# Cell-specific Expression of $\alpha_1$-Antitrypsin in Human Intestinal Epithelium

**Ernesto P. Molmenti, David H. Perlmutter, and Deborah C. Rubin**
*Departments of Pediatrics, Internal Medicine, Surgery, Cell Biology and Physiology,*
*Washington University School of Medicine, St. Louis, Missouri 63110*

## Abstract

$\alpha_1$-Antitrypsin ($\alpha_1$-AT) is an acute phase plasma protein predominantly derived from the liver which inhibits neutrophil elastase. Previous studies have suggested that $\alpha_1$-AT is also expressed in human enterocytes because $\alpha_1$-AT mRNA could be detected in human jejunum by RNA blot analysis, and $\alpha_1$-AT synthesis could be detected in a human intestinal adenocarcinoma cell line Caco2, which spontaneously differentiates into villous-like enterocytes in tissue culture. To definitively determine that the $\alpha_1$-AT gene is expressed in human enterocytes in vivo, we examined tissue slices of human jejunum and ileum by in situ hybridization. The results demonstrate specific hybridization to enterocytes from the bases to the tips of the villi. Although there was no hybridization to enterocytes in most of the crypt epithelium, there was intense specific hybridization in one region of the crypt. Double-label immunohistochemical studies showed that $\alpha_1$-AT and lysozyme co-localized to this region, indicating that it represented Paneth cells. Finally, there was a marked increase in hybridization to $\alpha_1$-AT mRNA in villous enterocytes and Paneth cells in Crohn's disease. The results of this study provide definitive evidence that $\alpha_1$-AT is expressed in human jejunal and ileal enterocytes in vivo, and show that $\alpha_1$-AT is also a product of Paneth cells. Together with the results of other studies, these data raise the possibility that $\alpha_1$-AT detected in fecal $\alpha_1$-AT clearance assays for diagnosing protein-losing enteropathies is predominantly derived from sloughed enterocytes. (*J. Clin. Invest.* 1993. 92:2022–2034.) Key words: $\alpha_1$-antitrypsin • enterocytes • Paneth cells • inflammatory bowel disease • intestine

## Introduction

$\alpha_1$-Antitrypsin ($\alpha_1$-AT)[1] is a $\sim$55-kD glycoprotein, an acute phase plasma protein, and prototype serine proteinase inhibitor (serpin), which inhibits neutrophil elastase. Genetic and acquired forms of $\alpha_1$-AT deficiency are associated with premature development of emphysema. The most common genetic form of $\alpha_1$-AT deficiency, homozygous PiZZ $\alpha_1$-AT deficiency,

is also associated with chronic liver disease and hepatocellular carcinoma (1).

Studies of changes in $\alpha_1$-AT allotypes after orthotopic liver transplantation have shown that liver is the predominant site of synthesis of blood-borne $\alpha_1$-AT (2, 3). There is evidence for extrahepatic sites of synthesis, notably that in blood monocytes and tissue macrophages (4). Results of transgenic mice studies have suggested that the human $\alpha_1$-AT gene is expressed in other extrahepatic tissues and cell types (5, 6). For instance, RNA blot analysis has demonstrated the presence of $\alpha_1$-AT mRNA in the small intestine of mice transgenic for human $\alpha_1$-AT and in human small intestine (5, 7). These studies did not, however, exclude the possibility that such $\alpha_1$-AT mRNA was derived from macrophages resident in the lamina propria or submucosal layers of the intestine. Synthesis of $\alpha_1$-AT has been demonstrated in a human intestinal adenocarcinoma cell line Caco2 (8). There is a marked increase in synthesis of $\alpha_1$-AT in Caco2 cells as they spontaneously differentiate from cryptlike to villouslike enterocytes. It could be argued, however, that expression of $\alpha_1$-AT in Caco2 cells is a result of the transformation of these cells and not necessarily a reflection of the physiological function of enterocytes in vivo. Ribonuclease protection assays designed to map transcriptional initiation sites for $\alpha_1$-AT mRNA indicate that the predominant $\alpha_1$-AT mRNA in jejunum is similar to that of Caco2-derived enterocytes and HepG2-derived hepatocytes, and entirely distinct from the alternative upstream transcriptional sites used in blood monocytes and bronchoalveolar macrophages (7). However, these results do not exclude the possibility that intestinal macrophages use the downstream transcriptional initiation site. In the current study, we subjected specimens of human intestine to in situ hybridization analysis to definitively determine that the $\alpha_1$-AT gene is expressed in enterocytes and to examine other cell-specific sites of $\alpha_1$-AT gene expression in intestinal epithelium.

## Methods

*Materials.* Ribonuclease A was purchased from Sigma Chemical Co. (St. Louis, MO), ribonuclease T1 was purchased from Bethesda Research Laboratories (Gaithersburg, MD), and DNAse I and ribonuclease inhibitor were purchased from Promega Biotech (Madison, WI). $^{35}$S-UTP and [$^{32}$P]CTP were purchased from Du Pont-New England Nuclear (Boston, MA).

*Cell culture.* Primary cultures of human peripheral blood monocytes were established according to previously described methods (8). Maintenance of Caco2 cells has also been previously described (9).

*Ribonuclease protection assays.* The DNA probe used (probe C) is a 158-bp BamHI-BamHI fragment derived from L17.3 and spanning the hepatocyte-specific transcriptional initiation site that had been subcloned into the pGEM4z plasmid (7). For protection assays, $^{32}$P-labeled antisense complementary RNA probe C was synthesized using linearized plasmid (5 $\mu$g), [$^{32}$P]CTP (50 $\mu$Ci), and SP6 RNA polymerase. From this reaction mixture, 1/1,000 of the final product was allowed to hybridize overnight to 10 $\mu$g total cellular RNA in 50% form-

1. *Abbreviations used in this paper:* $\alpha_1$-AT, $\alpha_1$-antitrypsin; IGSS, immunogold-silver enhancement.

amide at 55°C. RNAse digestion was performed at room temperature with RNAse A and T1, and the protected fragments were separated on 6% polyacrylamide gels.

*In situ hybridization.* Jejunal, ileal, and colonic tissue was obtained from two different organ donors through Mid America Transplant Association (St. Louis, MO). These studies were approved by the Human Studies Committee of Washington University School of Medicine. Ileal tissue from three patients with Crohn's disease was obtained at the time of ileocolonic resection. Sections of ileum with active inflammation were compared to adjacent uninflamed sections from each patient. In situ hybridization was performed as previously described (10) with the following modifications: immediately after harvesting, tissues were divided into 2-cm-long fragments and snap-frozen in liquid nitrogen. Cryostat sections (5–8 $\mu$m) of tissue were fixed in 4% paraformaldehyde and prehybridized by proteinase K digestion (1 $\mu$g/ml for 10 min). Hybridization with the $^{35}$S-UTP–labeled antisense probe C was performed at 55°C for 24 h in 60% formamide, 15% dextran sulfate, 1× Denhardt's solution, 1 mM EDTA, 10 mM Tris Cl (pH 8), and 400 mM NaCl. Sections were then subjected to RNAse A digestion to remove all nonhybridized single-stranded RNA, washed at stringencies of up to 0.1× SSC at 55°C for 30 min, and exposed to photoemulsion at 4°C. The slides were developed 26 d after the emulsion dipping. Cellular expression of mRNA is indicated by the presence of silver grains. "Background" signal was defined as the density of grains on the glass slide, where no tissue was present. To be certain that the hybridization signal was specific for probe C, sequential sections of the same tissue were hybridized under identical conditions using $^{35}$S-UTP–labeled sense probe C. Using the sense probe C, scattered grains were found over tissue and slide but no specific hybridization signal was ever found.
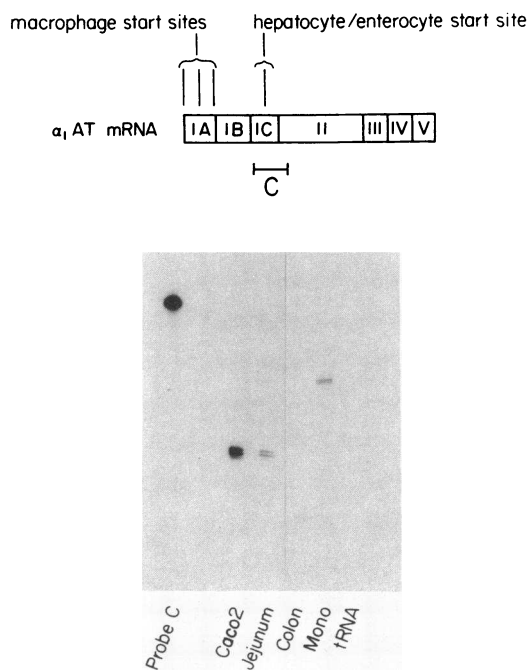


*Figure 1.* Ribonuclease protection assay to detect $\alpha_1$-AT mRNA in human intestine. A map of the presumed $\alpha_1$-AT mRNA precursor and the DNA probe used for the assay is shown at the top. For the autoradiogram shown at the bottom, 15 $\mu$g of total cellular RNA from each tissue or cell line were used to hybridize the corresponding $^{32}$P-labeled antisense cRNA probe. Ribonuclease protection assays followed the protocol described in Methods. Probe C is a 158-bp BamHI-BamHI fragment derived from macrophage $\alpha_1$-AT cDNA L17.3, which spans the hepatocyte-specific $\alpha_1$-AT cap site. It also has 60 bases of vector sequence.

*Immunohistochemical studies.* Cryostat sections (5–8 $\mu$m thick) of jejunum and colon (obtained as described above) were fixed in 4% paraformaldehyde and exposed to 70% ethanol for 5 min. Two different polyclonal sera were used, including rabbit anti–human $\alpha_1$-AT (1:2,000) (Dako Corp., Carpinteria, CA) and rabbit anti–human lysozyme (1:500) (Dako Corp.). The specificity of the antisera has been previously described (4, 11) or documented by the manufacturer. Detection of antigen was performed using immunogold-silver enhancement (IGSS) or immunofluorescent staining techniques, as previously described (11–13) and as outlined here. Tissues were 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. For IGSS, 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 H$_2$O, silver enhancement solution was added according to the manufacturer's protocol (Amersham International, Amersham, United Kingdom). Silver precipitation was monitored under the light microscope. For immunofluorescent studies, Texas-red–labeled donkey anti–rabbit immunoglobulin was used as a secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Substitution of the primary antibodies with nonimmune rabbit serum produced no specific labeling.

*Double-label immunohistochemistry.* Since the sera directed against $\alpha_1$-AT and lysozyme were both derived from rabbit, we used sequential immunogold silver staining and fluorescence techniques to detect these two antigens in the same tissue section. Precipitation of silver around the antibody-immunogold complex abolishes its immunoreactivity, making this technique ideally suited for double-label analysis as previously described (11–13). Sections were first immunostained for a particular antigen using the immunogold silver staining method. The same section was then incubated with a second primary antibody; antigen-antibody complexes were detected by Texas-red–labeled donkey anti–rabbit serum. Slides were then examined under epipolarization and fluorescence microscopy. The immunogold silver staining did not interfere with subsequent immunofluorescent staining.

## Results

*Ribonuclease protection assays.* First, we examined the possibility of detecting $\alpha_1$-AT mRNA in specimens of human intestine by ribonuclease protection assay. A 158-nt cRNA probe that corresponds to a region spanning the downstream "hepatocyte"-specific transcriptional initiation site was used (Fig. 1). The results indicate that there is a single fragment in human jejunum that comigrates with the fragment present in Caco2 cells. The same fragment is present in human hepatoma HepG2 cells and in human liver (7). There is no evidence of a protected fragment in human colon. The fragment that is protected in human jejunum and Caco2 cells is much smaller than a single fragment present in human peripheral blood monocytes. In other studies, this larger fragment found in cells of mononuclear phagocyte origin, which is fully protected by the probe, has been shown to be derived from transcripts that initiate at upstream alternative transcriptional initiation sites (7). Examination of three other donors yielded identical results (data not shown). These data provided evidence that $\alpha_1$-AT mRNA could be detected in intestinal specimens with cRNA probe C.

*In situ hybridization studies.* Next, we examined human liver by in situ hybridization with cRNA probe C to determine that both our previously established technique for in situ hybridization and our probe could be used in the highest $\alpha_1$-AT–producing tissue. Cryostat sections of normal human liver were hybridized with $^{35}$S-labeled cRNA probe C (Fig. 2). The results
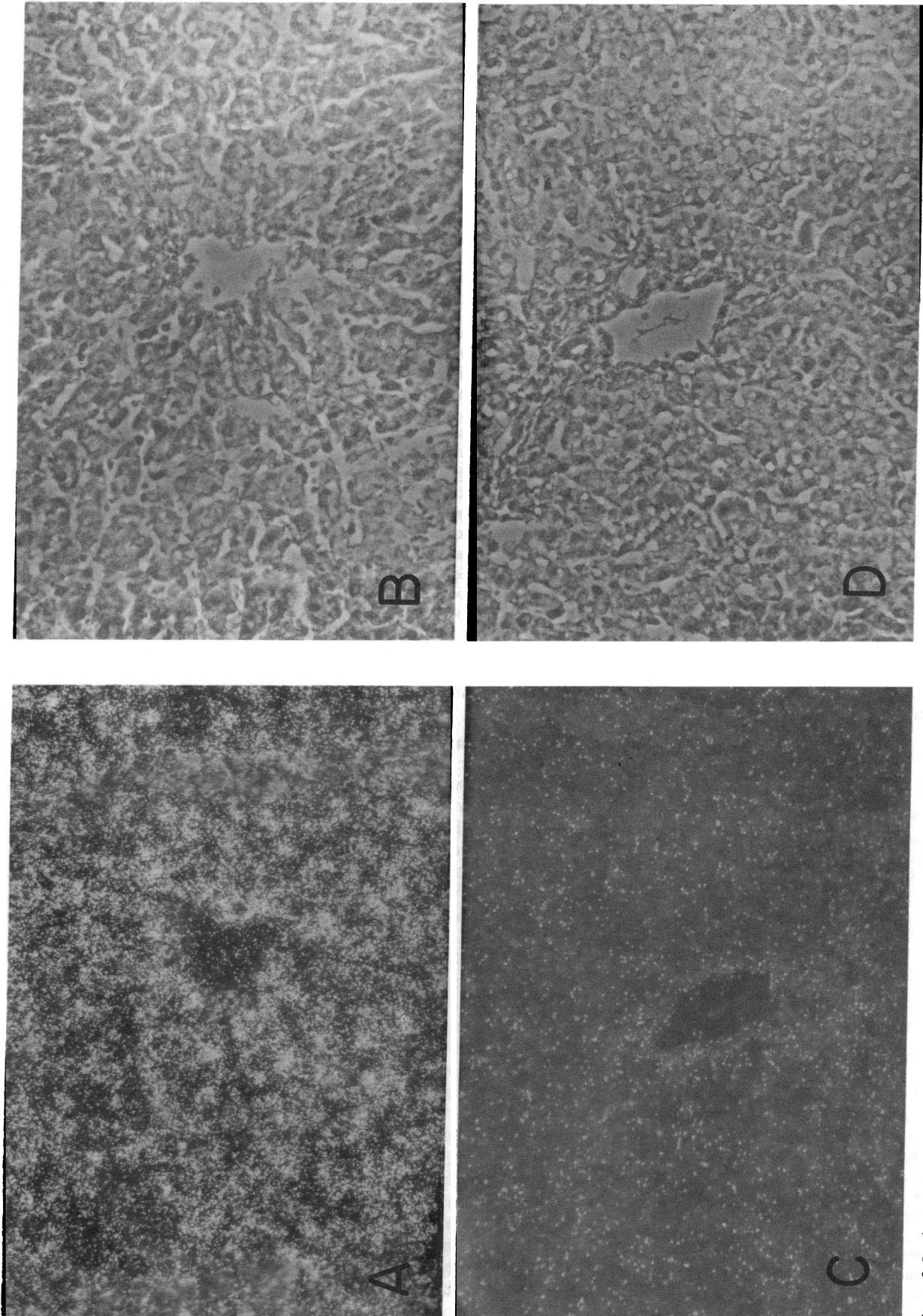
*Figure 2.* In situ hybridization to $\alpha_1$-AT mRNA in human liver. Serial cryostat sections of normal human liver from organ donors were examined by dark-field microscopy ($\times 100$) after hybridization with $^{35}$S-antisense cRNA probe C (*A*). (*B*) Same section as in *A* viewed with light-field microscopy. (*C*) Section of human liver hybridized with $^{35}$S sense cRNA probe C ($\times 100$). (*D*) Same section as in *C* under light-field microscopy.
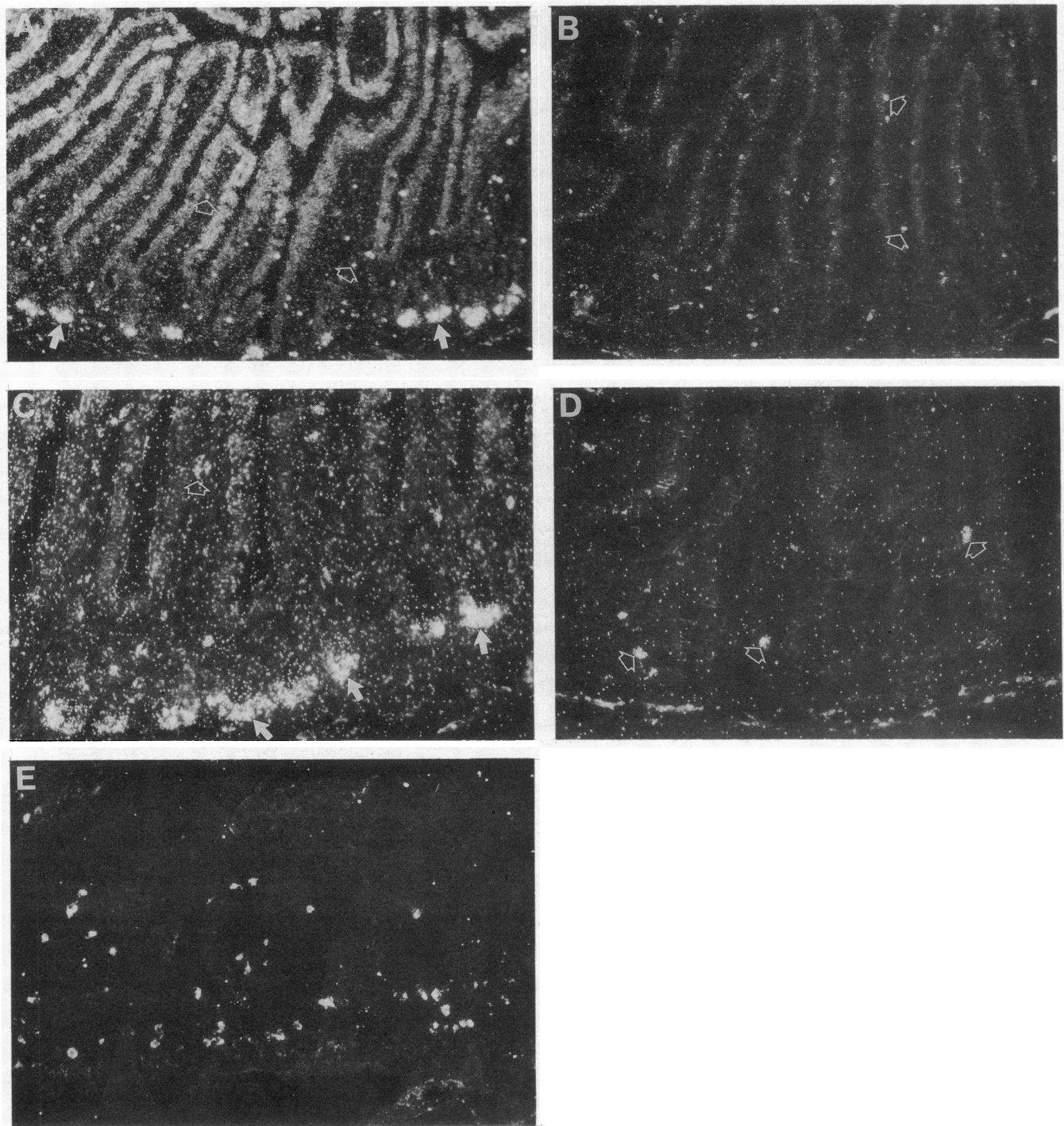
*Figure 3.* Analysis of cellular expression of $\alpha_1$-AT mRNA in normal human jejunum using in situ hybridization techniques. (*A*) A cryostat section of human jejunum was hybridized with $^{35}$S-labeled antisense cRNA probe C and then examined by dark-field microscopy (×80). The luminal side is located toward the top and the seromuscular side toward the bottom of the panel. White grains depict the presence of $\alpha_1$-AT mRNA. (*C*) High power view (×125) of crypt epithelium from *A*. (*B* and *D*) Specificity of in situ hybridization to $\alpha_1$-AT mRNA in human jejunum. A serial cryostat section of human jejunum was hybridized with $^{35}$S sense cRNA probe C and examined by dark-field microscopy under low (×80) and high (×125) powers. (*E*) Specificity of in situ hybridization to $\alpha_1$-AT mRNA in human jejunum. A serial cryostat section of human jejunum was examined by dark-field microscopy under low (×125) power without any hybridization to cRNA probes. This section was subjected to the same pre- and posthybridization conditions as those for *A–D*.

demonstrate extensive hybridization to parenchymal cells of the liver with the antisense probe (*A* and *B*) as compared to the negligible staining of adjacent liver sections with the sense probe (*C* and *D*). These studies established that our in situ hybridization technique with this cRNA probe was specific

and, therefore, could be used to examine human intestinal epithelium.

Cryostat sections of human jejunum from an organ donor were hybridized with $^{35}$S antisense cRNA probe C (Fig. 3, *A* and *C*). There was dense localization of silver grains to epithe-
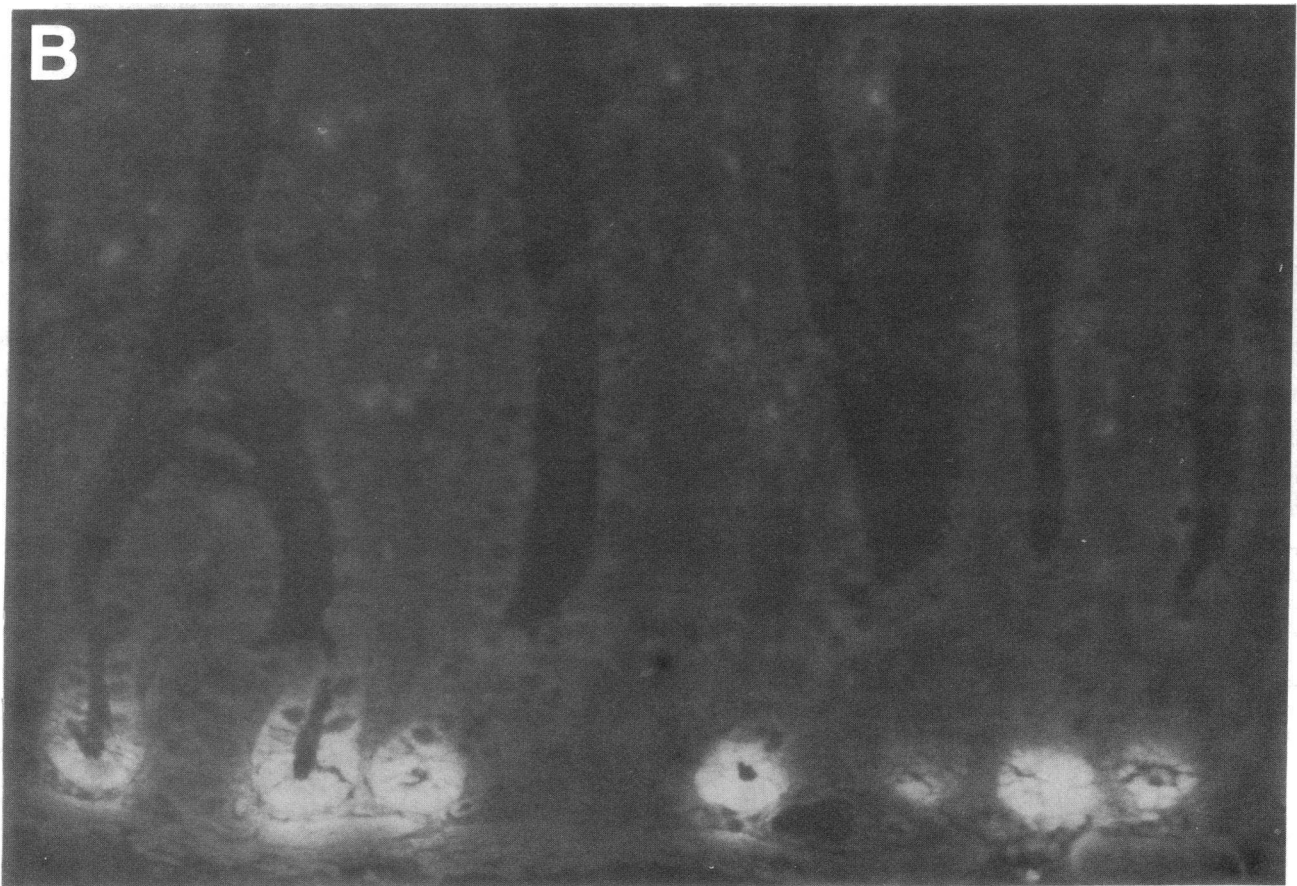
*Figure 4.* Immunofluorescence staining of human jejunum for lysozyme (*A*) and $\alpha_1$-AT (*B*). Serial sections of human jejunum were incubated with rabbit anti–human lysozyme (*A*) or rabbit anti–human $\alpha_1$-AT (*B*), and then with Texas red–conjugated donkey anti–rabbit Ig. These sections were then viewed by fluorescence microscopy ($\times 100$).
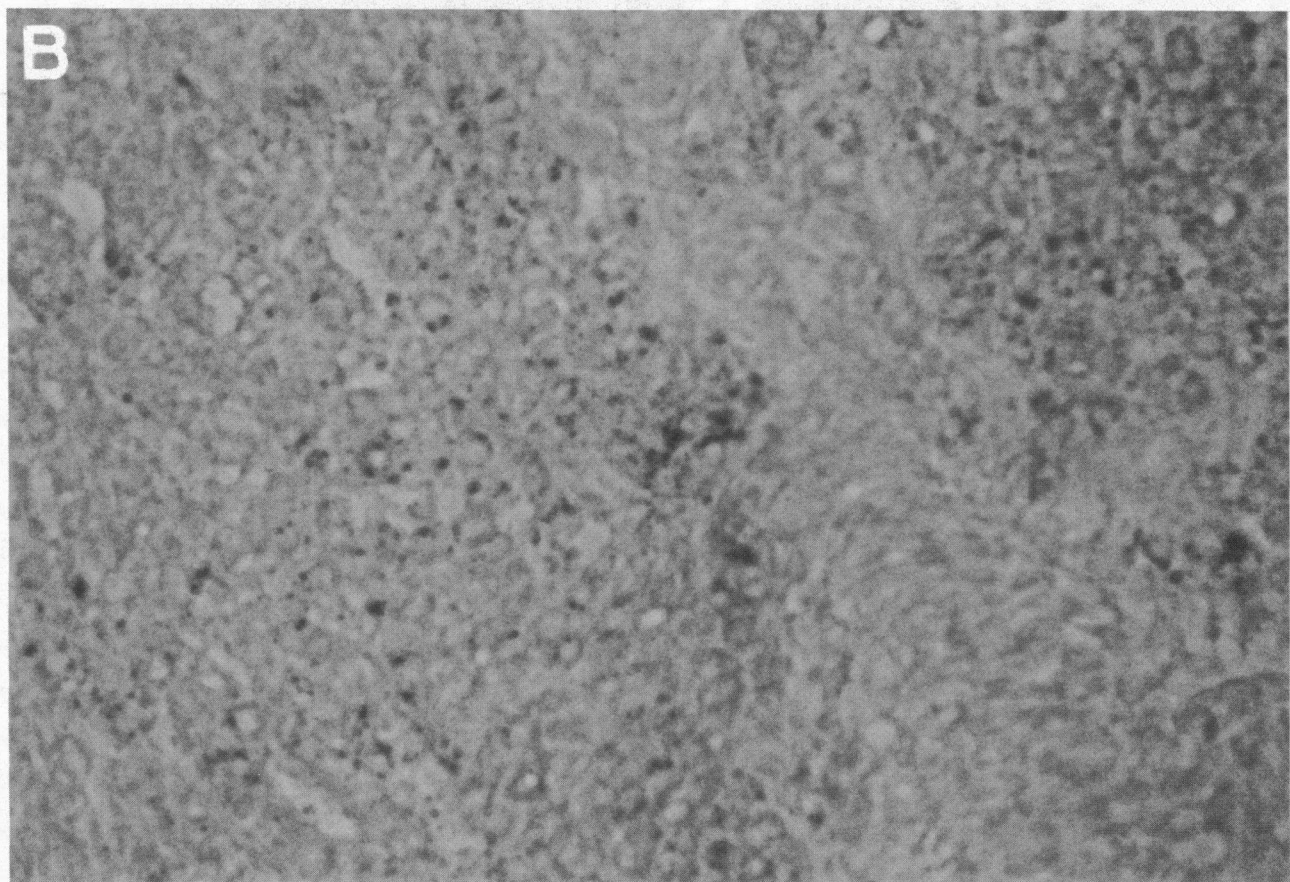
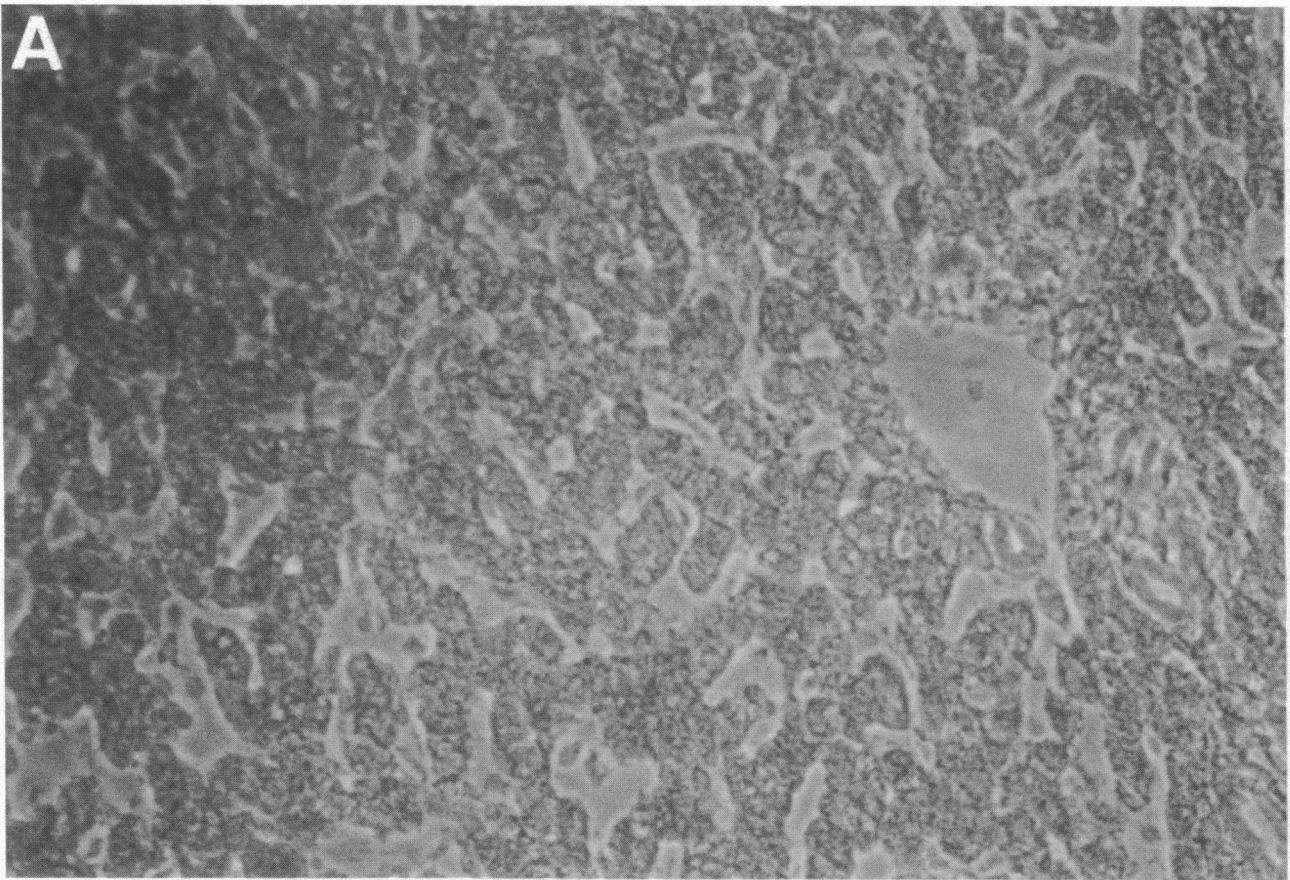*Figure 5.* Immunogold silver enhancement staining of normal and $\alpha_1$-AT-deficient liver for $\alpha_1$-AT. Sections of liver from a PiMM organ donor (*A*) and from an individual with PiZZ $\alpha_1$-AT deficiency who underwent liver transplantation (*B*) were incubated with rabbit anti–human $\alpha_1$-AT. Antigen-antibody complexes were detected by IGSS and viewed under light microscopy (×200).
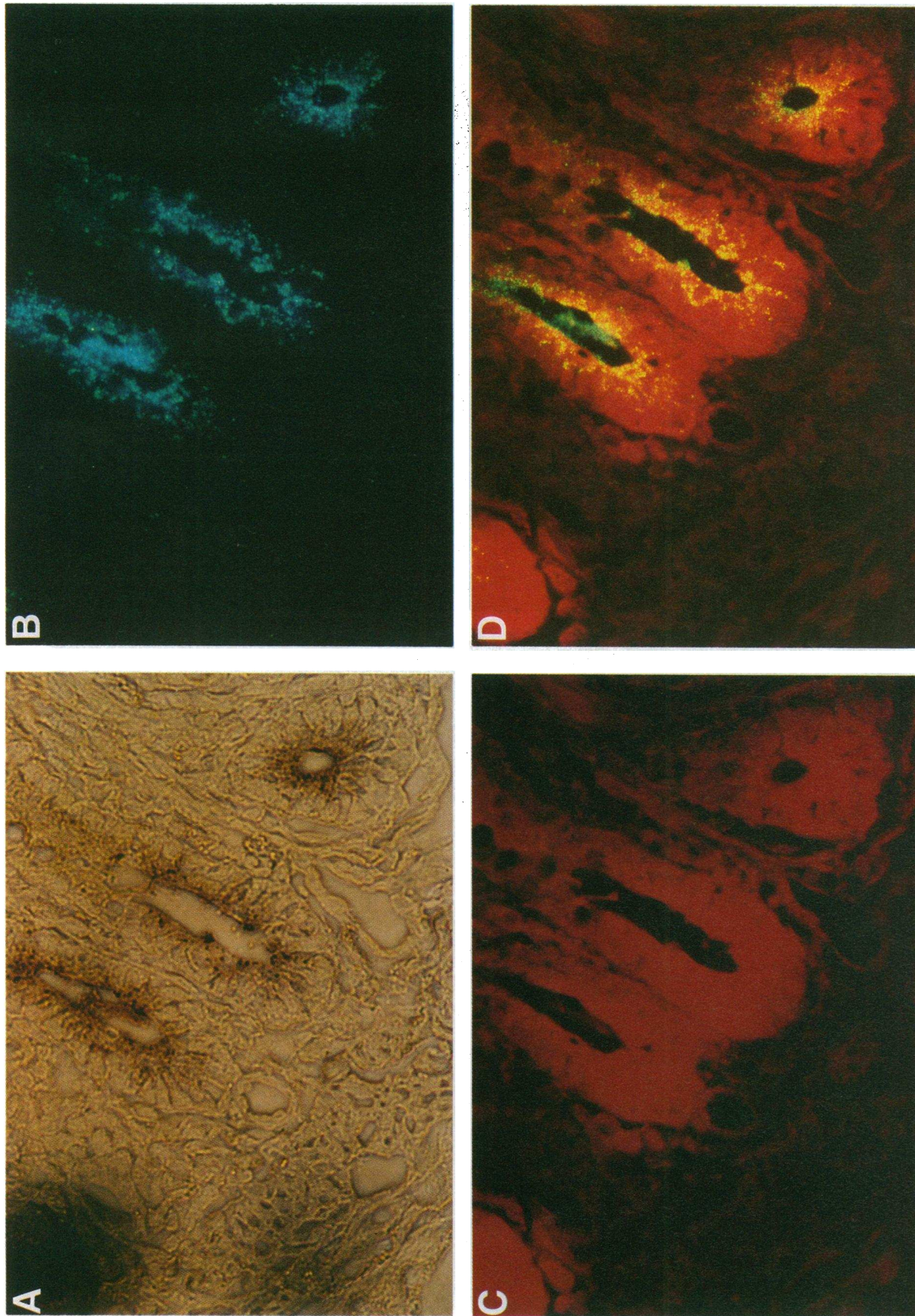
*Figure 6.* Immunohistochemical staining of $\alpha_1$-AT and lysozyme in normal human jejunum. A section of normal human jejunum was incubated with rabbit anti–human $\alpha_1$-AT, detected by IGSS and then viewed under light-field microscopy (*A*) or reflected polarized light (*B*). The same section was then incubated with rabbit anti–human lysozyme, labeled with Texas red–conjugated donkey anti–rabbit Ig and viewed under fluorescent light microscopy (*C*). A doubly exposed photomicrograph reveals coexpression of lysozyme and $\alpha_1$-AT in the crypt base epithelium (*D* [×400]).

lial cells lining the villi, from villus base to tip. There was also an impression that the density of silver grains increased toward the villus tip. Remarkably, although there were no silver grains in the upper epithelial cells of the crypt, an especially intense accumulation of grains was consistently found at the bases of the crypts (closed arrows). This is shown clearly in a higher power view in Fig. 3 C. On a purely anatomical basis, this domain is usually occupied by Paneth cells. Examination of a second, completely unrelated organ donor yielded identical results (data not shown). The hybridization to villous enterocytes and to cells at the bases of the crypts (presumably Paneth cells) with $^{35}$S-labeled antisense cRNA probe C was shown to be specific as evidenced by the absence of silver grains in these cells when serial sections of jejunum were incubated with $^{35}$S-labeled "sense" probe C (Fig. 3, B and D).

As depicted in Fig. 3, there were also cells in the lamina propria that were prominent because of their refractile properties (open arrows), but did not contain silver grains above background found on the slide. This was seen with both sense and antisense cRNA probes (Fig. 3, A and C), as well as with sense and antisense cRNA probes for an upstream transcriptional initiation site (data not shown). In fact, these refractile cells can often be seen on dark-field examination, even in sections that have not been subject to in situ hybridization (Fig. 3 E).

Cryostat sections of human colon from the same donors were also subjected to in situ hybridization (data not shown). Silver grains were not detected in cells lining the epithelium. These observations are consistent with results of ribonuclease protection assays (Fig. 1 and reference 7).

*Immunohistochemical studies.* To determine whether cells within the jejunal crypt epithelium that express $\alpha_1$-AT mRNA might indeed be Paneth cells, we subjected serial sections to immunohistochemical staining with antibodies directed against lysozyme, a protein that is abundantly expressed in Paneth cells (11, 14), and against $\alpha_1$-AT. First, cryostat sections of human jejunum were incubated with rabbit anti-human lysozyme (Fig. 4 A) or with rabbit anti-human $\alpha_1$-AT (Fig. 4 B) and detected using Texas red–conjugated donkey anti-rabbit sera. There was an intense staining of cells at the base of the crypt epithelium. The region was identical to that which had been specifically detected by in situ hybridization (Fig. 3, A and C).

Next, we used double-label immunocytochemical techniques to determine whether these cells coexpressed $\alpha_1$-AT and lysozyme. Since we planned to use immunogold with silver enhancement and fluorescence for double-antigen detection, we first examined the specificity of the immunogold silver detection system for $\alpha_1$-AT using human liver as positive control (Fig. 5). The results showed that we could not detect $\alpha_1$-AT in normal liver (A)/ but did find it in liver from individuals with homozygous PiZZ $\alpha_1$-AT deficiency, in which $\alpha_1$-AT is known to accumulate within hepatocytes (Fig. 5 B). There were brown-stained hepatic parenchymal cells scattered through the hepatic lobule but were most prominent adjacent to a broad band of fibrosis. These results are completely consistent with previous immunocytochemical analyses of human liver for $\alpha_1$-AT (15). It is unclear why one cannot stain $\alpha_1$-AT in normal human liver, but it has been suggested that the rapid secretion of this protein leads to steady state levels of $\alpha_1$-AT within the liver cells that fall below the limits of detection of most immu-

nocytochemical techniques. In contrast, intracellular accumulation of the mutant (Z) $\alpha_1$-AT molecule leads to steady state levels of $\alpha_1$-AT within some liver cells that are detectable by immunocytochemical techniques. Thus, these results demonstrate the potential for misleading conclusions when using immunocytochemistry as an indicator of biosynthesis for $\alpha_1$-AT. In any case, the fact that we could stain $\alpha_1$-AT in a specific manner in the deficient liver using IGSS as the detection system did indicate that it was possible to use this detection system for colocalization studies in human intestinal epithelium.

Thus, a cryostat section of human jejunum from one of the two organ donors that were used for in situ hybridization was incubated with rabbit anti–human $\alpha_1$-AT and then subjected to immunogold with silver enhancement staining (Fig. 6). Staining of epithelial cells within the crypt base could be detected by light microscopy (Fig. 6 A), as well as under reflected light polarization microscopy (Fig. 6 B). The fact that these cells from a normal human donor can be stained for $\alpha_1$-AT must indicate that they either secrete $\alpha_1$-AT at a slower rate than liver cells, or that there is a higher level of synthesis of $\alpha_1$-AT per cell. In situ hybridization studies in Fig. 3, A and C are consistent with the latter hypothesis but cannot be used as definitive evidence. The section used for Fig. 6, A and B was then incubated with rabbit anti–human lysozyme and Texas red–conjugated donkey anti–rabbit serum (Fig. 6 C). An identical region within the crypts stained red, as viewed under fluorescent microscopic examination. A doubly exposed photograph (Fig. 6 D) shows that there is colocalization of Texas red and immunogold staining. These results, therefore, provide further evidence that the $\alpha_1$-AT gene is expressed in Paneth cells.

*Intestinal $\alpha_1$-AT gene expression in Crohn's disease.* Next, we examined cryostat sections of inflamed ileum from a patient with Crohn's disease and compared them to sections of adjacent uninflamed ileum from the same patient and to sections of ileum from a normal organ donor. To make the analysis strictly comparable, these sections were hybridized together with the same prep of $^{35}$S-labeled antisense and sense cRNA probes, washed together, subjected to autoradiography together for the same length of time and photographed together (Fig. 7). The results indicate that there is hybridization to the epithelial cells of the villus in each case but there is an increase in intensity in the inflamed Crohn's ileum (Fig. 7 B) and in adjacent uninflamed Crohn's ileum (Fig. 7 C) as compared to normal ileum (Fig. 7 A). There were no silver grains in villus epithelial cells using the sense probe, in normal ileum (data not shown), inflamed Crohn's ileum (data not shown), or uninflamed Crohn's ileum (Fig. 7 D). The difference in density of silver grains between normal ileum and Crohn's ileum was also confirmed by quantitation of the number of silver grains per high power field. For this analysis silver grains per unit area were counted on 20 different villi from the normal ileum, on 20 different sites from inflamed ileum of two different patients with Crohn's disease and on 20 different villi from uninflamed adjacent ileum of one of the Crohn's patients. The results showed counts of $11.25\pm2.5$ for normal, $36.05\pm5.3$ for one inflamed specimen, $48.10\pm6.9$ for another inflamed specimen, and $23.35\pm4.6$ for the uninflamed specimen. The differences between normal and inflamed, normal and uninflamed, and inflamed and uninflamed were statistically significant ($P < 0.001$ by ANOVA; $P < 0.001$ by Tukey's test of multiple comparisons in each case).

Examination of the crypts showed silver grains in a localized region (closed arrows) in normal ileum (Fig. 7 $E$) as compared to silver grains which almost completely line the crypt epithelium of inflamed Crohn's ileum (Fig. 7 $F$). There is no increase in the length of the crypt epithelium from uninflamed Crohn's ileum which hybridizes to the antisense cRNA probe (Fig. 7 $G$). There were no silver grains above background in the crypt epithelium with the sense cRNA probe (Fig. 7 $H$). There was a qualitative impression that there was less intense hybridization to Paneth cells in the normal ileum than in the normal jejunum. A remarkable increase in inflammatory cells was noted in Crohn's ileum (open arrows). These cells have similar refractile properties as those indicated in Fig. 3 (open arrows) but do not demonstrate $\alpha_1$-AT mRNA, since there are no silver grains overlying them. All of these results were replicated in another series of in situ hybridization analyses with the same normal ileum compared to another inflamed Crohn's ileum (data not shown).

To determine whether $\alpha_1$-AT gene expression in the crypt epithelium of Crohn's ileum was attributable to Paneth cells and/or enterocytes, serial sections from the same specimens were subjected to immunohistochemical analysis for Paneth cells (Fig. 8). The results indicate that in the normal ileum, there is staining by antibody to lysozyme at the base of the crypts, the usual location of Paneth cells (Fig. 8 $A$). In the inflamed Crohn's ileum, staining with antibody to lysozyme extends a considerable distance up the length of the crypt (Fig. 8 $B$), but extension up the crypt epithelium is patchy (Fig. 8 $C$). In addition, the staining does not extend as far up the length of the crypt epithelium as does the specific hybridization with $\alpha_1$-AT antisense cRNA (data not shown). Taken together, these data suggest that there is induction of $\alpha_1$-AT gene expression in crypt enterocytes, as well as expression of $\alpha_1$-AT in proliferating Paneth cells in the crypts of inflamed Crohn's ileum. The induction of $\alpha_1$-AT in crypt enterocytes is great enough to be detected by in situ hybridization analysis but not enough to be detected by immunofluorescence (data not shown). There was no increase in the length of crypt epithelium stained with antibody to lysozyme in the uninflamed Crohn's ileum (Fig. 8 $D$).

## Discussion

These data establish that the $\alpha_1$-AT gene is expressed in human jejunal and ileal enterocytes in vivo and under physiologic conditions. Previous studies using immunohistochemical techniques, RNA blot analyses, and ribonuclease protection assays to detect cell-specific $\alpha_1$-AT mRNA transcripts had suggested, but could not prove that $\alpha_1$-AT was synthesized in enterocytes (5, 7). Previous in situ hybridization studies had detected a similar distribution of human $\alpha_1$-AT gene expression in the small intestine of mice transgenic for the human $\alpha_1$-AT gene (6). These animals had been generated with a DNA fragment that spanned the structural gene for $\alpha_1$-AT, $\sim$ 8 kb of its upstream flanking region and $\sim$ 20 kb of its downstream flanking region. Previous immunohistochemical studies had detected a roughly similar distribution of human $\alpha_1$-AT gene expression in the intestine of mice transgenic for a considerably smaller fragment encoding the $\alpha_1$-AT gene (5). In these mice, a DNA fragment that spanned the structural gene for $\alpha_1$-AT, 2 kb of its upstream flanking region and 2.3 kb of its downstream flank-

ing region was used as transgene. Although our study indicates that it can be misleading to draw conclusions about the expression of this gene from immunocytochemical analyses alone, taken together the above data suggest that the structural elements responsible for expression of the human $\alpha_1$-AT gene in enterocytes and Paneth cells are localized within 2 kb upstream and 2.3 kb downstream of the $\alpha_1$-AT structural gene. Moreover, the data indicate that transacting factors and/or mechanisms that are required for the same spatial distribution of human $\alpha_1$-AT gene expression are conserved from the mouse to the human system, even though there is no evidence for expression of the murine $\alpha_1$-AT gene in the small intestine.

The results of this study indicate that the expression of the $\alpha_1$-AT gene in the human intestinal adenocarcinoma cell line Caco2 reflects physiologic expression in the small intestine. There was no evidence for $\alpha_1$-AT mRNA in colonic epithelial cells or in small intestinal crypt epithelial cells other than Paneth cells, but $\alpha_1$-AT mRNA was detected in small intestinal villous epithelial cells. We have previously shown that there is a marked increase in expression of the $\alpha_1$-AT gene during differentiation of Caco2 cells from crypt-like to villous-like enterocytes (7, 9). The differentiation-dependent increase in Caco2 cell $\alpha_1$-AT gene expression could also represent a recapitulation of late fetal development as suggested by the expression of $\alpha$-fetoprotein in the cell line. The initiation site for transcription of the $\alpha_1$-AT mRNA in jejunum is identical to that of differentiated Caco2 cells (7).

The increase in $\alpha_1$-AT gene expression in epithelial cells of inflamed intestine from a patient with Crohn's disease is also of great interest. We have recently found that the acute phase cytokine interleukin-6 mediates an increase in $\alpha_1$-AT gene expression in Caco2 cells (16). Moreover, other cytokines mediate induction or upregulation of the expression of many of the positive acute phase reactants in Caco2 cells and do so in a manner that is almost identical to that which occurs in liver cells in the plasma during the host response to inflammation in vivo. For instance, IL-1$\beta$ and IL-6 induce the synthesis of serum amyloid A and $\alpha_1$-ACT, increase synthesis of complement proteins factor B and C3, increase synthesis of ceruloplasmin while leading to decreased synthesis of transferrin and $\alpha$-fetoprotein (16). IFN-$\gamma$ mediates an increase in complement protein C4 in Caco2 cells. These effects may be particularly relevant to Crohn's disease in that Ahrenstedt et al. have shown that there is increased local production of factors B, C3, and C4 in uninvolved intestine of patients with Crohn's disease, actively affecting other segments of bowel (17).

Results of this study also raise the possibility that fecal $\alpha_1$-AT quantitation for the diagnosis of protein-losing enteropathy (18) actually measures $\alpha_1$-AT derived from intestinal epithelial cells. We have previously shown that Caco2-derived enterocytes secrete $\alpha_1$-AT into the apical (luminal) fluid, as well as into the basolateral fluid. Even more important in this regard, however, is $\alpha_1$-AT within the continuously sloughed enterocytes. Enterocytes are sloughed from the tip of the villus $\sim$ 3 d after initiating migration at the base of the villus (17), and it is widely believed that the rate of sloughing increases during conditions in which there is local inflammation/injury. A study by Grill et al. showed that there is increased fecal clearance of $\alpha_1$-AT in individuals with homozygous PiZZ $\alpha_1$-AT deficiency (19). These individuals have only $\sim$ 15% of normal concentrations of $\alpha_1$-AT in their blood, but accumulate the abnor-
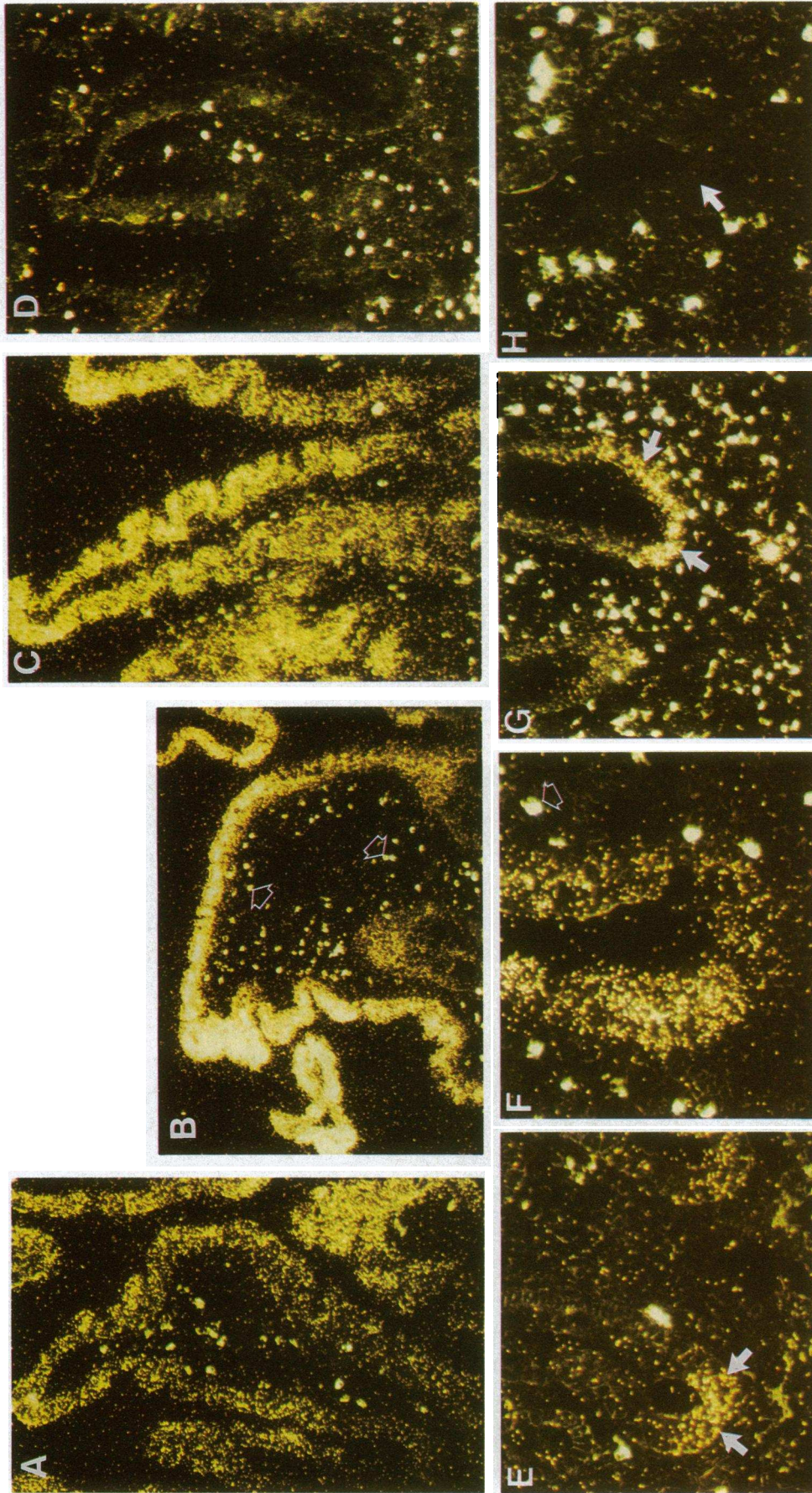
*Figure 7.* In situ hybridization of normal ileum (*A* and *E*), inflamed ileum from a patient with Crohn's disease (*B* and *F*), and adjacent uninflamed ileum from the same patient with Crohn's disease (*C, D, G,* and *H*). Cryostat sections were hybridized together with the same prep of $^{35}$S antisense cRNA probe C and were examined under dark-field microscopy. The lumen is located toward the top of the panels, and the serosa is located toward the bottom. Adjacent cryostat sections from the specimens for *C* and *G* were also hybridized with $^{35}$S sense cRNA probe C (*D* and *H*) and were examined under dark-field microscopy. Closed arrows point to localized regions of silver grains in the crypts. Open arrows point to cells in the lamina propria that have refractile properties and, therefore, not sites of specific hybridization. *A* and *G*, ×200; *B*, ×125; *C* and *D*, ×160; *E, F,* and *H*, ×400.
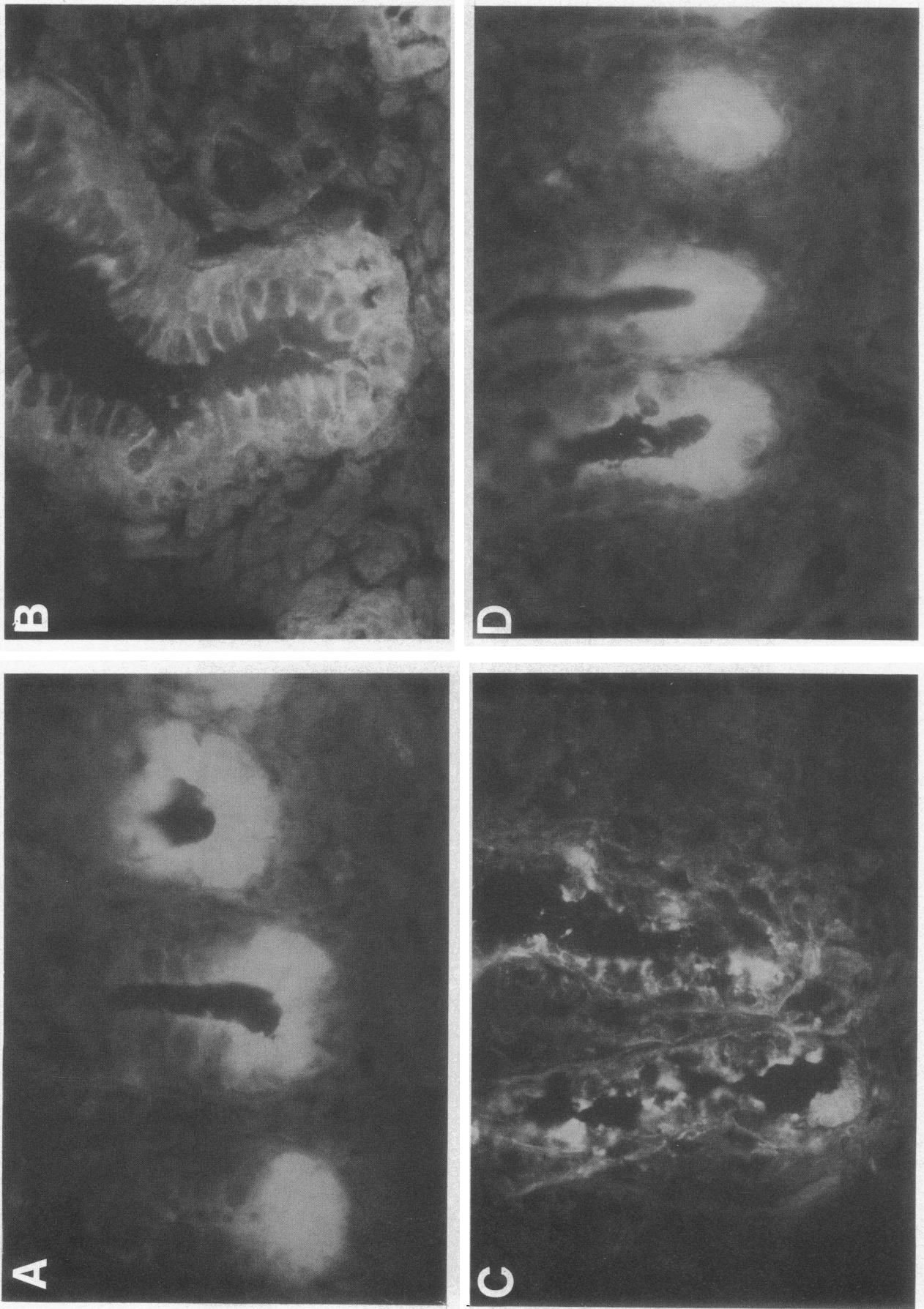
*Figure 8.* Immunohistochemical staining of normal ileum (*A*), inflamed ileum from a patient with Crohn's disease (*B* and *C*), and adjacent uninflamed ileum from the same patient with Crohn's disease (*D*). Cryostat sections were exposed to rabbit anti–human lysozyme and detected with Texas red–labeled donkey anti–rabbit serum. The sections were viewed under high power with fluorescence microscopy. *A, B,* and *D,* ×400; *C,* ×250.

mally folded $\alpha_1$-AT within $\alpha_1$-AT–synthesizing cells (20). Taken together with the current study, this observation makes it likely that $\alpha_1$-AT in feces is largely derived from $\alpha_1$-AT in sloughed enterocytes and unlikely that it diffuses or leaks into the intestinal lumen from the bloodstream. The increase in fecal $\alpha_1$-AT demonstrated in various protein-losing enteropathies could, in turn, represent the effect of small intestinal inflammation on the enterocyte extrusion rate.

It is also probable that $\alpha_1$-AT is expressed in the intestinal epithelium by resident macrophages. Previous studies have shown that blood monocytes and macrophages from two other tissues, respiratory epithelium and breast epithelium, synthesize $\alpha_1$-AT (4). Nevertheless, it was not possible to definitively demonstrate expression of the $\alpha_1$-AT gene in macrophages of the intestine by in situ hybridization because of the relatively high background. Ribonuclease protection assays have also been unable to detect macrophage-specific $\alpha_1$-AT transcripts in human intestinal homogenates, probably because of the relatively low numbers of macrophages ordinarily present in this tissue on a per unit area basis (7). Moreover, our recent studies have indicated that transcripts initiated at the alternative upstream, so-called "macrophage-specific" initiation sites may be detected in Caco2-derived enterocytes activated by interleukin-6 (7). Thus, even if these transcripts derived from the upstream alternative "macrophage-specific" initiation sites were detected by ribonuclease protection assays, it would not exclude the possibility that they were derived from activated enterocytes.

One of the most striking results of this study is the intense hybridization to $\alpha_1$-AT mRNA in Paneth cells. Several recent observations have led to greater understanding of this apparently highly specialized cell type. First, it is known that Paneth cells are derived from the same multipotent stem cell that also gives rise to enterocytes, goblet cells, and enteroendocrine cells (21–27). Enterocytes and goblet cells arise as they are rapidly translocated to the apical extrusion zone. Paneth cells differentiate as they descend to the base of the crypt. Second, several observations have suggested that Paneth cells play a role in host defense at the mucosal surface. In the rat, Paneth cells have been shown to have phagocytic activity against at least two different species of intestinal microorganisms (14). Lysozyme, an enzyme found in mononuclear phagocytes and that has antibacterial properties, has been detected in secretory granules of mature Paneth cells (28, 29). It has been considered a specific marker of these cells. Cryptidin, a peptide that is related to the defensins of classical phagocytic cells, has been specifically localized to Paneth cells (30). The appearance of cryptidin mRNA in mouse small bowel coincides with gut closure and with the establishment of a barrier to bacterial translocation; therefore, it has been implicated in antibacterial activity. Paneth cells are also similar to other phagocytic cells in that they express TNF-$\alpha$ (31). Paneth cells have recently been shown to be the site of expression within the intestine for guanylin (32), a peptide which represents the endogenous ligand for the intestine-specific receptor guanylyl cyclase (33). Expression of guanylin may be the most important function of Paneth cells in that the heat-stable enterotoxins of *Escherichia* coli and *Yersinia* are thought to induce diarrhea by competing with guanylin for binding to guanylyl cyclase (34). Third, Paneth cells may play a role in enzymatic digestion of luminal nutrients. There are abundant carboxylic ester hydrolases in secretory

granules of Paneth cells, similar to those of pancreatic exocrine cells (35, 36). Expression of $\alpha_1$-AT, a molecule that is capable of inhibiting bacterial serine proteases, as well as certain digestive proteases, and which otherwise plays an important role in the host response to inflammation/tissue injury, may therefore be especially relevant to the Paneth cell.

## Acknowledgments

## References

1. Perlmutter, D. H. 1991. The cellular basis for liver injury in alpha-1-antitrypsin deficiency. *Hepatology (Baltimore)*. 13:172–185.

2. Alper, C. A., D. Raum, Z. L. Awdeh, B. H. Petersen, P. D. Taylor, and T. E. Starzl. 1980. Studies of hepatic synthesis in vivo of plasma proteins including orosomucoid, transferrin, alpha-1-antitrypsin, C8 and factor B. *Clin. Immunol. Immunopathol.* 16:84–88.

3. Hood, J. M., L. J. Koep, R. L. Peters, G. P. J. Schroter, R. Weil, A. G. Redeker and T. E. Starzl. 1980. Liver transplantation for advanced liver disease with alpha-1-antitrypsin deficiency. *N. Engl. J. Med.* 302:272–275.

4. Perlmutter, D. H., F. S. Cole, P. Kilbridge, and T. Rossing. 1985. Expression of the alpha-1-proteinase inhibitor gene in human monocytes and macrophages. *Proc. Natl. Acad. Sci. USA.* 82:795–799.

5. Carlson, J., B. B. Rogers, R. N. Sifers, H. K. Hawkins, M. J. Finegold and S. L. Woo. 1988. Multiple tissues express alpha-1-antitrypsin in transgenic mice and man. *J. Clin. Invest.* 82:26–36.

6. Koopman, P., S. Povey and R. H. Lovell-Badge. 1989. Widespread expression of human alpha-1-antitrypsin in transgenic mice revealed by in situ hybridization. *Genes & Dev.* 3:16–25.

7. Hafeez, W., G. Ciliberto and D. H. Perlmutter. 1992. Constitutive and modulated expression of the human alpha-1-antitrypsin gene. Different transcriptional initiation sites used in three different cell types. *J. Clin. Invest.* 89:1214–1222.

8. Barbey-Morel, C., J. A. Pierce, E. J. Campbell and D. H. Perlmutter. 1987. Lipopolysaccharide modulates the expression of alpha-1-proteinase inhibitor and other serine proteinase inhibitors in human monocytes and macrophages. *J. Exp. Med.* 166:1041–1054.

9. Perlmutter, D. H., J. D. Daniels, H. S. Auerbach, K. De Schryver-Kecskemeti, H. S. Winter and D. H. Alpers. 1989. The alpha-1-antitrypsin gene is expressed in a human intestinal epithelial cell line. *J. Biol. Chem.* 264:9485–9490.

10. Rubin, D. C. 1992. Spatial analysis of transcriptional activation in fetal gut jejunal and ileal epithelium. *Am. J. Phys.* 263:G853–G863.

11. Rubin, D. C., K. A. Roth, E. W. Birkenmeier and J. I. Gordon. 1991. Epithelial cell differentiation in normal and transgenic isografts. *J. Cell Biol.* 113:1183–1192.

12. Roth, K. A., J. M. Hertz and J. I. Gordon. 1990. Mapping enteroendocrine cell populations in transgenic mice reveals an unexpected degree of complexity in cellular differentiation within the gastrointestinal tract. *J. Cell Biol.* 110:1791–1801.

13. Roth, K. A. and J. I. Gordon. 1990. Spatial differentiation of the intestinal epithelium: an analysis of enteroendocrine cells containing immunoreactive serotonin, secretin and substance P in normal and transgenic mice. *Proc. Natl. Acad. Sci. USA.* 87:6408–6412.

14. Satoh, Y., K. Ishikawa, H. Takana, Y. Oomori and K. Ono. 1988. Immunohistochemical observations of lysozyme in the Paneth cells of specific-pathogen-free and germ-free mice. *Acta Histochem.* 83:185–188.

15. Palmer, P. E., R. A. DeLellis and H. J. Wolfe. 1974. Immunohistochemistry of liver in alpha-1-antitrypsin deficiency. A comparative study. *Am. J. Clin. Pathol.* 62:350–354.

16. Molmenti, E. P., T. Ziambaras and D. H. Perlmutter. 1993. Evidence for an acute phase response in human intestinal epithelial cells. *J. Biol. Chem.* 268:14116–14124.

17. Ahrenstedt, O., L. Knutson, B. Nilsson, K. Nilsson-Ekdahl, B. Odlind and R. Hallgren. 1992. Enhanced local production of complement components in the small intestine of patients with Crohn's disease. *N. Engl. J. Med.* 322:1345–1349.

18. Strygler, B., M. J. Nicar, W. C. Santangelo, J. L. Porter and J. S. Fordtran. 1990. Alpha-1-antitrypsin excretion in stool in normal subjects and in patients with gastrointestinal disorders. *Gastroenterology.* 99:1380–1387.

19. Grill, B. B., T. Tinghitella, C. Hillmeier and J. Gryboski. 1983. Increased

intestinal clearance of alpha-1-antitrypsin in patients with alpha-1-antitrypsin deficiency. *J. Pediatr. Gastroenterol. Nutr.* 2:95–98.

20. Perlmutter, D. H., R. M. Kay, F. S. Cole, T. H. Rossing, D. VanThiel and H. R. Colten. 1985. The cellular defect in alpha-1-proteinase inhibitor (a-1-PI) deficiency is expressed in human monocytes and in Xenopus oocytes injected with human liver mRNA. *Proc. Natl. Acad. Sci. USA.* 82:6918–6921.

21. Gordon, J. I. 1989. Intestinal epithelial cell differentiation: new insights from chimeric and transgenic mice. *J. Cell Biol.* 108:1187–1194.

22. Schmidt, G. H., D. J. Winton and B. A. J. Ponder. 1988. Development of the pattern of cell renewal in the crypt villus unit of chimeric mouse intestine. *Development (Camb.)* 103:785–790.

23. Cohn, S. M., K. A. Roth, E. H. Birkenmeier and J. I. Gordon. 1991. Temporal and spatial patterns of transgene expression in aging adult mice provide insights about the origins, organization and differentiation of the intestinal epithelium. *Proc. Natl. Acad. Sci. USA.* 88:1034–1038.

24. Winton, D. J., and B. A. J. Ponder. 1990. Stem cell organization in mouse small intestine. *Proc. R. Soc. Lond. Ser. B. Biol. Sci.* 241:13–18.

25. Potten, C. S., and M. Loeffler. 1987. A comprehensive model of the crypts of the small intestine of the mouse provides insight into the mechanisms of cell migration and the proliferation hierarchy. *J. Theor. Biol.* 127:381–391.

26. Potten, C. S., and M. Loeffler. 1990. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lesson for and from the crypt. *Development (Camb.).* 110:1001–1020.

27. Schmidt, G. H., M. M. Wilkinson and B. A. J. Ponder. 1985. Cell migration pathway in the intestinal epithelium: an in situ marker system using mouse aggregation chimeras. *Cell.* 40:425–429.

28. Erlandsen, S. L., J. A. Parsons and T. D. Taylor. 1974. Ultrastructural immunocytochemical localization of lysozyme in the Paneth cells of man. *J. Histochem. Cytochem.* 22:401–413.

29. Geyer, G. 1973. Lysozyme in Paneth cell secretions. *Acta Histochem.* 45:126–132.

30. Ouellette, A. J., R. M. Greco, M. James, D. Frederick, J. Naftilan and J. T. Fallon. 1989. Developmental regulation of cryptidin, a corticostatin/defensin precursor mRNA in mouse small intestinal crypt epithelium. *J. Cell Biol.* 108:1687–1695.

31. Keshav, S., L. Lawson, L. P. Chung, M. Stein, V. H. Perry, and S. Gordon. 1990. Tumor necrosis factor mRNA localized to Paneth cells of normal murine intestinal epithelium by in situ hybridization. *J. Exp. Med.* 171:327–332.

32. de Sauvage, F. J., S. Keshav, W.-J. Kuang, N. Gillett, W. Henzel and D. Goeddel. 1992. Precursor structure, expression, and tissue distribution of human guanylin. *Proc. Natl. Acad. Sci. USA.* 89:9089–9093.

33. Currie, M. G., K. F. Fok, J. Kato, R. Moore, F. K. Hamra, K. L. Duffin and C. E. Smith. 1992. Guanylin: an endogenous activator of intestinal guanylate cyclase. *Proc. Natl. Acad. Sci. USA.* 89:947–951.

34. Schulz, S., C. K. Green, P. S. T. Yuen and D. L. Garbers. 1990. Guanylyl cyclase is a heat-stable enterotoxin receptor. *Cell.* 63:941–948.

35. Lechene de la Porte, P., H. Lafont and D. Lombardo. 1986. Immunocytochemical localization of pancreatic carboxylic ester hydrolases in human Paneth cells. *Histochemistry.* 86:211–214.

36. Mason, D. Y. and C. R. Taylor. 1974. The distribution of muramidase (lysozyme) in human tissues. *J. Clin. Pathol. (Lond.)* 28:124–132.