Ontogenic Regulation of Components of Ileal Bile Acid Absorption

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The apical sodium-dependent bile acid cotransporter (ASBT) and the ileal bile acid binding protein (IBABP) are two components of ileal bile acid absorption. During the third postnatal week of the rat, there is a dramatic increase in ASBT and IBABP expression. The goals of this study were to examine the role of hormones on the ontogenic expression of ASBT mRNA and the role of weaning for both ASBT and IBABP mRNA. Administration of various doses of dexamethasone during the second postnatal week induced ASBT mRNA levels, and this effect was significantly increased with concomitant thyroxine treatment. Early weaning and weaning prevention were utilized to investigate the influence of dietary factors. ASBT and IBABP mRNA levels were significantly elevated by early weaning and were decreased by weaning prevention compared with littermate controls. Thus, glucocorticoids and thyroxine appear to play a role in the ontogenic expression of ASBT mRNA and weaning appears to participate in both ASBT and IBABP expression.

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Bile acids play an important role in the solubilization and digestion of dietary lipids. The total bile acid pool is maintained by the enterohepatic circulation. In this system, bile acids are synthesized in the liver, conjugated to amino acids, and then secreted into the biliary tree. They enter the lumen of the proximal small intestine where they function in fat assimilation. Ultimately, they reach the distal small intestine and are reabsorbed into the portal circulation to return to the liver, thus completing the cycle (1, 2).

The intestinal absorption of conjugated bile acids is an essential step in the enterohepatic circulation. The major site at which this efficient process occurs is the distal ileum (1). The initial step is believed to involve an apical sodium-dependent bile acid cotransporter (ASBT) located in the brush border membrane of ileal epithelial cells (3). After entering the cell, bile acids are bound to cytosolic binding proteins, which shuttle them to the basolateral membrane where they exit the enterocyte through an anion exchanger (1).

The identification of two of the three proteins involved in ileal bile acid transport has allowed us to study the regulation of bile acid transport at the molecular level. ASBT is a 48-kDa protein primarily expressed in the ileum of rats (3). Ileal bile acid binding protein (IBABP) is a 14-kDa protein that is the major cytosolic bile acid binding protein in rat ileal enterocytes (4). Both of these proteins have been shown to markedly increase during the third postnatal week of rat development (3, 5), the same time at which functional bile acid active transport is first seen (6–9). For both ASBT and IBABP, ontogenic changes in protein levels are the consequence of parallel increases in their respective mRNAs (3, 10). Thus, knowledge regarding factors that help control ASBT and IBABP mRNA levels during normal development would provide insight into the regulation of ileal bile acid transport.

Several physiological changes occur during the second and third postnatal week of the rat that suggest candidate regulators of ASBT and IBABP expression. There are surges in serum levels of glucocorticoid (mainly corticosterone) and thyroid hormone (11). Because these increases in both glucocorticoid and thyroxine levels begin prior to the dramatic increase in ASBT and IBABP mRNA levels, they are logical candidates in regulating their mRNA expression. In addition to these hormonal changes, the third postnatal week is also characterized by a dietary change in which suckling rats make a transition from milk to solid food. Examination of stomach contents in different-aged rats reveals the presence of solid food starting on about day 16 (12). We have previously shown that physiologic doses of glucocorticoids can precociously induce IBABP, whereas thyroxine administration did not have a significant effect on IBABP mRNA levels (10). However, there are no studies

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examining the role of these hormones on the developmental expression of ASBT mRNA. Moreover, there are no investigations on the influence of dietary factors associated with weaning on the ontogenic expression of either ASBT or IBABP. Thus, the goals of this study were to examine the potential roles of glucocorticoids and thyroxine on ASBT mRNA expression and of dietary factors on both ASBT and IBABP mRNA expression.

Materials and Methods

Animals. Rats of the Sprague-Dawley strain (Crl:DC[SD]BR) were obtained from Charles River Laboratories (Portage, MI). Three adult male rats were utilized to generate a pooled reference sample of ileal RNA. The experiments used timed-pregnant dams. Animals were maintained in our facility using a 12:12-hr light: dark cycle with lights on at 0600 hr. Adults were provided with deionized water and Rodent Chow 5001 (Purina, Inc.) *ad libitum*. The day of birth was designated as Day 0. Litters were culled to eight to nine pups within 48 hr after birth. Hormones were administered as subcutaneous injections using doses as indicated in the individual experiments. All animal protocols were reviewed and approved by our Institutional Animal Care and Use Committee.

Dexamethasone (DEX) Dose Response. Two litters with nine pups per litter were used. Pups received either vehicle (0.9% NaCl) or one of several DEX doses on Days 10 through 13. DEX (Sigma, St. Louis, MO) was the selected form of glucocorticoid to be used because it does not bind to corticosteroid-binding globulin and thus its circulating concentration is not influenced by other factors (13, 14). DEX was administered at the following doses: 0.005, 0.02, 0.1, and 0.4 μg/g body wt (BW). By pair-weighing, each litter had two pups assigned to each DEX dose and one pup who received vehicle. A stock solution of DEX was made in 100% ethanol at a concentration of 4 mg/ml and then diluted with sterile normal saline such that the final ethanol content was <5%. On Day 14, the entire ileum (defined as the distal half of the small intestine between the ligament of Treitz and the ileocecal valve) was collected, flushed with ice-cold normal saline, and rapidly placed in liquid nitrogen.

Thyroxine Study. Two litters of suckling rats were placed into four treatment groups: vehicle, DEX, thyroxine, and simultaneous DEX and thyroxine. Animals were assigned by pair-weighing such that there were two pups in each treatment group per litter. Pups received daily injections on Days 8 through 13. A stock of L-thyroxine (Sigma) was made in 5 mM NaOH, from which a dilution with sterile saline to a concentration of 0.3 mM NaOH was made. L-Thyroxine was administered at a dose of 0.1 μg/g body wt, which results in circulating concentrations approximately 3-fold higher than in normal rats of comparable age (15). DEX was given at 0.01 μg/g body wt, a dose that is submaximal from the prior study and therefore would allow detection of synergism with thyroxine. Whole ileum was collected on Day 14 as described in an earlier section.

Early Weaning (EW) Study. Two litters of animals were placed into four treatment groups: control, DEX, EW, and EW + DEX. Pair-weighing was performed so that each litter had two pups assigned to each treatment group. EW was accomplished by separating the pups from their dams on Day 15 and placing them into other cages supplied with deionized water and pulverized chow. The time of separation was chosen based on a previous report that showed 100% mortality in animals prematurely weaned on Day 10, whereas animals weaned on Day 14 had an acceptable survival rate (16). An additional litter of pups was used to replace the removed EW pups such that the dams always nursed eight pups. A DEX group was included to account for the stress effects of prematurely separating the EW pups from their dams (17–20). DEX was given daily at a dose of 0.02 μg/g body wt on Days 15 through 19. Whole ileum was collected on Day 19 as described in an earlier section.

Weaning Prevention. To prevent normal weaning, we used the classical approach of a "16 plus 8" feeding schedule (21) in which the pups nurse for 16 hr/day and the dam feeds for 8 hr/day in a separate cage. Two litters were used in this study in which one litter was the experimental group and the other was the control group. On Day 14, these two litters were placed into new cages. The cage with the experimental litter contained deionized water without chow, whereas the cage with the control litter had both water and chow. Each dam was separated from her pups at 2200 hr daily and was placed into a separate cage that contained chow and water. At 0600 hr, the dams were returned to their pups. This design prevented the experimental pups from having access to chow. Whole ileums of four pups from each litter were collected on Day 19, and from the remaining pups on Day 22 as described in an earlier section.

Northern Blot Analysis. RNA was isolated from the collected ileums by the guanidine isothiocyanate-cesium chloride method (22). Northern blotting was performed from 0.8% or 1.0% formaldehyde agarose gels loaded with 20 μg of total RNA in each lane. The fractionated RNA was transferred to a nylon membrane. Membranes were hybridized with the insert of the rat ASBT cDNA donated by Dr. Benjamin Shneider (3). The cDNA probes were ^{32}P-labeled using the method of random-primed oligolabelling (23). Membranes were hybridized and washed as described previously (10). Some of the membranes were reprobed with the linearized plasmid of the mouse IBABP cDNA donated by Dr. Sherrie Hauft (24). Blots were exposed to Kodak XAR-5 film at −70°C. Equal loading of RNA samples was assessed by comparing the ethidium staining of the ribosomal bands. If the staining appeared unequal in the gel, then the Northern blot was reprobed with the cDNA for the constitutive marker, β-actin (25).

Quantitation. The adult pooled reference sample was included on each Northern blot as a standard. ASBT and IBABP mRNA hybridization signals were quantitated

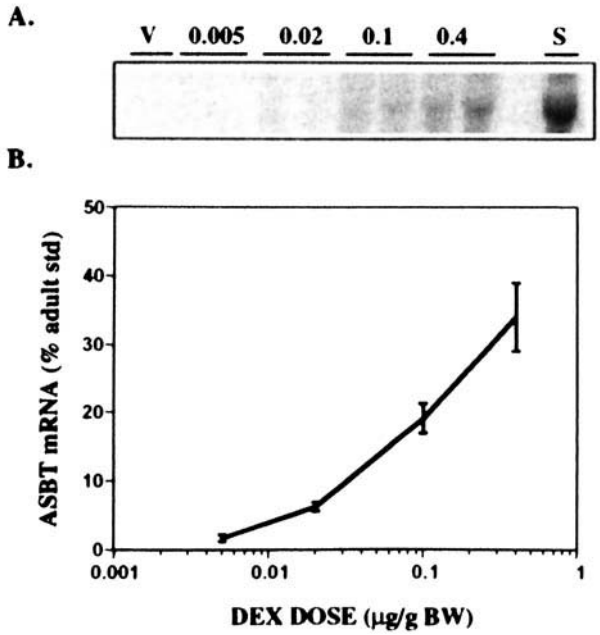

Figure 1. DEX dose response in Day 14 rats. (A) Representative Northern blot of ASBT mRNA detected in animals receiving different doses of DEX on Days 10 through 13. Numbers represent the dose given in micrograms per gram of body weight. V = vehicle and S = adult standard. (B) Quantitative data from Northern blot analysis. ASBT mRNA is expressed as a percentage of the pooled adult standard. Values are given as the means ±SE. (n = 4 at all DEX doses).

by phosphorimaging. Values were expressed as a percentage of the adult standard from the same blot. If RNA loading correction was done, then the ASBT or IBABP signal was first expressed as a ratio with the β-actin signal from the same sample lane before being expressed as a percentage of the standard. Data are expressed as means ±SE. Statistical analysis was performed by one- or two-way analysis of variance (ANOVA). If significance was detected (P < 0.05), then further *post hoc* comparisons were performed using either Fisher's least significant differences or Student's t-test.

Results

DEX Dose Response. To investigate the role of glucocorticoids in the developmental expression of ASBT mRNA in the ileum, suckling rats were administered physiologic doses of DEX on Days 10 through 13. Figure 1A reveals that ASBT mRNA is precociously induced in Day 14 animals by DEX administration with appreciable levels first seen at a dose of 0.1 μg/g body wt. Longer exposure of the autoradiogram revealed detectable ASBT mRNA at both of the lower doses of DEX, but not in the vehicle-injected animals. Quantitation of Northern blots (Fig. 1B) shows increasing amounts of ASBT mRNA with larger doses of DEX. Statistical analysis by one-way ANOVA supports a significant effect of DEX dose on ASBT mRNA levels (P < 0.001). The amount of ASBT mRNA induced continued to increase without evidence of reaching a plateau in this dose range.

Thyroxine Study. Because serum thyroxine levels also increase during the second postnatal week of the rat, the next experiment examined whether thyroxine could play a role in the normal ontogeny of ASBT mRNA. Figure 2 shows that although no ASBT mRNA was detected when suckling rats received thyroxine alone, there was a higher magnitude of mRNA levels seen when thyroxine was simultaneously given with DEX compared with levels achieved with DEX alone. Two-way ANOVA revealed a significant DEX effect as expected (P < 0.001) as well as a thyroxine effect (P = 0.03). Synergism between DEX and thyroxine was confirmed by the detection of an interaction between the two treatments (P < 0.04). ASBT mRNA levels were more than 2-fold higher in the DEX plus thyroxine group than the DEX only group.

Early Weaning. Since the parallel changes in both ASBT and IBABP mRNA levels normally occur at the time of weaning, the following experiment was done to explore the role of this dietary transition in ASBT and IBABP mRNA expression. A DEX treatment group was included in this study to account for the stress-induced effects seen from early weaning (17–20). Figure 3A shows that EW did elevate ASBT mRNA levels in Day 19 animals. Statistical analysis detected a significant effect of both DEX (P <

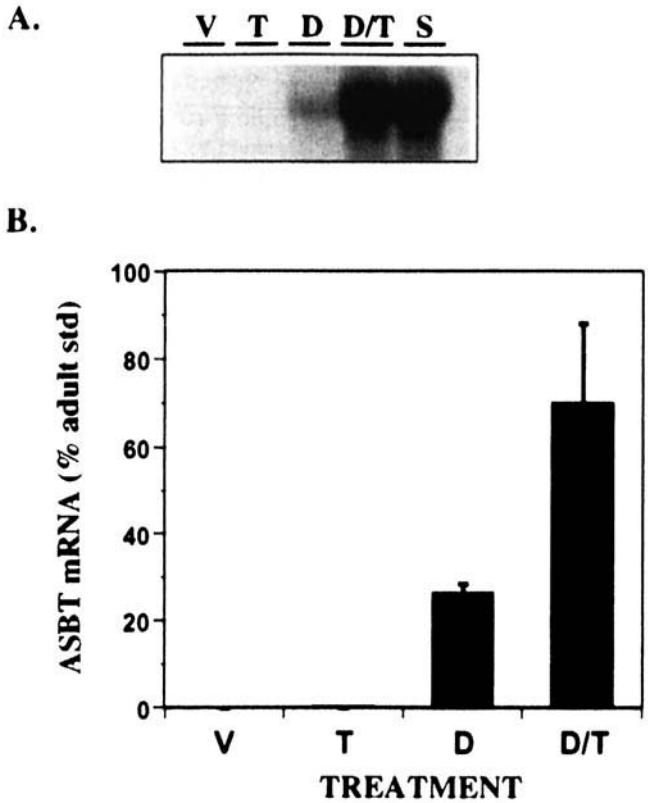

Figure 2. The effects of thyroxine, DEX, and simultaneous DEX + thyroxine in Day 14 rats. (A) Representative Northern blot of ASBT mRNA detected in animals administered vehicle (V), thyroxine (T), DEX (D), or both DEX and thyroxine (D/T) on Days 8 through 13. S = adult standard. (B) Quantitative data from Northern blot analysis. ASBT mRNA is expressed as a percentage of the pooled adult standard. Values are given as the means ±SE (n = 4).

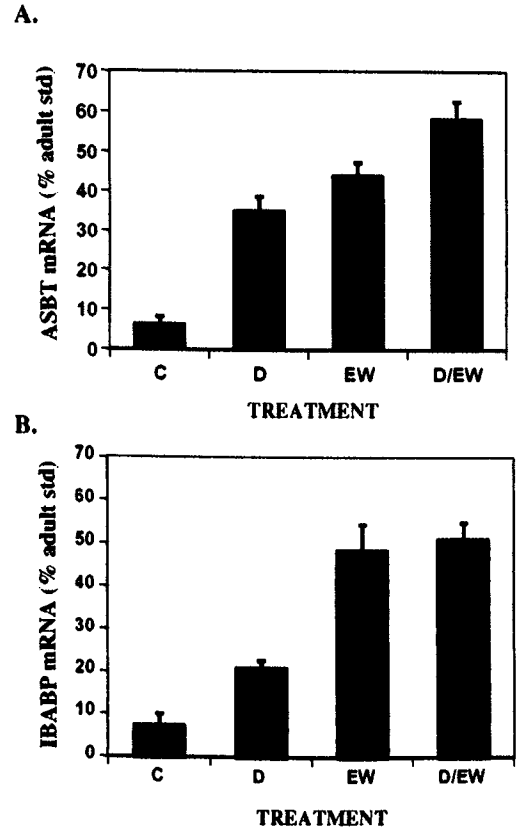

Figure 3. The effect of early weaning on ASBT and IBABP mRNA expression in Day 19 rats. (A) Quantitative data for ASBT mRNA expressed as a percentage of the pooled adult standard. Values are given as the means ±SE (n = 4). C = control, D = DEX, EW = early weaning, and D/EW = DEX and early weaning. (B) Quantitative data for IBABP mRNA expressed as a percentage of the pooled adult standard. Values are given as the means ±SE (n = 4).

0.001) and EW ($P < 0.001$) with no evidence of interaction between the two treatments ($P = 0.06$). The magnitude of ASBT mRNA elicited by EW was similar to that elicited by DEX with no significant difference in levels seen between these groups ($P = 0.07$). However, there was a significant difference detected between the EW and EW plus DEX group ($P < 0.03$). This suggests that EW may be an independent factor in ASBT mRNA expression.

In contrast to these results, Figure 3B shows that IBABP mRNA levels seen in the EW animals are higher than in the control and DEX groups. ANOVA verified that EW had a significant effect on IBABP mRNA levels ($P < 0.001$). Further statistical analysis revealed that there is a significant difference between the DEX and EW groups for IBABP mRNA ($P = 0.01$), suggesting that the induced IBABP mRNA levels seen in EW animals is not totally due to a stress effect, but that the dietary change was a significant factor.

Weaning Prevention. To further support that the transition from milk to solid food has an impact on ASBT and IBABP mRNA expression, the final experiment utilized a different design, namely weaning prevention. Figure 4A shows that weaning prevention inhibited ASBT mRNA expression. Statistical analysis revealed that both age ($P < 0.001$) and weaning prevention ($P = 0.001$) had a significant effect on ASBT mRNA levels. There was no interaction detected between age and weaning prevention ($P = 0.58$), indicating that there was no difference in the amount of reduction seen in ASBT mRNA levels on Days 19 and 22. Reprobing of these same Northern blots with IBABP (Fig. 4B) showed similarly that age ($P < 0.001$) and weaning prevention ($P < 0.001$) have a significant effect on IBABP mRNA levels without evidence of synergism between the two ($P = 0.23$). Thus, these results suggest that the absence of solid food in the diet reduces, but does not abolish, the developmental rise of both ASBT and IBABP mRNA.

Discussion

The enterohepatic circulation is an efficient system involving multiple interrelated steps that preserves the body's total bile acid pool. Many of its components are not fully developed in the human infant, including the intestinal absorption of conjugated bile acids (26, 27). This is also true in the developing rat, which has served as an animal model in the study of the enterohepatic circulation (6–9, 26). With the identification and characterization of some of the pro-

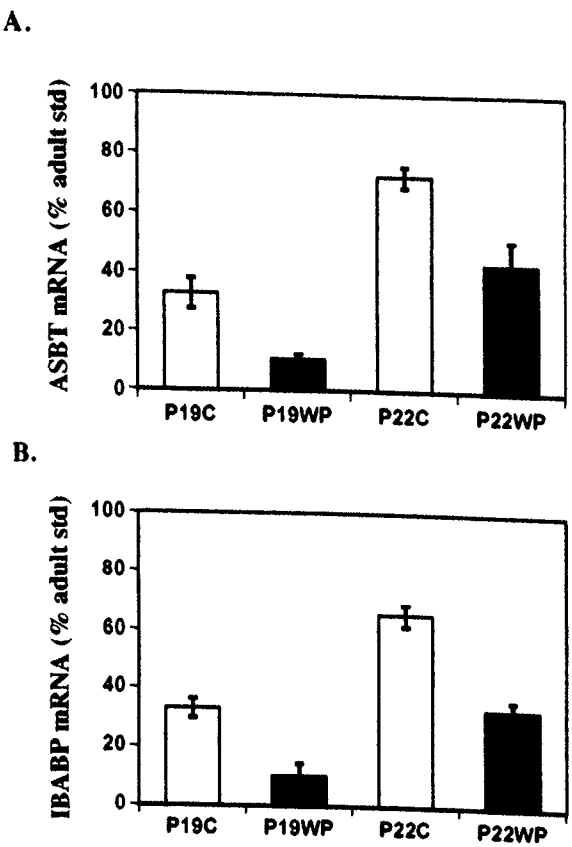

Figure 4. The effect of weaning prevention on ASBT and IBABP mRNA expression in Day 19 and 22 rats. (A) Quantitative data for ASBT mRNA expressed as a percentage of the pooled adult standard. Values are given as the means ±SE (n = 4–5). P19C = Day 19 control, P19WP = Day 19 weaning prevention, P22C = Day 22 control, and P22WP = Day 22 weaning prevention. (B) Quantitative data for IBABP mRNA expressed as a percentage of the pooled adult standard. Values are given as the means ±SE (n = 4–5).

teins involved in intestinal bile acid transport, namely ASBT and IBABP, the investigations of the regulation of intestinal bile acid transport can occur at the molecular level. This is supported by the fact that the normal developmental patterns of ASBT and IBABP mRNA and protein coincide with that of ileal active bile acid transport measured by functional assays (3, 5–10, 28). This report is the first to examine the role of hormones and diet in the developmental expression of ASBT mRNA, and is the first to examine the role of diet in the developmental expression of IBABP mRNA.

Glucocorticoids have been shown to play a role in the regulation of several intestinal genes (11, 29). Our data show that administration of glucocorticoids during the second postnatal week can elicit a surge of ASBT mRNA expression in the rat ileum. The response of ASBT mRNA increases linearly within the physiological dose range of DEX given in our study. Because we are interested in the possible role of glucocorticoids in the onset of ASBT mRNA expression, the DEX dose that equates to peak serum corticosterone levels attained during the second week of the rat is the one of physiologic importance. This DEX dose has been calculated to be approximately 0.1 μg/g body wt (10), a dose that elicited ASBT mRNA to 20% of adult levels. Glucocorticoids may mediate these effects by interacting with the regions in the rat ASBT promoter found to be corticosteroid responsive *in vitro* (30).

These results agree with prior studies involving glucocorticoid effects on the ileal bile acid transport system. Precocious induction of IBABP mRNA and protein has been shown with DEX treatment in suckling rats (10, 28). Interestingly, the magnitude of IBABP mRNA and protein levels elicited by a DEX dose of 0.1 μg/g body wt was also approximately 20% of adult levels (10). Barnard and Ghishan (31) reported early detection of sodium-dependent bile acid transport by brush border membrane vesicles of Day 14 rats after 3 days of methylprednisolone treatment. Little and Lester (6) used the technique of ileal rings to demonstrate increased taurocholate mucosal uptake in suckling rats who received DEX. Heubi and Gunn (32) revealed a linear increase in bile acid transport by about 20% in corticosterone-treated rats. The glucocorticoid-induced bile acid transport seen in these studies is most likely due to inductions of both ASBT and IBABP.

Because serum levels increase during the second postnatal week of the rat, thyroid hormone was another logical candidate regulator of ASBT mRNA expression. Prior studies have reported the ability of thyroid hormone to synergize with glucocorticoids in the maturation of several intestinal genes (33–36). Our results indicate that this holds true for ASBT mRNA as well. The study was designed so that extraneous effects of thyroid hormone on glucocorticoid kinetics were excluded. Thyroxine has been shown to increase circulating levels of corticosteroid-binding globulin, which confounds studies with natural glucocorticoids (13, 14). DEX was chosen for our study since it does not interact with corticosteroid-binding globulin and thus would not be influenced by thyroxine effects on corticosteroid-binding globulin. Therefore, the synergism observed between thyroxine and DEX on ASBT mRNA reflects a true interaction between the two hormones.

The response of ASBT mRNA to thyroxine agrees with a functional study on thyroid hormone effects on bile acid transport. Heubi (37) detected an induction of sodium-dependent bile acid transport in Day 16 rats treated with thyroxine. However, administration of thyroxine did not have a significant effect on IBABP mRNA expression in the suckling rat (10). These studies suggest that the induction of ASBT and IBABP is not required for increased bile acid transport and that the developmental regulation of these two proteins is not identical. Evidence of discoordinate regulation of ASBT and IBABP has also been reported in adult rodents (38, 39).

Since hormonal influences could not fully account for the developmental increase of ASBT mRNA, the next candidate that we examined was the dietary change associated with weaning. In general, the switch from milk to solid food does not appear to control the timing of intestinal maturation (11, 29, 40). However, for certain proteins (41–44) there is evidence that luminal cues from the weanling diet do play a role in the regulation of their developmental expression. Our study is the first to examine the impact of dietary factors on the ontogeny of any of the components of ileal bile acid absorption. Early weaning of suckling rats resulted in significantly increased levels of both ASBT and IBABP mRNA. Because early weaning has been known to cause stress in the animals (17–20), it was difficult to determine how much of the early weaning induction was from a glucocorticoid effect, especially for ASBT. However, the significant differences detected between the early weaning and early weaning with DEX groups suggests that there is an effect from luminal cues in the diet. The induction of IBABP mRNA from EW was significantly higher than the DEX group and more clearly suggested a role for dietary factors. To further support the possible role of dietary factors, an inverse experiment was done to prevent weaning (prolonged suckling). At both 19 and 22 days, the levels of ASBT and IBABP mRNA were significantly reduced when weaning was prevented. The effect may actually be more dramatic if we consider that separation from the dam is associated with significant increases of circulating corticosterone (17–20), which would act to elevate ASBT and IBABP mRNA. Thus, the true extent of the effect of weaning prevention may have been masked by a countereffect of stress-induced elevation of glucocorticoid. Taken together, these results support a role for dietary factors in the ontogenic expression of both ASBT and IBABP mRNA.

Our work focused on the regulation of ASBT and IBABP mRNA expression during ontogeny because there is evidence that variations in their mRNA levels may have a strong impact on ileal BA transport. During normal development of the rat, increases in ASBT and IBABP mRNA

levels coincide with increases in protein levels and the onset of functional BA transport (3, 5, 10, 28). In addition, we have shown that glucocorticoid administration to suckling rats results in precocious induction of IBABP mRNA and protein to comparable levels (10). The alterations in ASBT and IBABP steady-state mRNA levels seen in response to hormones and dietary changes in the current work may reflect changes in gene transcription, mRNA stability, or both. Shneider et al. (3) have revealed that increases in both gene transcription and mRNA stability account for the increased ASBT mRNA levels seen during normal development. Whether hormones and dietary factors affect ASBT and IBABP mRNA by increasing gene transcription, mRNA stability, or both has not been ascertained and will need to be investigated in future studies.

The data from these studies and our prior work (10) suggest that the regulation of the developmental expression of ASBT and IBABP mRNA is multifactorial. Administration of glucocorticoids can induce both ASBT and IBABP mRNA, whereas thyroxine treatment enhances the glucocorticoid response of ASBT mRNA only. Both ASBT and IBABP mRNA are precociously increased by early weaning and delayed by weaning prevention. Thus, hormones and the dietary transition from milk to chow seem to participate in the expression of ASBT and IBABP during normal development. The dietary effect on ASBT and IBABP may be due to the different forms of fat in the suckling and weanling diets. Milk fat is found in membrane-bound globules that are believed to be absorbed essentially intact in the pup intestine (45). However, fat in solid food does not have this feature and requires the presence of bile acids for micellar formation for digestion and absorption (46). Whether these physico-chemical differences in luminal fats serve as cues for developmental increases of ASBT and IBABP expression remains to be determined.

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