Mapping Enteroendocrine Cell Populations in Transgenic Mice Reveals an Unexpected Degree of Complexity in Cellular Differentiation within the Gastrointestinal Tract

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Abstract. The gastrointestinal tract is lined with a monolayer of cells that undergo perpetual and rapid renewal. Four principal, terminally differentiated cell types populate the monolayer, enterocytes, goblet cells, Paneth cells, and enteroendocrine cells. This epithelium exhibits complex patterns of regional differentiation, both from crypt-to-villus and from duodenum-to-colon. The “liver” fatty acid binding protein (L-FABP) gene represents a useful model for analyzing the molecular basis for intestinal epithelial differentiation since it exhibits cell-specific, region-specific, as well as developmental stage specific expression. We have previously linked portions of the 5’ nontranscribed domain of the rat L-FABP gene to the human growth hormone (hGH) gene and analyzed expression of the fusion gene in adult transgenic mice. High levels of hGH expression were noted in enterocytes as well as cells that histologically resembled enteroendocrine cells. In the present study, we have used immunocytochemical techniques to map the distribution of enteroendocrine cells in the normal adult mouse gut and to characterize those that synthesize L-FABP. In addition, L-FABP/hGH fusion genes were used to identify subsets of enteroendocrine cells based on their ability to support hGH synthesis in several different pedigrees of transgenic mice. The results reveal remarkable differences in transgene expression between, and within, enteroendocrine cell populations previously classified only on the basis of their neuroendocrine products. In some cases, these differences are related to the position occupied by cells along the duodenal-to-colonic and crypt-to-villus axes of the gut. Thus, transgenes appear to be sensitive tools for examining the cellular and regional differentiation of this class of intestinal epithelial cells.

The mouse intestine is lined with a complex monolayer composed of a number of distinct, differentiated cell types. The four principal ones are polarized, absorptive enterocytes, goblet cells, Paneth cells, and enteroendocrine cells. Tritiated thymidine labeling studies as well as histochemical analyses of normal and chimeric mouse intestine indicate that these terminally differentiated cells arise from pluripotent stem cells (2, 9). In adult mice, each intestinal crypt contains a population of stem cells located near its base that are derived from a single progenitor (9). Descendants of these crypt stem cells differentiate during a well organized, rapid, and bipolar migration process (15). Cell-specific and region-specific differences in gene expression are established and maintained in this continuously proliferating and differentiating gut epithelium. Regional “differentiation” occurs in several dimensions: from the crypts of Lieberkühn to the villus tip and from the proximal duodenum to the colon. The enteroendocrine population provides the most dramatic illustration of this point. A large number of enteroendocrine cell types have been identified in the intestinal epithelium based on their geographic location, their neurosecretory granular morphology, and their neurohormonal content (3, 6, 10, 18).

Recent studies have shown that transgenic mice represent a useful tool for exploring the mechanisms responsible for regulating region-specific, cell-specific and developmental stage-specific expression of genes in the intestine (5, 14, 20, 22). The mouse “liver” fatty acid binding protein (L-FABP) gene is efficiently expressed in hepatocytes, enterocytes, and in some cells that histologically resemble enteroendocrine cells (22). Functional mapping studies of the 5’ nontranscribed region of the L-FABP gene using transgenic mice have shown that distinct cis-acting elements, including orientation independent suppressor(s), are necessary for maintaining appropriate duodenal-to-colonic differences in its expression. However, these elements are

1. The proper designation for the mouse liver fatty acid binding protein gene is fabpl (see reference 21). For convenience, in this and previous publications (20, 22), we have used L-FABP when referring to the gene and its mRNA and protein products.
not sufficient to establish an appropriate crypt-to-villus distribution of L-FABP, at least in enterocytes (22). In this report, we have conducted a series of immunocytochemical analyses to extend these L-FABP promoter mapping studies to enteroendocrine cells. In so doing, we have identified a previously undefined level of differentiation within the gut epithelium, based on the ability of cellular populations to support expression of L-FABP-containing transgenes.

**Materials and Methods**

**Transgenic Mice**

Several pedigrees of transgenic mice were used for these studies. Some contained nucleotides -4,000 to +21 of the rat L-FABP gene (22) linked to the hGH gene (17) beginning at its nucleotide +3 (5, 20, 22). Mice heterozygous for this transgene were derived from two founders (G1 and G46 in reference 22). The other pedigrees contained nucleotides -596 to +21 of the rat L-FABP gene linked to human growth hormone (hGH)2. Obligate heterozygotes for this transgene were obtained from founders 13 and 19 in reference 22. Mice were fed a standard chow diet ad libitum and maintained under strictly cycled lighting conditions (lights on between 0600-1800 h). They were killed at 4-6 mo of age by cervical dislocation. Their tissues were rapidly dissected and then fixed in Bouin's solution (22).

**Immunocytochemical Methods**

5-µm-thick sections were cut from regions of the intestine that had been embedded in paraffin. Sections were subsequently treated with xylene to remove the paraffin, rehydrated in graded ethanols, and preincubated for 30-min in PBS containing 0.2% nonfat powdered milk, 2% BSA, and 0.3% Triton X-100. Primary antisera were diluted in the same buffer and applied to the sections. Sections were incubated overnight at 4°C, washed in PBS, and then incubated for 1 h with gold labeled goat anti-rabbit serum. After washes in PBS and H2O, silver enhancement solution was added according to the manufacturer's protocol (Jannsen Life Sciences Products, Piscataway, NJ). Silver precipitation was monitored under the light microscope. Sections were subsequently batted in H2O and lightly counterstained with hematoxylin.

A panel of antisera was used to survey the enteroendocrine population of the intestine. Their origins and final dilution were as follows: rabbit anti-L-FABP (1:1000; reference 22), goat anti-hGH (1:1000; reference 8), rabbit anti-IGF (1:1000; Daco Corp., Santa Barbara, CA), rabbit or goat antiserotonin (1:200, 1:600; Incstar Corp., Stillwater, MN), rabbit antit-ß-endorphin (1:400; reference 4), rabbit antityrosine (residues 1-8) (1:400; reference 12), rabbit antianterenalin (1:200; Incstar), rabbit antinorenergic peptide (1:200; Incstar), rabbit antipeptide histidine isoleucine (PHI) (1:400, Dako Corp.), rabbit antihuman polypeptide (PP) (1:400, Dako Corp.). The immunostaining characteristics and unique specificities of the L-FABP and hGH antisera have been demonstrated in previous reports (22, 23). The specificity of the remaining antisera was verified using sections prepared from tissues containing cells known to support synthesis of the relevant antigen and by the use of nonimmune rabbit and goat sera. Note that in control experiments, no hGH immunoreactivity was found in normal mouse intestine, demonstrating that the hGH antisera did not cross-react with any of the neuroendocrine peptides present in enteroendocrine cells.

To quantify expression of neuroendocrine products in the enteroendocrine cells of transgenic mice and their normal littermates, from 6 to 16 cross sections of the intestine were examined from duodenum, proximal jejunum, distal jejunum, ileum, plus the proximal and distal halves of the colon (see reference 22 for a description of how these regions were operationally defined). The total number of positive cells/complete cross section was counted. Moreover, each positive cell was classified as being either crypt or villus associated. As emphasized in Results, no qualitative or quantitative differences in the distribution of any endogenous neuroendocrine product was found between animals in any of the four transgenic pedigrees and between transgenic animals and their normal littermates. Therefore, results from all animals were averaged for each segment of the proximal to distal axis surveyed.

**Double Labeling: Simultaneous Immunofluorescence**

Double label immunofluorescence studies were performed on individual sections by coinucitation of primary antisera raised in two different species: rabbit and goat. Two separate secondary antisera raised in donkeys against rabbit and goat immunoglobulin were used to detect the two primary antisera. These secondary antibodies were labeled with either fluorescein or Texas red (Jackson Immunoresearch Laboratories, West Grove, PA). After an overnight incubation at 4°C with the two primary antisera, sections were washed in PBS, and then incubated for 1 h at room temperature with donkey anti-rabbit and donkey anti-goat labeled sera diluted 1:160. Tissue sections were subsequently washed with PBS three times, mounted with PBS/glycerol (1:1), and examined under the fluorescence microscope using filters of the appropriate wavelength. Note that control studies showed no cross-reactivity between mismatched primary and secondary antisera.

**Double Labeling: Sequential Immunogold Silver/Immunofluorescence Staining**

We used sequential immunogold silver staining and fluorescence microscopy to label two separate antigens in a single tissue section when the only available antisera were raised in a single species (rabbit). Sections were first immunostained for a particular antigen using the immunogold silver staining method described above. This was followed by application of the second primary antisera and then a fluorescent-labeled secondary antisera. As previously reported by Scopsi and Larsson (16), we found that the immunogold silver staining method did not interfere with subsequent immunofluorescence staining. Most importantly, we found that deposition of silver around the first primary antisera-gold labeled secondary antisera complex abolished its immunoreactivity. This phenomenon is similar to the masking of immunoreactivity by DAB tetrahydrochloride in the unlabeled antibody peroxidase-antiperoxidase method (19). This “masking” of the first rabbit primary antisera by silver deposition allowed us to add a second primary rabbit primary antisera to the sections that could then be visualized by adding fluorescent-labeled anti-rabbit secondary antibodies. The specificity of this procedure was verified in control studies by substituting normal rabbit serum for either one, or both, of the primary rabbit antisera. When both rabbit and goat antisera were available, we compared the results obtained using this sequential immunogold/immunofluorescence method with the results of the double label immunofluorescence procedure described in the preceding paragraph. Both double labeling procedures produced identical results. In other control experiments, we found that deposition of silver around the first primary antisera-gold labeled secondary antisera complex destroyed its subsequent antigenicity without precluding colocalization of a second antigen to the silver stained cell. Thus, this procedure could easily demonstrate colocalization of two antigens in a single cell.

**Triple Labeling: Sequential Immunogold Silver/Simultaneous Immunofluorescence Staining**

A logical next step was to perform double labeling immunofluorescence studies on sections previously stained by the immunogold silver method. This allows localization of three antigens in a single tissue section by using two antisera raised in one species and a third antisera produced in a different species. The immunogold silver staining method is performed first. As described above, deposition of silver around the primary rabbit antisera-gold labeled goat anti-rabbit serum complex destroys the antigenicity of the primary antisera. In addition, the antigenicity of the goat anti-rabbit secondary antisera is also effectively masked. After silver deposition, sections are processed for double label simultaneous immunofluorescence. Control experiments using sections previously stained with primary rabbit antisera and the immunogold-silver detection method showed that the sequential addition of normal rabbit serum and/or normal goat serum followed by Texas red-labeled donkey anti-rabbit serum and fluorescein-labeled donkey anti-glutamic acid decarboxylase (GAD) primary antisera could easily demonstrate colocalization of two antigens in a single cell.

2. **Abbreviations used in this paper:** CCK, cholecystokinin; GIP, gastric inhibitory peptide; GLP, glucagon-like peptide; hGH, human growth; NPY, neuropeptide tyrosine; PHI, peptide histidine isoleucine; PP, pancreatic polypeptide.
anti-goat serum resulted in no labeling of the previously deposited immunogold-silver staining.

Results

Description of Transgenic Mice Used to Map Enteroendocrine Populations in the Small and Large Intestine

We previously noted (22) that the intact, endogenous mouse L-FABP gene (fabp7) is expressed at highest levels in the proximal jejunum of adult mice. The steady-state concentrations of its mRNA and protein products steadily decline as one proceeds to the distal ileum. These gene products are not detectable in the colon. Within the small intestine, L-FABP is not expressed until epithelial cells emerge from the crypts of Lieberkühn. Functional mapping studies of the rat L-FABP promoter in transgenic mice revealed that cis-acting elements contained between nucleotides −596 and +21 are sufficient to produce a pattern of reporter gene (hGH) expression that recapitulates the duodenal-to-ileal gradient noted with the intact, endogenous mouse L-FABP gene (22). However, they are not sufficient to prohibit hGH expression in the crypts of Lieberkühn nor to repress its synthesis in the colon. Addition of nucleotides −597 to −4,000 in either orientation “restores” an appropriate proximal-to-distal pattern of hGH accumulation by suppressing colonic expression, but these additional sequences do not prevent its “anomalous” accumulation in the crypts of Lieberkühn (22). Up to several hundred copies of either transgene/haploid genome have no effect on the cell-specific or regional patterns of expression of the endogenous mouse L-FABP gene, suggesting that the trans-acting factors that are responsible for regulating its expression are present in high concentrations within intestinal epithelial cells.

We initiated an analysis of transgene expression in the enteroendocrine population of transgenic mice containing either one of these two L-FABP/hGH transgenes: i.e., L-FABP+4,000 to +21/hGH or L-FABP−596 to +21/hGH. Animals from four different pedigrees were analyzed (two pedigrees/transgene). In this way, we could assess whether cellular patterns of hGH expression were influenced by the site of insertion of these L-FABP/hGH fusion genes. The enteroendocrine population was selected for these studies for two reasons. First, it is remarkably complex with approximately 15 different subtypes, some of which exhibit dramatic geographic differences in their distribution along the crypt-to-villus and duodenal-to-colonic axes of the gut (3, 6, 10, 18). Second, we had previously noted (22) that while L-FABP immunoreactivity is primarily limited to small intestinal villus-associated enterocytes (Fig. 1 A), rare, intensely staining (L-FABP positive) cells with the histologic appearance of enteroendocrine cells are present in duodenal villi. The nature of this enteroendocrine cell population had not been defined in these earlier studies. Other than enterocytes and these rare enteroendocrine appearing cells, the L-FABP gene is quite restricted in its expression: L-FABP is not detectable in Paneth cells or in goblet cells by light microscopic immunocytochemistry using either the peroxidase antiperoxidase staining method (22) or the more sensitive immunogold-silver enhancement technique (data not shown). Moreover, no immunoreactive L-FABP can be detected by immunogold-silver staining in either colonic or stomach epithelium.

The Regional Distribution of Enteroendocrine Cells in the Mouse Gut

Since there was little information available about the peptide content of enteroendocrine cells in the mouse (10, 25), we first conducted a survey of enteroendocrine cell types present in normal adult C57BL/6J × LT/SV mice and their transgenic littermates. Sixteen different antisera were used for this analysis. Enteroendocrine cells were stained with antisera directed against serotonin, GIP, CCK, secretin, GLP-1, PP, NPY, somatostatin, and neurotensin. Galanin, vasoactive intestinal peptide, PHI, met-enkephalin, gastrin releasing peptide/bombesin, β-endorphin, and dynorphin antisera failed to stain enteroendocrine cells although enteric nerves were frequently positive (data not shown).

Serotonin containing enterochromaffin cells represent the largest population of enteroendocrine cells in this strain of mice (Fig. 1 B). These cells are found in high numbers throughout the length of the intestine and are present in both crypts and villi (Table I). CCK (Fig. 1 C) and GIP immunoreactive cells are both found in highest concentration in the duodenum and proximal jejunum. Approximately 25% of each of these cell types are located in the crypts of Lieberkühn (Table I). Secretin containing cells are also most abundant in the duodenum and proximal jejunum. However, unlike cells that reacted with our CCK and GIP antisera, secretin-immunoreactive cells are confined to villi. GLP-1 cells have a bimodal distribution along the proximal to distal axis of the gut: they are found with highest frequency in the proximal jejunum and colon (Table I) with only rare immunoreactive cells located in the duodenum, ileum, and distal colon. GLP-1 producing cells are almost evenly distributed between the crypt and villus. NPY and PP immunoreactive cells are rare in the small intestine but are present in relatively high numbers in the colon (Table I and footnote). Somatostatin and neurotensin immunoreactive cells are present in this strain of mice but their numbers are too few to confidently define their geographic distribution.

It is important to reemphasize (see Materials and Methods) that the distribution of these different enteroendocrine cell types is not affected by the presence of multiple copies of either transgene: there were no qualitative or quantitative differences in the regional distribution of these enteroendocrine populations between any members of the various L-FABP/hGH transgenic pedigrees and their normal littermates. Overall, the geography of the enteroendocrine cell populations in the mouse gut is similar to that reported in humans (3, 6, 18).

L-FABP Is Restricted to a Small Subset of Enteroendocrine Cells

Double labeling methods were used to characterize the enteroendocrine cell population that synthesizes L-FABP. Since serotonin immunoreactive cells are the most abundant enteroendocrine cell type, we used simultaneous double label immunofluorescence methods to initially determine if L-FABP is expressed in serotonin producing enteroendocrine cells. In all normal (as well as transgenic) mice examined, only rare serotonin immunoreactive cells contained L-FABP (Fig. 2, A–C). These cells are largely limited to villi located in the duodenum and proximal jejunum. They repre-
**Table I. Enteroendocrine Cells in the Mouse Gastrointestinal Tract**

|               | Duodenum | Proximal Jejunum | Distal Jejunum | Ileum   | Proximal Colon | Distal Colon |
|---------------|----------|------------------|----------------|---------|----------------|--------------|
| Serotonin     | 72 ± 9   | 80 ± 6           | 40 ± 3         | 44 ± 10 | 120 ± 12       | 131 ± 22     |
| CCK           | 20 ± 2   | 19%              | 19%            | 15%     | 38%            | 36%          |
| GIP           | 15 ± 3   | 22 ± 2           | 24 ± 5         | -       | -              | -            |
| Secretin      | 17 ± 4   | 14 ± 4           | -              | -       | -              | -            |
| GLP-1         | -        | 14 ± 2           | 13 ± 3         | -       | -              | 22 ± 2       |
| NPY (PYY)*    | -        | 43%              | 33%            | -       | 34 ± 8         | 48 ± 4       |
| PP            | -        | 0%               | 0%             | -       | 21 ± 3         | 16 ± 3       |
| hGH           | 85 ± 7   | 58 ± 4           | 30 ± 5         | -       | -              | -            |
| (-4000 to +21)| 11%      | 21%              | 21%            | -       | 20%            | 52%          |
| hGH           | 97 ± 9   | 88 ± 8           | 55 ± 8         | 7 ± 1   | 53 ± 5         | -            |
| (-596 to +21) | 14%      | 17%              | 17%            | 16%     | 25%            | -            |

Distribution of enteroendocrine cells in the adult mouse gastrointestinal tract. (Number of cells per cross section [± SEM]; percentage of cells in crypts.) To quantitate the expression of neuroendocrine products in enteroendocrine cells, 6-16 complete cross sections of the intestine were examined from each of the indicated regions. The number of immunoreactive cells per complete cross section were counted and the results averaged. The location of each immunoreactive cell in either crypt or villus was noted. The percentage of immunoreactive enteroendocrine cells found in the crypts is presented below the mean of the total number of cells per section. A dash indicates that only rare positive cells (<10 cells/cross section) were found in this gut segment. Neurotensin and somatostatin immunoreactive cells were found too infrequently for precise quantitation.

* The peptide species that react with these two antisera have not been definitively established. Antisera raised against PP and NPY frequently show cross-reactivity with the structurally related peptide tyrosine tyrosine (PYY) (7).

sent <5% of the serotonin immunoreactive cells in these regions.

The antisera we had available against L-FABP and the other peptide products of enteroendocrine cells were all raised in rabbits. We therefore used the sequential immunogold-silver-immunofluorescence technique described in Materials and Methods to determine if other, nonserotonergic enteroendocrine cells synthesize L-FABP. We validated this method by incubating sections of intestine with rabbit anti-L-FABP serum followed by rabbit anti-serotonin serum. The resulting patterns of cellular staining were identical to those obtained with the simultaneous double label immunofluorescence technique described above that used goat antisera to serotonin (compare Fig. 2, A–C, to Fig. 2, D–F, and to Fig. 5, E–H).

There was a striking lack of L-FABP coexpression in the other enteroendocrine cell populations surveyed including those which synthesize CCK, GIP, secretin, and GLP-1. Fig. 2, G–I, demonstrates the "typical" result: fluorescently labeled enteroendocrine cells produce a "blank" signal in the L-FABP immunogold-silver-stained sections. (This lack of coexpression was not an artifact of silver deposition into L-FABP producing cells that somehow precluded localization of a second antigen to the "blocked" cell. As noted below, colocalization of other antigen combinations could easily be demonstrated using the same sequential immunogold silver/immunofluorescence method.)

We concluded that the L-FABP positive enteroendocrine cells first noted in our earlier study of normal and transgenic mouse intestine (22), represented a small subpopulation of serotonergic cells and that few, if any, nonserotonergic enteroendocrine cells contain high concentrations of immunoreactive L-FABP. Thus, L-FABP immunoreactivity subdivides the enterochromaffin cell population into two distinct subsets. The function of L-FABP in this small subpopulation of enterochromaffin cells is unclear although it could be involved in mediating the effects of luminal contents (i.e., long chain fatty acids) on synthesis and/or release of their basal granular contents.

**Distribution of hGH Positive Enteroendocrine Cells in Mice Containing L-FABP/hGH Transgenes: Lack of Coexpression of L-FABP and hGH**

Two patterns of cellular staining for hGH were noted in the

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Figure 1. Immunoreactive cell populations in the mouse gastrointestinal tract. A–C are from normal adult C57BL/6J × LT/SV mice. D is from a L-FABP<sup>−/−</sup> hGH transgenic mouse. L-FABP immunoreactivity (A) is found primarily in villus enterocytes; "empty" L-FABP negative goblet and enteroendocrine cells are scattered between the positive villus enterocytes. Enteroendocrine cells are clearly labeled with antisera directed against serotonin (B) or specific peptide products (e.g., CCK in C). In transgenic mice containing L-FABP/hGH fusion genes, two patterns of hGH immunoreactivity are seen in gut epithelial cells. An intense "Golgi-like" pattern of staining is present in enterocytes while a second class of cells, histologically resembling enteroendocrine cells, contain strong, diffuse cytoplasmic staining often with prominent basally oriented granular staining (D). Bar, 25 μm.
Figure 2. Use of multiple label immunocytochemical methods to characterize L-FABP containing enteroendocrine cells. In each set of panels the arrow identifies the enteroendocrine cell of interest. A–C show an L-FABP<sup>-106 to +21</sup>/hGH transgenic mouse. (A) Section of proximal jejunum stained with goat antiserotonin and rabbit anti-L-FABP antisera. The primary goat serum was visualized with fluorescein-labeled donkey anti-goat serum and the primary rabbit serum with Texas red-labeled donkey anti-rabbit serum. The immunoreactive serotonin cell seen in A corresponds to the cell that was not labeled with L-FABP antisera in B. Double exposure of the section in C clearly demonstrates the lack of serotonin/L-FABP coexpression. D–F show an L-FABP<sup>-106 to +21</sup>/hGH transgenic mouse: the lack of serotonin/L-FABP coexpression can also be demonstrated by sequential immunogold silver and immunofluorescence staining techniques. This section of proximal jejunum was first incubated with rabbit anti-L-FABP serum followed by gold-labeled goat anti-rabbit serum and silver development (D). Goat antiserotonin serum was subsequently applied to the section and visualized with fluorescein-labeled donkey anti-goat serum (E). Note that the silver development completely masks the antigenicity of the rabbit primary antiserum-goat secondary antiserum complex. Double exposure of the section shows that the serotonin immunoreactive cell corresponds to an L-FABP negative enteroendocrine cell (F). Identical results are obtained if rabbit antiserotonin serum and Texas red-labeled donkey anti-rabbit serum are substituted for goat antiserotonin serum and fluorescein-labeled donkey anti-goat serum (see Fig. 5, E–H). (G–I) The lack of enteroendocrine cell/L-FABP colocalization is demonstrated here for CCK cells in a normal littermate. A single section of proximal jejunum was first stained with immunogold-silver for L-FABP immunoreactivity (G) followed by rabbit anti-CCK serum and Texas red-labeled donkey anti-rabbit serum (H). The CCK immunoreactive cell corresponds to an L-FABP negative cell (I). J–L show a section of proximal jejunum from a L-FABP<sup>-106 to +21</sup> transgenic mouse stained for L-FABP immunoreactivity using immunogold silver staining (J) followed by goat anti-hGH serum detected by fluorescein-labeled donkey anti-goat serum (K). The cell containing diffuse cytoplasmic hGH immunoreactivity corresponds to a L-FABP negative cell (L). Bar, 25 μm.
intestine epithelium of transgenic mice when viewed by light microscopic techniques. The antisera produced strong signals in the Golgi apparatus of enteroocytes while cells with the histologic appearance of enteroendocrine cells had intense cytoplasmic staining (Fig. 1D). In mice containing the rat \( L-FABP^{-4,000 \to +21}/hGH \) transgene, large numbers of \( hGH \) containing enteroendocrine cells were found in the duodenum and proximal jejum (up to 100 cells/compleate cross section). Their numbers progressively declined in the distal jejum and ileum (see Table I). However, numerous \( hGH \) immunoreactive enteroendocrine cells appeared in the proximal colon (53 cells/cross section compared to 7 cells/cross section in the ileum). <5 cells were encountered per cross section in the distal half of the colon. The percentage of \( hGH \)-containing enteroendocrine cells in crypts was similar from the proximal to distal intestine (14–25%; see Table I). Mice heterozygous for the \( L-FABP^{-4,000 \to +21}/hGH \) transgene showed a more restricted distribution of \( hGH \) immunoreac-

tive enteroendocrine cells along the proximal-to-distal axis of the gut: they had fewer \( hGH \) immunoreactive cells in the distal jejunum and ileum and none in their colon. These proximal-to-distal differences in the distribution of \( hGH \) containing enteroendocrine cells between the different types of transgenic mice were not the result of insertion site effects: each of the two pedigrees surveyed containing the \( L-FABP^{-4,000 \to +21}/hGH \) (or \( L-FABP^{-596 \to +21}/hGH \)) transgene had similar distributions of \( hGH \) immunoreactive cells. Interestingly, the percentage of \( hGH \) immunoreactive enteroendocrine cells along the crypt-to-villus axis was similar in both sets of transgenic mice (Table I).

There was virtually no overlap between \( hGH \) positive immunoreactive enteroendocrine cells and \( L-FABP \) immunoreactive cells in any member of any of the four pedigrees of transgenic mice surveyed (Fig. 2, J–L). This lack of colocalization was evident throughout the entire gastrointestinal tract (i.e., from duodenum to colon) and did not reflect a change in the total number or distribution of \( L-FABP \)-positive enteroendocrine cells in transgenic animals. These observations suggest that cis-acting elements in addition to those contained between nucleotides \(-4,000\) and \(+21\) are required to restrict expression of the reporter \( (hGH) \) to the appropriate small subpopulation of enteroendocrine cells that normally transcribe the intact, endogenous mouse \( L-FABP \) gene.

**Mapping Subtle Differences within Enteroendocrine Cell Populations Using Transgenes**

The data presented above suggested that transgene expression might represent a new way of operationally defining different subpopulations of enteroendocrine cells in the intestine. Double labeling simultaneous immunofluorescence studies were therefore performed on mice containing the \( L-FABP^{-596 \to +21}/hGH \) and \( L-FABP^{-4,000 \to +21}/hGH \) transgenes using goat anti-\( hGH \) serum together with our panel of rabbit antisera prepared against the various peptide and amine products of enteroendocrine cells. The percentage of each enteroendocrine cell type that supported synthesis of \( hGH \) was determined and their location in the duodenal-to-colonic and crypt-to-villus axes of the intestine noted. The results are tabulated in Fig. 3 and illustrated in Figs. 4 and 5.

**Serotonin**

In mice containing either one of the transgenes, \( \sim 30\% \) of the serotonin immunoreactive cells in the duodenum contained \( hGH \) (Fig. 4G). These serotonin/hGH cells were found primarily along villi although occasional double-labeled cells were seen in crypts. The percentage of serotonin immunoreactive cells with colocalized \( hGH \) declined sharply along the length of the small intestine with virtually no overlap present in serotonin cells located in the distal jejunum, ileum, and colon (Fig. 3 and Fig. 4, H–I).

**CCK**

In transgenic pedigrees containing the \( L-FABP^{-596 \to +21}/hGH \) transgene, \( hGH \) was present in \( \sim 95\% \) of CCK immunoreactive enteroendocrine cells. These double labeled cells were found in both crypts and villi. Mice containing the \( L-FABP^{-4,000 \to +21}/hGH \) transgene also had a high percentage (\( \sim 70\% \)) of \( hGH \) expression in their duodenal and prox-
Figure 4. Transgene expression in enteroendocrine cell subpopulations. Multilabeling studies were performed on individual sections using simultaneous immunofluorescence (A–I) and/or sequential immunogold silver/immunofluorescence staining (J–L). (A–C) Section of distal jejunum from a L-FABP -596 to +21/hGH transgenic mouse. The section was coincubated with rabbit anti-GIP serum and goat anti-hGH serum followed by Texas red-labeled donkey anti-rabbit and fluorescein-labeled donkey anti-goat sera. The red GIP immunoreactive cell in A corresponds to the diffusely stained green hGH immunoreactive cell shown in B. Double exposure of the GIP and hGH immunostained section shows the double labeled cell as yellow (C). (D–F) Double labeling of CCK and hGH immunoreactivity in the duodenum (D), proximal jejunum (E), and distal jejunum (F) of a L-FABP-4000 to +21/hGH transgenic mouse. CCK immunoreactive cells without hGH appear red (E, closed arrow) while those that coexpress hGH immunoreactivity appear yellow (E, open arrow). The percentage of CCK immunoreactive cells, which also contain hGH immunoreactivity, decreases along the proximal-to-distal axis of the intestine. In contrast to mice with this type of transgene, mice that contain nucleotides -596 to +21 of the rat L-FABP gene linked to hGH had near total overlap of hGH with CCK throughout the small intestine (see Table I). (G–L) Double staining for hGH and serotonin immunoreactivity in the duodenum (G), distal jejunum (H), and proximal colon (I) of a L-FABP-4000 to +21/hGH transgenic mouse. About 30% of the serotonin immunoreactive cells in the duodenum contained coexpressed hGH immunoreactivity, seen here as a double labeled “yellow cell” in G. There was a striking lack of serotonin/hGH overlap in the remainder of the gastrointestinal tract. Note the lack of overlap in H and I between red serotonin immunoreactive cells and green hGH immunoreactive cells. (J–L) Sequential staining of a proximal jejunum section from a L-FABP-4000 to +21/hGH transgenic mouse using rabbit anti-CCK and rabbit anti-human growth hormone sera. Sections were first incubated with rabbit anti-hGH serum followed by gold-labeled goat anti-rabbit serum and silver development. An intensely labeled hGH immunoreactive cell is seen in J. Sections were subsequently incubated with rabbit anti-CCK and Texas red–labeled donkey anti-rabbit serum (K). Double exposure shows the expression of CCK and hGH immunoreactivity in a single cell (L). By comparing J–L, it’s clear that deposition of silver around the first rabbit antiserum (hGH) does not preclude the immunolocalization of a second antigen (CCK) to the same cell using a second rabbit antiserum. Bar, 25 μm.
Figure 5. Sequential immunogold silver/simultaneous immunofluorescence staining demonstrating L-FABP-hGH-enteroendocrine cell relationships. Sections from the proximal (A–H) and distal (I–L) jejunum of a transgenic mouse containing the L-FABP–596 to +21/hGH fusion gene were first incubated with a rabbit primary antiserum. Antigen–antibody complexes were detected using the immunogold silver technique. This was followed by coincubation of the silver-stained section with a new primary rabbit antiserum and a primary goat antiserum. These antisera were detected using fluorescein-labeled donkey anti–goat and Texas red–labeled donkey anti–rabbit sera. (A–D) Multilabeling using rabbit anti-L-FABP (A), rabbit antisecretin (B), and goat anti-hGH (C) sera. The triple exposure of this section shows colocalization of secretin and hGH immunoreactivity in an L-FABP negative cell (D) indicated by an arrow. A second hGH positive cell, indicated by an arrowhead, lacks both L-FABP and secretin immunoreactivity. (E–H) Multilabeling using rabbit anti-L-FABP (E), rabbit antiserotonin (F), and goat anti-hGH (G) sera. Triple exposure (H) shows a total lack of overlap between serotonin immunoreactive cells, indicated by arrows, an hGH immunoreactive cell, indicated by an arrowhead, and the L-FABP immunoreactive enterocytes seen in E. (I–L) Multilabeling using rabbit anti-CCK (I), rabbit antiserotonin (J), and goat anti-hGH (K) sera. Triple exposure shows that the CCK and hGH immunoreactivity is coexpressed in a cell, indicated by an arrow, which lacks serotonin immunoreactivity (L). The serotonin immunoreactive cell indicated by the arrowhead in J and L lacks both CCK and hGH immunoreactivity. Bar, 25 μm.

imal jejunal CCK enteroendocrine cells. However, the percent colocalization declined sharply as one proceeded along the proximal to distal axis of the small intestine, reaching a value of 30% in the distal jejunum and ileum (Fig. 3 and Fig. 4, D–F, and J–L). The decreased colocalization affected both villus- and crypt-associated CCK immunoreactive cells.

Gastric Inhibitory Peptide

Virtually all GIP immunoreactive cells were found to contain hGH regardless of their location in the proximal-to-distal or crypt-to-villus axis of the gut or the L-FABP 5' nontranscribed region represented in the fusion gene (Fig. 3, and Fig. 4, A–C).

Secretin

In transgenic pedigrees containing the L-FABP–596 to +21/hGH transgene, nearly all secretin immunoreactive enteroendocrine cells were also positive for hGH. Addition of nucleotides −597 to −4,000 of the rat L-FABP gene, decreased the percent colocalization of secretin and hGH to ~60% in both the duodenum and proximal jejunum (Fig. 3, and Fig. 5, A–D). Since secretin producing enteroendocrine cells are not found in crypts, this reduction in colocalization only affected villus-associated cells.

GLP-1

For transgenic mice with nucleotides −596 to +21 of the L-FABP gene, nearly 100% of the GLP-1 immunoreactive cells located in the proximal jejunum contained immunoreactive hGH. Addition of nucleotides −4,000 to −597 reduced this number to 70% (Fig. 3). GLP-1/hGH positive cells were present in small intestinal crypts and villi in all pedigrees of transgenic mice surveyed. Since transgenic mice containing the additional 3.3 kb from the 5' nontranscribed domain of L-FABP do not express hGH in their large intestine, GLP-1 immunoreactive cells located in their colon did not contain any detectable hGH.

PP and NPY

PP and NPY cells are largely limited to the colon (Table I). Therefore, we observed no colocalization of PP or NPY with hGH in mice containing the L-FABP–4,000 to +21/hGH transgene. By contrast, mice containing the L-FABP–596 to +21/hGH
transgene displayed virtually 100% colocalization of hGH with PP and NPY in their proximal colons (Fig. 3). Colocalization was rarely encountered in PP and NPY enteroendocrine cells located in the distal half of the colon.

**Somatostatin and Neurotensin**

Although there are only a few somatostatin and neurotensin cells noted in the mouse intestinal tract, colocalization of hGH immunoreactivity in neurotensin positive cells was frequently seen in all pedigrees of transgenic mice. Both hGH positive and negative somatostain immunoreactive cells were found in mice containing either of the two different types of transgenes. Precise quantitation was not possible because of the limited number of somatostatin and neurotensin producing enteroendocrine cells available for scoring.

The results of triple labeling studies further illustrate the interrelationships of these cell types and more specifically the value of using transgenes to operationally define subpopulations of enteroendocrine cells. As predicted from the “individual” double label comparisons, these triple labeling studies directly show that L-FABP immunoreactivity is absent in hGH positive and negative enteroendocrine cell subsets (Fig. 5, A–D and E–H). They also underscore the heterogeneity between enteroendocrine cell populations as defined by their ability to support transgene expression (see Fig. 5, I–L).

**Discussion**

Our studies of hGH expression in the enteroendocrine cell population of adult transgenic mice containing L-FABP/hGH fusion genes indicate that transgene expression represents a new way to define populations of this terminally differentiated cell type that is different from previous methods/schemes. Immunocytochemical studies of the colocalization of hGH in cells that produce different neuroendocrine products reveals an unexpected degree of complexity in the differentiation of this remarkably heterogeneous but geographically well-organized gut epithelial cellular population.

Our initial survey of the geographic organization of the enteroendocrine cell population in normal adult mouse intestine indicated that each cell type (defined by its neuroendocrine product) had an easily discernible regional distribution along the proximal to distal axis of the gut. For example, PP-immunoreactive cells are essentially limited to the colon. CCK and GIP producing enteroendocrine cells are numerous in the small intestine but are rarely found in the colon. Within the small intestine, both CCK and GIP populations exhibit a sharp, decreasing gradient from proximal jejunum to ileum.

Some enteroendocrine cell types not only display differences in their distribution from duodenum-to-colon but also from crypt-to-villus tip. In the proximal jejunum, nearly all enteroendocrine cells that produce secretin are found in villi. By contrast, almost half of the GLP-1 immunoreactive cells in this region of the small bowel are found in the crypt while other enteroendocrine cell types (e.g., those producing CCK and GIP) show an intermediate percentage distribution between crypt and villus.

The factors that permit a given enteroendocrine cell type to differentiate in the “appropriate” region of the crypt-to-villus and duodenal-to-colonic axes of the gut are unknown. Maintenance of a precise geographic distribution of a given enteroendocrine cell type is all the more remarkable given the fact that the intestinal epithelium undergoes perpetual renewal. Although titrated thymidine labeling studies performed by Cheng and Leblond (2) suggested that enteroendocrine cells are derived from the same multipotent stem cells as the other principal, terminally differentiated, cell types of the gut epithelium, definitive data about their lineage relationships are still lacking (e.g., see reference 9).

Analysis of the L-FABP producing enteroendocrine cell population has provided some interesting clues about elements that are necessary to achieve appropriate enteroendocrine cell-specific expression. L-FABP appears to be restricted to a very small subpopulation of serotonin producing enteroendocrine cells located in the duodenal and proximal jejunal villi. Studies of transgenic mice indicate that while nucleotides −4,000 to +21 of the L-FABP gene contain cis-acting sequences that can direct expression of a reporter (hGH) to enterocytes, they lack those elements which limit L-FABP expression in enteroendocrine cells. In other words, distinct cis-acting sequences appear to be responsible for regulating transcription of this gene in enterocytes and enteroendocrine cells.

Functional mapping studies of the L-FABP gene allowed us to determine that different portions of its 5' nontranscribed region will direct different patterns of reporter expression within a given enteroendocrine cell population. In some instances, these differences appear to be related to the region of the intestine where the enteroendocrine cell subpopulation is located. For example, in transgenic pedigrees containing the L-FABP−596 to +21/hGH transgene, the hGH reporter was coexpressed in most or all (70-100%) GLP-1, CCK, secretin, and GIP-immunoreactive cells situated in the small intestine. A similar degree of coexpression was found for GLP-1, PP, and NPY immunoreactive cells located in the proximal colon. Although these latter two types of enteroendocrine cells are also present in the distal colon, few contained hGH. There was also region-dependent coexpression of hGH in serotonin producing enteroendocrine cells: in the duodenum and proximal jejenum ~10-30% of the serotonin immunoreactive cells contained hGH but the percent colocalization declined sharply along the length of the intestine with virtually no overlap present in the ileum and colon. Thus, analysis of these pedigrees of transgenic mice indicate that for the GLP-1, PP, NPY, and serotonin producing enteroendocrine cell populations, cis-acting sequences present between nucleotides −596 and +21 of the rat L-FABP gene are sufficient to restrict hGH expression in some regions of the intestine but not in others. This transgene thus subdivides these once apparently homogeneous enteroendocrine cell types (as defined by their neuroendocrine product) into subsets (now defined by their ability to support reporter expression).

A further level of complexity was revealed when we noted that addition of nucleotides −597 to −4,000 of the rat L-FABP gene affected expression of hGH in some, but not all, enteroendocrine cells. For example, its cis-acting elements did not alter the percentage of serotonin or GIP immunoreactive cells that synthesized hGH. They did, however, limit hGH expression to a smaller proportion of GLP-1, CCK, and secretin containing cells in the small intestine. Different degrees of restriction of hGH expression were documented within different regions of the small intestine. These upstream DNA
sequences reduced hGH coexpression in proximal jejunal GLP-1 cells slightly (from 100% to 70%) while in the proximal colon the percent colocalization of hGH and GLP-1 decreased from nearly 100% to essentially zero. A similar proximal-to-distal axis-dependent decrease in colocalization of CCK and hGH was also found within the small intestine. While the addition of nucleotides −4,000 to −597 produced some marked differences in the expression of hGH within certain enteroendocrine subsets located in different regions of the proximal to distal axis of the gut, they did not alter reporter expression along the crypt-to-villus axis.

Overall, our analyses of hGH expression in these enteroendocrine cell populations have uncovered subtle differences in expression of the two types of L-FABP/hGH transgenes. This allows us to define three subsets of enteroendocrine cells based on the ability of nucleotides −597 to −4,000 to restrict hGH expression: (a) those cells unaffected by the additional cis-acting elements (GIP and serotonin); (b) those enteroendocrine cells showing a reduction in transgene expression independent of their location in the proximal-to-distal axis of the intestine (secretin, NPY, and PP) and (c) those cells in which transgene expression is dependent upon their location along the duodenal-to-colonic axis (CCK and GLP-1).

In summary, expression of L-FABP/hGH transgenes in different mouse enteroendocrine cell populations has revealed a heterogeneity in their regulatory environments which had not been previously appreciated. The results confirm that the 5′ nontranscribed region of the rat L-FABP gene is a sensitive indicator of these differences. Differences that could reflect regional and cell-specific variations in the levels of as yet undefined positive or negative trans-acting factors. The data emphasize that transgenes represent powerful probes not only for examining the relationships between enteroendocrine cell subsets (I), but also for analyzing the molecular mechanisms that establish and maintain the enormously complex patterns of cellular differentiation in the continuously proliferating intestinal epithelium.

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