

Use of Transgenic Mice to Study the Routing of Secretory Proteins in Intestinal Epithelial Cells: Analysis of Human Growth Hormone Compartmentalization as A Function of Cell Type and Differentiation

Jeffrey F. Trahair, Marian R. Neutra, and Jeffrey I. Gordon

Department of Anatomy and Cellular Biology and Harvard Digestive Diseases Center, Harvard Medical School, Boston, Massachusetts 02115; and Departments of Medicine, Biochemistry, and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. The intestinal epithelium is a heterogeneous cell monolayer that undergoes continuous renewal and differentiation along the crypt-villus axis. We have used transgenic mice to examine the compartmentalization of a regulated endocrine secretory protein, human growth hormone (hGH), in the four exocrine cells of the mouse intestinal epithelium (Paneth cells, intermediate cells, typical goblet cells, and granular goblet cells), as well as in its enteroendocrine and absorptive (enterocyte) cell populations. Nucleotides -596 to +21 of the rat liver fatty acid binding protein gene, when linked to the hGH gene (beginning at nucleotide +3) direct efficient synthesis of hGH in the gastrointestinal epithelium of transgenic animals (Sweetser, D. A., D. W. McKeel, E. F. Birkenmeier, P. C. Hoppe, and J. I. Gordon. 1988. *Genes & Dev.* 2:1318-1332). This provides a powerful *in vivo* model for analyzing protein sorting in diverse, differentiating, and polarized epithelial cells.

Using EM immunocytochemical techniques, we demonstrated that this foreign polypeptide hormone entered the regulated basal granules of enteroendocrine

cells as well as the apical secretory granules of exocrine Paneth cells, intermediate cells, and granular goblet cells. This suggests that common signals are recognized by the "sorting mechanisms" in regulated endocrine and exocrine cells. hGH was targeted to the electron-dense cores of secretory granules in granular goblet and intermediate cells, along with endogenous cell products. Thus, this polypeptide hormone contains domains that promote its segregation within certain exocrine granules. No expression of hGH was noted in typical goblet cells, suggesting that differences exist in the regulatory environments of granular and typical goblet cells. In enterocytes, hGH accumulated in dense-core granules located near apical and lateral cell surfaces, raising the possibility that these cells, which are known to conduct constitutive vesicular transport toward both apical and basolateral surfaces, also contain a previously unrecognized regulated pathway. Together our studies indicate that transgenic mice represent a valuable system for analyzing trafficking pathways and sorting mechanisms of secretory proteins *in vivo*.

EXOCRINE cells share with endocrine and neuronal cells the ability to store and concentrate specific secretory products in membrane-limited cytoplasmic granules whose release is regulated by external secretagogues. These secretory cells also can export other products, including plasma membrane components, that are not stored or externally regulated, but are transported rapidly to the cell surface in small vesicles. The terms "regulated" and "constitutive" have been applied to the former and latter pathways, respectively, referring to the two modes of release (for review, see Kelly, 1985; Burgess and Kelly, 1987).

A few model cultured cell systems have been used to examine the mechanisms underlying entry of secretory protein into these two pathways. For example, Kelly, Moore and col-

leagues have transfected the mouse pituitary cell line AtT-20, and the rat pheochromocytoma-derived cell line PC-12, with genes encoding proteins that normally enter the regulated pathway in their cells of origin (Moore et al., 1983; Moore and Kelly, 1985; Schweitzer and Kelly, 1985; Burgess et al., 1985). They demonstrated that these cells can package both polypeptide hormones and an exocrine cell product (pancreatic trypsinogen) into their secretory granules (for review, see Kelly, 1985; Burgess and Kelly, 1987). One foreign protein, kappa light chain (that is normally secreted constitutively) failed to enter regulated granules in AtT-20 cells (Matsuuchi et al., 1988). On the other hand, transfection experiments in one of our laboratories showed that human (pro)apolipoprotein A-1, a constitutive secretory product of

hepatocytes and enterocytes, was sorted primarily into the regulated pathway of AtT-20 cells (Fennewald et al., 1988). This raises questions about the validity of using results obtained in these model cell systems to define the mechanisms that regulate protein sorting into regulated and constitutive pathways in cells *in vivo*.

There is evidence that some type of positive signal present in polypeptide hormones is required for their selective sorting into the regulated granule (Moore and Kelly, 1986). The observation that a regulated exocrine cell product was directed into stored, dense-core granules in endocrine cells (Burgess et al., 1985) implies that regulated exocrine and endocrine proteins share the putative molecular sorting signal(s), and that exocrine cells may use the same sorting mechanism as do endocrine and neuronal cells to direct proteins that bear this signal into their regulated secretory granules (Kelly, 1985). If this hypothesis is correct, then exocrine cells should recognize and sort foreign endocrine cell products into storage granules, but this has not been tested using transfected cells. Moreover, hypotheses derived from examination of model cultured cells concerning protein sorting have not been independently audited in other systems.

Transgenic mice offer a promising alternative to *in vitro* systems, and they have the advantage of providing expression of genes encoding foreign secretory proteins in normal, highly polarized epithelial cells present in complex tissues. A study of the control of gene expression in the pancreas of transgenic mice showed that the elastase gene promoter could direct specific expression of a reporter, human growth hormone (hGH)¹, in pancreatic acinar cells (Ornitz et al., 1985). Using light microscopic immunocytochemistry, these workers noted that hGH was concentrated in the apical cytoplasm, presumably in zymogen granules, but they could not determine to what degree hGH entered other compartments with light microscopy methods. Nor was it determined how the foreign protein would have been sorted by other types of exocrine, endocrine, and nonregulated secretory cells in the same animal.

The intestinal epithelium of adult mice consists of diverse cell types of differing polarities, with both regulated and constitutive secretory pathways. Moreover, this epithelium undergoes perpetual proliferation and differentiation along the crypt-villus axis (Cheng and Leblond, 1974; Leblond and Cheng, 1976; Potten and Hendry, 1983; Simon et al., 1979). Transgenic mice have recently been generated in one of our laboratories that carry fusion genes consisting of a portion of the 5' nontranscribed region (nucleotides -596 to +21) of the rat "liver" fatty acid binding protein (L-FABP) gene linked to the gene encoding hGH (Sweetser et al., 1987, 1988a, 1988b). In these animals, specific expression of hGH was observed in epithelial cells throughout the intestine. This provided for the first time a system in which the fate of a single foreign gene product could be analyzed and compared in multiple polarized cell types at various stages of differentiation (Gordon, 1989). Light microscopy immunoperoxidase labeling of this epithelium revealed hGH expression in enterocytes that have both apical and basolateral constitutive secretory pathways and also in entero-endocrine cells, a heterogeneous cell population that conducts regulated secretion of peptide hormones and neurotransmitters toward their

1. *Abbreviations used in this paper:* hGH, human growth hormone; L-FABP, liver fatty acid binding protein.

basal cell surfaces (Sweetser et al., 1988a). The intracellular compartments that contained the foreign gene product could not be resolved in these preparations, however, and hGH was not detected in any of the four exocrine cell types in the epithelium.

We therefore applied high resolution EM immunocytochemical methods to determine to what extent hGH is expressed in intestinal exocrine cells in these mice, and to visualize directly the secretory pathways that hGH enters in the various polarized cells of the epithelium. Our results reveal that the L-FABP promoter directs expression of hGH in intestinal exocrine cells as well as endocrine cells and enterocytes. In both endocrine and exocrine cells, the foreign hormone is concentrated in regulated secretory granules despite their opposite polarities. This is consistent with the idea that exocrine cells use the same or similar sorting mechanisms as do endocrine and neuronal cells to direct selected proteins into the regulated secretory pathway.

Materials and Methods

Four transgenic mice were used in this study, all derived from founders 13 and 19 described in Sweetser et al., 1988a. 4-8-mo-old adult males of the F 2-4 generation were examined, and all specimens showed the same patterns of hGH distribution in intestinal cells. This pedigree contained nucleotides -596 to +21 of the rat L-FABP gene linked to the hGH gene beginning at nucleotide +3 (Fig. 1). Mice of this pedigree carry ~490-530 copies of the hGH gene, and show serum hGH levels up to 548 μ g/ml. Mice were maintained on a standard chow diet and strictly cycled light conditions (0600-1,800 lights on). They were killed by cervical dislocation, and their tissues were rapidly excised and fixed.

Light Microscopy Immunocytochemistry

Intestinal mucosal samples were fixed in Bouin's fluid, embedded in paraffin, and sectioned in 5 μ m. Sections were incubated with nonspecific goat anti-hGH serum (Daughaday et al., 1987) followed by HRP-conjugated anti-goat IgG, and a peroxidase-antiperoxidase conjugate (Sternberger, 1979).

To determine optimal fixation conditions for EM immunocytochemistry of hGH, samples of small intestinal mucosa were fixed in three solutions: PLP (periodate-lysine-2% paraformaldehyde; McLean and Nakane, 1974); 2% formaldehyde and 0.2% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4; or 0.2% picric acid and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. After 18-h fixation at 23°C, tissues were rinsed in the appropriate buffer, infiltrated with 1 M and 2.3 M sucrose (for 5 h each), mounted with OCT embedding compound on metal supports, frozen in partially solidified Freon 22, and stored in liquid nitrogen. Cryosections (1 μ m) were stained with the goat anti-hGH serum described above, followed by rabbit anti-goat IgG coupled to rhodamine (Hyclone Laboratories, Logan, UT). Specific hGH immunoreactivity was equally well preserved after all three fixatives (not shown).

EM Immunocytochemistry

The 2% formaldehyde/0.2% glutaraldehyde fixative provided the best ultrastructural preservation in tissues embedded in LR Gold resin (Polysciences Inc., Warrington, PA). Thin sections were mounted on nickel grids previously coated with formvar and carbon, and nonspecific binding sites were blocked with 1-10% nonimmune serum of the same species as the secondary antibody, (shown to be devoid of anti-hGH immunoreactivity by ELISA). Two primary antibodies were used at 1:100 dilution, with identical results: the goat anti-hGH serum described above, and rabbit anti-hGH (Dako Corp., Santa Barbara, CA). Anti-hGH immunoreactivity was visualized either with protein A 10 nm colloidal gold (Janssen Life Science Products, Piscataway, NJ), or with anti-goat or anti-rabbit antibodies conjugated to 5 or 10 nm colloidal gold (Life Science Products Sigma Chemical Co., St. Louis, MO).

To establish the specificity of anti-hGH immunoreactivity in small intestinal cells of transgenic mice, two control preparations were examined in parallel with all immunolabeled grids. First, primary anti-hGH antiserum

Rat L-FABP/Human Growth Hormone Transgene

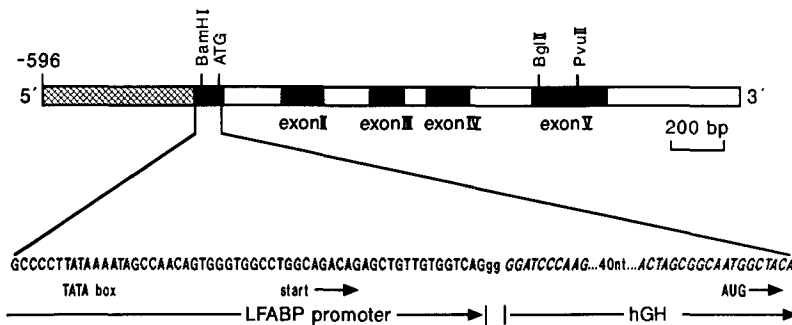


Figure 1. Schematic diagram of the L-FABP-hGH fusion gene. The nucleotide sequence of the L-FABP-hGH junction is shown. Two guanosine residues derived from the M13 vector DNA are noted by lowercase letters. For additional details, see Sweetser et al., 1988a.

was replaced by nonimmune or preimmune serum. Second, goat anti-hGH serum was twice preabsorbed with purified hGH (Sigma Chemical Co.) coupled to Sepharose 4B, a procedure that removed at least 95% of anti-hGH activity as assessed by ELISA, before EM immunolabeling.

Results

Cell Types of the Mouse Intestinal Epithelium

The cell types that comprise the small intestinal epithelium of the mouse have been described elsewhere (Cheng and Leblond, 1974) and are illustrated diagrammatically in Fig. 2 A. The crypt base contains exocrine Paneth cells with large, dense apical granules, undifferentiated cells (including both multipotent, proliferative cells and undifferentiated enterocytes), and various types of entero-endocrine cells whose small, dense secretory granules accumulate basally. The wall of the crypt is populated by differentiating and differentiated cells that are in the process of upward migration toward the villus, including crypt enterocytes, entero-endocrine cells, and three distinct exocrine cell types: intermediate cells, granular goblet cells, and typical goblet cells.

The four exocrine cell types in the epithelium contain distinct secretory granules that are readily distinguished by EM. *Paneth cells* synthesize multiple secretory proteins including lysozyme (Lopez-Lewellyn and Erlandsen, 1980) and defensin (Ouellette et al., 1989) and package them in large, dense apical granules that are rapidly released in response to acetylcholine (Troughton and Trier, 1969). *Intermediate cells*, present primarily in the crypts and lower villi, contain large, apical secretory granules typical of regulated exocrine cells, but the factors regulating their secretion have not been defined (Troughton and Trier, 1969; Calvert et al., 1988). The large dense central region of the granule matrix presumably contains protein but does not include Paneth cell products; the fibrillar peripheral portion of the granule content contains glycoconjugates but fails to label with typical mucin markers (Calvert et al., 1988). *Typical goblet cells* are characterized by large, closely packed apical secretory granules containing a highly glycosylated mucin product whose release, at least in crypts, is regulated by acetylcholine (Neutra and Forstner, 1987). A second population of mucin-producing cells in the mouse epithelium, the *granular goblet cells*, are distinguished from typical goblet cells by the presence of a single small dense core embedded within the mucin matrix of each granule (Merzel and Leblond, 1969; Cheng and Leblond, 1974). The nature of the material in the core is not known, but its electron density suggests that it consists of aggregated, nonmucin protein products.

Fig. 2 B shows a typical paraffin section of small intestinal mucosa from a transgenic mouse containing the L-FABP promoter-hGH fusion gene, and expressing the foreign hormone along the crypt-villus axis. Light microscopic immunocytochemistry shows that highest levels of intracellular hormone were present in entero-endocrine cells. hGH immunoreactivity was also prominent in villus absorptive cells, concentrated in the supranuclear Golgi complex. The apical mucin granules of villus goblet cells did not contain detectable levels of hGH in the HRP-labeled preparation shown in Fig. 2 B, but in immunofluorescently stained frozen semithin sections, some goblet cells were hGH-positive (data not shown). We were unable to distinguish granular goblet cells from typical goblet cells and intermediate cells in HRP-labeled or fluorescently-labeled preparations.

Ultrastructural Localization of hGH in Entero-endocrine Cells

The majority of entero-endocrine cells, both in crypts and on villi, possessed abundant, immunoreactive hGH concentrated in the basal dense granules that contain the cell's regulated hormonal product (Fig. 3 A). hGH was also detected in Golgi cisternae in these cells. The morphology of hGH-containing granules varied among cells, indicating that multiple endocrine cell types expressed the hormone. Control sections exposed to anti-hGH serum that had been depleted of specific antibody by preabsorption with pure hGH, showed no labeling of endocrine cell granules (Fig. 3 B). Other control preparations (see Materials and Methods) also showed no immunolabeling above background in any epithelial cell type, confirming that immunoreactivity of granule content was due specifically to the presence of hGH.

Distribution of hGH in Exocrine Cells

Paneth cell granules are filled with a homogeneous material that showed artifactual shrinkage with the fixation and embedding protocol used here (Fig. 4). Concentrations of anti-hGH serum that produced intense, specific staining of endocrine cell granules, produced much sparser labeling of Paneth cell granules; however, the control preparations described above confirmed that this label was hGH-specific. The very sparse hGH immunoreactivity observed over rough ER and the light labeling of Golgi cisternae confirmed that Paneth cells synthesize hGH, but at relatively low levels.

The pattern of anti-hGH immunoreactivity in intermediate cells was similar to that in Paneth cells. In these cells, hGH

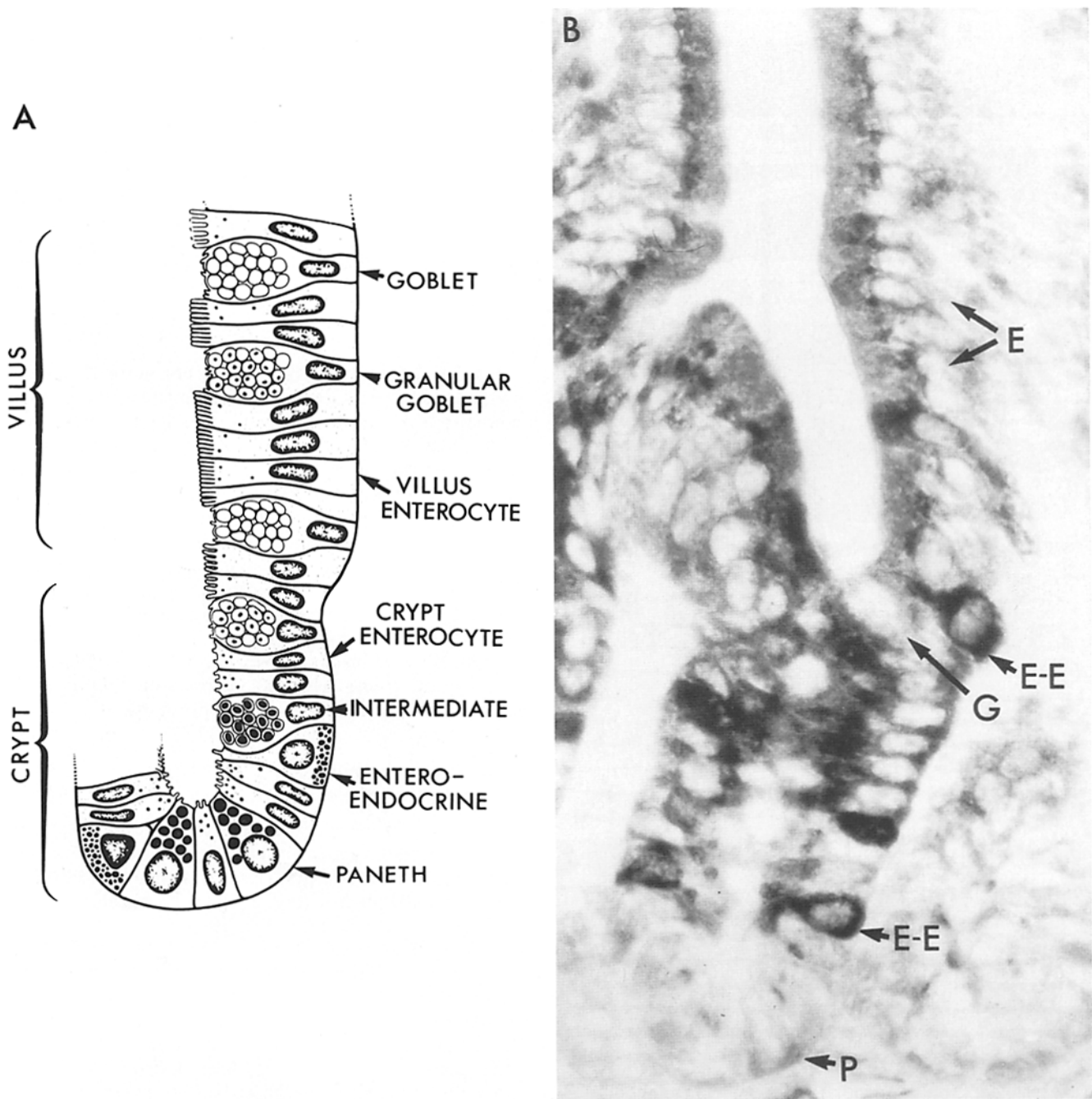


Figure 2. *A*, Diagrammatic illustration of the polarized cell types of the mouse small intestinal epithelium. A portion of a crypt-villus axis is shown. Enterocytes are present in the crypt and on the villus. Entero-endocrine cells contain basally oriented secretory granules. There are four distinct exocrine cell types: Paneth cells, intermediate cells, granular goblet cells, and typical goblet cells. *B*, Expression of hGH in the small intestine of a transgenic mouse. 5- μ m paraffin section immunostained with anti-hGH and peroxidase-antiperoxidase. Both crypt and villus enterocytes (*E*) show hGH immunoreactivity; in villus cells, hGH is concentrated in the supranuclear Golgi region. Entero-endocrine cells (*E-E*) also contain abundant hGH. In this preparation, Paneth cells (*P*) and goblet cells (*G*) appear negative, and other exocrine cell types are not identified. Bar, 100 μ m.

was concentrated in the dense central material of apical secretory granules while the peripheral glycoconjugate-containing "halo" was devoid of hGH (Fig. 5).

Typical goblet cells in these preparations were devoid of hGH immunoreactivity: their apical granules, Golgi complex and rough ER were not labeled by the procedures used here (data not shown). Granular goblet cells, in contrast, were

consistently labeled. This cell type was recognized by the small electron-dense "cores" contained within the mucin matrix of its apical secretory granules (Fig. 6 *A*). hGH immunoreactivity was associated with the dense cores, but not with the mucin matrix (Fig. 6 *B*). The supranuclear Golgi region of a granular goblet cell is shown in Fig. 6 *C*. Golgi cisternae and associated small dilations or vesicles were

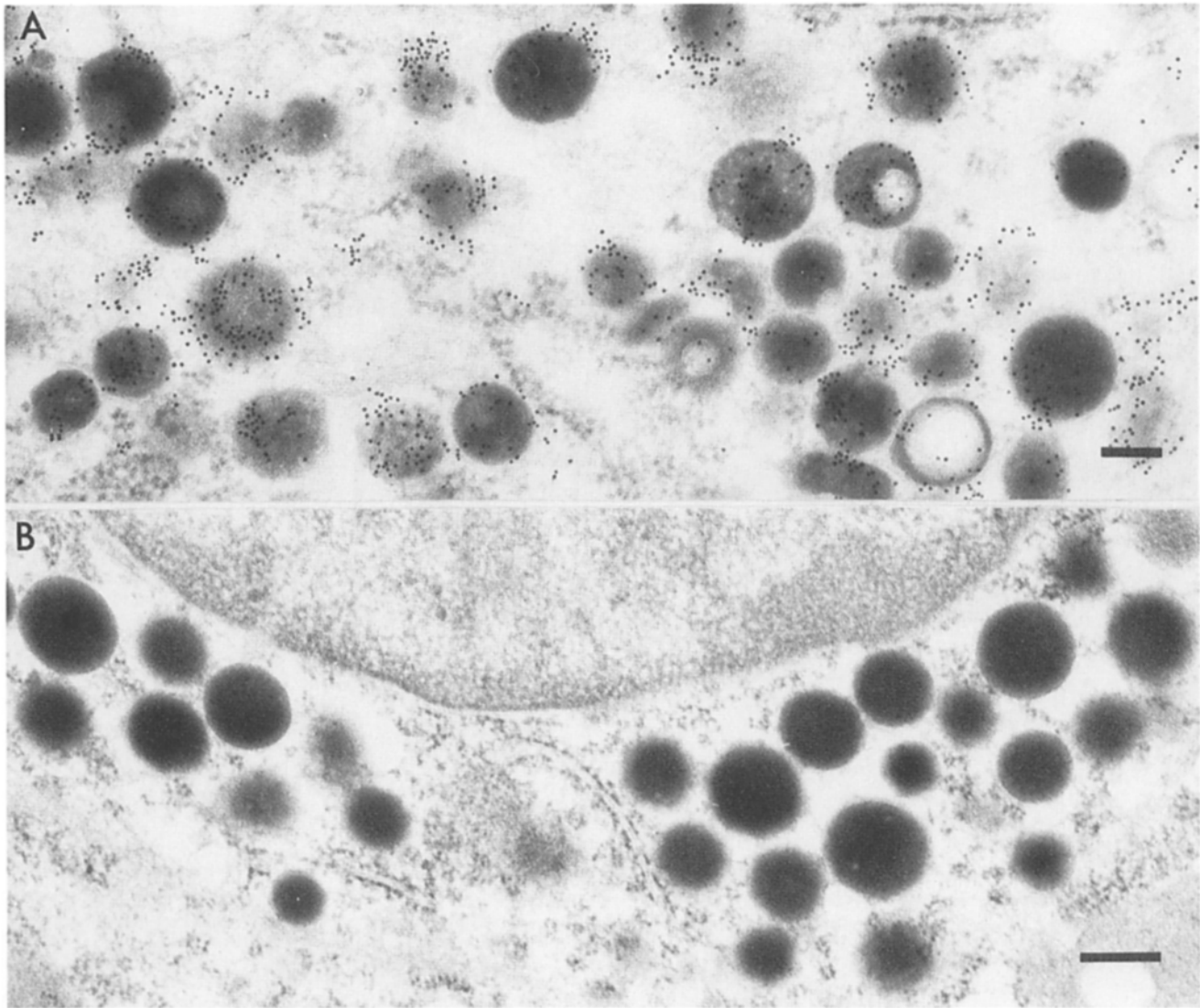


Figure 3. Immunolocalization of hGH in basal secretory granules of entero-endocrine cells. *A*, Immunolabeling with goat anti-hGH and secondary antibody: 10-nm colloidal gold shows that hGH is concentrated in the dense granule content. *B*, Control section treated as in *A*, but using goat anti-hGH serum preabsorbed with purified hGH, shows no label. Bar, 0.2 μm .

hGH-positive. In mucin granules adjacent to the Golgi complex, hGH-positive dense cores were embedded in material of intermediate density that also showed hGH immunoreactivity. The electron-lucent periphery of these granules, like that of mature granules, was unlabeled.

Distribution of hGH in Enterocytes

Enterocytes have no known exocrine secretory product. These cells were examined with two principal questions in mind. First, could we deduce the pathways of export of hGH in these cells by defining its distribution in intracellular compartments? Second, does compartmentalization of hGH change as enterocytes differentiate along the crypt-villus axis? In terminally-differentiated enterocytes situated on villi, stacked Golgi cisternae and clear, Golgi-associated vesicles contained abundant hGH immunoreactivity (Fig. 7 *A*). These vesicles were much more abundant in the enterocytes of transgenic mice than in those of normal mice. Such a change may be related to the very high levels of hGH expression pro-

duced by the L-FABP promoter, plus the high copy number of the transgene (Sweetser et al., 1988*a*). The lack of concentrated content in these vesicles and the fact that they did not accumulate in the cytoplasm outside the Golgi region suggests that their contents were constitutively released.

In some Golgi complexes, small vesicles with dense content also contained hGH (Fig. 7 *B*). These hGH-positive dense vesicles were commonly observed near the apical cell membrane (Fig. 7 *C*), and occasionally, near the lateral membrane. Accumulation of hGH in these small, dense-core vesicles that are a normal feature of enterocytes suggests that a previously unrecognized regulated secretory pathway may operate in these cells. hGH immunoreactivity was also present in the lateral intercellular spaces (not shown) and this may have been derived from secretion of the clear Golgi-associated vesicles. With these methods, however, we could not positively identify the vesicles destined for fusion with the lateral cell surface. In crypt enterocytes, the level of hGH in Golgi cisternae was lower than in villus cells and relatively

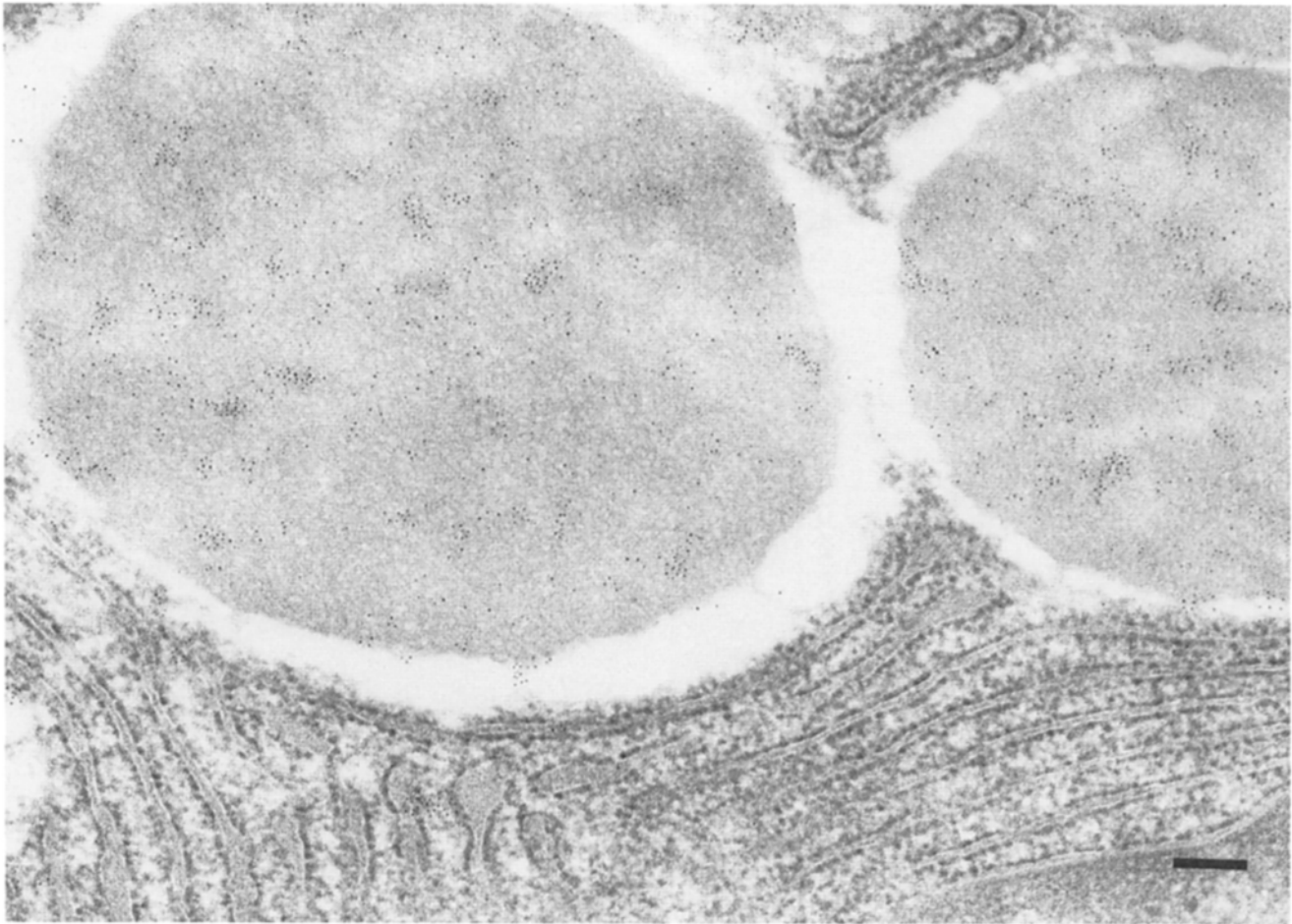


Figure 4. Immunolabeling of a Paneth cell with goat anti-hGH and secondary antibody, 5 nm gold. hGH is present throughout the contents of apical secretory granules. Clear space surrounding granule content is because of shrinkage during tissue processing. Bar, 0.2 μm .

few clear Golgi-associated vesicles were observed, but hGH again appeared in lateral intercellular spaces. Apical dense core vesicles were abundant, as they are in normal crypt cells (Madara and Trier, 1987) and these were consistently hGH-positive (Fig. 8).

Discussion

We have shown that transgenic mice containing a gastrointestinal tract-specific promoter linked to the gene encoding hGH provide a valuable model system for examining the sorting and intracellular transport of secretory proteins in diverse, highly polarized intestinal epithelial cells. This foreign polypeptide hormone was sorted into basal granules of polarized entero-endocrine endocrine cells, as expected. In addition, hGH, an endocrine cell product, entered regulated apical secretory granules in three types of exocrine cells. Finally, we demonstrated accumulation of hGH in dense-core granules of enterocytes, suggesting that these cells possess a hitherto-unrecognized regulated secretory pathway.

The Use of Transgenic Mice to Study Protein Sorting in the Intestinal Epithelium

The intestinal epithelium is a continuously regenerating system that arises from pluripotent stem cells that are function-

ally anchored deep in the crypt. Although the cells of each crypt and the adjacent villus surfaces represent a clone derived from a single progenitor cell (Ponder et al., 1985), the process of differentiation results in an epithelium containing several types of entero-endocrine cells and four types of exocrine cells in addition to crypt and villus enterocytes (absorptive cells) (Cheng and Leblond, 1974; Madara and Trier, 1987). This heterogeneous system of highly polarized cells has not been reproduced in monolayer culture (Neutra and Louvard, 1989). Thus, it has not been possible to use *in vitro* gene transfection methods to investigate patterns of protein sorting in its diverse secretory cells. The regulated secretory pathway of exocrine cells has been especially difficult to reproduce *in vitro*, since normal formation, storage, and secretion of exocrine granules requires prior establishment of a specialized apical membrane domain and other conditions that have been achieved only recently in monolayer cultures of neoplastic pancreatic acinar cells (Logsdon et al., 1984; Ingber et al., 1986) and neoplastic intestinal goblet cells (Huet et al., 1987; Phillips et al., 1988). In this study, we demonstrate the ability of nucleotides -596 to $+21$ of the rat L-FABP promoter to direct efficient expression of a foreign protein not only in polarized endocrine and absorptive cells of the intestinal epithelium of transgenic mice, but also in three of the four exocrine cell types in this epithelium. Moreover, expression is not limited by this promoter to ter-

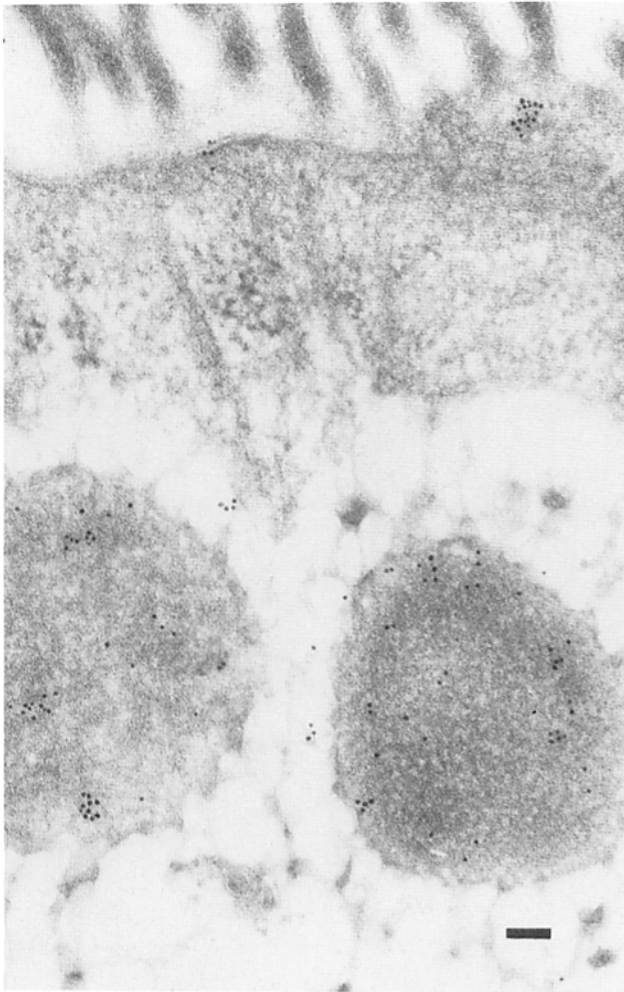


Figure 5. Immunolocalization of hGH in apical secretory granules of an intermediate cell. hGH is concentrated in the dense central material of each granule, but is not present in the fibrillar peripheral material that is known to contain glycoconjugates. Granule membranes are not visualized in these preparations. Bar, 0.1 μm .

minally differentiated cells: efficient expression was also noted in cells of the crypts. Because these ~ 600 nucleotides appear to be able to direct "global" expression of foreign proteins in the gut, they are ideally suited for *in vivo* analysis of protein sorting.

Entero-endocrine Cells

Entry of hGH into regulated granules of entero-endocrine cells shows that polarized epithelial endocrine cells have the capacity for recognizing, sorting, and storing regulated polypeptide hormones, as previously demonstrated in cultured nonpolarized endocrine cells derived from nonepithelial tissues (for review, see Kelly, 1985; Burgess and Kelly, 1987). Over six types of entero-endocrine cells have been identified in mouse small intestinal epithelium, each with a distinct granule morphology and hormone product (Solcia et al., 1987). In the transgenic mice, diverse entero-endocrine cell types packaged hGH in their basal regulated granules, lending added support to the idea that a common mechanism for sorting of polypeptide hormones is shared among endocrine cells. It should be noted, however, that a subpopulation

($\sim 10\%$) of intestinal entero-endocrine cells failed to synthesize detectable levels of hGH, indicating that there are subtle differences in the regulation of the L-FABP promoter within the entero-endocrine cell population. The identity of this subpopulation could not be established on the basis of granule morphology in our EM immunocytochemical preparations and further double-labeling studies will be required to define nonexpressing cell type(s).

Exocrine Cells

Of the four exocrine cell types in mouse intestinal epithelium, only one type (the typical goblet cells) did not synthesize detectable levels of hGH. This suggests that the regulatory environment in goblet cells differs from that of all other exocrine cells in the epithelium. In contrast, the other three exocrine cell types synthesized hGH and concentrated the foreign protein in apical regulated secretory granules.

While sorting appeared to be comparable in the exocrine cell types that expressed hGH, the subsequent segregation of hGH within the granule matrices seemed to be influenced by the nature of their endogenous secretory products. For example, in Paneth cells, hGH was uniformly distributed within apical granules, whereas in granular goblet and intermediate cells, hGH partitioned into a central, dense domain of the granule matrix along with other undefined endogenous proteins. This implies that hGH possesses the molecular features that permit aggregation of specific proteins within secretory granules. Using EM immunocytochemistry, we were able to visualize this process in newly formed secretory granules in the Golgi region of granular goblet cells (Fig. 6 C). It will be interesting in future studies to test how other prototypic secretory proteins are segregated within these diverse secretory granules.

The concentration of hGH in apical secretory granules of Paneth cells, intermediate cells, and granular goblet cells, along with previous observations of pancreatic exocrine cells (Ornitz et al., 1985), support the hypothesis that these exocrine cells use the same sorting mechanism as do endocrine and neuronal cells to selectively direct protein and glycoprotein products into regulated secretory granules (Kelly, 1985). It is also possible, however, that hGH was directed into exocrine granules because it interacts with mucin, lysozyme, or other exocrine cell products in the environment of the trans-Golgi cisternum and rides "piggy-back" into the regulated secretory pathway. Still another alternative is that hGH is not "recognized" or "sorted" at all by exocrine cells, and enters all available secretory pathways indiscriminately. In this case, hGH would be expected to enter the high volume regulated pathway where it would be stored and concentrated and thus readily detectable by immunocytochemical methods. If constitutive pathways exist in intestinal exocrine cells as demonstrated in pancreatic and parotid cells (Von Zastrow and Castle, 1987; Arvan and Chang, 1987), and if hGH entered these pathways, then it should be rapidly released and thus be undetectable in static immunocytochemical images. The nonsorting possibility cannot be ruled out in this transgenic mouse system because it is not possible to separately collect regulated and nonregulated apical and basolateral secretions from single cell types in the heterogenous intestinal epithelium. Further studies following the fates of non-regulated proteins in these same cell types are needed to resolve this issue.

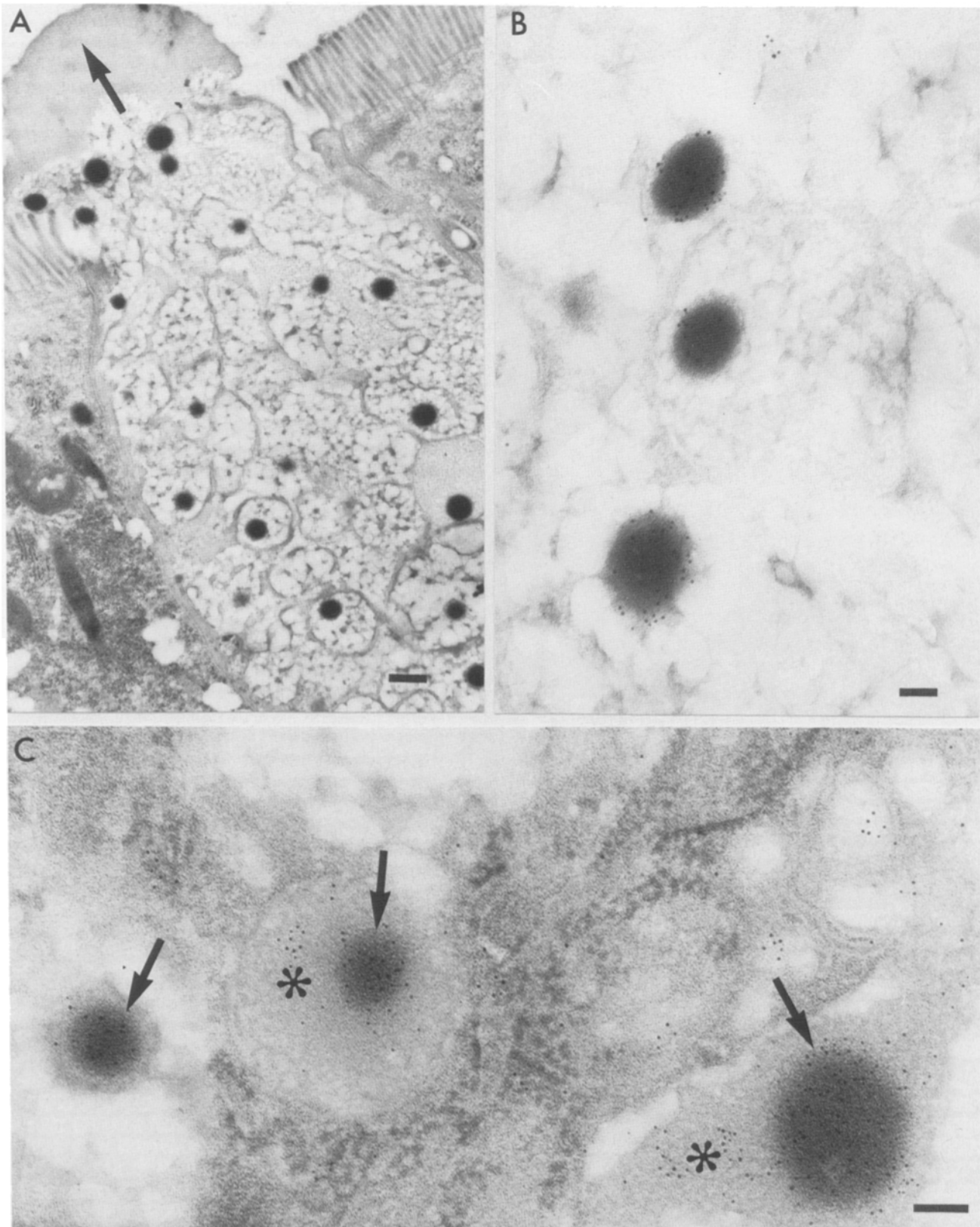


Figure 6. Immunolocalization of hGH in granular goblet cells. *A*, Control section, treated with nonimmune goat serum and secondary antibody gold, shows no immunolabel. A single dense core is embedded in the electron-lucent mucin content of most granules. Core material and mucin are released together at the apical cell pole (*arrow*). *B*, Section immunolabeled with anti-hGH serum and 10 nm gold shows that within apical granules, hGH was concentrated in the dense cores, but not in the mucin matrix. Granule membranes are poorly preserved. *C*, Newly formed secretory granules in the Golgi region show hGH immunoreactivity in dense cores (*arrows*) and also in associated less dense material (*asterisks*). Such images suggest that the dense core is formed by progressive segregation and aggregation of nonmucin proteins. Bar, (*A*) 0.5 μm ; (*B* and *C*) 0.1 μm .

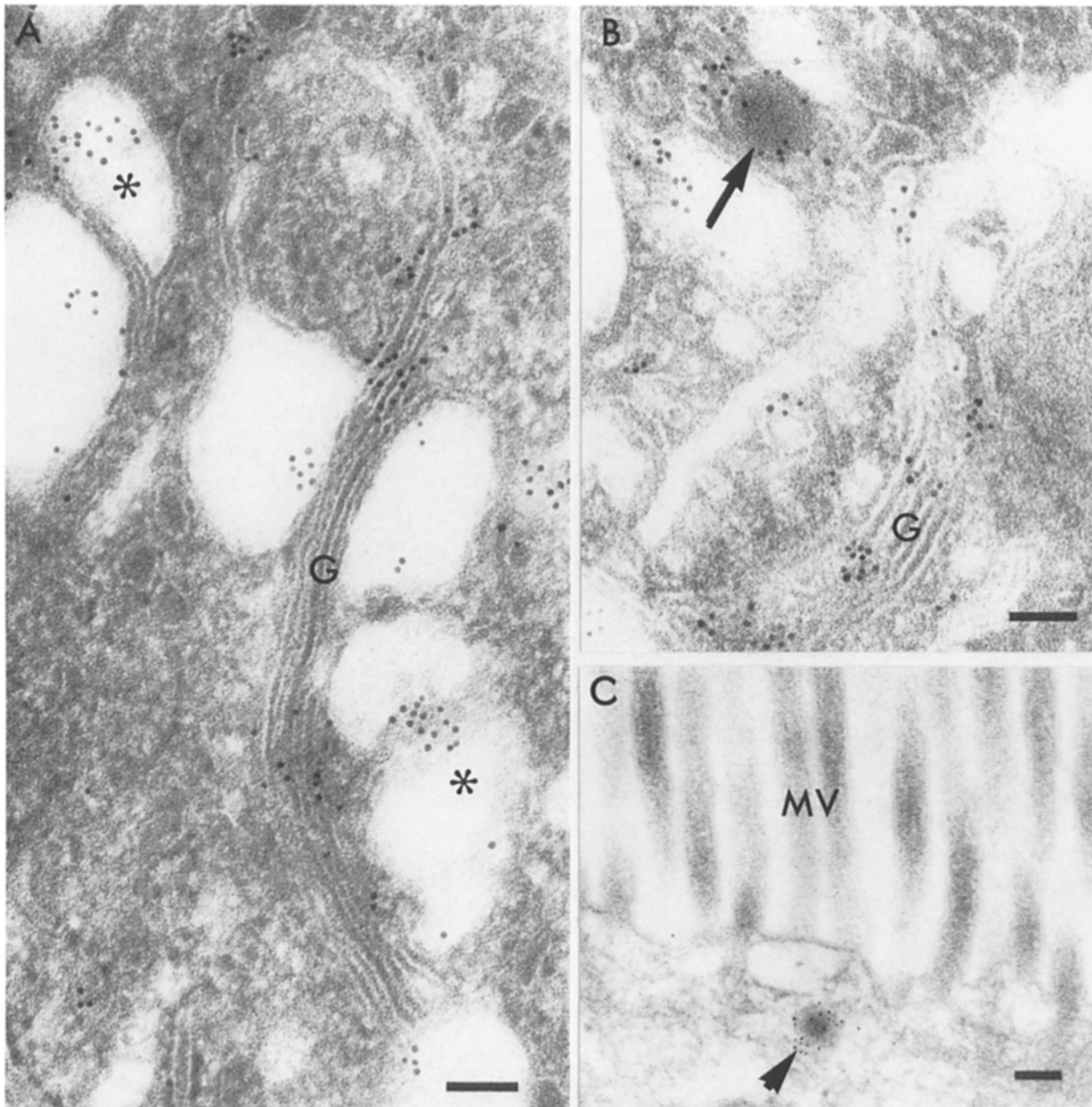


Figure 7. Immunolocalization of hGH in villus enterocytes, using goat anti-hGH and secondary antibody, 10 nm gold (*A* and *B*) or 5 nm gold (*C*). *A*, Golgi cisternae (*G*) and Golgi-associated clear vesicles (*asterisks*) contain hGH. *B*, Dense core vesicles (*arrow*) in the Golgi region also showed hGH immunoreactivity. *C*, Dense core vesicles containing hGH (*arrow*) were present near apical cell surfaces. *MV*, microvilli. Bar, 0.1 μm .

Secretory Pathways in Enterocytes

Absorptive enterocytes conduct constitutive vesicular transport of membrane glycoproteins toward both apical and basolateral surfaces, and most (but not all; Moktari et al., 1986; Massey et al., 1987) of the available evidence indicates that sorting of membrane constituents into one or the other pathway occurs in the Golgi complex (Danielson and Cowell, 1985; Fransen et al., 1985; Hauri et al., 1985). Studies of two cultured human colon adenocarcinoma-derived cell lines, Caco-2 and HT29-18C1, that develop enterocyte-like features in confluent monolayer culture (for review, see Rousset, 1986; Neutra and Louvard, 1989), showed that basolateral membrane components are continuously exported

to the cell surface, even in undifferentiated, unpolarized cells (LeBivic et al., 1988a; Godefroy et al., 1988). In contrast, apical membrane glycoproteins, including microvillar membrane enzymes, enter a vesicular pathway that seems to be selective in that it functions only in polarized cells that have tight junctions and an intact, polarized cytoskeleton (Blok et al., 1981; Bennett et al., 1984; Trugnan et al., 1987; LeBivic et al., 1988b; Achler et al., 1989). Small, Golgi-derived vesicles that deliver brush border glycoproteins to the apical membrane have been identified in the apical cytoplasm of normal enterocytes by EM autoradiography (Bennett et al., 1974; Michaels and Leblond, 1976; Bennett et al., 1984), and EM immunocytochemistry (Fransen et al., 1985; Lorenzsonn et al., 1987). The basolaterally directed vesicles are

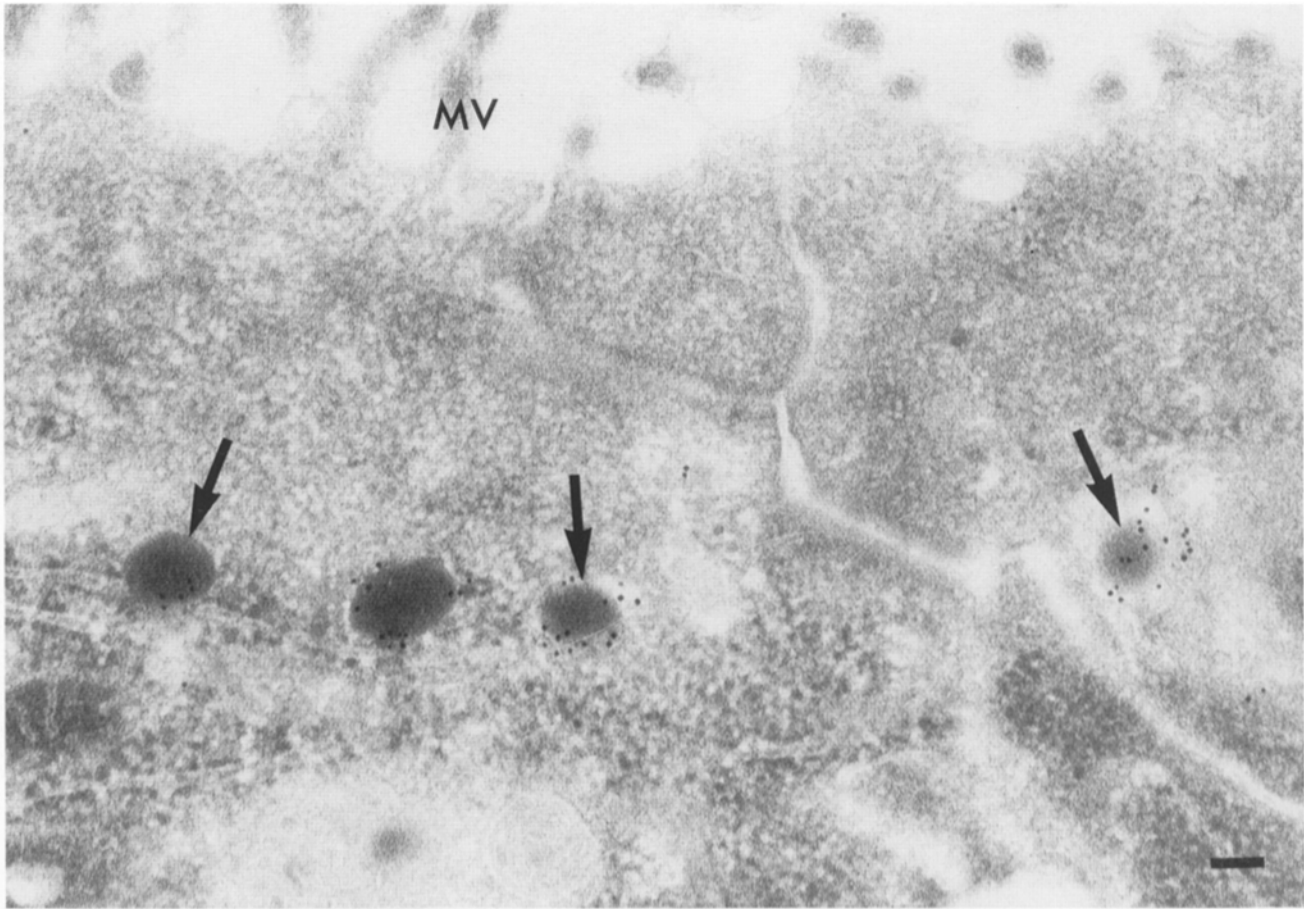


Figure 8. Immunolocalization of hGH in crypt enterocytes, labeled as in Fig. 7. hGH was concentrated in numerous dense core vesicles (arrows) in the apical cytoplasm. Bar, 0.2 μm .

difficult to visualize under normal physiological conditions, presumably because they move rapidly and can be released anywhere along the extensive lateral or basal membrane. It is assumed that these vesicles also deliver the soluble secretory proteins of enterocytes (e.g., apolipoproteins) basolaterally (Cardell et al., 1967; Traber et al., 1987; Rindler and Traber, 1988). No apical or exocrine secretory products have been identified, either in Caco-2 cells or in normal enterocytes (Hauri et al., 1985; Rindler and Traber, 1988).

Caco-2 cells that were induced by gene transfection to synthesize rat growth hormone, secreted the protein basolaterally, indicating that in Caco-2 cells the "default" pathway for both secretory and membrane proteins is exclusively basolateral (Rindler and Traber, 1988). These results also confirmed that Caco-2 cells lack the sorting machinery to selectively withdraw secretory products such as hGH out of constitutive secretory pathways. This is consistent with the apparent absence of a regulated secretory pathway in these cells. In our transgenic mice, the distribution of hGH in enterocytes and in lateral intercellular spaces also suggested that the hormone was secreted in a constitutive, basolateral pathway. High blood levels of hGH and the large size of these animals indicates basolateral secretion of hormone from some abundant cell source, but this source could theoretically have been enterocytes, entero-endocrine cells, hepatocytes, kidney tubule cells or all of these (Sweetser et al., 1988*a,b*).

Enterocytes *in vivo*, however, may have specialized secretory pathways not present in Caco-2 cells. In the enterocytes of normal mice (especially those in crypts) small vesicles with concentrated, electron-dense content are consistently present in the apical cytoplasm and in the Golgi region. Although their position in the apical pole of the cell implies apical secretion, the nature of the dense content is unknown and no studies have yet been done to establish the kinetics, regulation, or direction of their release (Madara and Trier, 1987). We have observed that they do not contain detectable levels of sucrase-isomaltase, an apically directed microvillar membrane enzyme (data not shown). In both crypt and villus enterocytes of the transgenic mice, these dense core vesicles contained relatively high levels of hGH. Indeed, in crypt cells they were the only non-Golgi compartment that was consistently labeled with anti-hGH antibodies. Since concentration of vesicle content is one hallmark of a regulated secretory pathway (Kelly, 1985), accumulation of hGH in the dense content of these vesicles suggests that they may represent a regulated apical secretory pathway in normal enterocytes that is not present in neoplastic intestinal cell lines. This observation again underscores the value of the transgenic mouse system for studying fates of foreign secretory proteins in the specialized, highly polarized cells of this native epithelium.

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