Weanling, but not adult, rabbit colon absorbs bile acids: flux is linked to expression of putative bile acid transporters

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Weanling, but not adult, rabbit colon absorbs bile acids: flux is linked to expression of putative bile acid transporters. Am J Physiol Gastrointest Liver Physiol 290: G439–G450, 2006. First published September 15, 2005; doi:10.1152/ajpgi.00163.2005.—Intestinal handling of bile acids is age dependent; adult, but not newborn, ileum absorbs bile acids, and adult, but not weanling or newborn, distal colon secretes Cl− in response to bile acids. Bile acid transport involving the apical Na+-dependent bile acid transporter (Asbt) and lipid-binding protein (LBP) is well characterized in the ileum, but little is known about colonic bile acid transport. We investigated colonic bile acid transport and the nature of the underlying transporters and receptors. Colon from adult, weanling, and newborn rabbits was screened by semiquantitative RT-PCR for Asbt, its truncated variant t-Asbt, LBP, multidrug resistance-associated protein 3, organic solute transporter-α, and farnesoid X receptor. Asbt and LBP showed maximal expression in weanling and significantly less expression in adult and newborn rabbits. The ileum, but not the colon, expressed t-Asbt. Asbt, LBP, and farnesoid X receptor mRNA expression in weanling colon parallel the profile in adult ileum, a tissue designed for high bile acid absorption. To examine their functional role, transepithelial [3H]taurocholate transport was measured in weanling and adult colon and ileum. Under short-circuit conditions, weanling colon and ileum and adult ileum showed net bile acid absorption: 1.23 ± 0.62, 5.53 ± 1.20, and 11.41 ± 3.45 nmol·cm−2·h−1, respectively. However, adult colon secreted bile acids (~1.39 ± 0.47 nmol·cm−2·h−1). We demonstrate for the first time that weanling, but not adult, distal colon shows net bile acid absorption. Thus, increased expression of Asbt and LBP in weanling colon, which is associated with parallel increases in taurocholate absorption, has relevance in enterohepatic conservation of bile acids when ileal bile acid recycling is not fully developed.

Bile acids are amphipathic steroid molecules that are essential for the solubilization and absorption of lipids and fat-soluble vitamins. In humans, ≥95% of the bile acids are recycled to the liver via the enterohepatic circulation, chiefly by active reabsorption in the ileum (52). Thus, under physiological conditions, only ∼5% of bile acids enter the colon. Active bile acid transport in the ileum is developmentally regulated: it is absent in the newborn and increases in the weanling and adult animal (13, 21–23, 37). However, the colon plays an important role in bile acid metabolism. Intestinal bacteria, especially in the colon, deconjugate bile acids and dehydroxylate primary bile acids at position 7 to form secondary bile acids (25). In certain pathophysiological states, such as irritable bowel syndrome, Crohn’s disease, and surgical ileal resection, active bile acid absorption in the ileum is compromised (26, 52). Under these conditions, more bile acids enter the colon, where dihydroxy bile acids, such as chenodeoxycholic acid and deoxycholic acid, stimulate net electrolyte and fluid secretion, resulting in diarrhea (8). In vitro studies have demonstrated that, in human colonic cell lines (15, 16) and in rabbit (44) or rat (41) colonic mucosa mounted in Ussing chambers, taurodeoxycholate (TDC) activates Cl− secretion. Interestingly, this action of TDC is age specific in rabbits: it affects only the adult distal colon and has no effect in newborns (44). These findings were extended by our previous investigations showing TDC- and taurochenodeoxycholate-activated Cl− flux in isolated adult colonocytes but not in colonocytes from newborn (7–10 day old) or weanling (~4 wk old) rabbits (14, 56). Although bile acid transport and the underlying transporters and receptors in the small intestine and liver are well characterized, there are only a few studies in the mammalian colon. For example, by sampling portal blood, Fujii et al. (17) showed a small, but measurable, absorption of taurocholate (TC) from the colon of adult mongrel dogs.

In the ileum, the secondary active Na+-dependent bile acid transporter (Asbt/iASBT) is localized to the apical membrane and transports conjugated primary and secondary bile acids with high affinity (10). Asbt is also expressed in the kidney and in cholangiocytes (10, 33, 59). Although Na+-dependent organic anion transporter 3 (Oatp3) is not as prominent as ileal Asbt, transport of bile acids by Oatp3 in the jejunum has been reported (7, 19, 57). Bile acids also interact with cytosolic binding proteins and members of the nuclear receptor superfamily in liver and small intestine. In enterocytes, a 14-kDa cytosolic protein, termed ileal bile acid-binding protein (iBABP) in most species but named lipid-binding protein (LBP) in rabbits, has been identified (31, 51); here, the term LBP is used for rabbits and iBABP for other species. Preand postnatal expression of Asbt and iBABP/LBP has been examined in a few species. In the rabbit, uptake of radioactive bile acids into the ileum increases from weanlings (6 wk old) to adults (1 yr old) (53). Postnatally, expression of Asbt and iBABP/LBP

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shows dramatic increases at around the time of weaning. For
example, Asbt mRNA first appears at postnatal day 16 in rat ileum, followed by protein expression by day 21 (47), with a
parallel, steep increase of iBABP mRNA expression from day 16 to day 22 (27). On the basis of photoaffinity labeling and
target size analysis of rabbit ileal proteins, it has been sug-
gested that a complex of four iBABP molecules and two Asbt
dimers play a critical role in transcellular bile salt movements
(29–32). The coexpression of Asbt and iBABP during postna-
tal development, as well as their parallel expression in large
collangiocytes (4), supports the hypothesis that Asbt and
iBABP are essential for ileal bile acid uptake. In contrast to its
absence in the early postnatal stage, Asbt expression is high in
the rat and mouse in the late fetal stage (47). A similar biphasic
developmental pattern is seen with respect to iBABP in the
mouse; however, in the rat, iBABP expression is not seen until
the postnatal stage, i.e., it is monophasic. Because iBABP is
absent in the rat during late fetal development when Asbt is
present (47) and iBABP is absent in other tissues possessing
Asbt, such as renal tissues (18), the hypothesis that iBABP is
always essential for transcellular bile acid transport is ques-
tionable.

Once transported into the cell, bile acids elicit their regula-
tory effects via their interaction with members of the nuclear
receptor superfamily. In the ileum, bile acids act via the
farnesoid X receptor (FXR) to increase expression of iBABP/
LBP (55) and decrease expression of Asbt in mice, rabbits, and
the human colonic cell line Caco-2 (8, 36). The influence of
FXR on expression of the basolateral membrane (BLM) trans-
porters is unknown. Four isoforms of FXR, FXR-α1, FXR-α2,
FXR-β1, and FXR-β2, have been described in mice, with the
intestines showing very low expression of FXR-α (62).
FXR-β1 and FXR-β2 differ by an insertion of four amino acids
(MYTG) between the DNA- and ligand-binding domains of
FXR-β2, and it has been postulated that activation of FXR
target genes may depend on the ratio of FXR-β2 to FXR-β1
(62). Bile acids also bind to and activate the pregnane X
receptor and the vitamin D3 receptor (VDR). When activated,
VDR and pregnane X receptor have largely been shown to
upregulate pathways that detoxify bile acids (38, 50). Prelimi-
ary studies imply a role for VDR in regulation of Asbt and
LBP (9).

In contrast to the well-characterized Asbt mechanism on the
apical membrane, the mechanism by which bile acids exit the
BLM of the ileal enterocytes is not fully understood. In
addition, whether expression of the putative basolateral trans-
porters is age dependent is not known. Functionally, exit via an
anion exchanger has been shown in rat small intestinal BLM
vesicles (58). In 2000, Lazaridis et al. (34) provided evidence
that the heteromeric organic solute transporter-α (Ost-α)-Ost-β may be the basolateral transporter in mouse ileum.

Although age-related changes in bile acid absorption and
Asbt and iBABP/LBP expression have been well documented in
the small intestine (5, 13, 27, 35, 37, 42, 47), little is known
about these processes in the developing distal colon, despite
the relevance of bile acids to colon physiology. In the present
study, we used adult (>6 mo old), weanling (4–8 wk old), and
newborn (5–8 day old) rabbits as a model to examine age-
related differences in bile acid transport and expression of
iBABP/LBP in mammalian colon. In rabbits, weaning occurs
at around day 21 postpartum, and the animals were used 4–7
days later. We report significant age-related differences in
mRNA expression of bile acid transporters/receptors and dem-
onstrate parallel changes in function determined by transpi-
ethelial fluxes. In particular, mRNA expression of Asbt, LBP,
and FXR in weanling colon parallels the profile found in adult
ileum, a tissue designed for high bile acid flow. We demon-
strate for the first time that weanling, but not adult, distal colon
shows net bile acid absorption. We postulate that weanling
colon has a salvage system to preserve the loss of valuable bile
acids during this critical stage of intestinal development.

**MATERIALS AND METHODS**

**Materials.** TRIzol reagent, SuperScript II RNase H− reverse transcriptase, oligo(dT)12–18 primer, RNaseOUT, and recombinant RNase inhibitor were obtained from Invitrogen (Carlsbad, CA); First Choice RLM-RACE (rapid amplification of cDNA ends) kit and RNA-Later from Ambion (Austin, TX); RedTaq polymerase, 10 mM dNTP mix, DNase kits, and protease inhibitor cocktail from Sigma (St. Louis, MO); oligonucleotide primers from Sigma Genosys (St. Louis, MO); polyclonal anti-Mrp3 antibody from Santa Cruz Biotechnology (Santa Cruz, CA); donkey anti-goat secondary antibody from Chemi-
con (Temecula, CA); SuperSignal West Pico Chemiluminescent Sub-
strate Kit from Pierce (Rockford, IL); 3H-labeled TC (2 μCi/μg) from Perkin-Elmer (Boston, MA); and scintillation cocktail from JT Baker (Phillipsburg, NJ). All other reagents were of analytic grade and were purchased from Sigma Chemical.

**Animals and tissue procurement.** New Zealand White adult (>6 mo old), weanling (25–28 day old; weaned at day 21), and newborn (5–8 day old) rabbits of either sex were purchased from New Franken Rabbity (New Franken, WI) and housed at the institutional Biologic Resources Laboratory according to guidelines of the American Asso-
ciation for Accreditation of Laboratory Animal Care. Animal proto-
cols were approved by the Institutional Animal Care Committee.

Rabbits were anesthetized with ketamine (10 mg/kg) and xylazine
(2.5 mg/kg) given intramuscularly and then euthanized by pentobar-
bital sodium overdose (75 mg/kg iv). Distal colon from the anal verge
to the splenic flexure and distal ileum were excised and flushed with
ice-cold (4°C) lactated Ringer solution containing 5% glucose (LRG)
and opened along the mesenteric border. Tissues were rinsed in LRG,
and the mucosal surface was gently blotted to remove mucus and
maintained in oxygenated LRG. For RNA isolation and Western blot
analyses, mucosae were separated from underlying muscles by me-
chanical scraping. Tissues for RNA isolation were placed in RNA-
Later and stored at −80°C until use. For flux studies, muscle layers of
the ileum and distal colon were stripped using curved forceps to avoid
mechanical damage to the epithelium.

**Semiquantitative RT-PCR.** Total RNA was isolated using TRIzol reagent, and contaminating DNA was eliminated using a DNase kit according to the manufacturer’s instructions. Total RNA was quanti-
tated photometrically at 260 nm. Before the RT reaction, DNase-
purified RNA was checked for purity in a high (i.e., 36) cycle PCR
using a primer pair designed to amplify GAPDH (see Table 1 in
supplemental data for this article at http://ajpgi.physiology.org/cgi/
content/full/00163.2005/DC1). Only RNA samples that showed no
amplification products were used in subsequent RT assays, which
employed 2 \( \mu \)g of total RNA and Superscript II reverse transcriptase.

For each primer pair (see Table 1 in supplemental online data),
optimal PCR amplification conditions were determined employing a
hot-start protocol and RedTaq polymerase. In all PCR assays com-
paring mRNA expression levels of different age groups or tissues,
equal amounts of template (RT product) were employed. To verify
amplification of the desired target gene, PCR products were se-
quenced at the Marine DNA Sequencing Center (Mount Desert Island
Biological Laboratory, Salisbury Cove, ME). For semiquantitative
RT-PCR, the sampled PCR product strictly depended on the abun-
dance of the target cDNA, and cycle numbers were chosen in which
the PCR ran in the exponential phase. Thus, for each set of primers,
the PCR using RT products from the three age groups were run for
different cycles, and the optimal cycle number in the exponential
phase was determined. For GAPDH, this was 24 cycles (cycle num-
bers for the other target genes are provided in Figs. 1–3 and 5; see
representative diagram in Fig. 1 addendum in supplemental online
data). PCR products (12 \( \mu l \)) were separated on 1–2% agarose gels
and visualized by ethidium bromide staining. The band densities were
determined under UV light by digital analysis using a Kodak Digital
Science DC 120 Zoom digital camera and Kodak Digital Science ID
2.0.2 software (Eastman Kodak, Rochester, NY). For each PCR assay,
a parallel PCR was performed employing GAPDH primers (see Table
1 in supplemental online data) at an annealing temperature of 55°C
and 24 PCR cycles. GAPDH is equally expressed in rabbit intestinal
tissues of all age groups (see Figs. 1–5) and served as an internal
control.

**Cloning of rabbit Mrp3, Ost-\( \alpha \), t-Asbt, and FXR.** Rabbit Mrp3,
Ost-\( \alpha \), t-Asbt, and FXR have not been cloned; therefore, we used the
following strategy to identify these rabbit transcripts. Degenerate
primers, based on conserved regions of published sequences of the
cDNA of interest (see Table 1 in supplemental online data), were
designed and used to amplify the rabbit transcript by PCR. Products
of the predicted size were gel purified, sequenced, and subjected to
Basic Local Alignment Search Tool (BLAST) searches to determine
homology with known transcripts. This sequence was then used to
design specific primers for use in semiquantitative PCR to compare
different age groups and to obtain the entire open reading frame
(ORF) of t-Asbt and FXR with use of the RACE technique (Ambion).

**Protein preparation and Western blotting.** With the use of previ-
ously published methods (56), ileum from adult animals and distal
colonic mucosal epithelia from adult, weaning, and newborn animals
were homogenized in a buffer containing (in mM) 1 EDTA, 2 MgCl2,
5 \( \beta \)-mercaptoethanol, 1 DTT, and 25 Tris-HCl, pH 7.4, and protease
inhibitor cocktail. The homogenates were further processed by soni-
washed in Tris-buffered saline (TBST: 50 mM Tris, pH 7.4, 150
mM NaCl, and 0.05% Tween 20) for 10 min and then blocked with
Blotto (5% Carnation nonfat dry milk in TBST) for 1 h at room
temperature. The blots were incubated with primary polyclonal goat
anti-human MRP3 antibodies generated against a peptide correspond-
ing to the COOH-terminal region of human MRP3 (Santa Cruz
Biotechnology). The antibodies were used at a dilution of 1:200 in
Blotto overnight at 4°C on a shaker. After incubation with the primary
antibody, the blots were washed three times for 10 min each in TBST
and then incubated with donkey anti-goat secondary antibodies con-
jugated to horseradish peroxidase in Blotto at room temperature for
1 h. The blots were washed three times for 15 min each in TBST, and
horseradish peroxidase was visualized using a SuperSignal West Pico
Chemiluminescent Substrate Kit.

**Measurement of transepithelial TC fluxes.** Distal colonic and distal
ileal epithelia were dissected from the underlying muscle layers and
mounted in 0.33-cm\(^2\) Ussing chambers (Physiological Instruments,
San Diego, CA). The tissues were bathed in HCO\(_3\)-Ringer buffer (buffer
\( \alpha \): 4 ml/chamber) containing (in mM) 141.8 Na\(^+\), 125.6 Cl\(^-\), 3.0
HPO\(_4\)\(^2-\) + H\(_2\)PO\(_4\)\(^-\), 1.2 Ca\(^2+\), 1.2 Mg\(^2+\), 5.4 K\(^+\), and 21.0 HCO\(_3\)\(^-\)
(pH 7.4). The tissues were oxygenated with 95% O\(_2\)-5% CO\(_2\) and main-
tained at 37°C. The mucosal bath solution contained 10 mM mannitol
and the serosal bath solution contained 10 mM glucose to circumvent the
compounding effects of glucose-stimulated short-circuit current (\(I_{sc}\))
in the ileum. Transmural \(I_{m} \) (mA/cm\(^2\)) was measured using an auto-
matic voltage-clamp apparatus (model VCC-MC6, Physiologic In-
struments). The electrode offset and the fluid resistance were compen-
sated between the potential-monitoring electrode bridges. The po-
tential difference (mV) was measured via a pair of calomel half-
cells connected to agar-salt bridges (3% agarose and 3 M KCl),
whereas the tissue was short-circuited via a pair of Ag-AgCl elec-
trodes that connected to separate agar-salt bridges. All experiments
were conducted under short-circuit conditions. Total tissue conduc-
tance (\(G_{t} \), mS/cm\(^2\)) and tissue resistance (\(R_{t} \), \(\Omega\)-cm\(^2\)) were obtained by
induction of a 5-mV bipolar pulse at 10-s intervals and calculated
by Ohm’s law.

\(^3\)H-labeled TC was used to measure unidirectional and net tran-
sepithelial bile acid fluxes in paired tissues from the same animal with
comparable \(R_{t} \) (±25%). From each rabbit, two pairs of distal colonic
tissue were studied, and the values were averaged to yield \(n = 1\). A
single pair of distal ileal tissue from the same animal was studied in
parallel. After stabilization of electrical parameters in each tissue pair
(−30 min after mounting), 100 \( \mu \)M \(^3\)H-labeled TC (1 \( \mu \)Ci) was added
to the mucosal or serosal side for measurement of mucosal-to-serosal
(\(J_{m} \rightarrow \)s) or serosal-to-mucosal (\(J_{s} \rightarrow \)m) flux. Immediately after radio-
isojoin addition, 10 \( \mu \)l were sampled from the radioactive side to
determine the specific activity. TC fluxes were determined from a
400-\( \mu \)l sample obtained from the nonradioactive side every 30 min
beginning with TC addition (time 0) and continuing for 3 h. The
samples were replaced with another 400 \( \mu \)l of fresh nonradioactive
buffer \( \alpha \), and this dilution was factored into the calculations. At
the end of the experiment, viability of the tissues was tested by determi-
nation of the \(I_{m} \) response to mucosal addition of 10 mM glucose to the
ileum or serosal addition of 10 \( \mu \)M forskolin to the distal colon.
Scintillation fluid (10 ml) was added to all samples, and radioactivity
was assessed in a Tri-Carb liquid scintillation analyzer (Perkin-
Elmer). TC fluxes are expressed as nanomoles per square centimeter
per hour, and net flux (\(J_{m} \rightarrow \)s) is the difference between \(J_{m} \rightarrow \)s and
\(J_{s} \rightarrow \)m (see Figs. 8–10).

**Statistics.** Each \( n \) value depicts a separate animal. If more than one
sample was tested from one animal, the values were averaged to
represent \( n = 1 \). Values are means ± SE. Independent \( t \)-test and one-
way ANOVA (for \( >2 \) means) followed by the least-significant
difference were used to evaluate statistical significance. For \(I_{m} \) and
\(G_{t} \) significance was determined by least-squares regression analysis
and a one-sample \( t \)-test. \( P \leq 0.05 \) was considered significant.

**RESULTS**

**Asbt.** To examine Asbt expression in rabbit colon, specific primers (RbAsbtF/RbAsbtR; see Table 1 in supplemental on-
line data) were employed in semiquantitative RT-PCR. The antisense primer corresponded to exon 2, a region unique to the Asbt sequence and not found in t-Asbt. Expression of colonic Asbt mRNA varied with age: it was highest in the weanling, lower in the newborn, and below detection in the adult colon (Fig. 1A). Although mean values for the newborn appeared to be >0, these values, as assessed by a one-sample t-test, were not different from 0 (see DISCUSSION). In contrast, adult ileum showed significantly higher Asbt mRNA abundance than weanling ileum, and Asbt was not detectable in the newborn at 28 cycles (Fig. 1B). At all ages, 36 cycles were needed for amplification in the linear phase for colonic samples, whereas only 28 cycles were needed for ileal samples.

**LBP.** Because expression of iBABP/LBP closely corresponds with that of Asbt in developing rat ileum (27, 47), we evaluated mRNA expression of LBP in developing rabbit distal colon. Although weanling distal colon showed a robust expression of LBP at 28 cycles, equivalent amounts of RNA in adult and newborn distal colon showed no detectable expression of LBP (Fig. 2A). At 32 cycles, LBP was detectable in the adult, but not in the newborn (data not shown). Expression of LBP mRNA was greater in the ileum than in the colon, with only 18 cycles needed to obtain a strong amplification signal for adult and weanling animals. Differences in expression between adult and weanling ileum were not statistically significant. LBP was not detectable in newborn ileum at 18 cycles (Fig. 2B).

**FXR.** Because FXR has been shown to regulate expression of mouse and rabbit ileal Asbt and iBABP/LBP mRNA, we examined mRNA expression of FXR in newborn, weanling, and adult rabbit distal colon and adult ileum. Rabbit FXR has not been cloned, so we used the RACE technique (see MATERIALS AND METHODS) to obtain the full-length cDNA rabbit FXR sequence. Amplification of the 3' untranslated region (UTR) employing the primer combination RbFXRF2 (see Table 1 in supplemental online data) and the inner 3'-RACE primer (provided by the kit) gave two distinct cDNA fragments (data...
not shown). The cDNA sequences of these forms contained identical ORFs coding for proteins of 482 amino acids and differed only with respect to position of the poly(A) tail at nucleotide 1824 in the longer isoform, 3\textsuperscript{\textit{H}11032}-UTR isoform \textit{L} (GenBank Accession No. AF497630), and at nucleotide 1717 in the shorter isoform, 3\textsuperscript{\textit{H}11032}-UTR isoform \textit{S} (GenBank Accession No. AF497631). The deduced amino acid sequence for rabbit FXR was 94\% identical with human FXR-\textit{H9252} (GenBank Accession No. AAM53550) and 90\% identical to golden hamster FXR-\textit{H9252} (GenBank Accession No. AAM53548).

Semiquantitative RT-PCR employing the primer pair RbFXRFA/RbFXRRA (pan FXR primers; see Table 1 in supplemental online data), designed to amplify a 267-bp sequence of all FXR isoforms, showed an age-dependent decline in FXR mRNA expression in distal colon, with maximal expression in the newborn (Fig. 3). In addition, expression was 2.55-fold greater in adult ileum than in adult distal colon (1.18 ± 0.11 vs. 0.46 ± 0.17 relative units) but less than in weaning and newborn colon (Fig. 3).

Recent evidence (62) suggests that higher expression of the ratio of FXR-\textit{\beta}_2 to FXR-\textit{\beta}_1 may be important in regulating the activation of target genes, such as LBP, and may parallel LBP expression. Using specific FXR-\textit{\beta}_2 and FXR-\textit{\beta}_1 primers, we determined that FXR-\textit{\beta}_1 is the dominant intestinal isoform; however, the ratio of FXR-\textit{\beta}_2 to FXR-\textit{\beta}_1 expression did not parallel LBP expression. Thus ratios of FXR-\textit{\beta}_2 to FXR-\textit{\beta}_1 were lower in adult ileum than in adult distal colon (data not shown).

t-Asbt. To determine whether rabbit ileum and colon possess the putative basolateral bile acid transporter t-Asbt, we designed primers based on the rat sequence (34). Exon 2 was missing in t-Asbt, and there was a frame-shift event at the splicing site. Primer pairs (Rbt-AsbtF/Rbt-AsbtR; see Table 1 in supplemental online data) specific for the rabbit were designed to span the putative splicing site for t-Asbt, such that, if present, Asbt (302 bp) and t-Asbt (183 bp) could be detected in the same PCR. A band corresponding to the predicted size of t-Asbt product (183 bp) was found in adult rabbit ileum but not adult or weanling distal colon. As reported for the rat (34), high (i.e., 40) cycles of PCR amplification (Fig. 4A) were needed to detect t-Asbt in the rabbit. No product was seen when RNA was used as the template, confirming that our samples were free of DNA contamination. In contrast, with use of the same primers, a band corresponding to Asbt (302 bp), as would be predicted from Fig. 1, was prominent in adult ileum and weanling distal colon and less prominent in adult distal colon.

The relative expression of Asbt to t-Asbt in adult ileum was 13.5:1.

The adult ileal RT-PCR sample product was further gel purified and sequenced. Specific primers were designed from this sequence to determine the entire ORF (see MATERIALS AND METHODS). As in the rat (34), this rabbit t-Asbt sequence (GenBank Accession No. AY292653) represented a splice variant of the ileal Asbt (GenBank Accession No. Z54354).

Fig. 4. Ethidium bromide-stained agarose gel of PCR products of truncated apical Na\textsuperscript{+}-dependent bile acid transporter (t-Asbt) and Asbt. A: cDNA and total RNA of adult ileum and adult and weanling distal colon were amplified (40 cycles) using the primer pair Rbt-AsbtF/Rbt-AsbtR. Ratio of Asbt to t-Asbt in adult ileum was 13.5:1. GAPDH (24 cycles) served as internal control. B: nucleotide sequence of predicted open reading frame and corresponding deduced amino acid sequence. Potential sites of N-glycosylation. Putative transmembrane domains are underlined.
with the omission of exon 2 (119 bp) and a frame-shift event. However, the deduced ORF of rabbit t-Asbt lacked the COOH-terminal 12 amino acids found in rat t-Asbt (34). The coding region of the deduced rabbit sequence consisted of 143 amino acids with 3 predicted transmembrane domains and 2 N-glycosylation sites at the extracellular NH$_2$ terminus (Fig. 4B; see TMHMM online at http://workbench.sdsc.edu and Prosite at http://www.expasy.org).

**Mrp3.** We next examined whether Mrp3, another putative basolateral transporter, was present in the colon. Using the strategy described in MATERIALS AND METHODS, we identified and cloned a partial rabbit Mrp3 sequence (GenBank Accession No. AY289920; see Table 1 in supplemental online data for primers). A GenBank BLAST search revealed that the predicted partial amino acid sequence of rabbit Mrp3 had 91% identity to the published human MRP3 sequence (GenBank Accession No. NP_003777) and 84% identity to rat Mrp3 (GenBank Accession No. NP_542148). With use of specific rabbit primers (RbMrp3F1/RbMrp3R1, see Table 1 in supplemental online data) and semiquantitative RT-PCR, Mrp3 expression was found to be significantly higher in adult than in newborn colon, with intermediate values in weaning colon (Fig. 5A); however, values in weaning and newborn colon were not significantly different. Although semiquantitative analyses were not described in an earlier study, the rat ileum expression was greater in the ileum than in the colon, whereas Ost-α may be the candidate transporter in the ileum. To determine whether Mrp3 protein expression paralleled the RT-PCR observations, Mrp3-specific polyclonal antibodies were used to probe ileal and colonic tissues from different age groups by immunoblotting. As shown in Fig. 7, adult and weaning distal colon expressed Mrp3, a 190-kDa protein, in high abundance. In marked contrast, Mrp3 protein expression in newborn distal colon (Fig. 7A) and adult ileum (Fig. 7B) was very low. In contrast, in the rat, although mRNA expression was lower, as determined by RT-PCR, Mrp3 protein expression was equivalent in the ileum and colon (45).

**Bile acid flux.** The increased mRNA expression in weaning distal colon of proteins such as Asbt, LBP, and FXR characteristically involved in ileal bile acid reabsorption suggested that developing distal colon may also be designed for transepithelial bile acid uptake. To test this hypothesis, bile acid transport was measured across stripped adult and weaning distal ileal and colonic mucosa mounted in Ussing chambers. $J_m$ and $J_{td}$ of 3H-labeled TC (100 μM) were measured under short-circuit conditions ($n = 4−7$), and $I_{sc}$ and $G_I$ were monitored. TC was selected because it is readily available.

Fig. 5. Semiquantitative mRNA expression of multidrug resistance-associated protein 3 (Mrp3) in developing rabbit distal colon and adult ileum. **A**: colonic samples. **B**: comparison of expression in adult distal colon and adult ileum. **Top**: representative agarose gels of ethidium bromide-stained PCR products from exponential phase of Mrp3 (32 PCR cycles) and GAPDH (24 PCR cycles). **Bottom**: quantitation of Mrp3-to-GAPDH mRNA expression (means ± SE; $n = 3$) calculated as described in Fig. 1. $^{*}P < 0.05$. 

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**A**

**Distal colon**

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**B**

**Adult intestines**

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Pixel (Mrp3/GAPDH)

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commercially and has been utilized in a number of studies to examine intestinal absorption in the rabbit (3); however, the major bile acids reported in the rabbit circulation are secondary bile acids, especially glycodeoxycholic acid (20). Another reason 3H-labeled TC is an appropriate marker is that, in a perfused rabbit model, ileal and hepatic rates of saturable uptake were much greater for TC than for TDC (2). TC caused a slight, but significant, increase in $G_t$ (14%) in weanling ileum, but not in any of the other tissues examined (Figs. 8 and 9). TC also did not affect $I_{sc}$ in adult (Fig. 9A2) or weanling (Fig. 9B2) colon. In adult (Fig. 8A2) and weanling (Fig. 8B2) ileum, TC caused a gradual increase in $I_{sc}$ over time, whether it was added to the mucosal or serosal side. By least-squares regression analysis and one-sample t-test, this increase was found to be significant ($P < 0.02$) in adult and weanling ileum but not in adult ($P = 0.13$) or weanling ($P = 0.06$) distal colon.

Viability was assessed at 180 min as the $I_{sc}$ response to addition of mucosal glucose in the ileum (arrow in Fig. 8, A2 and B2) or serosal forskolin in the colon (arrow in Fig. 9, A2 and B2). TC uptake increased with time and was linear, beginning anywhere from 30 min (weanling ileum and adult colon) to 60–90 min (adult ileum and weanling colon) and continuing for ≥3 h. If we consider the differences in expression of transporters in these tissues, it is not surprising that different times were needed to achieve steady state. On the basis of these flux rates and to achieve uniformity for comparison purposes, $J_{m \rightarrow s}$ and $J_{s \rightarrow m}$ were calculated from the slope between 90 and 180 min for all four tissues, and $J_{net}$ values were calculated as the difference ($J_{net} = J_{m \rightarrow s} - J_{s \rightarrow m}$). Net uptake of TC was observed in adult and weanling ileum: $11.41 \pm 3.45$ ($n = 4$) and $5.53 \pm 1.20$ nmol·cm$^{-2}$·h$^{-1}$ ($n = 6$; Fig. 10), respectively. When calculated as the mean of the steady-state fluxes between 90 and 180 min, $J_{m \rightarrow s}$ and $J_{net}$ in adult and weanling ileum were statistically significantly different. On the other hand, the adult distal colon exhibited a small, yet significant, net TC secretion ($-1.39 \pm 0.47$ nmol·cm$^{-2}$·h$^{-1}$, $n = 3$; Fig. 10). In marked contrast to the adult colon and as predicted by the expression of bile acid transporters, weanling distal colon showed a significant, although much smaller, net TC absorption than the ileum.
Fig. 8. Transepithelial unidirectional \(^{3}H\)-labeled taurocholate (TC) fluxes and electrical parameters in developing ileal epithelia stripped of underlying muscles. \(A1\) and \(B1\): mucosal-to-serosal and serosal-to-mucosal flux (\(J_{m-s}\) and \(J_{s-m}\)) of TC over 30-min periods from addition of TC (time 0) in adult (\(A1; n = 4\) or 5) and weanling distal ileum (\(B1; n = 6\) or 7). \(A2\) and \(B2\): transmural short-circuit current (\(I_{sc}\)) and total conductance (\(G_t\)) measured in parallel with fluxes in adult (\(A2; n = 5\)) and weanling distal ileum (\(B2; n = 7\)). Arrows, addition of 10 mM glucose to mucosal bath to assess tissue viability. *Significantly different from time 0 (\(P < 0.05\)).

(1.23 ± 0.62 nmol⋅cm\(^{-2}\)⋅h\(^{-1}\), \(n = 6\); Fig. 10). \(J_{m-s}\) and \(J_{s-m}\) of colonic tissues were significantly different from those of ileal tissues.

**DISCUSSION**

*Asbt and LBP.* The present study demonstrates that the array of bile acid transporters and receptors expressed in weanling distal colon is very different from that expressed in adult or newborn rabbit. The mRNA expression of LBP and Asbt in distal colon appears to be biphasic with respect to age, with no (LBP) or moderately low (Asbt) expression in newborns, followed by a clear expression in weanlings and disappearance in adults (Figs. 1 and 5). Asbt and LBP are key proteins in the enterohepatic circulation of bile acids. Mutations or specific inhibition of Asbt lead to malabsorption of primary bile acids, a steep decrease of the bile acid pool, and an increase in fecal bile acid secretion (11, 43, 46). Studies by Kramer et al. (32) using photoaffinity labeling suggested that LBP may be the only physiologically relevant bile acid-binding protein in the rabbit ileal cytosol. Whether this holds true for all species and during different stages in development remains to be established, because, when Asbt is high in the fetal rat, there is no expression of iBABP (46), and iBABP is not found in all tissues expressing Asbt (18). Although the overall mRNA expression of Asbt and LBP was lower in weanling distal colon than in adult ileum (Figs. 1 and 4), the presence of these two transcripts suggests that weanling distal colon is designed for bile acid absorption.

Fig. 9. Transepithelial unidirectional TC fluxes and electrical parameters in developing distal colonic epithelia stripped of underlying muscles. \(A1\) and \(B1\): \(J_{m-s}\) and \(J_{s-m}\) of TC over 30-min periods from addition of TC (time 0) in adult (\(A1; n = 3\) or 4) and weanling distal colon (\(B1; n = 6\) or 7). \(A2\) and \(B2\): transmural \(I_{sc}\) and \(G_t\) measured in parallel with fluxes in adult (\(A2; n = 4\)) and weanling distal colon (\(B2; n = 7\)). Arrows, addition of 10 \(\mu\)M forskolin to serosal bath to assess tissue viability.
This study demonstrates for the first time that the distal colon of weanling mammals transports bile acids from lumen to serosa at a net rate that is ~11% that of adult ileum and 22% that of weanling ileum (Figs. 8 and 9). In sharp contrast to these findings, the distal colon of adults, where neither mRNA of Asbt nor mRNA of LBP can be detected by PCR, promotes a small, but significant, net secretory bile acid flux. Perhaps other members of the Mrp superfamily underlie this secretion (Fig. 11). The concentrations of bile acids at the serosal surface under normal physiological conditions may be low because of efficient clearance by capillary flow; therefore, these secretory mechanisms of bile acids may come into play under conditions of low serosal clearance.

Although the total bile acid pool increases steeply during weaning, as shown for rats and guinea pigs (40, 60), the ileum has not yet reached the adult morphology. In weanling rabbits, the mucosal surface area is ~9 mm²/mm² serosa compared with ~25 mm²/mm² serosa in adults, with a villus surface area of 88 and 278 μm²/villus, respectively (53). As shown in the present study, Asbt and LBP mRNA expression in weanling ileum is also lower than adult levels (Figs. 1 and 2). Furthermore, this is borne out by the functional studies which demonstrate that Jₘ→ₙ and Jₑₙ are significantly lower in weanling than in adult ileum (Fig. 10). A lower bile acid uptake into ileal enterocytes in weanling rabbits was also shown earlier (53).

FXR. An increased interaction between weanling distal colon and bile acids is also indicated by higher abundance of the mRNA of the nuclear bile acid sensor FXR in weanling than in adult distal colon (Fig. 3). FXR cloned from adult rabbit colon represents FXR-β₁, which is also the predominant isoform in mouse small intestine (62). All rabbit intestinal tissues also express FXR-β₂ mRNA, although at much lower levels than FXR-β₁ (data not shown). FXR is involved in the regulation of LBP and also, indirectly, Asbt (9). The high expression of Asbt and LBP in distal ileum and the distinctive increase in their expression in weanling colon (Figs. 1A and 2A), together with the similar expression levels of FXR mRNA (Fig. 3) in these two tissues, corroborate the speculation that, as in the ileum, FXR may play a role in ensuring that bile acid absorption occurs in weanling distal colon.

t-Asbt, Mrp3, and Ost-α. Transcellular bile acid transport also requires basolateral exit mechanisms, and candidate transporters are anion exchangers (58): t-Asbt (34) and Mrp3 (45) in the rat and Ost-α-Ost-β (12) in the mouse. The evidence is largely based on immunohistochemical and functional studies in heterologous cell systems. The physiological role of these transporters remains to be established. In the present study, we demonstrate that rabbit ileum expresses mRNA for t-Asbt, and, as in the case of the rat (34), t-Asbt mRNA abundance is rather low. However, critical to this study is that t-Asbt mRNA was not detected in adult or weanling distal colon (Fig. 4).

Ost-α-Ost-β has recently been described as the putative basolateral bile acid transporter in the mouse, with strong expression in the ileum but not the distal colon (12). Similarly, our results show that, in the rabbit, Ost-α is strongly expressed in adult ileum (Fig. 6A), with much lower expression in adult distal colon. Equally important, we demonstrate an age-dependent expression of Ost-α (adult > weanling > newborn) in the ileum but uniformly low expression at all ages in the colon. We postulate that although Ost-α may play an important role in ileal bile acid absorption in rabbit ileum, it may have a small, if any, role in weanling colon.

In contrast, in terms of mRNA and protein, Mrp3 exhibits an age-dependent (adult > weanling > newborn) expression in distal colon, whereas it exhibits very low expression in adult ileum compared with adult colon (Figs. 5B and 7). These results also suggest parallel expressions of Mrp3 protein and mRNA in rabbit tissues. Earlier studies in the rat (45) also showed a greater expression of colon than ileal Mrp3 mRNA by RT-PCR but a similar Mrp3 protein expression in the two tissues. However, the PCR data were not quantitated in that study. Furthermore, immunohistochemistry in rat small and large intestine showed that Mrp3 is localized at the BLM of the villar and surface cells, respectively (45). Preliminary results from our laboratory also show a similar distribution in rabbit ileum and colon. Ost-α may play an important role in ileal bile acid absorption in rabbit ileum, it may have a small, if any, role in weanling colon.

**Bile acid transporters in distal colon of newborn rabbits.**

The lack of detectable LBP mRNA in distal colon of newborn rabbits is consistent with studies in rat small intestine, where iBABP mRNA was not detectable in postnatal week 1 (48). In contrast, mRNA expression of Asbt in newborn distal colon was variable (see standard error in Fig. 1), although by one-sample t-test the mean values were not different from 0. A
biphasic expression pattern of ileal Asbt was also noted during prenatal/early postnatal development of the rat (48). Thus Asbt mRNA was expressed in rats on embryonic day 22, decreased within postnatal week 1, and then increased steeply at weaning. It is possible that the variances in expression in the colon of newborn rabbits are due to the time span (postnatal days 5–8) in which the animals were killed. Together, these observations indicate that newborn distal colon is not designed for transcellular bile acid uptake. Although the physiological function of high FXR expression in the newborn remains unclear, a pre-adaptive high abundance might be advantageous in initiation of LBP expression in distal colon as the animal is weaned.

**Transport of bile acids.** Bile acid transport has been reported to be regulated at the transcriptional level. This is the first report of a detailed examination of age-related changes in unidirectional and net transepithelial fluxes of bile acids in the small and large intestine. The gradual increase in $I_{sc}$ in adult and weanling rabbit ileum is intriguing and may be due to the electrogenic TC flux; because the flux is of much smaller magnitude in weanling distal colon, a similar trend is not seen in that tissue. The changes we report during development might implicate a regulatory adaptation to balance the changing milieu of the gut in the transition from newborn to weanling to adult. The alterations in mRNA expression in ileum and distal colon with age in the present study occur in concert with the changes in bile acid flux. The great absorptive capability for bile acid in adult ileum may be balanced by the potential for secretion in the distal colon, whereas the lower absorption in weanling ileum may be supported by a secondary salvage absorptive process in the distal colon. It is conceivable that such a salvage mechanism may be present in a variety of mammalian species, inasmuch as most species exhibit age-dependent expression of ileal Asbt. To put this in the context of rabbit physiology, it is very useful to the animal to have a mechanism for conserving bile acids in the colon during development. The adult rabbit is a coprophagic animal and ingests soft feces with a high content of fibrous material; therefore, unconjugated bile acids can be found in its proximal intestinal lumen. However, with the increase of cereal grains in standard rabbit chow, the contribution of soft feces to total protein intake may be <10% (6). On the other hand, in the developing animal fed rabbit milk, the diet is high in fat and low in fiber. Under these circumstances, especially when ileal absorption has not reached adult levels, a colonic salvage pathway allowing for bile acid reabsorption to optimize nutrient (fat) absorption would be highly advantageous to the animal.

In summary, the present study provides new insights into bile acid transport and expression of transporter/receptor proteins in developing mammalian distal colon. We demonstrate for the first time that weanling distal colon possesses the necessary bile acid transporter machinery and exhibits net bile acid absorption. We postulate that, in weanling distal colon, active bile acid absorption occurs via Asbt and increases intracellular bile acid concentrations, because the ability of weanling ileum to absorb bile acids is below that of the adult. This in turn might activate FXR, which then leads to an increased LBP expression. We propose that bile acids exit the BLM via Mrp3. In contrast, adult colon has no Asbt and significantly lower FXR expression than weanling colon and, therefore, cannot absorb bile acids. Physiologically, this may be relevant in the enterohepatic conservation and recycling of bile acids at a stage when the ileal capacity for bile acid absorption is not maximal. The mechanisms regulating these changes remain to be elucidated.

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