

# Epithelial Cell Differentiation in Normal and Transgenic Mouse Intestinal Isografts

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**Abstract.** Transgenes consisting of segments of the rat liver fatty acid-binding protein (L-FABP) gene's 5' non-transcribed domain linked to the human growth hormone (hGH) gene (minus its regulatory elements) have provided useful tools for analyzing the mechanisms that regulate cellular and spatial differentiation of the continuously renewing gut epithelium. We have removed the jejunum from normal and transgenic fetal mice before or coincident with, cytodifferentiation of its epithelium. These segments were implanted into the subcutaneous tissues of young adult CBY/B6 nude mouse hosts to determine whether the bipolar, migration-dependent differentiation pathways of gut epithelial cells can be established and maintained in the absence of its normal luminal environment. Immunocytochemical analysis of isografts harvested 4–6 wk after implantation revealed that activation of the intact endogenous mouse L-FABP gene (*fabpl*) in differentiating enterocytes is perfectly recapitulated as these cells are translocated along the crypt-to-villus axis. Similarly, Paneth and goblet cells appear to appropriately differentiate as they migrate to the crypt base and villus tip, respectively. The enteroendocrine cell subpopulations present in intact 4–6-wk-old jejunum are represented in these isografts. Their precise spatial distribution along the crypt-to-villus axis mimics that seen in the intact gut. A number of complex interrelationships between enteroendocrine subpopulations are also recapitulated. In both "intact" and isografted jejunum, nucleotides –596 to +21 of the rat L-FABP gene were sufficient to direct efficient expression of the hGH

reporter to enterocytes although precocious expression of the transgene occurred in cells located in the upper crypt, before their translocation to the villus base. Inappropriate expression of hGH occurred in a high percentage (>80%) of secretin, gastrin, cholecystokinin, and gastric inhibitory peptide producing enteroendocrine cells present in the intact jejunum of 4–6-wk-old L-FABP<sup>–596 to +21</sup>/hGH transgenics. Addition of nucleotides –597 to –4,000 reduced the percentage of cells co-expressing this reporter four- to eightfold in several of the subpopulations. Jejunal isografts from each transgenic pedigree studied contained a lower percentage of hGH positive enteroendocrine cells than in the comparably aged intact jejunum. Together our analyses suggest that (a) the complex program of enteroendocrine cell differentiation can be expressed in the absence of extracellular luminal contents; (b) L-FABP/hGH transgenes appear to be useful reporters of a subtle heterogeneity in the regulatory environments of enteroendocrine cell subpopulations and a sensitive marker of the fact that luminal contents can regulate gene expression within and between subpopulations; (c) in contrast to enteroendocrine cells, transgene expression in enterocytes does not appear to be modulated by luminal factors; and (d) transgenic intestinal isografts represent a general and powerful tool not only for assessing the influence of extracellular factors on *cis*-acting elements contained in specific genes during epithelial differentiation but also for mapping these elements.

THE mouse small intestinal epithelium can be distinguished from many other epithelia by its ability to establish and maintain complex spatial patterns of cellular differentiation in the face of remarkably rapid and continuous renewal. Each adult intestinal crypt contains a monoclonal population of cells (Ponder et al., 1985; Schmidt et al., 1988) which are probably derived from a single renewing multipotent stem cell (Griffiths et al., 1988; Winton et al., 1988, 1989; Winton and Ponder, 1990; Schmidt et al.,

1990; Cohn et al., 1990). The progeny from this stem cell (which appears to be functionally anchored near the base of the crypt) undergo four to six divisions (Potten and Loeffler, 1987; 1990) within a well-demarcated proliferative zone. The amplified clonal descendants undergo terminal differentiation to four principal cell types during a bipolar migration: enterocytes and goblet cells arise as they are rapidly translocated in vertical coherent bands to the apical extrusion zone (Schmidt et al., 1985). Paneth cells differentiate

as they descend to the crypt base, while enteroendocrine cells arise both during ascent and descent from the proliferative zone. The containment of this perpetual process to well-demarcated anatomic units provides a unique opportunity to not only infer the biological properties of stem cells (Cohn et al., 1990) but also to analyze the processes of proliferation/commitment/differentiation in space. Spatial differentiation of the small intestinal epithelium occurs not only along its crypt-to-villus axis but also from duodenum to ileum (reviewed in Gordon, 1989). An additional temporal dimension must also be superimposed on this process since fundamental changes in gene expression occur in the terminally differentiated cells of this renewing epithelium throughout development (Roth et al., 1991; Cohn et al., 1990).

We have previously used transgenic mice containing rat liver fatty acid-binding protein (L-FABP)<sup>1</sup>/human growth hormone (hGH) fusion genes as models for analyzing how developmental stage specific, region- and cell-specific patterns of gene expression are established and maintained in the gut epithelium (Sweetser et al., 1988; Hauff et al., 1989; Roth et al., 1990a,b, 1991). The mouse liver fatty acid binding protein gene (*fabpl*) located on chromosome 6 (Sweetser et al., 1987) is efficiently transcribed in two tissues: in the liver where its expression is limited to hepatocytes, and in the gut where its mRNA and protein products are confined to villus-associated enterocytes and a small subpopulation of enteroendocrine cells (Sweetser et al., 1988; Roth et al., 1990a). The *fabpl* gene is not expressed in crypt epithelial cells. Not only are there differences in *fabpl* expression along the crypt-to-villus axis, but regional differences in steady state mRNA and protein levels are maintained along the duodenal-to-ileal axis (highest in the proximal jejunum with a progressive decrease occurring towards the ileum and no detectable gene products in the colon.) It is notable that these geographic differences in gene expression are established at the very time of the gene's first activation in late fetal life (days 17 and 18; Rubin et al., 1989; Hauff et al., 1989; Roth et al., 1991). *Fabpl* activation coincides with the time that the gut's poorly differentiated stratified epithelium undergoes a dramatic morphologic transformation to a monolayer overlying nascent villi (Trier and Moxey, 1979).

Analyses of young adult transgenic mice containing nucleotides -596 to +21 or -4,000 to +21 of rat *fabpl* (Sweetser et al., 1989) linked to hGH or a different reporter indicated that distinct *cis*-acting elements regulate expression of *fabpl* along the crypt-to-villus and duodenal-to-ileal axes of the gut. Inappropriate expression of the transgenes was noted in crypt epithelial cells as well as in numerous enteroendocrine cells (Sweetser et al., 1988; Roth et al., 1990a; Hansbrough, J. R., D. M. Lublin, K. A. Roth, E. H. Birkenmeier, and J. I. Gordon, manuscript submitted for publication). By contrast, analysis of fetal transgenic mice indicated that nucleotides -596 to +21 of the rat L-FABP gene are sufficient to reproduce an appropriate temporal, cellular, and regional pattern of hGH expression (with the exception of a small subset of enteroendocrine cells; Roth et al., 1991). These differences between the fetal and young adult gut epithelium could represent (a) a process of replacement of (committed) crypt stem cells during the life of the animal; (b) developmental

changes that are programmed into stem cells which permanently reside in the crypts; or (c) changes in the differentiation pathways of stem cell progeny and/or alterations in the levels of critical (intracellular) *trans*-acting factors that affect *fabpl* expression. This latter possibility (c) could arise from changes in the external cellular or luminal environment (e.g., luminal factors such as dietary nutrients, bile salts, and pancreatic secretions have been proposed to regulate enterocyte proliferation and differentiation (Altmann and Leblond, 1970; Jolma et al., 1980; Dowling, 1982).

In the present study, we have examined the cellular and regional patterns of expression of *fabpl* and L-FABP/hGH transgenes in small intestinal isografts. Gut segments were harvested from transgenic mice or their normal littermates before, or coincident with, cytodifferentiation of its epithelium and implanted into the subcutaneous tissues of young adult nude mouse recipients. A comparison of the patterns of *fabpl* and L-FABP/hGH expression along the crypt-to-villus axis of these grafts 4-6 wk after implantation permitted us to directly assess whether lumenally derived molecules serve as essential *trans*-activating factors that regulate expression of these model genes or whether their expression is determined by an "intrinsic" program encoded in the proliferating/differentiating epithelial cells of the small intestine.

## Materials and Methods

### Transgenic Mice

Male transgenic mice containing nucleotides -4,000 to +21 of the rat L-FABP gene linked to the hGH gene beginning at its nucleotide +3 (L-FABP<sup>-4,000 to +21</sup>/hGH) were derived from founder (G<sub>0</sub>) 46 described in Sweetser et al. (1988). Mice containing nucleotides -596 to +21 of the rat L-FABP gene linked to hGH (L-FABP<sup>-596 to +21</sup>/hGH) were from a pedigree derived from G<sub>0</sub>13 (Sweetser et al., 1988). Obligate heterozygotes for one or the other transgene were maintained under a strict light cycle and fed a standard chow diet ad libitum. They were mated to normal C57BL/6J females. The day when a vaginal plug was first noted was designated day 0 of gestation.

### Preparation and Implantation of Intestinal Isografts

The method used for preparing and implanting isografts was adapted from previous reports (Ferguson et al., 1973; Leapman et al., 1974; Kendall et al., 1977, 1979; Montgomery et al., 1981). Timed pregnant female mice were sacrificed at day 16, 17, or 18 of gestation by injection of a lethal dose of sodium pentobarbital. Fetuses were delivered by Caesarian section and anesthetized with sodium pentobarbital. Their gastrointestinal tracts were carefully removed and placed into RPMI medium maintained at 4°C. The stomach and colon were then dissected away from the small intestine at the pylorus and ileocecal junction, respectively. Segments of the duodenum and ileum were removed for histological analysis to assess the extent of morphologic differentiation of the gut epithelium (and also to confirm gestational age). The remainder of the small intestine was subsequently implanted into 4-5 wk-old male nude mice (strain CBY/B6) as follows. Recipient mice were anesthetized with sodium pentobarbital. A midline incision was made along the dorsal paravertebral surface and the subcutaneous fascia gently dissected. The fetal small intestinal isograft was placed into this subcutaneous space (tunnel) and its ends were fastened with 7-0 prolene sutures. The incision was closed with surgical clips. Grafts were removed from the subcutaneous "tunnel" 4-6 wk later.

Southern blots of EcoRI-digested fetal carcass DNA were probed with a <sup>32</sup>P labeled 150-bp BglII-PvuII fragment derived from exon V of the hGH gene (Sweetser et al., 1988) to determine which isograft had been harvested from a transgenic mouse or from its normal (nontransgenic) littermate.

### Immunocytochemical Studies

Sloughed cells and secretions accumulated in the distal part of the isograft

1. Abbreviations used in this paper: CCK, cholecystokinin; GIP, gastric inhibitory polypeptide; hGH, human growth hormone; L-FABP, rat liver fatty acid binding protein.

and often produced pressure necrosis. Therefore, our immunocytochemical studies were performed only on jejunal segments taken from the proximal half of the grafts. Frozen sections (5–8  $\mu$ m) were prepared. 11 different polyclonal antisera were used. Their sources and final dilutions were: rabbit anti-rat L-FABP (1:1,000; Sweetser et al., 1988); rabbit anti-serotonin (1:4,000; Incstar, Stillwater, MN); goat anti-serotonin (1:1,000; Incstar); rat anti-serotonin (1:2,000; Eugenetech, Allendale, NJ); rabbit anti-cholecystokinin (CCK, residues 1–39; 1:1,000, Peninsula Laboratories, Belmont, CA), rabbit anti-gastric inhibitory peptide (GIP 1:1,000; Peninsula Laboratories); rabbit anti-substance P (1:2,000; from J. Krause, Washington University, St. Louis, MO; see McDonald et al., 1989); rabbit anti-gastrin (1:2,000, Dako Corp., Santa Barbara, CA); goat anti-hGH (1:2,000, McKeel and Askin, 1978); rabbit anti-hGH (1:2,000, Dako); and rabbit anti-human lysozyme (1:500; Dako Corp.). The immunostaining characteristics and specificities of these sera have been described by the manufacturers and in our previous reports (Roth et al., 1990a,b). Nonimmune rabbit, goat, and rat sera were used as negative controls for all tissues examined. Antigen-antibody complexes were detected with either gold-labeled second antibodies and silver enhancement (Amersham Corp., Arlington Heights, IL) or with fluorescent labeled second antibodies as previously described (Roth et al., 1990a,b).

To quantitate the number of enteroendocrine cells in 4–6-wk-old isografts and the corresponding jejunal segments from intact gut, the total number of immunoreactive cells were counted per complete cross section and each positive cell was classified as crypt or villus associated (as defined in Roth et al., 1990a). At least two cross sections of proximal intestine were examined from 10 transgenic ( $n = 5$  animals from each pedigree) and three normal (littermate) isografts. We also examined "intact" jejunal segments harvested from nonoperated 4–6-wk-old transgenic mice ( $n = 2$  animals from each pedigree) as well as four normal animals. Finally, the small intestine from the nude mouse host was recovered and surveyed for the presence of hGH<sup>+</sup> epithelial cells.

### Radioimmunoassays of Serum hGH Levels

Serum was obtained from nude mice by retroorbital phlebotomy at the time of their sacrifice 4–6 wk after implantation of isografts. Details of the hGH radioimmunoassay and its ability to differentiate between hGH and mouse GH are described in a previous paper (Sweetser et al., 1988). Each serum sample was assayed in triplicate.

## Results and Discussion

### Morphology of 4–6-Wk-old Normal Mouse Small Intestine Isografts

Small intestinal isografts harvested from fetal C57BL/6J  $\times$  LT/Sv mice grew several centimeters in length during the 4–6 wk following implantation and occasionally demonstrated active peristalsis. An abundant vascular supply extended from the subcutaneous tissue into the isografts. Its lumen was typically filled with mucoid secretions and cellular debris. Routine enterocutaneous drainage of these secretions improved graft growth and morphology. The characteristic appearance of a 5-wk-old jejunal isograft is shown in Fig. 1 A. Typical small intestinal villi and crypts of Lieberkühn are present in addition to a well-developed lamina propria, enteric nervous system, and smooth muscle layer. Villus-associated enterocytes and goblet cells are readily apparent. Cellular debris located at the villus tips suggests normal exfoliation at the apical extrusion zone. Cells at the crypt base contain immunoreactive lysozyme (Fig. 1 B), a product characteristic of terminally differentiated Paneth cells (Peeters and Vantrappen, 1975). No morphologic differences were observed between isografts derived from 16-, 17-, or 18-d-old fetuses (data not shown).

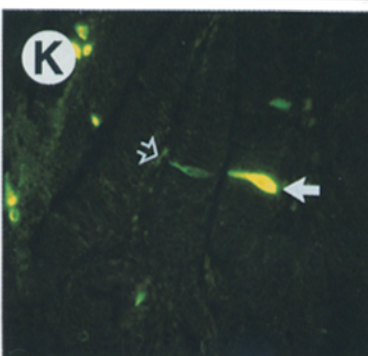
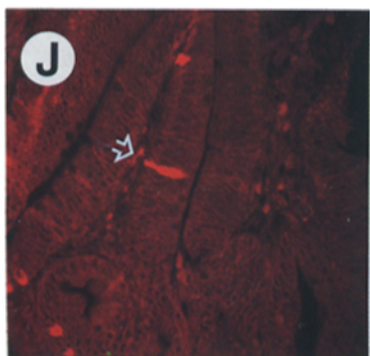
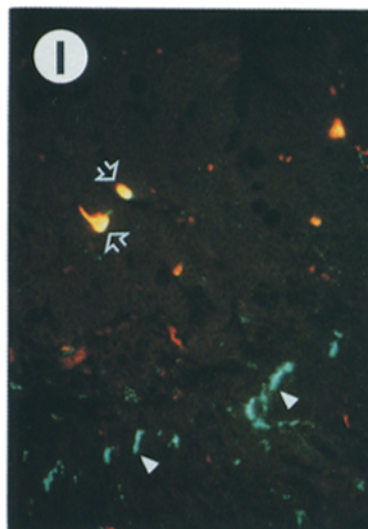
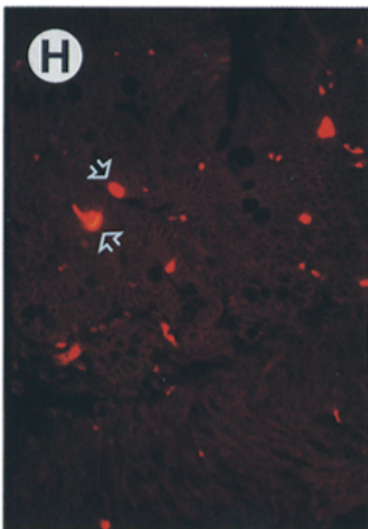
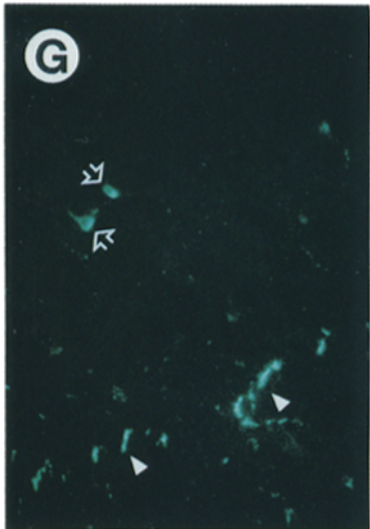
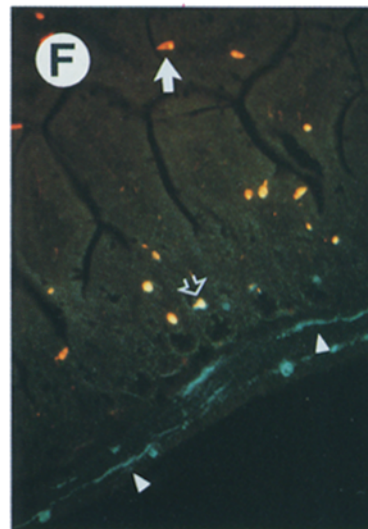
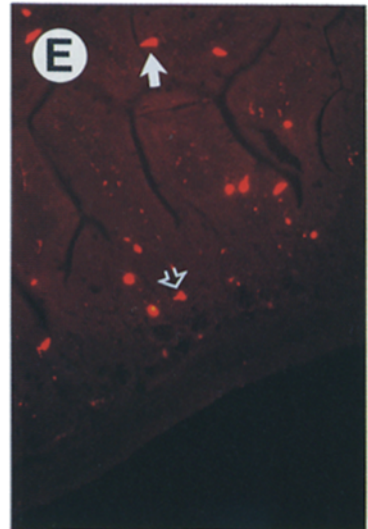
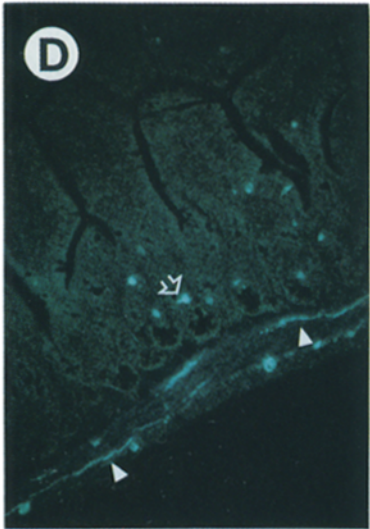
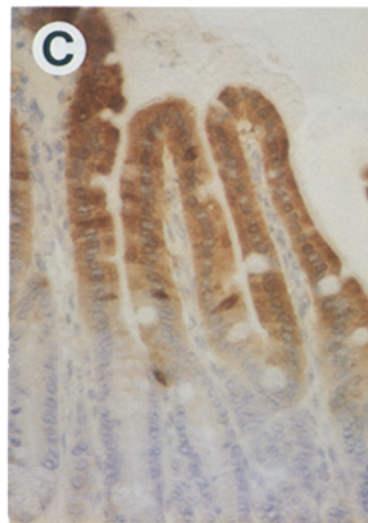
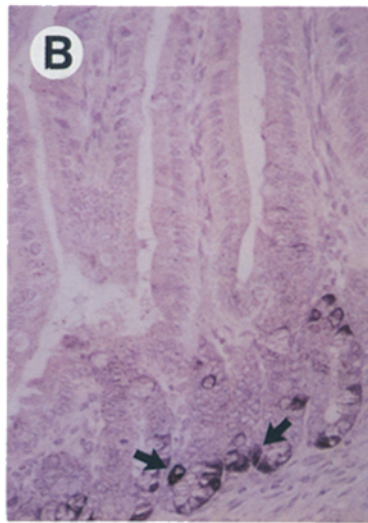
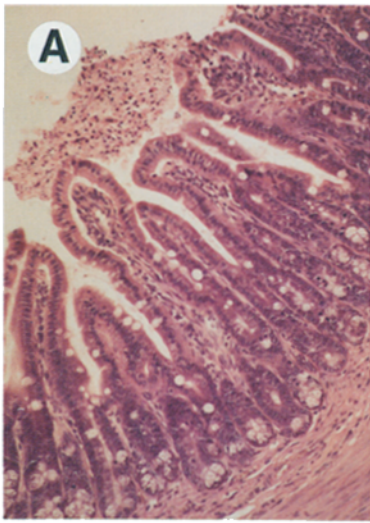
### Analysis of Enterocytic and Enteroendocrine Cell Differentiation in Normal Mouse Jejunal Isografts

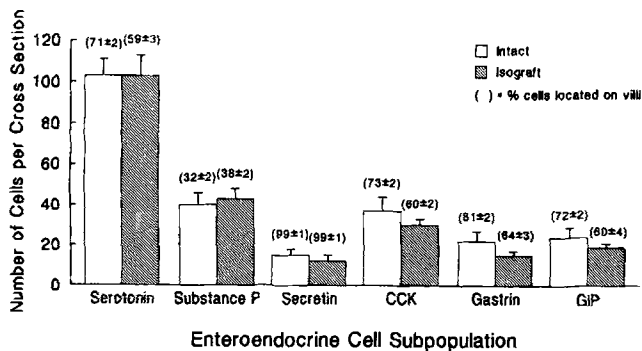
The histologic survey suggested, but did not prove, that the migration-dependent cellular differentiation programs that operate along the crypt-to-villus axis are recapitulated in these isografts even in the absence of (a) luminal contents and (b) the normal surge in glucocorticoids and thyroid hormone which occurs in the mouse at the suckling-weaning transition (postnatal days 12–14; Henning, 1987). This hypothesis was examined further.

Enteroendocrine cells represent <1% of the terminally differentiated cells in the mouse small intestine. Nonetheless, they provide a remarkably sensitive marker of the gut epithelium's complex differentiation pathways as well as its ability to establish geographic differences in gene expression (Roth et al., 1990a,b). At least 15 different subpopulations have been defined on the basis of their principal neuroendocrine product, each presumably descended from the same multipotent stem cell which gives rise to enterocytes, goblet, and Paneth cells (Cheng and Leblond, 1974a,b; Ponder et al., 1985). Each subpopulation maintains a distinctive pattern of distribution along the duodenal-to-colonic and/or crypt-to-villus axis (reviewed in Roth et al., 1990a,b).

We used immunocytochemical methods to determine if enteroendocrine cells are able to fully recapitulate their differentiation program(s) in the developing isograft. Fig. 2 summarizes the results of our comparison of enteroendocrine cell populations present in jejunal isografts and in jejunal segments obtained from "intact" 4–6-wk-old intestine. Each of the six principal subpopulations present in the normal, intact 4–6-wk-old mouse jejunum (i.e., those containing immunoreactive serotonin, substance P, secretin, CCK, gastrin, and GIP) are represented in comparably aged jejunal isografts. There are no significant differences between the number of each of these six cell types/cross section in jejunal isografts and in the intact 4–6-wk-old small intestine (Fig. 2). Moreover, the distribution of each cell type along the crypt-to-villus axis is not apparently affected by the absence of luminal contents or by the gestational day when the grafts were prepared. For example, virtually all secretin immunoreactive cells are associated with villi in both intact jejunum and jejunal isografts. In contrast, two thirds of all substance P immunoreactive cells are located in the crypt region of both isografted and intact jejunum (Fig. 1, D and G and Fig. 2). Fig. 1, D and G also shows that substance P is located in the well developed myenteric plexus of jejunal isografts just as it is in normal intact jejunum.

Previous multilabel immunocytochemical studies of normal adult mouse jejunum revealed complex interrelationships between substance P, and serotonin-, and secretin-immunoreactive cells along the crypt-villus axis (Roth et al., 1990b). For example, the majority (60–80%) of jejunal crypt-associated substance P immunoreactive cells and virtually all villus-associated cells also contain colocalized serotonin. The percentage of cells which produce serotonin and secretin is also affected by position along this axis;  $\sim$ 40% of jejunal secretin cells coexpress serotonin while <20% coexpress substance P. Assuming that these cells are derived from a common precursor, the results suggest a (migration-dependent) differentiation pathway that may involve sequential expression of substance P, serotonin, and secretin. What-





**Figure 2.** Enteroendocrine cell subpopulation frequency and distribution within intact jejunum and jejunal isografts. The number of immunoreactive enteroendocrine cells per cross section and their distribution along the crypt-to-villus axis was determined in eight "intact" jejunal segments and 13 isografts (see Materials and Methods). Since no quantitative differences in the distribution of each enteroendocrine subpopulation (defined by its principal neuroendocrine product) were noted between transgenic or normal isografts (of either pedigree) or between normal and transgenic 4–6-wk-old intact intestine, the data obtained from all isografts were averaged and compared with data obtained from all of the "intact" jejunal segments. The average percent of cells located on villi  $\pm$  SEM is given in parentheses.

ever the underlying pathway, these three cell types represent a superb marker of the gut's spatial differentiation along the crypt-to-villus axis.

Our analysis of jejunal isografts prepared from 16–18-d-old fetuses indicated that the substance P/serotonin/secretin cell interrelationships are established and maintained in the absence of luminal contents. Table I and Fig. 1, *D–I* disclose that 80–90% of substance P immunoreactive cells in 4–6-wk-old intact jejunum and isografts also contain serotonin, while ~50% of secretin immunoreactive cells contain colocalized serotonin.

Other enteroendocrine cell interrelationships are recapitulated in the isografts including some that normally change

**Table I. Colocalization of Serotonin in Other Enteroendocrine Cells in "Intact" and Isografted Jejunum**

Principal enteroendocrine product	Immunoreactive cells containing serotonin	
	Intact jejunum	Isograft
	%	
Secretin	54 $\pm$ 2	40 $\pm$ 10
Gastrin	5 $\pm$ 2	13 $\pm$ 1
CCK	4 $\pm$ 1	5 $\pm$ 1
GIP	0	0
Substance P	80 $\pm$ 4	94 $\pm$ 3

Four to six-wk-old jejunal isografts or corresponding segments of intact small intestine were examined using double-label immunocytochemical techniques to quantitate colocalization of serotonin in each of the five cell types listed. Six "intact" intestines and from six to eight isografts (derived from 16–18-d fetuses) were analyzed. At least two complete cross sections from each animal were examined. The mean value  $\pm$  SEM is shown.

quite dramatically during development. For example, gastrin and GIP immunoreactive cells are limited to the proximal small intestine on fetal day 17 and show a progressive increase in number during late gestation (Roth et al., 1991). Approximately 30% of gastrin-immunoreactive cells in the late gestation (day 18 to 19) fetal mouse jejunum contain serotonin while in adult animals this number declines to <5% (Roth et al., 1991). Table I and Fig. 1, *J–L* show that an "adult" pattern of rare gastrin/serotonin colocalization is present in a 6-wk-old jejunal isograft prepared from a 16-d-old fetus. Finally, GIP immunoreactive cells do not contain detectable levels of serotonin whether they are present in 17 to 18 day fetal jejunum (data not shown), intact 4–6-wk-old jejunum, or in 4–6-wk-old isografts (Table I).

### *The Intact Endogenous Mouse fabpl Gene Shows Appropriate Cellular Patterns of Expression in Normal Mouse Jejunal Isografts*

Previous studies had indicated that *fabpl* is a sensitive marker of enterocytic differentiation (Sweetser et al., 1988; Hautt

**Figure 1.** Immunocytochemical analysis of normal mouse intestinal isografts. (A) Histologic examination of hematoxylin and eosin-stained sections of a nontransgenic mouse jejunal isograft shows normal small intestinal architecture. Well-developed crypts, villi, lamina propria, muscularis, and enteric nerves are present. Eosinophilic cellular debris (in the upper left hand corner) can be seen in the intestinal lumen near the apical extrusion zone. Numerous goblet cells are evident. (B) Paneth cells are revealed using anti-lysozyme antiserum and immunogold with silver enhancement staining. The arrows point to the darkly labeled crypt-associated lysozyme immunoreactive Paneth cells. Enterocytic differentiation in isografts is illustrated in C with anti-L-FABP serum and immunogold with silver enhancement staining. L-FABP immunoreactivity in both intact and isografted jejunum is limited to villus-associated enterocytes. *D–F* illustrate the position-dependent overlap of substance P and serotonin immunoreactivity in an intact, normal jejunum. Substance P immunoreactivity, visualized in *D* with reflected light polarization microscopy, is found in the myenteric plexus (*arrowheads*) and in enteroendocrine cells (a crypt-associated substance P cell is indicated by an *open arrow*). *E* demonstrates serotonin immunoreactive enteroendocrine cells in the same section as *D*. Rat anti-serotonin and  $\beta$ -phycoerythrin-labeled donkey anti-rat sera were used to visualize these cells. They are frequent in both jejunal crypts and villi (examples are indicated by an *open* and *closed arrow*, respectively). Double exposure of the section in *F* shows only substance P staining in the myenteric plexus (*arrowheads*), both substance P and serotonin staining of a crypt-associated enteroendocrine cell (*open arrow*) and only serotonin staining of a villus-associated enteroendocrine cell (*closed arrow*). This organized pattern of substance P and serotonin coexpression is recapitulated in jejunal isografts: *G–I* are from a single section of isografted jejunum stained for substance P (*G*) and serotonin (*H*). Substance P immunoreactivity in the myenteric plexus is indicated by *arrowheads* and immunoreactive enteroendocrine cells are indicated by *open arrows*. Double exposure of this (isograft) section in *I* shows dual-labeled crypt-associated substance P/serotonin enteroendocrine cells (*open arrows*) and myenteric nerves which stain only for substance P (*arrowheads*). Numerous villus-associated serotonergic cells devoid of substance P immunoreactivity were also seen (data not shown). Other complex enteroendocrine interrelationships are also recapitulated in jejunal isografts. In *J–L* a single section of jejunal isograft was stained with rabbit anti-gastrin and Texas red-labeled donkey anti-rabbit sera (*J*) and rat anti-serotonin and fluorescein-labeled donkey anti-rat sera (*K*). *L* demonstrates that the gastrin immunoreactive cell, indicated by an *open arrow*, also contains serotonin immunoreactivity while an adjacent serotonergic cell, indicated by a *closed arrow*, is devoid of gastrin immunoreactivity. Such dual positive cells occurred with a similar frequency in both intact and isografted jejunum.

et al., 1989; Roth et al., 1991). In all 4–6-wk-old jejunal isografts examined (whether derived from 16-, 17-, or 18-d-old fetuses), high levels of this cytoplasmic long chain fatty acid-binding protein (Lowe et al., 1987; Cistola et al., 1989) are present in villus-associated enterocytes. No immunoreactive protein is detectable in crypt-associated epithelial cells (Fig. 1 C). The “abrupt” induction of *fabpl* expression which occurs in enterocytes “poised” at the crypt/villus junction of these jejunal isografts recapitulates the pattern seen in the intact gut (Sweetser et al., 1988).

We had previously used double label immunocytochemical methods to establish that the *fabpl* gene is not expressed in either the substance P, secretin, CCK, gastrin, or GIP immunoreactive enteroendocrine subpopulations present in the normal young adult mouse jejunum and only rarely in serotonergic cells (Roth et al., 1990a). Comparable searches using anti-L-FABP sera and antibodies directed against these neuroendocrine products failed to disclose any enteroendocrine cells in our jejunal isografts that contained detectable L-FABP (data not shown). This result, together with the data shown in Fig. 1 C, indicate that the mouse *fabpl* gene does not require luminal contents to produce its normal cell- and region-specific pattern of expression along the jejunal crypt-to-villus axis.

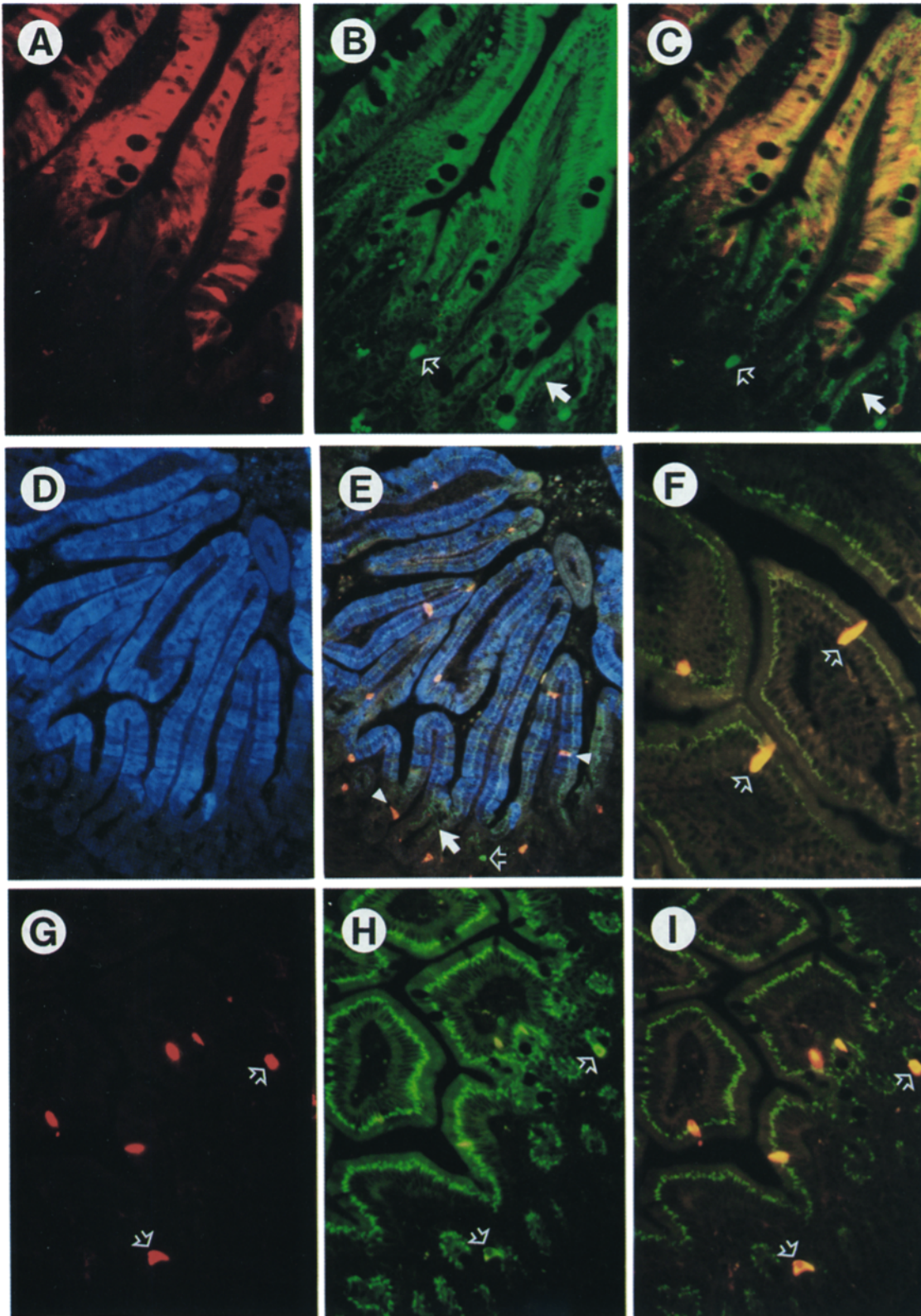
### Comparison of *fabpl* and L-FABP/hGH Gene Expression in Jejunal Isografts Prepared from Transgenic Mice

**hGH Production has no Apparent Effect on Intestinal Epithelial Differentiation in Isografts or in the Intact Gut of the Nude Mouse Host.** Analysis of serum obtained at the time of sacrifice of nude mice containing 4–6-wk-old jejunal isografts from L-FABP<sup>-4,000 to +21</sup>/hGH and L-FABP<sup>-596 to +21</sup>/hGH transgenic animals indicated that the hGH reporter was synthesized in these grafts and exported. The mean ( $\pm$  SEM) values for selected animals containing isografts derived from 16–18-d fetal L-FABP<sup>-4,000 to +21</sup>/hGH or L-FABP<sup>-596 to +21</sup>/hGH transgenics was  $77 \pm 45$  ng/ml, (range = 26–168,  $n = 4$  animals) and  $135 \pm 36$  ng/ml, (range = 75–200,  $n = 4$  animals), respectively. By contrast, our radioimmunoassay was not able to detect hGH in nude mice containing isografts prepared from normal (nontransgenic) littermates (i.e., levels were  $<0.7$  ng/ml). These values can be put into perspective by noting that the normal mouse growth hormone (mGH) levels during postnatal weeks 4–6 are 7–10 ng/ml (Sinha et al., 1974).

Several studies have indicated that growth hormone may be important for normal growth and development of the intestine (Kendall et al., 1977; Cooke et al., 1986). In one report (Cooke et al., 1986), segments of fetal rat intestine were implanted under the renal capsule of either normal or hypophysectomized syngeneic hosts. Over the course of the 11-d experiment, grafts implanted into hypophysectomized hosts failed to develop villi or smooth muscle. Administration of growth hormone to the hypophysectomized host restored normal isograft growth and histologic development. The young adult male nude mouse host we used has a normal endocrine system. The presence of either the L-FABP<sup>-596 to +21</sup>/hGH or L-FABP<sup>-4,000 to +21</sup>/hGH transgene had no apparent effect on the growth of jejunal isografts when measured 4–6 wk after implantation (data not shown). The histologic appearance of the isografts was indistinguishable from the normal mouse shown in Fig. 1, A and B. Immunocytochemical studies using antibodies directed against L-FABP, lysozyme, serotonin, substance P, secretin, CCK, gastrin, and GIP indicated that enteroendocrine, enterocytic, and Paneth cell differentiation in transgenic mouse isografts was qualitatively and quantitatively similar to that seen in (a) the intact jejunum harvested from 4–6-wk-old members of these pedigrees; (b) the intact jejunum of normal mice; and (c) normal mouse isografts of comparable age (Fig. 2; and data not shown). Single- and double-label immunocytochemical studies (Sweetser et al., 1988) also revealed that the presence of multiple ( $>100$ ) copies of either transgene had no qualitative or quantitative effect on either the cellular or spatial patterns of expression of the intact endogenous *fabpl* gene in either the intact jejunum or the isograft: L-FABP was only found in villus-associated enterocytes (e.g., Fig. 3 A) and none of the enteroendocrine cell subpopulations contained detectable levels of this fatty acid-binding protein (data not shown).

No histologic abnormalities were noted in the young adult CBY/B6 nude mouse host intestine. Our transgenic isografts provided a unique opportunity to determine whether stem cells located in the fetal gut epithelium are able to circulate and “home” to the intact host intestine (see Tavassoli and Hardy, 1990 for a review of the molecular basis of homing of hematopoietic stem cells). Inspection of multiple (30) sections prepared from the duodenum, jejunum, or ileum failed to reveal any hGH positive cells either in the epithelium or in the lamina propria ( $n = 10$  mice; six containing isografts from L-FABP<sup>-4,000 to +21</sup>/hGH transgenics, four containing isografts from L-FABP<sup>-596 to +21</sup>/hGH transgenics). This was true whether the CBY/B6 host received jejunal isografts from

**Figure 3.** Comparison of hGH expression in the enterocytic and enteroendocrine cell populations in “intact” and isografted transgenic small intestine. A–C are from a single section of a jejunal isograft from a L-FABP<sup>-4,000 to +21</sup>/hGH transgenic mouse. L-FABP immunoreactivity was detected in A with rabbit anti-L-FABP and Texas red-labeled donkey anti-rabbit sera. L-FABP immunoreactivity is limited to villus-associated enterocytes. hGH expression was detected in B with goat anti-hGH and fluorescein-labeled donkey anti-goat sera. Strong Golgi-associated enterocytic immunoreactivity (closed arrow) and diffuse cytoplasmic staining of occasional enteroendocrine cells (open arrow) are seen. Double exposure of the section in C clearly shows the discordant expression of hGH vs L-FABP in the upper crypt. Similar results were found in jejunal isografts from L-FABP<sup>-596 to +21</sup>/hGH transgenic mice (D and E). L-FABP was visualized in D and E with 7-amino-4-methyl coumarin-3-acetic acid-labeled donkey anti-rabbit serum. It is limited to villus-associated epithelial cells. Simultaneous incubation of this section with goat anti-hGH and rat anti-serotonin sera and their subsequent detection with fluorescein-labeled donkey anti-goat and  $\beta$ -phycoerythrin-labeled donkey anti-rat sera, respectively, illustrates the interrelationships between L-FABP, hGH, and serotonin immunoreactivity (E). Golgi-associated hGH immunoreactivity is found in the upper crypt region (closed arrow) and extends into L-FABP-labeled villus enterocytes. Occasional hGH positive enteroendocrine cells (open arrow) are found; these cells are independent of serotonergic enteroendocrine cells (arrowheads). Transgenic mice containing either L-FABP/hGH construct show frequent coexpression of hGH in GIP immunoreactive cells: F is a dual exposure of a single section of intact proximal jejunum from a mouse containing the L-FABP<sup>-4,000 to +21</sup>/hGH transgene incubated with rabbit anti-GIP and goat anti-hGH sera which were subsequently detected with Texas



red-labeled donkey anti-rabbit and fluorescein-labeled donkey anti-goat sera. The open arrows point to two enteroendocrine cells that contain both GIP and hGH and appear yellow because of the overlap of red and green fluorescence. Note the Golgi-associated (green) hGH immunoreactivity in enterocytes. *G-I* illustrate the relationship between GIP and hGH immunoreactivity in a jejunal isograft derived from an L-FABP<sup>-4,000 to +21</sup>/hGH transgenic mouse. Numerous GIP immunoreactive cells are seen in *G*; two crypt-associated GIP immunoreactive cells are indicated by open arrows. hGH immunoreactivity in *H* is found in both enterocytes and enteroendocrine cells. Double exposure of the section (*I*) shows the colocalization of hGH and GIP in several cells including the two crypt-associated cells indicated by open arrows.

16-, 17-, or 18-d-old L-FABP/hGH fetal transgenic mice. However, complete serial sectioning of the entire host intestine will be necessary to make an absolute statement about the absence of homing.

### Precocious Expression of L-FABP/hGH Transgene Expression during Enterocytic Differentiation

Fig. 3 compares the cellular patterns of expression of L-FABP and hGH in a jejunal isograft prepared from an L-FABP<sup>-4,000 to +21</sup>/hGH transgenic mouse and harvested 5 wk after implantation. In contrast to the diffuse cytoplasmic staining of L-FABP (Fig. 3 A), prominent hGH staining is seen in the supranuclear Golgi apparatus of villus-associated enterocytes (Fig. 3 B). Double-label immunofluorescence microscopy (Fig. 3 C) demonstrates that the transgene is inappropriately expressed in epithelial cells located in the upper crypt. Similar results were obtained when we analyzed jejunal isografts from 4–6-wk-old L-FABP<sup>-596 to +21</sup>/hGH transgenics (Fig. 3, D and E) as well as the intact jejunum of both pedigrees. Within each transgenic pedigree, no differences were noted in the regional (crypt-to-villus) patterns of enterocytic L-FABP and hGH accumulation if grafts were prepared from either 16-, 17-, or 18-d-old fetal animals.

These observations, together with our earlier analyses of multiple pedigrees of fetal and young adult mice containing either of the two transgenes, suggest two conclusions. First, *cis*-acting elements located outside of nucleotides -4,000 to +21 of the rat L-FABP gene are needed to produce a completely appropriate differentiation-dependent pattern of *fabpl* expression in enterocytes as they are translocated upwards along the crypt-to-villus axis. (It is important to note that studies in young adult animals containing nucleotides -596 to +21 of the rat L-FABP gene linked to a different reporter [a chimeric cDNA encoding human decay accelerating factor plus the transmembrane domain of HLA-B44] revealed a pattern of transgene expression in enterocytes and enteroendocrine cells located along the crypt-to-villus axis of their intact small bowel which was identical to that observed in comparably aged L-FABP<sup>-596 to +21</sup>/hGH transgenics [Hansborough, J. R., D. M. Lublin, K. A. Roth, E. H. Birkenmeier, and J. I. Gordon, manuscript submitted for publication]. This suggests that the hGH gene does not contain *cis*-acting elements which influence L-FABP/hGH expres-

sion in the gut epithelium). Second, "induction" of *fabpl* and L-FABP/hGH gene expression during differentiation of these cells does not apparently require "trans-acting" factors derived from dietary substances, pancreatic secretions, or biliary contents.

### Differences in Transgene Expression Occur within Enteroendocrine Cell Populations Present in Intact Jejunum and Jejunal Isografts

Expression of hGH in enteroendocrine cells is clearly time dependent in the intact gut. In an earlier study we noted that there is little hGH expression in fetal enteroendocrine cells (Roth et al., 1991). In this present study we found that the frequency of hGH positive enteroendocrine cells in the 4–6-wk-old intact jejunum of both L-FABP/hGH transgenic pedigrees (Table II) is also less than that previously reported for 3–5-mo-old transgenic mice (Roth et al., 1990a).

The total number of hGH immunoreactive enteroendocrine cells in both our L-FABP<sup>-596 to +21</sup>/hGH and L-FABP<sup>-4,000 to +21</sup>/hGH jejunal isografts and their relative frequency compared to individual enteroendocrine subpopulations is much greater than in intact fetal jejunum but is less than that encountered in intact 4–6-wk-old jejunum: i.e., mice containing the L-FABP<sup>-596 to +21</sup>/hGH transgene have  $76 \pm 6$  hGH<sup>+</sup> cells/cross section ( $n = 2$  animals) in the intact jejunum and  $14 \pm 8$  cells (range 2–39,  $n = 5$ ) in isografts. L-FABP<sup>-4,000 to +21</sup>/hGH transgenic mice have  $23 \pm 8$  ( $n = 2$ ) hGH immunoreactive enteroendocrine cells/cross section in the intact jejunum and  $13 \pm 4$  cells (range 6–27,  $n = 5$ ) in the jejunal isografts.

Double- and triple-label immunocytochemical methods were used to compare L-FABP/hGH transgene expression in specific enteroendocrine cell subpopulations represented in 4–6-wk-old intact jejunum and jejunal isografts (Table II). Nucleotides -596 to +21 of the rat L-FABP gene direct inappropriate expression of the transgene in >80% of the secretin, gastrin, CCK, and GIP immunoreactive enteroendocrine cells present in intact jejunum. By contrast, this transgene is expressed in <5% of cells containing substance P-like immunoreactivity or serotonin (Table II). Remarkably, the frequency of hGH expression is lower in all of the enteroendocrine cell subpopulations present in comparably aged isografts.

Addition of nucleotides -597 to -4,000 from the rat

Table II. Colocalization of hGH in Enteroendocrine Cells of "Intact" and Isografted Jejunum from Mice Containing L-FABP/hGH Transgenes

	Immunoreactive cells containing hGH					
	Secretin	Gastrin	CCK	GIP	Substance P	Serotonin
	%					
L-FABP <sup>-596 to +21</sup>						
Intact	81 ± 13	94 ± 8	84 ± 1	96 ± 1	2 ± 2	3 ± 1
Isograft	37 ± 15	47 ± 3	21 ± 2	65 ± 11	0	1 ± 1
L-FABP <sup>-4,000 to +21</sup> /hGH						
Intact	22 ± 2	25 ± 8	10 ± 2	89 ± 2	4 ± 1	2 ± 2
Isograft	21 ± 13	7 ± 7	5 ± 1	56 ± 20	3 ± 3	1 ± 1

Sections of "intact" jejunum and jejunal isografts prepared from mice containing the L-FABP<sup>-4,000 to +21</sup>/hGH and L-FABP<sup>-596 to +21</sup>/hGH transgenes were analyzed for coexpression of hGH in the six enteroendocrine cell subpopulations listed. Two 4–6-wk-old animals from each pedigree were used as a source of intact jejunum. Similarly, two or three isografts derived from 16–18-d fetal L-FABP<sup>-4,000 to +21</sup>/hGH or L-FABP<sup>-596 to +21</sup>/hGH mice were analyzed 4–6 wk after implantation. At least two cross sections from each animal were studied using each multilabel combination. Data are expressed as the mean ± SEM.



L-FABP gene results in 4–8-fold reductions in the percent colocalization of hGH in secretin-, gastrin-, and CCK-immunoreactive cells present in intact jejunum. However, these upstream sequences produce little silencing of transgene expression in GIP immunoreactive cells (Table II). Isografts from L-FABP<sup>-4,000 to +21</sup>/hGH transgenics also exhibit reduced percentage co-localization of the reporter in several of their enteroendocrine cell subpopulations compared to corresponding segments of intact L-FABP<sup>-4,000 to +21</sup>/hGH jejunum (Table II).

This reduction of transgene expression in the isografts' enteroendocrine subpopulation is selective and not due to a general reduction in the steady state levels of the reporter or to any apparent effect of the surgical procedure: (a) it does not involve enterocytes; (b) it affects some but not all members of a given enteroendocrine subpopulation; and (c) as noted above, there are no (other) detectable qualitative or quantitative differences between enteroendocrine cells present in intact jejunum or jejunal isografts (harvested from transgenic mice or their normal littermates; Fig. 2).

Differentiation of these enteroendocrine subpopulations thus appears to involve a program that does not require extracellular luminal contents (if the appearance of a cell's principal neuroendocrine product[s] is used to define the endpoint of this process [program]). Our results not only reveal a heterogeneity within these enteroendocrine subpopulations that is operationally definable by cellular differences in L-FABP/hGH expression, but they also indicate that these transgenes can be good reporters of the influence of luminal factors on gene expression within and among these subpopulations. Certainly they provide evidence that fundamental differences exist in the signaling pathways available to enterocytes and enteroendocrine cells which allow luminal "cues" to affect accumulation of the hGH reporter. The molecular basis for these differences (e.g., availability of specific *trans*-acting factors) remains to be defined but should provide a paradigm as to how certain enteroendocrine populations are able to sense and respond to changes in their luminal environment.

Our analyses suggest that transgenic intestinal isografts may be useful for examining a number of other questions related to enteric biology. They could be used as models for analyzing the pathways of reporter protein secretion in epithelial cell populations (i.e., is the foreign protein detected in the blood of the nude mouse recipient?). These results could then be correlated with "static" electron microscopic immunocytochemical studies of reporter trafficking in the exocrine and endocrine cells of the gut (e.g., Trahair et al., 1989). Isografts can be used to determine whether positional information regulating the expression of specific genes along the duodenal-to-colonic axis is encoded in stem cells before initial cytodifferentiation of the fetal gut epithelium. Transgenic isografts can be used to map *cis*-acting elements in genes known to be regulated by luminal factors. Other potential applications of transgenic intestinal isografts include (a) assessment of the effects of expressing certain products on gut epithelial cells when such expression in the intact gut may be lethal to the postnatal animal; and (b) an analysis of the role of extracellular factors in modulating abnormalities in proliferation/differentiation produced by oncoproteins (or specific genetic mutations, e.g., Moser et al., 1990).

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