

The Ontogeny of α -fetoprotein Gene Expression In the Mouse Gastrointestinal Tract

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Abstract. The ontogeny of α -fetoprotein (AFP) gene expression has been examined in the fetal and adult mouse gastrointestinal tract. AFP mRNA constitutes $\sim 0.1\%$ of total mRNA in the fetal gut. The transcripts were localized by in situ hybridization to the epithelial cells lining the villi of the fetal gut. At birth, AFP mRNA declines rapidly to achieve low adult basal levels, which are not affected by different alleles of *raf*, a gene that determines the adult basal level of AFP mRNA in the liver. The basal level in the adult gut is the consequence of continued AFP transcription in a small number of enteroendocrine cells that are distributed infrequently on the villi. These cells were identified by double antibody staining with antibodies to chromogranin A, an enteroendocrine cell marker and AFP. Previous studies resulted in the generation of a line of transgenic mice containing an internally

deleted AFP gene that was greatly overexpressed in the fetal gut. The basis for the inappropriately high level expression of the transgene was shown to be the consequence of very high levels of transcription in the epithelial cells of the villi rather than to expression in inappropriate cell types. The *cis*-acting DNA sequences required for expression of the AFP gene in the gut were investigated using Caco-2 cells, a human colon adenocarcinoma cell line. These experiments indicated that, with one exception, the regulatory elements required in both the promoter and enhancer regions of the gene coincided with those that are necessary for high level expression in the liver. The one exception was enhancer II, located 5 kbp of DNA upstream of the gene, which exhibited no activity in Caco-2 cells.

THE gastrointestinal tract is a complex organ that provides an excellent system for studying aspects of cellular differentiation during development. At day 15 of gestation in the fetal rat, the small intestine is a circular layer of undifferentiated stratified epithelial cells surrounding a tiny lumen (29). By day 19 of gestation, this epithelium converts to the simple columnar type, the lumen expands, and villi are formed (30). Crypts develop between the villi immediately after birth, and cell proliferation becomes confined to these crypts. The developing crypts are initially polyclonal in nature but become monoclonal by the age of 2 wk in the mouse (45).

The crypts and the villi compartmentalize the predifferentiated and differentiated cells of the adult intestinal epithelium. Stem cells localized at the base of the crypts give rise to proliferative cells that differentiate into at least four different cell types: villus columnar cells (enterocytes), goblet cells, enteroendocrine cells, and Paneth cells (26). Within ~ 3 d, all of the newly differentiated cells, except the Paneth cells, which remain at the base of the villi, migrate up the villi and are extruded at the tip (44). The adult intestinal epi-

thelium is constantly undergoing rapid renewal to the extent that each crypt in the mouse small intestine produces ~ 275 cells per day (41).

The marked changes in cell population that occur in the gut during late gestation and early neonatal development are accompanied by changes in the synthesis of α -fetoprotein (AFP),¹ the major serum protein of the mammalian fetus. This protein, which is expressed at high levels in the fetal liver and yolk sac, constitutes 0.1% of the total mRNA in the fetal gut (50). At birth, AFP mRNA declines precipitously in both liver and gut to levels that are barely detectable (51). The decline in liver has been extensively studied and attributed to a dominant *cis*-acting silencer element that lies between the promoter and three enhancers of the gene (4, Vacher, J., and S. Tilghman, manuscript submitted for publication). In addition, the adult basal level in liver is determined, at least in part, by a *trans*-acting locus termed *raf*, for regulation of AFP (3, 36).

To understand the basis for the ontogeny of AFP transcription in the gut and to ask whether it is mediated by the same positive and negative regulatory elements that govern its expression in liver, we investigated the nature of the cells that

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1. *Abbreviations used in this paper:* AFP, α -fetoprotein; TK, thymidine kinase; TTP, ribosylthymine triphosphate.

transcribe the AFP gene in both fetal and adult gut. We also used both transgenic mice and transient expression assays in a human colon adenocarcinoma cell line to identify elements in the AFP gene that contribute to its expression in the gut.

Materials and Methods

Tissue Preparation and Sectioning

ICR:Ha (icr) mice or mice from the transgenic line 164-6A (21) were killed, and intestines were immediately dissected. For *in situ* hybridizations, tissues were washed in PBS, blotted dry, and frozen in O.C.T. (Miles Laboratories Inc., Elkhart, IN) in liquid nitrogen. 8- μ m sections were cut on a cryostat, placed on room temperature poly-L-lysine-coated slides, and stored at -70°C . Immediately before the prehybridization and hybridization steps, tissue was thawed and fixed for 2 min in 4% paraformaldehyde in PBS. For immunohistochemistry, tissues were fixed for 1–2 h in Bouin's fixative and paraffin embedded, and 8- μ m sections were collected on poly-L-lysine-treated slides.

Preparation of Radiolabeled Probes

A 440-bp Hinc II mouse genomic DNA fragment containing AFP exon 1 and a 907-bp AFP cDNA clone spanning exons 3–8 were cloned into SP65 and pGEM 2, respectively. SP6 or T7 polymerase (Promega Biotech, Madison, WI) was used to generate ^{35}S -labeled cRNA probes. A 900-bp Pst I fragment from a human cDNA clone (42), a 1.9-kb Pvu II fragment of the herpes simplex virus thymidine kinase (TK) gene (31), and a 1.3-kb Hind III fragment from the ribosomal protein gene rpl32/4A (13) (provided by Dr. Robert Perry, Institute for Cancer Research, Philadelphia, PA) were gel purified, and ^{32}P -labeled DNA probes were prepared by nick translation.

In Situ Hybridizations

In situ hybridizations were conducted essentially according to the method of Cox et al. (12), with some modifications. Briefly, prehybridization treatment included rehydration of tissue and treatment with acetic anhydride (0.25% vol/vol) in 0.1 M triethanolamine, pH 8.0. After dehydration, tissues were incubated with 10^5 cpm [^{35}S] cDNA/ μ l in 50% formamide, $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl, 0.015 M Na citrate), 10 mM DTT, 1 mg/ml yeast tRNA, 1 mg/ml sheared denatured salmon sperm DNA, and 2 mg/ml

nuclease-free BSA at 55°C overnight. After hybridization, tissue was treated with 10 $\mu\text{g}/\text{ml}$ RNase A at 37°C for 30 min to remove any unhybridized probe. Sections were washed at 55°C in $0.1\times$ SSC, 10 mM β -mercaptoethanol. Autoradiography was performed using NTB-2 photographic emulsion (Eastman Kodak Co., Rochester, NY).

Immunohistochemical Staining

Immunohistochemical studies with rabbit anti-mouse AFP (ICN Biochemicals, Inc., Irvine, CA) and rabbit anti-bovine chromogranin A (kindly provided by Dr. Ruth Angeletti, Albert Einstein College of Medicine, Bronx, NY) antibodies were performed using the avidin-biotin technique (23) with a Vectastain kit from Vector Laboratories, Inc. (Burlingame, CA). Double antibody staining with sheep anti-rat AFP serum (Nordic Immunological Laboratories, Tilburg, The Netherlands) and the chromogranin A antibody was visualized by immunofluorescence. Tissues were treated with a 5:1 mixture of methanol and peroxide. Slides were blocked with 0.5 M Tris-HCl, pH 7.5, 2% FCS, 2% BSA. AFP and chromogranin A antibodies were used in 1:100 dilution. FITC-labeled goat anti-rabbit IgG was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL), biotin-conjugated goat anti-sheep IgG was from Vector Laboratories, Inc., and avidin-rhodamine was prepared by Dr. Tim Manser (Princeton University, Princeton, NJ).

In Vivo ^3H Labeling

[^3H]ribosylthymine triphosphate (TTP) (Amersham Corp., Arlington Heights, IL) in 50% ethanol was dried down and resuspended in 0.85% NaCl. ICR:Ha (icr) females at day 19 of pregnancy were anesthetized with avertin and injected via the retinal orbital route with 2–3 μCi of label per gram body weight. Animals were killed 60–90 min after injection. Adult and fetal intestines were dissected, fixed in Bouin's fixative for 2 h, paraffin embedded, and sectioned. Sections were incubated with rabbit anti-mouse AFP antibodies and then dipped in Eastman Kodak Co. NTB-2 photographic emulsion. Slides were exposed for 16 d.

Cell Culture and Transient Transfection Assays

The four human gut cell lines, LoVo, Caco-2, Ht-29, and HuTu 80, were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in BIO-RICH medium containing 10% FCS (Gibco Laboratories, Grand Island, NY). The cells were regularly subcultured immedi-

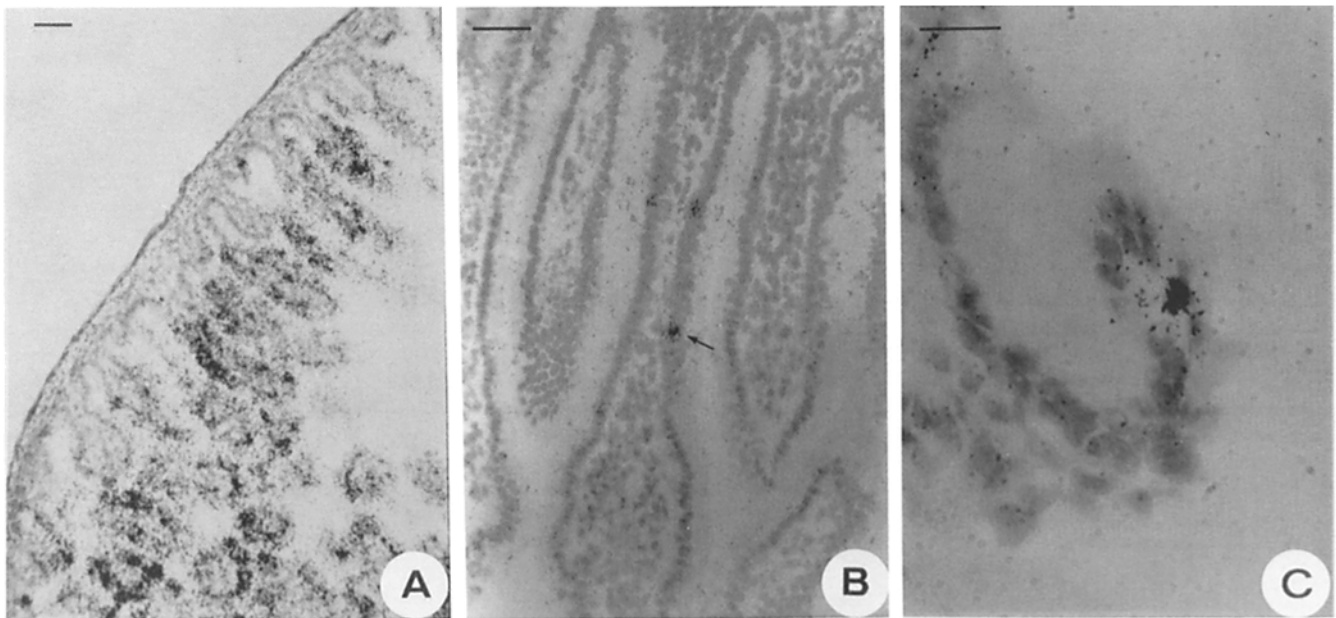


Figure 1. *In situ* hybridizations of ^{35}S -labeled AFP exon 1 probe to sections of fetal and adult mouse small intestine. Sections of small intestine dissected from fetal mice at 19 d of gestation (A) or from adults at 3–4 wk of age (B and C) were hybridized with ^{35}S -labeled 907-nucleotide AFP cRNA probe. Exposures were for 4 d. Bars: (A and B) 50 μm ; (C) 25 μm .

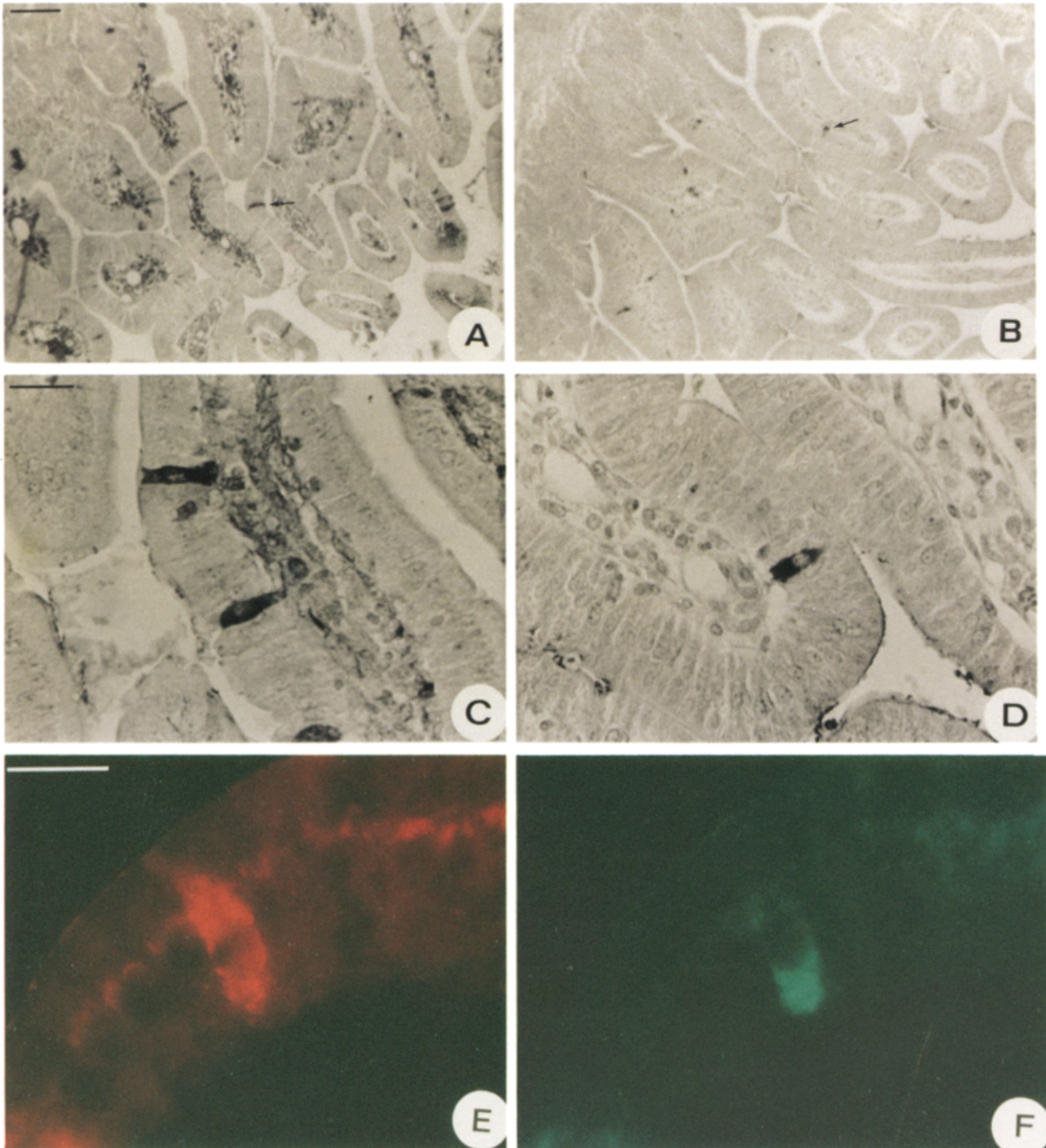


Figure 2. AFP is expressed in a subset of enteroendocrine cells that express chromogranin A. Small intestines removed from adult mice were fixed in Bouin's fixative, paraffin embedded, and sectioned. Sections were incubated with rabbit anti-mouse AFP antibody (A and C) or rabbit anti-bovine chromogranin A antibody (B and D), and antibody reactivity was visualized by the biotin-avidin technique. The low magnification views (A and B) demonstrate that cells expressing AFP or chromogranin A are found at approximately equal frequency. Examples of cells expressing either antigen are denoted by arrows. At higher magnification, a difference in intracellular localization of the two antigens can be noted. AFP is generally found distributed throughout the length of the cell (C), while chromogranin A (D) is often found localized toward the basal end. Tissue sections were also incubated with both sheep anti-rat AFP and the rabbit anti-chromogranin antibody. AFP reactivity was detected by incubation with a biotinylated goat anti-sheep secondary antibody and avidin-rhodamine (E). Chromogranin A reactivity was detected by incubation with an FITC-conjugated goat anti-rabbit secondary antibody (F). The differential distribution of the two antigens in the same cell can be seen in E and F. Bars: (A and B) 50 μ m; (C-F) 25 μ m.

ately before confluency. Transient expression assays using the Caco-2 cell line were performed as previously described (17, 19). In summary, 10 μ g of plasmid DNA was added to each plate using the calcium phosphate precipitation method. The DNA was left on the cells for 16 h, after which the cells were washed in PBS and then harvested at 48 h. RNA was prepared using the hot phenol method and enriched for poly (A)⁺ RNA by oligo (dT) cellulose chromatography (2). Poly (A)⁺ RNAs were electrophoresed in a 6% formaldehyde, 1.5% agarose gel in MOPS buffer (20 mM morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) after heat denaturation at 55°C for 15 min in 70% formamide, 1 \times MOPS. The gel was blotted onto nitrocellulose in 20 \times SSC, and the filters were hybridized in 50% formamide containing dextran sulfate (49).

For the isolation of plasmid DNA from transfected cells, 10% of the cells harvested 48 h after transfection were lysed by the method of Hirt (22). After proteinase K digestion, Hirt supernatants were phenol and chloroform extracted and ethanol precipitated. Equivalent portions of each sample were digested with restriction enzymes and electrophoresed on 1% agarose gels in 40 mM Tris-acetate, 1 mM EDTA, pH 7.2. Gels were blotted by the method of Southern (46) and hybridized as described (54).

Nuclear Run-on Assay

Nuclei were purified from fetal intestines (day 19 of gestation) of ICR:Ha (icr) mice and transgenic line 164-6A. Intestines were homogenized in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40 (Particle Data Inc., Elmhurst, IL), 2.75 mM DTT, 20 U/ml RNasin (Promega Biotech), and 0.25 M sucrose. Tissue homogenate was layered over a 3-ml cushion of 2.6 M sucrose in homogenization buffer, and nuclei were pelleted by centrifugation for 45 min at 25,000 rpm at 0°C. Assays were performed according to Konieczny and Emerson (24). Filters were hybridized in 4 \times SET (1 \times is 150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 5 mM EDTA), 1 \times Denhardt's solution (0.02% BSA, 0.02% polyvinyl pyrrolidone 40, 0.02% Ficoll 400), 100 mg/ml sheared salmon sperm DNA, and 50% formamide at 42°C. The filters were washed three times for 30 min in 0.1 \times SET and 0.1% SDS at 60°C.

Results

Localization of AFP Transcripts in the Fetal and Adult Small Intestine

It has been demonstrated, both by immunohistochemistry (25) and in situ hybridization techniques (14, 40), that AFP is synthesized by most hepatocytes in the fetal liver and that postnatal repression is accomplished by a uniform diminution in its transcription rate in all cells (25, 51). To identify the cells that transcribe the AFP gene in the gastrointestinal tract, a ³⁵S-labeled cRNA probe was hybridized with cross sections of intestine from fetal mice at day 19 of gestation (1 d before birth) and of postweaning mice, 3–4 wk of age. In the fetus, grains due to hybridization were visible over most of the cells lining the villi (Fig. 1 A). After birth, however, grains were restricted to single isolated cells along the adult villi that continued to express high levels of AFP RNA (Fig. 1, B and C). These cells constituted <1% of the total cells lining the villi.

AFP Is Expressed in a Subset of Enteroendocrine Cells in the Adult Small Intestine

The cells expressing AFP mRNA in the adult gut were distributed in a pattern and frequency that suggested that they could be of enteroendocrine origin (8, 9). To confirm their identity, an antibody against chromogranin A, an acidic glycoprotein that has been used as a marker for the enteroendocrine system, was used (34). Separate sections of adult small intestine were incubated with rabbit anti-mouse AFP or rabbit anti-bovine chromogranin A. The pattern and frequency of AFP- and chromogranin A-expressing cells in homolo-

gous sections were very similar (Fig. 2, A and B) but not identical in all sections examined. At a higher magnification, differences in the intracellular localization of AFP and chromogranin A could be seen as well. AFP was normally distributed evenly throughout the cells, while the chromogranin A reactivity was localized more basally (Fig. 2, C and D).

Double antibody staining was conducted with a sheep anti-rat AFP serum and the rabbit anti-bovine chromogranin A antibody to ascertain whether the AFP and chromogranin A antibodies colocalized to the same cells. The two antibodies reacted with the same cells ~85% of the time, indicating that AFP is indeed expressed in enteroendocrine cells. An example of the same cell reacting with both antibodies is shown in Fig. 2, E and F. A subset of cells showed positive staining for either AFP or chromogranin A alone. Since AFP is presumably secreted by the enteroendocrine cells (secretion can be observed in the lamina propria in Fig. 2 A), the chromogranin A positive and AFP negative cells could have been a result of AFP's recent secretion. On the other hand, it may be that AFP and chromogranin A are differentially expressed in different subclasses of enteroendocrine cells.

AFP Transcription Is Not Associated with Cell Division in the Gut

Enteroendocrine cells are present in the fetal gut during late gestation, as well as in the adult gut, at approximately the same frequency (32, 33). Thus, only a small proportion of the cells that transcribe the AFP gene in the fetal gut can be of enteroendocrine origin. The identity of the cells that express AFP in the fetal gut and their relationship to the well-defined cells of the adult gut is not clear. In the case of the liver, it has been established that the fetal hepatocytes that transcribe AFP are dividing and that repression in AFP transcription is coincident with a cessation of DNA synthesis in neonatal hepatocytes (51). This suggested to us that a similar relationship between AFP transcription and DNA synthesis might exist in the gut, in that it had been proposed that the cells lining the villi in the fetal gut are mitotically active, unlike their counterparts in the adult gut. To ask whether this was the case, female ICR:Ha (icr) mice at day 19 of pregnancy were injected with [³H]TTP and killed 60–90 min later, and intestines were removed from both the adults and fetuses. Before exposure to photographic emulsion, tissue sections were incubated with antibodies against mouse AFP, and binding was detected with the biotin-avidin technique. In the fetal gut, the cells that had incorporated [³H]TTP were localized to the base of the villi where crypt formation would occur after birth (Fig. 3 B). These cells did not stain with AFP antibodies (see arrows in Fig. 3, A and B). Rather, the AFP antibodies reacted in a diffuse manner to nascent villi (Fig. 3 A), consistent with the pattern of RNA localization observed for the in situ hybridization experiments (Fig. 1 A). The distribution of dividing cells in the day 19 fetus is highly reminiscent of the pattern of incorporation of [³H]TTP label observed in the adult gut (Fig. 3, C and D). From this we conclude that the expression of AFP in the cells lining the fetal villi is not correlated with mitotic activity.

Postnatal Repression of AFP Transcription Is Not Regulated in the Gastrointestinal Tract

The basal level of AFP mRNA in the postpartum mouse liver

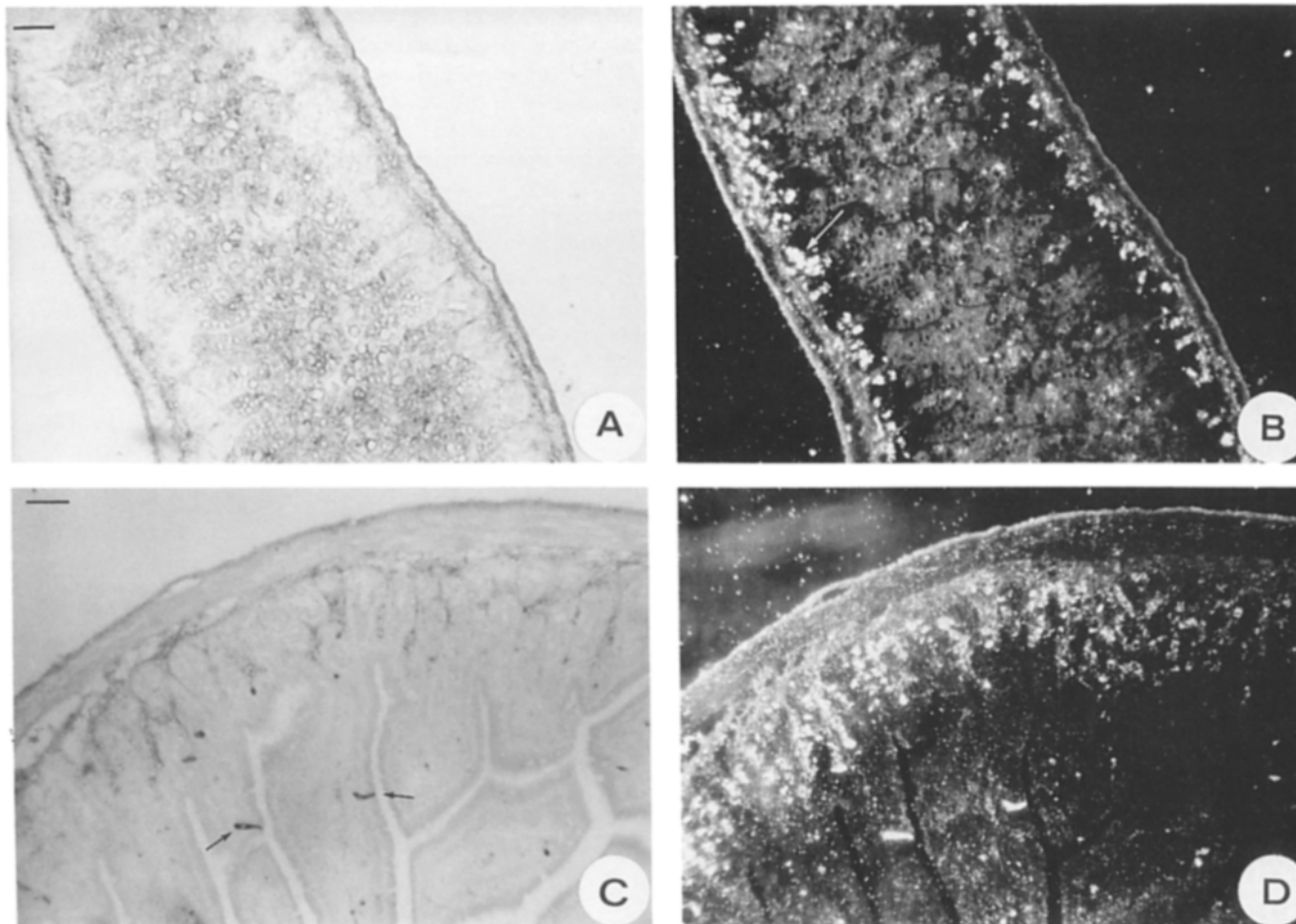


Figure 3. AFP is not expressed in the proliferating cells of the fetus or adult. Mice at 19 d of pregnancy were injected with 2–3 μCi of [^3H]dTTP per gram of body weight and killed 60–90 min later. The intestines of the fetuses and adult females were fixed in Bouin's fixative, paraffin embedded, and sectioned. Sections were incubated with an anti-mouse AFP antibody, and reactivity was visualized by the avidin-biotin method (23). Sections were then exposed to photographic emulsion for 17 d. Brightfield and darkfield views of the fetal gut (A and B) and adult gut (C and D) are shown. Diffuse AFP staining can be seen along the nascent villi of the fetus (A), but grains indicating cell division are localized to the rudimentary crypts as can be clearly seen in the darkfield view (B). In the adult, a few cells denoted by arrows in C strongly react with AFP antibody. The peroxidase reaction product deflects light in the darkfield view, and, although no ^3H grains are visible above these cells, they appear bright in D. Cell division is localized primarily to the crypts in the adult animals (D). Bars, 50 μm .

is regulated in part by a genetically defined *trans*-acting locus named *raf*, for regulation of AFP. Two alleles have been identified for the *raf* locus, *raf^a* and *raf^b* (36). BALB/cJ mice are homozygous for the rare recessive *raf^b* allele, resulting in 10–15-fold higher basal level AFP transcription in livers of adult BALB/cJ mice (3). To determine if *raf* affects the postpartum repression of AFP transcription in the gut, poly (A)⁺ RNA was isolated from the gastrointestinal tracts of BALB/cJ mice (*raf^b/raf^b*) and C3H/HeJ mice (*raf^a/raf^a*) at different times before and after birth (Fig. 4). At day 19 of gestation, relatively high levels of AFP mRNA could be detected in both strains, and no differences were observed as the RNA levels dropped rapidly after birth. Thus, the *raf* gene does not appear to affect the repression of AFP mRNA in the gut.

AFP Is Expressed in the Human Gut Cell Line Caco-2

To investigate the *cis*-acting elements that are necessary for

transcription of the AFP gene in the gut, we first identified a gut cell line that could be an appropriate recipient for gene transfer experiments. RNA was isolated from three cell lines derived from human colon adenocarcinomas, LoVo, Caco-2, and Ht-29 (not shown), and one line derived from an adenocarcinoma of the duodenum, HuTu 80. These RNAs were subjected to electrophoresis through a denaturing gel, blotted to nitrocellulose, and hybridized with an AFP-specific probe (Fig. 5). Only the Caco-2 cell line expressed AFP mRNA. The level of AFP RNA expressed in Caco-2 cells was comparable with that in Hep3B hepatoma cells, but significantly less than in another human hepatoma cell line, HepG2.

AFP Enhancers I and III Are Active in Caco-2 Cells

The expression of the AFP gene in liver cells has been attributed to the action of three cellular enhancers located 2.5, 5.0, and 6.5 kbp of DNA upstream of the transcriptional start site

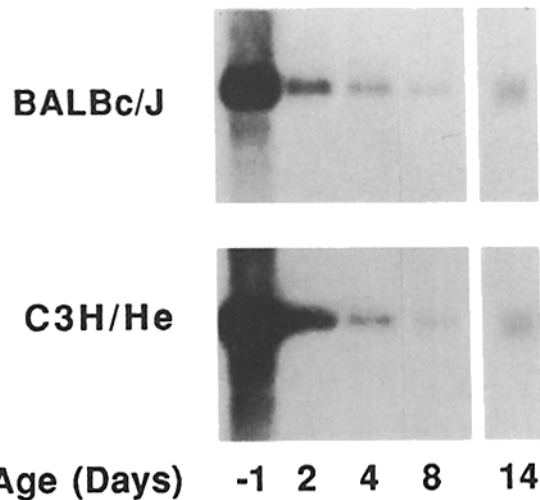


Figure 4. AFP expression in the gut is not *raf* regulated. Poly (A)⁺ RNAs were isolated from the intestines of BALBc/J (*raf^b/raf^b*) and C3H/HeJ (*raf^a/raf^a*) mice at day 19 of gestation (lane -1) and 2, 4, 8, and 14 d after birth (lanes 2, 4, 8, and 14, respectively). 10 μ g of each sample was electrophoresed through a 1.5% agarose formaldehyde gel and transferred to nitrocellulose by blotting. Blots were hybridized with a 907-bp ³²P-labeled AFP cDNA probe. Exposure time was 1 wk, except for day 14 which was exposed for 3 wk.

(17, 56). The three enhancers, which were each localized to 200–300 bp segments of DNA, exhibited nonadditive, equivalent activities when tested by transient transfection into human hepatoma cells. To examine the activities of these enhancers in Caco-2 cells, segments of DNA containing them were ligated to the herpes simplex virus TK gene and 200 bp of its promoter. These were introduced as calcium phosphate precipitates into Caco-2 cells, and, 48 h later, cells were harvested and RNA and Hirt DNA were prepared. The levels of TK transcription were determined by Northern gel analysis, using a hybridization probe internal to the TK structural gene. The blots were also hybridized to a ribosomal protein L32 probe to control for loading of RNA onto the gels. To control for transfection efficiency, the concentration of episomal TK plasmid in Hirt extracts was monitored. The experimental results of the transfections are shown in Fig. 6 A, with the gel lanes corresponding to the constructs as numbered in the diagram (Fig. 6 B).

Enhancer I was originally defined as a Bam HI fragment spanning from -1.0 to -3.9 kb upstream of the AFP gene cap site. As shown in Fig. 6 A, lane 2, it enhanced transcription of the TK gene alone (lane 1) ~20-fold. When the minimal region of 308 bp (MER I; see 18) required for full enhancer I function in liver cells was tested in Caco-2 (lane 3), a similar degree of enhancement was observed. Therefore, enhancing activity in enhancer I localized to the same sequence in both liver and gut cells. This conclusion is reinforced by the behavior of a duplication of a 195-bp subregion of MER I, which produced a 20-fold stimulation of transcription in liver cells (18) as well as in Caco-2 cells (lane 4).

In contrast to enhancer I, enhancer II exhibited no activity in Caco-2 cells, whether tested as a 1.5-kb Bam HI fragment (lanes 5 and 6) or as a 181- (lane 7) or a 420-bp (lanes 8 and 9) minimal region. All these constructs exhibited 15–30-fold enhancing activity in hepatoma cells (18). Finally, enhancer III behaved in an intermediate manner, showing a 10-fold en-

hancement in Caco-2 cells whether it was assayed as a 2.25-kb Eco RI–Bam HI fragment or a 380-bp minimal region (Fig. 6, A, lanes 10 and 11). This level was consistent with its activity in liver cells (18). Thus, the major difference in the behavior of the three enhancers in liver and gut cells is the absence of activity of enhancer II.

An HNF-1 Binding Site in the AFP Promoter Is Required for Efficient Transcription in Caco-2 Cells

The functional domains of the promoter of the AFP gene in liver have been studied extensively in both the rat and the mouse genes (15, 17, 20). These studies have identified two binding sites for the transcription factor HNF-1, one centered at -50 bp and the other at -110 bp. This factor was originally identified as a liver-specific transcription factor that interacts with the promoters of a variety of genes transcribed in the liver (6, 10, 11, 27). It was recently demonstrated that HNF-1 binding to the mouse AFP promoter at -110 bp is essential for its tissue-specific transcription in human hepatoma cells (15), and it can be inferred that binding downstream at -50 bp is also crucial for the activity of the rat AFP gene promoter (11, 20).

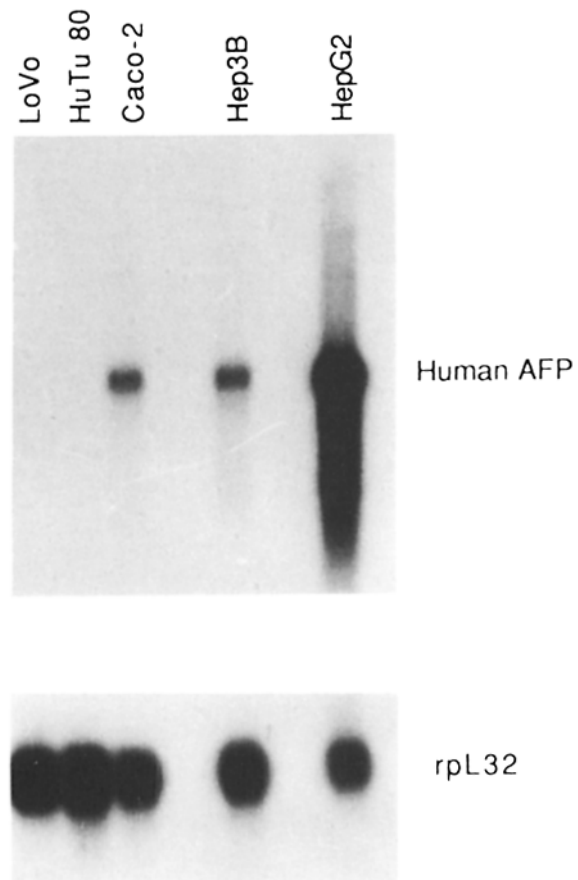
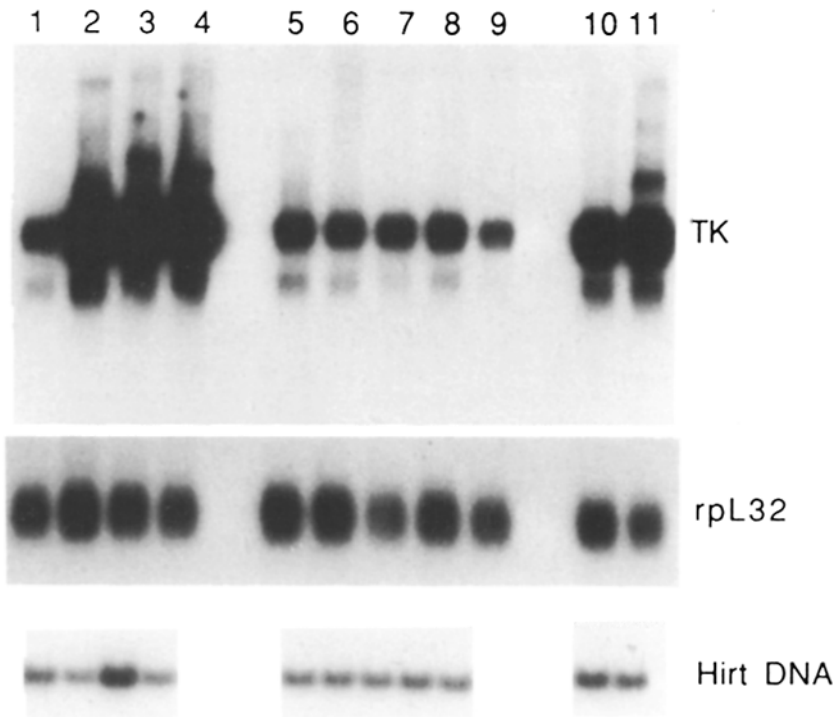


Figure 5. Expression of human AFP mRNA in colon carcinoma cell lines. Poly (A)⁺ RNA was isolated from three human cell lines derived from the gut and two cell lines derived from human hepatomas. 4 μ g of each RNA was electrophoresed through a 1.5% agarose gel in formaldehyde and transferred onto a nitrocellulose filter. The filter was hybridized with a human AFP cDNA fragment, the probe was washed off, and the blot was rehybridized to a labeled fragment derived from the mouse rpl32 gene (13).

A



B

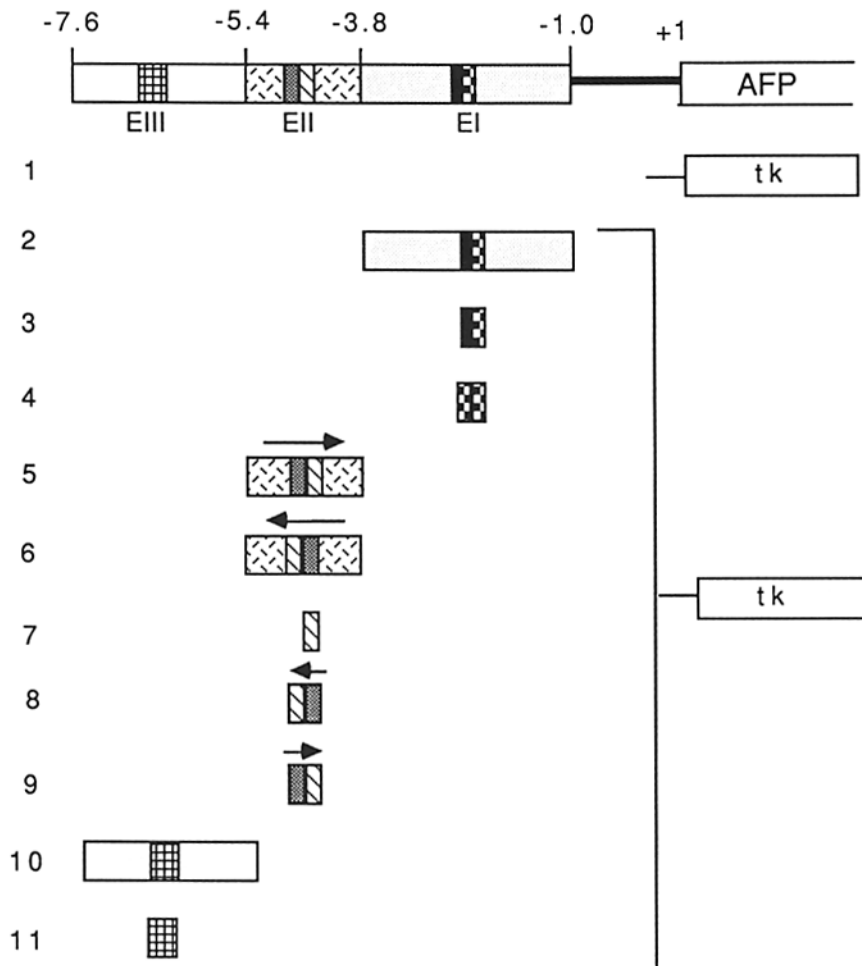


Figure 6. Functional analysis of the AFP enhancers in Caco-2 cells. Different segments of the 5' flanking region of the mouse AFP gene (top line in *B*) that had been previously shown to contain liver-specific enhancer function (18) were ligated to the TK gene, along with its promoter (construct 1 in *B*). These segments were a Bam HI fragment from -1 to -3.9 kb (enhancer I; construct 2); a 308-bp subfragment of it that contained all enhancer I activity (construct 3); a duplication of 195 bp of the 3' end of the subfragment (construct 4); a Bam HI fragment from -3.9 to -5.4 kb (enhancer II; constructs 5 and 6; two orientations); a 181-bp subfragment of enhancer II (construct 7); a 420-bp subfragment in enhancer II that contains all of its activity in liver cells (constructs 8 and 9; two orientations); an Eco RI-Bam HI fragment that spans -5.4 to -7.6 kb (enhancer III; construct 10); and a 380-bp subfragment of enhancer III that contains all its activity in liver cells (construct 11). All of these constructs are described in detail (18). These constructs were introduced into Caco-2 cells, and RNA and Hirt DNA were prepared 48 h later. The poly (A)⁺ RNA was electrophoresed through a 1.5% agarose gel and transferred to nitrocellulose, and blots were probed with a 1.9-kb fragment containing the TK structural gene (31), stripped, and rehybridized to rpL32 gene fragment (13). The Hirt DNA preparations were digested with Pst I and electrophoresed through a 1.0% agarose gel, transferred to nitrocellulose, and hybridized to an 800-bp DNA fragment from within TK structural gene. The lanes in *A* are numbered according to the constructs diagrammed in *B*.

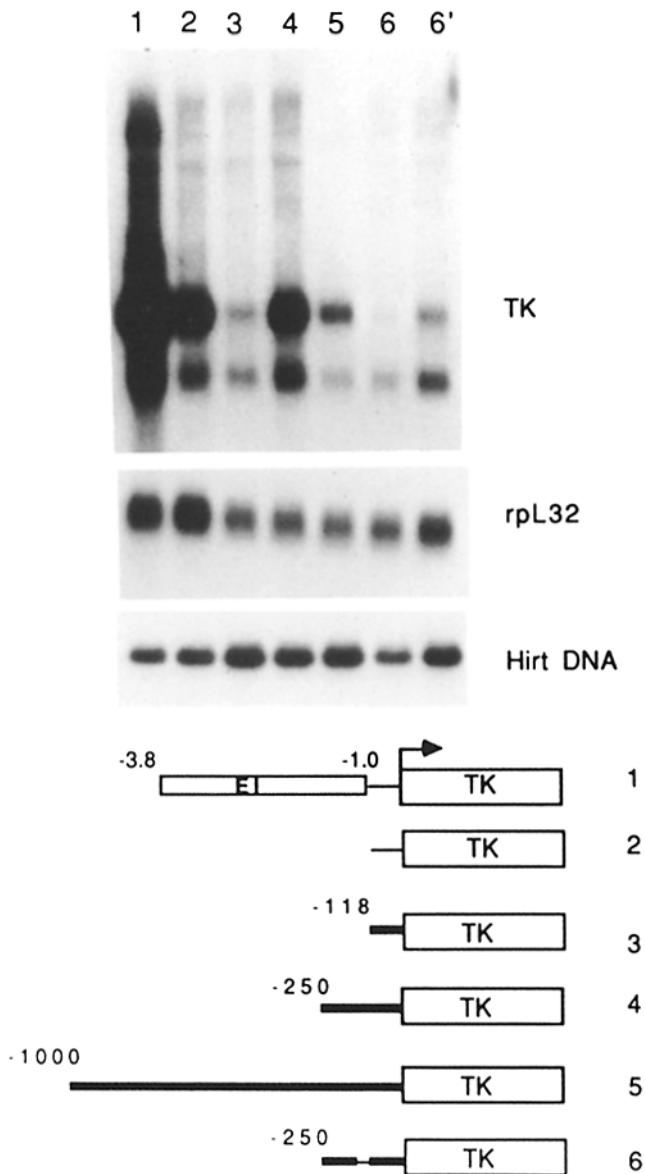


Figure 7. Functional analysis of the AFP gene promoter in Caco-2 cells. Segments of the AFP promoter (indicated by the dark line), which share a 3' endpoint at +1 and 5' endpoints indicated in the figure, were linked to the cap site of the TK structural gene (constructs 3-6). In construct 6, four base changes were made in an HNF-1 site between -120 and -100, as indicated by the break in the dark line. These were transfected, as was the TK gene driven by 200 bp of its own promoter region (construct 2) or the TK gene and promoter linked to enhancer I of the AFP gene (construct 1) into Caco-2 cells. 48 h later RNA and Hirt DNA were analyzed as described in the legend to Fig. 6. The lanes of the gel are numbered according to the numbers of the constructs below. Lanes 6 and 6' represent two different transfections of construct 6.

To determine if HNF-1 plays a role in transcription in the gut, transcription of the TK structural gene under the direction of varying segments of the AFP promoter was monitored in Caco-2 cells. Fig. 7 depicts the results of these transfection experiments, along with a diagram indicating the constructs used. The level of transcription directed by 250 bp of the AFP promoter was comparable with that of the TK promoter alone (Fig. 7, lanes 2 and 4). This level of transcription decreased substantially when the distal HNF-1 was disrupted by a deletion to -118 bp (lane 3). Introduction of a 3-base substitution and 1-base deletion in this HNF-1 site resulted in an equivalent decrease in transcription in Caco-2 cells (lanes 6 and 6'), further suggesting that the HNF-1 site at -110 bp plays an important role in gut-specific transcription.

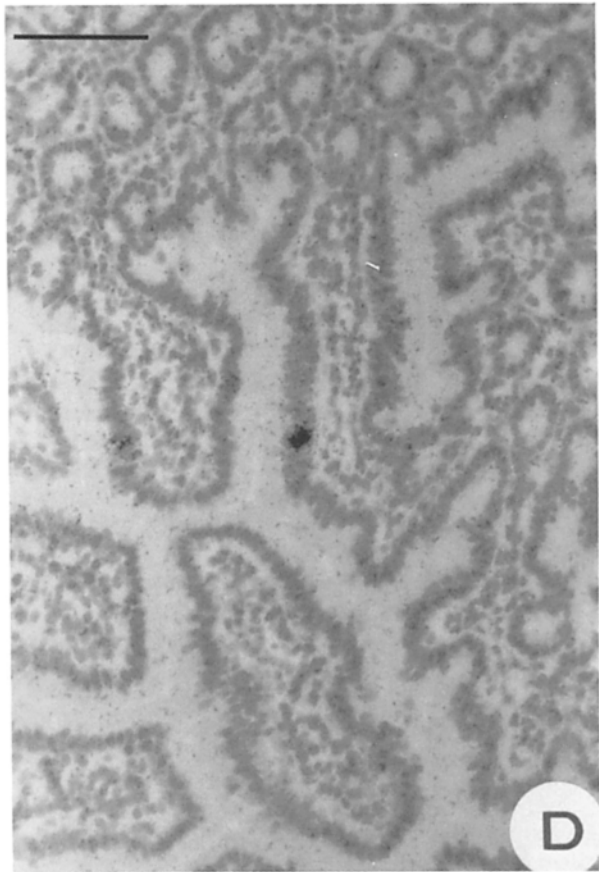
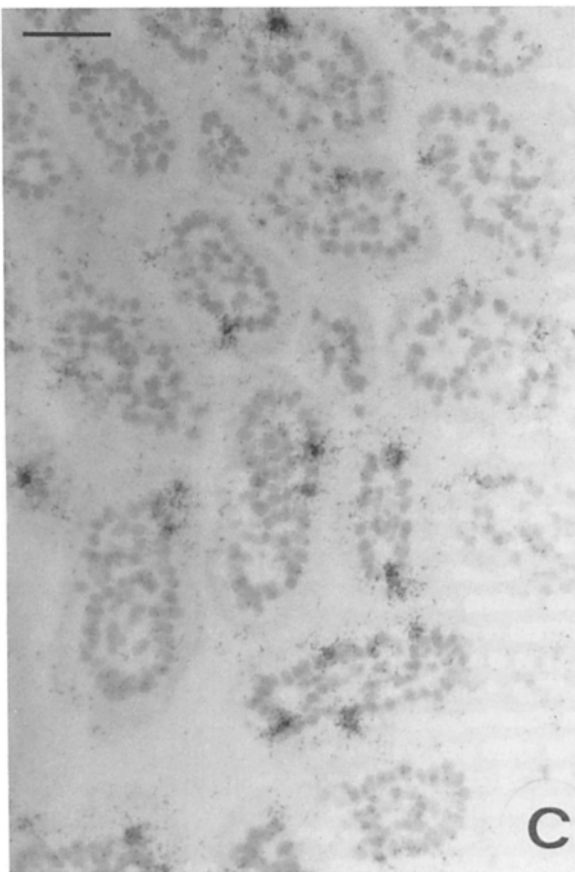
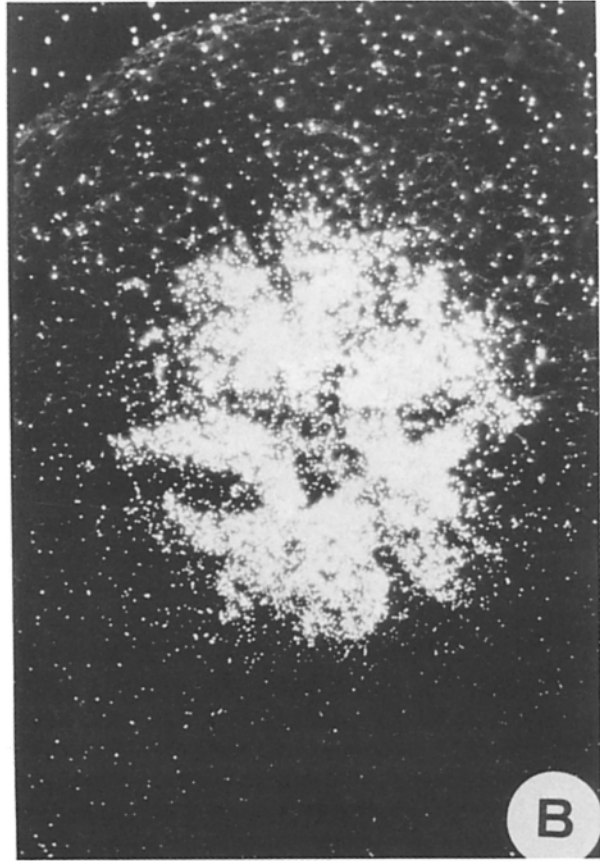
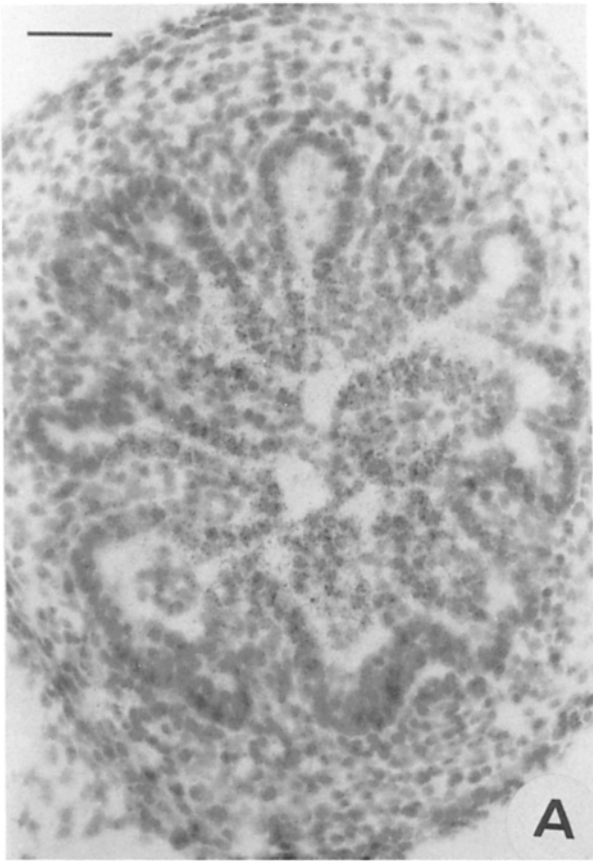
Interestingly, the addition of sequences extending from -250 to -1,000 bp resulted in a decrease in transcription in gut cells, a result that was not observed in liver cells (17). This suggests that sequences upstream of the promoter may play a role in negatively modulating the transcription rate of the gene in the gut cells. This region contains the dominant acting silencer that is necessary and sufficient for repression of the AFP gene in liver after birth (Vacher, J., and S. Tilghman, manuscript submitted for publication).

An Overexpressed AFP Transgene Is Expressed in the Same Cells as the Endogenous AFP Gene in the Fetal and Adult Gut

The elements responsible for AFP gene expression in vivo have been examined using the microinjection of genes into the mouse zygote (21). One of the most surprising results to come from these studies was the observation that a transgene consisting of 6.6 kb of 5' flanking DNA, including the three cellular enhancers and the tissue-specific promoter, linked to an internally deleted AFP "minigene" consisting of the first 3 and the last 2 exons of the 15-exon gene, was expressed at an inappropriately high level in the fetal gut. That is, independent of the copy number of the transgene in the mouse germline or its site of integration, transgenic mice expressed many-fold more AFP minigene RNA than endogenous AFP mRNA in the fetal gut. Two explanations were contemplated. First, it was possible that the overexpression was the consequence of inappropriate expression in the wrong cell types in the gut. Second, it was possible that the transgene transcript was selectively stabilized in the fetal gut, relative to the other expressing tissues in the mouse.

To examine these possibilities, a ³⁵S-labeled AFP exon 1 probe was hybridized to tissue sections from one line of transgenic mice (164-6A) overexpressing the AFP minigene. The exon 1 probe will hybridize with equal affinity to the endogenous and transgene transcripts, but because of the extraordinary levels of the transgene transcripts, and the

Figure 8. Transgenic mice overexpressing an AFP minigene maintain appropriate cell type expression of the transgene. Transgenic mice (line 164-6A) containing 6.6 kb of 5' AFP upstream sequence linked to an AFP minigene consisting of the first two and last three exons of the AFP gene greatly overexpress the minigene in the fetal gut. Sections of fetal gut at day 19 of gestation (A and B; brightfield and darkfield views, respectively), at 2 wk postpartum (C), and at adulthood (D) were hybridized with a ³⁵S-labeled 440-nucleotide AFP exon 1 cRNA probe. This probe contains only 129 nucleotides of coding sequence and will hybridize equally with both the endogenous AFP gene transcripts and the minigene transcripts. Exposures were for 2 d. In the fetus, a dense concentration of grains can be visualized along the villi in darkfield (B). In the postpartum transgenic animal (C and D), single cells appearing at the same frequency as the enteroendocrine cells are found to express the transgene product. Bars, 50 μm.



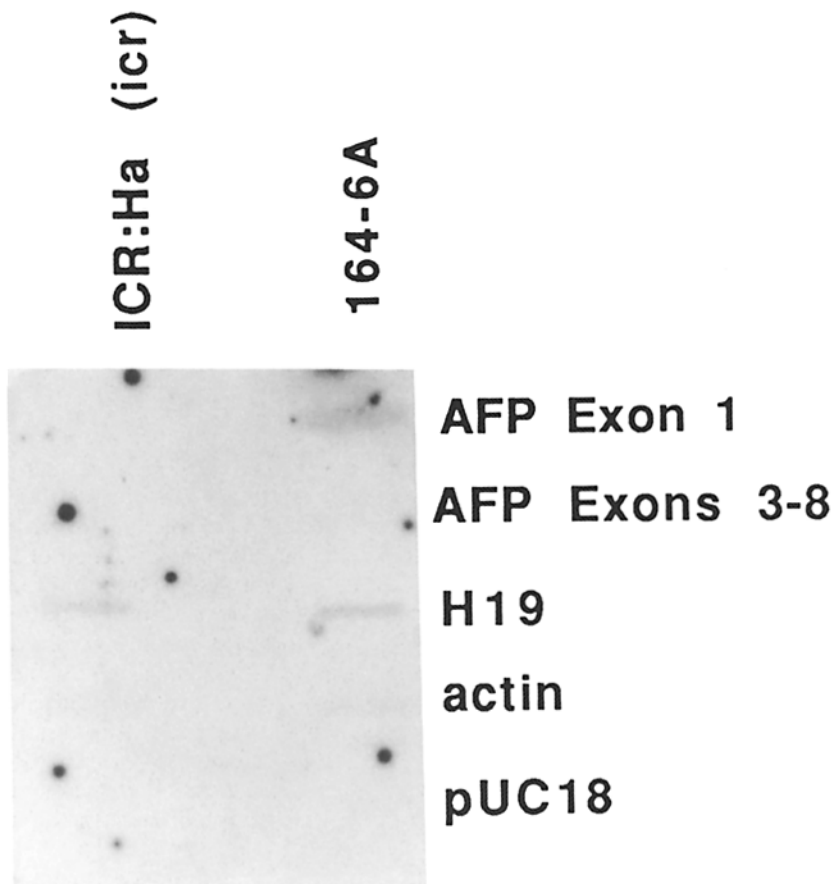


Figure 9. The increased expression of the AFP minigene in the transgenic mice occurs at the transcriptional level. Purified nuclei from fetal intestines from ICR:Ha (icr) and transgenic 164-6A mice were incubated with a nucleotide mix containing [³²P]UTP. Radiolabeled RNA was extracted from the nuclei and hybridized with filters containing 10 μg of each of the following DNAs: AFP exon 1; AFP exons 3–8; H19, a gene expressed at high levels in the fetal gastrointestinal tract; actin; and pUC18.

shorter exposure times used, the probe detects primarily the transgene product. After autoradiography, sections were stained with hematoxylin and eosin. In Fig. 8, *A* and *B*, sections of fetal gut are viewed under brightfield and darkfield microscopy, and it is clear that although the transgene is overexpressed ~50-fold in this line (21), the transcripts are restricted to the appropriate cells lining the villi. In the postpartum animal at 2 wk of age (Fig. 8 *C*) and in the adult (Fig. 8 *D*) expression is restricted to single cells.

The high level of expression of the transgene was examined by nuclear run-on experiments to determine whether it was the consequence of increased transcription or stabilization of the transcript. Nuclei were prepared from fetal transgenic and nontransgenic animals and incubated for a short period of time in the presence of [³²P]UTP. The radioactive transcripts were hybridized with filters onto which plasmid DNA containing AFP exon 1 DNA, and DNA encoding AFP exons 3–8 was immobilized. The exon 1 DNA will hybridize with both the transgene product and the endogenous gene product, while exons 3–8 will hybridize only with the endogenous gene product. As shown in Fig. 9, transcripts homologous to AFP exon 1 were detected at high levels in the transgenic progeny but not in the nontransgenic animals, leading to the conclusion that the overexpression arises from high level transcription of the transgene in the appropriate cell types.

Discussion

Localization of AFP-producing Cells in the Gut

These studies present the first detailed description of the on-

togeny of AFP gene expression in the gastrointestinal tract. AFP synthesis had previously been localized to the endodermal cells of the visceral yolk sac (14) and to all hepatocytes of the fetal liver (25, 40). By using *in situ* hybridization to tissue sections, we demonstrated that AFP is expressed in most epithelial cells lining the villi of the fetal gut, but after birth it becomes restricted to a subpopulation of enteroendocrine cells. Sweetser et al. (48) have also noted a strikingly high level of expression in enteroendocrine cells of another endoderm-specific gene, that encoding liver fatty acid binding protein. However, unlike AFP, the fatty acid binding protein is also expressed in adult enterocytes, in a horizontal gradient that is highest in the proximal duodenum and lowest in the colon.

The cells transcribing the AFP gene in the fetal gut are a mixture of immature enterocytes and goblet and enteroendocrine cells. Enteroendocrine cells have been detected by day 19 of gestation in the rat intestine, 3 d before birth (30), and in the human as early as 12 wk of gestation (32). We also observed distinct staining of cells with the chromogranin A antibody at day 19 of gestation in the mouse, suggesting the presence of differentiated endocrine cells at this stage. However, their frequency was not significantly different from that in the adult gut, arguing that the global AFP transcription in the fetal gut cannot be attributed to a more prevalent distribution of these cells alone.

There are more than 15 different classes of endocrine cells in the gastrointestinal tract, distinguishable by different morphological characteristics of their secretory granules and by the different products they secrete (for a review see 47). Chromogranin A, a large acidic glycoprotein with no known

function, is found in the secretory granules of many types of these endocrine cells and is stored along with a variety of polypeptide hormones (1, 28, 35). At the present time, we do not know the nature of the peptide hormones that are co-expressed with AFP and chromogranin A. AFP is a soluble glycoprotein that has the ability to bind many ligands including fatty acids, metals, steroids (estrogens), thyroxin, and tryptophan (38, 50). Although the function of AFP is unknown, further characterization of its expression in enteroendocrine cells may help us discern a function in the gastrointestinal tract.

Thus, two distinct patterns of AFP gene expression coexist in cells derived from a common precursor stem cell in the adult gut; in the majority of cells the AFP gene is repressed, however, in a subset of enteroendocrine cells it continues to be transcribed at a rate that approximates that in the fetal cells. Unlike the liver, where there is a strict correlation between DNA synthesis and AFP gene transcription, the cessation of AFP transcription in the gut after birth cannot be attributed to a decrease in the number of mitotically active cells. This conclusion arose from our observation that DNA synthesis is restricted to the rudimentary crypts before the loss of AFP transcripts along the villi in the day 19 fetus.

Transcriptional Control of the AFP Gene in the Gut

AFP is transcribed in the developing mouse in multiple cell types, all of endodermal origin. That these cell types might respond differently to the regulatory regions of the gene was first suggested by experiments whereby the gene was reintroduced into the mouse germline as an internally deleted minigene (21). The presence of all three enhancers of the gene generated high level, tissue-specific transcription in the visceral endoderm of the yolk sac, the liver, and gut. However, when the enhancers were tested separately, it was observed that each had a unique tissue specificity. Enhancer I activated transcription of the minigene in all three tissues, enhancer II activated transcription in liver and visceral endoderm, and enhancer III only activated transcription in the visceral endoderm (21). We postulated that this differential activity of the three enhancers played an important role in determining the different levels of expression of the gene, with the highest levels in the yolk sac resulting from the activity of all three enhancers in that tissue and conversely the low level in the gut the consequence of the activity of only one of the enhancers.

The importance of enhancer I in gut-specific transcription was reinforced by the transfection experiments performed in this study. Of the three enhancers tested, enhancer I exhibited the highest activity, enhancer II had no activity, and enhancer III enhanced TK transcription to an intermediate level. Based on the results of the transgenic experiments, the activity of enhancer III in the Caco-2 cell line was unexpected. This suggests that the minigene, activated only by enhancer III, might be susceptible to position effects due to the heterologous sites of integration in vivo. In the transgenic experiments, enhancer III was assayed as a 1.25-kb Cla I–Bam HI fragment, and, although the minimal region for expression in vitro is intact in this sequence, it is also possible that additional sequence is required for enhancer III activity in vivo. The transfection experiments demonstrated that the enhancers that were originally identified using liver-derived cell lines could account entirely for the enhancer activities in Caco-2 cells.

The failure of enhancer II to activate transcription in either transgenic mice or Caco-2 cells needs to be reconciled with its strong hypersensitivity to DNase I in the chromatin of fetal gut (16). This hypersensitivity could arise from the binding of a subset of proteins necessary to activate enhancer II or it could suggest a role for enhancer II that has not been apparent from the experiments we have performed.

The analysis of *cis*-acting elements necessary to activate the AFP gene promoter in Caco-2 cells revealed few differences between liver- and gut-specific domains. Although the *trans*-acting factor HNF-1 has only been associated with liver-specific transcription to date, we have demonstrated the necessity of the HNF-1 binding site at –110 bp for efficient transcription of AFP in a gut cell line. In fact, the effect of removing the HNF-1 site at –110 bp had a far more profound effect on transcription in Caco-2 cells than in liver cells, where the loss in the HNF-1 site resulted in a three- to five-fold reduction in TK transcription (15). This is consistent with experiments in transgenic mice, where a deletion of a portion of the HNF-1 site had a minor effect on liver gene expression but completely abolished AFP synthesis in the fetal gut (Vacher, J., and S. Tilghman, manuscript submitted for publication). Hepatocytes and the epithelial cells of the gut are both derived from the embryonic foregut. Indeed, many genes that are expressed in the liver are also expressed in the small intestine (liver fatty acid binding protein, AFP, glutamate dehydrogenase, apolipoproteins). It is therefore not surprising that both of these organs would share common regulatory motifs.

One difference in the behavior of the AFP gene promoter in liver and gut cell lines was the repressive effects of sequences between –250 and –1,000 bp in Caco-2 cells. Within this region is a dominant *cis*-acting silencer of AFP transcription in neonatal liver (Vacher, J., and S. Tilghman, manuscript submitted for publication). It is possible that the negative effects we observe in Caco-2 cells are the consequence of the same sequence acting as a negative regulator in gut cells. Further experiments in this cell line, as well as in vivo, will be necessary to clarify this point.

Although the experiments described here and elsewhere (5, 15, 17, 18, 20, 21, 43, 55, 56) have shed considerable light on the acquisition of tissue specificity of the AFP gene, we still do not understand what determines the rate of transcription in each cell type. For example, why is the rate of transcription in liver and yolk sac ~100-fold more than in the gut? The striking overexpression of the AFP minigene in the gastrointestinal tract of fetal transgenic mice served to highlight this interesting problem. The overexpression depends upon the presence of enhancer I (21) and the HNF-1 site in the promoter (Vacher, J., and S. Tilghman, manuscript submitted for publication) since deletion of either abolishes the high level expression. In this report, we have shown that the overexpression is the consequence of significantly higher rates of transcription of the transgene in the correct cells of the fetal animal, and not due to ectopic expression, or selective stabilization of the minigene in the gut. Yet despite the high level expression in the fetus, the transgene is regulated appropriately and is repressed in all but the enteroendocrine cells of the adult small intestine.

The rapid loss of AFP transcription in the majority of neonatal villus cells could occur by the absence of positive transcription factors or the imposition of a dominant negative mechanism, such as the one that underlies the repression in

neonatal liver (Vacher, J., and S. Tilghman, manuscript submitted for publication). Whatever the mechanism, it does not appear to be affected by *raf*, a regulatory locus that had been previously shown to affect the adult basal level of AFP mRNA in the liver. This conclusion arose from an examination of the levels of AFP mRNA in the guts of inbred mouse strains harboring different alleles of *raf*. Although the product of the *raf* locus was found to act in a cell autonomous manner in the liver (53), this result did not exclude the possibility that *raf* was affecting transcription in other tissues. It must be kept in mind that only two alleles of *raf* have been identified to date, and therefore it is possible that the effect of the rare *raf^b* allele in BALB/cJ mice is liver specific and that examination of other alleles would reveal effects in the gut as well. However, this seems unlikely given that another gene, H19 (37), has been shown to be under *raf* regulation in the liver but not in any other tissues in which its expression has been examined.

It is possible that the Caco-2 cell line may provide a useful model for understanding the late fetal maturation of the AFP-producing cells since it can undergo spontaneous differentiation in vitro. Differentiation of these cells, which is accompanied by cell polarization, the formation of tight junctions and apical microvilli, and the expression of brush border enzymes and apolipoproteins (7, 39), occurs in a growth rate-dependent manner and is particularly evident at postconfluent stages. We have recently observed that this differentiation is accompanied by a significant increase in AFP mRNA (Tyner, A., unpublished observations), suggesting that the cells are mimicking late fetal development rather than the postnatal period when AFP transcription is repressed. We are currently investigating the molecular basis for the increase in AFP transcription in differentiated Caco-2 cells.

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