Expression of SV-40 T Antigen in the Small Intestinal Epithelium of Transgenic Mice Results in Proliferative Changes in the Crypt and Reentry of Villus-associated Enterocytes into the Cell Cycle but Has No Apparent Effect on Cellular Differentiation Programs and Does Not Cause Neoplastic Transformation

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Abstract. The mouse intestinal epithelium represents a unique mammalian system for examining the relationship between cell division, commitment, and differentiation. Proliferation and differentiation are rapid, perpetual, and spatially well-organized processes that occur along the crypt-to-villus axis and involve clearly defined cell lineages derived from a common multipotent stem cell located near the base of each crypt. Nucleotides −1178 to +28 of the rat intestinal fatty acid binding protein gene were used to establish three pedigrees of transgenic mice that expressed SV-40 large T antigen (TAg) in epithelial cells situated in the uppermost portion of small intestinal crypts and in already committed, differentiating enterocytes as they exited these crypts and migrated up the villus. T antigen production was associated with increases in crypt cell proliferation but had no apparent effect on commitment to differentiate along enterocytic, enteroendocrine, or Paneth cell lineages. Single- and multilabel-immunocytochemical studies plus RNA blot hybridization analyses suggested that the differentiation programs of these lineages were similar in transgenic mice and their normal littermates. This included enterocytes which, based on the pattern of [^{3}H]thymidine and 5-bromo-2′-deoxyuridine labeling and proliferating nuclear antigen expression, had reentered the cell cycle during their migration up the villus. The state of cellular differentiation and/or TAg production appeared to affect the nature of the cell cycle; analysis of the ratio of S-phase to M-phase cells (collected by metaphase arrest with vincristine) and of the intensities of labeling of nuclei by [^{3}H]thymidine indicated that the duration of S phase was longer in differentiating, villus-associated enterocytes than in the less well-differentiated crypt epithelial cell population and that there may be a block at the G_2/M boundary. Sustained increases in crypt and villus epithelial cell proliferation over a 9-mo period were not associated with the development of gut neoplasms—suggesting that tumorigenesis in the intestine may require that the initiated cell have many of the properties of the gut stem cell including functional anchorage.

THE manner in which epithelial cell renewal occurs in the mouse intestine provides a unique opportunity to address questions about the relationships between cell division and differentiation programs in a mammalian system. The opportunity arises because of three features: (*a*) proliferation and differentiation in the gut are spatially well organized along the crypt-to-villus axis; (*b*) the processes occur rapidly (completed in ~3 d) and perpetually; and (*c*) cell lineage relationships are well known and involve derivation from a single, active multipotent stem cell.

Each adult small intestinal villus contains a steady state level of several thousand surface epithelial cells (7,000–2,000 depending upon its location along the duodenal-to-ileal axis) and is surrounded by ~14 (duodenum) to 6 (ileum) flask-shaped crypts of Lieberkühn (Wright and Irwin, 1982).

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The crypts represent the proliferative compartment of the small intestine. Each contains 350–550 epithelial cells. Approximately 150 of these cells, situated in the mid-portion of the crypt, pass through the cell cycle every 12 h resulting in an average production rate of 300 new cells/day/crypt (Potten and Loeffler, 1990). In adult animals, each crypt is monoclonal (Ponder et al., 1985; Schmidt et al., 1988): a multipotent stem cell, functionally anchored near the base of the crypt, gives rise to descendants (a transit stem cell population) that proliferate and ultimately yield terminally differentiated, nonproliferating columnar absorptive enterocytes, mucus-producing goblet cells, a complex population of enteroendocrine cells, and defensin/lysozyme-producing Paneth cells (Cheng and Leblond, 1974; Winton et al., 1988, 1990; Roth et al., 1991*b*). Paneth cells complete their differentiation program as they descend to the base of each crypt. Enterocytes, goblet cells, and enteroendocrine cells undergo differentiation/maturation as they migrate in vertical, coherent bands up adjacent (polyclonal) villi (Cheng and Leblond, 1974; Schmidt et al., 1985). 12 cells exit the crypt per hour and move up the villus at the remarkable velocity of 0.75 cell diameters per hour. It takes ~3 d to complete the journey to the apical portion of the villus where ~1,400 cells subsequently undergo exfoliation into the gut lumen/villus/day (Potten and Loeffler, 1990).

A number of intriguing questions about intestinal epithelial differentiation/proliferation can be posed. Is passage through the cell cycle a major regulator of the ill-defined process of commitment? Does such passage serve as a "punctuation" point by which committed cells are able to mark their entrance into a differentiation program? Should differentiation (i.e., the noncyclic acquisition of new gene products; Potten and Loeffler, 1990) be viewed as a series of irreversible binary decisions or the result of continuous active regulation (Blau and Baltimore, 1991)? What are the relative dependencies of the differentiation program on position occupied along the crypt-to-villus axis or on time? If the temporal factor is important, is it measured from the moment of passage through the cell cycle, from the time of exit from the crypt, or do other parameters serve as markers? Is there a way of separating the effects of time and space (position) on differentiation? Finally, when the balance between proliferation and differentiation is lost during neoplastic transformation, how is the initiated cell able to achieve functional anchorage for a period sufficient to allow clonal amplification?

We have generated a transgenic mouse model to explore several of these questions. Expression of the mouse intestinal fatty acid binding protein gene (*Fabpi*)[1] is gut-specific, cell-specific, region-specific, and developmental stage-specific: (*a*) expression is activated as enterocytes emerge from the crypt and sustained throughout their subsequent translocation to the apical extrusion zone of the villi; (*b*) highest steady state levels of its mRNA and protein products are attained in the jejunum, with a progressive fall occurring as one proceeds to the proximal colon; and (*c*) transcription is initiated in late gestation coincident with cytodifferentiation of the epithelium and the emergence of nascent villi (Sweetser et al., 1988*b*; Rubin et al., 1991).

1. *Abbreviations used in this paper*: BrdUrd, 5'-bromo-2'deoxyuridine; *Fabpi*, fatty acid binding protein gene; I-FABP, intestinal fatty acid binding protein; L-FABP, liver FABP; PCNA, proliferating cell nuclear antigen; TAg, T antigen.

Previous studies in transgenic mice indicated that nucleotides −1178 to +28 of rat *Fabpi* (I-FABP −1178 to +28; Sweetser et al., 1987) contain all of the necessary *cis*-acting elements to recapitulate the differentiation-dependent patterns of expression of the intact, endogenous mouse gene as well as its geographic specificity along the duodenal-to-colonic axis (Sweetser et al., 1988*b*). SV-40 T antigen (SV-40 TAg) is a multifunctional transforming protein that binds to two tumor suppressors - p53 and the retinoblastoma susceptibility gene product, Rb (Finlay et al., 1989; DeCaprio et al., 1988; Dyson et al., 1990; Ludlow et al., 1990). Large T antigen can eliminate proliferation controls which operate in the G_1 phase of the cell cycle (reviewed in Pardee, 1989). We reasoned that by linking nucleotides −1178 to +28 of rat *Fabpi* to the portion of the SV-40 genome encoding TAg and using the resulting chimeric gene to produce transgenic mice, production of this 708 residue polypeptide could be induced coincident with exit of enterocytes from the crypt. Such differentiation-dependent activation of transgene expression would, in turn, offer the opportunity for determining whether committed/differentiating enterocytes can reenter the cell cycle even if they occupy a position in the lower to mid-villus, whether such reentry affects the differentiation program, and finally whether functional anchorage of such cells can occur in the continuously renewing epithelium and tumor formation ensue.

Materials and Methods

Production of Transgenic Mice with I-FABP$^{-1178\ to\ +28}$/SV-40 TAg Fusion Genes

A 2.7-kb BamHI/BglI fragment spanning nucleotides 2533–5235 of the SV-40 genome was ligated to a 5.6-kb BamHI fragment obtained from pIFhGH2 (Sweetser et al., 1988*b*) using a BamHI/BglI adaptor (5'GATCCCT3'). A 3.9-kb EcoRI/SalI fragment containing the I-FABP$^{-1178\ to\ +28}$/SV-40 TAg fusion gene was then injected into recently fertilized B6D2F_1 × B6D2F_1 or FVB/N oocytes. Four founders (G_o13 and G_o30 from B6D2F_1 × B6D2F_1 oocytes and G_o48 and G_o103 from FVB/N [Taketo et al., 1991] oocytes) were identified after Southern blot analyses of tail DNA obtained from 135 live born mice (32 from B6D2F_1 × B6D2F_1 oocytes and 103 from FVB/N oocytes). Lines were established from each of these founders. Southern blot analysis of tail DNAs obtained from F_1 mice indicated that the transgene copy number was <5 per haploid genome in G_o13-, G_o30-, and G_o48-derived animals and 100 in the G_o103 pedigree. F_1–F_3 male and female mice from all lines were maintained on a standard chow diet given ad libitum and under a strictly controlled light cycle (lights on at 0700, off at 1900 h). They were subsequently sacrificed by cervical dislocation at ages ranging from 50 to 260 d for one or more of the studies described below.

RNA Blot Hybridization Studies

After the mice were killed, the gastrointestinal tract was rapidly subdivided as described in Sweetser et al. (1988*a*) and the eight segments were frozen in liquid N_2. Nine extra-intestinal tissues were also recovered from each mouse (brain, heart, lung, skeletal muscle, kidney, liver, spleen, pancreas, and gonads). Total cellular RNA was prepared from each tissue or intestinal segment using the guanidine isothiocyanate/cesium chloride method (Chirgwin et al., 1979). RNA integrity was established by denaturing methylmercury (Thomas, 1980) or formaldehyde agarose gel electrophoresis. Northern or quantitative RNA dot blot hybridizations were performed using [^{32}P]-labeled rat I-FABP cDNA, rat liver fatty acid binding protein (L-FABP) cDNA, and SV-40 TAg DNA probes plus the hybridization and washing conditions detailed in our earlier publications (Sweetser et al., 1988*a*,*b*). The steady state level of a specific mRNA in samples of total cellular RNA was calculated from the intensities of signals generated by in vitro transcribed mRNA standards (0.1–500 pg) that were included in each dot blot (Sweetser et al., 1988*a*,*b*). Blots were scanned with a laser den-

sitometer. Only signals in the linear range of film sensitivity were used for calculations of mRNA abundance.

Immunocytochemical Studies

Analysis of I-FABP, L-FABP, Alkaline Phosphatase, Lysozyme, and Neuroendocrine Gene Expression in Intestinal Epithelial Cells. 5-10-mm sections were recovered from the mid-portions of each intestinal segment before freezing and RNA extraction. These sections were fixed in Bouin's fluid and embedded in paraffin. 5-μm-thick sections were cut from material that had been embedded in paraffin, processed as described in Roth et al. (1990), and then surveyed with a panel of polyclonal antisera that have been extensively characterized (Roth et al., 1990, 1991*a,b*; Roth and Gordon, 1990; Rubin et al., 1991; Young et al., 1981*a,b*). Antigen-antibody complexes were visualized using gold-labeled goat anti-rabbit IgG serum and silver enhancement (Amersham, Arlington Heights, IL) or secondary antibodies labeled with either fluorescein or Texas red (Jackson ImmunoResearch Laboratories, West Grove, PA). Double-label immunofluorescence studies were also performed according to Roth et al. (1990). To quantitate expression of neuroendocrine products in the enteroendocrine cells of transgenic mice (n = 5) and their normal littermates (n = 4), 5-10 sections from the proximal jejunal segment of each animal were examined with each antibody and the total number of positive cells per cross section was determined. Each positive cell was also classified as being either crypt- or villus-associated (see Roth et al., 1990).

Distribution of SV-40 TAg. 10-μm-thick cryostat sections, prepared from duodenal, jejunal, and ileal segments that had been frozen in OCT compound, were fixed for 10 min at −20°C in acetone. Sections were subsequently incubated for 1 h at room temperature with either a mouse mAb or a hamster antiserum directed against SV-40 TAg (mAB 421 and the hamster antiserum were kindly supplied by J. Pipas [University of Pittsburgh]; final dilution of each = 1:1,000). Antigen-antibody complexes were visualized using the ABC peroxidase method or by fluorescein-labeled rabbit anti-hamster serum (Jackson ImmunoResearch Laboratories).

Studies Involving Proliferating Cell Nuclear Antigen. Segments of duodenum, proximal and distal jejunum, and ileum were removed from three control and three transgenic mice (3.5-9 mo of age, derived from G_o13, see below), fixed in 10% buffered formalin, and embedded in paraffin. 5-μm sections were cut, deparaffinized in xylene, hydrated, and then washed in PBS. Sections were incubated with 10% normal rabbit serum for 10 min at room temperature, washed in PBS, and overlaid with a 1:10 dilution of a mAb to proliferating cell nuclear antigen (PCNA) (PC-10; Dako, Santa Barbara, CA; Hall et al., 1990). After a 35-min incubation followed by a PBS wash, sections were overlaid (for 30 min) with a 1:300 dilution of a biotinylated rabbit anti-mouse immunoglobulin sera (Dakopatts, Denmark). They were then washed in PBS and incubated with peroxidase-labeled avidin. After another series of PBS washes, sections were incubated with diaminobenzidine (0.5 mg/ml) containing 0.03% hydrogen peroxide for 3 min at room temperature. Sections were subsequently washed and counterstained with hematoxylin. The PCNA labeling index of crypt- and villus-associated cellular populations was determined by dividing the number of PCNA-labeled nuclei by the total number of nuclei present in longitudinally oriented crypt or villus units (Hall et al., 1990).

Multilabel Immunocytochemical Studies of the Colocalization of I-FABP and Bromodeoxyuridine in Villus-associated Enterocytes Labeled in S-phase with the Nucleotide Analogue. Mice were given an intraperitoneal injection of a solution containing 5'-bromo-2'-deoxyuridine (BrdUrd, 120 mg/kg) and 5'-fluoro-deoxyuridine (12 mg/kg) 90 min before they were killed. Tissues were removed, fixed in Bouin's fluid, embedded in paraffin and 5-μm sections prepared. After removal of paraffin, sections were incubated overnight at 4°C with goat anti-BrdUrd serum (Cohn and Lieberman, 1984) and rabbit anti-I-FABP serum (Sweetser et al., 1988*b*). After a series of washes with PBS, antibody-antigen complexes were identified with fluorescein-labeled donkey antigoat and Texas red-labeled donkey antirabbit sera, respectively.

Calculation of Duodenal Mitotic and [^{3}H]Thymidine Labeling Indices

Three G_o13-derived transgenic mice (age = 3.5, 8, and 9 mo) and three normal littermates were injected intraperitoneally with 1 μg/g vincristine sulfate (David Bull Laboratories, UK) at 0900 h followed by an injection of [^{3}H]thymidine (2 Ci/mmol; 1 μCi/g of body weight, Amersham) 1 h later. 1 h after the radiolabeled nucleotide was infused, animals were killed by cervical dislocation. The entire gastrointestinal tract (i.e., stomach to distal colon) was removed, rapidly blotted, and weighed. After regional dissection, segments were either fixed and stored in neutral buffered formalin or fixed in Carnoy's fluid and stored in 70% (vol/vol) ethanol.

To determine the mitotic index, fixed duodenal segments were hydrated by incubation in a solution of 50% ethanol for 10 min at room temperature followed by 25% ethanol (again for 10 min). Segments were then hydrolyzed in 1 M HCl at 60°C for 12 min and subsequently stained in Schiff's reagent. After this last step, tissue was transferred to 45% acetic acid, gently teased apart under a dissecting microscope and squashed with a coverslip. The number of arrested metaphases was noted in 20 duodenal crypts and villi (Goodlad and Wright, 1982) for each of the three transgenic and three normal mice surveyed.

To calculate the labeling index, formalin-fixed segments of duodenum harvested from the three G_o13 transgenics and their normal littermates were embedded in paraffin. 5-μm sections were cut, mounted, and then dipped in Ilford K2 emulsion (Ilford Ltd., Cheshire, UK). After a 3-wk exposure, autoradiographs were developed and stained with hematoxylin and eosin. The number of labeled cells was quantitated in at least 10 duodenal villi per animal after first using an eyepiece graticule to divide the villus into five zones of equal length. A labeling index (percent of cells labeled) was also derived after scoring 20 crypts per mouse.

Grain Counts

Autoradiographs of the duodenum were examined using a 100× objective and the number of silver grains directly overlying the nuclei of 50 duodenal villus- or 20 crypt-associated epithelial cells was scored in each of the three transgenic animals studied. All cells were selected at random.

Morphometric Analysis

Microdissected crypts were examined under a compound microscope and the outline of villi or crypts traced using a drawing tube. Villus or crypt outlines were then digitized with a Macintizer ADB graphics tablet (SSI Ltd., Pewsey, Wiltshire) connected to an Apple Macintosh computer running MacSterology software (Ranfurly Microsystems, Airdrie, UK). The crypt-to-villus ratio was determined by counting the number of crypt necks or villus bases per low power field (16X objective) in duodenal, proximal jejunal, distal jejunal, and ileal segments after they were bulk stained with the Feulgen reaction.

Gross and Microscopic Surveys of the Gastrointestinal Tracts of I-FABP$^{-1178\ to\ +28}$/SV-40 TAg Mice for Neoplasms

Male and female transgenic mice (n = 21 mice, 2-9 mo of age, from the G_o13, G_o48, and G_o103 pedigrees) were killed by cervical dislocation and their gastrointestinal tracts removed. After fixation, the epithelium in each longitudinally incised segment was inspected under the dissecting microscope. 5-10-μm sections were also prepared from paraffin embedded or frozen samples of these segments and stained with hematoxylin and eosin for histologic analysis.

Results

Nucleotides 1178 to +28 of rat Fabpi Direct Expression of SV-40 TAg to the Intestinal Epithelium of Transgenic Mice

Four pedigrees of transgenic mice were established containing I-FABP$^{-1178\ to\ 28}$/SV-40 TAg. Tissue-specific and cell-specific patterns of transgene expression were analyzed in adult F_1-F_2 mice from each of the lines. Dot and Northern blot hybridization studies of total cellular RNA isolated from stomach, small intestine, colon, pancreas, brain, heart, lung, liver, spleen, kidney, skeletal muscle, and testes indicated that TAg mRNA was confined to the intestine of adult animals derived from G_o13, 48, and 103 (data not shown). Adult mice from the other line (G_o30) did not contain detectable levels of TAg mRNA in any of these 12 tissues. Regional dissection of the gastrointestinal tract allowed us to

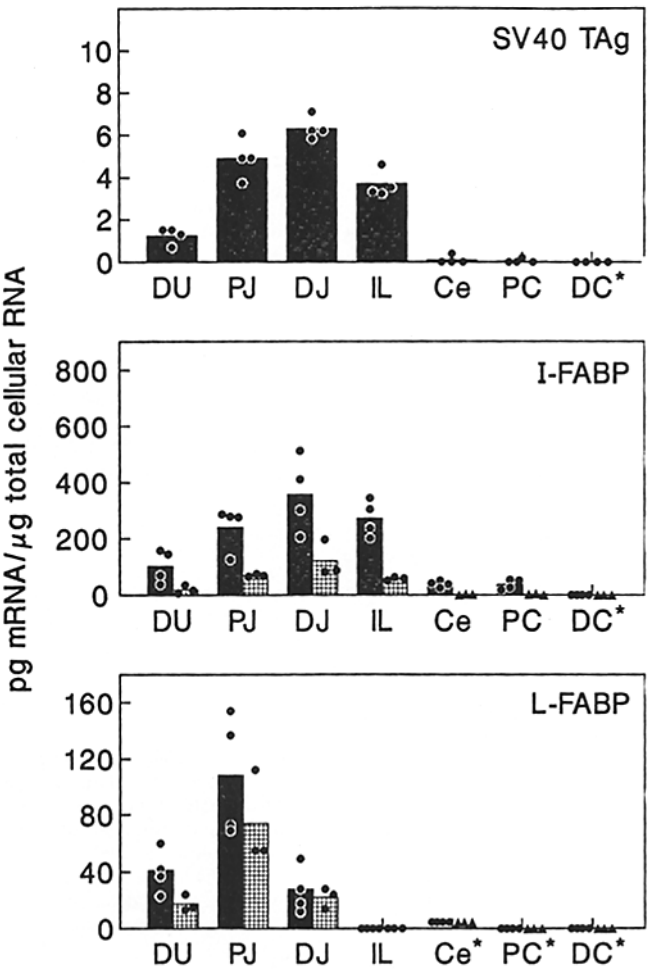


Figure 1. Distribution of SV-40 TAg, I-FABP, and L-FABP mRNAs along the duodenal to colonic axis of G_o13-derived I-FABP$^{1178\text{ to }+28}$/SV-40 TAg transgenic mice (■) and their normal littermates (▦). Values were determined by quantitative RNA dot blot hybridizations. Results obtained from each animal (n = four transgenic mice 3–9 mo of age and four of their normal littermates) are indicated by a ●. The asterisk indicates that the mRNA was not detectable in total cellular RNA prepared from this segment in any of the animals surveyed (limits of detection = 0.2 pg/μg). *DU*, duodenum; *PJ*, proximal jejunum; *DJ*, distal jejunum; *IL*, ileum; *CE*, cecum; *PC*, proximal colon; *DC*, distal colon. A two-tailed t-test revealed no significant differences in L-FABP mRNA levels between transgenic and normal mice in any of the intestinal segments surveyed ($P > 0.05$). While the relative distribution of I-FABP mRNA along the duodenal-to-colonic axis was not significantly different between the two groups, statistically significant ($P < 0.01$) quantitative differences were found in the proximal jejunal and ileal segments of their small bowels. These differences are not accompanied by appreciable differences in the steady state concentration of immunoreactive I-FABP in enterocytes located at comparable positions along the crypt-to-villus axis (see text).

define the distribution of SV-40 TAg mRNA along the duodenal-to-colonic axis. The results of quantitative dot blot studies of RNAs obtained from four 90–260-d-old mice from line 13 are shown in Fig. 1. The duodenal-to-colonic distribution of SV-40 TAg mRNA closely approximated the distribution of I-FABP mRNA (compare the upper and middle panels). Highest steady state levels of SV-40 mRNA were encountered in the distal jejunum (range = 6–7 pg/μg total cellular RNA) with a progressive fall occurring from this segment to the ileum (3–5 pg/μg) and the cecum (≤0.4 pg/μg). No TAg mRNA was detectable in colonic RNA (limits of detection of the assay = 0.2 pg/μg total cellular RNA). It is remarkable that the concentration of SV-40 TAg mRNA in a given segment varied so little between animals even over a wide range of ages (3–9 mo).

Northern blot analyses of duodenal, jejunal, and ileal RNAs prepared from adult (3-mo-old) descendants of G_o48 and G_o103 revealed (*a*) a unique mRNA species corresponding to the known size of SV-40 TAg mRNA in members of these transgenic pedigrees but not in RNA prepared from their normal littermates; and (*b*) a similar distribution of TAg mRNA along the duodenal-to-ileal axis compared to G_o13-derived transgenics despite their different genetic backgrounds (data not shown).

Immunocytochemical staining of proximal jejunum harvested from 3–9-mo-old transgenic mice from the three "expressing" pedigrees showed that SV-40 TAg production was confined to enterocytes. Immunoreactive protein was first detectable in the nuclei of cells located in the uppermost quarter of the crypt and in the basal portion of the villus (Fig. 2 *A*). Steady state levels were higher in cells situated in the middle and upper thirds of small intestinal villi. Fig. 2 *B* illustrates that there was little apparent change in the concentration of this transforming protein as enterocytes migrated from the mid to apical portions of several villi situated in the proximal jejunum. The distribution of immunoreactive SV-40 TAg along the crypt-to-villus axis was similar to that of I-FABP (Fig. 2 *C*). Control experiments established that no nuclear (or cytoplasmic) signal was present after incubation of the anti-TAg sera with sections of proximal jejunum harvested from normal littermates or from transgenic mice from the SV-40 TAg mRNA-negative line 30 (data not shown).

Effect of SV-40 TAg Expression on Intestinal Epithelial Cell Proliferation

There were no significant differences in the percent body weight of the small intestine or colon of adult 3–9-mo-old G_o13-derived transgenic mice compared to their normal littermates or members of the nonexpressing transgenic pedigree (two-way ANOVA, $P > 0.05$).

[^{3}H]Thymidine pulse labeling studies revealed that there were no significant differences in the duodenal crypt labeling indices of G_o13-derived control mice compared to their TAg-expressing transgenic littermates (32 ± 2% versus 33 ± 4%, respectively, see Fig. 3 *A*). However, the duodenal crypts in transgenic mice were significantly longer (Fig. 3 *B*), and thus the number of labeled cells per crypt was also significantly increased (Fig. 3 *C*). The 2-h collection of metaphases (see Materials and Methods) was also significantly increased (from 19 ± 1 to 35 ± 5 metaphases per duodenal crypt, $P < 0.05$, Fig. 3 *D*). The area of the duodenal crypts was also significantly greater in transgenic mice (Fig. 3 *E*). However, there was no difference in the number of crypts supplying the duodenal (or jejunal, or ileal) villi between transgenic animals and their normal littermates (see crypt to villus ratio in Fig. 4 *A*) nor was there a significant difference in the area of their villi (Fig. 4 *B*).

No [^{3}H]thymidine labeling of villus-associated enterocytes was noted in nontransgenic littermates (i.e., the labeling index was 0%, see Fig. 5 *A*). In contrast, there was

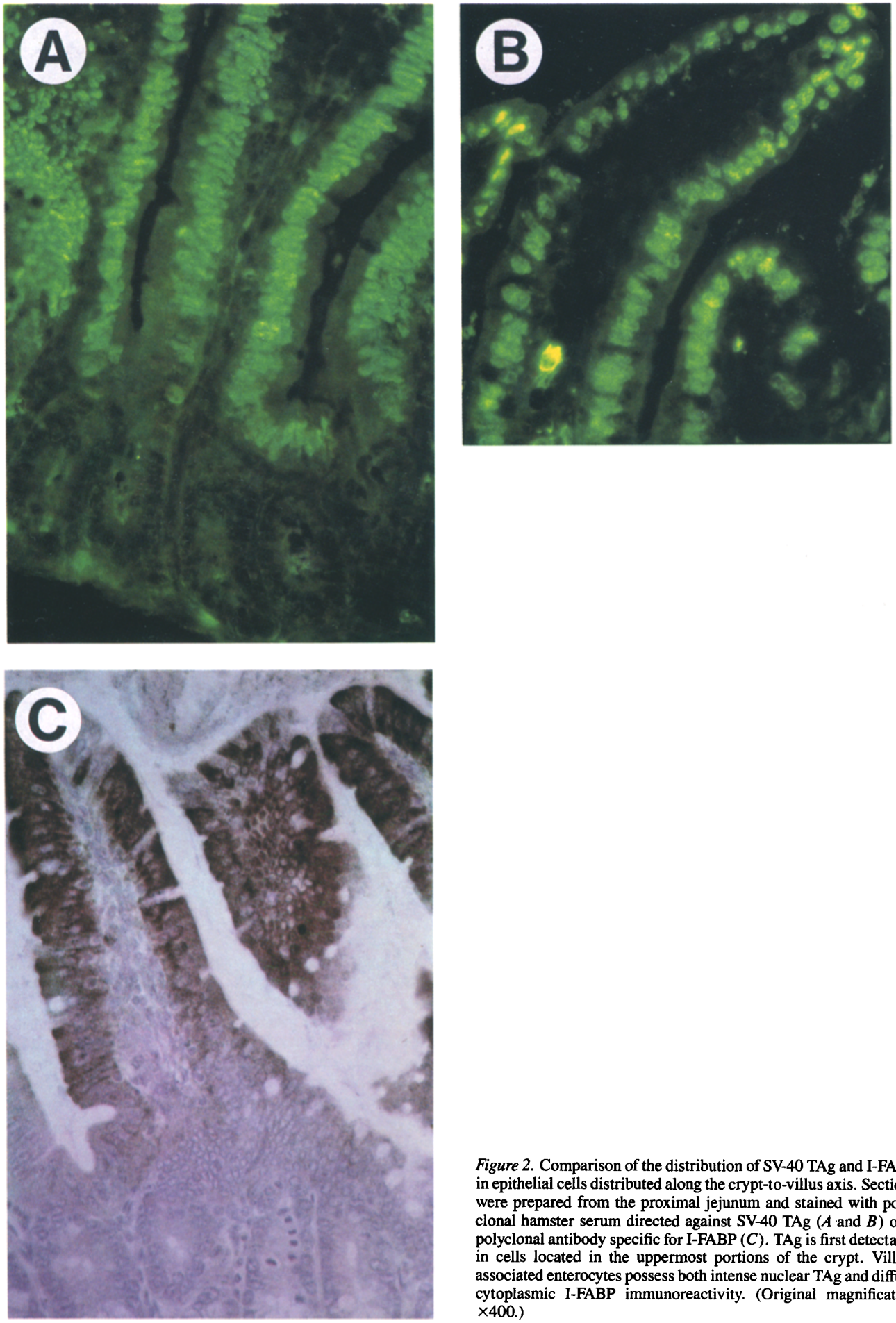


Figure 2. Comparison of the distribution of SV-40 TAg and I-FABP in epithelial cells distributed along the crypt-to-villus axis. Sections were prepared from the proximal jejunum and stained with polyclonal hamster serum directed against SV-40 TAg (*A* and *B*) or a polyclonal antibody specific for I-FABP (*C*). TAg is first detectable in cells located in the uppermost portions of the crypt. Villus-associated enterocytes possess both intense nuclear TAg and diffuse cytoplasmic I-FABP immunoreactivity. (Original magnification ×400.)

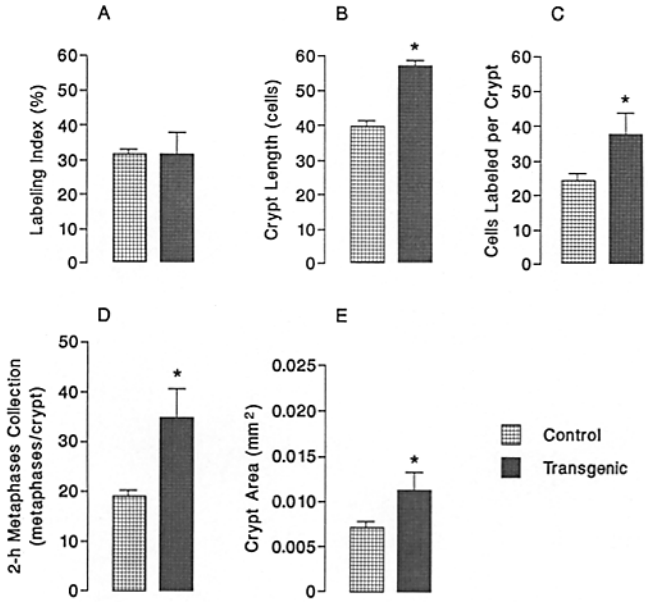


Figure 3. Comparison of duodenal crypt labeling and morphometric indices in I-FABP$^{1178\text{ to }+28}$/SV-40 TAg mice and their normal littermates. See Materials and Methods for a description of how these values were obtained from three G_o13 transgenic mice aged 3–9 mo and three normal littermates. The asterisks (*) indicate that the difference between transgenic and normal mice is statistically significant ($P < 0.01$).

extensive labeling of enterocytes on the duodenal villi of G_o13-derived transgenic animals (Fig. 5 *B*). The total duodenal villus labeling index for these mice was 12 ± 2% (Fig. 4 *C*). The majority of labeled cells were located in the mid-portion of duodenal villi where the labeling index averaged 20% (Fig. 4 *C*). This latter value approaches the labeling index found in normal and transgenic crypts (Fig. 3 *A*). Moreover, the mean number of silver grains overlying a nucleus in a transgenic villus-associated duodenal epithelial cell was 28 ± 2% while the mean number of silver grains over a labeled nucleus situated in the crypt was 52 ± 4%. The number of villus-associated cells labeled in S phase during the 1-h pulse of [^{3}H]thymidine decreased as a function of their position along the duodenal-to-ileal axis of the small intestine (Fig. 5, *B–D*), a decrease which paralleled the decrease in TAg expression (Fig. 1).

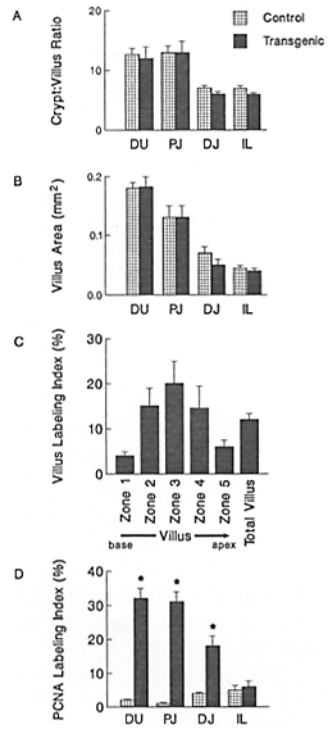


Figure 4. Comparison of villus labeling and morphometric indices in transgenic mice and their normal littermates. See Materials and Methods for details. Note that crypt/villus ratio refers to the number of crypts that surround duodenal (*DU*), proximal jejunal (*PJ*), distal jejunal (*DJ*), and ileal (*IL*) villi. The asterisks in *D* indicate that the differences in PCNA labeling indices between transgenic and normal mice are statistically significant ($P < 0.01$) in the segment surveyed.

The duodenal villus mitotic index in G_o13-derived transgenic mice was 2 ± 1% compared to a value of 18 ± 2% in their duodenal crypts. This ninefold difference between the duodenal villus' mitotic index and the duodenal crypt's mitotic index is greater than the threefold difference in [^{3}H]thymidine labeling indices determined simultaneously in the same sections (total villus labeling index = 12%, crypt = 33%). Expressed another way, there was a greater discrepancy between the number of cells in S and M phase in the villus (ratio of labeling index to mitotic index = 6) compared to the crypts (ratio of labeling index = 1.8, see Fig. 3, *A* and *D*). The significance of these observations is discussed below.

Proliferating cell nuclear antigen (PCNA) is a 36-kD nuclear phosphoprotein that functions as a cofactor for DNA polymerase-δ. It is synthesized in greater amounts during S-phase and has a half-life of ~20 h (Bravo and Celis, 1980; Celis and Celis, 1985; Bravo and Macdonald-Bravo, 1987; Jaskulski et al., 1988; Morris and Mathews, 1989; Chang et al., 1990). PCNA expression can be used to identify the proliferative compartment in normal human and rodent intestine (Hall et al., 1990; Yamada, K., K. Yoshitake, M. Sato, and D. Ahnen, unpublished observations). The PCNA labeling index was therefore determined for at least 8 (range = 8–16) longitudinally oriented crypts and villi in each of the four intestinal segments (duodenum, proximal and distal jejunum and ileum) of G_o13-derived transgenic mice and their normal littermates. In normal mice, PCNA-positive cells were largely confined to the crypts. Only occasional cells were present in the basal portion of villi (Fig. 6 *A*). No significant differences were noted in the duodenal, jejunal, or ileal crypt cell PCNA labeling index were noted between control and transgenic animals, just as there were no significant differences in the crypt cell [^{3}H]thymidine labeling index (Fig. 3 *A* and data not shown). A highly significant ($P < 0.001$) increase in the villus cell PCNA labeling index was found in the duodenum, proximal, and distal jejunum of transgenic compared to control mice while no significant differences in PCNA expression were detected in their ileums or colons (Fig. 4 *D* and data not shown). PCNA-positive cells were present in highest concentration in the mid-portion of duodenal and jejunal villi (Fig. 6, *B* and *C*). Thus the altered expression of PCNA parallels the expression of SV-40 TAg along both axes of the gut. The abnormal pattern of PCNA production did not appear to represent a "simple" expansion of the PCNA-positive crypt cell population to the villus since levels of this long-lived protein decrease markedly from the upper crypt to the lower portion of the villus before rising abruptly in mid-villus (Fig. 6, *B* and *C*).

F_1 and F_2 transgenic mice derived from G_o48 and G_o103 were given an intraperitoneal injection of BrdUrd (plus

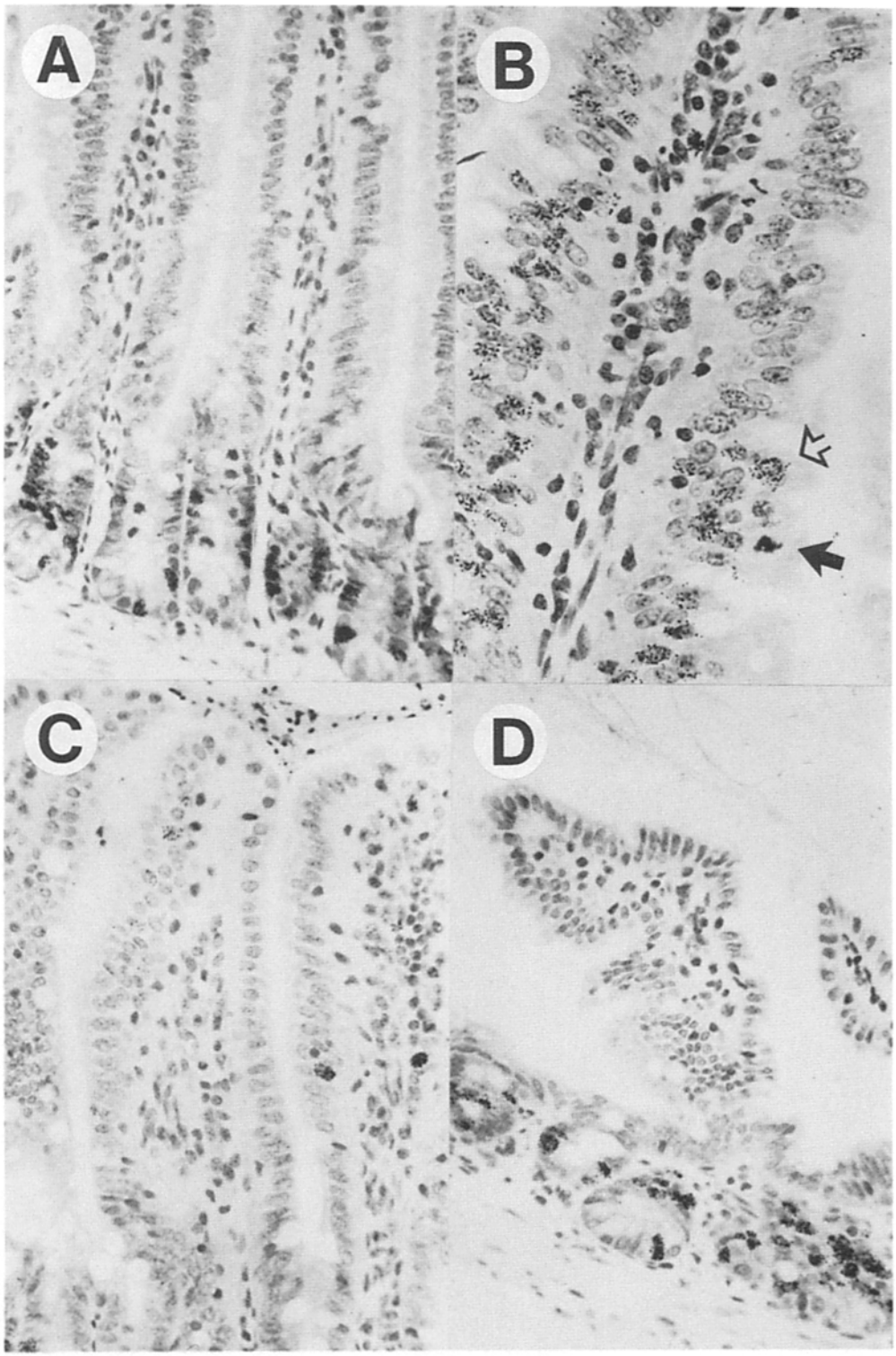


Figure 5. [^{3}H]Thymidine labeling of villus enterocytes in an 8-mo-old transgenic mouse producing SV-40 TAg. (*A*) Section from a normal littermate's duodenum showing densely labeled cells in the crypt but not on the villus (×300); (*B*) section from the duodenum of the transgenic animal. The open arrow points to a heavily labeled nucleus in a mid-villus enterocyte. The closed arrow points to a mitotic figure (×500); (*C*) section from the distal jejunum of the same transgenic mouse shown in *B*. Fewer epithelial cells overlying the villus are labeled compared to the duodenal section (×300); (*D*) section taken from the ileum of the $G_0$13-derived transgenic mouse. Silver grains are confined to crypt-associated cells (×300).

5'-fluoro-deoxyuridine to inhibit endogenous thymidylate synthetase activity; Brinkman and Dormier, 1972) 1.5 h before killing. Sections of their proximal small intestine were stained with a goat antibromodeoxyuridine serum (Cohn and Lieberman, 1984) or the TAg mAb. The results confirmed that TAg producing cells located in the mid-portion of duodenal and jejunal villi (Fig. 7 *A*) were also able to enter S-phase.

Together the data suggest a number of conclusions. First, the similar distributions of TAg, PCNA, and I-FABP along duodenal and jejunal villi together with the observed cellular patterns of [^{3}H]thymidine and BrdUrd labeling suggests that expression of TAg in differentiating enterocytes results in their "aberrant" reentry into S-phase (proliferation). Second, the twofold difference in the mean number of silver grains overlying a duodenal crypt nucleus versus a villus nucleus (53 v 28, $P = 0.0065$) indicates that the uptake of tritiated thymidine during S-phase in the crypts was almost

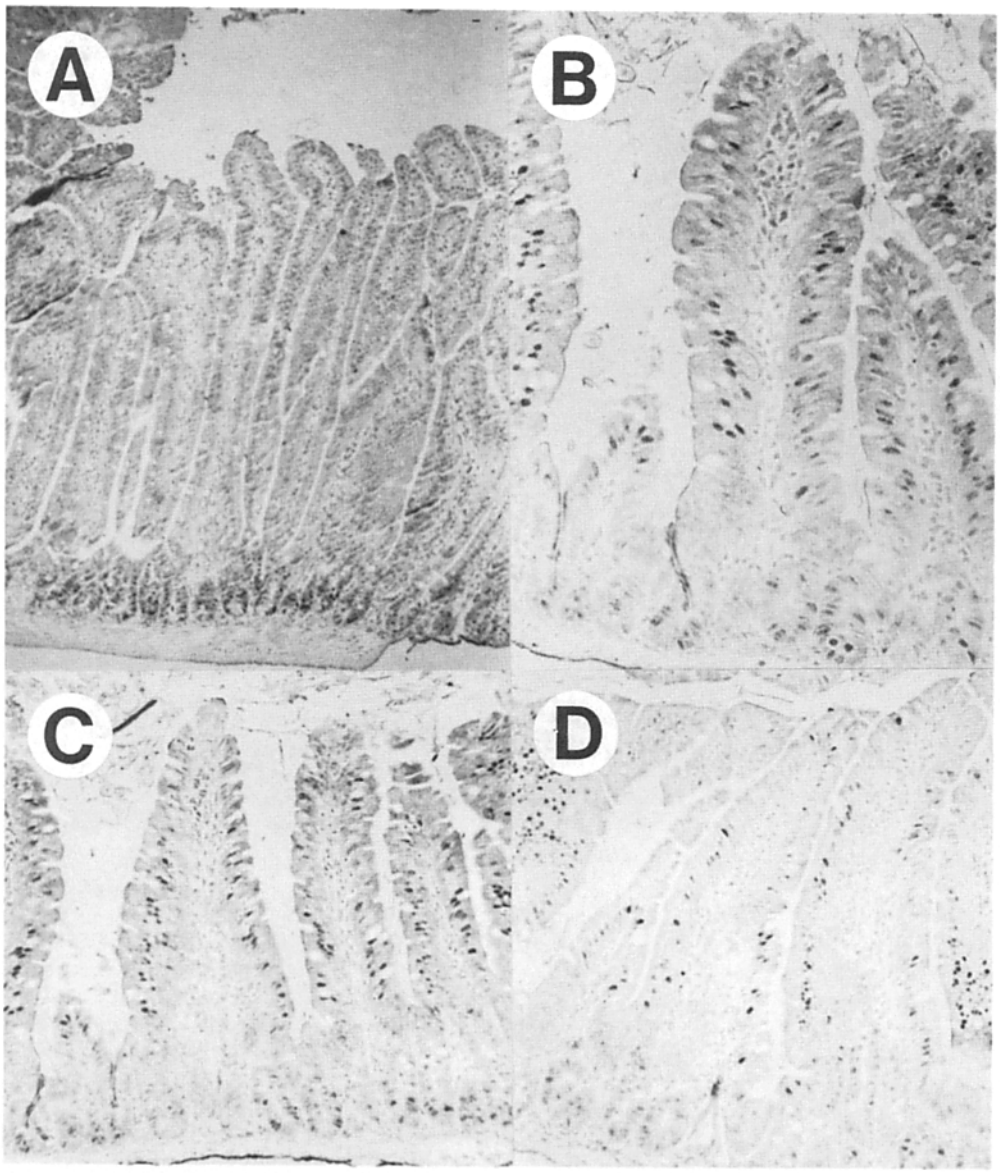


Figure 6. Immunocytochemical survey of PCNA expression along the crypt-to-villus and duodenal-to-ileal axis of an I-FABP$^{1178\ to\ +28}$/SV-40 TAg transgenic mouse and its normal littermate. *A* shows a section taken from the normal littermate's duodenum. Immunoreactive PCNA is limited to crypt epithelial cells; *B* shows a section from the duodenum of the transgenic animal illustrating intense PCNA staining of enterocytes distributed from the mid to upper villus. No nuclear staining is detectable in enterocytes located in the lower portion of the villus. (*C* and *D*) Sections obtained from proximal and distal jejunum, respectively. The concentration of PCNA-positive villus epithelial cells is lower in the distal compared to proximal portions of the small intestine. Note that there were no significant differences in the crypt cell PCNA labeling indices between transgenic mice and/or their normal littermates in any of the small intestinal segments surveyed (i.e., duodenum, jejunum, or ileum).

twice that in villus enterocytes and suggests that cellular DNA synthesis was proceeding more slowly in villus enterocytes. The marked differences in duodenal crypt and villus mitotic indices "despite" the similarities in their [^{3}H]thymidine and PCNA labeling indices also supports the fact that there may be differences in the kinetics of their cell cycle programs (e.g., differentiated TAg-producing villus enterocytes may tend to arrest at the G_2/M boundary). Third, cell proliferation in the crypt was increased in transgenic mice. Since transgene expression was barely detectable in the upper quarter of the crypt, this suggests that crypt epithelial cell proliferation may be influenced by levels of SV-40 TAg that are below the limits of detection by immunocytochemical methods and/or that most of the proliferative increases are due to the presence of the reporter in the upper portions of the crypt. Fourth, the fact that there was no increase in villus area despite the increases in cell proliferation in the crypt raises the possibility that there may be increased cell loss on the villus. When normal crypt cells go into mitosis, they reduce their contact with the basement membrane and are "squeezed" towards the crypt lumen (Tannock, 1967; Loehry et al., 1969). However, there was no evidence for this phenomenon along the villi of transgenic mice. The presence of similar numbers of enterocytes containing the long-lived PNCA in the mid and upper portions of duodenal and jejunal villi "despite" the sharp drop in nuclear labeling with [^{3}H]thymidine suggests that reentry into the cell cycle is not associated with cellular exfoliation at mid-villus but rather with continued migration to the apical extrusion zone.[2]

The Effects of SV-40 TAg Production on Epithelial Cell Differentiation Programs

Since SV-40 TAg production was associated with changes in crypt epithelial proliferation, two questions arise: (*a*) does TAg production have any effect on the processes of commitment and differentiation of the other, nonenterocytic, gut epi-

2. While cell migration pathways can normally be directly quantified by following the fate of a "wave" of labeled cells as they migrate through a villus, the abnormal mitotic activity of villus enterocytes makes the results of such an experimental approach difficult, if not impossible, to interpret.

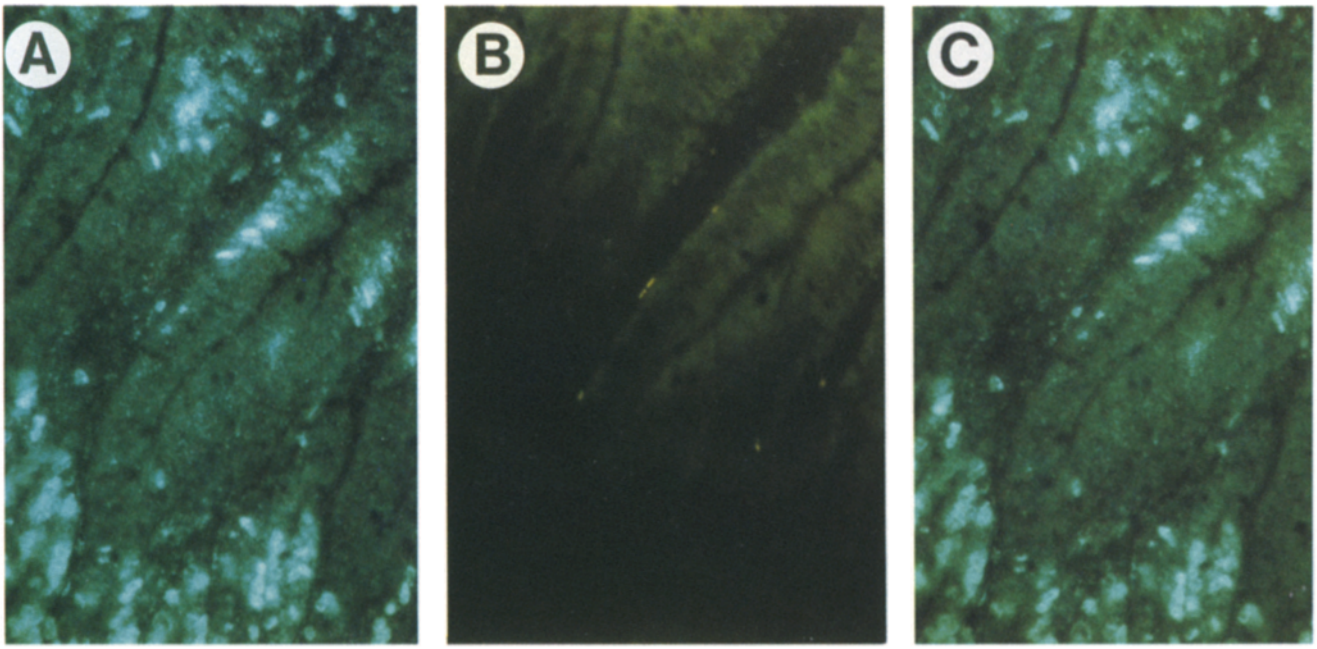


Figure 7. Single and multi-label immunocytochemical studies of enterocytes in S-phase. Sections were prepared from a 9-mo-old female I-FABP$^{1178\ to\ +28}$/SV-40 TAg transgenic mouse (derived from G_o48) 1.5 h after receiving an intraperitoneal injection of 5′-bromo-2′deoxyuridine (BrdUrd). (*A*) BrdUrd-positive crypt epithelial cells are seen as is a cluster of S-phase cells in mid-villus; (*B*) distribution of I-FABP immunoreactive cells in the same section; (*C*) dual exposure shows I-FABP positive-enterocytes in S-phase in the mid and upper portion of jejunal villi (original magnification ×300).

thelial cell lineages; and (*b*) what effects does reentry into the cell cycle have on differentiation of enterocytes?

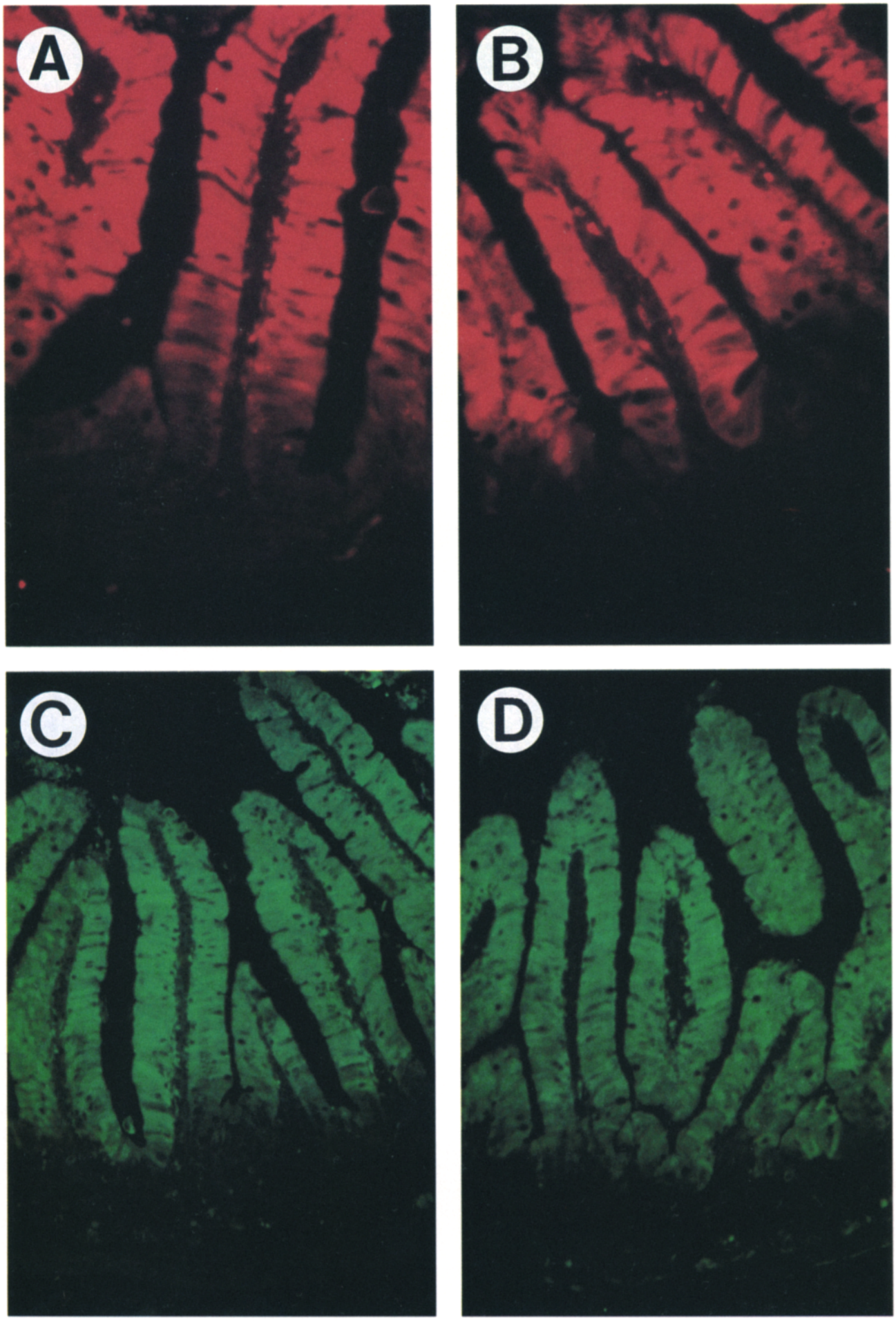
Enterocytes. Production of SV-40 TAg had no apparent effect on the enterocytic differentiation program. Synthesis of SV-40 TAg was not associated with any apparent change in the cellular distribution, or levels, of immunoreactive I-FABP along the crypt-to-villus axis (Fig. 8, *A* and *B*). Double label experiments using antibodies directed against BrdUrd and I-FABP revealed that entry of villus-associated enterocytes into S-phase was not associated with any discernible change in the level of this cytoplasmic long chain fatty acid binding protein (Fig. 7, *A–C*). [Control experiments using both normal and transgenic mice demonstrated that bromodeoxyuridine-positive cells located in the mid and lower portions of the crypt were I-FABP negative.) The distribution of I-FABP mRNA along the duodenal-to-colonic axis was also comparable in transgenic mice and their normal littermates (middle panel of Fig. 1). The cellular and regional patterns of expression of several other markers of enterocytic differentiation appeared to be unaffected by SV-40 TAg production and by entry into S-phase. These markers include liver fatty acid binding protein (Figs. 1 and 8, *C* and *D*) as well as alkaline phosphatase (Fig. 8, *E* and *F*).

Enteroendocrine Cells. Expression of SV-40 TAg had no discernible effect on enteroendocrine cell differentiation programs. *Fabpi* is not normally expressed in enteroendocrine cells (Sweetser et al., 1988*b*). While enteroendocrine cells and enterocytes are apparently derived from the same multipotent, crypt-associated stem cell (Cheng and LeBlond, 1974; Roth et al., 1991*b*), it is not known when, or how, the descendants of this stem cell become committed to differentiate along distinct enterocytic and enteroendocrine lineages. In addition, each of the ~15 different enteroendocrine cell subpopulations have characteristic distributions along both the crypt-to-villus and duodenal-to-colonic axes of the gut. Therefore, they collectively provide a sensitive marker for positional differentiation in this continuously renewing epithelium (Roth et al., 1990, 1991*a*; Roth and Gordon, 1990; Rubin et al., 1991).

We used single and multilabel immunocytochemical techniques to survey the proximal jejunal segments of G_o13-derived I-FABP$^{-1178\ to\ +28}$/SV-40 TAg transgenic mice (n = 5 animals 3–9 mo of age) and their normal littermates (n = 4) for enteroendocrine subpopulations known to reside in this portion of the proximal-to-distal axis of the intestine. The results, presented in Table I, indicate that there were no statistically significant differences in the number, or crypt-to-villus distribution, of serotonin-, substance P-, secretin-, gastric inhibitory peptide, cholecystokinin, and gastrin-immunoreactive cells between the two groups of animals.

Previous studies (Roth et al., 1990, 1991*a*; Roth and Gordon, 1990) have suggested that differentiation-dependent interrelationships exist between enteroendocrine subpopulations. For example, there appears to be an ordered sequential, migration-dependent expression of substance P, serotonin, and secretin as these cells are translocated along the crypt-to-villus axis (Roth and Gordon, 1990). The percentage of secretin cells containing colocalized serotonin immunoreactivity (Fig. 8, *G–I*) was the same in both groups: 53 ± 6% in transgenic jejunum versus 54 ± 3% in normal littermates. Moreover, secretin-immunoreactive cells were confined to the villus whether or not SV-40 TAg was expressed in the gut epithelium (Table 1 and Fig. 8 *G*).

Paneth Cells. As noted in the introduction, Paneth cell differentiation, unlike enterocytic and enteroendocrine cell differentiation, proceeds during descent to the base of the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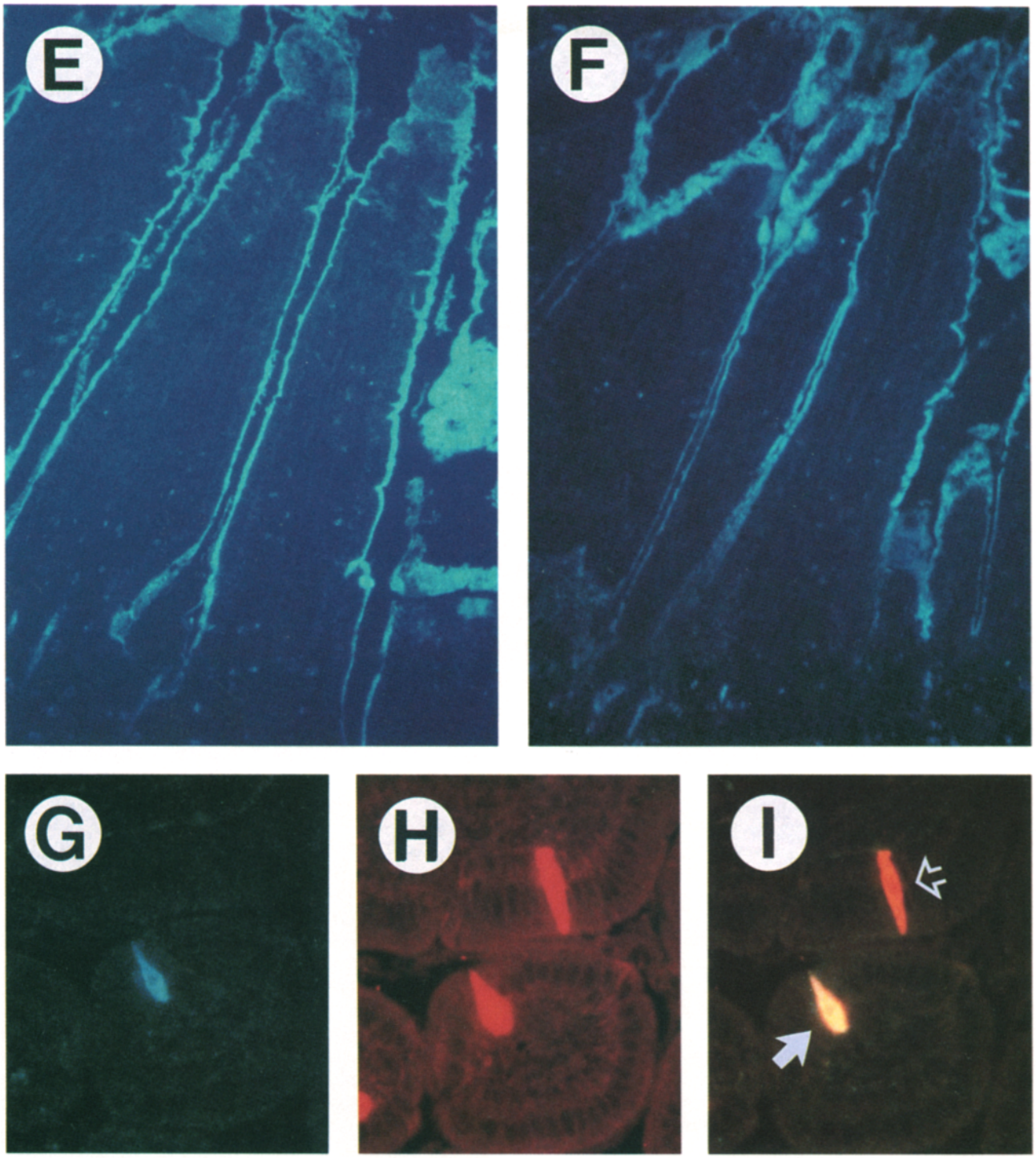

Figure 8. Expression of cellular differentiation markers along the crypt-to-villus axis in the jejunum of an 8-mo-old normal (*A*, *C*, and *E*) and I-FABP$^{1178\ to\ +28}$/SV-40 TAg transgenic mouse (*B*, *D*, and *F*). Sections were incubated with antisera against I-FABP (*A* and *B*), L-FABP (*C* and *D*), and alkaline phosphatase (*E* and *F*) and detected with Texas red-, fluorescein-, and gold-labeled secondary antibodies (with subsequent silver intensification and visualization with reflected light polarization microscopy), respectively. In all cases, the distribution and intensity of immunoreactivity appeared identical between control and transgenic mice. (Original magnifications ×400 [*A* and *B*] and ×200 [*C*, *D*, *E*, and *F*].) Complex enteroendocrine cell interrelationships appear to be maintained in I-FABP/SV-40 TAg transgenic mice. A section from the jejunum of an 8-mo-old transgenic mouse shows a single secretin immunoreactive cell in *G* (visualized with reflected light polarization microscopy). The identical section was subsequently stained for serotonin immunoreactivity and shows several positive cells (*H*). Dual exposure of the secretin and serotonin immunoreactivities (*I*) demonstrates colocalization of serotonin and secretin (indicated by an *arrow*). Several other serotonin-immunoreactive cells lack secretin immunoreactivity. (Original magnification ×400).

Table I. Comparison of Enteroendocrine Cell Differentiation in the Proximal Jejunum of I-FABP/SV40 T Ag Transgenic Mice and their Normal Littermates

	Serotonin	Substance P	Secretin	GIP	CCK	Gastrin
	Number of cells/cross section (x ± SEM)					
Transgenic	128 ± 25	52 ± 13	40 ± 11	32 ± 6	47 ± 7	41 ± 8
Control	120 ± 22	31 ± 8	21 ± 4	24 ± 3	42 ± 3	38 ± 5
	Percentage of cells associated with villus (x ± SEM)					
Transgenic	61 ± 3	42 ± 4	100 ± 0	64 ± 2	67 ± 3	71 ± 5
Control	66 ± 5	42 ± 4	100 ± 0	63 ± 5	71 ± 4	69 ± 5

See Materials and Methods for further details.

crypt. Therefore, examination of the distribution of Paneth cells in the crypts of normal and transgenic mice provided an opportunity to assess whether the proliferative changes produced in the crypts by SV-40 TAg affected this lineage. They did not. Staining of duodenal and jejunal sections with an antilysozyme antibody (Moser et al., 1992) indicated Paneth cell number and location were comparable in transgenic mice and their normal littermates (data not shown).

Expression of SV-40 TAg in the Gut Epithelium of Transgenic Mice Is Not Accompanied by Neoplastic Transformation

The studies described above suggested that SV-40 TAg expression did not result in any detectable dedifferentiation of the gut epithelium. Gross and microscopic inspection (see Materials and Methods) of duodenal, jejunal, ileal, cecal and colonic segments harvested from 21 male and female mice derived from the three pedigrees and ranging in age from 2 to 9 mo failed to reveal any neoplastic lesions. The relationship between these findings and current concepts of the mechanisms which underlie intestinal epithelial tumorigenesis is discussed below.

Discussion

As noted in the introduction, the crypt-to-villus axis of the mouse intestinal epithelium represents an attractive mammalian system for examining the relationships between cell division and differentiation. In the transgenic model described here, differentiation-activated production of TAg provided an intracellular switch that led to the entry of enterocytes into S phase. Such an entrance presumably reflects the fact that SV-40 TAg binds to, and inactivates, the dephosphorylated form of the retinoblastoma susceptibility gene product (Ludlow et al., 1989; 1990). RB is phosphorylated at the G_1/S boundary. The dephosphorylated form is thought to inhibit entry into S phase (Buckovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989). TAg contains a domain that has been identified in two cellular proteins (RBP-1 and RBP-2) and two viral transforming proteins (human papilloma virus-16 E7 and adenovirus E1A). This conserved motif mediates binding of all four proteins to the same region of RB (Defeo-Jones et al., 1991).

Induction of SV-40 TAg expression at a defined temporal and spatial point of epithelial cell differentiation/translocation along the crypt-to-villus axis allowed us to assess (*a*) whether the state of differentiation affected the nature of the cell cycle; (*b*) whether reentry into the cell cycle affected differentiation of a specific lineage (enterocytes); and (*c*) whether proliferative changes in the crypt affected other lineages.

We were not able to determine whether enterocytes that entered S-phase in the mid-villus passed through the cell cycle in its entirety. However, there was a marked discrepancy between the number of cells in S-phase and in M-phase on the villus compared to the crypt (in the absence of any evidence for abnormal exfoliation of enterocytes before their arrival at the apical extrusion zone). This discrepancy could reflect a number of mechanisms. First, the state of differentiation could influence cell cycle kinetics (prolonging S phase or affecting passage through the G_2/M boundary) by altering the levels of key regulators of cell cycle induction and cell cycle transitions (see Murray and Kirschner, 1989; McIntosh and Koonce; 1989; Laskey et al., 1989; Hartwell and Weinert, 1989 for overviews of these processes). For example, progression from G_2 to M phase depends on activation of a protein kinase composed of $p34^{cdc2}$ and G_1-specific cyclins (Pines and Hunter, 1990). The levels of this kinase and its state of phosphorylation may be quite different in crypt compared to villus epithelial cell populations. Second, SV-40 TAg may itself influence cell cycle kinetics independent of, or dependent upon, the state of enterocytic differentiation. Crossing transgenic animals with I-FABP$^{-1178\ to\ +28}$/SV-40 TAg to mice that contain I-FABP$^{-1178\ to\ +28}$ linked to specific cyclins or $p34^{cdc2}$ may provide a means for examining the mechanisms which regulate cell cycle kinetics in enterocytes as they traverse the crypt-to-villus axis.

Single and double label immunocytochemical studies suggested that TAg-induced entry into S-phase was not associated with any apparent effect on enterocytic differentiation. However, this conclusion must be viewed with caution. Migration of enterocytes up the villus and exfoliation at the apical extrusion zone may occur too rapidly to allow us to detect changes in the state of differentiation that could have been manifested if these cells had a longer residence time on the villus. Alternatively, changes may have been detectable if the turnover times of the differentiation markers used for the analysis was more rapid than those of L-FABP, I-FABP, or alkaline phosphatase.

Studies of the *Drosophila string* locus have suggested that cell-division programs can be uncoupled from commitment and differentiation programs (Edgar and O'Farrell, 1989, 1990; O'Farrell et al., 1989). It will be important to establish what proportion of villus-associated enterocytes that have entered S-phase subsequently complete the journey

through the cell cycle. If division of these enterocytes is symmetric (i.e., yielding two daughters with similar phenotypes) it would contrast with the asymmetric nature of the division of crypt stem cells and their immediate progeny (i.e., yielding one daughter that remains undifferentiated while the other undergoes commitment to differentiate). Increasing symmetry of cell division as a function of position along the spatially well organized crypt-to-villus axis could be viewed as one way of defining commitment and/or progression through a differentiation program. To date, our studies indicate that SV-40 TAg–induced changes in crypt cell proliferation affect a population that is already committed since there was no discernible change in the representation of terminally differentiated members of the enterocytic, enteroendocrine, or Paneth cell lineages. Expression of SV-40 TAg in the mid to lower crypt would represent a way of inducing division in cells that are less differentiated and perhaps less committed.[3] This could provide a strategy for defining where decisions about lineage allocation (commitment) are made.

Sustained expression of SV-40 TAg in the small intestinal epithelium is associated with sustained increases in crypt and villus epithelial cell proliferation without formation of gut epithelial cell neoplasms. This contrasts with results obtained in other transgenic mice where TAg production in nonrenewing cellular populations has yielded a variety of tumors (e.g., Brinster et al., 1984; Hanahan, 1985; Ornitz et al., 1987) or where production of other oncoproteins in more slowly renewing epithelia produces neoplasia (Bailleul et al., 1990). The absence of neoplasms in our transgenic mice raises several questions concerning initiation, promotion, and progression of neoplasia in the gut epithelium. Current hypotheses concerning the origin of human colorectal carcinomas suggest that they arise from a series of accumulated genetic mutations that affect both protooncogenes and tumor suppressor genes (Fearon and Vogelstein, 1990; Bodmer et al., 1987; Leppert et al., 1987; Kinzler et al., 1991*a*,*b*; Groden et al., 1991; Joslyn et al., 1991; Fearon et al., 1987; Forrester et al., 1987; Bos et al., 1987; Vogelstein et al., 1988; Farr et al., 1988; Baker et al., 1989; Nigro et al., 1989; Fearon et al., 1990). Studies of human colorectal tumors (Kirkland, 1986, 1988; van den Ingh et al., 1986; Carroll et al., 1990) as well as several mouse models of gut neoplasia (Oomen et al., 1984; Moser et al., 1990, 1992) have shown that all four epithelial cell lineages may be represented in a given lesion suggesting that the site of initiation of tumorigenesis may be the multipotent stem cell or one of its immediate descendants. The lack of gut neoplasms in I-FABP$^{-1178\ to\ +28}$/SV-40 TAg animals could reflect the inability of a rapidly migrating "initiated" (TAg producing) cell to become functionally anchored and to acquire multiple independent or interdependent somatic mutations required for dedifferentiation and clonal expansion. In contrast, expression of SV-40 TAg in gut stem cells or one of their immediate descendants may represent the equivalent of a single germline mutational event that is sufficent to produce a neoplasm since this initiated cell can already "provide" an enormous division potential, functional anchorage and multipotency. It will be interesting to note whether expression of SV-40 TAg in gut epithelial cells located outside of the stem cell compartment but with longer residence times than enterocytes (e.g., certain enteroendocrine cell populations or Paneth cells) is sufficient to result in tumor formation (see Rindi et al., 1990). The I-FABP/SV-40 TAg mouse should provide a sensitive genetic substrate for auditing the ability of gene products to affect such fundamental processes as cell anchorage, for assessing structure/activity relationships of proteins thought to play a role in the multistep journey to colorectal carcinoma, for obtaining cell lines that more closely resemble enterocytes than currently available systems, and for testing the potential mutagenic effects of therapeutic agents that are being developed and require chronic oral administration.

3. Recent work with I-FABP/hGH chimeric genes indicate that reporter expression can be precociously activated in epithelial cells located in the lower and middle thirds of intestinal crypts by removing elements upstream of nucleotide - 184 of rat *Fabpi*, Cohn, S., T. Simon, K. Roth, E. Birkenmeier, and J. Gordon, unpublished observations). Thus, comparison of mice with I-FABP$^{184\ to\ +28}$/SV-40 TAg and I-FABP$^{1178\ to\ +28}$/SV-40 TAg transgenes may be useful for these types of analyses.

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References

Bailleul, B., M. A. Surani, S. White, S. C. Burton, K. Brown, M. Blessing, J. Jorcano, and A. Balmain. 1990. Skin hyperkeratosis and papilloma formation in transgenic mice expressing a *ras* oncogene from a suprabasal keratin promoter. *Cell.* 62:697–708.

Baker, S. J., E. R. Fearon, J. M. Nigro, S. R. Hamilton, A. C. Preisinger, J. M. Jessup, P. vanTuinen, D. H. Ledbetter, D. F. Barker, Y. Nakamura, R. White, and B. Vogelstein. 1989. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science (Wash. DC).* 244:217–221.

Blau, H. M., and D. Baltimore. 1991. Differentiation requires continuous regulation. *J. Cell Biol.* 112:781–783.

Bodmer, W. F., C. J. Bailey, J. Bodmer, H. J. R. Bussey, A. Ellis, P. Gorman, F. C. Lucibello, V. A. Murday, S. H. Rider, P. Scambler, D. Sheer, E. Solomon, and N. K. Spurr. 1987. Localization of the gene for familial adenomatous polyposis on chromosome 5. *Nature (Lond.).* 328:614–616.

Bos, J. L., E. R. Fearon, S. R. Hamilton, M. Verlaan-de Vries, J. H. van Boom, A. J. van der Eb, and B. Vogelstein. 1987. Prevalence of *ras* gene mutations in human colorectal cancers. *Nature (Lond.).* 327:293–297.

Bravo, R., and J. E. Celis. 1980. A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells. *J. Cell Biol.* 84:795–802.

Bravo, R., and H. Macdonald-Bravo. 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J. Cell Biol.* 105:1549–1554.

Brinkmann, W., and P. Dormer. 1972. *In vitro*-verfahren zur bestimmung der DNS-synthese-Dauer einzelner Zellen. Biochemische voraussetzungen und ergebnisse. *Histochemie.* 30:335–343.

Brinster, R. L., H. Y. Chen, A. Messing, T. van Dyke, A. J. Levine, and R. D. Palmiter. 1984. Transgenic mice harboring SV40 T-antigen genes develop characteristic brain tumors. *Cell.* 37:367–379.

Buchkovich, K., L. A. Duffy, and E. Harlow. 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell.* 58:1097–1105.

Carroll, S. L., K. A. Roth, and J. I. Gordon. 1990. Liver fatty acid binding pattern: a marker for studying cellular differentiation in gut epithelial neoplasms. *Gastroenterology.* 99:1727–1735.

Celis, J. E., and A. Celis. 1985. Cell cycle-dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: Subdivision of S phase. *Proc. Natl. Acad. Sci. USA.* 82:3262–3266.

Chang, C.-D., L. Ottavio, S. Travali, K. E. Lipson, and R. Baserga. 1990. Transcriptional and post-transcriptional regulation of the proliferating cell nuclear gene. *Mol. Cell. Biol.* 10:3289–3296.

Chen, P.-L., P. Scully, J.-Y. Shew, J. Y. J. Wang, and W.-H. Lee. 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell.* 58:1193–1198.

Cheng, H., and C. P. Leblond. 1974. Origin, differentiation, and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial cell types. *Am. J. Anat.* 141: 537–561.

Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

Cohn, S. M., and M. W. Lieberman. 1984. The use of antibodies to 5-bromo-2'-deoxyuridine for the isolation of DNA sequences containing excision-repair sites. *J. Biol. Chem.* 259:12456–12462.

DeCaprio, J. A., J. W. Ludlow, J. Figge, J. Y. Shew, C. M. Huang, W. H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell.* 54:275–283.

DeCaprio, J. A., J. W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica-Worms, C.-M. Huang, and D. M. Livingston. 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. *Cell.* 58:1085–1095.

Defeo-Jones, D., P. S. Huang, R. E. Jones, K. M. Haskell, G. A. Vuocolo, M. G. Hanobik, H. E. Huber, and A. Oliff. 1991. Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product. *Nature (Lond.).* 352:251–254.

Dyson, N., R. Bernards, S. H. Friend, L. R. Gooding, J. A. Hassell, E. O. Major, J. M. Pipas, T. VanDyke, and E. Harlow. 1990. Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein. *J. Virol.* 64:1353–1356.

Edgar, B. A., and P. H. O'Farrell. 1989. Genetic control of cell division patterns in the Drosophila embryo. *Cell.* 57:177–187.

Edgar, B. A., and P. H. O'Farrell. 1990. The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by *string*. *Cell.* 62:469–480.

Fairman, M. P. 1990. DNA polymerase delta/PCNA: actions and interactions. *J. Cell Sci.* 95:1–4.

Farr, C. J., C. J. Marshall, D. J. Easty, N. A. Wright, S. C. Powell, and C. Paraskeva. 1988. A study of ras gene mutations in colonic adenomas from familial polyposis coli patients. *Oncogene.* 3:673–678.

Fearon, E. R., and B. Vogelstein. 1990. A genetic model for colorectal tumorigenesis. *Cell.* 61:759–767.

Fearon, E. R., S. R. Hamilton, and B. Vogelstein. 1987. Clonal analysis of human colorectal tumors. *Science (Wash. DC).* 238:193–197.

Fearon, E. R., K. R. Cho, J. M. Nigro, S. E. Kern, J. W. Simons, J. M. Ruppert, S. R. Hamilton, A. C. Preisinger, G. Thomas, K. W. Kinzler, and B. Vogelstein. 1990. Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science (Wash. DC).* 247:49–56.

Finlay, C. A., P. W. Hinds, and A. J. Levine. 1989. The p53 proto-oncogene can act as a suppressor of transformation. *Cell.* 57:1083–1093.

Forrester, K., C. Almoguera, K. Han, W. E. Grizzle, and M. Perucho. 1987. Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. *Nature (Lond.).* 327:298–303.

Goodlad, R. A., and N. A. Wright. 1982. Quantitative studies on epithelial replacement in the gut. *In* Techniques in Life Sciences. Digestive Physiology. T. A. Titchen, editor. Elsevier Biomedical Press, Ireland. 212/1–212/23.

Green, R. P., S. M. Cohn, J. C. Sacchettini, K. E. Jackson, and J. I. Gordon. 1992. The mouse intestinal fatty acid binding protein gene: nucleotide sequence, pattern of developmental and regional expression, and proposed structure of its protein product. *DNA Cell Biol.* 11:31–41.

Griffiths, D. F. R., S. J. Davies, D. Williams, G. T. Williams, and E. D. Williams. 1988. Demonstration of somatic mutation and colonic crypt clonality by X-linked enzyme histochemistry. *Nature (Lond.).* 333:461–463.

Groden, J., A. Thliveris, W. Samowitz, M. Carlson, L. Gelbert, H. Albertsen, G. Joslyn, J. Stevens, L. Spirio, M. Robertson, L. Sargeant, K. Krapcho, E. Wolff, R. Burt, J. P. Hughes, J. Warrington, J. McPherson, J. Wasmuth, D. LePaslier, H. Abderrahim, D. Cohen, M. Leppert, and R. White. 1991. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell.* 66:589–600.

Hall, P. A., D. A. Levison, A. L. Woods, C. C.-W. Yu, D. B. Kellock, J. A. Watkins, D. M. Barnes, C. E. Gillett, R. Camplejohn, R. Dover, N. H. Waseem, and D. P. Lane. 1990. Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: An index of cell proliferation with evidence of deregulated expression in some neoplasms. *J. Pathol.* 162: 285–294.

Hanahan, D., 1985. Heritable formation of pancreatic β-cell tumors in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature (Lond.).* 315:115–122.

Hartwell, L. H., and T. A. Weinert. 1989. Checkpoints: Controls that ensure the order of cell cycle events. *Science (Wash. DC).* 246:629–634.

Jaskulski, D., C. Gatti, S. Travali, B. Calabretta, and R. Baserga. 1988. Regulation of the proliferating cell nuclear antigen cyclin and thymidine kinase mRNA levels by growth factors. *J. Biol. Chem.* 263:10175–10179.

Joslyn, G., M. Carlson, A. Thliveris, H. Albertsen, L. Gelbert, W. Samowitz, J. Groden, J. Stevens, L. Spirio, M. Robertson, L. Sargeant, K. Krapcho, E. Wolff, R. Burt, J. P. Hughes, J. Warrington, J. McPherson, J. Wasmuth, D. LePaslier, H. Abderrahim, D. Cohen, M. Leppert, and R. White. 1991. Identification of deletion mutations and three new genes at the familial polyposis locus. *Cell.* 66:601–613.

Kinzler, K. W., M. C. Nilbert, B. Vogelstein, T. M. Bryan, D. B. Levy, K. J. Smith, A. C. Preisinger, S. R. Hamilton, P. Hedge, A. Markham, M. Carlson, G. Joslyn, J. Groden, R. White, Y. Miki, Y. Miyoshi, I. Nishisho, and Y. Nakamura. 1991*a*. Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. *Science (Wash. DC).* 251:1366–1370.

Kinzler, K. W., M. C. Nilbert, L.-K. Su, B. Vogelstein, T. M. Bryan, D. B. Levy, K. J. Smith, A. C. Preisinger, P. Hedge, D. McKechnie, R. Finniear, A. Markham, J. Groffen, M. S. Boguski, S. F. Altschul, A. Horii, H. Ando, Y. Miyoshi, Y. Miki, I. Nishisho, Y. Nakamura. 1991*b*. Identification of FAP Locus Genes from Chromosome 5q21. *Science (Wash. DC).* 253:661–665.

Kirkland, S. C. 1986. Endocrine differentiation by a human rectal adenocarcinoma cell line (HRA-19). *Differentiation.* 33:148–155.

Kirkland, S. C. 1988. Clonal origin of columnar, mucous, and endocrine cell lineages in human colorectal epithelium. *Cancer.* 61:1359–1363.

Laskey, R. A., M. P. Fairman, and J. Blow. 1989. S Phase of the cell cycle. *Science (Wash. DC).* 246:609–614.

Leppert, M., M. Dobbs, P. Scambler, P. O'Connell, Y. Nakamura, D. Stauffer, S. Woodward, R. Burt, J. Hughes, E. Gardner, M. Lathrop, J. Wasmuth, J.-M. Lalouel, and R. White. 1987. The gene for familial polyposis coli maps to the long arm of chromosome 5. *Science (Wash. DC).* 238:1411–1413.

Loehry, C. A., D. N. Croft, A. K. Singh, and B. Creamer. 1969. Cell turnover in the rat small intestinal mucosa: an appraisal of cell loss. I. Cell loss in rats with a normal mucosa. *Gut.* 10:13–18.

Ludlow, J. W., J. A. DeCaprio, C.-M. Huang, W.-H. Lee, E. Paucha, and D. M. Livingston. 1989. SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. *Cell.* 56:57–65.

Ludlow, J. W., J. Shon, J. M. Pipas, D. M. Livingston, and J. A. DeCaprio. 1990. The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. *Cell.* 60:387–396.

McIntosh, J. R., and M. P. Koonce. 1989. Mitosis. *Science (Wash. DC).* 246: 622–628.

Morris, G. F., and M. B. Matthews. 1989. Regulation of proliferating cell nuclear antigen during the cell cycle. *J. Biol. Chem.* 264:13856–13864.

Moser, A. P., H. C. Pitot, and W. F. Dove. 1990. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science (Wash. DC).* 247:322–324.

Moser, A. R., W. F. Dove, K. A. Roth, and J. I. Gordon. 1992. The *Min* (multiple intestinal neoplasia) mutation: its effect on gut epithelial cell differentiation and interaction with a modifier system. *J. Cell Biol.* 116:1517–1526.

Murray, A. W., and M. W. Kirschner. 1989. Dominoes and clocks: the union of two views of the cell cycle. *Science (Wash. DC).* 246:614–621.

Nigro, J. M., S. J. Baker, A. C. Preisinger, J. M. Jessup, R. Hostetter, K. Cleary, S. H. Bigner, N. Davidson, S. Baylin, P. Devilee, T. Glover, F. S. Collins, A. Weston, R. Modali, C. C. Harris, and B. Vogelstein. 1989. Mutations in the p53 gene occur in diverse human tumour types. *Nature (Lond.).* 342:705–708.

O'Farrell, P. H., B. A. Edgar, D. Lakich, and C. F. Lehner. 1989. Directing cell division during development. *Science (Wash. DC).* 246:635–640.

Oomen, L. C., M. A. van der Valk, and P. Emmelot. 1984. Stem cell carcinoma in the small intestine of mice treated transplacentally with *N*-ethyl-*N*-nitrosourea: Some quantitative and histological aspects. *Cancer Lett.* 25: 71–79.

Omitz, D. M., R. E. Hammer, A. Messing, R. D. Palmiter, and R. L. Brinster. 1987. Pancreatic neoplasia induced by SV40 T-antigen expression in acinar cells of transgenic mice. *Science (Wash. DC).* 238:188–193.

Pardee, A. B. 1989. G_1 events and regulation of cell proliferation. *Science (Wash. DC).* 246:603–608.

Pines, J., and T. Hunter. 1990. $p34^{cdc2}$: the S and M kinase? *New Biol.* 2:389–401.

Ponder, B. A. J., G. H. Schmidt, M. M. Wilkerson, M. J. Wood, M. Monk, and A. Reid. 1985. Derivation of mouse intestinal crypts from single progenitor cells. *Nature (Lond.).* 313:689–691.

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

Rindi, G., S. G. N. Grant, Y. Yiangou, M. A. Ghatei, S. R. Bloom, V. L. Bautch, E. Solcia, and J. M. Polak. 1990. Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice. Heterogeneity of hormone expression. *Am. J. Pathol.* 136:1349–1363.

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.

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.

Roth, K. A., D. C. Rubin, E. H. Birkenmeier, and J. I. Gordon. 1991*a*. Expression of liver fatty acid binding protein/human growth hormone fusion genes within the enterocyte and enteroendocrine cell populations of fetal transgenic mice. *J. Biol. Chem.* 266:5949–5954.

Roth, K. A., M. L. Hermiston, and J. I. Gordon. 1991*b*. Use of transgenic mice to infer the biological properties of small intestinal stem cells and to examine the lineage relationships of their descendants. *Proc. Natl. Acad. Sci. USA.* 88:9407–9411.

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

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.

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 small intestine. *Development (Camb).* 103:785–790.

Sweetser, D. A., E. H. Birkenmeier, I. J. Klisak, S. Zollman, R. S. Sparkes, T. Mohandes, A. J. Lusis, and J. I. Gordon. 1987. The human and rodent intestinal fatty acid binding protein genes. A comparative analysis of their structure, expression and linkage relationships. *J. Biol. Chem.* 262:16060–16071.

Sweetser, D. A., E. H. Birkenmeier, P. C. Hoppe, D. W. McKeel, and J. I. Gordon. 1988*a*. Mechanisms underlying generation of gradients in gene expression within the intestine: an analysis using transgenic mice containing fatty acid binding protein/human growth hormone fusion genes. *Genes Dev.* 2:1318–1332.

Sweetser, D. A., S. M. Hauft, P. C. Hoppe, E. H. Birkenmeier, and J. I. Gordon. 1988*b*. Transgenic mice containing intestinal fatty acid binding protein/human growth hormone fusion genes exhibit correct regional and cell specific expression of the reporter gene in their small intestine. *Proc. Natl. Acad. Sci. USA.* 85:9611–9615.

Taketo, M., A. C. Schroeder, L. E. Mobraaten, K. B. Gunning, G. Hanten, R. R. Fox, T. H. Roderick, C. L. Stewart, F. Lilly, C. T. Hansen, and P. A. Overbeek. 1991. FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc. Natl. Acad. Sci. USA.* 88:2065–2069.

Tannock, I. F. 1967. A comparison of the relative efficiencies of various metaphase arrest agents. *Exp. Cell Res.* 47:345–356.

Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77:5201–5205.

van den Ingh, H. F., L. J. van den Broek, A. A. J. Verhofstad, and F. T. Bosman. 1986. Neuroendocrine cells in colorectal adenomas. *J. Pathol.* 148: 231–237.

Vogelstein, B., E. R. Fearon, S. R. Hamilton, S. E. Kern, A. C. Preisinger, M. Leppert, Y. Nakamura, R. White, A. M. M. Smits, and J. L. Bos. 1988. Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.* 319:525–532.

Vogelstein, B., E. R. Fearon, S. E. Kern, S. R. Hamilton, A. C. Preisinger, Y. Nakamura, and R. White. 1989. Allelotype of colorectal carcinomas. *Science (Wash. DC).* 244:207–211.

Winton, D. J., M. A. Blount, and B. A. J. Ponder. 1988. A clonal marker induced by mutation in mouse intestinal epithelium. *Nature (Lond.).* 333: 463–466.

Wright, N. A., and M. Irwin. 1982. The kinetics of villus cell populations in the mouse small intestine. I. Normal villi: the steady state requirement. *Cell Tissue Kinet.* 15:595–609.

Young, G. P., S. Friedman, S. T. Yedlin, and D. H. Alpers. 1981*a*. Effect of fat feeding on intestinal alkaline phosphatase activity in tissue and serum. *Am. J. Physiol.* 241:G461–G468.

Young, G. P., S. T. Yedlin, and D. H. Alpers. 1981*b*. Independent biosynthesis of soluble and membrane-bound alkaline phosphatases in the suckling rat ileum. *Biochem. J.* 200:645–654.