The Mouse Ileal Lipid-binding Protein Gene: A Model for Studying Axial Patterning during Gut Morphogenesis

Michael W. Crossman,** Sherrie M. Haufl,† and Jeffrey I. Gordon*
Departments of *Molecular Biology and Pharmacology and †Pediatrics, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. Normal, chimeric-transgenic, and transgenic mice have been used to study the axial patterns of ileal lipid-binding protein gene (Ilbp) expression during and after completion of gut morphogenesis. Ilbp is initially activated in enterocytes in bidirectional wave that expands proximally in the ileum and distally to the colon during late gestation and the first postnatal week. This activation occurs at the same time that a wave of cytodifferentiation of the gut endoderm is completing its unidirectional journey from duodenum to colon. The subsequent contraction of Ilbfs expression domain, followed by its reexpansion from the distal to proximal ileum, coincides with a critical period in gut morphogenesis (postnatal days 7-28) when its proliferative units (crypts) form, establish their final stem cell hierarchy, and then multiply through fission. The wave of reactivation is characterized by changing patterns of Ilbp expression: (a) at the proximal most boundary of the wave, villi contain a mixed population of scattered ileal lipid-binding protein (ILBP)-positive and ILBP-negative enterocytes derived from the same monoclonal crypt; (b) somewhat more distally, villi contain vertical coherent stripes of wholly ILBP-positive enterocytes derived from monoclonal crypts and adjacent, wholly ILBP-negative stripes of enterocytes emanating from other monoclonal crypts; and (c) more distally, all the enterocytes on a villus support Ilbp expression. Functional mapping studies of Ilbp's promoter in transgenic mice indicate that nucleotides -145 to +48 contain cis-acting elements sufficient to produce an appropriately directed distal-to-proximal wave of Ilbp activation in the ileum, to maintain an appropriate axial distribution of monophenotypic wholly reporter-positive villi in the distal portion of the ileum, as well as striped and speckled villi in the proximal portion of its expression domain, and to correctly support reporter production in villus-associated ileal enterocytes. Nucleotides -417 to -146 of Ilbp contain a "temporal" suppressor that delays initial ileal activation of the gene until the second postnatal week. Nucleotides -913 to -418 contain a temporal suppressor that further delays initial activation of the gene until the third to fourth postnatal week, a spatial suppressor that prohibits gene expression in the proximal quarter of the ileum and in the proximal colon, and a cell lineage suppressor that prohibits expression in goblet cells during the first two postnatal weeks.

The mouse gut epithelium is an attractive model system for studying how regional diversity or axial patterning is established and maintained. Its four component cell lineages are rapidly renewed through a geographically well-organized sequence of proliferation, commitment, and migration-associated differentiation. This renewal occurs perpetually throughout the lifespan of the mouse and is fueled by a stem cell hierarchy that is maintained in an anatomically distinct unit known as the crypt of Lieberkühn. Morphogenesis of the gut epithelium occurs relatively late in development, from embryonic day (E) 15 through postnatal day (P) 21 (1, 3, 9, 49). This allows the process of axial patterning (establishment of region-specific differences in the distribution and differentiation of its component cell lineages) to be studied in fetal and postnatal animals.

The mouse gut endoderm undergoes rapid remodeling from E15 to E19 as a proximal-to-distal wave of cytodifferentiation converts it from a pseudostratified to a simple columnar epithelium. This monolayer covers nascent villi that are separated from one another by a proliferative compartment...
known as the intervillus epithelium. The intervillus epithelium represents the precursor to intestinal crypts. Studies of mouse aggregation chimeras indicate that the intervillus epithelium is polyclonal, i.e., supplied by several active stem cells with distinct genotypes (68). A poorly understood process of cell selection occurs during crypt formation (3, 9), which converts them to monoclonality by P14. Crypt number is dramatically increased between the second and third postnatal week through fission (3, 9). The adult mouse small intestine contains ~1.1 million crypts (25), each of which is supplied by one or more active multipotent stem cells (22, 27, 41, 80). The descendants of these stem cells undergo several rounds of cell division in the lower and middle thirds of each crypt, forming a transit cell population (54). Cellular differentiation occurs during a bipolar migration along the crypt-to-villus axis. Enteroendocrine cells, and enteroendocrine cells differentiate as they are rapidly translocated in vertical coherent bands from a crypt to the apex of a surrounding villus (5, 7, 8, 67), after which they are exfoliated. Proliferation, upward migration/differentiation, and exfoliation are completed in 2–5 d (82). In contrast, Paneth cells differentiate during descent to the base of the crypt, where they reside for ~20 d (6).

Axial patterning of the mouse intestinal epithelium is evident at the time of its initial cytodifferentiation in late fetal life. Studies with El5 intestinal isografts implanted into the subcutaneous tissues of young adult syngeneic or nude mouse recipients indicate that this patterning can occur without exposure to normal luminal contents, e.g., the microflora, biliary and pancreatic secretions (17, 62, 63). The nature and location of the epithelium's positional address is unknown. Region-specific differentiation could reflect a cell autonomous process or it could be programmed/maintained by interactions between epithelial, stromal, and/or mesenchymal compartments.

The different cephalocaudal and developmental patterns of activation of three homologous fatty acid-binding protein genes in villus-associated enterocytes provide an opportunity for identifying cis- and trans-acting factors that regulate regional specification of the enterocytic lineage during gut morphogenesis (12, 56–60, 70, 74, 75). The intestinal fatty acid binding protein gene (Fabpi) is activated on El5. Fabpi's expression domain is fully established by the first postnatal week and extends from the proximal duodenum to the proximal colon with highest steady-state levels of its mRNA and protein products occurring in the distal jejunum (12). The liver fatty acid binding protein gene (Fabpl) is also activated on El5 (26). Like Fabpi, its expression domain in the gut is established coincident with movement of the proximal-to-distal wave of endodermal cytodifferentiation (57). The concentration of liver fatty acid binding protein (L-FABP) mRNA and protein is highest in the proximal jejunum of suckling, weaning, and adult mice (56, 57, 70). The ileal lipid binding protein gene (Ilbp) encodes a protein that appears to be the cytosolic receptor for bile acids that have undergone sodium-dependent active transport into the enterocyte (37, 81). Its mRNA is confined to the ileum of adult mice and is not detectable in total cellular RNA prepared from the intact intestine until after birth (64). In the current study, we have characterized the developmental patterns of expression of the mouse Ilbp gene and Ilbp/reporter transgenes. The results have allowed us to identify cis-acting elements that regulate axial patterning of the distal small intestine during completion of gut morphogenesis and that are responsive to temporal factors that operate over a time scale of weeks to months.

**Materials and Methods**

**Isolation of Mouse ILBP-cDNA**

An oligo (dT)-primed cDNA library was prepared in lambda ZapII (Stratagene, La Jolla, CA) using poly(A) + RNA isolated from the distal third of adult male FVB/N mouse small intestine. The library was screened with a full-length porcine ILBP cDNA (20, 64). 10 probe-positive recombinant phages were isolated with inserts ranging in size from 200 to 560 bp. The largest (560 bp) was subcloned into pBluescript SK(-) (yielding pMC4), and both strands were sequenced (66). Restriction endonuclease digestion and nucleotide sequence analyses of double-stranded cDNAs contained in the other recombinant phage revealed that they were all represented within pMC4's 560-bp insert.

**Isolation and Sequencing of the Mouse Ilbp Gene**

An adult DBA/2J mouse liver genomic library (Clontech, Palo Alto, CA) was screened with the full-length ileal lipid-binding protein (ILBP) cDNA. Five unique probe-positive phages were recovered from the 300,000 plaques that were surveyed. The insert in one of these phages (XILBP.5) was analyzed further. Two apparently contiguous pieces of DNA consisting of a 3-kb EcoRI/BamHI fragment and a 4-kb BamHI/EcoRV fragment (Fig. 1 A) were each subcloned into pBluescript. The nucleotide sequences of both strands of these DNA were determined using oligonucleotide primers and the diodeoxynucleotide chain termination method. Two sets of oligonucleotide primers flanking the presumed junction between these two fragments were used together with XILBP.5 DNA for PCR. 5'-GCATGATATGATAAGTGCGCTTG-3' and 5'-GCTAGAGGAGGAGGCCTGCAAG-3' produce a 156-bp PCR fragment from both XILBP.5 and FVB/N spleen DNA while 5'-TAACTGCCTTGATGGTGTC-3' and 5'-AGAAATCGGCTGCTGCTTGGC-3' produce a 494-bp PCR product from the same template DNAs. These results allowed us to conclude that there were no additional sequences in this region of XILBP.5 DNA that were not represented in the 3-kb EcoRI/BamHI and 4-kb BamHI/EcoRV fragments. Intron/exon boundaries in mouse Ilbp were subsequently identified by comparing the deduced genomic sequence with the sequence of the 560-bp mouse ILBP cDNA.

**Primer Extension Analysis**

To identify the start site of transcription of Ilbp, an oligonucleotide, 5'-GCCCTATGAAACTCTGCTT3', encoding Asp66→Arg of mouse ILBP (Fig. 1 B), was labeled with 32p at its 5' end (65) and annealed to total cellular RNA isolated from FVB/N ileum. Primer extension with avian myeloblastosis virus reverse transcriptase was carried out according to a previously published protocol (73). Negative template controls included yeast transfer RNA and mouse liver RNA. Reaction products were analyzed on urea-polyacrylamide sequencing gels, and their sizes were compared to the sizes of products obtained from sequencing the mouse Ilbp genomic clone with the same primer.

**Computer-assisted Sequence Analysis**

Dot matrix comparisons were performed using the GCG software package (15). Quality scores >55 were considered potentially significant when using the Fit Consensus algorithm. Transcription factor databases (16, 21) were searched with the program supplied in Geneworks (IntelliGenetics, Mountain View, CA).

**Generation and Analysis of Transgenic Mice**

**Construction of ILBP/hGH +J Fusion Genes.** A 966-bp NcoI fragment, encompassing nucleotides -913 to +52 of mouse Ilbp was subcloned into NcoI-digested pBluescript KS, yielding pBSIL0. pBSIL0 was digested with BamHI/Ksp6321 yielding a 960-bp fragment spanning nucleotides -913 to +48. pBSGH is a recombinant pBluescript KS plasmid that contains the human growth hormone (hGH) gene beginning at its nucleotide +3 with all of its exons and introns (hGH +J). pBSGH was linearized with BamHI, ligated to the 0.96-kb BamHI/Ksp6321 Ilbp fragment, and the remaining Ksp6321 end was filled in with Klenow fragment of DNA poly-

---

The Journal of Cell Biology, Volume 126, 1994

1548
merase before a second, blunt end ligation reaction. This yielded plLhGH containing nucleotides -913 to +48 of mouse ilbp linked to hGH3 (ilBP -913 to +48/hGH3). IlBP -913 to +48/hGH3 was excised from plLhGH using Scal and EcoRI. IlBP -471 to +48/hGH3 and IlBP -142 to +48/hGH3 were obtained by digesting plLhGH with XcmI/EcoRI and AflII/EcoRI, respectively. All IlBP/hGH fusion genes were purified by agarose gel electrophoresis followed by glass bead extraction (GeneClean II; BIO 101, Inc.). IlBP was off transcriptionally in vitro and sequenced to probe three (32P-labeled) DNAs: (a) the 150-bp hGH exon V fragment; (b) a 540-bp PstI/EcoRV fragment encompassing the full-length mouse IlBP cDNA from PMc; and (c) an 870-bp RsaI fragment from human glyceroldehyde-3-phosphate dehydrogenase cDNA (pHGAP; American Type Culture Collection, Rockville, MD). Washed blots were subsequently scanned using a storage phosphor imager to calculate the steady-state levels of specific scRNAs. Labelled probes were removed by incubating the blot at 100°C for 5 min in 0.01X SSC/0.01% SDS. The stripped blot was then reprobed with the next 32P-labeled DNA.

Single- and Multilabel Immunocytochemical Studies. Normal FVB/N mice were killed by decapitation at E18 and by cervical dislocation at P2, P5, P7, P9, P11, P13, P15, P17, P19, P21, and P28 (n = 2–4 littermates/time point). Transgenic mice and their normal littersmates were killed at P7, P14, P28, P45, P64, P78 (12 wk), and P168 (24 wk) by cervical dislocation. Pronuclear injection and identification of transgenic animals. FVB/N mice were obtained from Taconic Farms, Inc. (Germantown, NY). Purified plLhGH and plLhGTG-3 DNA were injected into pronuclei of fertilized FVB/N eggs. Injected eggs were transferred to pseudopregnant Swiss Webster mice (30). Live born animals were screened at the time of weaning for the presence of the transgenes using the polymerase chain reaction, tail DNA as a template, and two oligonucleotide primers, 5'-GAC-CAACCCTTTGTTGGAGCTGTCGTCG-3' (sense primer representing nucleotides -1 to +29 of mouse ilbp) and 5'-GGGGATCCGGGCTGTTGGAGCTGTCGTCG-3' (antisense primer representing nucleotides +351 to +332 of hGH; reference 69). The PCR mixture (total vol. = 50 µl) contained Tris (20 mM, pH 8.5), MgCl2 (2.5 mM), (NH4)2SO4 (16 mM), bovine serum albumin (150 µg/ml), oligonucleotide primers (2 µM), dNTPs (100 µM), KlenTaq DNA polymerase (Ab Peptides), and mouse genomic (tail) DNA (1 µg). The cycling conditions were used: denaturation = 1 min at 94°C, annealing = 2 min at 56°C, and extension = 2 min at 72°C for a total of 30 cycles. Each of the three IlBP/hGH3 transgenes yields a 205-bp amplified product with these primers.

Calculation of Transgene Copy Number. Transgene copy number was determined by Southern blot analysis of genomic DNA prepared from the liver or spleen of F0 or F1 mice. 10 µg of DNA was digested with PvuII, which produces a 1.5-kb internal fragment from each IlBP/hGH3 transgene. The PvuII digests were fractionated by agarose gel electrophoresis and transferred to nylon membranes (GIBCO/BRL, Gaithersburg, MD). Southern blots were probed with a 32P-labeled, 150-bp BglII/PvuII fragment obtained from exon V of hGH (74). The intensity of the hybridization signal was quantitated by a storage phosphorimaging system (Molecular Dynamics, Sunnyvale, CA). Signal intensities produced from PvuII-digested genomic DNA were compared to those obtained from known amounts of PvuII-digested plLhGH DNA included as internal standards in each blot. Only signals in the linear range of film sensitivity were used to calculate copy number.

Maintenance of Mice. Each transgenic pedigree was established and subsequently maintained by crosses to normal FVB/N littermates (Table I). All mice were caged in microisolators and kept under a strictly controlled light cycle (lights on at 0600 h, off at 1800 h). Animals were given a standard autoclavable Chow diet (no. 5010; Ralston Purina, St. Louis, MO) ad libitum. The mice used in this study were free of pathogens including murine hepatitis virus. For developmental studies, gestational age was calculated from the day a vaginal plug was first noted (designated day 0). Postnatal age was computed with day 1 equal to the day of birth.

Analysis of the Developmental, Cellular, and Axial Patterns of IlBP/hGH3 Expression. Measurement of IlBP and hGH mRNA Levels in Normal and Transgenic Mice. FVB/N mice were killed between 1200 and 1400 h at E15, E16, E17, E18, P2, P5, P7, P9, P11, P13, P15, P17, P19, P28, and P42, and P84 (n = 8 mice from one litter for each fetal time point surveyed; n = 2 littersmates for each postnatal time point). For E15–E18 mice, the entire length of the intestine (i.e., from the gastroduodenal junction to the rectum) was removed en bloc, frozen in liquid nitrogen, the pooled samples from each time point were pulverized and RNA was extracted with RNAzol (10). For postnatal time points, the small intestine was divided into three equal-length segments (designated duodenum, jejunum, and ileum). The cecum was removed by making incisions at the ileal-cecal and cecal-colonic junctions, and the colon was divided into two equal-length portions (proximal and distal colon). Comparably positioned segments of gut were pooled from each of the two littersmates killed at each postnatal time point surveyed, and total cellular RNA was recovered.

Transgenic mice and their normal littersmates were killed by cervical dissection between 1200 and 1400 h at P7, P14, P21, and P28. Their gastrointes- tinal tracts were rapidly subdivided into seven segments (stomach, duodenum, jejunum, ileum, cecum, proximal, and distal colon). Each segment of gut was snap frozen in liquid nitrogen. 11 additional tissues were also obtained from each mouse (brain, submaxillary glands, thymus, heart, lung, kidney, liver, spleen, pancreas, adrenal, and gonads). Total cellular RNA was extracted from frozen pulverized tissue using RNAzol.

Samples of total cellular RNA (10 µg) were fractionated by denaturing formaldehyde-agarose gel electrophoresis (65) and were transferred to nylon membranes. Each blot contained IlBP and hGH mRNA standards (10 µg) prepared by in vitro transcription (47) from PvuII-digested IlBP/hGH3 DNA and sequenced to probe three (32P-labeled) DNAs: (a) the 150-bp hGH exon V fragment; (b) a 540-bp PstI/EcoRV fragment encompassing the full-length mouse IlBP cDNA from PMc; and (c) an 870-bp RsaI fragment from human glyceroldehyde-3-phosphate dehydrogenase cDNA (pHGAP; American Type Culture Collection, Rockville, MD). Washed blots were subsequently scanned using a storage phosphor imager to calculate the steady-state levels of specific scRNAs. Labelled probes were removed by incubating the blot at 100°C for 5 min in 0.01X SSC/0.01% SDS. The stripped blot was then reprobed with the next 32P-labeled DNA.

Confocal Microscopy. A Molecular Dynamics Multimprobe 2001 inverted confocal laser scanning confocal microscope system equipped with a 60x oil immersion objective lens with a numerical aperture of 1.4 was used to scan sections of Bouin's fixed ileum prepared from P7 ILBP -471 to +48/hGH3 transgenic mice and their normal littersmates. These sections were

Crossman et al. Axial Patterning in the Mouse Gut Epithelium
gene sequence is available from EMBL/Genbank/DDBJ under accession number U00938. (C) Both strands of
-1051 to +1
stained as described in the legend to Fig. 9 and were scanned using a confocal optical plane of 0.6 μm.

Generation and Characterization of a Rabbit Anti-ILBP Sera. Porcine ILBP was expressed in and purified from Escherichia coli as previously described (64). The purity of the protein preparation was established by SDS-PAGE and by automated sequential Edman degradation. The purified protein was emulsified with Freund’s complete adjuvant (Sigma Immunonochemicals) and used to immunize New Zealand white rabbits. The specificity of the resulting antisera was established by Western blot analysis. Jejunum, ileum, cecum, colon, lung, and liver were recovered from P7 and P42 FVB/N mice and were washed with ice cold 0.9% saline before freezing in liquid nitrogen. After lyophilization for 24 h at -60°C, the tissues were ground to a fine powder and resuspended in extraction buffer (30 mg dry wt of tissue/ml of Tris [0.125 M, pH 6.8], SDS [4%], β-mercaptoethanol [10%], glycerol [20%], aprotinin [5 μg/ml], phenylmethylsulfonyl fluoride [0.05 M], leupeptin [5 μg/ml], pepstatin A [1 μg/ml], and EDTA [5 mM]). The suspension was boiled for 5 min and then spun for 5 min in a microfuge at room temperature to remove insoluble debris. The protein content was determined on the supernatant fraction (52).

Intestinal Isografts

Intestinal isografts were prepared from E15 FVB/N mice (63). The entire intestine plus cecum and colon were implanted into the dorsal subcutaneous fascia of P22 FVB/N male recipients (1 graft/recipient). 2 wk after implantation, mucoid material was aspirated from the lumen of each isograft using a sterile syringe. Isografts (n = 2) were subsequently harvested 6 wk after implantation. The luminal fluid obtained from each isograft at the time of its recovery was devoid of bacteria as determined by Gram stain and by culture on Luria agar at 37°C under aerobic and anaerobic conditions (17). Duodenal, jejunal, ileal, and colonic segments from the grafts were fixed in Bouin’s solution.

Generation of Chimeric-Transgenic Mice

D3 embryonic stem (ES) cells (23) were used at passages 8-11 and maintained on mouse embryonic fibroblasts. 10 μg of HindIII-digested pLNDon DNA, containing nucleotides -596 to +21 of Fabp1 linked to the hGH gene beginning at its nucleotide +3 (L-FABP -596 to +21/hGH+3) followed by a neomycin selection cassette in the same transcriptional orientation (29), were introduced into ES cells by electroporation. Stably transfected, G418-resistant clones were isolated and Southern blot hybridization plus PCR analysis of their genomic DNA used to establish that complete integration of L-FABP -596 to +21/hGH+3/neo had occurred. Chimeric-transgenic mice were generated by injecting 10-12 ES cells into 3.5 d postcoitum C57BL/6 blastocysts (2).

C57Bl/6 (control), 129/Sv (control), and C57Bl/6→D3(pLNDon) chimeric-transgenic mice were killed at 12 wk of age by cervical dislocation, the entire intestine was removed en bloc, flushed with cold PBS, followed by Bouin’s solution. The gut was then fixed in Bouin’s for 6 h. Swiss rolls of the entire intestine were prepared for single and multilabel immunocytochemical analyses.

Results

Mouse Ilbp Has an Organization Similar to that of Other Members of the Fabp Family

Fig. 1 B shows the sequence of mouse Ilbp including 1051 nucleotides of its 5′ nontranscribed domain and 285 nucleotides of its 3′ flanking region. Like other members of the Fabp family, Ilbp contains four exons (117, 176, 89, and 54 bp) and three introns (2726, 995, and 1295 bp). Exon size and intron location are similar to that encountered in other family members (13, 24, 32, 72). Primer extension analyses using adult FVB/N ileal RNA as a template revealed that Ilbp has a single start site of transcription located 23 bp downstream from a TATA box (Fig. 1 B). Nucleotides -1051 to -1 of mouse Ilbp contain a number of sites that are similar to published consensus sequences for the binding of transcriptional factors (Fig. 1 C).

ILBP mRNA contains an open reading frame of 384 nucleotides encoding a protein of 128 amino acids which has 95% primary sequence identity with the orthologous rat protein (19, 34) and 70% identity with porcine ILBP (20).

Southern blot analysis performed using a variety of hybridization and washing stringencies indicate that Ilbp is a single-copy gene (data not shown). Mapping studies using a M. splendens panel disclosed that Ilbp is located on mouse chromosome 11, 10.2 cM from Emvl4, a retroviral insertion site (Birkenmeier, E. H., and J. I. Gordon, unpublished observations). Ilbp is not linked to any other Fabp family members or to other reported mapped genes encoding proteins involved in lipid metabolism and/or bile acid transport.
Ilbp Is Only Expressed in the Ileum of FVB/N Mice and Undergoes a Complex Pattern of Activation during a Critical Period of Gut Morphogenesis

Surveys of E15-E18 FVB/N mice indicated that ILBP mRNA was first detectable in total intestinal RNA at E18 (Fig. 2), coinciding to the time that the proximal-to-distal wave of cytodifferentiation of the pseudostratified gut epithelium to a monolayer has reached the ileum. Throughout postnatal development, a unique 600-nucleotide ILBP mRNA was isolated from the distal third of the small intestine for all of the postnatal time points surveyed (n = 2 littermates/time point). Note that tissues from littersmates were pooled before RNA extraction.

The concentration of this mRNA remains essentially unchanged at least through the next 5 mo of postnatal life, nor is it detectable in any of the 12 extraintestinal tissues surveyed. Steady-state levels of ileal ILBP mRNA rise from P2 to P5 and then fall abruptly (Fig. 2). Beginning at the suckling/weaning transition (P13), mRNA concentrations rise rapidly to a peak value of 65 pg/μg total ileal RNA on P19. Levels remain constant throughout weaning (P19-P28), and they fall slightly as animals reach sexual maturity (~35 pg/μg at P42). The concentration of this mRNA remains essentially unchanged at least through the next 5 mo of postnatal life (Fig. 2 and data not shown).

Single- and multilabel immunocytochemical analyses reveal a distal-to-proximal wave of activation of the Ilbp gene (Fig. 3). At E18, scattered ILBP-positive columnar epithelial cells are evident overlying nascent villi in the distal region of the small intestine (Fig. 4 A), as well as in the colonic epithelium. During the first postnatal week, ILBP production expands bidirectionally to involve additional columnar epithelial cells in the distal half of the ileum and in the cecum and colon (Fig. 3). By P7, clusters of ILBP-positive cells are interspersed among large areas of ILBP-negative cells in the proximal colon and cecum. These cells coexpress known markers of the enterocytic lineage such as I-FABP (data not shown). In the distal half of the ileum, most villus-associated enterocytes are ILBP positive. ILBP is not detectable in proliferating (BrdUrd-positive) cells located in the P2-P7 intervillus epithelium or in members of the goblet or enteroendocrine cell lineages (Fig. 4 B plus data not shown).

Just after P7, the expression domain of ilbp abruptly "collapses," resulting in confinement of ILBP-positive, villus-associated enterocytes to the distal ileum. The extinction of ilbp expression in the proximal colon and mid-ileum occurs during P7-P11 (Fig. 3). This is followed by a wave of reactivation that moves from the distal to proximal portions of the ileum over a 2-wk period. There are three characteristic cellular patterns of ILBP staining found at this stage of gut morphogenesis: (a) distal ileal villi contain a wholly ILBP-positive population of enterocytes (Figs. 3 and 4 C); (b) more proximal villi contain vertical coherent bands or stripes of wholly ILBP-positive enterocytes, each emanating from a given crypt and adjacent vertical coherent bands of wholly ILBP-negative stripes derived from adjacent crypts (Fig. 4, D and E); and (c) at the leading proximal portion of the gene's expression domain, villi generally contain a scattered population of ILBP-positive and ILBP-negative cells, giving them a speckled or mosaic appearance (Fig. 4 F). The distribution of wholly positive, striped, and speckled villi follows the wave of activation of ilbp from P11 to P28; i.e., at any one point in time, the proximal portion of ilbp expression domain contains striped villi while the leading edge consists of a predominant population of speckled villi composed of ILBP-positive and -negative cells with only a few striped and essentially no wholly positive villi (Fig. 3). The ILBP-positive cells encountered in the P11-P28 ileum were defined as enterocytes, based on their morphologic appearance and by their ability to support expression of a variety of well-characterized lineage-specific markers including I-FABP and L-FABP (data not shown).

Figure 2. Developmental changes in ileal ILBP mRNA levels. mRNA concentrations were determined using RNA prepared from the entire intestine of E15-E18 mice (n = 8 littermates/time point). Total cellular RNA was isolated from the distal third of the small intestine for all of the postnatal time points surveyed (n = 2 littermates/time point). Note that tissues from littermates were pooled before RNA extraction.

Figure 3. Summary of the distal-to-proximal wave of ilbp activation in the developing FVB/N mouse intestine. The ileum has been operationally defined as the distal third of the small intestine. IL1 and IL4 refer to the proximal most (IL1) and distal most (IL4) quarters of the ileum. CE, cecum; PC, proximal colon; DC, distal colon. Segment containing scattered ILBP-positive epithelial cells; striped villi with vertical coherent bands of ILBP-positive enterocytes; villi that contain >95% ILBP-positive enterocytes; ILBP not detectable in epithelial cells.
Figure 4. Immunocytochemical studies of the cellular patterns of ILBP expression in the developing FVB/N mouse intestine. (A) Cross-section of the distal small intestine of an El8 mouse, incubated with rabbit anti-ILBP followed by Texas red–labeled donkey anti-rabbit Ig. Scattered ILBP-positive columnar epithelial cells (arrows) are present on nascent villi. (B) Section prepared from P7 ileum incubated with rabbit anti-ILBP and goat anti-BrdUrd Ig. Antigen-antibody complexes were detected with goat anti-rabbit secondary antibody followed by gold-labeled secondary antibodies with silver enhancement and Texas red–labeled donkey anti-goat Ig. ILBP is confined to villus-associated enterocytes (green-colored cells) and is not detectable in proliferating BrdUrd-positive cells located in nascent crypts (red-colored cells). (C–F) Sections were incubated with goat anti-BrdUrd and rabbit anti-ILBP sera. Antigen-antibody complexes were then detected with fluorescein-labeled donkey anti-goat and Texas red–labeled donkey anti-rabbit sera, respectively. (C) ILBP is expressed in all villus-associated enterocytes located in the distal quarter of the ileum (IL4) of P28 FVB/N mice. (D) A villus located in the proximal quarter of the ileum (IL1) of this P28 mouse exhibits striping with a vertical coherent band of wholly ILBP-positive enterocytes (open arrows) located next to a vertical coherent band of wholly ILBP-negative enterocytes (closed arrows). (E) Coherent bands of ILBP-positive and -negative enterocytes are evident in cross-sections of villi located in the proximal quarter of the P28 ileum. (F) At the proximal-most border of Ilbp's expression domain, villi contain a scattered population of ILBP-positive (closed arrows) and ILBP-negative enterocytes (open arrows). (G) Section prepared from the distal quarter of a P28 ileum stained with rabbit anti-ILBP and Texas red–labeled donkey anti-rabbit sera. (H) Double exposure of the same section shown in G after addition of FITC-conjugated UEA-I. Villus-associated UEA-I-positive goblet cells (green, open arrows) do not coexpress ILBP (seen as orange staining material in enterocytes). (I) Section prepared from the distal third of a P42 intestinal isograft incubated with rabbit anti-ILBP sera and immunogold-labeled goat anti–rabbit serum followed by silver enhancement. The section has been visualized using reflected light polarization microscopy. ILBP is present in villus-associated enterocytes (aqua-colored cells). (J) I-FABP detected in the same section as shown in I. Rabbit anti–I-FABP sera and Texas red–labeled donkey anti–rabbit Ig were used to identify immunoreactive cells (red colored). (K) Double exposure shows a population of enterocytes that coexpress both ILBP and I-FABP (gray-green) and a population of villus-associated enterocytes that only expresses I-FABP (orange). Bar, 25 μm.
By the conclusion of the weaning period (P28), the distal-to-proximal wave of ILbp activation has completed its journey and the expression domain becomes fixed, remaining unchanged for at least the first 6 mo of postnatal life. ILBP is confined to enterocytes in the ileum and cecum. No immunoreactive protein can be detected in proliferating and nonproliferating crypt epithelial populations or in members of the Paneth cell, enteroendocrine, or goblet cell lineages (Fig. 4, G and H plus data not shown). Immunoreactive protein is not detectable in any epithelial cell population located in the proximal colon.

**Intestinal Isografts Reveal that Establishment of ILbp’s Expression Domain Is Not Dependent on Exposure to Luminal Contents**

To assess the effect of luminal components on establishment and/or maintenance of ILbp’s expression domain, E15 FVB/N small intestine and colon were implanted into the subcutaneous tissue of young adult male FVB/N recipients. After 6 wk, the isografts were removed and analyzed for the presence of ILBP using immunocytochemistry. The results demonstrate that the cell lineage-specific, differentiation-dependent, and regional patterns of ILbp expression are not dependent on signaling pathways activated by exposure to pancreatic and biliary secretions, the microflora, or components of the diet such as mother’s milk (Fig. 4, I–K).

**Chimeric-Transgenic Mice Indicate That a Mosaic Pattern of ILBP Accumulation Can Occur in Enterocytes Derived from the Same Monoclonal Crypt**

The monoclonal nature of villus stripping (i.e., all enterocytes in a stripe are either ILBP-negative or ILBP-positive) suggests that members of this lineage are programmed within the monoclonal crypt, perhaps at the level of the stem cell or a committed progenitor, to either express or to not express ILbp during their subsequent migration-associated differentiation program. In contrast, the presence of speckled villi containing isolated ILBP-positive enterocytes intermixed with ILBP-negative cells suggests a subtle heterogeneity in the differentiation program of enterocytes derived from the same monoclonal crypt or a heretofore unexpected degree of mixing of enterocytes from adjacent monoclonal crypts during their upward migration to the apical extrusion zone of ileal villi. If the latter were the case, we would also have to invoke a time-dependent change in this mixing because speckled villi are only evident at the leading edge of the proximally moving wave of ILbp activation (Fig. 3).

We used chimeric-transgenic mice to determine whether the ILBP-positive and -negative enterocytes of a “speckled” villus are derived from the same monoclonal crypt. These mice are created by introducing D3 embryonic stem cells (129/Sv origin) stably transfected with Fabp1 reporter DNAs into normal C57BL/6 blastocysts (29). The gut epithelium of the resulting chimeric animals will be composed of cellular populations derived from the stably transfected ES cell and cellular populations derived from the normal host blastocyst. A villus located at an ES/host cell border in the intestine of such a mouse can be composed of a discrete band of reporter-positive epithelial cells that emanate from a monoclonal, ES-derived crypt and an adjacent band of epithelial cells that do not produce the reporter encoded by the transgene because they emanate from a monoclonal, normal host blastocyst-derived crypt (Fig. 5 A). The 129/Sv ES- and C57BL/6 blastocyst-derived components of such a polyclonal villus can be differentiated from one another using the α1-fucose–specific lectin, UEA-I. C57BL/6 enterocytes and enteroendocrine cells are UEA-I negative, while Paneth cells and a subset of goblet cells bind this lectin. In contrast, 129/Sv Paneth cells are UEA-I-negative, while villus-associated 129/Sv enterocytes, goblet cells, and a subset of enteroendocrine cells are UEA-I positive (17, 29). Studies in transgenic mice have shown that a fusion gene consisting of nucleotides -596 to +21 of the rat Fabp1 linked to the human growth hormone gene beginning at its nucleotide +3 (L-FABP-596 to +21/hGH+) is expressed throughout the duodenal-to-ileal axis, in all four gut epithelial cell lineages, as well as in proliferating and nonproliferating cells located in the lower and upper halves of small intestinal and colonic crypts (70, 75, 77). Adult C57BL/6+/+D3(L-FABP-596 to +21/hGH+) chimeric-transgenic mice do not contain a mixture of hGH-positive and hGH-negative enterocytes within a given UEA-I-positive band of villus epithelial cells, providing independent support for the notion that each adult crypt is monoclonal (29). Moreover, the hGH and UEA-I phenotypes of villus-associated enterocytes are coincident, i.e., UEA-I-positive, ES-derived duodenal, jejunal, and ileal enterocytes are all hGH-positive, while a band of UEA-I-negative, blastocyst-derived enterocytes located on the same villus is wholly hGH-negative (29). With these findings in mind, sections prepared from the proximal ileum of 12-wk-old C57BL/6+/+D3(L-FABP-596 to +21/hGH+) animals were stained with anti-ILBP and anti-hGH sera. The results reveal that a monoclonal ES-derived crypt can give rise to a band of hGH-positive enterocytes with a mixture of ILBP-positive and -negative phenotypes (Fig. 5, B–D).

An additional feature of chimeric-transgenic mice is that the band of host blastocyst-derived epithelium can serve as an internal control when assessing the biological effects of the transgene, i.e., the effect of a single gene product can be assessed in a single villus, located at a particular position along the cephalocaudal and crypt-to-villus axes, in a single animal. Immunocytochemical surveys of wholly ILBP-positive villi containing stripes of ES- and blastocyst-derived enterocytes revealed that cellular ILBP concentrations were the same in the adjacent stripes of hGH-negative and hGH-positive enterocytes (data not shown). This finding established that hGH has no quantitative or qualitative effects on endogenous ILbp gene expression, and that it would be "safe" to use hGH as a reporter for functional mapping studies of ILbp’s promoter.

**Mapping cis-Acting Elements that Regulate the Cell Lineage-specific, Axial, and Developmental Patterns of ILbp Expression**

An initial assumption was made that the major determinants of ILbp’s expression patterns were located in its 5’ nontranscribed region. A series of successive deletions of ILbp’s 5’ nontranscribed domain were generated using a site selection strategy based solely on the availability of unique locations for cleavage by restriction endonucleases. Several pedigrees
Figure 5. Multilabeling studies of 12-wk-old C57BL/6→D3(L-FABP-596→+/hGH+3) chimeric-transgenic mice reveal that enterocytes derived from a single monoclonal crypt can display marked variations in ILBP levels. (A) Schematic view of a villus located at the border of ES cell- and host-blastocyst-derived gut epithelium in an adult C57BL/6→D3 chimeric mouse. The polyclonal villus receives contributions from a monoclonal ES-derived crypt, as well as from a monoclonal B6 crypt. (B) Cross-section of villi located in the proximal quarter of the ileum (IL1) of a 12-wk-old C57BL/6→D3 (L-FABP-596→+/hGH+3) mouse. The section is oriented similar to the cross section shown in A. Staining with rabbit anti-ILBP and fluorescein-labeled donkey anti-rabbit sera reveals ILBP-positive, villus-associated enterocytes as green-colored cells. (C) The same section as shown in B, incubated with goat anti-hGH followed by Texas red-labeled donkey anti-goat Ig. Golgi staining of hGH-positive enterocytes is evident (arrows). (D) A double exposure indicating that hGH-positive enterocytes derived from the same monoclonal ES crypt have both ILBP-positive (open arrows) and ILBP-negative phenotypes (closed arrows). Bar, 25 μm.
### Table I. Summary of Transgenic Mouse Pedigrees

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Liveborn mice screened</th>
<th>Founders*</th>
<th>Pedigrees expressing transgene</th>
<th>Pedigree number</th>
<th>Transgene copy no./haploid genome</th>
<th>[hGH] serum (ng/mL)~</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILBP^−913 to +48/hGH^+3</td>
<td>75</td>
<td>10</td>
<td>9</td>
<td>20</td>
<td>20</td>
<td>15–40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>ND</td>
<td>0.5–1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52</td>
<td>27</td>
<td>9–150</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>56</td>
<td>ND</td>
<td>15–65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>ND</td>
<td>21–120</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>66</td>
<td>ND</td>
<td>0.5–1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>68</td>
<td>38</td>
<td>12–40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>70</td>
<td>ND</td>
<td>2–4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>71</td>
<td>8</td>
<td>33–44</td>
</tr>
<tr>
<td>ILBP^−417 to +48/hGH^+3</td>
<td>58</td>
<td>3</td>
<td>3</td>
<td>11</td>
<td>32</td>
<td>9–50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45</td>
<td>45</td>
<td>2–200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>78</td>
<td>2–18</td>
</tr>
<tr>
<td>ILBP^−143 to +48/hGH^+3</td>
<td>64</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>ND</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>ND</td>
<td>1–4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17</td>
<td>ND</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>26</td>
<td>ND</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>94</td>
<td>16–60</td>
</tr>
</tbody>
</table>

* Founders were identified by PCR and Southern blot analyses of tail DNA.
† Expression was defined by radioimmunoassay of serum for hGH and by immunocytochemical surveys of tissue sections.
§ Serum was obtained by retroorbital phlebotomy from P28 F1 transgenic mice (n = 2–4 mice/pedigree). Each serum sample was assayed in duplicate using a standard radioimmunoassay kit (Nichols Institute Diagnostic, San Juan Capistrano, CA). The range of the values recorded are shown. Note that the limit of sensitivity of the radioimmunoassay is 0.5 ng hGH/ml serum.

![Figure 6](https://example.com/image6.png)

**Figure 6.** Summary of the spatial and temporal patterns of ILBP/hGH^+3 expression in 1–24-wk old transgenic mice. **A** Segment contains scattered populations of hGH-positive epithelial cells; **B** segment contains scattered populations of hGH-positive epithelial cells; **C** villi with predominantly hGH-positive enterocytes; **D** villi with ∼95% hGH-positive enterocytes; **E** no detectable hGH expression in epithelial cells.

The Journal of Cell Biology, Volume 126, 1994

---

ILBP^−913 to +48/hGH^+3

This transgene is only expressed in the small intestine of adult members of the nine pedigrees of mice we surveyed. Moreover, none of the extraintestinal tissues examined in P7, P14, P28, and P168 animals belonging to the two lines studied in detail (Table I, 20 and 17) contained detectable levels of reporter mRNA or protein.

ILBP^−913 to +48/hGH^+3 is silent in the duodenum, jejunum, ileum, cecum, and colon during the first 2 postnatal weeks. The transgene is first activated during the 4th postnatal week, well after the endogenous Ilbp gene (Fig. 6 B). Unlike Ilbp, initial activation occurs in the mid-section of the ileum, where the hGH reporter is confined to villus-associated enterocytes. The pattern of transgene expression is speckled with hGH-positive and hGH-negative enterocytes distributed in a seemingly random pattern along the basilar-apical axis of the villus (Fig. 7 A). However, both hGH-positive and hGH-negative enterocytes contain similar levels of ILBP (Fig. 7, B and C). Between P70 and P84, the transgene's expression domain expands to include the distal three quarters of the ileum (Fig. 6 B). During this expansion, villi that formerly demonstrated a speckled pattern of reporter...
production are "converted" to a monophenotypic pattern with wholly hGH-positive enterocytes. By the end of the 12th postnatal week, only wholly hGH-positive ileal villi are encountered (Fig. 7 D), with the exception of the very proximal and distal margins of ILBP-145 to +48 hGH expression domain (Fig. 6 B). The transgene shows appropriate differentiation-dependent activation, i.e., hGH (or ILBP) is not detectable in BrdUrd-positive or -negative crypt epithelial cells. Reporter production is not evident in the proximal quarter of the ileum, even in mice that are 24 wk old. Moreover, ILBP-143 to +48/hGH+3 is silent in the cecum and colon from 1 to 24 wk of age (Fig. 6 B). The absence of expression in the colon is appropriate, at least from the 2nd postnatal week through adulthood. The absence of transgene expression in the cecal epithelium is inappropriate, contrasting with the persistent expression of Ilpb in this portion of the gut.

The delayed temporal pattern of ILBP-93 to +48/hGH+3 activation in the ileum is also evident when measuring levels of reporter and ILBP mRNA. Between 4 and 12 wk of age, ILBP-93 to +48/hGH+3 transgenic mice exhibit a sevenfold increase in their ileal hGH mRNA levels (from 18 ± 3 pg/μg total ileal RNA to 128 ± 17 pg/μg), while their ileal ILBP mRNA levels remain constant (Fig. 8).

**ILBP-47 to +48/hGH+3**

Like ILBP-93 to +48, this transgene is not expressed in any extraintestinal cell lineages, at least during the first 24 wk of life. Detailed developmental studies of two pedigrees of mice (Table I, lines 45 and 50) revealed that ILBP-47 to +48/hGH+3 is activated before ILBP-93 to +48/hGH+3. At P7, the hGH reporter is only evident in the proximal colonic epithelium. There are two populations of hGH-positive cells in the colon: one belongs to the enterocytic lineage, based on its expression except that of UEA-I. The proximal wave of ILBP-145 to +~/hGH+3 activation in the ileum between the 1st and 2nd wk of postnatal life. This extinction mirrors the silencing of Ilbp expression except that it involves members of the goblet cell rather than enterocytic lineage. By P14, only a small segment of the distal quarter of the ileum (IL4) contains villus-associated hGH-positive cells (Fig. 6 D). Most of these cells appear to be enterocytes, i.e., they are ILBP positive and I-FABP positive but UEA-I negative (data not shown). The expression domain of ILBP-145 to +48/hGH+3 then expands between P14 and P28, traveling in a distal-to-proximal wave at a rate that is slower than that of Ilbp. For example, by P28, hGH is still limited to villi located in the distal half of the ileum (IL3 and IL4), whereas Ilbp expression extends to villi in IL1 (compare Figs. 3 and 6, A and D). At 4 wk of age, ILBP-145 to +48/hGH+3 is expressed in virtually all villus-associated enterocytes in IL3 and IL4 (Fig. 7, K and L). Proliferating cells in the crypts and goblet cells do not contain detectable levels of the hGH reporter. A speckled cellular pattern of hGH staining is limited to the very proximal and distal edges of the transgene's expression domain. The proximal wave of ILBP-145 to +48/hGH+3 activation continues to move slowly so that by the 12th postnatal week, IL2-IL4 are composed of villi with wholly hGH- (and ILBP-) positive enterocytes. At 12 and 24 wk, IL1 contains villi with a speckled pattern of reporter production in their enterocytes.

**ILBP-47 to +48/hGH+3**

Only one out of five pedigrees containing this transgene produced detectable levels of reporter mRNA or protein in the intestine (Table I, line 60). Two of the other lines (lines 17 and 26) did not express ILBP-145 to +48/hGH+3 in their gastrointestinal tracts or in any of the extraintestinal tissues surveyed. By 4 wk of age, members of pedigree 3 contained low levels of hGH mRNA in the kidney, but not in the stomach, small intestine, colon, or other tissues (data not shown). hGH mRNA was confined to the adrenal in 4-wk-old members of pedigree 5. Both of these patterns of transgene expression are anomalous: kidney and adrenal RNA do not contain detectable levels of ILBP mRNA in these transgenic mice or in their normal littermates (data not shown).

Analysis of pedigree 60 revealed that by P7, ILBP-145 to +48 is activated in scattered villus-associated, UEA-I-positive goblet cells located in the distal quarter of the ileum and in the proximal colonic epithelium (Figs. 6 D, 7 H-J, and 9 A-D). ILBP-145 to +48/hGH+3 is the only transgene among the three analyzed that exhibits an extinction of expression in the ileum between the 1st and 2nd wk of postnatal life. This extinction mirrors the silencing of Ilbp expression except that it involves members of the goblet cell rather than enterocytic lineage. By P14, only a small segment of the distal quarter of the ileum (IL4) contains villus-associated hGH-positive cells (Fig. 6 D). Most of these cells appear to be enterocytes, i.e., they are ILBP positive and I-FABP positive but UEA-I negative (data not shown). The expression domain of ILBP-145 to +48/hGH+3 then expands between P14 and P28, traveling in a distal-to-proximal wave at a rate that is slower than that of Ilbp. For example, by P28, hGH is still limited to villi located in the distal half of the ileum (IL3 and IL4), whereas Ilbp expression extends to villi in IL1 (compare Figs. 3 and 6, A and D). At 4 wk of age, ILBP-145 to +48/hGH+3 is expressed in virtually all villus-associated enterocytes in IL3 and IL4 (Fig. 7, K and L). Proliferating cells in the crypts and goblet cells do not contain detectable levels of the hGH reporter. A speckled cellular pattern of hGH staining is limited to the very proximal and distal edges of the transgene's expression domain. The proximal wave of ILBP-145 to +48/hGH+3 activation continues to move slowly so that by the 12th postnatal week, IL2-IL4 are composed of villi with wholly hGH- (and ILBP-) positive enterocytes. At 12 and 24 wk, IL1 contains villi with a speckled pattern of reporter production in their enterocytes.

**ILBP-47 to +48/hGH+3**

Expression of ILBP-145 to +48 in UEA-I-positive goblet cells is extinguished between P7 and P14 in the colon, just as it is in the distal ileum. However, by P14 and for at least the next 12–24 wk, expression of the transgene persists in colonic (but not cecal) enterocytes. These hGH-positive cells are clustered at the surface epithelial cuffs that surround the orifice of crypts (i.e., the colonic homologues of villi). Scat-
Figure 7. Immunocytochemical studies of the cellular patterns of ILBP/hGH expression during and after gut morphogenesis. (A-C) A section of ileum (IL3) recovered from a P28 ILBP-85 to +38/hGH-3 transgenic mouse was incubated with goat anti-hGH and rabbit anti-ILBP sera. Antigen-antibody complexes were detected with fluorescein-labeled donkey anti-goat and Texas red-labeled donkey anti-rabbit secondary antibodies, respectively. (A) Villi are composed of a scattered population of hGH-positive and hGH-negative enterocytes. (B) The same section shows the homogeneous pattern of ILBP expression in villus-associated enterocytes. (C) Double exposure demonstrates that the hGH-positive enterocytes coexpress ILBP (yellow cells). (D) A section prepared from IL3 of a P84 ILBP-85 to +38/hGH-3 mouse was incubated with goat anti-hGH and rabbit anti-ILBP sera. Antigen-antibody complexes were detected with CY3-labeled donkey anti-goat and fluorescein-labeled donkey anti-rabbit secondary Igs. The villi contain a wholly hGH-positive population of enterocytes (orange staining supranuclear Golgi apparatus) that coexpress ILBP (green staining material in cytoplasm). (E) Section from IL3 of a P28 ILBP-41 to +38/hGH-3 mouse stained as in D. A homogeneous pattern of hGH expression (orange) is evident in all villus-associated ILBP-positive (green) enterocytes located in this portion of the ileum. The arrows point to the crypt region. (F) Villi from the proximal ileum
Figure 8. Analysis of steady-state levels of ILBP and hGH mRNAs in the ileum of 4- and 12-wk-old ILBP/hGH+3 transgenic mice. mRNA concentrations were determined as described in Materials and Methods. The pedigrees used for these analyses are indicated in parenthesis. n = 2 mice/time point. The mean value ± 1 SD has been plotted. ⊗, hGH; □, ILBP.

Discussion

A Functional Map of Ilbp's Promoter

Fig. 10 summarizes the results of our functional mapping studies of Ilbp's 5' nontranscribed domain. A remarkably compact region of the gene that spans nucleotides -145 to +48 contains cis-acting elements sufficient to produce an appropriately directed distal-to-proximal wave of activation in the ileum and to subsequently correctly confine reporter production to villus-associated ileal enterocytes. In addition, these sequences can establish and maintain an appropriate axial distribution of wholly reporter-positive villi in the distal three quarters of the ileum, as well as striped and speckled villi in the proximal portion of Ilbp's expression domain. Nucleotides -145 to +48 are unable to reproduce four features of Ilbp expression: (a) the normal postnatal extinction (of reporter) expression in the colon is not evident; (b) cecal expression is never detectable; (c) the distal-to-proximal wave of ILBP-145 to +48 reactivation during P7 to P28 moves more slowly than the wave of activation of the intact endogeneous Ilbp gene; and (d) expression during the first 2 wk of postnatal life in the ileum and proximal colon occurs in goblet cells rather than being confined to enterocytes. However, additional pedigrees of mice containing ILBP-145 to +48/hGH+3 are needed to evaluate the significance of these differences. Nucleotides -417 to -146 appear to contain a 'temporal' suppressor of ileal expression that delays initial activation of the gene until the 2nd postnatal week. Nucleotides -913 to -417 have three types of suppressors: (a) a temporal suppressor that further delays initial activation of the gene until the 3rd to 4th postnatal week, but does not appear to modify the rate of movement of Ilbp's expression domain from IL4 to IL2; (b) a spatial suppressor that prohibits gene expression in the proximal quarter of the ileum and in the proximal colon; and (c) a cell lineage suppressor that pro-

Crossman et al. Axial Patterning in the Mouse Gut Epithelium

1559
Figure 9. Confocal microscopy of villi located in the distal quarter of the ileum of a P7 ILBP−/−/hGH+/+ mouse. The same villus is viewed in all four panels. A and B are on the same focal plane, and they are separated by 2 μm from the focal plane of C and D. Sections were incubated with fluorescein-labeled UEA-I and goat anti-hGH followed by detection with Texas red-labeled donkey anti-goat secondary Ig. A and C show the UEA-I staining pattern of villus-associated goblet cells. B and D are a double exposure and reveal that a subset of UEA-I-positive goblet cells coexpress hGH (arrows). Bar, 25 μm.
hibits expression in goblet cells during the first 2 postnatal weeks.

Sequence Comparison of Functionally Defined Regions of Homologous and Nonhomologous Genes Expressed in Ileal Villus-associated Enterocytes

Promoter mapping studies of Ilbp, Fabpi, and Fabpl conducted in transgenic mice indicate that their duodenal-to-ileal gradients of expression in villus-associated enterocytes can be established and maintained by remarkably compact sequences located within 103 bp (Fabpi), 132 bp (Fabpl), and 145 bp (Ilbp) of their start sites of transcription (references 12, 70; Fig. 10). Nucleotides -103 to +28 of rat Fabpi, like nucleotides -145 to +48 of Ilbp, are sufficient to restrict production of hGH to the enterocytic lineage in adult mice. This region contains one copy of a repeated 14-bp element that is conserved in the orthologous mouse and human genes (24, 73). Cotransfection studies in established epithelial cell lines indicate that hepatic nuclear factor-4 (HNF-4) and apolipoprotein regulatory protein-I, two members of the steroid hormone receptor superfamily of transcription factors that are produced in enterocytes, bind to this element and can activate I-FABP -1178 to +278/hGH +278 (61). Nucleotides -277 to -184 of Fabpi contains cis-acting suppressors of hGH expression in the ileum and proximal colon, and they include a 24-bp element spanning nucleotides -212 to -188 that binds nuclear factors present in colonic but not small intestinal epithelial cells (12). In contrast, nucleotides -1178 to +278 of Fabpi contain sequences that enhance ileal and colonic expression (12). We were unable to detect any similarities between nucleotides -913 to +48 of Ilbp and these functionally defined portions of Fabpi using dot matrix comparisons. These analyses also failed to disclose any obvious sequence similarities between ILBP -913 to +48 and nucleotides -3424 to +54 of the nonhomologous human sucrase isomaltase gene, which confine hGH expression to distal jejunal and ileal villus-associated enterocytes in adult transgenic mice (44). The functionally mapped portions of the Ilbp do contain matches to consensus binding sequences for a number of known transcription factors (compare Fig. 1 C and 10). Several of these sites are also predicted to occur in the Fabpi promoter (e.g., C/EBP, HNF-4, HNF-5, ANTP; see reference 12). However, the relevance and significance of these predictions are unknown at present. Higher resolution functional maps of the Ilbp and Fabpi promoters are needed, as are analyses of the cell lineage-specific, axial, and temporal patterns of expression of these transcriptional factors in the gut epithelium (44).

The Evolution of Ilbp's Expression Domain Occurs during a Critical Period in Gut Morphogenesis

Axial Patterning. Ilbp represents the only enterocytic-specific gene that we know of that has a distal-to-proximal wave of activation. The timing of this activation makes Ilbp a very attractive molecular marker of the intestine's axial patterning. Axial patterning appears to be expressed at different times and in different directions during intestinal development. As noted above, the initial cytodifferentiation of the intestinal endoderm to an epithelial monolayer proceeds in a proximal-to-distal wave during late gestation. Fabpi and Fabpl follow this wave during their activation in the duodenum, jejunum, and ileum. Establishment of Ilbp's expression domain occurs during postnatal life and proceeds in the opposite direction. The patterns of activation of other ileal-specific genes (81) need to be examined to determine whether this distal-to-proximal wave of differentiation is a general feature, or whether the ileum and other segments of the gut are capable of establishing their positional addresses through a bidirectional flow of information along the cephalocaudal axis.

The source of the information that directs Ilbp's regional patterns of activation remains unclear. The isograft experiments reported in this work suggest that the E15 intestine has sufficient information to direct establishment and maintenance of Ilbp's expression domain without instruction from luminal contents. However, these experiments do not answer the question of whether axial patterning reflects a cell autonomous process encoded entirely within the E15 endoderm, or whether it is programmed through instructive/ permissive interactions between the mesoderm and endoderm with initial patterning occurring in the mesoderm (as is the case with the midgut of Drosophila larvae; see references 31, 33, 30, 55, 76). Insights about the contribution of the mesoderm to axial patterning could be obtained by implanting recombinant xenografts, composed of endoderm derived from one region of E14/E15 mouse intestine and mesoderm derived from the same or other portions of its cephalocaudal axis, into the subcutaneous tissues or kidney capsules of young adult syngenlc recipients (35). A gene such as Ilbp should be a useful marker of regional identity in these types of experiments.

Homogeneous, Striped, and Speckled Villi. The mechanisms responsible for converting the enterocytic patterns of Ilbp and ILBP/hGH +3 expression in ileal villi from wholly negative to speckled/striped to wholly positive are unknown. Nonetheless, this phenomenon should be viewed in light of developmental changes in the migration/replacement rates of enterocytes and current hypotheses about the nature of the crypt's stem cell hierarchy.

[PH]Thymidine-labeling studies indicate that proliferating cells located in the intervillus epithelium of E17 mouse ileum do not complete their migration up and subsequent extrusion from villi until P14 (3). Thus, the initial cycle of activation and extinction of Ilbp expression in E18–P11 enterocytes occurs during a period that coincides with their "birth" in late fetal life and completion of their "death" (exfoliation) during the 2nd postnatal week. Cellular proliferation and migration rates increase between the sucking and weaning periods,
reaching a rough equilibrium state by P28. This equilibrium is achieved at the completion of a period of rapid crypt multiplication, changing crypt-to-villus ratios, and villus lengthening (1, 9). We have not measured the rate of enterocyte replacement at specific positions along the cephalocaudal axis of the ileum during P14-P28. We have determined that as the distal-to-proximal wave of l1bp (re)activation from P11 to P28 passes through a particular point along IL4→IL1, the cellular pattern of l1bp expression in a villus is converted from wholly negative to speckled/striped to wholly positive during a period of ~d (Fig. 3). Although we do not know whether the rate of conversion of l1bp expression patterns is more rapid, equivalent, or slower than the transit time of enterocytes on P13-P28 ileal villi, it is clear that evolution of the pattern of transgene expression can span a period of 70-84 d (i.e., ILBP-913 to +487/hGH+3; Fig. 6 B). Such a slow rate of change takes place over many generations of enterocytes; the ileal villus transit time in adult mice is only 29-35 h (82). This raises a question about whether these slowly evolving changes reflect an alteration in the properties (programming) of an existing population of active crypt stem cells, or whether it signifies a replacement of these stem cells through recruitment of other potential stem cells.

There are two current views of crypt stem cell organization in adult mice (summarized in reference 41). One view, known as the stem cell pedegree concept, postulates that a single, slowly dividing "master" stem cell ultimately maintains the epithelial cell population of each crypt with the help of descendants termed transient or temporary stem cells, i.e., active stem cells are arranged in a pedegree with the most slowly cycling master stem cell at the apex of this pedegree (41, 46). Mutational assays suggest that some of these temporary stem cells may maintain themselves for 8-12 wk in the small intestine (78, 79). In the other view, the adult mouse intestinal crypt contains several functionally equivalent, self-maintaining stem cells which, in turn, arise from a single stem cell during gut morphogenesis (39-41, 53). Our studies with adult chimeric-transgenic mice indicate that a given monoclonal crypt is capable of giving rise to a population of enterocytes with a mixture of ILBP phenotypes (i.e., the speckled villi at the proximal boundary of the gene's expression domain). If these cells are derived from multiple active stem cells in a crypt, it would appear that l1bp and ILBP/hGH+3 transgenes are reporting subtle differences in the regulatory environments of their descendants (and perhaps the stem cells themselves) both during and after completion of crypt morphogenesis/purification. The other view, i.e., that the decision to express ILBP (or hGH) is not programmed at the level of the stem cell or its immediate descendants, but rather arises from permissive/instructive interactions between villus-associated enterocytes and the underlying mesenchyme during cellular translocation along the crypt-to-villus axis, would imply a remarkable heterogeneity in the character of this mesenchyme.

Although the cellular and molecular mechanisms responsible for maintaining speckled and striped cellular patterns of ILBP and hGH production in the mouse intestine are not known, the pattern appears to be related to the location of the boundaries of the genes' expression domains. This is also true for Fabp1 and L-FABP/hGH+3 transgenes (70). Adult male and female adult transgenic mice containing one of five different L-FABP/hGH+3 fusion genes, each with successively smaller segments of Rabpl's 5' nontranscribed domain, exhibit a striped pattern of reporter expression in small intestinal villi (58, 70). The frequency of striped villi at a given location along the duodenal-to-ileal axis increases as more of the 5' nontranscribed domain of rat Fabp1 is removed, e.g., ~10% of duodenal villi have hGH-negative stripes in adult L-FABP-996 to +217/hGH+3 mice compared to 50% in L-FABP-132 to +217/hGH+3 animals. Although the endogenous mouse Fabp1 gene shows no evidence of striping in the duodenal and jejunal villi of transgenic mice or their normal littermates, ileal villi located at the distal boundary of its expression domain contain vertical coherent stripes of enterocytes with distinct levels of immunoreactive L-FABP. The proximal-most position where hGH striping first appears seems to coincide with the distribution of hGH mRNA levels, i.e., when the "volume" of transgene expression is lowered below a threshold level in the distal intestine, striping inevitably appears (70). These observations indicate that the capacity to express a striped pattern of reporter expression is not limited to ileal villi. Moreover, this capacity persists throughout adulthood and may provide a marker of variations in the biological properties of stem cells or their immediate descendants that populate different "monoclonal" crypts supplying the same villus.

Temporal Suppressor Elements in l1bp. The temporal patterns of l1bp activation appear to be modulated by a series of cis-acting suppressors. Removal of these temporal suppressor elements allows ILBP-144 to +476/hGH+3 expression to be initiated at a stage of development that is similar to that of the endogenous gene. However, the distal-to-proximal evolution of the transgene's expression domain is still slower than that of l1bp. l1bp contains a predicted glucocorticoid response element in intron 1 (5'-CAAACACTCGTTCT-3'; see Fig. 1 B). An increase in circulating glucocorticoid levels occurs at the suckling/weaning transition and mediates a number of changes in the enterocyte's differentiation program (28, 43). This increase coincides with the time of (re)activation of l1bp and subsequent rapid expansion of its expression domain. Although the hGH gene contains a GRE in its first intron (48), it may not be functionally equivalent to the GRE in the first intron of l1bp, thereby accounting for the delay in axial expansion of the transgene's expression domain. Characterization of the functional role of the GRE in l1bp plus further mapping of the cis-acting temporal suppressors located between nucleotides -913 and -146 should provide insights about the molecular mechanisms that regulate gene transcription during and after completion of gut morphogenesis.

Fabp1's Developmentally Regulated Goblet Cell Suppressor. The goblet cell--specific suppressor located between nucleotides -913 and -418 is remarkable for at least two reasons: (a) its function is only revealed during the first 2 wk of postnatal life; and (b) its existence provides additional evidence in support of the notion that enterocytes and goblet cells are derived from a common transit cell (51). The identification of a developmental stage-specific goblet cell specific suppressor in Fabp1 provides an opportunity to study the differentiation of these lineages at different stages of gut morphogenesis.

We are grateful to David O'Donnell for his participation in the generation and maintenance of transgenic mice, Michelle Hermiston for providing...
chimeric-transgenic animals, Per Falk for his help with intestinal isografts and lectin-staining protocols, Rob Murphy for preparing some of the RNA samples used in this study, plus Kevin Roth, Bill Coleman, and Elvive Taylor for their suggestions and technical assistance with the immunocytochemical analyses. We thank Ann Stone for her assistance in preparing this manuscript.

This work was supported by a grant from the National Institutes of Health (DK37960).

Received for publication 4 May 1994 and in revised form 22 June 1994.

References


