Gata4 and Hnf1α are partially required for the expression of specific intestinal genes during development

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Bosse T, Fialkovich JJ, Piaseckyj CM, Beuling E, Broekman H, Grand RJ, Montgomery RK, Krasinski SD. Gata4 and Hnf1α are partially required for the expression of specific intestinal genes during development. Am J Physiol Gastrointest Liver Physiol 292: G1302–G1314, 2007.—The terminal differentiation phases of intestinal development in mice occur during cytodifferentiation and the weaning transition. Lactase-phlorizin hydrolase (LPH), liver fatty acid binding protein (Fabp1), and sucrase-isomaltase (SI) are well-characterized markers of these transitions. With the use of gene inactivation models in mature mouse jejunum, we have previously shown that a member of the zinc finger transcription factor family (Gata4) and hepatocyte nuclear factor-1α (Hnf1α) are each indispensable for LPH and Fabp1 gene expression but are both dispensable for SI gene expression. In the present study, we used these models to test the hypothesis that Gata4 and Hnf1α regulate LPH, Fabp1, and SI gene expression during development, specifically focusing on cytodifferentiation and the weaning transition. Inactivation of Gata4 had no effect on LPH gene expression during either cytodifferentiation or suckling, whereas inactivation of Hnf1α resulted in a 50% reduction in LPH gene expression during these same time intervals. Inactivation of Gata4 or Hnf1α had a partial effect (~50% reduction) on Fabp1 gene expression during cytodifferentiation and suckling but no effect on SI gene expression at any time during development. Throughout the suckling period, we found a surprising and dramatic reduction in Gata4 and Hnf1α protein in the nuclei of absorptive enterocytes of the jejunum despite high levels of their mRNAs. Finally, we show that neither Gata4 nor Hnf1α mediates the glucocorticoid-induced precocious maturation of the intestine but rather are downstream targets of this process. Together, these data demonstrate that specific intestinal genes have differential requirements for Gata4 and Hnf1α that are dependent on the developmental time frame in which they are expressed.

lactase-phlorizin hydrolase; liver fatty acid binding protein; sucrase-isomaltase; intestinal differentiation; cytodifferentiation; suckling

THE MATURE MAMMALIAN SMALL intestine is lined by a continuously renewing epithelium that is established through a series of programmed developmental transitions (reviewed in Ref. 37). In mice, beginning on embryonic day (E) 8.5, anterior and posterior invaginations of the visceral endoderm occur that eventually fuse, forming a primitive gut tube. Between E9.5 and E14.5, the gut tube undergoes considerable longitudinal growth, and interactions between visceral endoderm and mesoderm result in organ specification. Between E14.5 and E17.5, the process of cytodifferentiation occurs in which the mouse gut endoderm that lines the presumptive small intestine is transformed from an undifferentiated, stratified epithelium to a highly differentiated, columnar epithelium with villus outgrowth. Dividing cells segregate to the inter villus regions, whereas differentiated cells migrate up the villi and begin to express proteins that are critical for intestinal function after birth. During the first week of life, crypts of Lieberkühn develop from the flat inter villus regions, resulting in the formation of a distinct proliferating compartment, and the differentiated cells on villi express proteins that are critical for the digestion and absorption of nutrients in milk (reviewed in Ref. 19). During the third week of life (corresponding to the weaning transition), the proteins expressed on villi undergo a final transition to an adult pattern designed for the efficient digestion and absorption of nutrients in solid foods. Although the precise timing of events in intestinal development differs between rodents and humans, the fundamental mechanisms underlying cytodifferentiation and the control of villus protein expression during postnatal development are thought to be highly conserved (reviewed in Ref. 29). Fundamental insight into these processes is essential for understanding gut function and the processes that fail in intestinal disease, as well as for creating possible avenues for therapeutic intervention.

Lactase-phlorizin hydrolase (LPH), liver fatty acid binding protein (Fabp1), and sucrase-isomaltase (SI) are intestinal proteins important for nutrition during different stages of development and are also established markers for the transitions that occur in intestinal development (18, 20, 25, 35, 36, 38, 40, 44, 45, 47). LPH and SI are microvillus membrane disaccharidases that hydrolyze milk lactose and α-disaccharides found in solid foods, respectively, whereas Fabp1 is a cytoplasmic protein important for intracellular lipid transport. In rodents, LPH and Fabp1 are first detected at the beginning of cytodifferentiation in preparation for a critical function after birth, continue to be expressed at high levels during the suckling period, and decline during weaning. In contrast, SI is undetectable before weaning and increases to adult levels during weaning. Thus, LPH and Fabp1 are markers of cytodifferentiation during fetal development, whereas LPH, Fabp1, and SI are indicators of the well-orchestrated patterns of

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absorptive enterocyte gene expression that occur during postnatal development.

Although much is known about the patterns of LPH, Fabp1, and SI gene expression during development, the mechanisms underlying these patterns remain to be fully elucidated. Transgenic studies have shown that the 5’-flanking regions of LPH, Fabp1, and SI direct appropriate tissue, cell-type, and temporal patterns of expression (21, 24, 26, 27, 40, 48), and highly conserved transcription factor binding sites in the proximal promoters of LPH, Fabp1, and SI have been identified for the Gata family of zinc finger transcription factors that bind the consensus DNA sequence (A/T)GATA(A/G), as well as the hepatocyte nuclear factor-1 (Hnf1) and caudal (Cdx) families of homeodomain proteins (5, 6, 8 –10, 12, 14, 15, 22, 28, 42, 46, 53). Gata4 and Hnf1α are the predominant members of their respective families in nuclear extracts from mouse intestinal epithelial cells that bind to the LPH and SI promoters (4, 5, 49), and both Gata4 and Hnf1α activate the LPH, Fabp1, and SI promoters in cell culture overexpression experiments (5, 6, 8, 9, 12, 22, 28, 42, 49). Gata4 and Hnf1α physically associate and synergistically activate the LPH, Fabp1, and SI genes (5, 9, 49) through an evolutionarily conserved pathway (49, 50), and we have postulated that this interaction is a means to achieve high levels of intestine-specific gene expression in vivo (20).

We recently investigated the importance of Gata4 and Hnf1α in vivo for intestinal gene expression by using gene-inactivation approaches (3, 4). Inactivation of Gata4 in adult mouse jejunal produces a shift to an ileal-like phenotype but no obvious consequences in weight, behavior, skin, or general physiology (3). Germine Hnf1α knockout mice survive into adulthood and demonstrate sterility, diabetes, delayed growth rate, and liver dysfunction (23). In both models, LPH and Fabp1 mRNA abundances in adult jejunum were reduced ~90%, whereas that of SI was surprisingly not affected by the inactivation of either Gata4 or Hnf1α (3, 4). These data thus indicate that, in adult mouse intestine, both Gata4 and Hnf1α are necessary for the expression of the LPH and Fabp1 genes, consistent with our model of coregulation, but are dispensable for SI gene expression. The goal of the present study is to use these models to define the requirement of Gata4 and Hnf1α for the regulation of LPH, Fabp1, and SI gene expression in the developing mouse small intestine, specifically focusing on cytodifferentiation and the weaning transition.

MATERIALS AND METHODS

Mice. All animal studies were performed under protocols approved by the Institutional Animal Care and Use Committee of Children’s Hospital Boston. Mice were housed in the Animal Research at Children’s Hospital facility under standard conditions with 12-h light-dark cycles and were given food and water ad libitum.

Two gene-inactivation models were used, including an inducible, intestine-specific Gata4 inactivation model (3) and a germline Hnf1α-null model (4, 23), all in a C56Bl/6 background. To inactivate Gata4 in the small intestinal epithelium, Gata4<sup>floxed/fox</sup> mice (33) were crossed with transgenic Villin-CreERT<sup>2</sup> mice (11) to generate Gata4<sup>fox/fox</sup>, Villin-CreERT<sup>2</sup> positive (Cre<sup>+</sup>) study animals (mutant) and Gata4<sup>floxo/fox</sup>, Villin-CreERT<sup>2</sup> negative (Cre<sup>−</sup>) controls. To inactivate Gata4 in the intestine, tamoxifen (1 mg/20 g body wt; Sigma-Aldrich, St. Louis, MO) was administered to timed-pregnant females for 5 consecutive days beginning at E12.5 or for 4 consecutive days beginning at postnatal day (P) 7, as described (2). Gata4-mutant mice produce a truncated, transcriptionally inactive form of Gata4 that is capable of site-specific binding to DNA elements and thus has the potential for dominant negative activity in vivo (3). Hnf1α<sup>−/−</sup> mice survive into adulthood but are sterile; therefore mating of Hnf1α<sup>−/−</sup> parents is required to generate both null and wild-type study animals (23). All mice were genotyped by using PCR on tail DNA as previously described (4).

Mice were killed for study at various time points beginning at E13.5 and extending throughout postnatal development. Study mice or pregnant females were anesthetized with Avertin anesthesia (2,2,2-tribromoethanol, 240 mg/kg body wt; Sigma) prior to dissection. For fetal mice, embryos were removed from the mother and transferred to a Petri dish containing 1× PBS, and tissue was isolated by using a dissecting microscope. For postnatal mice, tissue was extracted through a midline incision and transferred to a glass plate on a bed of wet ice. All tissues were collected between 1300 and 1600 to avoid any fluctuations in gene expression due to circadian cycles (34).

RNA isolation. RNA was isolated from snap-frozen fetal and postnatal mouse tissues. From fetal animals, RNA was isolated from either the entire small intestine or, in the case of selected E17.5 pups, from intestinal segments separated into equal lengths, where segment 1 was the most proximal 20%, segment 2 the next 20%, segment 3 the middle 20%, segment 4 the next 20%, and segment 5 the most distal 20% of the small intestine. From postnatal mice, RNA was isolated from 30–50 mg of small intestine (0.5–1.0 cm) obtained from the geometric center (segment 3, jejunum). RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). To ensure that all traces of DNA were removed, RNA samples were treated with DNase (DNA-free; Ambion, Austin, TX) for 1 h at 37°C, following the manufacturer’s instructions. RNA samples were quantified by optical density at 260 nm and were checked for absence of degradation on an agarose gel.

Semiquantitative and real-time RT-PCR. Semiquantitative and real-time RT-PCR were conducted as previously described (4, 49). For both PCR reactions, complementary DNA (cDNA) was synthesized by using iScript (BioRad). Primer pairs were designed by using Beacon Designer software (Premier Biosoft International, Palo Alto, CA) and were optimized as described (3, 4). Primer sequences are available upon request. Semiquantitative RT-PCR experiments were terminated in the linear range of amplification. Real-time RT-PCR was conducted by using an iCycler and iQ SYBRgreen Supermix (Bio-Rad, Hercules, CA). All real-time RT-PCR data were corrected for Gapdh and were expressed relative to the calibrator, which was adult jejunal RNA from a single mouse, unless otherwise indicated.

Immunofluorescence. Immunofluorescence was conducted on ileal tissue as previously described (3). Following dissection, mouse tissues were immediately immersed in a freshly made solution of buffered 4% paraformaldehyde and incubated for 4 h at 4°C, then resuspended in 70% ethanol overnight. Tissue was embedded in paraffin, and 5-μm sections were prepared for immunohistochemistry in the Department of Pathology, Children’s Hospital Boston. Following tissue deparaffinization and rehydration, antigen retrieval was conducted by boiling slides for 10 min in 10 mM sodium citrate (pH 6). The slides were then slow-cooled and washed in 1× PBS. After being blocked (10% donkey serum in 1× PBS) for 1 h in a humidified chamber, the primary antibody was pipetted onto slides and incubated overnight at 4°C. After being washed, the fluorescent secondary antibody was pipetted onto slides and incubated for 1 h at room temperature due to cross-reactivity, sequential addition of the two secondary antibodies with extensive washing in between was necessary for Gata4-Hnf1α coimmunofluorescence experiments.

The primary antibodies used were goat anti-Hnf1α (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Gata4 (1:100; Santa Cruz), goat anti-Gata4 (1:400; Santa Cruz), rabbit anti-Cdx2 (1:500; gift from D. Silberg, University of Pennsylvania), rabbit anti-LPH (1:500; gift from K. Y. Yeh, Louisiana State University), rabbit anti-Fabp1 (1:1,000; gift from J. Gordon, Washington University),
and rabbit anti-SI (1:500; gift from K. Y. Yeh, Louisiana State University). The secondary antibodies used were Alexa Fluor 594 donkey anti-goat IgG, Alexa Fluor 488 donkey anti-rabbit IgG, and Alexa Fluor 488 goat anti-mouse IgG (1:500; Molecular Probes). In most experiments, a solution containing 4’,6-diamino-2-phenylindole dihydrochloride (DAPI) nucleic acid stain (2 μg/ml; Molecular Probes) in PBS was added to reveal the nuclei.

Isolation of nuclear and nonnuclear extracts. Nuclear extracts were isolated as previously described (49) from pooled mucosal scrapings of 4-cm segments at the geometric center of the small intestine (midjejunum) from P4, P7, P14, P21, P28, and adult (6–12 wk) mice. In selected experiments, nuclear and nonnuclear fractions were isolated similarly from P10 mice. The epithelial scrapings were resuspended in hypotonic buffer [in mM: 10 HEPES (pH 7.9), 10 KCl, 1.5 MgCl2, 1.0 PMSF, and 1.0 DTT, plus protease inhibitor cocktail] and centrifuged at 5,000 rpm for 5 min at 4°C. The cell pellet was resuspended in hypotonic buffer, incubated on ice for 5 min, and homogenized in a precooled Dounce homogenizer with 20 strokes using the loose pestle. After centrifugation at 8,000 rpm for 5 min at 4°C, the supernatant in selected experiments was saved (~70°C) as the nonnuclear fraction. The nuclei were resuspended in low-salt buffer [20 mM HEPES (pH 7.9), 20 mM KCl, 1.5 mM MgCl2, 25% glycerol, protease inhibitor cocktail, 1.0 mM PMSF, and 1.0 mM DTT] followed by the slow addition of high-salt buffer [20 mM HEPES (pH 7.9), 1.2 M KCl, 1.5 mM MgCl2, 25% glycerol, protease inhibitor cocktail, 1.0 mM PMSF, and 1.0 mM DTT]. After extraction at 4°C for 30 min with vigorous mixing every 5 min, the sample was centrifuged at 14,000 rpm for 15 min at 4°C and the supernatant was saved as the nuclear fraction (~70°C).

Western blotting. Western blot analysis was performed as described previously (4) by using 20–80 μg of nuclear or nonnuclear extracts. The primary antibodies included affinity-purified goat polyclonal antibodies for Gata4 or Hnf1α (Santa Cruz), a mouse monoclonal antibody for Gata4 (Santa Cruz), or rabbit polyclonal antibodies for Fabp1 (gift of J. Gordon, Washington University). All blots were stripped and reprobed with anti-mouse β-actin.

EMSA. EMSAs were performed by using labeled, double-stranded oligonucleotides containing well-characterized binding sites for Gata or Hnf1 families of transcription factors, as described previously (22). These included the Gata binding site present in the Xenopus Fabp1 promoter (X-GATA; 5′-GGAGATCCCTGTA-CAGATGTGGGAGAC-3′) (17) and the Hnf1 binding site present in the rat β-fibrinogen promoter (β-Fib; 5′-CAACTGTCAATATA-TAATCAAAAGGAG-3′) (7). Supershift EMSAs were conducted by using affinity-purified goat polyclonal antibodies for Gata4 or Hnf1α (Santa Cruz).

Dexamethasone treatment. To investigate the role of Gata4 and Hnf1α in hormonally induced precocious weaning, a model was used in which wild-type, and Gata4-mutant Hnf1α−/− mice were treated with dexamethasone (Sigma-Aldrich) at P10 essentially as described (25). Dexamethasone was injected intraperitoneally at 1.0 μg/g body wt. Negative controls included littersmates injected with vehicle (0.8% ethanol in 1× PBS). After 4 or 24 h, mice were killed and the jejuna (segment 3) were collected for the isolation of RNA and nuclear extracts as well as for sectioning.

Statistical analyses. Statistically significant differences were determined by Student’s t-test or analysis of variance followed by the Tukey-Kramer multiple-comparison test.

RESULTS

Gata4 and Hnf1α differentially regulate LPH and Fabp1 gene expression during cytodifferentiation. The mouse intestinal epithelium undergoes rapid cytodifferentiation beginning at E14.5, resulting in the formation of nascent villi and synthesis of intestinal differentiation markers by E17.5. The mRNAs for the markers of cytodifferentiation, LPH and Fabp1 (18, 35, 44), were not detectable by real-time RT-PCR in whole intestine at E13.5 or E14.5, were just detectable at E15.5, and reached their highest levels by E17.5 (Fig. 1A). LPH and Fabp1 mRNAs were highest throughout the proximal half of the small intestine (segments 1–3) and declined to nearly undetectable levels in the distal gut (segment 5), as determined by semiquantitative RT-PCR (Fig. 1B). These data confirm that LPH and Fabp1 are markers for cytodifferentiation of the mouse midgut.

To begin to understand the regulation of target genes by Gata4 and Hnf1α during cytodifferentiation, the expression patterns of these transcription factors during this time frame were determined. Gata4 and Hnf1α mRNAs were expressed throughout cytodifferentiation, with the highest levels occurring at E17.5 (Fig. 2A). Along the cephalocaudal axis at E17.5, Gata4 mRNA displayed a declining proximal-to-distal gradient, being nearly undetectable in the most distal segment, whereas Hnf1α mRNA was nearly undetectable in the most proximal segment and demonstrated a generally increasing proximal-to-distal gradient, as shown by semiquantitative RT-PCR (Fig. 2B). Gata4 and Hnf1α protein were colocalized in...
epithelial cells of the E13.5 midgut (Fig. 2, C–E) and on nascent villi and in intervillus regions of the E16.5 (Fig. 2, F and G) and E17.5 (data not shown) midgut. Gata4 and Hnf1α were specifically localized to the nucleus at these ages, as determined by costaining with DAPI (data not shown). Together, these data demonstrate a topographic basis for possible coregulation by Gata4 and Hnf1α in the midgut during cytodifferentiation.

Inactivation of Gata4 or Hnf1α in the jejunum of adult mice results in an attenuation (25%) of both LPH and Fabp1 gene expression (3, 4). To define the importance of Gata4 or Hnf1α in the jejunum during cytodifferentiation, we quantified the expression of LPH and Fabp1 mRNAs in our knockout models during this time frame.

To inactivate Gata4 in the midgut, pregnant mothers carrying Gata4<sup>fl<sup>ox</sup>/fl</sup> embryos that were either positive (mutant) or negative (control) for the Villin-CreERT<sup>2</sup> transgene were treated with five daily doses of tamoxifen beginning at E12.5 (Fig. 3A). At E17.5, Gata4 was specifically inactivated in the midgut of Gata4-mutant mice (Fig. 3B), verifying the model.

Body size, gross intestinal structure, and overall intestinal histology as indicated by hematoxylin and eosin staining of Gata4-mutant mice were indistinguishable from controls. LPH mRNA abundance in the midgut of Gata4-mutant mice was also indistinguishable from controls, whereas Fabp1 mRNA abundance was reduced (~75%) (Fig. 3C, top). In Hnf1α<sup>−/−</sup> mice, both LPH and Fabp1 mRNA abundances were significantly reduced (P < 0.05) by ~50% compared with Hnf1α<sup>+/+</sup> wild-type controls (Fig. 3C, bottom). These data indicate that during cytodifferentiation, Gata4 is dispensable for LPH gene expression but partially required for Fabp1 gene expression, whereas Hnf1α is partially required for both LPH and Fabp1 gene expression.

Nuclear Gata4 and Hnf1α are paradoxically reduced before weaning despite high levels of their respective mRNAs. To begin to define the underlying mechanism by which Gata4 and Hnf1α differentially regulate intestinal genes during postnatal development, the patterns of expression of Gata4 and Hnf1α mRNAs and proteins were first determined in wild-type mice at P7, P14, P21, and P28. Gata4 mRNA was highest before weaning and gradually declined during weaning to significantly lower levels at P21 (P < 0.05) (Fig. 4A, top). Hnf1α mRNA was highest at P7 and was significantly lower at all time points thereafter (Fig. 4A, bottom). These patterns are generally consistent with recently published data (13).

To determine if the mRNA abundances correlate with nuclear protein levels, Western blot analyses using nuclear extracts from midjejunum of wild-type mice were performed. Gata4 and Hnf1α were surprisingly low at P7 and P14 but increased markedly at P21 and P28 (Fig. 4B), sharply contrasting with their decreasing mRNA levels during this time interval (see Fig. 4A). This was verified by using 40–80 µg of nuclear extracts isolated additionally from the jejunum of P4, P7, and P10 mice (Fig. 4C). Gata4 was detected at all time points but at much lower levels than in adults, with the lowest level occurring at P7, whereas Hnf1α could not be detected at P4 and P7. To determine if Gata4 and Hnf1α are localized outside of the nucleus before weaning, Western blot analyses were performed on nonnuclear fractions isolated from jejunal enterocytes at P10. As shown in Fig. 4D, neither Gata4 nor Hnf1α was detected in the nonnuclear fractions at this age. As controls, both Gata4 and Hnf1α were readily detected in nuclear extracts of wild-type jejunum from adult mice but not in the mature jejunum from the respective knockout models. The relative abundance of Fabp1 in the nonnuclear fraction verifies the enrichment of cytoplasmic protein in this fraction.
Gata4 and Hnf1α are readily detected in the nuclei of villi and intervillus regions of embryonic intestine by immunofluorescence (see Fig. 2). However, just after birth (P1), Gata4 was expressed only in the intervillus regions, not on villi (Fig. 5, A and B), and Hnf1α could not be detected in either compartment (Fig. 5C). From P4 to P10, neither Gata4 nor Hnf1α were detected, as exemplified by immunofluorescence of P7 intestine (Fig. 5, D–G). As a positive control, all sections during this time interval positively stained for Cdx2 (Fig. 5, H and I), an intestinal nuclear transcription factor that is expressed in the intestinal epithelium throughout development (39). At P14, Gata4 was detected only in the nuclei of cells in the crypts and lower villi (Fig. 5, J and K), whereas Hnf1α was not detected (data not shown). By P21, Gata4 and Hnf1α were coexpressed throughout the villus epithelium (Fig. 5, L–M), as in adults. Together, these studies demonstrate that both the Gata4 and Hnf1α genes are expressed at high levels throughout postnatal development, as indicated by their high levels of mRNA (see Fig. 4A), but their protein products are expressed at low levels during suckling.

Gata4 and Hnf1α differentially regulate target gene expression during postnatal development. During weaning, which in mice occurs throughout the third week of life, the proteins expressed on villi undergo a final transition from a suckling pattern optimized for the synthesis of enzymes important for the digestion of nutrients in milk to an adult pattern designed for the efficient digestion and absorption of nutrients in solid foods (reviewed in Ref. 19). After birth and throughout suckling, LPH and Fabp1 are highly expressed and decline during weaning, whereas SI is low before weaning and increases during weaning. To define the importance of Gata4 or Hnf1α for LPH, Fabp1, and SI gene expression during weaning, we quantified the mRNAs for these genes in our knockout models during this time frame. To inactivate Gata4 in jejunum, we employed a time course essentially as described previously (2) (Fig. 6A), whereby 7-day-old mice were treated for 4 consecutive days with a single daily injection of tamoxifen. Mice were killed for study at P10 (preweaning), P20 (midweaning), and P30 (postweaning) (Fig. 6A). Gata4 was expressed normally in heart, liver, and stomach (data not shown) but was absent in the jejunum at all ages (Fig. 6B), verifying the Gata4 inactivation model for the study of postnatal development.

Growth rate and overall intestinal structure and histology (data not shown) in Gata4-null mice were indistinguishable from controls throughout the weaning transition. Analysis of LPH gene expression in these mice revealed that LPH mRNA abundance in midjejunum at P10 and P20 of Gata4-null mice was similar to that in control mice but at P30 was <10% of that in control mice (P < 0.05, Fig. 7, top), a difference that is similar to that in adult mice (3). Fabp1 mRNA levels were reduced by the inactivation of Gata4 ~50% at P10 (P < 0.05, Fig. 7A, middle), similar to that observed at E17.5 (see Fig. 3C). Fabp1 mRNA abundance in Gata4-mutant mice at P20 and P30 was <10% of that in control mice (P < 0.05, Fig. 5A, middle), similar to that in adult mice (3). SI mRNA levels revealed an expected increase during weaning but no difference between control and Gata4-mutant mice at any postnatal time point (Fig. 7A, bottom).

Immunofluorescence for LPH, Fabp1, and SI in control and Gata4-null mice during weaning generally followed the
results of their respective mRNA abundances. LPH immunofluorescence was specific to the microvillus membrane in the P10 jejunum (Fig. 7B) and was not affected by the inactivation of Gata4 (Fig. 7C), consistent with mRNA levels. LPH immunofluorescence was also present in the jejunum of P30 control mice (Fig. 7D) but absent in the Gata4-mutant mice (Fig. 7E), which is also consistent with mRNA levels. Fabp1 immunofluorescence in the cytoplasm of villus enterocytes was reduced by the inactivation of Gata4 at both P10 and P30 (Fig. 7, F–I), consistent with its mRNA levels. SI was not detected at P10 in either control or Gata4-mutant mice (Fig. 7, J and K) and was localized to the microvillus membrane at P30 with no apparent difference in intensity between control and Gata4-mutant mice (Fig. 7, L and M), consistent with mRNA levels. These data demonstrate that Gata4 is not required for LPH gene expression before weaning but is indispensable after weaning, is at least partially required for Fabp1 gene expression throughout development, and is not required for SI gene expression at any time during development.

In Hnf1α−/− mice, LPH mRNA abundance was ~50% of that in wild-type jejunum at P7, P14, and P21, similar to that at E17.5, but was <10% of that in wild-type jejunum at P28 (Fig. 8A, top), similar to that in adult Hnf1α-null mice (4).

Fabp1 mRNA was reduced ~50% in the Hnf1α-null mice at P7 and P14, similar to that at E17.5 (see Fig. 3C), and was barely detectable at P21 and P28 (Fig. 8A, middle), similar to that in adults (4). SI mRNA increased during weaning in Hnf1α+/+ and Hnf1α−/− mice, with no significant difference between the two groups (Fig. 8A, bottom).

LPH immunofluorescence was most intense in the P7 jejunum of wild-type mice (Fig. 8B) and was consistently less intense in the P7 jejunum of Hnf1α-null mice (n = 3; Fig. 8C), in agreement with the decrease in LPH mRNA levels at this age. LPH immunofluorescence was present in the P28 jejunum of wild-type mice (Fig. 8D) but was not detectable in the jejunum of P28 Hnf1α-null mice (Fig. 8E), again consistent with mRNA levels. Fabp1 immunofluorescence was reduced in Hnf1α-null mice at both P7 and P28, consistent with its mRNA levels (Fig. 8, F–I). SI immunofluorescence was not detected in either control or Hnf1α-null mice at P7 (Fig. 8, J and K) and was similarly intense in the jejunum of both mice at P28 (Fig. 8, L and M), correlating with its mRNA levels. These data indicate that Hnf1α, although indispensable for LPH and Fabp1 gene expression after weaning (4), is only partially required before weaning. These data also show that Hnf1α is not required for the endogenous increase in SI gene expression during weaning.
Gata4 and Hnf1α do not mediate the precocious weaning induced by glucocorticoids. Glucocorticoids are known to induce maturation of the small intestine, resulting in the precocious induction of intestinal enzymes such as SI (25). However, because this induction is characterized by an 8-h lag, it is thought to be a secondary effect. The primary response is likely mediated by intestinal transcription factors, and Gata factors have been implicated (32). To define the possible role of Gata4 as well as Hnf1α in mediating the glucocorticoid response in preweaning mice, we characterized the dexamethasone-induced response in the context of null intestinal expression of Gata4 or Hnf1α. As shown in Fig. 9A (top), SI mRNA abundance was similarly induced ~80-fold 24 h after dexamethasone administration in wild-type, Gata4-mutant, and Hnf1α−/− mice, indicating that neither Gata4 nor Hnf1α is necessary to mediate the dexamethasone response on SI in vivo. LPH mRNA abundance was not affected by dexamethasone in wild-type mice but was significantly reduced by dexamethasone in both Gata4-mutant and Hnf1α−/− mice (Fig. 9A, bottom). These data indicate that dexamethasone induces a process in which Gata4 and Hnf1α become regulatory for LPH gene expression, as in the postweaning situation. In wild-type mice, the mRNAs for both Gata4 and Hnf1α were significantly reduced by dexamethasone, both at 4 h (data not shown) and 24 h (Fig. 9B), similar to that which occurs after weaning. Together, these data indicate that Gata4 and Hnf1α are not required for the precocious maturation process induced by glucocorticoids but are likely downstream targets of this process.

DISCUSSION

The establishment of a fully functioning mature mammalian gut is the result of a series of ordered developmental transitions...
The terminal differentiation phases of intestinal development, characterized in part by the expression of proteins necessary for the digestion and absorption of nutrients, occur during cytodifferentiation and the weaning transition (reviewed in Ref. 19). LPH, Fabp1 and SI are well-characterized markers of these transitions (18, 20, 25, 35, 36, 38, 40, 45), and the Gata and Hnf1 families of transcription factors have been implicated in their regulation (5, 6, 8–10, 12, 15, 22, 28, 42, 53).

In mature jejunum of mice, we have previously shown that Gata4 and Hnf1α are necessary for LPH and Fabp1 gene expression, but not for SI gene expression (3, 4). In the present study, we found that the regulation of these target genes by Gata4 and Hnf1α during development differs from that in adults (Fig. 10), in that before weaning, including during cytodifferentiation, Gata4 and Hnf1α are either not required or only partially required for LPH and Fabp1 gene expression, contrasting with their indispensability after weaning. The partial Gata4 requirement for Fabp1 gene expression during cytodifferentiation is consistent with data from E18.5 mosaic Gata4-knockout mice, in which Fabp1 gene expression is attenuated in intestinal epithelial cells that do not express Gata4, as indicated by in situ hybridization (9). We also found that Gata4 and Hnf1α are not required for SI gene expression at any time during development. During the suckling period, we found a surprising and dramatic reduction in Gata4 and Hnf1α protein in the nuclei of absorptive enterocytes of the jejunum despite high levels of mRNA. Finally, we show that neither Gata4 nor Hnf1α mediate the precocious maturation of the intestine induced by glucocorticoids. Together, these data demonstrate that specific intestinal genes, including LPH and...
Fabp1, have differential requirements for Gata4 and Hnf1α that are dependent on the developmental time frame in which they are expressed.

Our data show that Gata4 and/or Hnf1α is partially required for LPH and Fabp1 gene expression before weaning, which contrasts with their indispensability for LPH and Fabp1 gene expression after weaning. Functionally, LPH is necessary for the digestion of milk lactose and is thus critical for nutrition during suckling, while Fabp1 plays a role in intracellular lipid transport and is likely important for the transport of the lipid load present in milk. Thus a plausible hypothesis is that a redundant mechanism for the maintenance of gene expression during this critical developmental time interval is necessary. These data also indicate that other factors are involved in LPH and Fabp1 gene expression before weaning. Candidate transcription factors include Gata5, Gata6, Hnf1β, and members of the Cdx, Hnf3, and C/EBP families, all of which have been previously implicated as activators of LPH and/or Fabp1 gene transcription in vitro (5, 9, 10, 12, 14, 15, 22, 30, 43, 46, 51). Additional studies will be necessary to define the requirement in vivo of these transcription factors and/or identify other factors involved in the developmental regulation of LPH and Fabp1 gene expression.

These data also reveal differential mechanisms underlying the regulation of genes whose expression patterns during development are strikingly similar. Both LPH and Fabp1 are highly expressed in more proximal regions of small intestine than distal regions and at higher levels before weaning than after weaning (Figs. 1, 7, and 8 and Refs. 18, 20, 35, 36, 40), and their promoters contain binding sites for similar sets of transcription factors (8, 22). However, although both are similarly regulated by Gata4 and Hnf1α in mature intestine (3, 4), they are differentially regulated by these two transcription factors before weaning. Both LPH and Fabp1 mRNAs are reduced ~50% in Hnf1α-null mice before weaning, but only Fabp1 mRNA is reduced in the Gata4-mutant mice before weaning (Figs. 3, 7, and 8). Furthermore, Fabp1 mRNA abundance is attenuated ~90% by the inactivation of Gata4 at P20 or Hnf1α at P21, similar to that in adult mice, whereas LPH mRNA abundance is reduced ~50% at these time points, similar to preweaning mice, indicating a differential in the timing of regulation by Gata4 or Hnf1α. The differential regulation of LPH and Fabp1 by Gata4 before weaning highlights a target-specific gene regulation during development.

We have previously hypothesized that the induction of SI gene expression during postnatal development is regulated by the combinatorial effect of a complex of transcription factors, including Gata4 and Hnf1α (5, 22). In addition, the abundance of nuclear Gata4 and Hnf1α proteins in the jejunum increases during the weaning transition, paralleling SI gene expression (Fig. 4B and Refs. 5, 22). Despite these compelling data for a combinatorial role by Gata4 and Hnf1α in the activation of SI gene expression, we recently reported that the inactivation of Gata4 or Hnf1α had no effect on SI gene expression in adult mice (3, 4). Here, we show that the inactivation of Gata4 or Hnf1α had no effect on the initiation of SI gene expression.

Fig. 8. Null expression of Hnf1α demonstrates gene-specific, developmental regulation. A: LPH, Fabp1, and SI mRNA abundances are differentially affected by null expression of Hnf1α during development. Real-time RT-PCR for LPH (top), Fabp1 (middle), and SI (bottom) mRNAs were conducted on RNA isolated from jejunum (segment 3) of Hnf1α+/+ and Hnf1α−/− mice at P7, P14, P21, and P28. Data are means ± SD of n = 3–5 mice. *P < 0.05 compared with controls. B–M: immunofluorescence for LPH (B–E), Fabp1 (F–I), and SI (J–M) in P7 Hnf1α+/+ (B, F, J), P7 Hnf1α−/− (C, G, K), P28 Hnf1α+/+ (D, H, L), and P28 Hnf1α−/− (E, I, M).
Although Gata4 and Hnf1α are greatly reduced or absent in the intestinal epithelial nuclei during suckling, they are nevertheless partially required for LPH and Fabp1 gene expression during this time frame (Fig. 7 and 8). One explanation is that at least some Gata4 and Hnf1α is normally present in the nucleus, as suggested by Western blot analysis for Gata4 using 40 µg of protein (Fig. 4C). However, Hnf1α could not be detected at P4 and P7 on Western blots using 80 µg of protein (Fig. 4C), suggesting that Hnf1α is not present during this time interval. Thus a second explanation is that Gata4 and/or Hnf1α is required earlier in intestinal development for later expression of putative target genes. Although this is a plausible explanation for Hnf1α, in which a germline-null model was used, it is a less likely explanation for Gata4, which is inducibly inactivated after birth. Precedence for an early developmental requirement for later expression is shown in the liver, where embryonic, but not postnatal, reexpression of Hnf1α is capable of reactivating the silent phenylalanine hydroxylase gene in Hnf1α-deficient hepatocytes (52).

Interestingly, regulation in the knockout models at P7–P14 (Figs. 7 and 8), when Gata4 and Hnf1α nuclear protein is decreased, is virtually identical to that which occurs at E17.5 (Fig. 3), when Gata4 and Hnf1α are normally present in

![Fig. 9. Dexamethasone induces a precocious weaning response in jejunum in the presence or absence of Gata4 or Hnf1α. A: dexamethasone induces SI mRNA levels in preweaning mice independently of Gata4 or Hnf1α but attenuates LPH mRNA levels specifically in the absence of Gata4 or Hnf1α. Mice were treated with dexamethasone (1 µg/g body wt) on P10, and RNA was collected 24 h later from the jejunum of wild-type, Gata4-mutant, and Hnf1α−/− mice. SI (top) and LPH (bottom) mRNA levels were quantified by real-time RT-PCR in n = 3–5 mice. *P < 0.05 and **P < 0.01 compared with untreated mice. B: dexamethasone attenuates Gata4 and Hnf1α mRNA levels in jejunum of preweaning mice. Wild-type mice were treated with dexamethasone on P10, and RNA was collected 24 h later from the jejunum. Gata4 and Hnf1α mRNA levels were quantified by real-time RT-PCR in n = 3–5 mice. *P < 0.05 compared with untreated mice.]

![Fig. 10. Summary of Gata4 and Hnf1α regulation of LPH, Fabp1, and SI gene expression during intestinal development. Schematic representation of LPH (top), Fabp1 (middle), and SI (bottom) gene expression in wild-type (solid line), Gata4-mutant (dashed line), and Hnf1α−/− (dotted line) mice during development.]

during weaning. Together, these data demonstrate that Gata4 and Hnf1α are dispensable for SI gene expression throughout development. The future challenge, therefore, is to identify transcription factors essential for SI gene expression.

In our studies, we identified a paradoxical loss of Gata4 and Hnf1α protein in the nuclei of absorptive enterocytes beginning shortly after birth, continuing through the suckling period, and ending during the third week of life, when weaning occurs. At P4–P10, Gata4 and Hnf1α were greatly reduced in the nuclear fraction of jejunal extracts as determined by Western blot analysis (Fig. 4, B–D) and EMSA (Fig. 4E), and neither could be detected in the nuclei of villus enterocytes in the jejunum by immunofluorescence (Fig. 5, D–I). Interestingly, Gata4 and Hnf1α mRNA abundance remain high during this time interval (Fig. 4A), suggesting that the reduction in Gata4 and Hnf1α protein is not due to a decrease in transcription rate. We thus believe that either the mRNAs for these proteins are not transcribed and/or that their translation products are actively catabolized.
epithelial nuclei, suggesting that the loss of nuclear Gata4 and Hnf1α after birth is not a critical regulatory mechanism for LPH and Fabpl1 gene expression. Thus it is possible that the process of nuclear Gata4 and Hnf1α loss during suckling in mice is a regulatory process, but for other, as yet unknown, targets of Gata4 and Hnf1α.

Glucocorticoids like dexamethasone can induce the precocious maturation of the intestine, but the underlying mechanism has not been fully elucidated (1, 54). Characteristic of this process is a dramatic increase in SI mRNA abundance 24 h after the administration of glucocorticoids (25). Since the induction in SI mRNA is not apparent within the first 8 h (25), it is thought that the SI induction is not a direct response to glucocorticoids, but rather a secondary effect of early response genes on SI gene transcription (1). Recently, Oesterreicher and Henning (32) showed that in 8-day-old mice, Gata4 and Gata6 were both induced 4 h after dexamethasone treatment, as shown by supershift EMSAs and Western blot analysis, suggesting a role for Gata factors in the glucocorticoid-induced maturation of the intestine. To test the hypothesis that Gata4 or Hnf1α mediates this process, we conducted dexamethasone-induced precocious maturation experiments in our knockout models. SI mRNA was strongly induced in the presence or absence of Gata4 or Hnf1α (Fig. 9A), indicating that these proteins are not required for mediating the dexamethasone response on SI. We next defined the effect of dexamethasone on LPH mRNA abundance in our knockout models and found that LPH mRNA was reduced in the Gata4-mutant and Hnf1α−/− mice (Fig. 9A) but not in wild-type controls. Our interpretation of these data is that dexamethasone induces precocious maturation to the point where Gata4 and Hnf1α become more regulatory for LPH gene expression. We also found a decrease in both Gata4 and Hnf1α mRNA abundance with dexamethasone treatment (Fig. 9B), which is also consistent with a maturation of the intestine, because Gata4 and Hnf1α mRNAs decline normally during weaning (Fig. 4A). Therefore, we believe that neither Gata4 nor Hnf1α mediates the glucocorticoid response but rather are downstream targets of this response.

Cre-mediated inactivation of Gata4 in our current model results in the synthesis of a truncated, transcriptionally inactive form of Gata4 (mutant Gata4) that is missing its NH2-terminal activation domains but contains its functional zinc finger region (3). Since mutant Gata4 continues to bind DNA, it has the potential to act as a dominant negative Gata factor (3), masking the activity of other enterocyte Gata factors, such as Gata6, which is coexpressed with Gata4 in villus enterocytes (3, 9, 41). Furthermore, mutant Gata4 contains the functional zinc finger region, which has been shown to interact not only with DNA, but also with other proteins, such as Hnf1α (49) and friend of Gata (Fog) cofactors (16). Indeed, we have shown that, although the activation domains of Hnf1α are absolutely required for synergy, the Gata activation domains are dispensable for this activity (49, 50), suggesting that mutant Gata4 maintains the ability to mediate Gata4-Hnf1α synergy. Thus the phenotype attributed to the inactivation of Gata4 in our current model could represent a specific Gata4 function, a masked Gata6 function, and/or a function dependent on Gata4 activation domains. Furthermore, because Gata4 is inducibly inactivated rather than null for Gata4 from the earliest phases of intestinal development, it is possible that gene expression is dependent on the presence of Gata4 early in development (before administration of tamoxifen) may not be revealed in the current model. Nevertheless, since the same Gata4 model (induction of mutant Gata4) is used throughout our studies, the differential regulation of target genes at diverse developmental time points continues to support different underlying mechanisms of regulation during development.

Our data show that the induction and maintenance of terminal differentiation by Gata4 and Hnf1α is highly dependent on the developmental time frame under study. Although Gata4 and Hnf1α are indispensable for the maintenance of expression of specific genes in the mature intestine (3, 4), consistent with a mechanism of coregulation, these transcription factors are only partially required for the same genes prior to the final maturation that occurs at weaning, arguing against a coregulation mechanism and implicating other mechanisms in the development of gut function. Understanding the individual and combined effects of intestinal transcription factors during development will continue to reveal important regulatory pathways essential for intestinal differentiation.

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