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Hepatocyte Nuclear Factor 4 α Is Essential for Embryonic Development of the Mouse Colon

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Abstract

Background & Aims—Hepatocyte nuclear factor 4 alpha (HNF4 α) is a transcription factor that has been shown to be required for hepatocyte differentiation and development of the liver. It has also been implicated in regulating expression of genes that act in the epithelium of the lower gastrointestinal tract. This implied that HNF4 α might be required for development of the gut.

Methods—Mouse embryos were generated in which *Hnf4a* was ablated in the epithelial cells of the fetal colon by using Cre-*loxP* technology. Embryos were examined by using a combination of histology, immunohistochemistry, DNA microarray, reverse-transcription polymerase chain reaction, electrophoretic mobility shift assays, and chromatin immunoprecipitation analyses to define the consequences of loss of HNF4 α on colon development.

Results—Embryos were recovered at E18.5 that lacked HNF4 α in their colons. Although early stages of colonic development occurred, HNF4 α -null colons failed to form normal crypts. In addition, goblet-cell maturation was perturbed and expression of an array of genes that encode proteins with diverse roles in colon function was disrupted. Several genes whose expression in the colon was dependent on HNF4 α contained HNF4 α -binding sites within putative transcriptional regulatory regions and a subset of these sites were occupied by HNF4 α in vivo.

Conclusions—HNF4 α is a transcription factor that is essential for development of the mammalian colon, regulates goblet-cell maturation, and is required for expression of genes that control normal colon function and epithelial cell differentiation.

The primary functions of the gastrointestinal tract are to process ingested food, absorb nutrients and water, and regulate energy homeostasis.^{1–4} The gut tube consists of tissue layers called tunics that contribute to specific aspects of gut function. The innermost tunic is the mucosa, which contains an epithelial layer that interfaces with the lumen of the gut and is derived from the definitive endoderm that forms during gastrulation.^{5,6} At around embryonic day (E) 8.5 in mouse development, the endoderm invaginates to form 2 portals. An anterior portal generates the foregut, and the hindgut is formed from the caudal portal.⁵ Initially, the gut endoderm forms a simple tube consisting of a psuedostratified epithelium

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Appendix

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1053/j.gastro.2006.01.003.

surrounded by gut mesenchyme.^{5,7} At around E14.5, the epithelium transitions to a simple columnar epithelium and the topology of the mucosa changes to include villi and crypts within the small intestine and only crypts within the colon.⁵ The architecture and tissue patterning of the gut is established during embryonic development and involves cross-talk between the fetal gut endoderm and mesenchyme.^{2,8} Signaling pathways that control patterning of the gut epithelium have been intensely studied and include wingless-related MMTV integration site proteins, Hedgehog, fibroblast growth factor, bone morphogenic protein, Notch, and platelet-derived growth factor.^{2,7,9} In addition, several transcription factors have been identified that are required for epithelial cell differentiation. *Atoh1* (also called *Math1*) controls the commitment of cellular differentiation toward the secretory cell lineage, a process that is antagonized by *Hes1*^{10,11}; *Klf4* and *Ngn3* are required for establishing goblet and enteroendocrine cells,^{12–14} respectively; and *Elf3* is essential for the terminal differentiation of both columnar and goblet cells in the small intestine.¹⁵ Analyses of transcriptional regulatory elements within genomic DNA have suggested that hepatocyte nuclear factor 4 alpha (HNF4α) also regulates expression of many genes throughout the lower gastrointestinal tract.^{16–23} In addition, studies using the Caco2 intestinal epithelial tumor cell line have shown that HNF4α occupies the promoter of the *α1-antitrypsin* (*Serpina1*) gene coincident with the onset of expression of *α1-antitrypsin* during Caco2 cell differentiation.²⁴ Further support for HNF4α being an important developmental regulator comes from studies using knockout mice. Most relevant is the finding that ablation of *Hnf4a* in fetal livers prevents the hepatic parenchyma from establishing an epithelium and blocks hepatocyte differentiation.^{25–27}

To directly address the role of HNF4α in development of the colon, mice were derived in which the *Hnf4a* gene was ablated in the colonic epithelial cells by using the Cre-*loxP* system.²⁸ Examination of these embryos revealed that loss of HNF4α disrupts formation of crypt topology in fetal colons by E18.5. Loss of crypts was accompanied by reduced goblet cell maturation and proliferation of epithelial cells. Gene expression and HNF4α DNA-binding analyses revealed that loss of HNF4α is required for expression of several target genes that are central to colon function.

Materials and Methods

Animals

The derivation of *Hnf4a*^{+/-} (*Hnf4a*^{tm1Dnl}), *Hnf4a*^{loxP/loxP} (*Hnf4a*^{tm1Sad}), and *Foxa3Cre* (*Tg[Foxa3-cre]*^{1Khh}) mice has been described previously.^{28–30} Mating of *Hnf4a*^{+/-} and *Hnf4a*^{loxP/loxP} with *Foxa3Cre* mice produced *Hnf4a*^{+/-Foxa3Cre} and *Hnf4a*^{loxP/+Foxa3Cre} male mice that were used as studs. These males were mated with *Hnf4a*^{loxP/loxP} female mice to generate control *Hnf4a*^{loxP/+Foxa3Cre} or experimental *Hnf4a*^{loxP/loxPFoxa3Cre} and *Hnf4a*^{loxP/-Foxa3Cre} embryos. Analysis of HNF4α loss of function was restricted to the colon because deletion of HNF4α was inefficient in the small intestine. Noon on the day of the appearance of a vaginal plug was considered as 0.5 days postcoitum, and the genotype of all embryos was determined by polymerase chain reaction (PCR) analysis of genomic DNA. The Medical College of Wisconsin's IACUC committee approved all animal experiments and procedures.

Histology and Immunohistochemistry

Five millimeters of colon lying 5 mm distal to the cecum was dissected from E18.5 embryos in phosphate-buffered saline, fixed overnight with 4% paraformaldehyde (Sigma, St Louis, MO) in phosphate-buffered saline, and embedded in paraffin. Five-micrometer paraffin sections were used in all staining procedures. H&E, alcian blue, and β-galactosidase staining were performed by using standard histological protocols.³¹ Immunohistochemistry was

performed by using antigen retrieval in citrate buffer as described previously,²⁶ except for PECAM1 (platelet/endothelial cell adhesion molecule 1) staining in which sections were treated with 0.25% trypsin for 20 minutes at room temperature. Primary antibodies recognized HNF4 α (sc-6556, 1:500; Santa Cruz, Santa Cruz, CA), E-cadherin (#610181, 1:16,000; BD Transduction Laboratories, San Jose, CA), Ki-67 (sc-7846, 1:500; Santa Cruz), phosphoHistone H3 (#06-570, 1:1500; Upstate, Charlottesville, VA), acetylated tubulin (T6793, 1:1000; Sigma, St Louis, MO), α smooth-muscle actin (A2547, 1:1600; Sigma), laminin (L9393, 1:500; Sigma), and PECAM1 (#553370, 1:50; Pharmingen, San Jose, CA). Micrographs were collected by using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI) and images from control and experimental samples were processed identically using Adobe Photoshop 7.0 (Adobe, San Jose, CA). Cell counts were taken from digital images of 3 sections from each embryo analyzed. Thickness of the circular muscle was measured using Metamorph software (Molecular Devices, Sunnyvale, CA). Data were subjected to statistical analyses by using analysis of variance (ANOVA) (StatView 5.0.1; Adept Scientific Inc, Acton, MA) and were considered significant at $P < .001$.

DNA Microarrays and Reverse-Transcriptase PCR

Affymetrix GeneChip Mouse Expression 430A Arrays were used for the analyses. Total RNA was extracted from fetal colons using Qiagen-RNeasy kit (Qiagen, Valencia, CA). Biotin-labeled complementary RNA probes were generated from 5 μ g of total RNA by using a bioarray transcript labeling kit (Affymetrix). After hybridization, array data were analyzed by using DNA-Chip Analyzer (dChip) software (<http://www.dchip.org>). The microarray data (Supplementary Table 1; see supplemental material online at www.gastrojournal.org) presented in this manuscript have been deposited in National Center for Biotechnology Information Gene Expression Omnibus series accession number GSE3116. Reverse-transcriptase PCR (RT-PCR) was performed as described previously²¹ by using oligonucleotide sequences shown in Supplementary Table 2 (see supplemental material online at www.gastrojournal.org). Amplification of *Hprt* was used to standardize loading and reactions lacking reverse transcriptase were included to confirm the absence of contaminating DNA.

Electrophoretic Mobility Shift Assays

Radiolabeled probes were incubated at room temperature for 20 minutes with liver or Cos-7 cell nuclear extracts in the presence or absence of HNF4 α antiserum or a negative control antibody (anti-Pes1³²). For competition analyses, double-strand oligonucleotide sequence representing the HNF4 α -binding site (H4.21³³) from the ApoCIII promoter³⁴ was labeled by using [P32]-deoxyadenosine triphosphate and incubated with 1 μ g of nuclear extracts from Cos-7 cells or Cos-7 cells expressing exogenous HNF4 α in the presence or absence of 150-fold molar excess of each test binding site as described previously.³⁵ Alternatively, radiolabeled binding sites were incubated with mouse liver nuclear extract prepared as described previously.³⁴ Complexes were resolved by electrophoresis in 4% polyacrylamide gels in 0.25X Tris-borate-EDTA buffer at 400 V for 1 hour. The oligonucleotide sequences representing putative HNF4 α -binding sites used in electrophoretic mobility shift assay (EMSA) reactions are provided in Supplementary Table 2.

Chromatin Immunoprecipitation

Brains and colons were isolated from wild-type CD1 mice, fixed with 1% formaldehyde, and cells dissociated by using a BD Medimachine (BD Biosciences, #340587). Chromatin immunoprecipitation (ChIP) assays were performed using the Upstate ChIP Assay Kit (Upstate #17-295) following the manufacturer's instructions with anti-HNF4 α (Santa Cruz

sc-8987) or anti-Pes1³² antibodies. Oligonucleotide primers used for PCR amplification of precipitated genomic DNA are given in Supplementary Table 2.

Results

HNF4 α Protein Expression Is Restricted to the Epithelial Cells of the Developing Lower Gastrointestinal Tract

Hnf4a mRNA has been previously localized to the fetal gastrointestinal tract.^{36,37} However, to facilitate cell-specific disruption of the *Hnf4a* gene using the Cre-*loxP* system required clarification of which specific cell types in the developing gut expressed HNF4 α protein. Figure 1 shows the result of immunohistochemistry staining analyses of mouse embryos using an antibody (Santa Cruz, anti-HNF4 α C19) that specifically recognizes the carboxyl end of all major isoforms of HNF4 α .²⁶ At E9.5, HNF4 α protein was undetectable in the foregut but could be identified in the developing hepatic rudiment as well as in the epithelium of the midgut and hindgut (Figure 1A and B). By E11.5, staining became more pronounced in these tissues presumably reflecting an increase in the abundance of HNF4 α (Figure 1C and D). At this developmental time, HNF4 α protein was detected throughout the epithelium of the lower gastrointestinal tract with a rostral boundary of expression occurring within the developing stomach (Figure 1C). HNF4 α expression was maintained in all epithelial cells during the conversion of the gut epithelium from pseudostratified to simple columnar (Figure 1E–H). The restriction of HNF4 α protein to the epithelium of both small and large intestine continued throughout development into adulthood (not shown) with expression found in both absorptive and secretory cell lineages of the intestinal epithelium.

HNF4 α -Null Colons Were Recovered From Both *Hnf4a*^{loxP/loxP}*Foxa3Cre* and *Hnf4a*^{loxP/-}*Foxa3Cre* E18.5 Embryos

Development of *Hnf4a* knockout embryos arrests during gastrulation because of defects in visceral endoderm function.^{30,36} This has prevented the use of *Hnf4a*^{-/-} embryos to study the role of HNF4 α in gut development. The Cre-*loxP* system was therefore used to circumvent this early embryonic lethality and to generate embryos lacking HNF4 α in the fetal colonic epithelium. The production of mice (*Hnf4a*^{loxP/loxP}) that contain an allele of *Hnf4a* in which exon2 is flanked by *loxP* elements has been described previously.^{26,28} Mice that are homozygous for this allele are healthy and fertile. However, in the presence of Cre, recombination between *loxP* sites generates a null allele.^{26,28} A line of transgenic mice (*Foxa3Cre*) in which Cre recombinase is expressed from a yeast artificial chromosome encompassing all *Foxa3* transcriptional regulatory elements has also been previously described.²⁹ The tissue distribution of Cre activity in transgenic mice can be determined by breeding Cre mice to a LacZ reporter mouse (*Gt[ROSA]26Sor^{tm1Sho}*).³⁸ Using this approach confirmed that Cre recombinase activity was present from E8.5 onwards in the definitive endoderm and its derivatives including the epithelial cells of the colon (Figure 2A and B).²⁹ *Foxa3Cre* mice were therefore chosen to delete *Hnf4a* from the colon because Cre was active at the very onset of colon development in these mice. To disrupt HNF4 α in the gastrointestinal tract, *Hnf4a*^{loxP/loxP} mice or *Hnf4a*^{+/-} mice were mated to *Foxa3Cre* mice. Double-heterozygous offspring were then bred to *Hnf4a*^{loxP/loxP} mice to generate either *Hnf4a*^{loxP/loxP}*Foxa3Cre* and *Hnf4a*^{loxP/-}*Foxa3Cre* experimental embryos or *Hnf4a*^{loxP/+}*Foxa3Cre* control embryos. In contrast to control embryos, colons could be recovered from *Hnf4a*^{loxP/loxP}*Foxa3Cre* embryos at E18.5 in which HNF4 α protein was undetectable by immunohistochemistry (Figures 2C and D). E18.5 was the latest stage at which HNF4 α -null embryos could be recovered because *Hnf4a*^{loxP/loxP}*Foxa3Cre* newborn mice also lacked HNF4 α in the liver, which resulted in neonatal lethality as previously reported.²⁶ The efficiency of ablating HNF4 α in the colon of *Hnf4a*^{loxP/loxP}*Foxa3Cre* embryos was variable, with <10% of the mice showing complete absence of HNF4 α and the

remaining colons exhibiting varying levels of chimerism (Figure 2E). The observed variation in loss of HNF4 α in different *Hnf4a*^{loxP/loxP}*Foxa3Cre* embryos most likely reflects variation in recombination efficiency. In an attempt to increase the efficacy of recovering HNF4 α -null colons, *Hnf4a*^{loxP/-}*Foxa3Cre* embryos were derived in which 40% had undetectable levels of HNF4 α in the colon (Figure 2F). The continued presence of HNF4 α in a subset of fetal colons could potentially confound any phenotypic analyses and so only embryos in which either HNF4 α protein was judged absent by immunohistochemistry or *Hnf4a* mRNA was undetected by using RT-PCR were studied further. In addition, *Hnf4a*^{loxP/loxP}*Foxa3Cre* and *Hnf4a*^{loxP/-}*Foxa3Cre* embryos were used interchangeably because they displayed indistinguishable phenotypes.

HNF4 α Is Essential for Normal Development of the Colon

Examination of E18.5 HNF4 α -null colons by histochemistry uncovered abnormalities in lower gut topology and tissue organization. H&E-stained sections of control colons revealed that the epithelium contained characteristic crypts extending from a relatively compact lumen (Figure 2G). In contrast, HNF4 α -null colons showed no obvious sign of crypt formation with the mucosa arranged as a simple band surrounding a greatly expanded lumen (Figure 2H).

To more accurately define the consequence of the absence of HNF4 α on the development of the colon, the structure of various tunics was examined by using immunohistochemistry. Although H&E staining revealed that crypt formation was severely affected by loss of HNF4 α , it was also apparent from these analyses that the epithelium had successfully transitioned from pseudostratified to simple columnar in both control and HNF4 α -null E18.5 colons (Figure 2G and H). In support of the formation of a normal epithelium, the cell-adhesion protein, E-cadherin, could be readily detected between apposing epithelial cells regardless of the presence of HNF4 α (Figure 3A and B). It has been previously shown that loss of HNF4 α in the fetal liver results in reduced expression of E-cadherin.²⁶ Nevertheless, in contrast to HNF4 α -null livers in which E-cadherin (*Cdh1*) mRNA is barely detectable,²⁶ RT-PCR analyses revealed that the level of *Cdh1* mRNA in HNF4 α -null colons was reduced by a moderate 2-fold compared with controls (Figure 3K).

Although HNF4 α expression is restricted to epithelial cells, signaling between the mesenchymal and endodermal compartments coordinate development of all layers of the gastrointestinal tract.⁷ It was therefore important to determine whether development of the enteric nervous system, lamina propria, and intestinal smooth muscle was affected in HNF4 α -null colons. First, the integrity of the capillary network within the lamina propria of the colon was investigated by using antibodies to identify the endothelial cell marker PECAM1. In control colons, PECAM1-positive endothelial cells could be identified extending through the lamina propria to the top of the crypts (Figure 3C). In contrast, this network of capillaries was disrupted in colons lacking HNF4 α (Figure 3D). The lamina propria, in which the intestinal capillary network is housed, consists of loose connective tissue that secretes extracellular matrix proteins including laminin. It has been proposed that the extracellular matrix defines an environment that supports cell-cell communication, thereby contributing to the control of cell differentiation.^{39,40} Figure 3E shows that laminin extends throughout the mucosa occupying the spaces between the crypts of control colons. In HNF4 α -null colons, laminin was still clearly detectable but instead was organized as a lining at the base of the epithelium (Figure 3F). The observation that laminin is present in HNF4 α -null colons suggests that the disorganization of laminin localization is a consequence of defective crypt formation rather than complete absence of the lamina propria per se. Consistent with this proposal, RT-PCR analyses of colons lacking HNF4 α revealed no significant change in the levels of mRNAs encoding *Bmp4*, *Tcf21*, and *FoxI1* (Figure 3K), all of which are expressed in the lamina propria.⁴¹⁻⁴³

The development of the enteric nervous system and the smooth-muscle layers of HNF4 α -null colons were examined next. Immunohistochemical staining for neural acetylated tubulin, whose expression is restricted to the enteric neurons, identifies the myenteric plexus as indistinguishable between control and mutant colons (Figure 3G and H). The muscularis layers were detected by using antibodies against α smooth-muscle actin (Figure 3I and J). Although the muscularis mucosa and muscularis externa were identifiable in both control and HNF4 α -null colons, the muscularis externa appeared thinner in mutant colons compared with controls. The apparent difference in muscle volume was confirmed by morphometric analyses using Metamorph software, which calculated a 58% reduction (ANOVA, $P < .0001$) in the volume of the external smooth-muscle layer of HNF4 α -null colons compared with controls. In support of a reduction in overall muscle mass of the colon, RT-PCR analyses revealed that smooth muscle myosin heavy chain (Myh11) mRNA, whose expression is restricted to the muscle cells of the colon, was reduced by 2-fold in HNF4 α -null colons (Figure 3K).

The phenotype associated with loss of HNF4 α was similar in some aspects to that associated with loss of the signaling molecule Indian hedgehog (IHH). Like HNF4 α -null colons, *Ihh*^{-/-} embryos have a severely dilated intestine and display disrupted crypt formation.⁴⁴ Examination of *Ihh*^{-/-} embryos revealed a reduction in intestinal smooth-muscle content and loss of enteric neurons, and it was proposed that this contributed to the abnormal dilation of the colon.⁴⁴ Although loss of smooth-muscle volume was also identified in HNF4 α -null colons (Figure 3J), the observation that these colons contained neural cells (Figure 3H) suggested that IHH signaling was unlikely to be affected by loss of HNF4 α . This was confirmed by RT-PCR analyses (Figure 3K) that identified similar levels of *Ihh* mRNA in both control and HNF4 α -mutant colons. Therefore, we conclude that HNF4 α has an integral role in controlling multiple aspects of colon development and that this regulation is not mediated through control of IHH expression.

Loss of HNF4 α Results in a Reduction in Epithelial Cell Numbers in the Developing Colon

The observation that the colonic mucosa seemed thinner in the absence of HNF4 α could result from a reduction in the number of epithelial cells present within the mucosa. Epithelial cells were therefore counted in three independent H&E-stained sections per colon in either control or mutant embryos. The total number of epithelial cells counted per section was reduced by 40% (ANOVA, $P < .0001$) in HNF4 α -null colons compared with controls (Figure 4A). The reduction in HNF4 α -null epithelial cells did not appear to be caused by increased apoptotic cell death because immunohistochemistry using anti-cleaved Caspase 3 antibodies revealed few apoptotic cells in colons from either control or experimental embryos (not shown). To test whether cell proliferation was affected by loss of HNF4 α , sections were processed for immunohistochemistry to detect Ki-67 protein, which acts as marker of cells that are actively proliferating. Figure 4B shows that, as expected, immunohistochemistry detected Ki-67-positive cells located in the lower portion of the crypts in control colons. In contrast, in HNF4 α -null colons Ki-67 was identified in epithelial cells that were present as intermittent clusters throughout the epithelium (Figure 4C). This analysis shows that although crypt architecture is disrupted by loss of HNF4 α , proliferating cell compartments were still present, suggesting that some level of crypt to surface epithelium patterning was maintained in the absence of HNF4 α . Although proliferating cells were detected in HNF4 α -null colons, they appeared to be less numerous compared with control colons (Figure 4B and C). Cell counting revealed that the number of Ki-67-positive cells was reduced by 35% (ANOVA, $P = .0002$) in colons lacking HNF4 α compared with controls (Figure 4D). Similar results were also found by using antiphosphohistone H3 antibodies to detect mitotic cells (data not shown). Expression of transcription factors *c-Myc* and *Foxm1* has been shown to closely correlate with cell proliferation in the gut.⁴⁵⁻⁴⁷ RT-

PCR analyses (Figure 4E) showed that the levels of both *Myc* and *Foxm1* mRNAs were reduced by 2-fold in colons lacking HNF4 α , which is consistent with the reduction in proliferating cells observed using immunohistochemistry. These data imply that the observed reduction in the total number of epithelial cells associated with the absence of HNF4 α is at least in part a consequence of a reduction in epithelial cell proliferation.

Goblet-Cell Maturation in the Colon Is Dependent on HNF4 α

H&E staining indicated a reduction in the number of goblet cells present in HNF4 α -null colons (Figure 2G and H). Alcian blue histochemistry, which identifies the presence of acidic mucins, was therefore used to differentiate between goblet cells and the rest of the epithelial cells of the colon. In control colons, alcian blue-positive mature goblet cells and accumulation of mucin within the gut lumen could easily be detected (Figure 5A). A pregoblet cell can be distinguished from a mature goblet cell by the grouping of a limited number of mucous granules into a small theca. As the pregoblet cell matures, the number of mucus granules increases and the theca expands to occupy a large portion of the cytoplasm, which gives the mature goblet cell its characteristic morphology.⁴⁸ Examination of control colons revealed that the majority of the alcian blue-positive cells were mature goblet cells (Figure 5A). However, few mature goblet cells could be identified in HNF4 α -null colons, which instead had an increase in the abundance of epithelial cells containing small clusters of mucin (Figure 5B). It seems most likely that these cells are pregoblet cells that have failed to mature, although it is formally possible that they represent colonocytes that ectopically express mucins. In support of the view that cells containing small clusters of alcian blue material are pregoblet cells, cell counting revealed that the total number of alcian blue-positive cells was similar but that the ratio of presumptive immature to mature goblet cells was reversed between control and HNF4 α -null colons (Figure 5C). The apparent block in goblet cell maturation was reminiscent of the phenotype associated with loss of the zinc finger transcription factor *Klf4*.¹⁴ However, RT-PCR analyses revealed that *Klf4* mRNA levels remained unchanged in HNF4 α -null colons (Figure 5D). In addition, levels of *Trefoil factor 3* mRNA (*Ttf3*, also called *Itf*), which is considered a marker of differentiated goblet cells,⁴⁹ was unchanged in colons lacking HNF4 α (data not shown).

The finding that HNF4 α -null goblet cells failed to accumulate normal amounts of mucin (Figure 5B) raised the possibility that the expression of genes encoding mucin core proteins could be disrupted in HNF4 α -null colons. The major mucins found in the intestine include *Muc1*, *Muc2*, *Muc3*, and *Muc4*.⁵⁰ The steady-state levels of mRNA encoding these proteins in control and HNF4 α -null colons were therefore compared by RT-PCR. The level of *Muc3* mRNA was reduced by 18-fold in HNF4 α -null livers compared with controls (Figure 5D). However, somewhat surprisingly, expression of *Muc2* was only minimally reduced (2-fold), and *Muc1* and *Muc4* were unaffected in the mutant colons (Figure 5D). *Muc2* is the major gel-forming mucin in goblet cells and mucin production is undetectable by alcian blue staining in *Muc2*^{-/-} colons; in contrast, *Muc1*, *3*, and *4* are primarily transmembrane glycoproteins.^{49,51} The fact that *Muc2* mRNA is detected in reasonable abundance in HNF4 α -null colons implies that the observed loss of goblet cell morphology likely reflects a block in maturation of mucous granules as opposed to a loss of expression of secretory mucin core protein genes.

HNF4 α Is Required for Expression of Several Genes That Contribute to Colon Function

Although at E18.5 the colonic epithelium is not fully mature, it does express gene products related to the function of the adult colon. This implies that any analyses of HNF4 α -null E18.5 colons should offer insight into the potential contribution of HNF4 α in regulating both colon development and adult colon function. Microarray expression profiling was therefore used to comprehensively define the requirement for HNF4 α in controlling colon

gene expression. Analyses were performed independently on 2 control and 3 HNF4 α -null colons and results were analyzed for fold changes by dChip. Genes whose expression exhibited a mean 2-fold or more differential expression between control and null colons are listed in Supplementary Table 1. By using these criteria, expression of 20 genes was predicted to have increased and 85 to have decreased. However, only 2 mRNAs had a statistically significant (Student *t* test, *P* < .05) increase of >3-fold in the mutant colons suggesting that, like in the liver, HNF4 α is predominantly an activator of gene expression. Analyses of the gene ontology distribution of genes whose expression was predicted to be reduced revealed that a wide range in physiological and cell biological processes was likely affected by loss of HNF4 α in the colon (Supplementary Figure 1; see supplemental material online at www.gastrojournal.org). These included genes encoding proteins with roles in various aspects of cell and physiological metabolism, transport, and localization, all of which are crucial functions of the colon. A statistical comparison of the distribution of down-regulated genes to the occurrence of gene categories in the genome using GOTree Machine⁵² found few gene ontology categories to be overrepresented (Supplementary Figure 1). This implies that HNF4 α action is not restricted to regulating a specific process but rather is required for widespread control of colonic gene expression.

Known genes whose expression was predicted to be reduced 3-fold by array analyses were considered further because, based on past experience, such changes are most often verified by RT-PCR. Figure 6 shows that of the genes analyzed 16 were confirmed to express reduced steady-state mRNA levels in mutant colons. In control colons, *aquaporin 4 (Aqp4)*; *ATPase H+/K+ transporting nongastric alpha polypeptide (Atp12a)*; *apolipoprotein C-II (ApoC2)*; *serum amyloid 1 and 2 (Saa1&2)*; *selenoprotein P, plasma, 1 (Sepp1)*; *solute carrier family 39 (zinc transporter); member 4 (Slc39a4)*; *aldolase B (Aldob)*; *cytochrome P450, family 2, subfamily d, polypeptide 26 (Cyp2d26)*; *glycine amidinotransferase (Gatm)*; *neuraminidase3 (Neu3)*; *sulfotransferase family 1B, member1 (Sult1b1)*; *claudin2 (Cldn2)*; *mucin and cadherin like (Mucdhl)*; *mucin3 (Muc3)*; and *extracellular link domain-containing 1 (Xlkd1)* mRNAs were easily detectable. However, in fetal colons lacking HNF4 α , the level of each of these mRNAs was reproducibly reduced. In general, the fold differences in mRNA levels predicted by analyses of the microarray data closely correlated with the changes determined by phosphoimager quantification of the RT-PCR products. Several of the genes with reduced expression encode proteins that have crucial roles in gut function, including ion and water transporters, metabolic proteins, and cell-adhesion molecules. The dependence of gene expression on HNF4 α did not appear to be the consequence of disrupting expression of colonic epithelial transcription factors in general because expression of *Klf4* (Figure 5), *Atoh1*, *Hes1*, *Elf3*, *Cdx1*, and *Cdx2* (data not shown) was unaffected. These data show that HNF4 α has an integral role in controlling the developmental expression of genes that define a functioning colonic epithelium.

HNF4 α Directly Interacts With Multiple Genes Whose Expression Is Reduced in HNF4 α -Null Colons

To determine whether the 16 genes whose expression was reduced in HNF4 α -null colons were direct targets of HNF4 α , genomic sequences lying -10Kb to +10Kb relative to the transcriptional start site (+1) of each gene were searched for the presence of any of 169 known HNF4 α -binding-sites³³ by using a Knuth-Morris-Pratt exact match algorithm.⁵³ Of the 16 genes studied, 8 were found to contain nucleotide sequences that matched known HNF4 α -binding sites (Supplementary Figure 2; see supplemental material online at www.gastrojournal.org). These sites were positioned both upstream and downstream of the transcriptional start site and included intronic sequences (Supplementary Figure 2). All sequences were confirmed to interact with HNF4 α by EMSA (Supplementary Figure 2). Although these data showed that the majority of the 16 down-regulated genes contained

HNF4 α -binding sites, it also seemed possible that previously uncharacterized HNF4 α -binding sites could be present within the genes as well. Therefore, we used a permuted Markov model that was recently developed to predict any potential HNF4 α -binding sites⁵⁴ to search the same -10Kb to +10Kb of genomic sequence encompassing the 16 potential target genes. By using this approach, 35 binding sites were identified in 14 genes with several genes containing multiple sites (Supplementary Table 2). Eleven of 14 genes contained potential HNF4 α -binding sites within their putative promoter regions (Figure 7A). Further studies were restricted to these 11 genes because they seemed most likely to be responsible for regulating expression by HNF4 α . The ability of HNF4 α to interact with the predicted binding sites was assessed by EMSA. Nuclear extracts from Cos-7 cells expressing exogenous HNF4 α yielded a single specific shift complex when incubated with a ³²P-labeled oligonucleotide containing a well-characterized HNF4 α -binding site (Figure 7B, compare lanes 1 and 2). As expected, a 150-fold molar excess of the same unlabeled oligonucleotide specifically competed the complex (Figure 7B, compare lanes 2 and 3), whereas control oligonucleotides lacking a predicted HNF4 α -binding site did not (Figure 7B, lane 17). Of the 14 putative HNF4 α -binding sites analyzed in a similar fashion, 13 were shown to compete the known site and therefore interact with HNF4 α . Together with the identification of the previously characterized sites, these analyses show that 13 of the 16 genes whose expression in the colon was dependent on HNF4 α -contained sequences that could interact with HNF4 α in vitro.

If any of the sites identified by EMSA (Supplementary Figure 2 and Figure 7) facilitated HNF4 α -controlled transcription of the associated gene, then it could be predicted that these sites would be occupied by HNF4 α in the colon. To test this prediction, ChIP assays were performed on individual colons isolated from fasted mice. Antibody specific to HNF4 α precipitated colonic chromatin encompassing the HNF4 α -binding sites in 7 of 13 genes tested but not from a control gene (*Hprt*) that had no HNF4 α -binding site (Figure 8). Only those reactions that yielded a positive result, with the exception of Muc3 BS2, which serves as an example of a negative result, are shown. The positive precipitations were specific for the anti-HNF4 α antibody because an unrelated antibody (anti-Pescadillo) failed to precipitate genomic DNA from any samples. Moreover, the anti-HNF4 α antibody did not precipitate any genomic sequences from samples of brain tissue that does not express HNF4 α . These studies show that in the colon HNF4 α interacts with several genes whose expression is dependent on HNF4 α implying that they are direct targets of HNF4 α transcriptional control.

Discussion

The results presented here show that the nuclear receptor HNF4 α makes a crucial contribution toward establishing a mature epithelium within the large intestine during embryonic development. Loss of HNF4 α in the epithelial tissue of the colon results in a block to formation of normal crypt topology, thinning of muscle cell layers, loss of vascular tissue, a reduction in the number of epithelial cells, and diminished maturation of goblet cells. However, although these disruptions to gut tissue morphology and cell differentiation are quite striking, many aspects of colon development occur normally including specification of all cell types, generation of all tissue tunics, and the conversion of the primitive gut tube from a pseudostratified epithelium to a simple columnar epithelium. The observation that most early processes in colon development occur normally suggests that the predominant requirement for HNF4 α is to maintain a differentiated gene expression profile rather than to control early patterning events or cell-fate choices. Molecular studies have revealed that HNF4 α regulates expression of several transcription factors in the liver, and disruption to this transcription factor network in HNF4 α -null livers results in a widespread dysregulation of hepatic gene expression.^{25,26} However, in contrast to the liver, our studies

using expression profiling and RT-PCR uncovered only minor alterations in the expression of gut transcription factors in HNF4 α -deficient colons. Transcription factors whose expression is unaffected by loss of HNF4 α include several with well-defined roles in controlling differentiation of intestinal epithelial cells such as those encoded by *Klf4*, *Atoh1*, *Elf3*, and *Cdx2*.^{11,14,15,55,56} Although our studies show that the transcription factor profile in the colon is relatively unaffected by loss of HNF4 α , we identified a down regulation in expression of many genes that encode proteins with important roles in colon and epithelial cell function. Importantly, EMSA and ChIP analyses showed that many of these genes are direct targets of HNF4 α . Cumulatively, these studies suggest that HNF4 α acts to control terminal differentiation of the colonic epithelial cells by controlling gene expression downstream of other transcription factors. In this regard, it will be interesting to determine whether other developmental gut transcription factors such as *Klf4*, *Atoh1*, and *Elf3* control expression of HNF4 α in the gut epithelium.

Of the genes analyzed whose expression in the colon is dependent on HNF4 α , we have identified 7 that contained HNF4 α -binding sites, are bound by HNF4 α in vivo, and are therefore likely to be direct targets of HNF4 α . However, it seems likely that this is an underestimation of the total number of HNF4 α target genes in the colon resulting from the relatively stringent criteria that were applied. For example, we limited our binding-site search to genomic sequences that lay within 10 kb of the gene. Of the novel HNF4 α -binding sites identified, we chose to study only those that were in putative promoter regions and those with a sequence close to the established HNF4 α -binding-site consensus.^{33,54} This means that genes with unconventional HNF4 α -binding sites or sites downstream of +1, of which there are many (Supplementary Table 2), were excluded by our analyses. Even with these limitations, however, our data show that HNF4 α is required for expression of a significant number of genes whose products contribute to gut function. This set of genes includes those encoding proteins involved in a diverse array of physiological and cellular functions implying that HNF4 α is not only essential for colon development but will also make an important contribution toward maintaining adult gut physiology.

The phenotype associated with loss of HNF4 α function in the colon is clearly complex. Although HNF4 α expression is restricted to the epithelial cells of the gut, analyses of *Hnf4a*^{loxP/loxP}*Foxa3Cre* embryos revealed, in addition to defects in development of the epithelium, abnormal formation of the colon's muscle and vascular tissues. The phenotype associated with nonepithelial cells most likely reflects a secondary consequence of disrupting HNF4 α in the epithelium. Cross-talk between the mesenchymal and epithelial layers of the gut has been well documented and signaling between mesenchymal and epithelial layers is required for differentiation, cell proliferation, and patterning of the gut tube.^{2,7,9} A large array of growth factors secreted from one intestinal tissue type have been shown to assert their effects on the other including Indian and Sonic hedgehog, FGFs, BMPs, Wnts, Notch, and PDGF.^{2,7,9} Indeed, *Ihh*^{-/-} embryos share characteristics of the phenotype associated with loss of HNF4 α in the colon including a distended gut and reduced thickness of the muscularis layers.⁴⁴ However, no changes in expression of specific growth factors in the absence of HNF4 α were detected, including expression of *Ihh* (Figure 3K). Nevertheless, we did find that embryos in which HNF4 α was deleted in a subset of epithelial cells displayed an apparently normal morphology, including the generation of crypts (Figure 2E). The formation of morphologically normal crypts and tissue tunics in such chimeric embryos is consistent with at least some aspects of the phenotype being non cell-autonomous. Analyses of the genes whose expression is most significantly affected by loss of HNF4 α does not give any clear indication of a single specific gene product that would result in the pleiotropic effects of losing HNF4 α in the colon. Mice lacking *Aqp4*, *Atp12a*, or *Sepp1* have been described, and although some have phenotypes that could be associated with colon dysfunction, none display characteristics that are similar to those

associated with *Hnf4a^{loxP/loxP}Foxa3Cre* embryos.^{57–59} Although it remains possible that the phenotype associated with loss of HNF4 α could be caused by reduced expression of a specific target gene, it seems more likely that the cumulative loss of multiple proteins whose expression is regulated by HNF4 α in the epithelial cells results in an environment that is unable to sustain normal colon development. Nevertheless, as more gene knockouts in HNF4 α targets become available, it is likely that subsets of defects caused by loss of HNF4 α in the colon will be identified.

In summary, we have shown that HNF4 α is a critical regulator of gene expression in the epithelial cells of the colon. Ablation of the *Hnf4a* gene solely in the epithelial cells disrupts many aspects of normal colon development affecting multiple tissue layers. Analyses of expression of other transcription factors with known roles in colon development in *Hnf4a^{loxP/loxP}Foxa3Cre* embryos suggest that HNF4 α is an endpoint mediator of colon epithelial cell gene expression. This notion is supported by the identification of several genes that are new targets of HNF4 α regulation in the colon. Based on this, we conclude that HNF4 α is not only necessary for colon development but is also likely to act as a crucial regulator of colon physiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

ANOVA	analysis of variance
E	embryonic day
HNF4α	hepatocyte nuclear factor 4 alpha
RT-PCR	reverse-transcription polymerase chain reaction

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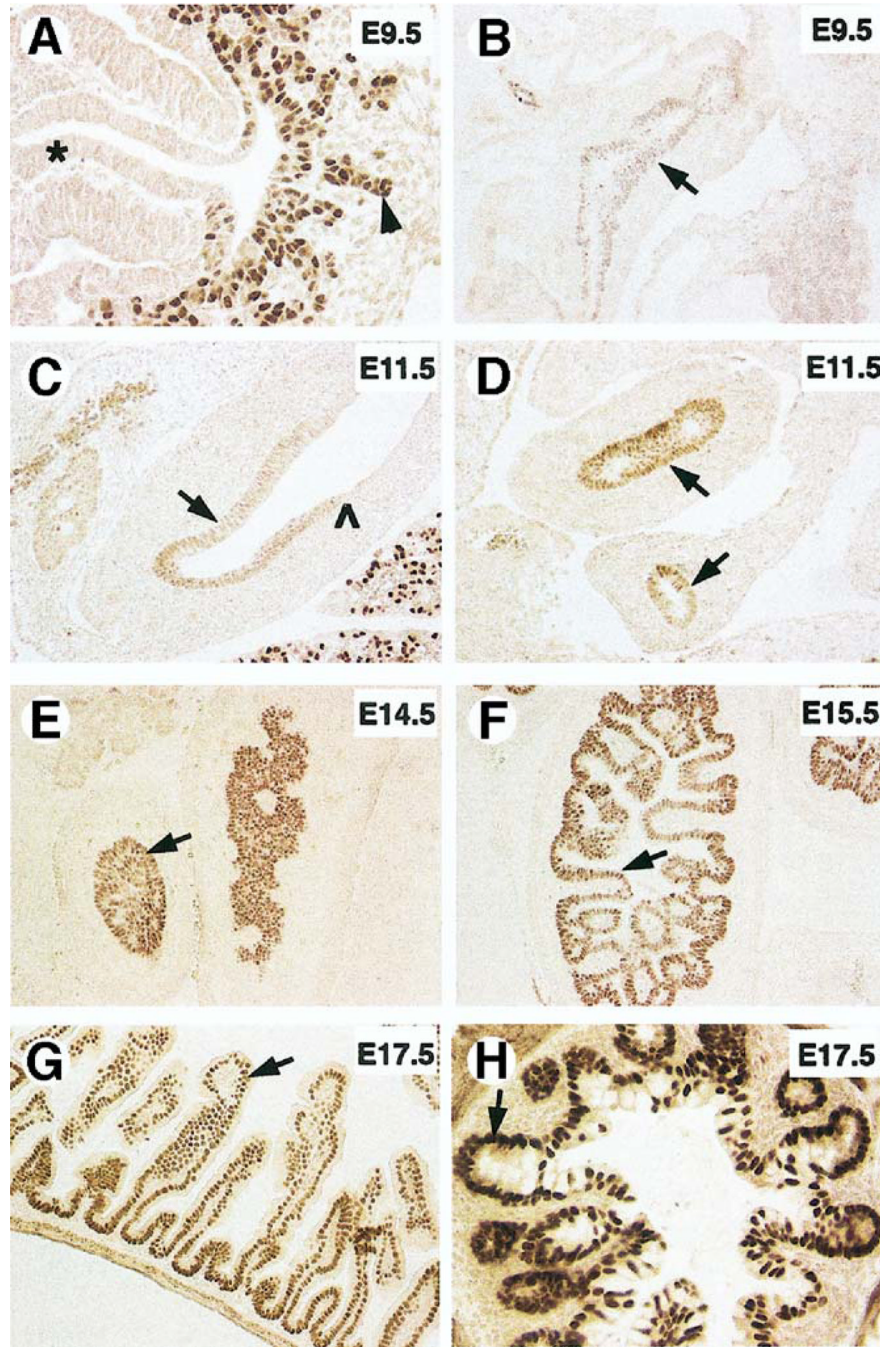


Figure 1. HNF4 α is expressed in the epithelium of the lower gastrointestinal tract during embryogenesis. Embryos were collected at E9.5 (A and B), E11.5 (C and D), E14.5 (E), E15.5 (F), and E17.5 (G and H) and transverse sections processed for immunohistochemistry using an anti-HNF4 α antibody. At E9.5, HNF4 α (brown nuclear staining) was identified in the hepatoblasts (A, arrowhead) as well as in the epithelium of the hindgut and midgut (B, arrow) but not in the foregut (A, *). There was a sharp rostral boundary of expression of HNF4 α within the stomach (C, ^). Caudal to this boundary, HNF4 α was restricted to the epithelium (C–H, arrows) of the gut throughout the remainder of development, including that of the small intestine (D–G) and colon (H).

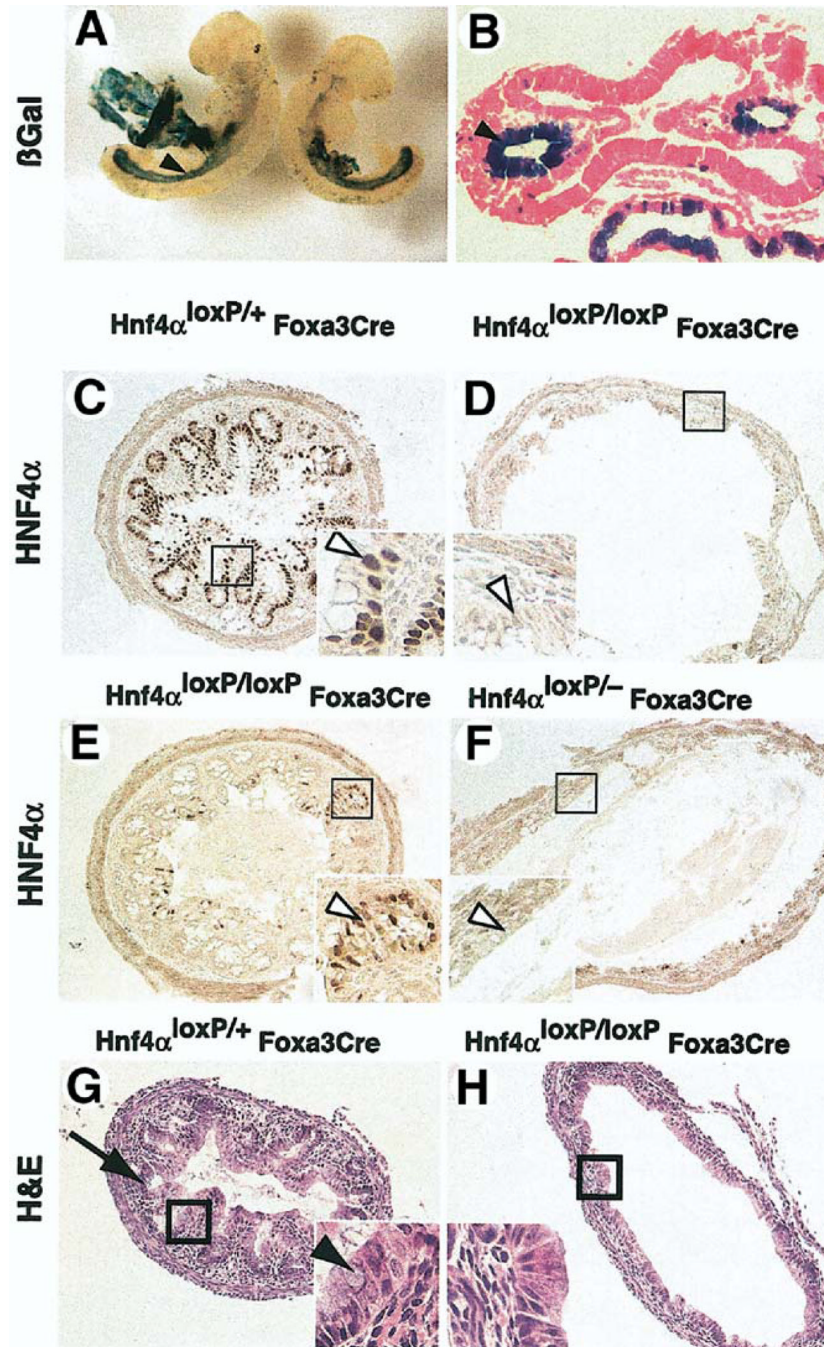


Figure 2. Loss of HNF4 α in the epithelial cells disrupts development of the colon. (A and B) *Foxa3cre* mice were bred to a line of transgenic mice, *Gt(ROSA)26Sor^{tm1Sho}*, that expresses β -galactosidase only after Cre-mediated deletion of loxP-flanked DNA sequences and resulting E9.5 embryos were stained for β -galactosidase expression. Expression of β -galactosidase was detected in the colon (arrowhead) of whole embryos (A) and sections (B) through these embryos revealed the expression to be restricted to the epithelial cells of the colon (arrowhead). (C–F) Micrographs of immunohistochemistry experiments using an anti-HNF4 α antibody. HNF4 α was identified in the nuclei (open arrowheads) of control (C, $Hnf4\alpha^{loxP/+} Foxa3Cre$) but was undetectable in a subset of experimental (D, $Hnf4\alpha^{loxP/loxP} Foxa3Cre$)

Hnf4a^{loxP/loxP}*Foxa3Cre*, *F*, *Hnf4a*^{loxP/-}*Foxa3Cre*) E18.5 colons. Some *Hnf4a*^{loxP/loxP}*Foxa3Cre* colons displayed a chimeric expression of HNF4 α (*E*). (*G* and *H*) H&E-stained sections of E18.5 embryos revealed the formation of crypts (*G*, *arrow*) and the presence of mature goblets cells (*G*, *arrowhead*) in control E18.5 colons, whereas crypt formation and goblet cell maturation was disrupted in colons lacking HNF4 α (*H*). Boxed areas are shown at a higher resolution in the inset.

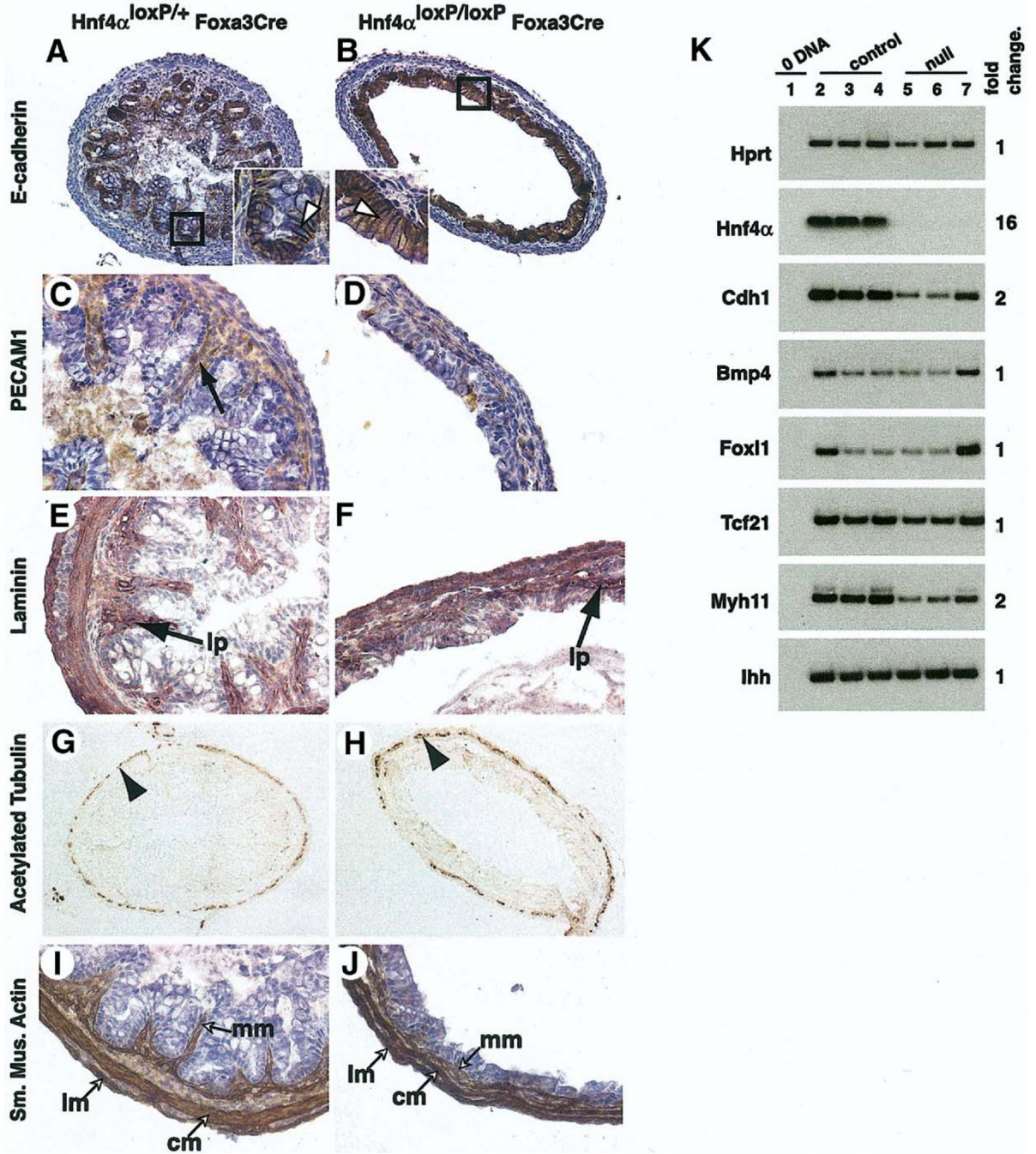


Figure 3.

HNF4 α is necessary for multiple aspects of colon development. (A–J) Micrographs showing results of immunohistochemistry (brown staining) performed on sections of control (A, C, E, G, I, *Hnf4 α ^{loxP/+}Foxa3Cre*) and mutant (B, D, F, H, J, *Hnf4 α ^{loxP/loxP}Foxa3Cre*) E18.5 embryos using antibodies that recognize E-cadherin (A and B), PECAM1 (C and D), laminin (E and F), neural acetylated tubulin (G and H), and α smooth-muscle actin (I and J). (K) RT-PCR analyses of mRNA isolated from *Hnf4 α ^{loxP/+}Foxa3Cre* (control, lanes 2–4) and *Hnf4 α ^{loxP/loxP}Foxa3Cre* (null, lanes 5–7) E18.5 colons using oligonucleotide pairs that amplified *Hnf4 α* , *Cdh1*, *Bmp4*, *Foxl1*, *Tcf21*, *Myh11*, and *Ihh* mRNAs. Amplification of *Hprt* mRNA was used as a loading control and reactions lacking input DNA (0 DNA, lane 1)

confirmed that amplicons originated from input RNA. Levels of amplicons were determined by phosphoimager analyses and were presented as an average fold difference (fold change) between control and mutant samples after normalizing to levels of the Hprt amplicon.

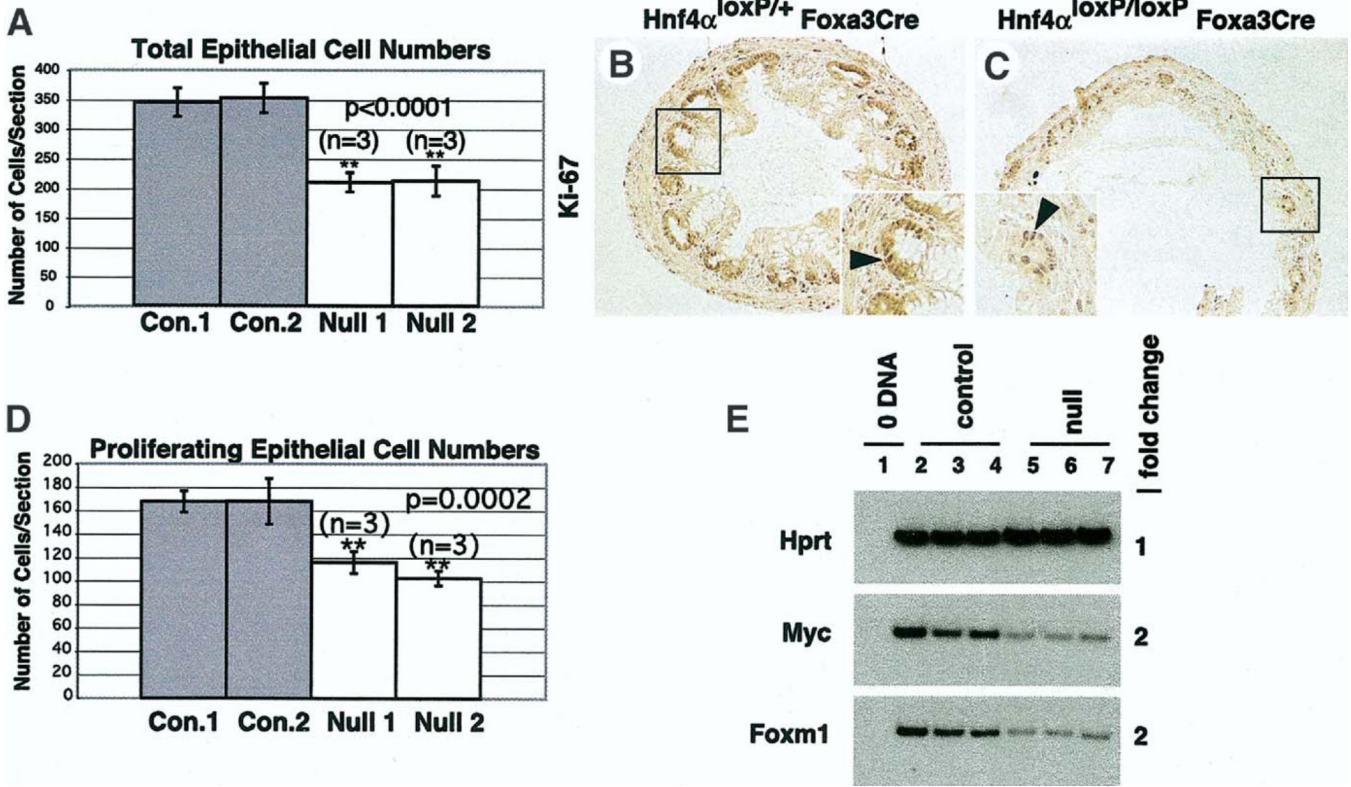


Figure 4. Epithelial cell proliferation is reduced in colons lacking HNF4 α . (A) Epithelial cells were counted in 3 H&E-stained colon sections from 2 *Hnf4a*^{loxP/+}*Foxa3Cre* (Con.1 and Con.2, shaded box) or 2 *Hnf4a*^{loxP/loxP}*Foxa3Cre* (Null 1, Null 2, open box) E18.5 embryos, and results are presented graphically. Statistical significance (**) was calculated by using ANOVA. (B and C) Proliferating cells were identified by using immunohistochemistry with anti-Ki-67 antibodies (brown staining, arrowhead) on control (B) and mutant (C) E18.5 colons. A high-resolution image of the boxed area is contained within inset. (D) Counts of proliferating cells were collected from 3 independent sections and are presented as in A. (E) RT-PCR analyses of mRNA isolated from *Hnf4a*^{loxP/+}*Foxa3Cre* (control, lanes 2–4) and *Hnf4a*^{loxP/loxP}*Foxa3Cre* (null, lanes 5–7) E18.5 colons using oligonucleotide pairs that amplified *Hprt* as a loading control, *Myc* and *Foxm1* mRNAs. 0 DNA (lane 1) was included to exclude the possibility of contaminating DNA. Average fold changes in amplicon levels between control and mutant colons are shown.

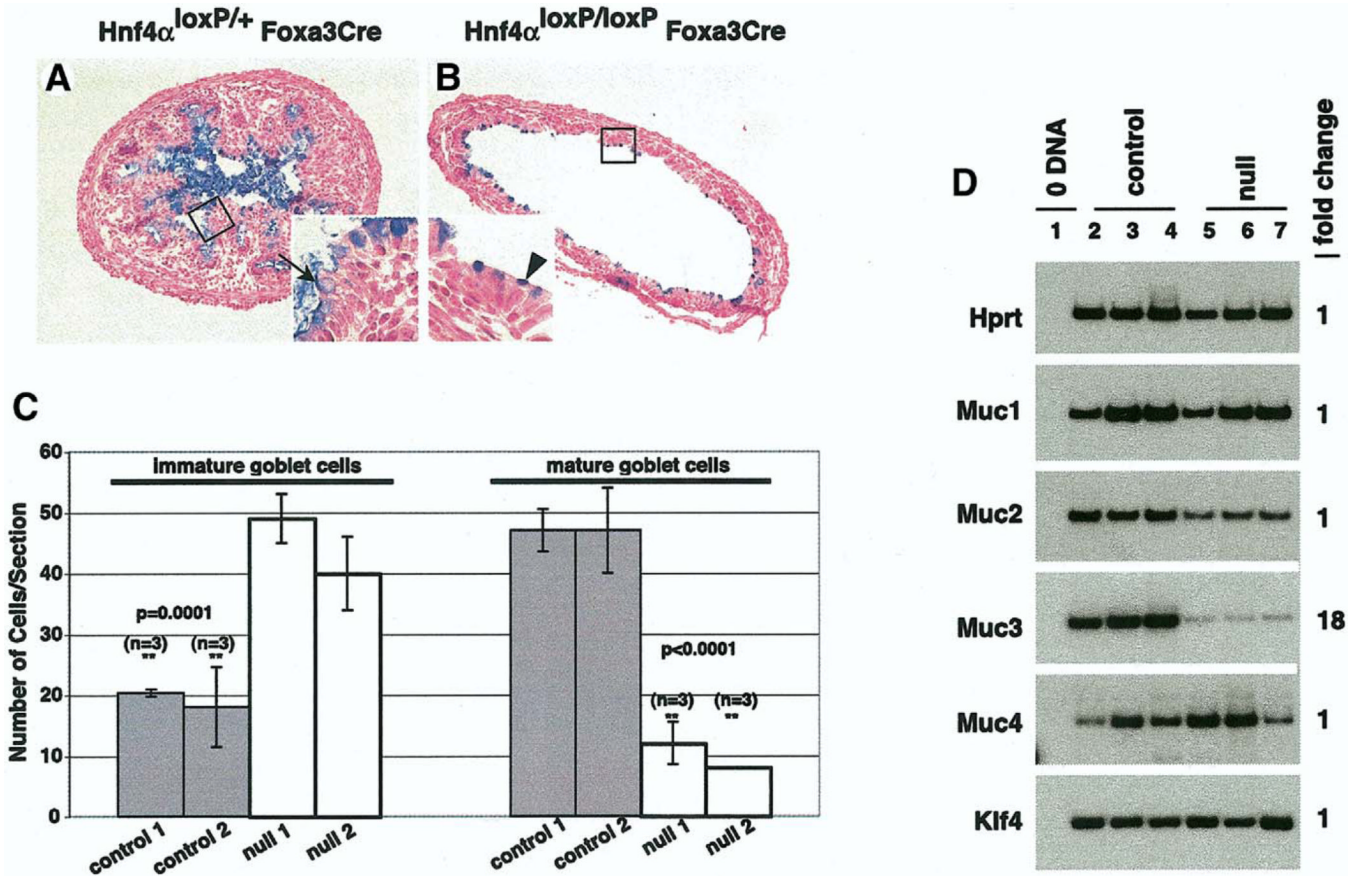


Figure 5. Goblet-cell maturation is blocked by the loss of HNF4 α . (*A* and *B*) Alcian blue histochemistry identified goblet cells in both control ($Hnf4\alpha^{loxP/+}Foxa3Cre$) and null ($Hnf4\alpha^{loxP/loxP}Foxa3Cre$) E18.5 colons (blue-stained cells). Insets contain high-resolution image of boxed regions showing that mature goblet cells (*arrow*) predominate in control colons, whereas immature goblet cells (*arrowhead*) are most abundant in HNF4 α -null colons. (*C*) The number of mature or immature goblet cells were counted in 3 sections from each of 2 $Hnf4\alpha^{loxP/+}Foxa3Cre$ (control 1, control 2; shaded boxes) and $Hnf4\alpha^{loxP/loxP}Foxa3Cre$ (null 1, null 2; open boxes) E18.5 colons and data are presented as a bar graph. Statistical significance (**) was calculated by using ANOVA. (*D*) RT-PCR analyses of mRNA isolated from $Hnf4\alpha^{loxP/+}Foxa3Cre$ (control, lanes 2–4) and $Hnf4\alpha^{loxP/loxP}Foxa3Cre$ (null, lanes 5–7) E18.5 colons using oligonucleotide pairs that amplified *Hprt* as a loading control; *mucins*-1, -2, -3, and -4; and *Klf4*. 0 DNA (lane 1) showed the absence of contaminating DNA, and average fold changes in amplicon levels between control and mutant colons are shown.

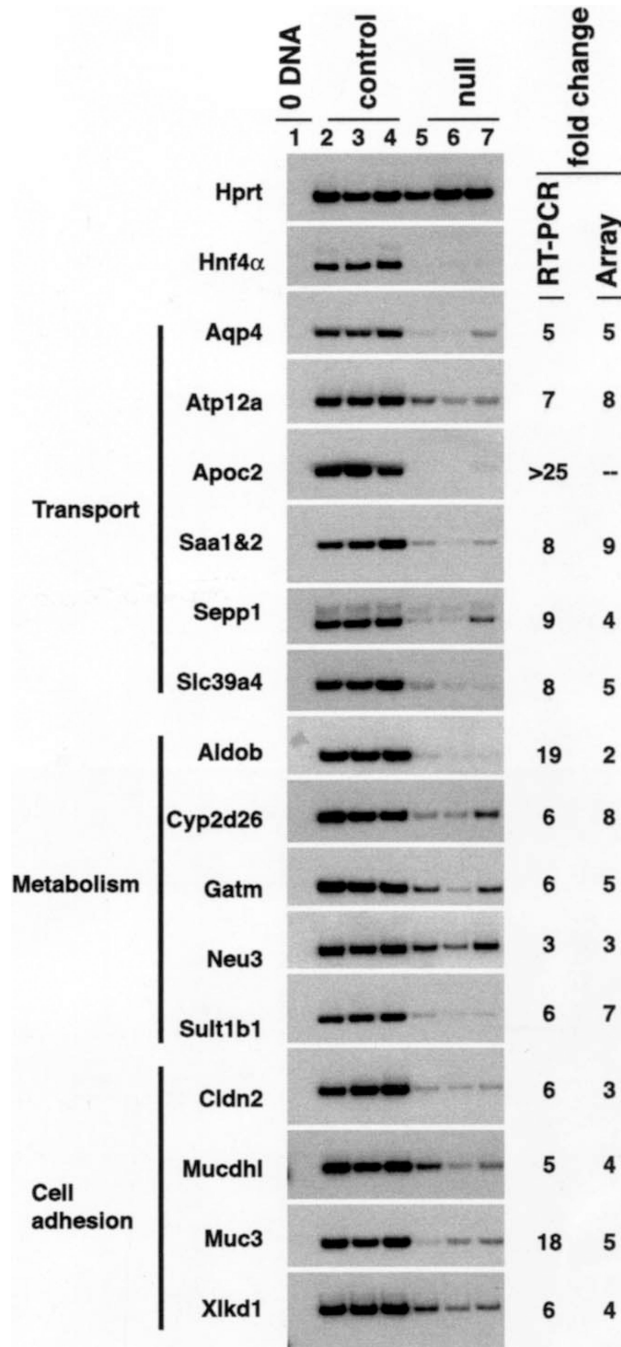


Figure 6.

Loss of HNF4α disrupts expression of multiple genes encoding proteins that contribute to colon function. RT-PCR analyses of mRNA isolated from *Hnf4a^{loxP/+}Foxa3Cre* (control, lanes 2–4) and *Hnf4a^{loxP/loxP}Foxa3Cre* (null, lanes 5–7) E18.5 colons using oligonucleotide pairs that amplified *Hprt* as a loading control; *Aqp4*, *Atp12a*, *Apoc2*, *Saa1* and *2*, *Sepp1*, and *Slc39a4*, which encode proteins with a variety of roles in transport; *Aldob*, *Cyp2d26*, *Gatm*, *Neu3*, and *Sult1b1* that encode proteins having diverse roles in metabolism; and *Cldn2*, *Mucdhl*, *Muc3*, and *Xlkd1* that encode proteins with various contributions to cell adhesion. 0 DNA (lane 1) confirmed the absence of contaminating DNA and average fold changes in

mRNA levels between control and mutant colons calculated from RT-PCR analysis or Affymetrix array analysis are shown.

A

Gene Symbol	Entrez ID	H4 Site	Location	Sequence
Aldob	230163	H4.170	-6969	agggtcaagggtta
Apoc2	11813	n/a	-539	agttcaggcccca
		H4.172	-122	aggccagagggtca
		H4.173	-2867	aggtcaagggtca
Aqp4	11829	H4.174	-7325	agagcaagggtct
Cldn2	12738	H4.175	-1996	agggtcagaggcct
Gatm	67092	H4.176	-6333	agggcaaggagca
Muc3	17832	H4.73	-2176	gggtcaagggtca
Mucdhl	72040	H4.177	-1439	ggttcaagggtca
Neu3	50877	H4.178	-241	ctagcaagggtca
Saa1*	20208	H4.179	-2349	gtaccaagggtcc
Saa2*	20209	H4.179	-6516	gtaccaagggtcc
Slc39a4	72027	H4.180	-8995	gggtccagggtca
		H4.181	-948	agggctagggtca

B

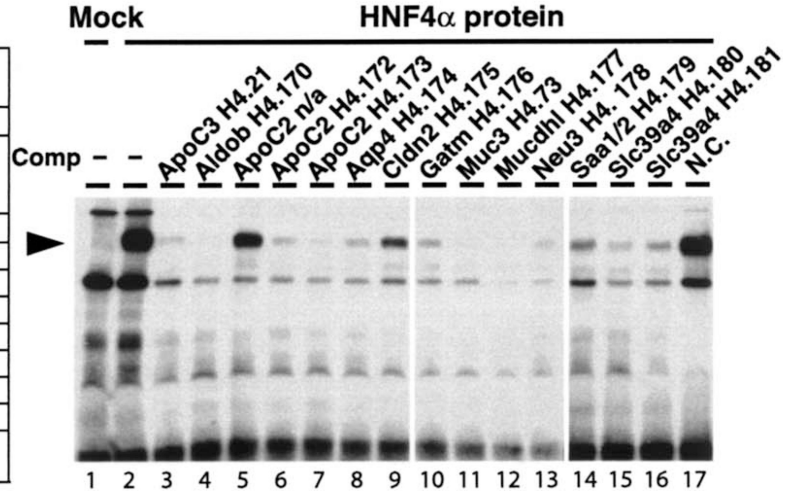


Figure 7.

HNF4 α -binding sites are present in genes whose expression is dependent on HNF4 α . (A) Table showing the position and sequence of putative HNF4 α -binding sites predicted to be present within genes whose expression is HNF4 α dependent. HNF4 α -binding site numbers (H4 sites) were assigned by using the criteria established by Yang et al.³³ (B) EMSA was performed by using an HNF4 α -binding site from the *Apoc3* promoter and extracts from Cos-7 cells transfected with a control plasmid (mock, lane 1) or a plasmid expressing exogenous HNF4 α (lanes 2–17). A specific shift caused by binding of HNF4 α to the *Apoc3* HNF4 α -binding site is indicated with an arrowhead. Inclusion of 150-fold molar excess of unlabeled oligonucleotides corresponding to HNF4 α -binding sites in either the *Apoc3* promoter (H4.21, lane 3) or binding sites predicted through computer modeling in Aldob (lane 4), *Apoc2* (lanes 6–7), *Aqp4* (lane 8), *Cldn2* (lane 9), *Gatm* (lane 10), *Muc3* (lane 11), *Mucdhl* (lane 12), *Neu3* (lane 13), *Saa1* and 2 (lane 14), and *Slc39a4* (lanes 15 and 16) all competed for binding of HNF4 α to the labeled *Apoc3* HNF4 α -binding site. One binding site (lane 5) in the *Apoc2* gene failed to compete, as did a negative control (N.C.) binding site for the transcription factor Foxa (lane 17).

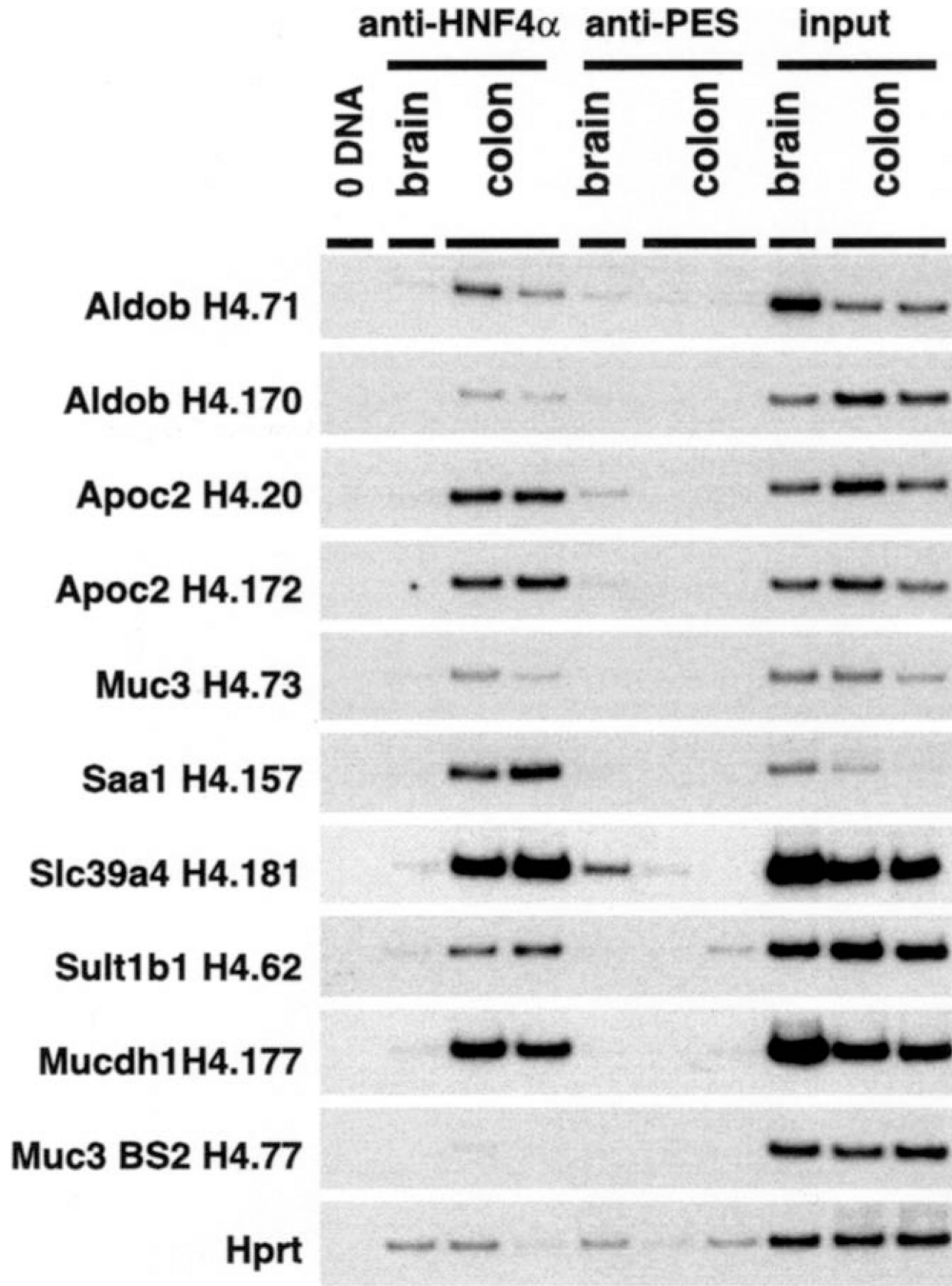


Figure 8. Genomic sequences within several genes whose expression is dependent on HNF4 α are occupied by HNF4 α within the colon. Chromatin immunoprecipitation was performed on 2 independent colons and a brain sample, a negative control tissue that does not express HNF4 α , by using antibodies that precipitated either HNF4 α or an unrelated protein, PES1. DNA sequences that coprecipitated with these proteins were identified by PCR by using primers that flanked the HNF4 α -binding sites within *Aldob*, *Apoc2*, *Muc3*, *Saa1*, *Slc39a4*, *Sult1b*, and *Mucdh1*. PCR of the *Hprt* gene or the HNF4 α -binding site 2 of the *Muc3* gene (*Muc3* H4.77) failed to show any enrichment in the colon, confirming that DNA sequences

that were precipitated with anti-HNF4 α were specific and reflected a bona fide interaction with HNF4 α in the colon.