Use of Transgenic Mice to Map cis-acting Elements in the Intestinal Fatty Acid Binding Protein Gene (Fabpi) That Control Its Cell Lineage-specific and Regional Patterns of Expression along the Duodenal–Colonic and Crypt–Villus Axes of the Gut Epithelium

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Abstract. The mouse intestinal epithelium is able to establish and maintain complex lineage-specific, spatial, and temporal patterns of gene expression despite its rapid and continuous renewal. A multipotent stem cell located near the base of each intestinal crypt gives rise to progeny which undergo amplification and allocation to either enterocytic, Paneth cell, goblet cell, or enteroendocrine cell lineages. Differentiation of these four lineages occurs during their geographically ordered migration along the crypt–villus axis. Gut stem cells appear to have a "positional address" which is manifested by differences in the differentiation programs of their lineal descendants along the duodenal–colonic (cephalocaudal) axis. We have used the intestinal fatty acid binding protein gene (Fabpi) as a model to identify cis-acting elements which regulate cell- and region-specific patterns of gene expression in the gut. Nucleotides -1178 to +28 of rat Fabpi direct a pattern of expression of a reporter (human growth hormone [hGH]) which mimics that of mouse Fabpi (a) steady-state levels of hGH mRNA are highest in the distal jejunum of adult transgenic mice and fall progressively toward both the duodenum and the mid-colon; and (b) hGH is confined to the enterocytic lineage and first appears as postmitotic, differentiating cells exit the crypt and migrate to the base of small intestinal villi or their colonic homologs, the surface epithelial cuffs. Nucleotides -1178 to -28 of rat Fabpi direct a pattern of expression of a reporter (human growth hormone [hGH]) which mimics that of mouse Fabpi (a) steady-state levels of hGH mRNA are highest in the distal jejunum of adult transgenic mice and fall progressively toward both the duodenum and the mid-colon; and (b) hGH is confined to the enterocytic lineage and first appears as postmitotic, differentiating cells exit the crypt and migrate to the base of small intestinal villi or their colonic homologs, the surface epithelial cuffs. Nucleotides -103 to +28, which are highly conserved in rat, mouse and human Fabpi, are able to correctly initiate transgene expression in late fetal life, restrict hGH to the enterocytic lineage, and establish an appropriate cephalocaudal gradient of reporter expression. This cephalocaudal gradient is also influenced by cis-acting elements located between nucleotides -1178 and -278, and -277 and -185 that enhance and suppress (respectively) expression in the ileum and colon and by element(s) located upstream of nucleotide -277 that are needed to sustain high levels of hGH production after weaning. Nucleotides -277 to -185 contain part of a domain conserved between the three orthologous Fabpi genes (nucleotides -240 to -159), a 24-bp element (nucleotides -212 to -188) that binds nuclear factors present in colonic but not small intestinal epithelial cells, and a portion of a CCAAT/enhancer binding protein footprint (C/EBPα, nucleotides -188 to -167). Removal of nucleotides -277 to -185 (yielding i_FABP-lS4 to +28/hGH+3) results in inappropriate expression of hGH in proliferating and nonproliferating epithelial cells located in the mid and upper portions of duodenal, jejunal, ileal, and colonic crypts without affecting the “shape” of the cephalocaudal gradient of transgene expression. Despite this precocious induction of hGH production, the hGH reporter remains restricted to the enterocytic lineage in both the small intestine and colon suggesting that these replicating crypt epithelial cells may already be allocated to this lineage at the point of induction of transgene expression. Alternatively, these replicating, hGH-positive cells may not be fully committed to the enterocytic lineage but nucleotides -184 to +28 contain cis-acting elements that bind factors which mediate lineage-specific expression. Together, these results indicate Fabpi will be a useful model system for describing the molecular mechanisms that maintain axial pattern formation in the perpetually renewed, lineal descendants of the multipotent crypt stem cell.
The adult mouse small intestine contains ~1.1 million flask-shaped crypts of Lieberkühn (Hagemann et al., 1970), each composed of an average of 530 cells (duodenum) to 360 cells (ileum) (Wright and Irwin, 1982). Cell division in the intestinal epithelium is confined to these crypts. Studies of normal, chimeric, and transgenic mice suggest that each crypt is monoclonal, containing a single active multipotent stem cell and its descendants (Cheng and LeBlond, 1974a; Ponder et al., 1985; Inoue et al., 1988; Winton et al., 1988, 1989; Potten and Loeffler, 1990; Winton and Ponder, 1990; Schmidt et al., 1985a, 1990; Cohn et al., 1991; Roth et al., 1991b). These descendants undergo amplification through several rounds of cell division (~ two thirds of the crypt's epithelial cell population pass through the cell cycle every 12 h) (Potten and Loeffler, 1987, 1990) and are allocated to four principal lineages. Differentiation occurs during a geographically ordered bipolar migration. Absorptive columnar enterocytes, mucus-producing goblet cells, and enteroendocrine cells differentiate as they migrate in vertical coherent bands from the crypt to the apex of a surrounding villus where they are exfoliated. Paneth cells differentiate during their descent to the base of the crypt (Cheng and Bjerknes, 1987; Schmidt et al., 1985b; Bjerknes and Cheng, 1981a,b). Proliferation, lineage allocation (commitment), differentiation/migration, and exfoliation are processes which occur perpetually and are completed rapidly in the small intestinal epithelium (e.g., it takes 2-3 d for an enterocyte to move from the upper crypt to the apical extrusion zone of a villus). A similar statement can be made about the colonic epithelium. A single active multipotent stem cell located at or near the base of each colonic crypt gives rise to each of the four epithelial cell lineages (Griffiths et al., 1988; Cohn et al., 1991; Roth et al., 1992b). The proliferative capacity of colonic crypts is comparable with that of small intestine crypts (Sunter et al., 1979). However, there are no villi in the colon. Cells migrate from colonic crypts to a hexagonal-shaped cuff of surface epithelial cells that surround the orifice of each crypt (gland) (Schmidt et al., 1985b).

Crypts form during the first two weeks of postnatal life. They arise from a polyclonal intervillus epithelium (i.e., one that has several active stem cells) through a complex process that involves: (a) progressive deepening (invagination); (b) a cell selection that results in "purification" of the nascent crypt from polyclonality to monoclonality; and (c) division (crypt fission) (Obuoforibo and Martin, 1977; Al-Nafussi and Wright, 1982; Cheng and Bjerknes, 1987; Schmidt et al., 1988). Studies of mouse aggregation chimerae indicate that each small intestinal villus in the adult mouse is supplied by several crypts, i.e., it is "polyclonal" (Schmidt et al., 1985b). The number of anatomically defined crypts that surround each villus varies as a function of location along the cephalocaudal (duodenal–ileal) axis: the crypt–villus ratio decreases from 14 in the duodenum to 6 in the ileum (Wright and Irwin, 1982). Moreover, a given crypt can supply cells to several adjacent villi. It is unclear whether one or more adjacent colonic crypts supply cells to a given surface epithelial cuff.

Since the intestinal epithelium undergoes perpetual and rapid renewal of its four terminally differentiated cell types along an anatomically well defined pathway (i.e., the crypt–villus or crypt–surface epithelial cuff axis), and since each crypt is composed of the progeny of a single active multipotent stem cell, the gut is an attractive system for: (a) inferring the biological properties of stem cells; (b) examining how stem cell hierarchies are established and maintained; (c) analyzing the relationships between passage through the cell cycle and lineage allocation (commitment); and (d) defining differentiation programs (Gordon et al., 1992). The mouse intestine is, in essence, a continuous developmental system. An intriguing additional feature is its capacity for morphoregulation (Edelman, 1992), i.e., to establish and maintain distinct regional differences in gene expression within specific cell lineages and in the relative proportions of the four lineages along the duodenal–colonic axis. Studies of jejunal, ileal, and colonic isografts, harvested just before conversion of the gut epithelium to a monolayer overlying nascent villi on embryonic days 15–16 and implanted into the subcutaneous tissues of young adult CB1/Bl nude mouse recipients, indicate that this regional differentiation of the gut epithelium can occur in the absence of luminal contents and suggest that a crypt stem cell has a "positional address" describing its location along the cephalocaudal axis (Rubin et al., 1991, 1992). Although axial pattern formation reflects position-dependent differences in the differentiation programs of crypt stem cell descendants, the molecular mechanisms that produce polarity in the gut epithelium remain unknown. The cellular basis is also unclear. For example, how much positional information is encoded in stem cells themselves? Do mesenchymal components, such as the pericystral fibroblasts that proliferate and migrate along the crypt–villus axis, help specify the crypt stem cell's positional address and/or influence the differentiation programs of its progeny (Marsh and Trier, 1974a,b; Maskens et al., 1979; Haffen et al., 1983, 1987; Kedinger et al., 1986)?

Transcription of the mouse intestinal fatty acid binding protein gene (Fabpi) located on chromosome 3 (Sweetser et al., 1987; Green et al., 1992) is confined to the gut epithelium: its mRNA and protein products first appear as differentiating enterocytes exit small intestinal and colonic crypts. Expression is sustained during subsequent migration to the apical extrusion zone of villi and to the surface epithelial cuff of colonic glands (Sweetser et al., 1988a). Distinct regional differences in the steady-state concentrations of these gene products also occur along the cephalocaudal axis: highest levels are present in the distal jejunum with progressive decreases occurring toward either the proximal duodenum or the mid-colon (Sweetser et al., 1988a). These cell-specific and region-specific patterns of Fabpi are evident at the time of its initial activation in late fetal life (E16—coinciding with the first appearance of the gut epithelial monolayer), and are maintained throughout adulthood (Sweetser et al., 1988a; Green et al., 1992). Analyses of intestinal isografts have shown that the differentiation-dependent induction of intestinal fatty acid binding protein (I-FABP) synthesis and the cephalocaudal variations in enterocyte I-FABP levels do not depend upon luminal signals (Rubin et al., 1991, 1992).

We have exploited these features of Fabpi to define cis-acting elements that regulate enterocyte-specific and region-specific patterns of gene expression in the mouse gut epithelium. Preliminary studies in transgenic mice indicated that

1. Abbreviations used in this paper: Fabpi, intestinal fatty acid binding protein gene; IGSS, immunogold/silver stained; hGH, human growth hormone.
Figure 1. Summary of conserved elements present in the 5' nontranscribed domains of human, rat, and mouse Fabpi. (A) Comparison of the orthologous Fabpi genes. Conserved domains I, II, and III are shown as stippled boxes and are described in Green et al., 1992. The positions of sequences homologous to a 14-bp element (consensus 5'-TGAACTTTGAACTT-3') found in Fabpi, other members of its gene family which are expressed in the gut, and in several nonhomologous genes that are also transcribed in the intestine (Sweetser et al., 1987) are indicated by thick, black, vertical lines. The dark hatching shows a B2 repetitive element (Krayev et al., 1982) present in rat, but not human or mouse, Fabpi. (B) Summary of sequence elements present in I-FABP/hGH transgenes.

nucleotides -1178 to +28 of rat Fabpi (Sweetser et al., 1987) are sufficient to produce a pattern of reporter expression in enterocytes distributed along the crypt-villus and duodenal-colonic axes which mimics that of the intact endogenous mouse and rat Fabpi genes (Sweetser et al., 1988a; Haufl et al., 1992). Removal of nucleotides -1178 to -278 restricted transgene expression to the proximal small intestine in the one pedigree that was available but did not affect expression along the crypt-villus axis, suggesting that distinct cis-acting regulatory elements may modulate gene expression along the two axes of the gut (Sweetser et al., 1988a). Recent comparison of the nucleotide sequences of mouse, rat and human Fabpi revealed three highly conserved domains in their 5' nontranscribed regions as well as multiple copies of a 14-bp element. This element (consensus 5'-TGAACATTGAACCTT-3') is a direct repeat of a 7-bp sequence and is present in comparable positions of the 5' nontranscribed domains of the orthologous Fabpi genes (see Fig. 1 A). It is also represented in the 5' nontranscribed domains of several other genes that are expressed in enterocytes including another member of the Fabpi gene family (Crhp2 which encodes cellular retinol binding protein II) (Sweetser et al., 1987; Demmer et al., 1987). We have used this information and transgenic mice to conduct a more detailed functional analysis of the 5' nontranscribed region of Fabpi to understand how its differentiation-dependent and region-specific patterns of expression are regulated in the enterocyte lineage.

Materials and Methods

Generation and Characterization of Transgenic Mice

Construction of I-FABP/hGH Fusion Genes. The I-FABP/human growth hormone (hGH) DNAs used to produce transgenic animals are illustrated in Fig. 1 B. I-FABP-1178 to +28/hGH+3, representing nucleotides -1178 to +28 of rat Fabpi (Sweetser et al., 1987) linked to the human growth hormone gene beginning at its nucleotide +3 (Seeburg, 1982), is described in Sweetser et al. (1988a). I-FABP-277 to +28/hGH+3 was obtained by digesting pIFhGH1 (Sweetser et al., 1988a) with EcoRI yielding a 2.4-kb EcoRI fragment. I-FABP-103 to +28/hGH+3 was produced as follows. A SalI-PvuII restriction fragment containing nucleotides -277 to +28 of rat Fabpi was sub-
cloned into pUC13 DNA that had been digested with SalI and Smal. The resulting recombinant plasmid DNA was digested with SphI and BamHI and a double-stranded oligonucleotide (5'-CAATTGACACGAGAAACGGTTC-GGGTCAAACAGTGGGACAGCGTCTGCTGCTGCGTTCTTACCCACCCCGCT- CATGGCTGCTCAGCTTCCATCCACCGTGCACGCTGTGGTTGCGTGCCTGTTGCCTT- CATGTTCTGGCTGATCATGATG-3') representing nucleotides −84 to +3 of the hGH gene (Seeberg, 1982) was inserted in place of the SphI-BamHI fragment that contained nucleotides −103 to +28 of rat Fabp1. Finally, a 3.7-kb BamHI-EcoRI fragment from pFpGH1 (i.e., a fragment which contains the hGH structure gene beginning at its nucleotide +3) was subcloned into the BamHI site of the recombinant plasmid, pFabpi. Partial digestion of this fragment with EcoRI was recovered as a 2.4-kb EcoRI fragment and used for pronuclear injections.

Production and Identification of Transgenic Mice. All restriction fragments used for pronuclear injections were purified by agarose gel electrophoresis followed by the GeneClean system (Biol01, Inc., La Jolla, CA). DNA fragments were injected into the pronuclei of oocytes obtained from mating C57BL/6J X C3H F1 mice. Oocytes were transferred on the day of injection to the infundibulum of the fallopian tubes of pseudopregnant Swiss Webster mice (Hogan et al., 1986). Live born mice were subsequently screened for the presence of the transgene by probing Southern blots of EcoRI digested tail DNA with a 32P-labeled (Feinberg and Vogelstein, 1984) 2.2-kb BamHI-EcoRI fragment of pFpGH1 containing the hGH gene beginning at its nucleotide +3. Founder mice were mated to C57BL/6J X C3H F1 animals to establish pedigrees that were obligate hemizygotes for the transgene. Littermates were subsequently maintained by littermate breeding.

Calculation of Copy Number. To determine transgene copy number, genomic DNA was prepared from the liver or spleen of F1 mice. 15 μg of DNA were digested with PvuII, an enzyme that cleaves the hGH gene at two sites, and a 1.6-kb internal fragment from each copy of the cloned hGH transgene. The products of EcoRI digestion were separated by agarose gel electrophoresis and transferred to nylon membranes (GeneScreen Plus, Dupont, New England Nuclear, Boston, MA). Southern blots were probed with the 32P-labeled 2.2-kb BamHI-EcoRI fragment of pFpGH1 described above, using hybridization and washing stringencies detailed in Sweetser et al. (1988b). The intensity of the hybridization signal generated by the 1-kb band was quantitated by scanning laser densitometry of the filter autoradiograph and compared to the intensities of signals produced by known amounts of cloned hGH DNA (added to 15 μg of liver or spleen DNA prepared from normal littersmates and included in the same Southern blots as internal controls). Only signals in the linear range of film sensitivity were used to calculate transgene copy number.

Caging. Suckling, weaning, and adult mice were maintained in microisolator cages under a strictly controlled light cycle (lights on at 0600 and off at 1800 h). Weaning and weaned mice were given a standard autoclaved chow diet ad libitum. For pregnant mice, gestational age was calculated from the day a vaginal plug was first noted (designated day 1).

Preparation of Tissue RNA. Transgenic mice and their normal littermates were sacrificed by cervical dislocation and their tissues rapidly removed and frozen in liquid nitrogen. Tissues used for single- and double-label immunocytochemical studies are described in previous publications (McKeel and Askin, 1978). Tissue samples were fixed in Bouin's solution (Sweetser et al., 1988b). Material obtained from the seven segments of intestine from a given animal were placed on a single slide together with samples of stomach, liver, pancreas, and kidney. All tissues were then embedded in paraffin and 5-10 μm sections were cut with a microtome. Sections were then deparaffinized and rehydrated using guanidinium isothiocyanate, phenol, and chloroform (Chomczynski and SACHER, 1992) and were probed with a 32P-labeled (Feinberg and Vogelstein, 1984) 2.2-kb BamHI-EcoRI fragment of pFpGH containing the hGH gene beginning at its nucleotide +3. Founder mice were mated to C57BL/6J X C3H F1 animals to establish pedigrees that were obligate hemizygotes for the transgene. Littermates were subsequently maintained by littermate breeding.

Measurement of NGH Levels in Serum by Radioimmunoassay. Serum was collected from transgenic mice at various ages. Serum from each animal was assayed in duplicate for hGH using a sensitive radioimmunoassay (Nicholas Institute, San Juan Capistrano, CA) whose limits of detection is 0.5 ng/ml of hGH. It does not recognize mouse growth hormone as assessed by surveys of sera obtained from nontransgenic littermates (data not shown).

Preparation of Nuclear Extracts from Intestinal Epithelial Cells

A modification of the procedure of Gorski et al. (1986) was used to isolate nuclear proteins from small intestinal and colonic epithelial cell populations. The intestine was removed en bloc (i.e., from the gastroduodenal junction to the rectum) from 2-3-month-old mice and then divided at the ileal-cecal border. A 22-gauge, blunt-end needle, attached to a 30-ml syringe, was inserted into the duodenum and the lumen of the small intestine perfused with an ice cold solution of TBS buffer (10 mM Tris, pH 7.6, 150 mM NaCl). The small bowel was then placed in ice cold homogenization buffer (10 mM Heps, pH 7.6, 25 mM KCl, 1 mM EDTA, 1 mM DTT, 2 M sucrose, 2 mM benzamidine, 0.5 mM phenylmethyisulfonyl fluoride, 0.5 mM aprotinin, 0.1 mM benzamidine, 0.1% glycerol [v/v]). The mucosa was
extruded by scraping the serosal side of the proximal two thirds of the small intestine with the blunt end of a spatula. The remaining fibrous tissue was discarded. After removal of luminal contents, the colon was minced in ice cold homogenization buffer rather than scraped. The scraped mucosa from each small intestine or the minced colonic tissue was brought to a total volume of 28 ml with homogenization buffer (maintained at 4°C). Typically, material obtained from the small intestine or colon of three animals was pooled and adjusted to a final volume of 28 ml. N-acetyl-l-cysteine (Sigma Chemical Co.) and Triton X-100 were then added to final concentrations of 50 mM and 0.5%, respectively. The mixture was homogenized with a Tissumizer (Teknmr Co., Cincinnati, OH) set at maximum speed for 30 sec. The resulting pellet was resuspended in 2 ml of ice cold nuclear extraction buffer (10 mM Hepes, pH 7.6, 0.1 mM EDTA, 3 mM MgCl₂, 100 mM KCl, 2 mM benzamidine, 0.2 mM phenylmethylsulfonylfluoride, 1 mM DTT, 10% glycerol) using a loose fitting pestle and Dounce homogenizer. NaOH was then added (final concentration = 10 M) to an aliquot of nuclei, and the absorbance of the resulting lysate determined at 260 nm. This information was used to adjust the volume of the remaining nuclear suspension to 10-20 A₂₆₀/ml using ice cold nuclear extraction buffer. One-tenth volume of 4 M (NH₄)₂SO₄, pH 8.0, was slowly added to the nuclear suspension and the mixture gently rocked for 30 min at 4°C. Chromatin was pelleted from the lysate by centrifugation at 100,000 g for 60 min at 4°C. The supernatant fraction was collected and 0.34 g of (NH₄)₂SO₄ added per milliliter. After an overnight incubation on ice, precipitated material was recovered by centrifugation at 100,000 g for 25 min. The pellet was resuspended in an ice cold buffer composed of 25 mM Hepes, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2 mM benzamidine, 0.2 mM phenylmethylsulfonylfluoride, and 10% glycerol and dialyzed against the same buffer at 4°C (three changes of 100 vol of dialysis buffer, 2-3 h per exchange). The concentration of protein in the nuclear extract was determined using the method of Bradford (1976). Aliquots were then frozen in liquid nitrogen. Nuclear extracts were also prepared from liver, kidney, and spleen using a protocol identical to that described above for colon.

**Results**

**Comparative Sequence Analyses of Orthologous Fabpi Genes and DNA Binding Assays Using Nuclear Extracts Prepared from Small Intestinal and Colonic Epithelial Cells Provide a Design Rationale for Conducting Functional Mapping Studies**

As noted in the introduction, earlier studies in transgenic mice indicated that nucleotides −1178 to +28 of rat Fabpi contain sufficient information to direct cellular and regional patterns of expression of at least two reporters, hGH (Sweetser et al., 1988a) and SV-40 T antigen (Hauft et al., 1988). Double-stranded oligonucleotide template spanning nucleotides −1178 to +28 of rat Fabpi was incubated with the indicated amount of protein from each of these nuclear extracts and the products analyzed by electrophoresis through 5 or 8% polyacrylamide gels (running buffer = 25 mM Tris, 190 mM glycine, 1 mM EDTA; final pH = 8.5). After electrophoresis, gels were dried and subjected to autoradiography at −80°C using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) and an intensifying screen.

**Mobility Shift Studies**

DNA binding assays were performed in 25-μl reactions. The mixture contained 50 fmol of template DNA labeled at one of its 5' ends with [³²P] using T4 polynucleotide kinase (New England Biolabs, Beverly, MA), 0.5-1.0 μg of poly dI-dC (Pharmacia Fine Chemicals, Piscatway, NJ), varying amounts of one of the double-stranded oligodeoxynucleotide competitors described in the legend to Fig. 3, 25 mM Tris, pH 8.0, 50 mM KCl, 25 mM Tris-HCl, and 5 mM 2-mercaptoethanol. The solution was incubated at room temperature for 30 min and the products analyzed by electrophoresis through 5 or 8% polyacrylamide gels (running buffer = 25 mM Tris, 190 mM glycine, 1 mM EDTA; final pH = 8.5). After electrophoresis, gels were dried and subjected to autoradiography at −80°C using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) and an intensifying screen.

**DNase I Footprint Analysis Using Recombinant C/EBPα and Fabpi**

_E. coli_ derived_C/EBPα was a kind gift of Steve McKeown (Carnegie Institution of Washington, Baltimore, MD) and was prepared as described in Landshulz et al. (1988). The template DNA used for these studies was prepared by digesting pFHGH with EcoRI. The resulting 2.4 kb EcoRI fragment was purified by agarose gel electrophoresis and labeled with [³²P] using T4 polynucleotide kinase. Digestion with BamHI yielded a 312-bp EcoRI-BamHI fragment containing nucleotides −277 to +28 of rat Fabpi. This DNA was purified by agarose gel electrophoresis. Alternatively, pFHGH was digested with BamHI, the 3.7-kb fragment labeled with [³²P] at its 5' ends, and then incubated with EcoRI yielding a 312-bp EcoRI-BamHI fragment that was labeled at nucleotide +28 of Fabpi rather than at nucleotide −277. <3000 cpm of either one of these [³²P]DNAs was added to a reaction mixture (45 μl) containing C/EBPα (final concentration = 30 ng/μl), 1 μg unlabeled poly dI-dC, 50 mM KCl, 25 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 0.5 mM EDTA, and 10% glycerol. After a 10-min incubation on ice and a 2-min incubation at 23°C, 5 μl of DNAase I (prepared in 25 mM CaCl₂) was introduced into the mixture at varying final concentrations which were determined empirically as a function of both the DNase I activity and the specific activity of the labeled DNA fragment. After a 1-min incubation at 23°C, DNAase I digestion was terminated by adding 110 μl stop buffer (1% SDS, 200 mM NaCl, 20 mM EDTA, and 200 μg/ml proteinase K), placing the mixture at 55°C for 30 min, and then extracting it with phenol and chloroform. The labeled DNA products were recovered by ethanol precipitation, dissolved in loading dye (0.1% bromophenol blue, 0.1% xylene cyanol, 80% formamide, 50 mM Tris-borate, pH 8.3, and 1 mM EDTA), and fractionated by electrophoresis through a 3% polyacrylamide gel containing 8 M urea.

**Figure 2.** Gel retardation studies using nuclear extracts prepared from the mouse proximal small intestinal epithelium or colon and nucleotides −241 to −188 of rat Fabpi. Nuclear extracts were prepared from duodenal and jejunal mucosal scrapings and from the colon as described in Materials and Methods. A [³²P]-labeled double-stranded oligonucleotide template spanning nucleotides −241 to −188 of rat Fabpi was incubated with the indicated amount of protein from each of these nuclear extracts and the products analyzed by electrophoresis through nondenaturing 8% polyacrylamide gels. An autoradiograph of the gel is shown. Note that extracts from these different regions of the cephalocaudal axis of the gut produce different patterns of retarded bands.
Figure 3. Localization of the binding site for colonic nuclear factor(s) to nucleotides -212 to -188 of rat Fabpi. (A) Nuclear extracts from colon (3 µg total protein) were incubated with 0.05 pmole of a 32P-labeled 54-bp fragment spanning nucleotides -241 to -188 plus the indicated amount of unlabeled oligonucleotide competitors. The reaction products were fractionated by nondeaturing PAGE. Note that all competitors which contain the nucleotide sequence 5'-TGCTGTAGTCGGAGACAGAGTAGGT-3' (nucleotides -212 to -188) compete for binding of colonic nuclear factors to bands a and b. Competition for binding to band c was only observed with the same oligonucleotide that was used as the labeled template (-241 to -188, data not shown) and, to a lesser extent, by the oligonucleotide which spans nucleotides -212 to -158. NE, no extract added; "-" no oligonucleotide competitor added. (B) Summary of the results presented in A, demonstrating the relationships of the various oligonucleotide competitors to the conserved sequence elements present in Fabpi and to a C/EBPα binding site identified by DNase I footprint analysis (see Fig. 4).

The sequences of the unlabeled double-stranded oligonucleotides used as competitors are as follows: -277 to -228, 5'-AATTCGACTCTCGATATTAAAGGTTGGAAGCCATCACCTTGACCCCTAAATTCTTGGA-3'; -248 to -220, 5'-CACTTGACCCTAATTCTTG-3'; -241 to -188, 5'-CCCTAATTCTTGGAATAAAAATGGCTCTACATGTGATTCGGGAGACAGAGTAGGT-3'; -219 to -188, 5'-GAATAAAAATGGCTCTACATGTGATTCGGGAGACAGAGTAGGT-3'; -212 to -188, 5'-GAATAAAAATGGCTCTACATGTGATTCGGGAGACAGAGTAGGT-3'; -219 to -188, 5'-GAATAAAAATGGCTCTACATGTGATTCGGGAGACAGAGTAGGT-3'; -229 to -188, 5'-GAATAAAAATGGCTCTACATGTGATTCGGGAGACAGAGTAGGT-3'; -241 to -188, 5'-GAATAAAAATGGCTCTACATGTGATTCGGGAGACAGAGTAGGT-3'; -248 to -220, 5'-CACTTGACCCTAATTCTTG-3'; -219 to -188, 5'-GAATAAAAATGGCTCTACATGTGATTCGGGAGACAGAGTAGGT-3'; -157 to -117, 5'-TCAGCAATAGATTCAAAAGGCACTAGAAGAGAAACTAA-3'.

1992), that mimic that of the intact endogenous Fabpi gene. The cellular and regional patterns of Fabpi expression are quite similar in humans, rats, and mice (Shields et al., 1986; Sweetser et al., 1988a; Green et al., 1992). Therefore, we reasoned that the three conserved sequence elements located in the 5' nontranscribed domains of the orthologous human, rat, and mouse Fabpi genes (labeled I-III in Fig. 1A) and sites in their 5' nontranscribed domains that specifically bind intestinal nuclear proteins are likely to contain cis-acting regulatory sequences important for establishing and maintaining cell-specific and/or region-specific patterns of expression.

Nuclear proteins were prepared from the duodenal/jejunal epithelium and from the colon of adult mice (see Materials and Methods). Gel mobility shift studies were then performed using these extracts, or control extracts from liver, kidney, and spleen, and DNA fragments derived from nucleotides -277 to +28 of rat Fabpi (i.e., those nucleotides sufficient to support reporter expression in villus-associated enterocytes located in the proximal half of the small intestine of transgenic mice). Nuclear extracts from the proximal small intestine and the colon produced distinct patterns of shifted bands when a 32P-labeled DNA fragment spanning nucleotides -241 to -188 was used as a template (Fig. 2). Markedly different patterns were noted with liver, kidney, and spleen extracts (data not shown). Moreover, incubation of proximal small intestinal or colonic nuclear extracts with another, double-stranded oligodeoxynucleotide templates derived from nucleotides -277 to +28 yielded a set of shifted bands that included subsets which migrated in a similar fashion to those shown in Fig. 2 and subsets that were distinct (data not shown). Analysis of these results revealed that two prominent shifted bands were only present when colonic extracts were incubated with nucleotides -241 to -188 (la-
beled a and b in Figs. 2 and 3 A). Addition of proteinase K to the reaction mixture containing this template DNA and colonic nuclear extracts resulted in a single band that had the same mobility as the free 32P-labeled 54-bp oligodeoxynucleotide, indicating that the shifted bands were likely due to the binding of proteins present in the colonic nuclear extract (data not shown).

The region(s) in the −241 to −188 fragment that reacted with colonic nuclear proteins to produce bands a and b was further defined in a series of competitive binding studies using unlabeled double stranded oligodeoxynucleotides (Fig. 3, A and B). All oligonucleotides that contained a 24-base sequence representing nucleotides −212 to −188 of rat Fabpi (5'-TGCTGTAGTCGGAGACAGAGTAGGT-3') were efficient competitors as judged by their ability to reduce the amount of labeled bands a and b but not band c (Fig. 3 A).

Other oligonucleotides that did not contain this 24-bp element (e.g., −248 to −220 or −157 to −117) were ineffective competitors even when present in >100-fold molar excess relative to the −241 to −188 template (Fig. 3, A and B). Finally, each of the oligonucleotides used in the competition experiments was labeled with 32P, incubated with colonic nuclear extracts, and the reaction products subjected to native gel electrophoresis. Their patterns of reactivity with colonic nuclear proteins (as judged by gel shifts) mirrored their ability to reduce the amount of labeled bands a and b (data not shown). Together, these data suggest that nucleotides −212 to −188 contain a binding site(s) for one or more proteins represented in colonic, but not proximal small intestinal, nuclear extracts.

Members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors (Cao et al., 1991; Williams et al., 1991; Ron and Habner, 1992) have been shown to affect the transcriptional activities of several genes during cellular differentiation (Umek et al., 1991), including which indicate that C/EBPα and related leucine zipper proteins were present in the recombinant C/EBPα used for these studies thereby precluding the formation of heterodimers with other C/EBP isoforms (Williams et al., 1991) or with other trans-acting factors that might, in turn, alter its binding site specificity (Lamb and McKnight, 1991). Unfortunately, we have been unable to obtain unambiguous footprints in this region of Fabpi using either colonic or small intestinal nuclear extracts. This failure may be due to a number of causes including, for example, (a) partial degradation of C/EBPα and other transcription factors by intracellular and/or luminal (pancreatic) proteases during preparation of our nuclear extracts and (b) C/EBPα’s extraordinarily low concentration in the gut epithelium.

Only the most sensitive immunocytochemical techniques such as the immunogold/silver intensification staining method combined with reflected light polarization microscopy are able to detect C/EBPα in jejunal villus-associated enterocytes. Moreover, enterocytic levels decrease markedly in the distal small intestine and the protein is undetectable in the colonic epithelium (C. Chandrasekaran, K. A. Roth, and J. I. Gordon, unpublished observations).

The results presented above provided us with a rationale for constructing several recombinant DNAs composed of elements from the 5' nontranscribed domain of rat Fabpi linked to the human growth hormone (hGH) gene beginning at its nucleotide +3 (hGH was selected because it had been used as a reporter in the earlier functional studies of Fabpi).

Figure 4. DNase I footprint analysis reveals a binding site for recombinant C/EBPα located between nucleotides −188 and −167 of rat Fabpi. E. coli-derived rat C/EBPα was incubated with a 32P-labeled, 312-bp fragment of rat Fabpi spanning nucleotides −277 to +28. DNase I was subsequently added to the reaction mixture and the digestion products were analyzed by electrophoresis through a 7% polyacrylamide gel containing 8 M urea. Parallel lanes contained the products of sequencing reactions (Maxam and Gilbert, 1977) performed on the same labeled DNA fragment. The sequence of Fabpi that was protected from DNase I digestion by C/EBPα is shown.

Nucleotides −178 to −278 Modulate Expression of Fabpi in the Distal Half of the Small Intestine and in the Colon

The highest steady-state level of I-FABP mRNA in 20-wk old nontransgenic mice is encountered in the distal jejunum and ileum (60–70 pg/μg total cellular RNA). Its concentration in duodenum and colon ranges from 10–15% of that achieved in the distal jejunum (Fig. 5 A).

Cohn et al. Axial Pattern Formation in the Gut Epithelium 33
Table I. Summary of Transgenic Pedigrees

| Transgene                   | Number of liveborn mice screened | Number of founders identified | Designation of pedigrees that expressed transgene | Transgene copy number/haploid genome |
|-----------------------------|----------------------------------|-----------------------------|--------------------------------------------------|-------------------------------------|
| I-FABP-1178 to +28/hGH+3   | 40                               | 2                           | 1*†                                               | 600                                 |
| I-FABP-277 to +28/hGH+3    | 200                              | 11                          | 54‡                                               | 60                                  |
| I-FABP-184 to +28/hGH+3    | 117                              | 20                          | 2‡                                                | 4                                   |
| I-FABP-277 to -104/hGH-84  | 111                              | 10                          | none                                              | ND                                  |
| I-FABP-183 to +28/hGH+3    | 190                              | 8                           | 36*                                               | 8                                   |

* Sterile founder.
‡ See Sweetser et al. (1988a).
§ Poor fertility in these transgenic animals limited the number of mice that were available for detailed analysis.

Figure 5. Comparison of the distribution of I-FABP and hGH mRNAs along the gastric–colonic axis of 21-wk-old I-FABP-1178 to +28/hGH+3 mice (a) and their normal littermates (c) representing two different pedigrees. Total cellular RNA was prepared from stomach (ST), duodenum (DU), proximal jejunum (PJ), distal jejunum (DJ), ileum (IL), cecum (CE), proximal colon (PC), and distal colon (DC). Steady-state levels of I-FABP and hGH mRNA were determined by quantitative RNA dot blot hybridizations. Two transgenic mice from I-FABP-1178 to +28/hGH+3 lines 1 (a) and 4 (A) (Sweetser et al., 1988a) were analyzed together with normal littermates (o, o). The bars represent the average of the data obtained from individual animals. The asterisks indicate that no hGH mRNA was detected (limits of sensitivity of the RNA blot hybridization assay = 0.2 pg hGH mRNA/µg total cellular RNA).

The Journal of Cell Biology, Volume 119, 1992 34
Figure 6. Distribution of hGH mRNA along the gastric–colonic axis of 2–21-wk-old I-FABP-277 to +28/hGH+3 transgenic mice from two different pedigrees. Total cellular RNA was prepared from intestinal segments harvested from members of I-FABP-277 to +28/hGH+3 lines 18 and 54 (see Table I). The concentration of hGH mRNA in these preparations was determined as described in Materials and Methods. N.D., not determined because the yield of RNA from the duodenum of these 2-wk-old transgenic mice was not sufficient to allow accurate quantitation of hGH mRNA levels. (*) Not detectable.

located between nucleotides −1178 to −278 support transgene expression in the distal intestine and colon and contribute to establishment of a proper cephalocaudal distribution of reporter mRNA.

Nucleotides −277 to −184 Contain cis-acting Elements That Suppress Transgene Expression in Distal Small Intestinal and Colonic Enterocytes and in Their Crypt Epithelial Cell Populations

Removal of nucleotides −277 to −185 (containing a portion of conserved domain II, the colonic nuclear factor binding site, and four nucleotides of the C/EBPα footprint) “restored” reporter expression in the distal small intestine and colon. Of the 20 founder mice containing I-FABP-184 to +28/hGH+3 that were identified, obligate hemizygous mice from six established lines that contained hGH-positive sera were characterized. Fig. 7 shows that the concentration of hGH mRNA in ileal and colonic segments harvested from 2–21-wk-old members of two representative I-FABP-184 to +28/hGH+3 pedigrees ranged from 20–80% of jejunal levels. Inappropriate expression of the transgene occurred in the stomach but in none of the other 8 tissues examined. (Immunocytochemical surveys of portions of the zymogenic, mucopartrial, and pure mucous zones of the stomach (defined according to Lee et al., 1982 and Roth et al., 1992a), failed to reveal hGH-positive cells. Our inability to find reporter-positive cells despite the presence of hGH mRNA in stomach RNA could either be due to a sampling error or the fact that the steady-state levels of hGH in gastric epithelial cell lineages (Karam and Leblond, 1992) were below the limits of detection by the IGSS technique.) The relative and absolute concentrations of I-FABP mRNA along the cephalocaudal axis were similar in I-FABP-277 to +28/hGH+3, I-FABP-184 to +28/hGH+3 transgenics and in their comparably aged normal littermates (data not shown).
Figure 8. Multilabel immunocytochemical studies of the distribution of I-FABP and hGH along the crypt-villus and crypt-surface epithelial cell cuff axes of adult I-FABP<sup>-1178 to +28</sup>/hGH<sup>+3</sup>, I-FABP<sup>-277 to +28</sup>/hGH<sup>+3</sup>, I-FABP<sup>-84 to +28</sup>/hGH<sup>+3</sup> transgenic mice and their normal littermates. (A–D) Fabpi is activated in enterocytes as they exit crypts and migrate to the base of small intestinal villi or their colonic homologues, the surface epithelial cuff. Sections from the small intestine (A–C) and proximal colon (D) from a nontransgenic mouse illustrate the normal cellular distribution of I-FABP. In A–C the section was incubated with goat anti-BrdUrd and rabbit anti-I-FABP sera. Antigen–antibody
Removal of nucleotides -277 to -185 also affects cellular patterns of transgene expression along the crypt-villus and crypt-surface epithelial cuff axis of the gut. Double-label immunocytochemical studies of normal and transgenic mice, treated with 5-bromo-2'-deoxyuridine (BrdUrd) 90 min before sacrifice to label cells in S phase, reveal that I-FABP is first detectable in nonproliferating enterocytes as they exit small intestinal and colonic crypts and migrate onto the base of villi or the surface epithelial cuffs (Green et al., 1992 and Fig. 8, A-D). (Comparative immunocytochemical and RNA blot hybridization studies of transgenic mice and their normal littermates treated with BrdUrd or vehicle alone indicated the nucleoside analog had no qualitative or quantitative effect on the cellular and regional patterns of Fabpi and I-FABP/hGH expression.) None of the proliferating, BrdUrd-positive cells located in the lower and middle thirds of these crypts contain I-FABP. Adult I-FABP-277 to +29/hGH-3 transgenics only express hGH in nonproliferating, villus-associated enterocytes located in the proximal two thirds of the small intestine (Fig. 8, G and H). In contrast to I-FABP-178 to +29/hGH-3 (Fig. 8, E and F) and I-FABP-277 to +29/hGH-3 transgenic animals, adult I-FABP-184 to +29/hGH-3 mice produce hGH in villus enterocytes and both proliferating and nonproliferating epithelial enterocytes located in the mid and upper thirds of the crypt (Fig. 8, I-L). The hGH reporter is also present in enterocytes located in the upper portion of colonic crypts and in the surface epithelial cuff (Fig. 8, M-P). Despite its inappropriate production in proliferating crypt epithelial cells, hGH was not detectable in differentiating or differentiated members of the goblet, Paneth, or enteroendocrine cell lineages.

Nucleotides -277 to -104 of Fabpi were linked to a neutral promoter/reporter (the hGH gene beginning at its nucleotide -84 [see above]) to test the possibility that this 174-bp fragment which contains conserved domain II, plus the colonic factor and C/EBP binding sites but no copies of the 14-bp element (Fig. 1 B), can modulate expression along the crypt-villus and/or duodenal-colonic axes. Ten I-FABP-277 to -104/hGH-44 pedigrees were established (Table I). No hGH expression was detected in intestinal or extraintestinal tissues harvested from one-wk-old and/or adult animals that were members of each of these lines. Thus, it appears likely that sequences contained within nucleotides -277 to -104 of Fabpi are not sufficient to direct intestinal expression of Fabpi. However, we could not exclude the possibility that these 174 bp could function as a tissue-specific element if it were linked to a different heterologous promoter/reporter.

In contrast, cis-acting elements contained within 103 bp of the start site of transcription of Fabpi appear to be sufficient to establish an appropriate cephalocaudal gradient of hGH expression and to confine this reporter to the enteroendocrine lineage. This conclusion is based on the following observations. We identified seven founder transgenic mice containing nucleotides -103 to +28 linked to nucleotide +3 of hGH. This sequence contains conserved domain III, an associated 14-bp element and the TATA and CCAAT boxes of Fabpi (Fig. 1 B). Three of these founders expressed hGH and
were used to establish pedigrees. F1 and/or F2 mice from two of these lines exhibited gastrointestinal-specific expression of the reporter (e.g., see Fig. 9 A) while another (derived from G46) demonstrated expression in stomach, small and large intestine, kidney, lung, brain, spleen and pancreas (data not shown). However, all three pedigrees demonstrated a duodenal–colonic distribution of hGH mRNA (Fig. 9 B) which mimicked that of I-FABP mRNA in the same preparation of total cellular RNA (data not shown). Peak levels of reporter mRNA were encountered in the distal jejunum of 2-wk-old suckling mice and ranged from 2–10 pg/μg total cellular RNA. Immunocytochemical studies demonstrated that removal of nucleotides −184 to −104 had no effect on the restriction of hGH expression to the enterocytic lineage (see Fig. 11 K–N). Moreover, the presence of 2–8 copies of the I-FABP−103 to +28/hGH+3 transgene had no qualitative or quantitative effect on the regional or cellular patterns of mouse Fabpi expression (see Fig. 11, L and N).

Developmental Patterns of Fabpi/hGH Expression

As noted in the introduction, Fabpi not only represents a sensitive marker of enterocytic differentiation along the crypt–villus and crypt–surface epithelial cuff axis, it is also a molecular marker of the process of cytodifferentiation that the gut undergoes during late fetal life. This process is initiated in the proximal small intestine during E15–E16 and proceeds in a proximal–distal “wave” that travels along the cephalic axis during late fetal life. This process is initiated in the proximal small intestine during E15–E16 and proceeds in a proximal–distal “wave” that travels along the cephalo-caudal axis that overlie nascent villi. Transcriptional activation of Fabpi occurs in villus-associated enterocytes located in the proximal small intestine at the same time that morphologic transformation of its endoderm is initiated (i.e., E15–16) (Green et al., 1992). Initiation of Fabpi expression follows the wave of cytodifferentiation that proceeds rapidly along the duodenal–colonic axis (Rubin et al., 1989, 1992; Green et al., 1992).

We measured serum hGH levels and performed multilabel immunocytochemical surveys of late gestation and suckling I-FABP−277 to +28/hGH+3, I-FABP−184 to +28/hGH+3, and I-FABP−103 to +28/hGH+3 transgenic mice to determine whether their transgenes exhibited appropriate developmental stage-specific patterns of activation and whether the cellular patterns of reporter accumulation along the evolving duodenal–colonic and crypt–villus axis mimicked those of Fabpi. hGH first appears in the serum of I-FABP−277 to +28/hGH+3 and I-FABP−184 to +28/hGH+3 mice at the time that Fabpi is activated in the proximal small intestine (Fig. 10, A and B). hGH is also present in the serum of I-FABP−103 to +28/hGH+3 mice on the day of birth, which was the earliest time point we surveyed (Fig. 10 C). Serum hGH concentration peaks during the suckling period (postnatal days 1–14) and then declines during weaning (postnatal days 15–28) in mice containing each of these three I-FABP/hGH transgenes (Fig. 10 A–C). The postnatal decreases in serum hGH levels documented during the suckling/weaning period are accompanied by decreases in the steady-state levels of hGH mRNA along the cephalocaudal axis of the gut (compare the upper two panels in Figs. 6 and 7 with Fig. 10, A and B). I-FABP mRNA concentrations do not fall during the suckling/weaning period (e.g., see Green et al., 1992). However, a number of gene products show marked changes in their levels at the suckling/weaning transition in both the rat and mouse. Many of these alterations have been ascribed to increases in glucocorticoid levels which occur at this stage of development (reviewed in Henning et al., 1987). Therefore, we wondered whether the glucocorticoid responsive element present in intron I of hGH (Moore et al., 1985) mediated the observed declines in I-FABP/hGH expression. However, administration of hydrocortisone acetate (25 μg/g body weight per day) to G18-derived I-FABP−277 to +28/hGH+3 mice, from postnatal days 7–10, produced only minimal (<50%) reductions in serum hGH levels compared with transgenic littersmates that had been treated with vehicle alone.

Surveys of 1–14-d-old I-FABP−277 to +28/hGH+3, I-FABP−184 to +28/hGH, and I-FABP−103 to +28/hGH+3 mice indicated that the presence of the transgene had no effect on the cellular patterns of I-FABP accumulation along the na-
Discussion

Fig. 12 describes the functional elements that have been identified in the 5' nontranscribed domain of Fabpi from the studies described above. Analysis of I-FABP:hGH transgenic mice have provided insights about: (a) the mechanisms which permit cephalocaudal differences in gene expression to be sustained in a continuously renewing gut epithelial cell lineage; (b) how gene transcription is regulated in enterocytes as they differentiate during their migration along the crypt-villus axis; (c) the relationship between proliferation and lineage allocation; and (d) the "temporal" gradient of gene expression that affects the gut epithelium.

Our results suggest that the spatial patterns of Fabpi expression observed along the duodenal–colonic and crypt-villus (or crypt–surface epithelial cuff) axes reflect the contributions of positive and negative cis-acting transcriptional regulatory elements which lie within 1 kb of its start site of transcription. Remarkably, nucleotides –103 to +28 of rat Fabpi are sufficient to direct lineage-appropriate expression and to establish a duodenal–colonic distribution of reporter mRNA accumulation in enterocytes which mimics that of mouse I-FABP mRNA. These 132 bp include (a) a sequence which extends from nucleotide –94 to –1 that is conserved between three orthologous Fabpi genes and (b) a 14-bp element which is represented in at least one other family member (Crbp2) whose enterocyte-specific and geographic patterns of expression resemble those of Fabpi. However, the functional significance of these elements remains to be defined. A detailed search of a relational database of known transcriptional regulatory elements (Ghosh, 1990) failed to reveal any members, other than TATA and CCAAT box elements, represented within nucleotides –103 to +28. The gradient of I-FABP mRNA levels observed along the cephalocaudal axis appears to be modulated by two additional cis-acting spatial regulatory elements: a domain or domains located between nucleotides –1178 and –277 which promotes expression in the ileum and colon and a domain or domains located between nucleotides –277 and –184, which operates to suppress ileal and colonic expression (Fig. 12). This latter portion of the 5' nontranscribed domain of Fabpi includes a 24-bp element, spanning nucleotides –212 to –188, that binds nuclear factors present in colonic but not proximal small intestinal epithelial cells. The suppressor and colonic factor binding domains have no obvious sites recognized by known transcription factors and are contained within a portion of Fabpi (nucleotides –277 to –104) that is not sufficient to support expression of the hGH reporter in any lineage in any portion of the gut epithelium. (The use of various combinations of a limited number of positive and negative elements could permit diverse regional patterns of expression of genes along the cephalocaudal axis of the gut. For example, it will be interesting to determine which of these elements in Fabpi, if any, are represented in a homologous gene encoding ileal lipid binding protein [ILBP]. Ilbp expression is limited to ileal, villus–associated enterocytes in weaning and adult mice [Sacchettini et al., 1990; M. Crossman, S. Hauft, K. Roth, and J. Gordon, unpublished observations].)

Figure 10. Serum hGH levels in E16-E20, suckling, and weaning I-FABP/hGH transgenic mice. 1-15 mice from the indicated pedigrees were examined at each of the ages shown. hGH concentrations were determined in each serum sample in duplicate and the results were averaged. Data are expressed as the average serum hGH concentration among comparably aged mice from a given line ± SEM. The limits of detection of the assay are 0.5 ng/ml serum. (A) Data derived from members of I-FABP-277 to +28/hGH+3 lines 18 and 54; (B) values documented in members of I-FABP-103 to +28/hGH+3 pedigrees 12 and 55; (C) serum hGH levels in members of I-FABP-103 to +28/hGH+3 lines 40. Further analysis of I-FABP-103 to +28/hGH+3 and I-FABP-188 to +28/hGH+3 transgenic mice from the same pedigrees indicated that hGH levels remained constant from the 35–250 days of life (data not shown).
Figure 11. Comparison of cellular distribution of I-FABP and hGH along the nascent crypt-villus axis of proximal small intestinal segments prepared from suckling I-FABP-277 to +28/hGH+3, I-FABP-184 to +28/hGH+3, I-FABP-103 to +28/hGH+3 transgenic mice and their normal litter-mates. (A-G) transgene expression in developing I-FABP-277 to +28/hGH+3 mice is limited to villus-associated enterocytes. A section of small intestine from a 1-d-old I-FABP-277 to +28/hGH+3 transgenic mouse was incubated with rabbit anti-I-FABP and goat anti-hGH sera and visualized with Texas red and fluorescein-conjugated secondary antibodies, respectively. Diffuse cytoplasmic I-FABP immunoreactivity (A) and intense Golgi staining for hGH (B) are seen in villus-associated enterocytes. The colocalization of I-FABP and hGH immunoreactivity and the lack of intervillus expression of either protein is seen in the double exposed photomicrograph in C (the intervillus region is indicated by arrows). In a 14-d-old I-FABP-277 to +28/hGH+3 transgenic mouse, the crypts are well formed and purification from polyclonality to monoclonality has been completed. The hGH reporter remains restricted to villus-associated enterocytes (D-G). BrdUrd-labeled cells, detected with fluoresceinated secondary antibodies, clearly define the proliferative zone in the crypt (D). I-FABP immunoreactivity, detected with Texas red-labeled secondary antibodies in E, is restricted to villus-associated enterocytes. hGH immunoreactivity, detected
As noted in the introduction, the differentiation programs of the four principal gut epithelial cell lineages are expressed as these descendants of a single, active multipotent crypt stem cell undergo a spatially constrained amplification and bipolar migration along the crypt-villus axis. Nucleotides $-277$ to $+28$ of rat Fabpi are sufficient to recapitulate the normal pattern of activation of mouse Fabpi in differentiating enterocytes as they exit the crypt and begin their upward journey in vertical coherent bands to the apical extrusion crypt epithelial cells. Bar, 25 µm.

The staining was generally too weak to make conclusions about whether the reporter is also present in proliferating and/or nonproliferating expression of I-FABP to $+28$ in a 2-wk-old mouse. A section of small intestine was incubated with antibodies against BrdUrd, I-FABP, and hGH. The fluorescein-labeled BrdUrd-immunoreactive cells in K define the crypt cell zone, while Texas red-labeled I-FABP is apparent in M (indicated by immunoreactive cells in L define the villus epithelium. Relatively weak, focal hGH immunoreactivity, visualized by the IGSS method, shows labeling of cells in the intervillus epithelium (indicated by I-FABP and hGH are not detectable in crypt epithelial cells. (H, I-FABP-immunoreactive cells in K define the crypt cell zone, while Texas red–labeled I-FABP immunoreactive cells in L define the villus epithelium. Relatively weak, focal hGH immunoreactivity, visualized by the IGSS method, is apparent in M (indicated by arrows). Multilayering of the section in N, indicates that hGH is limited to villus-associated enterocytes. The staining was generally too weak to make conclusions about whether the reporter is also present in proliferating and/or nonproliferating crypt epithelial cells. Bar, 25 µm.

| Structure: | Function: |
|---|---|
| -1178 | -277 | -185 | -103 | +28 |
| I | II | III |
| | Promotion of ileal and colonic expression | Ileal and Colonic suppression | Ileal and Colonic suppression |
| | Sustained expression through adulthood | Suppression of expression in proliferating crypt epithelial cells | Sustained expression through adulthood |
| | Proper differentiation of ileal and colonic segments in the 5' nontranscribed domain of rat Fabpi that regulate its patterns of expression along the duodenal–colonic and crypt–villus (or crypt–surface epithelial cuff) axes. See text for discussion. |
| Figure 12. Summary of elements in the 5' nontranscribed domain of rat Fabpi that regulate its patterns of expression along the duodenal–colonic and crypt–villus (or crypt–surface epithelial cuff) axes. See text for discussion. |
pressor factors produced by cells after they are subsequently allocated to enteroendocrine, goblet and Paneth cell lineages. Alternatively, suppression of hGH synthesis after allocation to these lineages could reflect loss of a positive factor, produced in uncommitted cells, that binds to elements located between nucleotides −184 to +28 (this loss would presumably occur during differentiation of enteroendocrine, goblet and Paneth cells but not enterocytes). The apparent restriction of I-FABP-184 to +28/hGH expression to the enteroendocrine lineage is interesting in light of our previous studies of transgenic mice that contain nucleotides −4000 to +21 or −596 to +21 of the homologous liver fatty acid binding protein gene (Fabpl) linked to a variety of reporters including hGH (Sweetser et al., 1988b; Hansbrough et al., 1991). The intact, endogenous mouse Fabpl gene is normally restricted to the enterocytic lineage, although a very small subpopulation of enterocytes in proximal small intestinal villi contains detectable levels of this cytoplasmic long chain fatty acid binding protein (Sweetser et al., 1988b; Roth et al., 1990). Like Fabpl, Fabpi is activated as enterocytes exit small intestinal crypts. L-FABP-4000 to +21 and L-FABP-596 to +21 direct inappropriate expression of these reporters in proliferating and nonproliferating small intestinal and colonic crypt epithelial cells (Sweetser et al., 1988b; Trahair et al., 1990; Roth and Gordon, 1990; Cohn et al., 1991; Hansbrough et al., 1991; Rubin et al., 1991, 1992; Roth et al., 1991b). The cellular distribution of hGH in the crypts of L-FABP-4000 to +28/hGH +3 and L-FABP-596 to +21/hGH +3 animals is somewhat different than the cellular distribution observed in comparably aged I-FABP-184 to +28/hGH +3 transgenic mice: the L-FABP/hGH transgenes are first activated in cells that are closer to the base of crypts where the multipotent stem cell is presumed to reside (Potten and Loeffler, 1990) and the steady-state level of their hGH product is maintained at higher levels in both proliferating and nonproliferating cells during their translocation through the crypts (Sweetser et al., 1988b and data not shown). Precocious expression of hGH in the crypts of multiple pedigrees of L-FABP-4000 to +28/hGH +3 and L-FABP-596 to +28/hGH +3 mice is associated with “appropriate” production of the reporter in enterocytes and inappropriate production in fully differentiated members of the enteroendocrine, the goblet cell, and the Paneth cell lineages (Roth et al., 1990; 1991a,b; 1992b; Roth and Gordon, 1990; Trahair et al., 1990; Cohn et al., 1991). One possible interpretation of these results is that nucleotides −4000 to +28 do not contain lineage-specific suppressor elements comparable with those postulated to be located between nucleotides −184 to +28 of Fabpl. Alternatively, these two L-FABP/hGH transgenes may be activated in crypt epithelial cells that are uncommitted, or less committed, than those cells which initially support I-FABP-184 to +28/hGH +3 activation (i.e., L-FABP/hGH may be initially expressed in the multipotent stem cell or one of its immediate descendants). Whatever the mechanisms, transgenes appear to be potentially useful probes for operationally defining where and how lineage allocation occurs in the crypt. For example, crossing I-FABP-184 to +28/hGH +3 transgenic mice with mice that contain transgenes composed of L-FABP-596 to +21 linked to a different reporter would allow assessment of which crypt epithelial cell populations support expression of one or both transgenes and their relative locations along the basal-apical axis. Moreover, the recent development of methods for preparing primary cultures of suckling crypt and villus epithelial cell populations (Evans et al., 1992), may allow mice containing both I-FABP-184 to +28/reporter and L-FABP-596 to +21/reporter transgenes to be used for isolation of gut stem cells, their early descendants, and/or members of specific lineages if the reporters encode gene products that allow for positive and negative selection ex vivo.

A fourth conclusion that can be made from the experiments described here is that nucleotides −103 to +28 of rat Fabpi contain cis-acting elements which are sufficient to produce appropriate developmental stage-specific activation of hGH expression along the cephalocaudal axis of the gut but lack elements necessary to sustain similar levels of expression beyond the suckling/weaning period. Addition of nucleotides −184 to −104 or −277 to −104 did not modify the proper temporal pattern of activation of reporter production during E16-20 nor did they "prevent" a several fold reduction in steady state hGH mRNA levels between postnatal weeks 2 and 21. Analyses of I-FABP-1778 to +28/hGH +3 or I-FABP-1778 to +28/SV-40 T antigen transgenic mice suggest that nucleotides −1178 to +28 of rat Fabpi contain cis-acting elements that are sufficient to sustain reporter mRNA levels through at least the first nine months of life (Sweetser et al., 1988a; Hauft et al., 1992 and data not shown). It is tempting to speculate that the conserved domain I, which spans nucleotides −594 to −420, and/or its associated 14-bp repeat (Fig. 12) may function as a positive transcriptional element which is involved in sustaining Fabpi expression during postnatal life. It appears unlikely that the postnatal fall in hGH mRNA and protein levels (the latter evident by immunocytochemical surveys of sections of intestine and by measurement of serum hGH concentrations) is mediated by glucocorticoids, thyroxine, or by luminal contents. Although marked increases in glucocorticoids and thyroxine levels occur at the suckling/weaning transition and mediate a number of changes in gene expression within the enteroendocrine lineage (Malo and Menard, 1983; Henning, 1987), analysis of normal mice and rats or mice with congenital hypothyroidism, produced by a recessive mutation known as hvt, indicates that neither precocious administration of glucocorticoids, adrenalectomy, nor thyroid hormone deficiency affects Fabpi expression (Green et al., 1989; N. Nathankumar and S. J. Henning, personal communication). Moreover, studies of intestinal isografts harvested from E16 mice and implanted into the subcutaneous tissues of adult CB6F1 recipients reveal that Fabpi expression is sustained for up to 2 mos in villus-associated enterocytes distributed along the duodenal-colonic axis (Rubin et al., 1992). One obvious question is what role the reporter plays in the postnatal decreases in I-FABP-4000 to +28/hGH +3, I-FABP-184 to +28/hGH +3 and I-FABP-103 to +28/hGH +3 expression. hGH production is extinguished in aging transgenic mice containing L-FABP/hGH fusion genes (Cohn et al., 1991). However, the temporal and spatial pattern of silencing of L-FABP-4000 to +21/hGH +3 or L-FABP-596 to +21/hGH +3 differs from that of I-FABP/hGH: it is restricted to the colon and occurs in a “wave” that progresses from the distal to proximal portions of the large bowel over a 6-12 mo period. The decision to extinguish L-FABP/hGH expression appears to be made at the level of the crypt stem cell or one of its immediate descendants, and is coordinated between adjacent colonic
crypts, producing patches of wholly reporter negative colonic glands (Cohn et al., 1991). In contrast, the reduction in I-FABP/hGH expression is more rapid, affects the entire cephalocaudal axis, and produces a diminution in the steady state levels of hGH in all villus-associated enterocytes. It does not involve the progressive appearance of coherent, wholly positive bands of hGH-negative enterocytes extending from a crypt to the apex of a villus, as would be expected if the decision to suppress transgene expression were made at the level of a single crypt stem cell that supplies a polycyonic villus (see introduction, and Roth et al., 1991b).

Although the mechanism of postnatal suppression of I-FABP/hGH expression is currently unknown, these observations emphasize that mice containing Fairpi/reporter transgenes represent powerful model systems for further defining how gene expression is regulated in specific gut epithelial cell lineages as a function of their "positional address" along the duodenal–colonic and crypt–villus axes and as a function of their "temporal address" in this rapidly yet continuously renewing system.

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References

Al-Nafussi, A. I., and N. A. Wright. 1982. Cell kinetics in the mouse small intestine during immediate postnatal life. Virch. Arch. B Cell Pathol. 40:51–62.

Birknerreiter, E. H., B. Gwynn, S. Howard, J. Jerry, J. I. Gordon, W. H. Landschultz, and S. L. McKnight. 1989. Tissue-specific expression, development regulation and genetic mapping of the gene encoding CAAAT/enhancer binding protein. Genes & Dev. 3:1146–1156.

Bjerknes, M., and H. Cheng. 1981a. The stem cell zone of the small intestinal epithelium. I. Evidence from Paneth cells in the adult mouse. Am. J. Anat. 160:51–63.

Bjerknes, M., and H. Cheng. 1981b. The stem cell zone of the small intestinal epithelium. II. Evidence from columnar, enteroendocrine, and mucus cells in the adult mouse. Am. J. Anat. 160:77–91.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

Bromma, W., and H. Dörrer. 1972. In vitro verfahren zur bestimmung der DNS-synthese-Dauer einzelner Zellen. Biochemische voraussetzungen un-...
