell line.

GATA-4 is capable of activating the LPH promoter independently as well as cooperatively through physical interactions with HNF-1α (26). To delineate the effect of FOG cofactors on the dual mechanisms of GATA-4 activation, the effect of FOG cofactors on the GATA-4-independent and GATA-4/HNF-1α cooperative activation were determined using transient co-transfection assays in HeLa cells. HeLa cells were used because they do not synthesize HNF-1α (12), which is necessary to distinguish GATA-4-independent from GATA-4/HNF-1α cooperative activation. As shown in Fig. 3, FOG-1 reduced the GATA-4-specific activation by approximately 50%, whereas FOG-2 reduced it virtually to baseline.

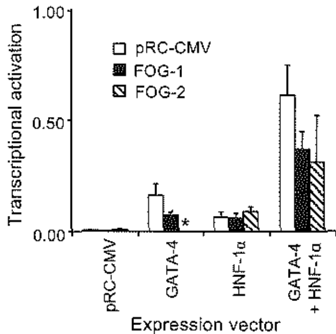

Fig. 3. FOG-1 and FOG-2 repress the GATA-4-specific and GATA/HNF-1α cooperative activation of the LPH promoter. Transient co-transfection assays were carried out in HeLa cells using h118wt and expression vectors for pRC-CMV (control), GATA-4, and HNF-1α as indicated, without (open box) and with expression vectors for FOG-1 (filled box) or FOG-2 (hashed box). *Significantly different than the non-FOG cotransfected control, $P < 0.05$.

Again, the FOG effects were GATA-specific because the FOG cofactors had no effect on HNF-1α activation of the LPH promoter. FOG cofactors also reduced the combined GATA-4/HNF-1α activation approximately 40% by FOG-1 and 50% by FOG-2. This experiment demonstrates that FOG cofactors, especially FOG-2, repress GATA-4-mediated activation of the LPH promoter in HeLa cells. This experiment also suggests that FOG cofactors repress the GATA/HNF-1α activation of the LPH promoter, although it is uncertain whether this repression is due to repression of cooperativity or to repression of the GATA-4-specific component of the combined activation.

To test the hypothesis that FOG-2 represses the GATA/HNF-1α cooperative activation of the LPH promoter specifically, two models of cooperativity were employed. In the first model (Fig. 4A), GATA-5 was used since GATA-5 demonstrates high levels of synergy with HNF-1α , but has no intrinsic activity of its own (12). As shown by the addition of FOG-2 to the combined activation, synergistic activation was reduced approximately 70%. In the second model (Fig. 4B), GATA-4 was used, but the GATA binding sites in the LPH promoter were mutated. As shown recently (26), GATA-4-specific activation requires intact GATA binding sites on the promoter, but GATA/HNF-1α cooperative activation does not. Cooperative activation requires only an intact HNF-1 binding site. Thus, by mutating the GATA sites, the GATA-specific component of cooperative activation is eliminated. As shown by the addition of FOG-2, synergistic activation is reduced approximately 50%.

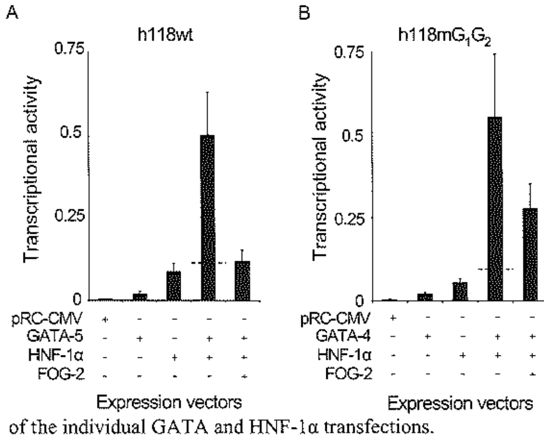

Fig. 4. FOG-2 represses the GATA/HNF-1α cooperative activation of the LPH promoter. Transient cotransfection assays were carried out in HeLa cells using two models of GATA/HNF-1α cooperativity. In the first model, A, GATA-5 was used since GATA-5 demonstrates high levels of synergy with HNF-1α in the activation of h118wt, but has no intrinsic activity of its own (12). In the second model, B, GATA-4 was used, but the LPH promoter in the promoter-reporter construct had GATA binding sites that were mutated (indicated as h118mG_1G_2). GATA-4-specific activation requires intact GATA binding sites on the promoter, but GATA/HNF-1α cooperative activation does not. The transfected expression vectors are indicated along the bottom of each figure. The dotted line represents the sum of the individual GATA and HNF-1α transfections.

This is parallel to the finding by Lu et al (16), who showed that the cooperative activation of the atrial natriuretic factor (ANF) promoter by GATA-4 and Nkx-2.5 is repressed by FOG-2. Together, these data are support the hypothesis that FOG-2 represses not only the GATA-specific activation, but also the GATA/HNF-1α cooperative activation of the LPH promoter.

It has been previously shown that FOG cofactors physically associate with the N-terminal zinc finger of GATA factors to modulate GATA activation of target genes (16, 19, 25). To test the hypothesis that FOG cofactors mediate repression of the LPH gene through interactions with the N-terminal zinc finger of GATA-4, a previously characterized amino acid substitution (E215K) that disrupts physical interactions with FOG-2 (19) was introduced into GATA-4 (mut1). As shown in Fig. 5, GATA-4 mut1 activates the LPH promoter similar to that of wild type GATA-4 shown in Fig. 3.

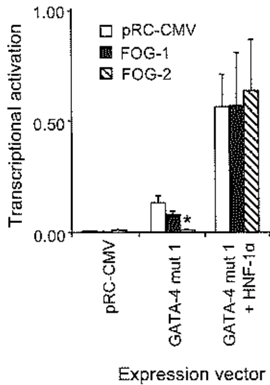

Fig. 5. A mutation in the N-terminal zinc finger of GATA-4 disrupts GATA-4/HNF-1α cooperative, but not GATA-4-specific activation of the LPH promoter. Transient co-transfection assays were carried out in HeLa cells using h118wt and expression vectors for pRC-CMV (control) and GATA-4 mut1 individually and in combination with HNF-1α as indicated, without (open box) and with expression vectors for FOG-1 (filled box) or FOG-2 (hashed box). *Significantly different than the non-FOG cotransfected control, $P < 0.05$.

As with wild-type GATA-4, GATA-4 mut1 binds DNA (EMSA, not shown). In the absence of HNF-1α, GATA-4 mut1 mediates FOG repression similarly to that of wild type GATA-4, but in the presence of co-transfected HNF-1α, GATA-4 mut1 is unable to mediate repression of GATA/HNF-1α cooperative activation (in contrast to wild type GATA-4). Based on these findings, it is hypothesized that in the absence of HNF-1α, GATA-4 mut1 is capable of binding FOG cofactors through the intact C-terminal zinc finger, whereas in the presence of HNF-1α, which we have previously shown binds to this zinc finger (27), is no longer capable of binding FOG cofactors and mediating FOG repression, perhaps because of competition with HNF-1α.

These data demonstrate that FOG cofactors are expressed in adult mouse small intestine and repress the GATA-mediated activation of the LPH promoter. Although FOG-1 and FOG-2 are both expressed in fetal intestine (3, 16, 22, 25), this is the first report demonstrating

expression and function of these two proteins in adult intestine. Previous studies have shown that depending on promoter and cell lines, FOG cofactors can act as either activators or repressors of the GATA activation of target gene expression (10, 16, 24, 25). For example, the α-myosin heavy chain (α-MHC) promoter is activated by FOG-2 in COS cells, but repressed by FOG-2 in neonatal rat cardiomyocytes (16). In the present study, we found both FOG-1 and FOG-2 repress the GATA-specific activation of the LPH promoter in intestinal Caco-2 cells as well as in non-intestinal HeLa cells. Thus, FOG cofactors may regulate the specific expression of intestinal genes by repressing GATA-activated gene transcription in vivo. This repression likely occurs via physical associations between GATA and FOG proteins that involve, in part, the N-terminal zinc fingers of GATA factors.

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Chapter 8

HNF-1α is a critical transcription factor for the regulation of LPH gene expression in vivo.

(Manuscript in preparation)

INTRODUCTION

These data are outside the scoop of this thesis. However, these experiments were carried out during the process of the thesis preparation and demonstrate a significant part of the hypothesis of this thesis and therefore were appropriate to mention.

HNF-1α is expressed in the intestine and activates the promoters of several intestine-specific genes, including that of the intestinal differentiation marker gene, lactase-phlorizin hydrolase (LPH). LPH, an intestinal disaccharidase necessary for the digestion of milk lactose, is expressed only in absorptive enterocytes on villi [1-4], and thus is a model for tissue-, cell-type-, and differentiation-specific gene expression. In rats, LPH is first expressed at the time of villus formation [5, 4], and thus is a marker for this event in the developing gut. The LPH gene is expressed throughout the small intestine at birth and during suckling, but is reduced and also restricted to the jejunum and proximal ileum after weaning [2]. Together, these data demonstrate an intricate, highly regulated pattern of expression that coincides with critical mechanisms of gene regulation in the mammalian small intestine. Accordingly, the LPH gene is used as a model for defining the mechanisms of intestine-specific gene expression.

Initial characterization of LPH gene expression revealed that the developmental pattern of lactase activity in rats [6, 7, 2] and the genetic pattern in humans [8] are both coordinated with the abundance of LPH mRNA. Using sophisticated pre-mRNA analyses and nuclear run-on assays, the PI then demonstrated that the horizontal and developmental patterns of LPH gene expression are transcriptionally regulated [2]. Transcriptional control was further supported by in vivo studies by the PI [3] and others [9, 10] who showed using transgenic mice that the LPH promoter directs reporter expression to absorptive enterocytes on small intestinal villi. These studies revealed that information for tissue-, cell-type-, and differentiation-specific LPH gene expression is contained in its 5′-flanking region.

Sequence analysis of the LPH 5′-flanking regions of the human, rat, mouse, and pig LPH genes [11-13] reveal binding sites for the transcription factors, Cdx-2, GATA, and HNF-1, all of which are expressed in the intestinal epithelium. The conserved position of these binding sites in the LPH promoter among different species, and the close proximity of these sites to the TATA-box and to each other have led to our hypothesis that the factors that bind these sites are important and act in concert to modulate LPH gene expression in vivo. Indeed, we have shown that HNF-1α independently and cooperatively (with GATA factors) activates the LPH promoter, and that the HNF-1 binding site at -90 to -70 bp is critical for these activations [14].

These data have led us to hypothesize that HNF-1α act as a linker for other proteins in order to act in concert with these proteins for the regulation of LPH gene expression in adult mice in vivo.

Methods

Knock out mice-To test the hypothesis that HNF-1α is required for LPH gene expression in vivo, we have obtained frozen tissues from adult *hnf-1α-/-* and -/+ littermates (gift from Drs. T. Akiyama and F. Gonzalez, NIH). The *hnf-1α* null mice were produced in the laboratory of Dr. F. Gonzalez (NIH, Bethesda, MD) using the Cre-*lox*P recombination system whereby mice homozygous for an *hnf-1α* gene containing introduced *lox*P sites were bred with a homozygous *cre* transgenic mouse line, EIIa-*cre* [5], which express Cre in all cells of early embryos. Lack of HNF-1α expression in the livers and kidneys of these mice has been verified [15, 16].

Plasmids– To construct plasmids templates for the synthesis of RNA probe for RNase protection assays. Single-stranded ^{32}P-labeled antisense RNA probe for mouse LPH was synthesized and gel purified. To identify endogenous mouse LPH mRNA, a template was derived from the 3.3-kb *Eco*RI (located at -823 in the 5'-flanking region)/Hind III (located at +584 in the coding region) fragment into the *Eco*RI/*Hind* III sites of pSP73 (Promega Biotec, Madison, WI). The template was linearized with *Nco*I, which is present in the first exon (+398) of the rat LPH gene [17]. The antisense RNA probe was synthesized using T7 RNA polymerase.

RNase protection assays-The quantitative pattern of GATA and HNF-1α expression was compared to that of LPH using RNase protection assays as previously described [2, 3]. Total RNA was isolated from mouse tissues using Trizol Reagent™ (Invitrogen), quantified by optical density at A260 nm, and checked on an agarose gel. [^{32}P]-labeled probes were hybridized at 68°C in 50% formamide overnight, digested with RNase A and T1, and the protected fragments were separated on 6% denaturing polyacrylamide gels and revealed by autoradiography.

Results

To characterize the effect of the absence of HNF-1α on LPH gene expression in vivo, LPH mRNA abundance was determined by RNase protection assays in RNA isolated from jejunum of adult mice. As shown in Fig. 1, LPH mRNA was reduced by 85% in the hnf-1α-/- mice as compared to the -/+ control, indicating that LPH gene expression requires HNF-1α in vivo.

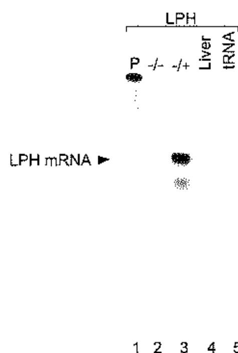

Fig. 1. **LPH mRNA expression is reduced in adult mice homozygous for the *hnf-1α* null allele.** RNase protection assay using a probe specific for mouse LPH mRNA [3] was carried out on RNA isolated from the small intestine of mice homozygous (-/-, *lanes 2*) and heterozygous (-/+, *lanes 3*) for the *hnf-1α* null allele. Mouse liver RNA (*lanes 4*) and tRNA (*lanes 5*) were used as negative controls. Intact ribosomal bands on ethidium bromide-stained gels indicated that the RNA was not degraded (not shown). LPH mRNA abundance was reduced by 85% in the small intestine of an *hnf-1α* -/- mouse (*lane 2*) as compared to that of a *hnf-1α* -/+ mouse (*lane 3*) (determined by the cutting and counting of the protected fragment from dried gels).

These data demonstrate that HNF-1α is a critical component of the LPH gene expression in adult mice in vivo.

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Chapter 9

Summary

9. SUMMARY

9.1 LPH and SI are marker genes

Here, I have defined the function of Cdx-2, HNF-1α, and GATA-4, -5, and -6 transcription factor family members in the transcriptional regulation of the intestinal marker genes, LPH and SI. Through a series of elegant experiments in the laboratory of R. J. Grand, as well as experiments in other laboratories, lactase and sucrase were shown to be excellent markers of intestine-specific gene expression (chapter 1). These disaccharidases are absorptive enterocyte membrane anchored glycoproteins. Lactase digests lactose, the main carbohydrate in milk, whereas sucrase hydrolyzes sucrose, which is derived mainly from sugar cane and beet which are found in many solid foods. LPH and SI are both expressed in absorptive enterocytes on villi, and therefore display tissue-, cell type-, and differentiation specific patterns. The expression patterns along the horizontal and developmental gradients for LPH and SI are different from each other. LPH first appears at the time of villus formation, is highly expressed at birth, and remains high until weaning, when LPH expression is reduced and restricted to the mid jejunum and proximal ileum. During this period, the intestine undergoes a biochemical and morphological transition that prepares the intestine for solid food nutrients. At weaning, SI increases from low levels to adult levels and therefore is an excellent marker for this transition. The abundance of LPH and SI proteins coincides with the levels of their mRNAs, pre-mRNAs, and transcriptional rates, demonstrating that LPH and SI protein abundances are regulated at the transcriptional level. Transgenic studies have revealed that a 2 kb promoter of the rat LPH gene directs specific expression along the vertical, horizontal, and developmental gradients. For SI, a short180 bp evolutionarily conserved region of the SI promoter is sufficient to direct its developmental and differentiation-dependent expression patterns. Together, these data establish that LPH and SI are excellent marker genes for intestine-specific-gene expression, and that these expression patterns coincide with important transitions in the intestine of developing mammals. Therefore, understanding the mechanisms of LPH and SI expression during these important transitions should reveal critical processes in the regulation of intestinal differentiation and development. This thesis reveals fundamental control mechanisms for the transcriptional processes of the LPH and SI marker genes.

9.2 Differential activation of rat and human LPH and human and mouse SI promoter

In chapter two, it is revealed that approximately the first 118 bp of the rat and human LPH promoters and 183 bp of the human SI promoter contain binding sites for three intestinal transcription factor families, Cdx-2, HNF-1 and GATA. The effects of members of these transcription factor families on the transcription of rat and human LPH and human SI promoters linked to a human growth hormone reporter were studied using transient co-transfection assays in Caco-2 cells, which are derived from a human colon adenocarcinoma and are a model for absorptive enterocytes. GATA and HNF-1α transcription factors were strong activators of the human LPH promoter, whereas Cdx-2 was a weak activator. In contradistinction to the LPH promoter, the Cdx-2 and HNF-1α transcription factors were strong activators of the human SI promoter, whereas GATA factors were weak activators. Cdx-2, HNF-1α, and GATA factors co-transfected together cooperatively activated both LPH and SI promoters. However, a key finding of this study was that LPH demonstrated maximal levels of functional synergy in response to a combination of GATA-5 and HNF-1α. Indeed, GATA-5 was inactive in cell lines that do not

make endogenous HNF-1α, but demonstrated synergistic activation of the human LPH promoter when HNF-1α was co-transfected together with GATA-5 in these cells. Functional synergy is defined as a higher activation by a combination of transcription factors than the additive activation of each transcription factor co-transfected individually. In the human LPH promoter, an intact HNF-1 site was required for functional synergy, but not intact GATA sites. In this chapter, it is demonstrated that: (1) LPH and SI promoters are differentially activated by multiple transcription factors co-transfected singly and in combination. (2) GATA-5 was unable to activate the human LPH promoter in the absence of HNF-1α. (3) GATA-5 and HNF-1α cooperatively activate the human LPH promoter, which requires an intact HNF-1 binding site. Synergistic activation is a mechanism by which higher levels of tissue-specific-gene expression might be attained by overlapping expression of specific transcription factors.

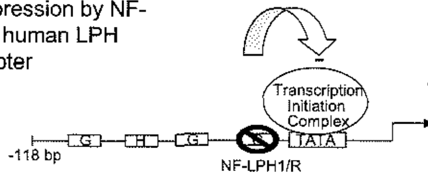
The SI gene is strongly induced in differentiated small intestinal villus epithelium during the suckling-weaning transition. In chapter two, we have demonstrated that specific cis-acting elements within a short evolutionarily conserved human SI promoter contain binding sites for HNF-1α, GATA and Cdx-2 and that their *cis*-acting elements are necessary for the transcriptional activation of SI. In the report that is described in chapter three, it is suggested that from the GATA-4, -5, and -6 subfamily, GATA-4 is more important for the mouse SI promoter. However, the nature and profile of expression and interactions among HNF-1α, GATA-4, and Cdx-2 proteins had not been previously investigated before, during and after the weaning period, and this is the main focus of this report (chapter 3). In chapter three, we present data consistent with the hypothesis that HNF-1α, GATA-4, and Cdx-2 are the main transcription factors in the adult mouse intestinal epithelium that interact with SIF3, GATA, and SIF1 elements, respectively, and regulate the SI promoter. Immunohistochemistry revealed that HNF-1α is weakly detected in rare epithelial cells on the villi of suckling mice and becomes progressively expressed in all epithelial cells along the entire villi during the weaning period, similar to the up-regulation of SI during this transition. GATA-4 protein is expressed in differentiated epithelial cells on villi of the proximal small intestine, and its expression is decreased in the ileum and is undetectable in the colon. Cdx-2 proteins are detected on the entire villi and in colon at all ages. This pattern parallels that of the horizontal SI expression of adult mice. HNF-1α, GATA-4 and Cdx-2 interact in vitro and it is hypothesized that they co-exist in a complex in vivo. In addition, these factors activate SI promoter activity in co-transfection assays where GATA-4 requires the presence of both HNF-1α and Cdx-2 to influence this activity. These findings imply a combinatory role of HNF-1α, GATA-4, and Cdx-2 for the temporal and position-dependent regulation of SI transcription during intestinal development.

9.3 Regulation of LPH transcription

9.3.1.1 Cdx-2 and NF-LPH1/R

The last two chapters characterize the mechanisms of functional activation of the LPH and SI promoters by Cdx-2, HNF-1α, and GATA-4, -5, and -6 transcription factors individually as well as cooperatively. In chapter four, we characterize the Cdx-2-mediated activation of the LPH promoter at CE-LPH1a and the TATA-box, and have identified a non-Cdx-2 repressor complex that binds CE-LPH1a. Cdx-2 is capable of activating a short human LPH construct that contains the first 37 bp of the 5'-flanking region and does not contain CE-LPH1a but just the TATA-box. Using EMSAs, Cdx-2 was found to bind a Cdx-2 binding site overlying the TATA-box. A specific mutation introduced into the TATA-box which binds TBP, necessary for

transcription (chapter 1), but does not bind Cdx-2, does not disrupt the Cdx-2 activation of the LPH promoter, suggesting that Cdx-2 does not activate the LPH promoter through interaction at the TATA-box, but might rather be regulated through direct physical interaction with proteins that are part of the transcription initiation complex, such as TBP (Fig. 1). Characterization of CE-LPH1a using EMSAs confirmed Cdx-2 binding, but also revealed the presence of a novel, non-Cdx-2 complex in multiple cell lines that has a slower mobility than that of Cdx-2. This complex binds to a site that is different from the Cdx-2 binding site. Mutational analysis in transient co-transfection assays using Caco-2 cells revealed a repressor function for this protein (NF-LPH1/R). Transfer of four copies of this element onto the active TK promoter revealed a ~50% reduction of transcriptional activity as compared to TK alone, using NIH/3T3 cells, which do not produce Cdx-2, demonstrating that this repressor function is transferable to a heterologous promoter. Together, these data are consistent with the hypothesis that NF-LPH1/R represses LPH gene expression in non-Cdx-2-producing cells, and that this repression is overcome by Cdx-2 in cells that synthesize Cdx-2 such as those in the intestinal epithelium (Fig. 1).



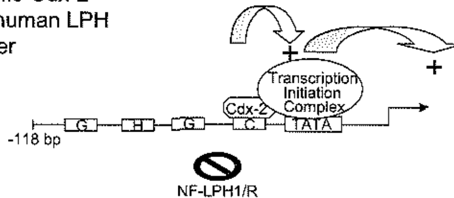


Fig. 1. Model for CE-LPH1a regulation of LPH by Cdx-2 and NF-LPH1/R (Top panel). *NF-LPH1/R binds at CE-LPH1a independently of the Cdx-2 binding site.* The line indicates the first 118 bp of 5'-flanking region of the human LPH gene. The TATA-box binds the transcription initiation complex, which is necessary for gene transcription. C, H, and G are the binding sites for Cdx-2, HNF-1, and GATA transcription factors, respectively. **(Bottom panel)** *Cdx-2 is present in intestinal epithelial cells and binds to the CE-LPH1a site.* Since the binding sites for NF-LPH1/R and Cdx-2 overly each other, it is hypothesized that Cdx-2 preferably binds CE-LPH1a and replaces NF-LPH1/R in intestinal epithelial cells, which results in activating the human LPH promoter.

As shown in Fig. 1, NF-LPH1/R represses the human LPH promoter in cells that do not contain Cdx-2 (i.e. non-intestinal cells). Since the binding sites for both proteins overlie each other the binding of Cdx-2 and NF-LPH1/R might be mutually exclusive. The presence of Cdx-2 in intestinal epithelial cells may therefore block binding of NF-LPH1/R and release its repressional function, allowing the transcription of the LPH promoter to be activated by Cdx-2 (bottom panel). In addition, there is evidence that Cdx-2 might activate the human LPH promoter through direct physical interaction with the transcriptional initiation complex. Cdx-2 is expressed in mid jejunum where LPH is also expressed (chapter 3 for Cdx-2 expression). However, Cdx-2 is also expressed in the colon where LPH is not, therefore Cdx-2 might be necessary but is not sufficient for the cell-specific transcriptional activation in vivo.

9.3.1.2 Future directions

Since these studies identified a potential critical repressor pathway that may mediate intestine-specific gene expression, it would be important in future studies to characterize this repressor protein. This could be done by isolating the complex and micro sequencing it. The

peptide analysis will facilitate searches in protein databases for matches. If the protein is cloned, expression vectors might be available or construction of expression vectors will be possible. Antibodies might be available or developed. Probes for in situ hybridization and RNase protection assays, and expression vectors, will than be available for future experiments. To define the expression of NF-LPH1/R in cells as well as the expression along the vertical (in situ hybridisation) and horizontal (RNase protection assays) gradients, in situ hybridization and RNase protection assays should be carried out on intestinal tissues, using non-intestinal tissues as controls. To test affinity differences of CE-LPH1a for Cdx-2 and NF-LPH1/R EMSAs will be carried out, using nuclear extracts from cells that were co-transfected with different amounts of expression vectors for Cdx-2 and NF-LPH1/R and a CE-LPH1a as a probe. To test for functional mutual exclusivity of these proteins, transient co-transfection assays need to be carried out using different amounts of expression vectors of these proteins and compare the transcriptional activities. To explore the expression of NF-LPH1/R, immunohistochemistry experiments could be carried out. By defining the independent and overlapping expressions of NF-LPH1/R and Cdx-2, these studies will provide insights into the function of NF-LPH1/R for the down-regulation of the human LPH gene resulting in the tissue-specific, vertical and horizontal expression patterns of LPH.

9.3.2.1 Physical association between GATA and HNF-1α transcription factors

In chapter five, we characterized the mechanism of GATA-5/HNF-1α cooperativity in regulating the human LPH promoter. GATA-5 and HNF-1α physically associate both in vivo and in vitro, which is necessary for GATA-5/HNF-1α cooperative activation (Fig. 2). Furthermore, physical association is mediated by the C-terminal zinc finger and basic region of GATA-5 and the homeodomain of HNF-1α. Interruption of HNF-1 binding sites in the human LPH promoter or a deletion of the HNF-1α activation domains result in a complete loss of cooperativity, whereas an interruption of GATA binding sties or a deletion of GATA-5 activation domains results in a reduction, but not an elimination, of cooperativity. These results led to a model for GATA/HNF-1α-cooperativity in which activation requires HNF-1α bound to the DNA and is mediated by HNF-1α through its activation domains which are oriented for high levels of activation through the physical association with GATA factors (Fig. 2). In addition, GATA-4 also physically associates with HNF-1α, and co-transfection of GATA-4 and HNF-1α together also results in cooperative activation of the human LPH promoter (Chapter 5). Parallel mechanisms in other tissue of *vertebrates* as well as in *Drosophila* suggest that zinc finger/homeodomain interactions are an efficient pathway of cooperative activation of gene transcription that has been conserved throughout evolution (chapter 1). These data suggest a paradigm whereby intestine-specific gene expression is regulated by unique interactions among tissue-restricted transcription factors co-expressed in the intestine.

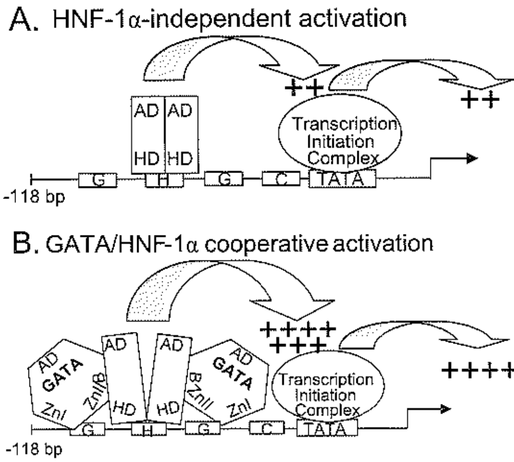


Fig. 2. Upstream elements of the human LPH promoter direct independent as well as cooperative activation by HNF-1α and GATA-4, -5 transcription factors. *A. HNF-1α binds to the cis-acting element of the human LPH promoter as a dimer, and independently activates the human LPH promoter (++).* C, H, and G are the binding sites for Cdx-2, HNF-1, and GATA transcription factors, respectively. DNA binding is regulated through its homeodomain (HD). The independent activation requires the HNF-1α activation domains *B. GATA/HNF-1α cooperative activation (+++++++).* Both GATA-4 and GATA-5 demonstrate cooperative activation of the human LPH promoter with HNF-1α. This mechanism requires HNF-1α binding to DNA, but not GATA binding. The physical interaction between GATA and HNF-1α is critical for the cooperative activation and is mediated through the homeodomain of HNF-1α and the C-terminal zinc finger and basic regions of GATA proteins.

9.3.2.2 Future directions

LPH gene expression in adult mammals is hypothesized to be regulated by an HNF-1α dependent mechanism. Cell culture and other in vitro experiments (chapters 2, 3, and 5) demonstrated that HNF-1α is a critical transcription factor, which may interact with several intestinal transcription factors resulting in high levels of LPH transcription. These in vitro data are complemented by a critical finding (chapter 8) that demonstrates that HNF-1α is important for LPH expression of adult mice in vivo, using knockout studies. RNase protection assays revealed that in HNF-1α knockout mice, the level of LPH mRNA was reduced by 80% from that of wild type mice. HNF-1α is capable of recruiting histone-acetylase activity and other cofactors important for the general transcription machinery and is important for gene transcription in vivo (chapter 1). Thus, the goal of future experiments is to define the importance of HNF-1α and its binding site for the regulation of LPH and SI gene expression in vivo, and delineate the function of HNF-1α-dependent recruitment of histone acetylase activity. To test these hypotheses, the chromosomal immunoprecipitation (CHIP) assay, an accepted technique for the study of protein-DNA interaction in vivo, will be carried out. These experiments will demonstrate whether HNF-1α occupies the HNF-1 site in the human LPH promoter in vivo and the function of HNF-1α for the recruitment of histone acetylase activity to activate the LPH and SI promoters.

9.3.3.1 Differential activation between GATA-4 and GATA-5

The horizontal expression of GATA-4 mRNA coincided with that of the LPH mRNA, suggesting an important role for GATA-4 in the transcriptional regulation of LPH gene. Furthermore, similar to the GATA-5/HNF-1α cooperativity, GATA-4 and HNF-1α cooperatively activate the human LPH promoter as well. However, in contrast to GATA-5, which in the absence of HNF-1α does not appreciably activate the human LPH promoter (chapter 2 and 5), GATA-4 activates the human LPH promoter independently of HNF-1α. To define the

dual mechanisms of GATA-4 activation of the human LPH promoter, the function of critical domains in GATA-4 was mapped by first introducing mutations that delete or disrupt specific structures, and then characterizing specific functions such as physical association with HNF-1α (GST pull-down assays), binding to DNA (EMSAs), and transcriptional activation in HeLa cells (transient co-transfection assays). HeLa cells were used because GATA-specific and GATA/HNF-1α cooperative activation can be distinguished in cells that do not synthesize endogenous HNF-1α. This study revealed that in contrast to GATA-5, GATA-4 activates the human LPH promoter independently of HNF-1α by a mechanism that requires the activation domains and the C-terminal zinc finger which is responsible for binding to DNA (Fig. 3). However, similar to GATA-5, GATA-4 physically associates with HNF-1α and cooperatively activates the human LPH promoter by a mechanism that does not require the GATA-4 activation domains or GATA-4 binding to DNA. Cooperative activation does require physical association between GATA-4 and HNF-1α as well as an intact HNF-1 binding site on the promoter. Physical interaction involves the C-terminal zinc finger of GATA-4.

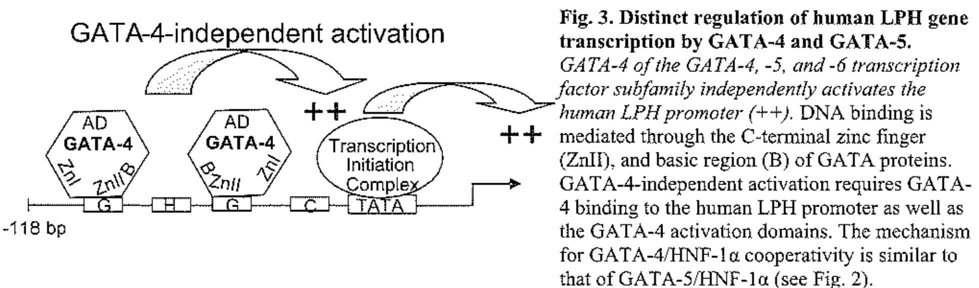


Fig. 3. Distinct regulation of human LPH gene transcription by GATA-4 and GATA-5. *GATA-4 of the GATA-4, -5, and -6 transcription factor subfamily independently activates the human LPH promoter (++).* DNA binding is mediated through the C-terminal zinc finger (ZnII), and basic region (B) of GATA proteins. GATA-4-independent activation requires GATA-4 binding to the human LPH promoter as well as the GATA-4 activation domains. The mechanism for GATA-4/HNF-1α cooperativity is similar to that of GATA-5/HNF-1α (see Fig. 2).

Indeed, EMSAs revealed that the GATA binding sites in the human LPH promoter have a higher affinity for GATA-4 than for GATA-5. The conclusions derived from these data are that GATA-4 demonstrates dual mechanisms of activation of the human LPH promoter that are characterized by: (1) HNF-1α-independent activation (in contrast to GATA-5), in which the DNA binding and activation domains are required, and (2) HNF-1α-dependent cooperative activation (similar to GATA-5), in which physical association is required but not the GATA-4 activation domains or GATA-4/DNA binding. It is hypothesized that the distinct functions in transcriptional activation between members of the GATA-4, -5, and-6 subfamily in part is mediated through the specific affinity of promoters for the individual GATA factors. This study is the first to demonstrate distinct regulatory mechanisms for activation of an intestinal gene promoter by two conserved GATA factors.

Although, LPH mRNA abundance is reduced in the absence of HNF-1α, as shown in chapter 8 by RNase protection assays, a residual amount of LPH mRNA was present in adult HNF-1α knockout mice. These data suggest that in adult mice the LPH expression is also regulated by a mechanism that is independent of HNF-1α. Since GATA-4 is expressed in the small intestine, its horizontal expression pattern coincides with that of LPH and finally GATA-4 independently activates the human LPH promoter, it is suggested that GATA-4 plays an important role in the expression of LPH in the absence of HNF-1α as well as in cooperativity with HNF-1α. As previously is shown in chapter 3, HNF-1α is low detectable during the suckling period when LPH is abundantly expressed. However, GATA-4 is expressed in small intestine during suckling (chapter 3) and GATA-4 independently activates the human LPH promoter (chapter 8). These data has led to the hypotheses that during the pre-weaning period LPH transcription is regulated by a mechanism independently of HNF-1α in which GATA-4

potentially plays a critical role and during and after weaning LPH expression is regulated by a mechanism that depends on HNF-1α, in which GATA factors play a role as cofactors.

9.3.3.2 Future directions

To characterize the role that GATA factors play in the expression of LPH and SI genes independently as well as cooperatively with HNF-1α in vivo, knockout studies need to be conducted. To complement the studies in this thesis, a critical technology that should be developed is tissue-specific knockouts. Fortuitously, HNF-1α knockout mice survive providing an opportunity to study the effect of the absence of HNF-1α on intestinal differentiation and LPH an SI gene expression. GATA-5 knockout mice also survive, and thus the role of GATA-5 for intestinal gene regulation can also be studied in vivo. In contrast, GATA-4 and GATA-6 knockout mice die during early embryogenesis without a foregut. Therefore, the conventional whole animal knockout system does not provide any data for the importance of these transcription factors for intestinal differentiation and intestine gene expression. Using Cre-loxP recombination technology, it may be possible to ablate the expression of GATA-4, -5, and -6 individually as well as in all combinations in limited tissues and provide in vivo data for their specific role in the intestinal development and intestinal genes expression.

To define the mechanism of the independent GATA-4 activation, detailed characterization of the domains that mediate affinity differences among GATA factors need to be carried out. To define binding sites specificities, a methodological approach should be used that depends on randomized oligonucleotides as was carried out for GATA-1 and GATA-6. Amino acid sequence analysis of the C-terminal zinc finger and basic region, which mediate protein-DNA binding, revealed only 10 residues that are not conserved among this subfamily (Fig. 4).

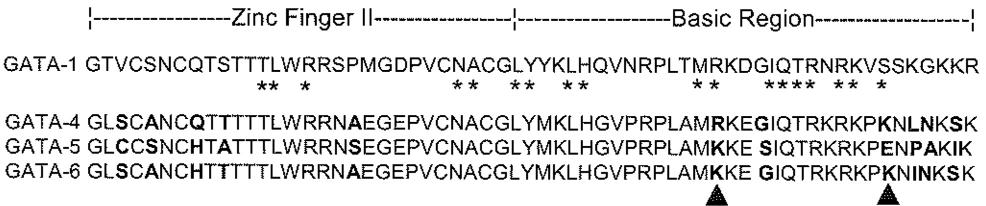


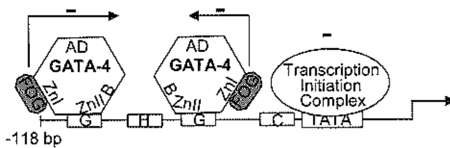
Fig. 4. The –100 to –20 bp region of the human (h) (1), rat (r) (2), mouse (m), and pig (p) (3) LPH 5'-flanking sequence contains conserved binding sites for GATA (A/TGATAA/G), HNF-1 (GTTAATNATTAAC), and Cdx-2 (TTTAT/C) (*underlined*). TATA-boxes are in *boldface* type. The mouse sequence was obtained from the genetic database of the Celera Discovery System (Rockville, MD) (Dr. M. Fleming, licensee, Dept. Pathology, Children's Hospital Boston). Cortesy of Stephen D. Krasinski

To define the importance of these and other non-conserved amino acids, specific residues in GATA-5 will be changed into GATA-4-like structures and tested for gain-of-affinity for the LPH GATA sites. Likewise for GATA-4 amino-acid, changes into GATA-5-like structures and tested for lost-of-affinity. This affinity differences for specific cis-acting elements might be a key regulatory pathway, by which proteins and cis-acting elements regulate promoter-specific and tissue-specific gene transcription.

9.3.4.1 Regulation of FOG on the LPH transcriptional activation

FOG is a family of multi-zinc finger cofactors that modulate the GATA activation of target genes. Two members have been identified thus far, FOG-1 and FOG-2. The aim of the study presented in chapter 7 was to define the role of FOG cofactors on the GATA-specific and GATA/HNF-1α cooperative activation of the LPH promoter. To demonstrate expression of FOG-1 and FOG-2 in the intestine, RT-PCR was carried out. To characterize the function of FOG cofactors, transient co-transfections were conducted in HeLa cells using a promoter-reporter construct containing 118 bp of the human LPH 5'-flanking region fused to the human growth hormone gene. Both FOG-1 and FOG-2 mRNAs were detected in RNA isolated from adult mouse jejunum using RT-PCR, demonstrating that these two cofactors are expressed in adult mammalian small intestine. FOG-1 and FOG-2 repressed the GATA-4-specific activation of the human LPH promoter to less than 50% (FOG-1) or less than 5% (FOG-2) of its activated value. FOG-1 and FOG-2 had no effect on the HNF-1α-stimulated activation, demonstrating that FOG repression is GATA-specific. FOG-1 and FOG-2 also had an effect on the GATA/HNF-1α cooperative activation of the LPH promoter although to a lesser extent than the effect on GATA-4 independent activation. Introduction of a critical amino acid substitution in the N-terminal zinc finger of GATA factors, which have been shown to disrupt physical interactions with FOG, results in the reduction of FOG-specific repression. FOG cofactors expressed in the intestine may modulate LPH gene expression by repressing the GATA-specific and GATA/HNF-1α cooperative activation of the LPH promoter (Fig 5). This repression likely occurs via physical associations between GATA and FOG proteins that involve the N-terminal zinc fingers of GATA factors.

Repression by FOG-2 of the GATA-4-independent activation



Repression by FOG-2 of GATA/HNF-1α cooperative activation

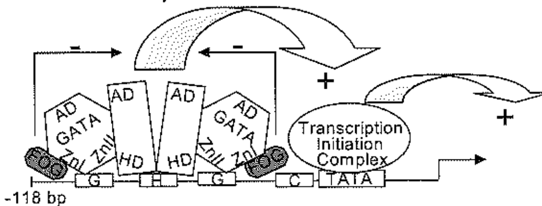


Fig. 5. The mechanisms of FOG mediated repression of the human LPH promoter. FOG-1 and FOG-2 co-factors do not bind DNA, but physically interact specifically with GATA proteins. This physical interaction is mediated by the N-terminal zinc finger of GATA factors. GATA-4 independent activation is reduced for 90% by FOG-2 (top panel). The GATA/HNF-1α cooperative activation of the human LPH promoter is also reduced by FOG-2 but to a lesser extent than without presence of FOG-2 (bottom panel).

9.3.4.2 Future directions

To delineate the mechanisms of FOG repression, the expression patterns need to be defined along the vertical, horizontal and developmental gradients using in situ hybridization, RNase protection assays, and immunohistochemistry. The hypothesis in these experiments is that

FOG-2, in the presence of intestinal GATAs, is a repressor of LPH gene transcription, and therefore demonstrates an inverse relationship in its pattern of expression of LPH in vivo.

9.4 Model for LPH expression in adult mammals

This thesis has described the important transcription factors for gene regulation of intestine-specific marker genes, LPH and SI. Members of the Cdx-2, HNF-1, and GATA-4, -5, and -6 transcription factor families have been demonstrated to be important for LPH and SI gene transcription, both in vitro and in vivo. The proximal promoters of the LPH and SI genes demonstrate differences in number and arrangement of binding sites for these transcription factors. Based on the data demonstrated in this thesis, I propose the following model for the LPH expression in adult mammals, which is demonstrated by a schematic representation in Fig. 6.

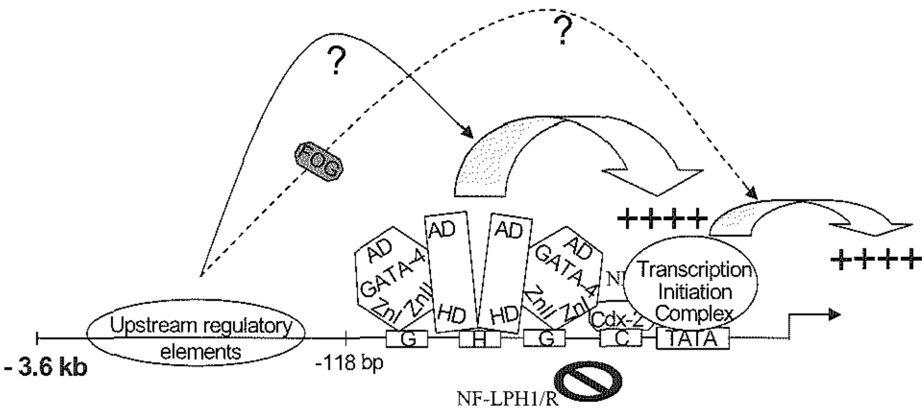


Fig. 6. Schematic representation of LPH specific gene expression. Straight and dotted lines indicate influences by upstream elements on the transcription process. The presence of Cdx-2 in intestinal cells relieves the repression of NF-LPH1/R that binds to CE-LPH1a in non-intestinal cells. Cdx-2 may then physically interact with the transcriptional initiation complex to stabilize the transcription initiation complex. An HNF-1α dimer binds to the DNA and links GATA factors for synergistic up-regulation of LPH gene expression, as well as proteins that have histone acetylase activity. GATA-4 is activates LPH promoter in absence of HNF-1α, which requires DNA binding as well as cooperatively activate with HNF-1α, which requires physical interaction with HNF-1α. FOG represses both GATA independent as well as GATA/HNF-1α cooperativity, which requires physical interaction with GATA transcription factors but not with DNA. All these elements are required for the appropriate LPH expression along the vertical, horizontal, and developmental gradients.

Cdx-2 binds to CE-LPH1a and blocks NF-LPH1/R from binding CE-LPH1a. Cdx-2 individually is capable of minimally activating the LPH promoter and may physically interact directly with the transcriptional initiation complex and stabilize this complex. HNF-1α binds as a dimer to its *cis*-acting element and activates transcription. HNF-1α may act as a linker protein that physically interacts with a multitude of proteins, such as GATA and Cdx-2 transcription factors. These physical interactions result in high levels of LPH expression, as well as recruitment of histone acetylase activity that facilitates LPH gene transcription. Since the expression patterns of GATA-4 and LPH coincide along the horizontal axis in adult small intestine, GATA-4 is suggested to play an important role in the GATA/HNF-1α cooperative activation of the LPH gene. Since Cdx-2 physically interacts with HNF-1α (4) and GATA-4 (chapter 3) it remains possible that these three transcription factors all form one complex that stabilizes the transcriptional initiation complex and recruits histone acetylase activity. Since, the transcription factors all are activating

LPH and SI transcription, repressors might play a role in the down-regulation of LPH and SI gene transcription. FOG cofactors are repressors of GATA-4 independent as well as the GATA/HNF-1α cooperative activation and might play a role in the regulation of the specific LPH expression patterns.

LPH is a marker gene which has a distinct expression pattern along the vertical, horizontal and developmental gradient. Members of the transcription factor families described in this thesis have independent and overlapping expression patterns. These expression patterns do not always coincide with that of the LPH and SI expression patterns, meaning that Cdx-2, HNF-1α and GATA might be expressed together in regions were LPH is not expressed. Therefore, upstream elements must contain more regulatory elements that cooperate with the proximal elements to direct the appropriate LPH expression pattern which is suggested in previous reports (5). Although, the upstream elements play a role in directing the specific vertical, horizontal and developmental expression patterns of LPH, the Cdx-2, HNF-1α and GATA-4,-5 transcription factors, which are characterized in this thesis, activate in concert the transcription of LPH and SI genes and turn on the transcription machinery for the LPH and SI intestine-specific-gene expression in vivo.

9.5 Significance

The studies presented in this thesis provide insight into the regulatory mechanisms that control LPH and SI protein expression in the intestine, and also elucidate some of the mechanism governing the complex processes of cell-specific gene expression, cellular differentiation, and intestinal development. As these mechanisms are defined, and general as well as gene-specific regulatory pathways are characterized, it may become possible to stimulate the expression of individual genes or panels of genes in specific regions of intestine resulting in restoration of lost or deficient intestinal function.

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Chapter 10

Samenvatting

10. SAMENVATTING

10.1 LPH en SI zijn marker genen

In dit proefschrift wordt de functie van Cdx-2, HNF-1α, en GATA-4, -5, en -6 transcriptie factoren gedefinieerd voor wat betreft de transcriptie regulatie van de intestinale marker genen, lactase-phlorizin hydrolase (LPH) en sucrase-isomaltase (SI). Door middel van een aantal elegante experimenten, is gebleken dat lactase en sucrase zeer goede markers zijn voor het intestinale differentiatieproces (Hoofdstuk 1). Deze disaccharidases zijn suiker-eiwitten die verankerd zijn in het membraanoppervlak van enterocyten (absorptieve cellen). Lactase splitst lactose, het belangrijkste koolhydraat in melk. Sucrase hydrolyseert sucrose. Sucrose wordt hoofdzakelijk gevonden in suikerriet dat voorkomt in vele soorten voeding. LPH en SI worden allebei tot expressie gebracht in enterocyten op villi (darmvlokken) en laten een weefsel-, cel, en differentiatie-specifiek verticale (crypt-villus) expressie patroon zien. De expressie patronen van LPH en SI langs de horizontale (proximaal-distaal) en ontwikkelings gradienten verschillen van elkaar. De LPH expressie begint al gedurende de uitrijping en differentiatie van de foetale darm, op het moment dat de eerste rudimentaire darmvlokken worden aangelegd. LPH gen expressie wordt aldus vlak voor de geboorte aangezet en komt vlak voor en direct na de geboorte maximaal tot expressie en blijft constant tot de periode van het spenen; daarna vindt er uitdoving plaats aan beide uiteinden van de dunne darm zodat na het spenen slechts in het midden-gedeelte van de dunne darm (jejunum) LPH wordt gevonden. Spenen is de periode waarin de darm van een zoogdier zich voorbereidt op de overgang van een dieet met alleen borstvoeding naar een dieet met ook vast voedsel. Daarentegen, SI wordt op een heel laag niveau tot expressie gebracht vanaf het stadium van de rudimentaire darmvlokken en dit blijft zo tot de periode van het spenen. Tijdens deze periode bereikt de SI expressie snel het niveau van het volwassen gen-expressie niveau. Het feit dat de expressie patronen van LPH en SI eiwitten parallel lopen aan die van hun messenger RNA, pre-mRNA en hun transcriptie activaties, wijst erop dat het niveau van de eiwitten voornamelijk wordt gereguleerd op het niveau van de transcriptie van deze respectievelijke genen. Experimenten met transgene muizen hebben aangetoond dat een 2 kb promoter regio van de rat LPH promoter een expressiepatroon dirigeert met een adequaat verticale, horizontale en ontwikkelings gradient. Een evolutionair geconserveerd 180 base paren (bp) tellende SI promoter is genoeg om een goed expressiepatroon te dirigeren langs de verticale en ontwikkelings gradienten. Al deze onderzoeksresultaten hebben er toe geleid dat LPH en SI heden ten dage als goede markers van intestinaal-specifieke-genexpressie kunnen worden beschouwd. Deze specifieke expressiepatronen lopen parallel met andere belangrijke veranderingen in de ontwikkeling van zoogdieren. Daarom kan een beter begrip van de mechanismen betrokken bij LPH en SI genexpressie gedurende deze belangrijke stadia van ontwikkeling leiden tot een beter inzicht in de intestinale differentiatie en ontwikkeling. Dit proefschrift karakteriseert enkele fundamentele controle mechanismen van de transcriptie van LPH and SI.

10.2 Differentiele activatie van het LPH gen bij de rat en de mens en van het SI gen bij de mens en de muis.

Zoals is beschreven in Hoofdstuk 2, blijkt dat de eerste 118 bp van de LPH promoter bij de rat en de mens en 183 bp van de SI promoter bij de mens bindingsplaatsen bevatten voor leden van drie intestinale transcriptiefactor families te weten Cdx-2, HNF-1 en GATA. De effecten van leden van deze transcriptiefactor families op de transcriptie van de LPH promoter

bij rat en mens en de SI promoter bij de mens zijn bestudeerd door middel van tijdelijke co-transfectie experimenten in Caco-2 cellen. Dit zijn cellen die zijn geisoleerd uit een humaan colon adenocarcinoom en zij vormen een model voor enterocyten. GATA en HNF-1α transcriptiefactoren zijn sterke activatoren van de LPH promoter van de mens, in tegenstelling tot Cdx-2 dat een zwakke activator is. In tegenstelling tot de LPH promoter wordt de humane SI promoter, sterker geactiveerd door Cdx-2 en HNF-1α transcriptiefactoren en zijn GATA factoren de zwakste activatoren. Alle drie de transcriptiefactoren samen activeren de LPH and SI promoteren synergistisch. Een van de hoofdpunten van dit hoofdstuk is dat LPH de maximale synergistische activatie ondervond door een combinatie van GATA-5 en HNF-1α transcriptiefactoren. Het bleek dat GATA-5 individueel niet in staat was om LPH te activeren in cellen die geen endogene HNF-1α produceren, en dat ook in deze cellen LPH synergistisch geactiveerd werd, wanneer HNF-1α tegelijk met GATA-5 werd getransfecteerd. Functionele synergie wordt gedefinieerd als een sterkere activatie door een combinatie van transcriptiefactoren dan de som van de activaties van iedere transcriptiefactor apart. Voor de GATA-5/HNF-1α synergie van de LPH promoter is een intacte HNF-1 bindingsplaats nodig, maar hoeven er geen intacte GATA bindingsplaatsen te zijn. Met andere woorden is in dit hoofdstuk aangetoond dat: (1) LPH en SI promoters verschillend worden geactiveerd door meerdere transcriptiefactoren, zowel individueel als ook in combinatie. (2) GATA-5 is niet in staat om de LPH promoter in afwezigheid van HNF-1α te activeren. (3) GATA-5 and HNF-1α zorgen voor een cooperatieve activatie (synergie) van de LPH promoter, waarvoor een intakte HNF-1 bindingsplaats noodzakelijk is. Synergie is een mechanisme om hoge niveaus van weefsel-specifieke-gen expressie te verkrijgen door gebruik te maken van overlappende expressie van transcriptiefactoren.

Tijdens de periode van het spenen wordt de SI gen expressie sterk geinduceerd in de epitheellaag van de villi in de dunne darm. Zoals in Hoofdstuk 2 is aangetoond blijkt dat een korte, evolutionair constante, SI promoter, bindingsplaatsen bevat die HNF-1α, GATA en Cdx-2 binden en dat deze bindingsplaatsen nodig zijn voor de transcriptie van de SI promoter van de muis. De experimenten zoals beschreven in Hoofdstuk 3, suggereren dat van de GATA-4,-5 en -6 familie GATA-4 het belangrijkst is voor de muis SI promoter. Buiten deze initiële constatering zijn de expressie profielen en de interactie tussen HNF-1α, GATA-4 en Cdx-2 nog niet eerder onderzocht. In Hoofdstuk 3 worden er gegevens gepresenteerd die consistent zijn met de hypothese dat in het intestinale epitheel van volwassen muizen, HNF-1α, GATA-4 en Cdx-2 de belangrijkste transcriptiefactoren zijn die binden aan SIF3, GATA en SIF1 elementen, respectievelijk, en zo de transcriptie van de SI promoter reguleren. Immunohistochemische experimenten hebben aangetoond dat HNF-1α slechts gering aanwezig is in epitheelcellen van intestinale villi gedurende de lactatie-periode (vóór spenen), maar steeds meer tot expressie komt tijdens de periode van het spenen om uiteindelijk, aan het einde van de periode van het spenen, in alle enterocyten in de dunne darm tot expressie te komen. Deze op-regulatie komt overeen met de op-regulatie van SI expressie tijdens dezelfde periode. GATA-4 wordt gevonden in gedifferentieerde epitheelcellen van villi in de proximale dunne darm, maar de expressie neemt langzaam in hoeveelheid af in de richting van het ileum, om uiteindelijk volledig afwezig te zijn in het colon. In volwassen muizen loopt het horizontale expressiepatroon (proximal-distal) van GATA-4 synchroon met die van SI. HNF-1α, GATA-4 en Cdx-2 hebben een interactie *in vivo* and *in vitro* eperimenten en de hypothese is dat deze dire factoren gezamelijk een complex vormen. Verder blijkt dat deze factoren de transcriptie van SI activeren, met dien verstande dat GATA-4 de andere twee factoren (HNF-1α en Cdx-2) nodig heeft. Deze resultaten suggereren een gecombineerde rol voor HNF-1α, GATA-4 en Cdx-2 in een positie afhankelijke regulatie van SI expressie tijdens de ontwikkeling van de dunne darm.

10.3 Regulatie van LPH transcriptie

10.3.1.1 Cdx-2 en NF-LPH1/R

In de vorige twee hoofdstukken werd het mechanisme besproken waarmee functionele activatie plaatsvindt van de LPH en SI promoters door Cdx-2, HNF-1α en GATA-4, -5, en -6 transcriptiefactoren (individueel als ook in combinatie). In het volgende hoofdstuk (Hoofdstuk 4) wordt de Cdx-2 gemedieerde activatie aan CE-LPH1a en de TATA-box van de LPH promoter beschreven. Ook wordt er een niet-Cdx-2 repressor complex geidentificeerd dat aan CE-LPH1a bindt. Cdx-2 kan een korte LPH promoter van de mens (37 bp) activeren die alleen een TATA-box en geen andere bindings elementen bevat. EMSAs (gel-shift experimenten) hebben aangetoond dat Cdx-2 aan een Cdx-2 bindingplaats bindt, die gedeeltelijk over de TATA-box sequentie heen ligt. Een specifieke mutatie die geintroduceerd werd in de TATA-box, die wel TBP bindt, wat nodig is voor transcriptie (Hoofdstuk 1), maar niet meer Cdx-2, verandert de Cdx-2 transcriptie activatie niet. Deze resultaten suggereren dat Cdx-2 niet door binding met de TATA-box, de LPH transcriptie reguleert, maar eerder door direkt eiwit-eiwit contact met eiwitten van het transcriptie initiatie complex, zoals TBP (Fig. 1). CE-LPH1a werd onderzocht door gebruik te maken van gel-shift experimenten. Deze experimenten bevestigde de binding van Cdx-2 aan CE-LPH1a, maar lieten ook binding van een nieuw niet-Cdx-2 complex zien. Dit complex was in intestinale asl ook in niet intestinale cellen te vinden. Dit complex beweegt zich langzamer door de gel dan Cdx-2. Dit complex bindt aan een bindingsplaats die verschillend is van die van Cdx-2. Analyse met behulp van constructies met mutaties in de CE-LPH1a in transcriptie experimenten, als ook transriptie experimenten met constructies met meerdere copieën van CE-LPH1a gebonden aan de actieve TK promoter, hebben aangetoond dat dit complex een repressor functie heeft (NF-LPH1/R). Alles bij elkaar genomen komen deze resultaten overeen met de hypothese, dat NF-LPH1/R, de expressie van de LPH promoter van de mens onderdrukt in cellen, die geen Cdx-2 produceren, zoals niet-intestinale cellen, maar dat deze onderdrukking wordt opgeheven in cellen, die wel Cdx-2 synthetiseren zoals intestinale cellen (Fig. 1). Omdat de bindingsplaatsen van respectievelijk Cdx-2 en NF-LPH1/R elkaar overlappen, wordt er verondersteld dat ze niet tegelijk kunnen binden aan CE-LPH1a. De aanwezigheid van Cdx-2 in intestinale cellen zou de binding van NF-LPH1/R aan CE-LPH1a dan kunnen blokkeren en de onderdrukking van NF-LPH1/R opheffen en zo de transcriptie van LPH activeren (Fig.1 onderste gedeelte).

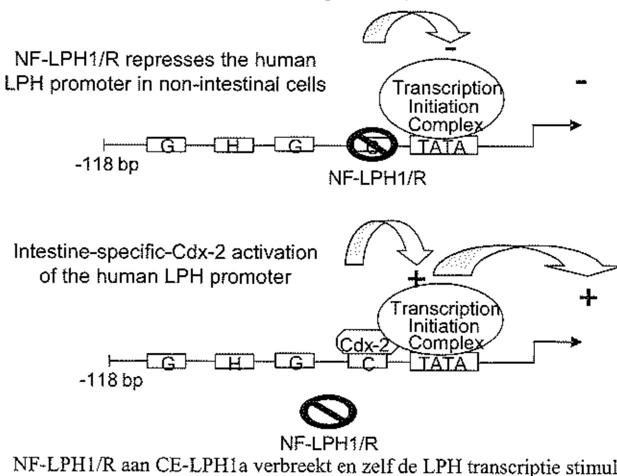


Fig. 1. Model van de LPH transcriptie regulatie door Cdx-2 en NF-LPH1/R via CE-LPH1a (bovendste). *A) NF-LPH1/R bindt aan CE-LPH1a onafhankelijk van de Cdx-2 bindingsplaats.* De doorgetrokken streep geeft de eerste 118 bp of 5'-flanking regio (gedeelte van de promoter) van het LPH gen van de mens weer. De TATA-box bindt het transcriptie-initiatie complex, wat nodig is voor de algemene gen transcriptie. C, H en G zijn de bindingsplaatsen respectievelijk voor de Cdx-2, HNF-1 en GATA transcriptie factor families.**(Onderste)** *Cdx-2 is aanwezig in intestinale epitheelcellen en bindt aan CE-LPH1a.* Omdat de bindingsplaatsen voor NF-LPH1/R en Cdx-2 gedeeltelijk over elkaar heen liggen, is de hypothese dat Cdx-2 de binding van NF-LPH1/R aan CE-LPH1a verbreekt en zelf de LPH transcriptie stimuleert.

Verder zijn er aanwijzingen dat Cdx-2 de LPH transcriptie activeert door middel van direkte eiwit-eiwit binding met gedeeltes van het transcriptie-initiatie complex. Deze eiwit-eiwit interactie zou het initiation complex kunnen helpen stabiliseren. Cdx-2 is aanwezig in het middelste gedeelte van dunne darm (jejunum) waar LPH ook tot expressie komt (zie Hoofdstuk 3 voor de Cdx-2 expressie). Echter, Cdx-2 is bijvoorbeeld ook aanwezig in het colon waar LPH niet tot expressie komt. De conclusie is dat Cdx-2 wel noodzakelijk is voor de transcriptie regulatie van LPH maar niet effectief is voor de expressiepatroon-specifieke transcriptie van LPH in vivo.

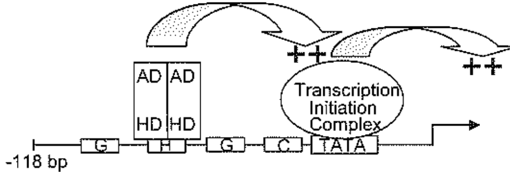
10.3.1.2. Toekomstbeeld

Vanwege de identificatie van een potentieel belangrijk repressor mechanisme, dat specifiek intestinale gen expressie zou kunnen reguleren, is het belangrijk om in toekomstige studies dit repressor eiwit te karakteriseren. Dit kan gedaan worden door dit eiwit te isoleren en de aminozuur sequentie te identificeren. Deze peptide analyse zal het mogelijk maken om een match te zoeken in de eiwit-databases. Als het eiwit gecloond is, kunnen expressievectoren gemaakt worden. Antilichamen tegen dit eiwit zullen ontwikkeld kunnen worden. Probes zullen gebruikt kunnen worden in in-situ hybridisatie en RNase protection experimenten en expressievectoren zijn te gebruiken in tijdelijke transfectie experimenten. Om de expressie van NF-LPH1/R in de verschillende weefsels en langs de horizontale en ontwikkelings gradienten te definieren, dienen RNase protectie en in situ hybridisatie experimenten te worden uitgevoerd op intestinale en niet intestinale weefsels. Om affiniteitsverschillen te meten kunnen gel-shift experimenten worden uitgevoerd, waarbij kern extracten worden gebruikt die zijn geco-transfecteerd met verschillende hoeveelheden expressievectoren voor Cdx-2 en NF-LPH1/R. De transcriptie activatie van deze co-transfectie experimenten met verschillende hoeveelheden expressievectoren van Cdx-2 en NF-LPH1/R kunnen worden uitgevoerd, waardoor de exclusiviteit waarmee deze eiwitten kunnen binden aan CE-LPH1a kunnen worden gekarakteriseerd. Om de expressie van NF-LPH1/R te onderzoeken moeten immunohistochemische experimenten worden uitgevoerd. Door de overlappende expressiepatronen van Cdx-2, NF-LPH1/R en LPH te bepalen zal er inzicht ontstaan over de eventuele functie van NF-LPH1/R in de regulatie ven de specifieke expressie patronen van het LPH gen.

10.3.2.1 Fysieke associatie tussen GATA en HNF-1α transcriptiefactoren

Het mechanisme van de GATA-5/HNF-1α cooperatieve activatie van het humane LPH gen wordt besproken in Hoofdstuk 5. GATA-5 en HNF-1α zijn fysiek met elkaar verbonden, zowel *in-vitro* als *in-vivo*. Deze fysieke interactie is nodig voor de GATA-5/HNF-1α synergie (Fig. 2). Verder wordt de fysieke interactie gemedieerd door de C-terminale zink vinger en basische regio van GATA factoren en het homeodomein van HNF-1α. Het verbreken van HNF-1α binding met het DNA of een verwijdering van de activatie domeinen in het HNF-1α eiwit resulteren in een volledige verdwijning van de synergie, maar een verbreken van de GATA binding aan de bindingsplaatsen of een verwijdering van de activatie domeinen in de GATA-5 eiwitten doen de synergie wel afnemen maar niet verbreken. Deze resultaten hebben tot het volgende model geleid voor de GATA/HNF-1α synergie: de synergistische activatie komt tot stand doordat de binding van HNF-1α aan zijn bindingsplaats en de activatie domeinen, welke zo is georienteerd, door de fysieke interactie met GATA factoren, dat hoge niveaus van LPH gen expressie kunnen worden bereikt (Fig. 2). Daarbij komt nog dat GATA-4 ook fysiek met HNF-

1α kan verbinden en dat in tijdelijke co-transfectie experimenten GATA-4 en HNF-1α samen ook het LPH gen van de mens synergistisch kunnen activeren. Overeenkomende mechanismen in zowel andere weefsels als ook in *Drosophila* suggereren dat dit geconserveerde zink vinger/homeodomein interactie een efficient mechanisme is om synergistische gen activatie te verkrijgen. Op basis van deze data wordt er een paradigma voorgesteld waarin intestinaal-specifieke gen expressie is gereguleerd door een unieke interactie tussen twee weefsel gebonden transcriptiefactoren in de dunne darm.



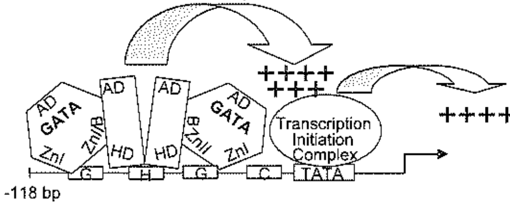


Fig. 2. Elementen in de 5'-flanking sequentie van het LPH gen van de mens sturen zowel onafhankelijke als ook cooperative activatie door HNF-1α en GATA-4, -5 transcriptie factoren. A. *HNF-1α vormt een dimeer en bindt zo aan zijn bindingsplaats in de LPH promoter van de mens, om zo onafhankelijk the LPH promoter te stimuleren (++).* C, H en G zijn de bindingsplaatsen voor respectievelijk Cdx-2, HNF-1 en GATA transcriptiefactoren. **B.** *GATA/HNF-1α synergistische activatie (+++++++).* Beide GATA-4 en GATA-5 kunnen samen met HNF-1α de LPH promoter synergistisch activeren. Dit mechanisme heeft debinding van HNF-1α aan het DNA nodig, maar niet dat van de GATA factoren. De fysieke interactie tussen GATA en HNF-1α is belangrijk voor de synergistische activatie en deze interactie wordt gemedieerd door de homeodomein van HNF-1α en de C-terminale zink vinger en basische regio van GATA eiwitten.

10.3.2.2 Toekomstbeeld

Het wordt verondersteld dat de LPH genexpressie in volwassen zoogdieren wordt gereguleerd door een mechanisme dat afhankelijk is van HNF-1α. Celkweek en andere *in vitro* experimenten (Hoofdstukken 2, 3 en 5) laten zien dat HNF-1α een belangrijke transcriptiefactor is, die met verschillende intestinale transcriptiefactoren associeert het geen resulteert in een verhoogde transcriptie activatie van LPH. Deze *in vitro* resultaten worden aangevuld door een bevinding (Hoofdstuk 8) waarin met behulp van HNF-1α knock-out muizen wordt aangetoond dat HNF-1α ook belangrijk is voor LPH expressie *in vivo*. RNase potectie experimenten laten zien dat in volwassen HNF-1α knock-out muizen de transriptie van LPH mRNA met 80% gereduceerd is in vergelijking met de transcriptie van LPH mRNA in wild type muizen. HNF-1α is belangrijk voor de gentranscriptie *in vivo*, omdat HNF-1α zowel histone acetylatie activiteit kan recruteren als ook andere cofactoren, die belangrijk zijn voor het transcriptie initiatie complex (Hoofdstuk 1). Volgende experimenten zijn noodzakelijk om het belang van HNF-1α en zijn bindingsplaats voor de LPH en SI gen expressie *in vivo* aan te tonen als ook om de functie van het recruteren van histone acetylatie activiteit door HNF-1α voor de LPH en SI gen expressie aan te tonen. Deze hypothese kan worden getest door middel van immunoprecipitatie (CHIP) experimenten. Dit is een geaccepteerde techniek voor het aantonen van eiwit-DNA interactie *in vivo*. Deze experimenten zullen aantonen of HNF-1α de HNF-1 bindingsplaats in de LPH en SI promoters van de mens bindt *in vivo* en ze zullen de functie van HNF-1α in het recruteren van histone acetylase activiteit voor de activatie van LPH en SI promoters aantonen.

10.3.3.1 Differentiele activatie tussen GATA-4 en GATA-5

Het horizontale expressie patroon van GATA-4 mRNA loopt synchroon met het expressiepatroon van LPH mRNA. Dit suggereert dat GATA-4 een rol speelt in de transcriptie regulatie van het LPH gen (Hoofdstuk 6). Gel-shift experimenten die gebruik maakten van nucleaire extracten van dunne darm epitheelcellen uit volwassen muizen, laten zien dat GATA-4 preferentieel aan de GATA bindingsplaatsen bindt in de LPH promoter van de mens (hoofstuk 6) en SI (hoofdstuk 3) promoter van de muis. Tenslotte kan GATA-4 ook met HNF-1α de LPH promoter synergistisch activeren wat overeenkomt met de GATA-5/HNF-1α synergie (Hoofdstuk 5). In tegenstelling tot GATA-5, dat de LPH promoter niet kan activeren zonder HNF-1α (Hoofdstuk 2), kan GATA-4 de LPH promoter wel individueel activeren (Hoofdstuk 6). Voor het karakteriseren van dit tweeledige mechanisme van de GATA-4 activatie aan de LPH promoter van de mens, werd de rol van de GATA bindingsplaatsen als eerste getest door middel van gemuteerde LPH promoters. Daarna werden de functies van kritieke domeinen in GATA-4 in kaart gebracht door ten eerste mutaties die specifieke domeinen verwijderen of dysfunctioneel maken te introduceren in GATA-4. Hierna werden de gemuteerde eiwitten getest op specifieke functies zoals; bindingsmogelijkheid aan DNA (gel-shift experimenten), de mogelijkheid om fysiek HNF-1α te binden (GST-pull down experimenten) en de mogelijke transcriptie activatie te meten in Hela cellen (tijdelijke co-transfectie experimenten). Hela cellen werden hier gebruikt omdat ze niet in staat zijn endogenen HNF-1α te produceren, zodat onderscheid gemaakt kan worden tussen de GATA-specifeke en de GATA/HNF-1α synergistische activatie. De studies besproken in Hoofdstuk 6 laten zien dat GATA-4, in tegenstelling tot GATA-5, de LPH promoter van de mens wel onafhankelijk activeert, via een mechanisme waarvoor intacte GATA bindingsplaatsen, de GATA-4 activiteits domeinen noodzakelijk zijn. De C-terminale zink vinger van GATA-4 reguleren de binding met het DNA (Fig. 3). Daarentegen kan GATA-4, gelijk aan GATA-5, samen met HNF-1α de LPH promoter van de mens synergistisch activeren. Deze activatie gaat via een mechanisme, waarbij GATA bindingsplaatsen of de GATA-4 activatie domeinen niet noodzakelijk zijn. De fysieke interactie tussen GATA-4 en HNF-1α als ook een intacte HNF-1 bindingsplaats zijn noodzakelijk voor de GATA-4/HNF-1α synergie. De C-terminale zink vinger van GATA-4 medieert de fysieke interactie met HNF-1α.

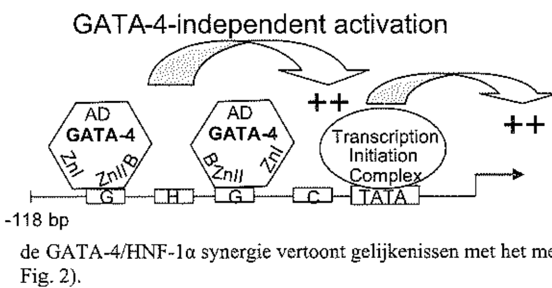


Fig. 3. GATA-4 en GATA-5 reguleren het humane LPH gen verschillend. *De GATA-4 van de GATA-4, -5 en -6 transcriptie factor familie kan de LPH promoter van de mens onafhankelijk activeren (++).* Binding op het DNA wordt gemedieerd door de C-terminale zink vinger (ZnII), en de basische regio (B) van de GATA eiwitten. Voor de onafhankelijke activatie moet GATA-4 met het DNA binden en heeft het zijn activatie domeinen nodig. Het mechanisme van de GATA-4/HNF-1α synergie vertoont gelijkenissen met het mechanisme van de GATA-5/HNF-1α synergie (zie Fig. 2).

Gel-shift experimenten hebben aangetoond dat de GATA bindingsplaatsen in de LPH promoter van de mens een hogere affiniteit hebben voor GATA-4 dan voor GATA-5. De conclusies gebasseerd op deze resultaten zijn, dat GATA-4 gebruik maakt van een tweeledig mechanisme om de humane LPH promoter transcriptie te activeren. Dit mechanisme wordt gekarakteriseerd door: (1) activatie die onafhankelijk is van HNF-1α (in tegenstelling tot GATA-5), waarbij binding aan het DNA en de GATA-4 activatie domeinen nodig zijn en (2) een activatie in samenwerking met HNF-1α (gelijk aan GATA-5), waarbij de fysieke associatie met

HNF-1α noodzakelijk is, maar intacte GATA bindingsplaatsen en de GATA-4 activatie domeinen niet noodzakelijk zijn. De veronderstelde verschillen in activatie door de GATA-4, -5 ranscriptie factoren voor een deel worden bepaald door de verschillende affiniteiten van de GATA bindingsplaatsen voor de GATA factoren. Dit is de eerste studie die heeft aangetoond dat er een verschil is in het transcriptie regulatie mechanisme van een intestinale gen door twee verschillende GATA factoren.

Alhoewel LPH mRNA is gereduceerd in de afwezigheid van HNF-1α zoals is beschreven in Hoofdstuk 8, wordt er een kleine hoeveelheid LPH mRNA geproduceerd in de volwassen knock-out muizen. Dit suggereert dat gedurende de lactatie-periode er een transcriptie regulatie mechanisme is dat onafhankelijk van HNF-1α de transcriptie van het humane LPH gen *in vivo* kan activeren. Vanwege de GATA-4 expressie in de dunne darm en het feit dat het horizontale expressiepatroon van GATA-4 parallel loopt aan die van LPH en omdat GATA-4 de LPH promoter van de mens individueel kan activeren, wordt er verondersteld dat GATA-4 een belangrijke rol speelt in de transcriptie van LPH zowel zonder als met HNF-1α. Zoals in Hoofdstuk 3 is beschreven wordt HNF-1α nauwelijks in de dunne darm aangetroffen gedurende de lactatie-periode, terwijl in deze periode LPH op de villi overal in de dunne darm kan worden aangetoond. Met behulp van de resultaten bij de knock-out muizen die in Hoofdstuk 8 beschreven zijn kan worden verondersteld dat het mechanisme van de LPH transcriptie *in vivo* tijdens de lactatie-periode onafhankelijk is van HNF-1α. Echter, GATA-4 eiwitten worden wel gevonden in de villi van de dunne darm gedurende deze periode (Hoofdstuk 3). Dit alles heeft tot de volgende hypothese geleid: (1) Tijdens de lactatie-periode wordt de LPH transcriptie gestuurd door een mechanisme dat onafhankelijk is van HNF-1α maar waarin GATA-4 potentieel een belangrijke rol zou kunnen spelen. (2) Gedurende de periode van het spenen en in de periode daarna verandert het mechanisme van HNF-1α onafhankelijk naar een HNF-1α afhankelijk mechanisme waarin samen met GATA factoren de LPH transcripite wordt gereguleerd.

10.3.3.2 Toekomstbeeld

Om de rol die GATA factoren zowel individueel als in samenwerking met HNF-1α bij de *in vivo* expressie van LPH en SI genen spelen duidelijk te maken, moeten knock-out experimenten worden uitgevoerd. Ter aanvulling van de onderzoeksresultaten in dit proefschrift moet er een belangrijke techniek worden ontwikkeld waarin weefsel specifieke knock-outs worden gecreëerd. Gelukkig overleven HNF-1α knock-out muizen waardoor de effecten van de afwezigheid van HNF-1α op de intestinale differentiatie en de LPH en SI gen expressie kunnen worden bestudeerd. In tegenstelling tot de GATA-5 knock-out muizen, die overleven en waarin de rol van GATA-5 kan worden bestudeerd voor de intestinale gen regulatie *in vivo*, gaan de GATA-4 en GATA-6 knock-out muizen dood zonder voor-darm te ontwikkelen. De conventionele knockout studies waarin de factor uit het hele dier wordt verwijderd, leveren dus geen informatie op over intestinale processen. Gelukkig is er een vrij nieuwe techniek ontwikkeld waarin gebruik gemaakt wordt van een Cre-loxP recombinatie technologie. Deze techniek maakt het mogelijk om de expressie van GATA-4, -5 en -6 zowel individueel als ook in combinatie specifiek uit de dunne darm te verwijderen. Dit model zal *in vivo* informatie opleveren over de rol die GATA-4, -5 en -6 spelen in de intestinale ontwikkeling en gen expressie.

Om het mechanisme van de onafhankelijke activatie van GATA-4 te bestuderen moet er een gedetailleerde karakterisatie worden uitgevoerd van de GATA bindingsplaatsen op de verschillende promoters en van de domeinen in de GATA eiwitten die de DNA binding reguleren. Een aanpak om de bindingsplaats karakteristieken te definieren is het gebruik van gerandomiseerde GATA bindingsplaatsen als oligonucleotiden in gel-shift experimenten. Dit is

al eerder gedaan voor GATA-1 (2). Aminozuur sequentie analyses van de C-terminale zink vinger en basische regio, die DNA binding reguleren, hebben aangetoond dat er ongeveer 10 aminozuren zijn die niet zijn geconserveerd tussen de GATA-4, -5 en -6 factoren (Fig.4).

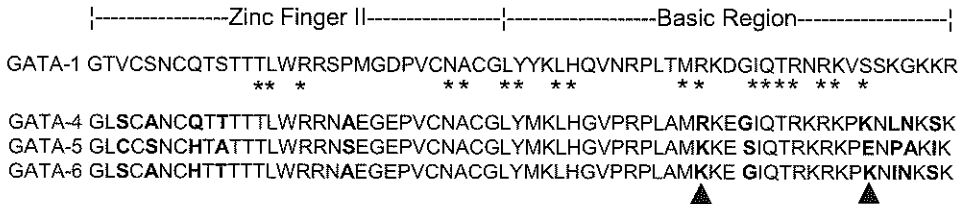


Fig. 4. Aminozuur sequentie van de C-terminale zink vinger en basische regio van GATA-1, -4, -5 en -6. Niet geconserveerde aminozuren tussen GATA-4, -5 en -6 zijn in dik gedrukte hoofdletters aangegeven. Een *astriks* (*) houdt potentiele contact punten met de DNA in voor GATA-1 (2). Een zwarte driehoek geeft niet geconserveerde aminozuren in de hele GATA familie aan die tegelijkertijd contact punten zijn met de DNA.

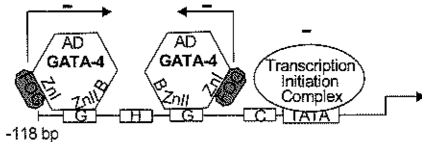
Om het belang van deze en andere niet geconserveerde aminozuren te bestuderen, kunnen specifieke aminozuren in GATA-5 worden veranderd in die van GATA-4 en worden getest voor een verhoogde bindingsaffiniteit. Natuurlijk zal het omgekeerde ook kunnen worden getest waarin GATA-4 structuren worden veranderd in die van GATA-5 en wordt er gekeken naar verlies van bindingsaffiniteit. Deze verschillen in bindingsaffiniteit voor specifieke bindings plaatsen zouden een belangrijk mechanisme kunnen zijn waarmee bindingsplaatsen op promoters voor eiwitten een weefsel specifieke gen transcriptie kunnen regelen.

10.3.4.1 FOG reguleert de transcriptie van het LPH gen van de mens.

FOG het geen een afkorting is van (Friend of GATA) zijn multipele zink vinger cofactoren die specifiek de GATA activatie van de door GATA gereguleerde transcriptie van genen beinvloeden. Twee leden zijn tot nu toe geidentificeerd, FOG-1 en FOG-2. Het doel van de studies beschreven in Hoofdstuk 7 is om de rol te definieren van FOG cofactoren op de GATA-specifieke en GATA/HNF-1α synergistische activatie van de LPH promoter. De FOG-1 en FOG-2 mRNA expressie in de dunne darm werd bestudeerd door middel van reverse transcriptase-PCR (RT-PCR) experimenten. Om de functie van FOG cofactoren te karakteriseren, werden transfectie experimenten gedaan in Hela cellen met een construct dat 118 bp van de LPH promoter van de mens bezit en aan de humane groeihormoon reporter was gekoppeld. Beide FOG-1 en FOG-2 mRNA werden gevonden in RNA dat was geisoleerd uit jejunum van volwassen muizen hetgeen suggereert dat beide cofactoren in de dunnen darm worden produceerd. FOG-1 onderdrukte de GATA-4 specifieke activatie van het LPH gen tot ongeveer een niveau van 50% en FOG-2 onderdrukte de activatie tot ongeveer 5% van de GATA-4 activatie. Daarnaast hebben FOG-1 en FOG-2 geen effect of de HNF-1α activatie, waarmee wordt aangetoond dat het effect inderdaad GATA specifiek is. Ook hebben beide FOG-1 en FOG-2 een effect op de GATA/HNF-1α synergie van de LPH promoter, alhoewel het effect minder drastisch is dan op de GATA-4 specifieke activatie. Een substitutie van een belangrijk aminozuur in de N-terminale zink vinger van GATA factoren, dat voorheen de fysieke interactie met FOG cofactoren onderbrak, resulteert in een reductie van de FOG-specifieke repressie maar niet een verlies van de repressie op de GATA-4-specifieke activatie. Deze mutatie voorkomt volledig de FOG-repressie op de GATA/HNF-1α synergie. FOG cofactoren die in de darm worden gevonden zouden een rol kunnen spelen in de expressie van het LPH gen door middel

van de onderdrukking van GATA-specifieke en GATA/HNF-1α synergistische activatie op de LPH promoter. Bij deze onderdrukking wordt gebruik gemaakt van onder andere de N-terminale zink vinger van GATA factoren.

Repression by FOG-2 of the GATA-4-independent activation



Repression by FOG-2 of GATA/HNF-1α cooperative activation

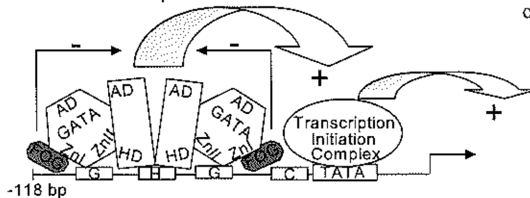


Fig. 5. Het mechanisme waarmee FOG cofactoren de LPH promoter van de mens onderdrukken. FOG-1 en FOG-2 cofactoren binden niet aan het DNA, maar associeren specifiek met GATA eiwitten. Deze fysieke associatie vindt plaats door middel van de N-terminale zink vinger van de GATA factoren. De onafhankelijke GATA-4 activatie wordt ongeveer met 95% gereduceerd (bovendste plaatje) De synergistische activatie van de LPH promoter tussen GATA en HNF-1α wordt ook gereduceerd maar minder sterk dan de reductie op de GATA-specifieke onderdrukking.

10.3.4.2 Toekomstbeeld

Om het mechanisme van de FOG onderdrukking te karakteriseren, moeten de expressie patronen van deze cofactoren langs verticale, horizontale en ontwikkelings gradienten worden onderzocht. Deze patronen kunnen worden gekarakteriseerd met behulp van RNase protectie experimenten, in situ hybridisatie en immunohistochemische experimenten. De hypothese voor deze experimenten is dat FOG-2, in de aanwezigheid van GATA factoren, een repressor is van de LPH gen expressie, en dat het patroon van FOG expressie in de dunne darm een omgekeerd evenredig patroon laat zien ten opzichte van LPH.

10.4 Model voor de LPH expression in volwassen zoogdieren

In dit proefschrift worden belangrijke transcriptie factoren beschreven die de transcriptie van intestinaal-specifieke genen zoals LPH en SI reguleren. Het is aangetoond dat leden van de Cdx-2, HNF-1 en GATA-4, -5 en -6 transcriptie factor families belangrijk zijn voor de LPH en SI gen transcriptie, zowel *in vivo* als *in vitro*. De proximale promoters van LPH en SI genen verschillen in het aantal en configuratie van de bindingsplaatsen voor deze transcriptie factoren. Gebaseerd op alle onderzoeksgegevens beschreven in dit proefschrift kan een model van de LPH gen expressie worden voorgesteld, zoals afgebeeld in Fig. 6.

Cdx-2 bindt op CE-LPH1a en blokkeert zo de binding van NF-LPH1/R. Cdx-2 op zichzelf activeert de LPH promoter marginaal maar zou met onderdelen van de transcriptie initiation complex een directe eiwit-eiwit contact aan kunnen gaan. Deze fysieke interactie zou het initiation complex kunnen stabiliseren en dus het uit elkaar vallen van het transcription initiation complex tegengaan en zo het transcriptie proces kunnen stimuleren. HNF-1α bindt als een dimeer aan het DNA en activeert de transcriptie van de LPH promoter.

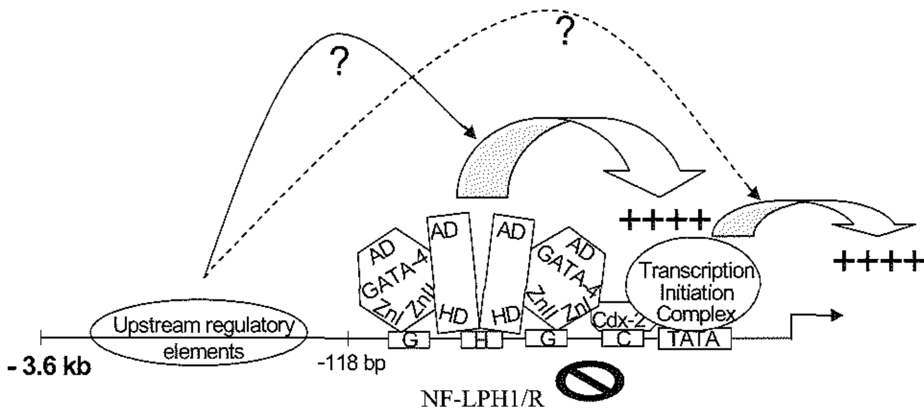


Fig. 6. Schematische weergave van de specifieke LPH gen expressie. De aanwezigheid van Cdx-2 in intestinale cellen verbreekt de onderdrukking van NF-LPH1/R dat anders wel bindt op CE-LPH1a (zoals in niet intestinale cellen). Cdx-2 zou ook een fysieke interactie kunnen aangaan met onderdelen van de transcriptie initiation. Een dimeer van HNF-1α bindt op het DNA en recrutreert zo GATA factoren als ook histone acytelatie activiteit, dat zorgt voor de op-regulatie van de LPH gen expressie. GATA-4 activeert individueel de LPH promoter in afwezigheid van HNF-1α, wat binding op het DNA nodig heeft. Maar GATA-4 zorgt ook met HNF-1α voor een synergistische activatie van de LPH transcriptie, wat de fysieke interactie tussen deze factoren nodigt heeft maar niet de binding van GATA factoren op het DNA. De doorgetrokken en gestippelde lijnen geven de invloeden weer die de upstream elementen hebben op het transcriptie proces in het specifieke expressie patroon van het LPH gen van de mens. Al deze factoren helpen in het transcriptie proces van de intestinale genen.

HNF-1α werkt als verbindings-eiwit die fysieke verbindingen aan kan gaan met vele eiwitten, waaronder GATA en Cdx-2. Deze fysieke interactie resulteert in hoge transcriptie activiteit van de LPH promoter en het zou ook de histone acetylatie activiteit kunnen aantrekken (hoofdstuk 1), hetgeen de LPH transcriptie faciliteert. Vanwege het parallel lopen van de horizontale expressie patronen van GATA-4 en LPH in de dunne darm van volwassen muizen en het feit dat de GATA bindings plaatsen in de proximale LPH promoter over het algemeen een voorkeur voor GATA-4 hebben, is de suggestie dat GATA-4 een belangrijke rol speelt in de horizontale LPH gen expressie. Daarenboven kan GATA binden met HNF-1α (Hoofdstukken 3, 5 en 6) en Cdx-2 (Hoofdstuk 3) en HNF-1α en Cdx-2 kunnen op hun beurt ook weer met elkaar binden (1). Deze interacties sugereren dat deze eiwitten een groot complex kunnen vormen, dat de transcriptional initiation complex kan helpen vormen en stabiliseren, maar ook histone acetylatie activiteit kan aantrekken. Het feit dat leden van deze trasncriptie factor families ook samen worden gevonden in cellen waar LPH niet aanwezig is moeten er ook repressoren een rol spelen in de regulatie van de LPH en SI transcriptie. FOG cofactoren zijn repressoren van de GATA-specifieke en GATA/HNF-1α synergistische activatie en zouden een rol kunnen spelen in de specifieke expressie patronen van LPH en SI genen.

LPH is een marker gen dat een specifiek expressiepatroon heeft volgens verticale, horizontale en ontwikkelings gradienten. Leden van de transcriptie families die in dit proefschrift zijn besproken hebben onafhankelijke maar ook gedeeltelijk overlappende expressie patronen. Alhoewel over het algemeen deze facoren een positief effect hebben op de transcriptie van het LPH gen, lopen deze expressie patronen niet altijd parallel aan die van LPH en SI. Daarom zijn deze eiwitten wel belangrijk maar waarschijnlijk niet voldoende om de LPH en SI expressiepatronen te verklaren. Dus spelen de "upstream elementen" in samenwerking met de proximale elementen spelen een belangrijke rol in het tot expressie komen van LPH. Hoewel de

"upstream elementen" naar alle waarschijnlijkheid een belangrijke rol spelen zijn de "down-sream" elementen uiterst belangrijk voor de stabilisatie van het transcriptie initiation complex en het faciliteren van de LPH transcriptie.

10.5. Significantie

De studies die hier zijn beschreven hebben nieuwe inzichten gegeven in het regulatie proces voor de transcriptie van LPH en SI genen. Deze regulatie mechanismen dirigeren de verschillende expressie patronen in de dunne darm. Zij hebben ook een bijdrage geleverd aan de opheldering van een aantal aspecten van de complexe processen van cel-specifieke genexpressie, intestinale-cellulaire differentiatie, en intestinale ontwikkeling. Wanneer deze mechanismen steeds meer bekend worden en zowel algemene als ook gen-specifieke regulatie mechanismen worden gekarakteriseerd, zal het ook mogelijk worden om de expressie van een individueel gen specifiek te kunnen stimuleren, hetgeen resulteert in de herstel van de expressie van het verloren of insufficiente functie van intestinale genen in de tractus digestivus.

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AMSTERDAM-BOSTON-AMSTERDAM

A continuing story: Lactase research in the Grand lab started around 1982 with some scribbling notes and drawing graphs on a paper napkin in a restaurant in Boston, where Prof. dr. Hans Büller en Prof. Richard J. Grand formed the lactase research collaboration. Edmond Rings in 1988 and many others have followed this initiative. From characterizing the lactase enzyme to describing the expression patterns of lactase in the intestine, I was honored when it was my turn to help continuing the project in 1999.

Through Mark Benninga, who helped me to contact Hans Büller, I was offered the opportunity to start with a clinical rotation at the New England Medical Center, Boston for three months in 1998. I gratefully took the opportunity that was offered to me the year after to do a research elective in Richard Grand's laboratory for six months. What started as a 6 month clerkship soon became a PhD-project. Three and a half years, some blood, sweat and tears later this thesis is the result of one of the best experiences I have had in my life so far! Now it is my turn to express my feelings and appreciation to the people who have had a hand in this experience.

The "Big Dig" project on I-93 in Boston that, if one has visited Boston in the last seven years, made an impression in one way (waiting forever to pass the construction scene) or the other (hotel taxes raised in order to finance this project). The project consists of digging a 8 lane highway underneath Boston (I-93) while the old highway directly above the construction stays open for regular traffic. As former project director Peter Zuk stated: "It's like performing open heart surgery on a patient who continues to work and play tennis". This project is a symbol for the work that is done in the laboratory. Some facts: Largest underground and under water urban highway project ever (18 years, construction started 1991); The project so far has cost $ 15 billion (the estimated cost in 1983 was $ 2.5 billion); 14 million cubic yards were dug, enough to fill a professional sports stadium….14 times; The control artery project will use up to 3.8 million cubic yards of concrete, enough to build a sidewalk 3 feet wide and 4 inches thick from Boston to San Francisco and back… 3 times!; During peak periods of construction (1998-2000) about $3 million of work is completed each day.

Richard J. Grand, Dick, is projected in the money (Cdx-2), for obvious reasons. As head of the Pediatric Gastroenterology you are guiding this project. In 1999 it was you who helped to plan my career and helped me decide to do my PhD before my clinical rotations. Your enthusiasm is very contagious and you're an example to me how one should deal with clinic, research, teaching and being just a very nice person. Thank you very much for the opportunity to learn from you. I'm proud to have you as one of my Promoters.

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Courtesy of Marcelo Lavallen

Courtesy of Marcelo Lavallen

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CURRICULUM VITAE

The author of this thesis was born on March 30, 1972 in Rotterdam, The Netherlands. He graduated from Het Nieuwe Lyceum, Hilversum, The Netherlands in 1992, and started medical school the same year at the Catholic University of Leaven, Belgium. In 1994 he received the First medical degree at the Free University of Brussels, Belgium, however in the same year he started medical school at the Free University of Amsterdam, The Netherlands. As a medical student, he performed from May, 1998 through August, 1998, a clinical research elective at the Floating Hospital for Children at New England Medical Center, Tufts University under Dr. R.J. Grand his supervision. This research involved statistical analysis of two data sets. One was a comparison of growth and nutritional status in two cohorts of children, a group diagnosed with IBD in 1979-1983, and another in 1984-1996. From 1999 to 2001, he was trained in basic research in the laboratory of Pediatric Gastroenterology and Nutrition (trained by Dr. S.D. Krasinski, Dr. R.K. Montgomery, and Prof. R.J. Grand) New England Medical Center, Tufts University School of Medicine, Boston MA, USA and worked on intestine specific gene expression. He received the American Digestive Health Foundation Student Research Fellowship Award for his work on lactase gene transcription. In 1999 he received the degree of Master of Science from the Free University of Amsterdam, The Netherlands. He continued and finished his thesis work, from 2001 till 2002 in the same laboratory at the Pediatric Gastroenterology and Nutrition Department at the Children's Hospital, Harvard Medical School, Boston MA, USA. In August 2002, Herbert van Wering started his clinical rotations in order to obtain his Medical Doctor degree from the Free University of Amsterdam, The Netherlands in 2004.

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