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The Role of AP-1 in the Transcriptional Regulation of the Rat Apical Sodium-dependent Bile Acid Transporter*

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Ileal reclamation of bile salts, a critical determinant of their enterohepatic circulation, is mediated primarily by the apical sodium-dependent bile acid transporter (ASBT=SLC10A2). We have defined mechanisms involved in the transcriptional regulation of ASBT. The ASBT gene extends over 17 kilobases and contains five introns. Primer extension analysis localized two transcription initiation sites 323 and 255 base pairs upstream of the initiator methionine. Strong promoter activity is imparted by both a 2.7- and 0.2-kilobase 5′-flanking region of ASBT. The promoter activity is cell line specific (Caco-2, not Hep-G2, HeLa-S3, or Madin-Darby canine kidney cells). Four distinct specific binding proteins were identified by gel shift and cross-linking studies using Caco-2 or rat ileal nuclear extracts. Two AP-1 consensus sites were identified in the proximal promoter. DNA binding and promoter activity could be abrogated by mutation of the proximal AP-1 site. Supershift analysis revealed binding of c-Jun and c-Fos to this AP-1 element. Co-expression of c-Jun enhanced promoter activity in Caco-2 cells and activated the promoter in Madin-Darby canine kidney cells. Region and developmental stage-specific expression of ASBT in the rat intestine correlated with the presence of one of these DNA-protein complexes and both c-Fos and c-Jun proteins. A specific AP-1 element regulates transcription of the rat ASBT gene.

The enterohepatic circulation of bile acids plays a key role in a number of physiologically essential functions, including promoting hepatic bile flow and enhancing intestinal assimilation of fats and fat soluble vitamins (1). In addition, bile acid excretion represents a major mechanism of cholesterol catabolism. The orderly movement of bile acids through the enterohepatic circulation is dependent upon an asymmetric array of vectorial transporters, which are located in the liver and intestine. A key member of this set of transporters is the apical sodium-dependent bile acid transporter (ASBT),[1] which is found on the apical surface of ileal enterocytes, renal proximal convoluted tubule cells, and large cholangiocytes (2–6). In the ileum, ASBT plays a critical role in the intestinal reclamation of bile salts that are secreted by the liver. Complete disruption of this process leads to congenital primary bile acid malabsorption (7, 8). Partial inhibition, by either ileal exclusion or pharmacologic blockade, leads to bile acid wasting and provides an important approach to the treatment of hypercholesterolemia and cholestasis (8–12). In contrast, to the well established physiological function of ASBT in the intestine, the importance of ASBT expression in renal and bile duct epithelial cells remains uncertain. No biliary or renal phenotype has been described in children with defects in the ASBT gene.

Regulation of ASBT expression is complex and has only been examined in recent descriptive studies. In the rat ileum, ASBT undergoes a biphasic pattern of regulation during normal development, with a fetal onset of expression, early post-natal down-regulation, followed by marked up-regulation at the time of weaning (3, 4, 13). In contrast, renal expression of ASBT is constitutive during the same developmental stages. The basis of the ileal regulation appears to be both transcriptional and post-transcriptional. ASBT has a distinct pattern of expression along the longitudinal axis of the intestine with a restriction of its expression to the terminal 30% of the small bowel (14). The bile acid responsiveness of the ASBT gene is a subject of unresolved controversy and may be species and experimental-condition specific (15–20). Intestinal inflammation appears to down-regulate ASBT expression, while glucocorticoid administration leads to up-regulation (21, 22).

Given the overall importance of ASBT function in health and human disease, precise understanding of the molecular mechanisms of its regulation is of great importance. Exploitation of endogenous mechanisms of up- and down-regulation of this gene will provide novel approaches to the treatment of inflammatory bowel disease and hypercholesterolemia, respectively. Identification of key elements involved in directing ileal-specific expression of ASBT will lead to new ways to treat patients with short gut, where ileal function has been irreversibly lost. Similarly, identification of elements involved in the developmental regulation of ASBT expression will lead to new methods to care for premature infants, where intestinal function is immature. The human and rabbit ASBT 5′-flanking regions have been cloned (GenBank™ accession number AJ002005) (8, 23). Recent knock-out studies have shown that hepatocyte nuclear factor-1α (HNF-1α) is critical for basal expression of ASBT (24). The following investigations provide a further description of the transcriptional machinery of the ASBT gene.

EXPERIMENTAL PROCEDURES

Genomic Cloning and Organization—A rat P1 genomic library (25) was screened using the polymerase chain reaction (PCR) and ASBT cDNA-derived oligonucleotide primers (sense primer 5′-CGAAGGTGATTCCTGCCTAG-3′, antisense primer 5′-CAGCAACCCATAATTAGCACC-3′; Genome Systems, Inc., St. Louis MO). One of three positive clones was purified, digested with either *Hin*dIII or *Bam*HI, and sub-

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[1] The abbreviations used are: ASBT, apical sodium-dependent bile acid transporter; HNF-1α, hepatocyte nuclear factor-1α; RT-PCR, reverse transcriptase-polymerase chain reaction; MDCK, Madin-Darby canine kidney; bp, base pair(s); kb, kilobase pair(s); DI, distal ileal.

cloned into a pZeRO™-1 vector (InVitrogen, Carlsbad, CA). Ninety-six randomly selected subclones of each digest were spotted onto nitrocellulose and screened by Southern blotting using three different cDNA probes generated from ASBT clone BS32C2 (4). The insert in clone BS32C2 was released by digestion with *Kpn*I and *Pst*I and was subsequently digested with *Eco*RI and *Bam*HI to yield 679-, 1206-, and 310-bp fragments which represented the 5′, middle, and 3′ portions, respectively, of the ASBT cDNA. Selected positive genomic clones were sequenced on both strands using a series of synthetic oligonucleotides, *AmpliTaq* DNA polymerase, and an ABI 373A automated sequencing system (ABI, Foster City, CA) (26). Oligonucleotide synthesis and DNA sequencing were performed at the William Keck Biotechnology Resource Laboratory at Yale University, New Haven, CT. Genomic sequence was compared with previously published cDNA sequence (Fig. 1) to determine the intron/exon organization of the rat ASBT gene (3). DNA sequence analysis was performed using software of the Genetics Computer Group (Madison, WI). Mapping of the site of polyadenylation of the ASBT gene was determined by Northern blot analysis of rat ileal RNA using *Apa*LI/HindIII and *Ssp*I/*Sca*I fragments of ASBT genomic clone BSB2F (Fig. 1).

Cell Lines and Culture Conditions—Caco-2 (HTB-37), HepG2 (HB-8065), MDCK (CCL-34) and HeLa-S3 (CCL-2.2) cells were obtained from the American Type Culture Collection (Rockville, MD). Caco-2, a human colon cancer cell line, was grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/liter glucose, 1 mM sodium pyruvate, 0.1 mg/ml human transferrin, and 10% fetal calf serum. The human liver tumor cell line HepG2 and the dog renal tubular epithelial cells MDCK were maintained in Eagle's minimum essential medium containing 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% fetal calf serum. HeLa-S3 cells were cultured in Ham's F12K medium with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, and 10% fetal calf serum. All cells were grown at 37 °C in 95% air and 5% CO_2.

Primer Extension Analysis—Primer extension assays were carried out in order to identify the ASBT transcription initiation site within the 5′-flanking sequence (27). The primer was a 27-mer oligonucleotide (5′-GCTAGTATGTCAGTTTCAAGAGGCTGC-3′) which hybridizes 180 bp upstream of the translational initiation codon (Fig. 2). Fifteen μg of total cellular RNA, extracted from rat ileum epithelial cells, was utilized as a template. The extension products were analyzed on a 6% polyacrylamide/urea gel and compared with the dideoxy sequencing reaction of plasmid BSB1C (Fig. 1).

Plasmid Construction—To define *cis* elements involved in the regulation of ASBT expression, the rat ASBT 5′-flanking sequence and its subfragments were subcloned into a mammalian expression vector pGL3-Basic (Promega, Madison, WI), upstream of a firefly luciferase gene. A 3.1-kilobase (kb) *Bgl*II/*Nco*I fragment containing the largest (2.7 kb) rat genomic ASBT 5′-flanking region (ASBT 5′) was derived from clone BSB1C. The hybrid construct pGL3-ASBT5′/$^{-2685/+384}$ (Fig. 2, *construct a*) was generated by cloning this fragment into the pGL3-Basic plasmid in an orientation such that the sense strand DNA of the firefly luciferase gene was transcribed. Based on pGL3-ASBT5′/$^{-2685/+384}$, ASBT 5′-deletion/hybrid constructs were prepared, in order to localize transcriptional *cis* elements. The construct pGL3-ASBT5′/$^{-2685/-830}$ (Fig. 2, *construct b*) was made by insertion of an upstream 1.9-kb sequence of the ASBT 5′ region into the polylinker of the pGL3-Basic vector. pGL3-ASBT5′/$^{-829/+384}$ (Fig. 2, *construct c*), was prepared by insertion of a downstream *Xho*I/*Nco*I 1.2-kb fragment containing 0.8-kb of the ASBT 5′ sequence.

Based upon the transcription initiation sites that were found by primer extension assay, two DNA fragments with 496 bp (P2) and 327 bp (P3), respectively, were generated by PCR, using BSB1C plasmid as a template. Constructs were sequenced to confirm the veracity of the amplification process. P2 and P3 contain the sequences flanking both sides of, and including, a TATA box, the transcription initiation sites and two AP-1 *cis* elements (Fig. 2, *constructs d* and *e*). These two fragments were then subcloned into *Xho*I/*Hin*dIII-nicked pGL3-Basic, forming the constructs pGL3-ASBT5′/$^{-378/+118}$ and pGL3-ASBT5′/$^{-208/+118}$, respectively. In order to precisely localize the *cis* element in ASBT 5′-flanking region, six DNA oligonucleotides (C1-C6) of 54–55 bp corresponding to different portions of P3 sequence (Fig. 2, *P3 subfragments*) were synthesized and tested for the *trans*-acting factor binding activity by band-shift assay. Additional 15-bp oligonucleotides were used to further define the *cis*- and *trans*-acting elements.

Site-directed PCR Mutagenesis of ASBT 5′ Sequence—The site-directed point mutagenesis of ASBT 5′-flanking region was performed by a QuikChange™ Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA), targeting to two AP-1 *cis* elements contained in P3 DNA fragment corresponding to the ASBT 5′-flanking region, positioned from −208 to +118 (28, 29). DNA oligonucleotide primers, P3mu-1 and P3mu-2 were synthesized, with sequences of 5′- GCCAATAAATGATTGATTATAACTTTCTGTCTTGG-3′ and 5′-GGACTTTTTATATTTATTATTTGTGC-3′, respectively, which contained mutations as shown by underlined nucleotides (A ↔ G, T ↔ C). The third primer P3mu-3 is the combination of the sequences and mutations of the primers P3mu-1 and P3mu-2. The PCR products, pGL3-ASBT5′/P3M1, pGL3-ASBT5′/P3M2, and pGL3-ASBT5′/P3M3, were examined for their sizes by electrophoresis, and the produced point mutations contained within the P3 fragment were confirmed by DNA sequencing.

Transient Transfection and Luciferase Analysis of Cells—Confluent cells (5×10^6/plate) were harvested and resuspended in 700 μl of phosphate-buffered saline containing 4 μg of the ASBT5′/luciferase hybrid plasmid constructs and 0.1 μg of a quantitation control plasmid containing a thymidine kinase promoter-driven Renilla luciferase gene (Promega, Madison, WI). Transfection was accomplished by electroporation (30) at 0.22 kV and 0.95 μF × 1,000 (Bio-Rad). After electroporation, the cells were resuspended in culture medium and returned to a plate for culturing for 40 h before dual luciferase assays (Promega, Madison, WI) using standard techniques as recommended by the manufacturer (31). The effect of c-Jun on promoter activity was assessed by co-transfection of a cytomegalovirus promoter driven c-Jun expression construct (32).

Preparation of Cell Nuclear Extracts and Band Shift Assays—Nuclear extracts were prepared from confluent cells or rat ileal mucosal scrapings using previously described techniques (33). Nuclear extracts were aliquoted into vials, quick-frozen, and stored at −80 °C. Five micrograms of nuclear proteins were incubated, with the appropriate ^{32}P-labeled ASBT 5′-flanking DNA fragments or oligonucleotides (34, 35). For band supershift analysis, specific antibodies were added together with the DNA probe and protein samples. Following digestion with 1 μl (127 units/μl, Life Technologies, Inc.) of DNase I at 37 °C for 30 min, samples were subjected to electrophoresis in a 7% native polyacrylamide gel. The rat small intestine was divided into four equal segments (proximal jejunum, distal jejunum, proximal ileum, and distal ileum) for analysis of the regional specificity in the rat small intestine. Rat terminal ileum (distal 25% of small intestine) was obtained from 10 (pre-weaning) and 28 (post-weaning)-day-old rats in order to assess developmental stage specificity.

Ultraviolet (UV) Cross-linking Assays—DNA-protein binding reactions were carried out as described above, using 2×10^5 cpm of ^{32}P-labeled DNA fragments or oligonucleotides and 5 μg of nuclear protein per reaction. Following incubation at 37 °C for 1 h, 1 μl of DNase I (127 units/μl) was added to the reaction mixture in order to digest the unbound DNA fragments. Reaction mixtures were transferred to wells of a 96-well microtiter plate, and irradiated on ice by UV light at 1,200 microjoules for 7 min, using a UV-Stratalinker chamber apparatus (Stratagene, La Jolla, CA). The samples were analyzed by electrophoresis through a 7% SDS-polyacrylamide gel.

RESULTS

Genomic Cloning and Organization—Transcriptional analysis required elucidation of the genomic organization of the ASBT gene and cloning/sequencing of its 5′-flanking region. The ASBT gene extends over ~17 kb and consists of six exons and five introns (Fig. 1 and Table I). Primer extension analysis using ileal total RNA identified two potential sites of transcription initiation, which were 323 (genomic ASBT bp = −68) and 255 (genomic ASBT bp = 0) bp from the translation start site (Figs. 2 and 3). A TATA box is located between these two sites (−12 to −9). Approximately 2800 bp of the 5′-flanking sequence have been cloned, sequenced, and utilized for the transcriptional studies described here (GenBank™ accession number AF285154). The sizes of the intronic regions of the ASBT gene were confirmed by a series of PCR reactions utilizing rat genomic DNA (data not shown). The entire 3.1 kb of 3′-untranslated sequence was found in a single intron (GenBank™ Accession number U07183). The first potential conserved polyadenylation signal was found 3116 bp from the cDNA stop codon (36). Two additional polyadenylation signals were found in the immediate 3′ region of the ASBT gene. Northern blot analysis of ileal total RNA mapped the polyadenylation signal to the first of these three potential sites (Fig. 4).

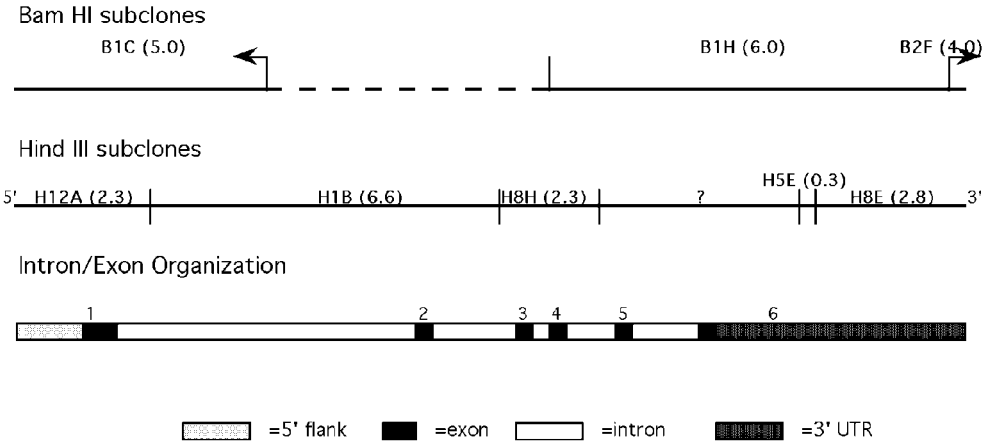

FIG. 1. **Schematic diagram of genomic organization.** Overlapping *Bam*HI and *Hin*dIII subclones of rat genomic DNA are indicated by the *top two lines*. Clone designations are *above* the line, and fragment sizes in kb are marked in the *parentheses* that follow the designation. The genomic organization of the ASBT gene is seen in the *bottom block diagram*. Exons are *shaded* and introns are *unshaded*.

TABLE I
Intron/exon boundaries and splice sites for the rat ASBT gene

The splice sites, intron and exon sizes of the ASBT gene are listed.

3′ Splice acceptor			5′ Splice acceptor		
Intron	Exon	Exon No. = bp	Exon	Intron	Intron length (kb)
	TATTTC or	Exon 1 = 699			
	TAGTTT	Exon 1 = 631	ACCTCAG	gtaaga	5.7
ttcag	TGTTAG	Exon 2 = 119	GCATTG	gtaagt	0.9
tgttag	GCATTTC	Exon 3 = 89	CTTAAA	gtaagt	0.7
ttccag	ATTGGA	Exon 4 = 176	GGTACAG	gtatga	2.0
tcccag	GTGCCG	Exon 5 = 158	TAGGAA	gtaagg	3.3
ttacag	TGTATG	Exon 6 = 3238	AAATAG	acac	

ASBT 5′-Flanking DNA Possesses Transcriptional Promoter Activity—A luciferase reporter assay system was utilized in order to investigate the promoter function of the ASBT 5′-flanking region. ASBT5′/luciferase hybrid constructs were made using a pGL3-Basic vector (Promega) which contains a firefly luciferase coding region as a reporter gene. Caco-2 cells were chosen as transfectants for reporter gene assays since they are an intestinally derived cell line and support sodium-dependent bile acid transport activity (37). They also express ASBT transcripts (data not shown) and thus have the necessary factors for ASBT transcription. Cells transfected with pGL3-Basic and pGL3-SV40 were employed as negative and positive controls, respectively, with the latter containing an SV40 promoter-driven firefly luciferase gene. Calibration of transfection efficiency was achieved by co-transfection of cells with pRL-TK plasmids that express Ranilla luciferase. To test whether ASBT5′ has promoter activity, reporter gene assays were carried out initially using the full-length ASBT5′ construct pGL3-ASBT5′/$^{-2684/+384}$ (Fig. 2, *construct a*). Results demonstrated that the cloned genomic ASBT5′ sequence possessed significant transcriptional activity, which was 61% of that of SV40 promoter, as reflected by luciferase activities (Fig. 5, *panel A*). The transcriptional activity of the downstream 0.8-kb ASBT 5′ sequence (pGL3-ASBT5′/$^{-829/+384}$) was 22% of that of the full-length 5′ sequence, however, still 30 times higher than that of the control construct pGL3-Basic which contains no promoter sequence. No luciferase activity was detected in cells transfected with pGL3-Basic as well as pGL3-ASBT5′/$^{-2685/-830}$ (data not shown) compared with the background measurement, indicating that the upstream 1.9-kb sequence has no transcriptional activity.

A 208-bp Region Preceding the Downstream Transcription Initiation Site Demonstrates Major Promoter Activity—Since a number of intestinal promoters have key regulatory elements near the transcription initiation start site, the proximal promoter of ASBT was carefully characterized. Two ASBT5′-related DNA fragments P2 and P3 with 496 bp (ASBT5′ nucleotide positions −378 to +118) and 327 bp (ASBT 5′ nucleotide positions −208 to +118), respectively, were generated by PCR and sequenced. Both fragments contain the transcription initiation sites and TATA box, and are part of the downstream 0.8-kb 5′ sequence which showed transcriptional activity (Fig. 2, *construct c*). P2 and P3 possess 40 and 34%, respectively, of the transcriptional activity of the full-length 5′ sequence (Fig. 5, *panel A*).

Nuclear Trans-acting Proteins Specifically Bind to ASBT 5′ Sequences—To test the hypothesis that *trans*-acting factors exist that can bind to *cis*-element(s) of ASBT5′, a band-shift assay was carried out initially with nuclear proteins extracted from Caco-2 cells and ^{32}P-labeled ASBT 5′ sequences, which included P2 and P3. Four DNA-ASBT-binding protein (ABP1-ABP4) complexes were detected as shown in the *panel B* of Fig. 5. Furthermore, a cross-linking assay was performed with radiolabeled P3 as the probe to measure the molecular weights of the ABPs found by the band-shift analysis. As shown in *panel C* of Fig. 5, four protein bands were detected with apparent molecular weights of 100,000, 73,000, 70,000, and 43,000.

To determine ABPs DNA binding specificity, the unlabeled P2 was tested for the ability to compete for ABPs binding to the ^{32}P-labeled P2 sustrate. Preincubation of Caco-2 nuclear proteins with an unlabeled 1.2-kb fragment of the ASBT 5′-flanking region (−829 to +384) followed by addition of the radiolabeled P2 substrate, resulted in a concentration-dependent reduction in the formation of the DNA-protein complexes (Fig. 5, *panel D*). Binding of ABP2 and ABP4 were markedly affected, with lesser but substantial effect observed for ABP1 and ABP3. Addition of up to 2 nM nonspecific competitor, a 1.2-kb *Xba*I fragment of pGL3-Basic plasmid had no effect on the

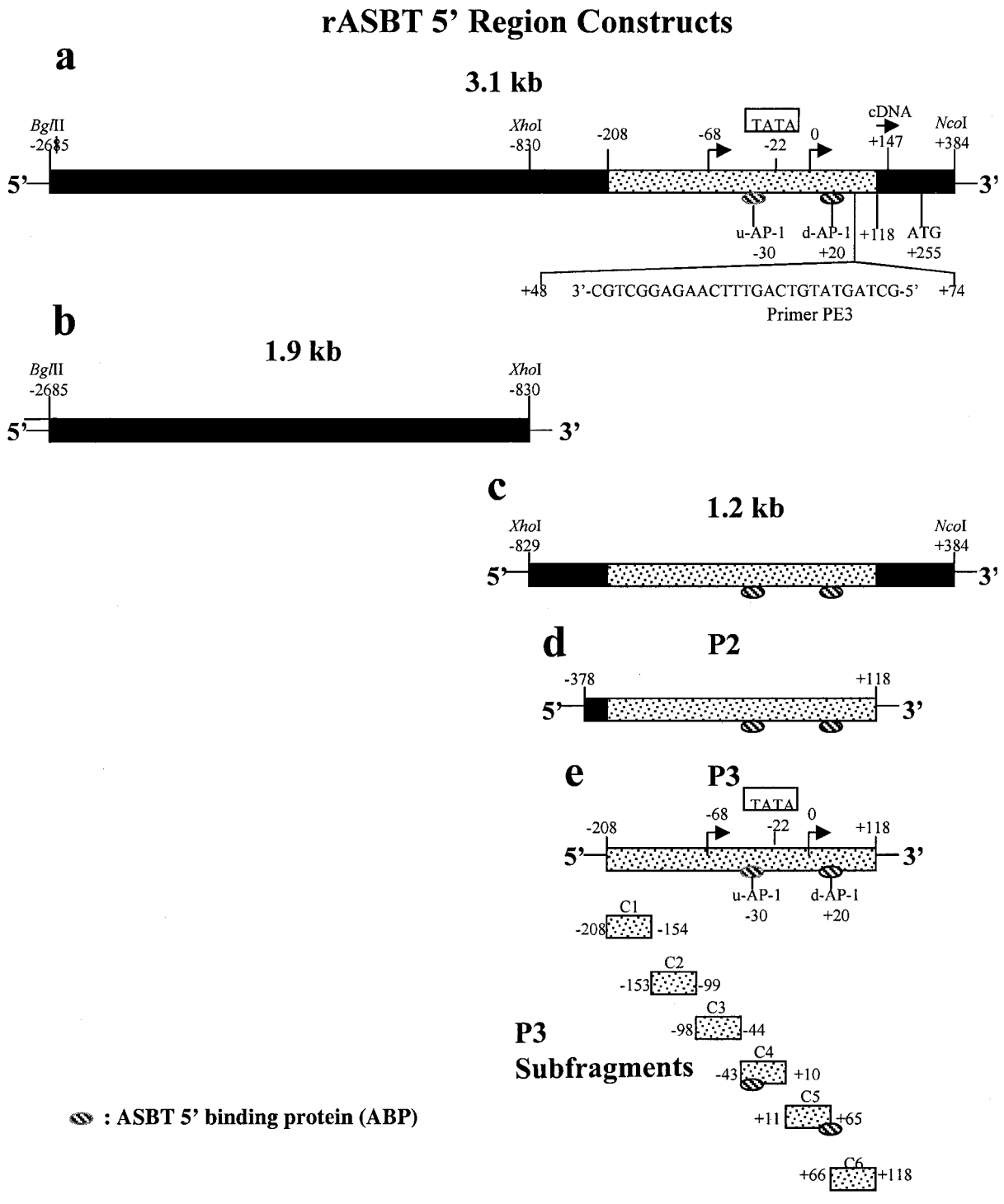

FIG. 2. **Schematic diagrams of ASBT 5′ constructs.** Construct a contains the full-length 2.7-kb ASBT 5′ sequence upstream of the reporter gene. Positions of the important sites are shown, including those identified in further experiments; *Primer PE3* indicates sequence utilized for primer extension assay. *Construct b* is a 1.9-kb upstream ASBT 5′ sequence restricted by *Bgl*II/*Xho*I and corresponding to the region from −2685 to −830. *Construct c* is a 1.2-kb fragment and covers the region of −829 to +384 obtained by digestion with *Xho*I/*Nco*I. *Constructs d* (P2) and *e* (P3) are PCR synthesized and correspond to the 5′ sequences of −378 to +118 and −208 to +118, respectively. P3 subfragments C1-C6 are *in vitro* synthesized. Each subfragment contains 54–55 base pairs corresponding to different portions of P3 sequence. All these ASBT 5′ fragments, except C1-C6, were subcloned into the polylinker of the expression vector pGL3-Basic, which contains a firefly luciferase gene downstream of the polylinker.

complex formation (Fig. 5, *panel D*), indicating that the DNA-protein interactions are specific. Further evidence of the specificity of the interaction is the fact that no nuclear proteins were bound by a radiolabeled *Xba*I fragment of the pGL3-Basic plasmid (Fig. 5, *panel B, Control*). Furthermore, the DNA-ABPs binding characteristics were examined. DNase I pretreatment of radiolabeled P3 inhibited the formation of ABPs-DNA complexes (data not shown) excluding the possibility of nonspecific [^{32}P]dCTP binding to ABPs. It was also shown that pretreatment of the nuclear preparation with either proteinase K or SDS could abolish the binding to P3 (data not shown), indicating that the binding activity of ABPs is proteinase K and SDS sensitive. These results confirm that the complexes detected by the band-shift assay are composed of DNA and

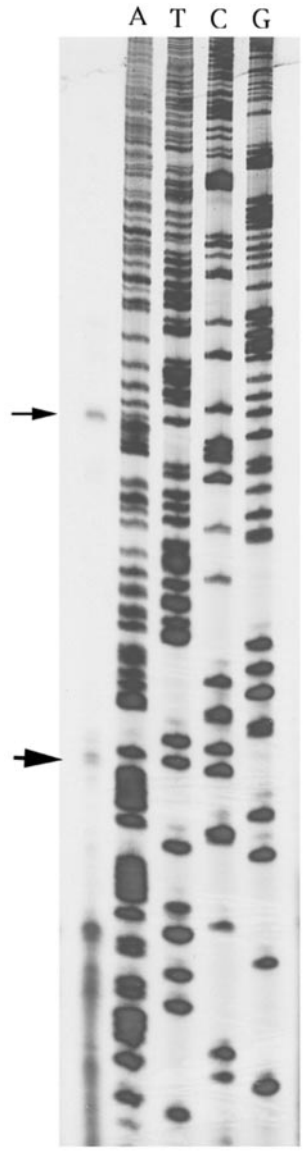

FIG. 3. **Identification of transcription initiation sites within the ASBT 5′ region.** To determine the transcription initiation site within the ASBT 5′ region, a primer (PE3) was synthesized, with the sequence of 5′-GCTAGTATGTCAGTTTCAAGAGGCTGC-3′ (located 180 bp upstream of the translation initiator ATG, Fig. 2, *construct a*). As shown in the figure, two sites (*arrow*) were found which correspond to nucleotide positions 323 and 255 bp upstream of the translation initiator ATG.

polypeptides.

Identification of 5′ cis-Elements Involved in ASBT Expression—To determine further the *cis*-element(s) of ASBT5′, six DNA fragments (Fig. 2, *C1-C6*) of 54–55 bp corresponding to different portions of P3 sequence were synthesized, radiolabeled, and incubated with the nuclear extracts for band-shift assay. Interestingly, we found that one of the ABPs, ABP2, only bound to C4 and C5, but failed to bind to other fragments (Fig. 6, *panel A*), suggesting that these two fragments may contain the *cis*-element(s) which interact with the *trans*-acting factor.

Further analysis of the *cis*-element(s) existing within P3 was carried out using MacVector software and transcription factor data bases. P3 contained two AP-1 *cis*-elements (ASBT5′ nucleotide positions −30 to −24 and +20 to +26, respectively). The proximal site (upstream = uAP-1) was located immediately upstream of the TATA box and the lower transcription initiation site. The distal AP-1 site (downstream = dAP-1) was located 18 nucleotides downstream of the lower transcription site (Fig. 2, *construct a*). Furthermore, the upper (5′-sided) AP-1 *cis*-element was contained in C4 and the lower (3′-sided) one contained in C5 fragment (Fig. 2, *P3 subfragments*). This finding was consistent with the result obtained in the band-shift assay, where we showed that ABP2 only bound to C4 and C5 and not the rest of the P3-related subfragments tested, as described above (Fig. 6, *panel A*).

These findings strongly suggested that AP-1 played a key role in the transcriptional regulation of the ASBT gene. This was confirmed by examination of plasmid constructs containing the AP-1-mutated P3, which were generated by a QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). pGL3-ASBT5′/P3M1 and pGL3-ASBT5′/P3M2 contained point-mutated upstream and downstream AP-1 element, respectively, each with other elements in normal sequence. pGL3-ASBT5′/P3M3 had the mutations of both pGL3-ASBT5′/P3M1 and pGL3-ASBT5′/P3M2. Results of the reporter gene assays, as shown in *panel B* of Fig. 6, demonstrated that in comparison with the normal P3 construct, mutation of the upper AP-1 element (M1) resulted in a 10-fold decrease in luciferase gene transcription. Mutations of both AP-1 sites (M3) lead to almost complete abrogation of promoter activity, compared with the basic construct. In contrast, mutation of the lower AP-1 element (M2) alone caused a negligible decline in the luciferase activity (Fig. 6, *panel B*). These results further support the finding that the upper AP-1 *cis*-element, which locates upstream of the lower transcription initiation site within the 5′-region, is responsible, at least in part, for ASBT gene transcription.

c-Jun Is a Component of ABP2 in Caco-2 Cells—The AP-1 transcriptional complex is composed of at least two major factors, c-Jun and c-Fos (38). AP-1 functions as a dimer in the form of Jun/Fos *or* Jun/Jun, depending on individual target genes. Because we demonstrated that an AP-1 *cis*-element controlled ASBT expression, supershift assays with monoclonal antibodies against c-Fos and c-Jun (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used to examine whether these transcription factors existed in ABPs identified by band-shift assay. As shown in *panel C* of Fig. 6, a supershifted band (*upper arrow*) was formed by addition to the reaction mixture of the c-Jun monoclonal antibody, but was not detected in the presence of c-Fos monoclonal antibody. As the supershifted complex was formed, the ABP2 band disappeared at the original position (*panel C* of Fig. 6, *lower arrow*), confirming that ABP2 contains c-Jun protein. This result is consistent with the findings obtained in the band-shift assay, which showed ABP2 only bound to AP-1-containing fragments C4 and C5 (Fig. 6, *panel A*). Electrophoretic mobility shift assay analysis using 15-bp oligonucleotides that included either the downstream (d-AP-1) or upstream (u-AP-1) AP-1 sites demonstrated that the ABP2 was the result of binding to the upstream AP-1 site (Fig. 6, *panel D*). The difference in electrophoretic mobility of the complexes formed with u-AP1 and d-AP1 suggests that there are significant differences in the proteins that bind to these two different elements.

To further determine the relationship between ABP2 and AP-1-binding protein, competitive band-shift assays were conducted with *in vitro* synthesized specific competitors, 15-bp fragments containing a normal or mutated AP-1 consensus sequence. Caco-2 cell nuclear proteins were preincubated with the unlabeled competitor, followed by addition of the radiolabeled normal P3 fragment as the probe. The band-shift assay demonstrated that ABP2 band was missing only after addition of the unlabeled 15-bp fragments containing the normal AP-1 element sequence, indicating a specific interaction between ABP2 and AP-1 *cis*-element (Fig. 6, *panel E*). The mutated AP-1

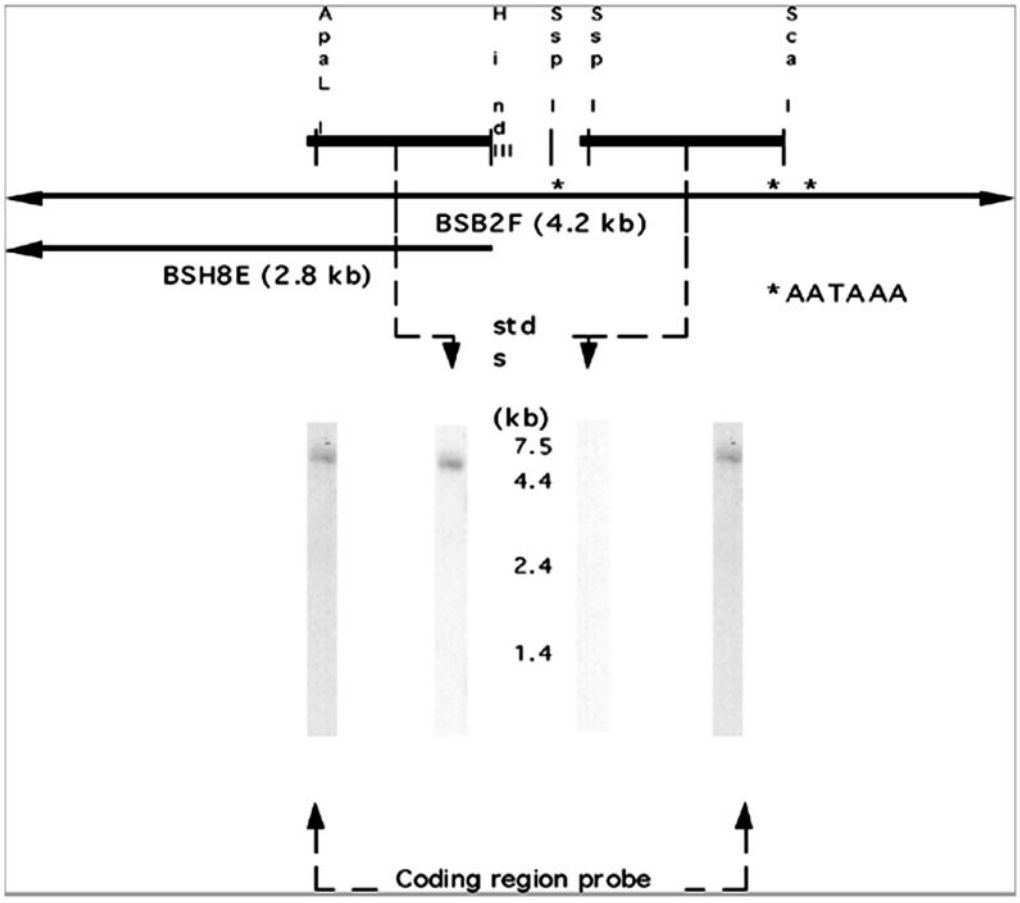

FIG. 4. **Mapping of the ASBT polyadenylation signal.** Overlapping clones BSB2F and BSH8E are depicted with *Apa*LI, *Hin*dIII, *Ssp*I, and *Sca*I sites identified. The locations of potential polyadenylation sites are indicated by an *asterisk*. Northern blot analysis of rat ileal total RNA was performed using a probe generated from either an *Apa*LI/*Hin*dIII or *Ssp*I/*Sca*I digest of BSB2F. The blots were reprobed with an ASBT coding region probe. The 5.0-kb ASBT mRNA was detected with the *Apa*LI/*Hin*dIII but not the *Ssp*I/*Sca*I digest of BSB2F, indicating that the first polyadenylation was utilized.

sequences failed to compete off ABP2 band (Fig. 6, *panel E*). In addition, there was no band shift observed using the mutated AP-1 element and Caco-2 nuclear extracts (data not shown). Taken together, these observations demonstrate that in Caco-2 cells ABP2 is a c-Jun-like, AP-1-binding factor or complex, which is involved in ASBT gene expression by interaction with AP-1 *cis*-elements contained within the downstream portion of the 5′ sequence. Transient co-transfection of Caco-2 cells with c-*jun* and pGL3-ASBT/$^{-208/+118}$ led to enhancement of luciferase activity (Fig. 6, *panel B*).

ASBT 5′-Directed Gene Expression Is Cell Line-specific—Cell lines have been utilized as a surrogate to analyze organ-specific expression of genes. This approach is especially useful for transient transfection analysis. As a preliminary stage of investigation, RT-PCR analysis was performed on several standard cell lines in order to identify candidates that expressed the ASBT mRNA. Previously described degenerate oligonucleotide primers that spanned intron 1 were utilized (3). Appropriate sized products (data not shown) were identified in MDCK and Caco-2 cells, while HepG2 and HeLa cells were negative. We then determined whether there was a correlation between cell line expression of ASBT and its ability to support promoter activity. In the experiments, Caco-2, HeLa-S3, HepG2, and MDCK cells were transfected with the pGL3-ASBT5′/$^{-208/+118}$ hybrid plasmid construct, followed by luciferase assays. Luciferase activity could only be detected in the Caco-2 cells (Fig. 6, *panel A*). These findings indicate that there is not an exact correlation between the ability of cell line to express ASBT and its ability to support ASBT promoter function. Co-transfection of MDCK cells with both a c-*jun* expression construct and pGL3-ASBT5′/$^{-208/+118}$ resulted in markedly enhanced luciferase activities that approximated that seen in Caco-2 cells.

In order to further understand the correlation between ASBT mRNA expression and transcriptional activity, ASBT5′ DNA binding activity was assessed. ABPs-DNA interactions were assessed by band-shift assay using the radiolabeled ASBT 5′-sequence and nuclear proteins from the ASBT^{+} and ASBT^{-} cells. Caco-2 nuclear proteins were used as control. Interestingly, the results demonstrated that ABP2, the AP-1-binding protein, was absent in HeLa and MDCK cells, whereas it was present in HepG2 cells (Fig. 7, *panel B*).

AP-1 Expression Correlates with Region and Developmental Stage-specific Expression of ASBT in the Rat Small Intestine—Two important aspects of the regulation of ASBT expression are region specificity and ontogeny. In order to assess the role of AP-1 in this aspect of regulation, gel-shift and Western blotting studies were performed using different segments of adult rat intestine and terminal ileum from pre- and post-weaning rat intestine. The pattern of four DNA-binding proteins observed by gel shift analysis with nuclear extracts from rat distal ileal (DI) mucosal scrapings was the same as that observed with Caco-2 nuclear extracts (Fig. 8, *panel A*). Interestingly, ABP2 was missing in the mucosa of the jejunum (PJ and DJ) and only trace amounts were detected in the proximal ileum (PI). Supershift analysis revealed the presence of both c-Jun and c-Fos in the DNA-protein complexes derived from rat distal ileal mucosal scrapings (Fig. 8, *panel B*). Western blot analysis revealed that c-Jun and c-Fos protein expression was

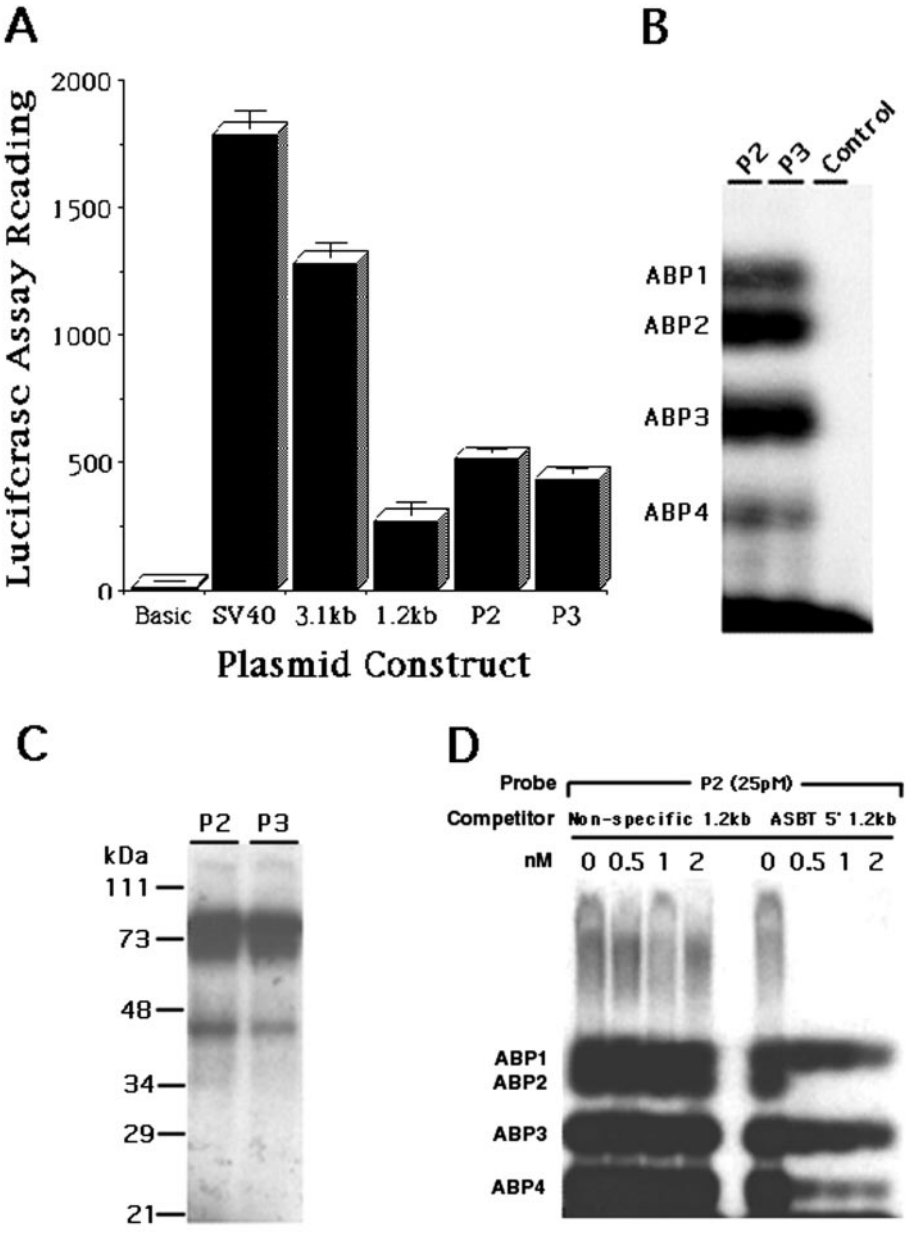

FIG. 5. **Analysis of the ASBT 5′-flanking region.** *A,* a Dual-Luciferase™ reporter assay system (Promega) was used to examine transcriptional activities of rat ASBT 5′ sequences. All activities were normalized to thymidine kinase promoter driven *Renilla* luciferase. The results show that cells transfected with the construct containing the full-length 2.7-kb ASBT 5′ sequence express the highest luciferase activity, ~60% of that induced by SV40 promoter. The promoter activities of the downstream 1.2 kb region, P2 and P3 are 22, 40, and 34%, respectively, of that of the full-length 5′ sequence. *B,* band-shift assays were carried out with 5 μg of Caco-2 cell nuclear extract and 2×10^5 cpm of radiolabeled ASBT 5′ sequences as indicated on the *top* of each lane. Four DNA-binding proteins, named as ABP1–4, were detected. The control reveals that there are no bands seen when a 1.2-kb *Xba*I fragment of pGL3 is utilized. *C,* to assess the molecular weights of ABPs, cross-linking assays were conducted with 5 μg of Caco-2 nuclear proteins and 2×10^5 cpm of ^{32}P-labeled ASBT 5′ sequences, as indicated on the *top* of each lane. Four protein bands with molecular weights of 100,000, 73,000, 70,000, and 43,000, respectively, were detected, which bound to all the sequences tested. *D,* to examine ABPs-DNA binding specificity, 5 μg of nuclear proteins from Caco-2 cells were preincubated with the unlabeled specific competitor (1.2 kb fragment from −829 to +384) or nonspecific competitor (a 1.2-kb pGL3-basic vector sequence), followed by addition of the radiolabeled P2 fragments, at the concentrations as shown on the *top* of the lanes. Binding of ABP2 and ABP4 were markedly affected, with lesser but substantial effects observed for ABP1 and ABP3. There was no inhibition of binding by the nonspecific competitor, indicating that the ABPs DNA binding activity is specific.

restricted to the distal ileum (Fig. 8, *panel A*). ASBT expression along the longitudinal axis of the small intestine correlated with the presence of ABP2, c-Jun, and c-Fos (Fig. 8, *panel A*). Other members of the AP-1 family of proteins, with the exception of FosB were not correlated with the expression of ASBT (Table II). The ontogenic expression of ASBT correlated with the expression of AP-1. ABP2, c-Fos, c-Jun, and ASBT were detected in terminal ileum from 28-day-old rats, but not from 10-day-old rats (Fig. 9). Additional Western blot analysis of rat nuclear extracts revealed significantly greater expression of HNF-1 in PI and DI compared with PJ and DJ and marked enhancement of expression in 28-day-old rats compared with 10-day-old rats (data not shown).

DISCUSSION

The complex nature of intestinal gene expression necessitates a highly regulated system. The intestinal mucosa undergoes marked and perpetual proliferation that requires careful control in order to maintain normal physiology. Intestinal gene expression is also highly regulated along the crypt to villus and longitudinal axes and in the specific cell types that make up the intestinal mucosa. As such, the transcriptional regulation of genes in the intestine is of great importance, and significant advances have been made in beginning to understand these complex systems (39–43). ASBT is an important candidate gene for analysis, as it provides information relevant to specific aspects of intestinal gene expression as well as to the enterohepatic circulation of bile acids. The tissue-specificity of ASBT expression is somewhat unusual in that it is found in intestine, bile duct epithelium, and kidney. In addition, ASBT has a distinctive pattern of regulation along the longitudinal axis of the intestine, with expression restricted to the terminal ileum (14, 44, 45). The ileal lipid-binding protein has a similar pattern of expression along the longitudinal axis of the intestine (46). Transgenic mouse analysis of the ileal lipid-binding protein promoter indicates that this pattern of expression is controlled by elements in the proximal promoter, although the specific molecular mechanisms underlying that regulation are unknown.

The rat ASBT gene was cloned from a P1 library and analysis of its genomic organization revealed that it consists of six

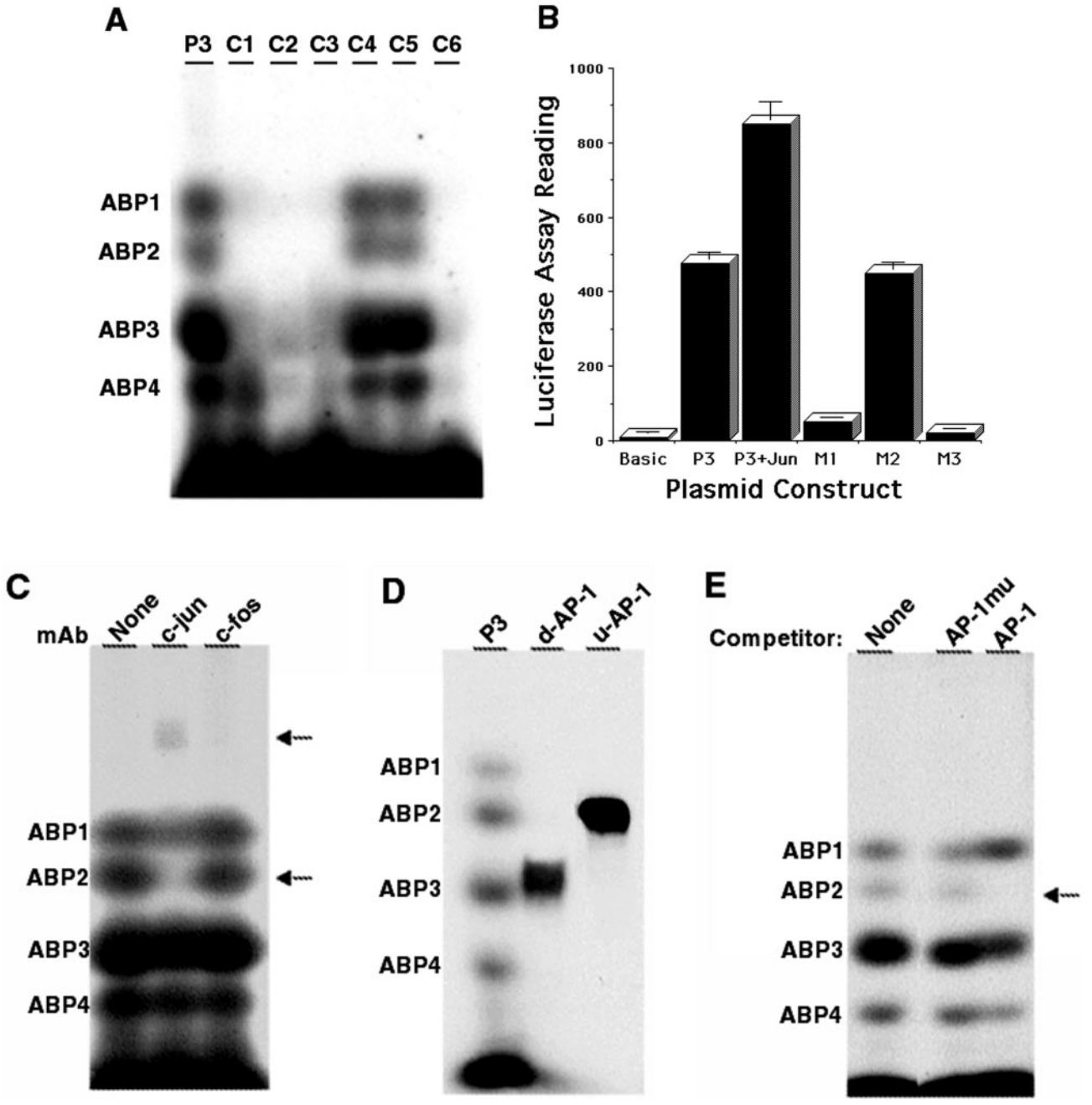

FIG. 6. **Localization of *cis*-elements within the ASBT 5′ region.** *A,* to further localize the nuclear protein-binding sites within P3 sequence, P3 subfragments were *in vitro* synthesized, each of which contained 54–55 bp and was incubated with the nuclear extracts, followed by the band-shift assay. C4 and C5 bound to all the ABPs; however, other subfragments failed to bind to ABP1 and ABP2, indicating that these two proteins bound to a *cis*-element which only exists in C4 and C5. *B*, point mutations of the upstream (M1) or both (M3) AP-1 elements resulted in inhibition of ASBT 5′-directed luciferase gene expression, as compared with the positive control (P3). Point mutations of the downstream (*M2*) AP-1 sequence had no significant effect on the gene expression. Co-expression of c-Jun with P3 lead to significant enhancement of luciferase activity. *C,* a supershifted band (*upper arrow*) was detected with c-Jun, but not c-Fos, monoclonal antibody. Correspondingly, the ABP2 band disappeared at the original position (*lower arrow*), indicating that the supershift band contains the DNA-binding protein that is recognized by c-Jun antibody. *D,* electrophoretic mobility shift assay analysis of a 15-bp oligonucleotide centered on the upstream AP-1 site (u-AP-1) revealed a single species that had the same electrophoretic mobility as ABP2. Similarly, the downstream AP-1 (d-AP-1) generated a band similar to ABP3. *E,* Caco-2 nuclear proteins were preincubated with a 15-bp fragment containing a normal (AP-1) or mutated AP-1 (AP-1 mu) consensus sequence, followed by addition of the ^{32}P-labeled P3 probe and band-shift analysis. Normal, but not mutated, AP-1 sequence can compete off P3 ABP2 binding activity, indicating that ABP2 binds to the AP-1 *cis*-element.

exons. This genomic organization is nearly identical to that observed in humans and rabbits (8, 23). In all three species the second through fifth exons are nearly identical in size. The 5′-and 3′-untranslated regions in the three species are variable, with the 3′-untranslated region in excess of 2.6 kb in all three. In rats and rabbits the first intron is large, 5700 and 7556 bp, respectively. Analysis of the 3′-untranslated region of the rat ASBT gene reveals three potential polyadenylation sites, which are all at least 3118 bp from the translation stop codon. Multiple AUUUA elements, which have been associated with mRNA instability, are present in this long 3′-untranslated RNA (47).

Analysis of the mechanisms underlying the transcriptional regulation of ASBT expression were performed using a cloned 3.1-kb DNA fragment containing 2.7 kb of ASBT 5′ genomic sequence. Primer extension analysis identified two transcription

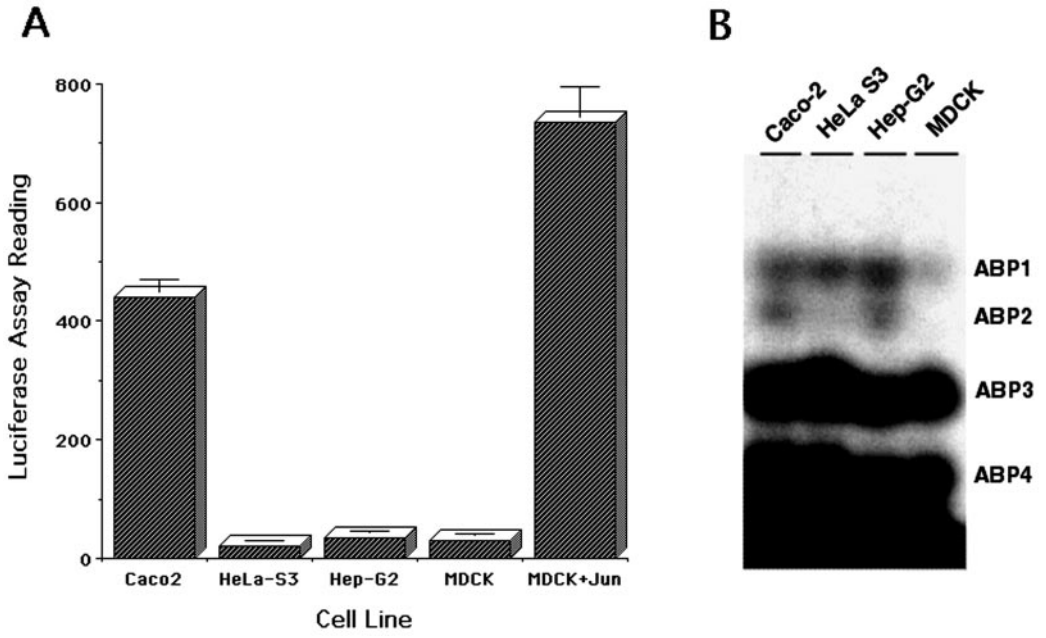

FIG. 7. **Transcriptional regulation of ASBT and ABPs-DNA interactions in various cell lines.** *A,* reporter gene assays were conducted to investigate the ASBT promoter functioning status in various cell lines as indicated in the figure. Cells were transfected with the construct pGL3-ASBT5′/$^{-208/+118}$, followed by measurement of luciferase activities in these cells. ASBT 5′-driven luciferase expression was only detected in Caco-2 cells, but not in HeLa, HepG2, and MDCK cell lines. Co-expression of c-Jun with P3 in MDCK cells led to luciferase activity, which was greater than that observed in native Caco-2 cells. *B,* band-shift assays were carried out with the radiolabeled P3 as probe and nuclear extracts from different cell lines as indicated on the *top* of each lane. Like Caco-2, all ABPs are present in HepG2 cells. However, ABP2 is missing in HeLa and MDCK cells.

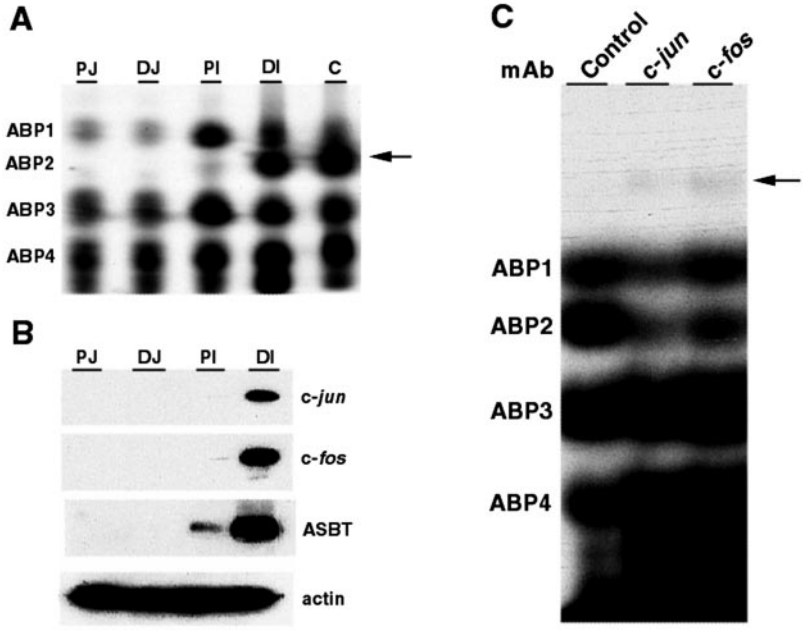

FIG. 8. **Analysis of ASBT DNA/protein interactions along the longitudinal axis of the rat small intestine.** *A,* band-shift assays were carried out with radiolabeled P3 as a probe and nuclear extracts from different quartiles of the rat small intestine (*PJ,* proximal jejunum; *DJ,* distal jejunum; *PI,* proximal ileum; *C,* Caco-2 (positive control)). ABP2 is only observed primarily in the distal ileum. Faint signal is detected in PI. *B,* Western blot analysis was performed using ileal homogenates or membrane vesicles. c-Jun (78 kDa), c-Fos (75 kDa), and ASBT (48 kDa) were detected primarily in the DI segment of rat intestine, although a small amount of the three proteins could be found in the PI segment. These proteins were absent in the PJ and DJ segments. Equivalent loading is demonstrated by signal intensity for the 45-kDa actin protein. *C,* supershift analysis of rat ileal mucosal nuclear extracts demonstrates the presence of both c-Jun and c-Fos. A phosphotyrosine antibody was used as a negative control.

initiation sites in the rat ASBT 5′-region. This finding is consistent with RNase protection assays which identified two different sized copies of rat ASBT transcripts (48). These two sites in the rat are significantly closer together than the two sites that have been identified in humans (8, 49). The 2.7-kb ASBT 5′-flanking sequence exhibited very strong promoter activity, ~60% of that of SV40 promoter. The downstream 0.8-kb fragment expresses moderate promoter activity, indicating the 1.9-kb fragment may contain enhancer element(s). Basic promoter activity was found within a 208-bp region immediately upstream of the lower transcription initiation site.

This minimal promoter contains two transcription initiation sites and a TATA box between the two sites (Fig. 2, *construct e*),

TABLE II
Pattern of expression of AP-1 proteins along the longitudinal axis of the rat intestine

The presence or absence of various AP-1 proteins as determined by Western blotting is indicated by a + or −, respectively.

	PJ	DJ	PI	DI
Jun	−	−	−	+
Jun B	−	−	−	−
Jun D	−	+	+	+
Fos	−	−	−	+
Fos B	−	−	−	+
Fra-1	−	+	−	+
Fra-2	−	−	−	−

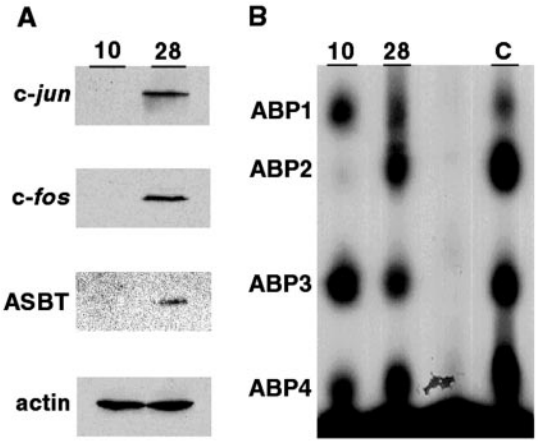

FIG. 9. **Analysis of ASBT DNA/protein interactions during post-natal development of the rat small intestine.** *A,* Western blot analysis was performed using ileal homogenates or membrane vesicles. c-Jun (78 kDa), c-Fos (75 kDa), and ASBT (48 kDa) were detected from 28 but not 10-day-old rat ileum. Equivalent loading is demonstrated by signal intensity for the 45-kDa actin protein. *B,* band-shift assays were carried out with radiolabeled P3 as a probe and nuclear extracts from the terminal ileum of 10- (pre-weaning) and 28 (post-weaning)-day-old rats. ABP2 is only observed in the 28-day-old rat ileum. Caco-2 cell nuclear extract was used as a positive control (*c*).

suggesting a potential core sequence for the origin of ASBT transcription. This is supported by the finding that this sequence, starting from −208, is highly conserved in the ASBT 5′-region of different species including rat, human, and rabbit, with an average homology of 88% (Fig. 10). Electrophoretic mobility shift assays revealed that four nuclear proteins ABP1-ABP4 bound this minimal promoter sequence in a specific fashion as determined by competition studies. The *trans*-acting binding activities, especially for ABP1 and ABP2, were further mapped to the P3-related subfragments C4 and C5, by band-shift assay with *in vitro* synthesized oligonucleotides. Both C4 and C5 contain an AP-1 consensus sequence. These sites were absolutely conserved in the proximal promoters in humans and rabbits. Furthermore, an *in vitro* synthesized 15-bp fragment containing a normal AP-1 consensus sequence was able to directly bind to ABP2 and efficiently competed off the protein binding activity of P3; whereas, a 15-bp sequence containing a mutated AP-1 element failed to have these abilities. These results indicate that AP-1 is the ABP2-binding *cis*-element. This was further strengthened by detection of a supershifted ABP2 with a c-Jun monoclonal antibody. Rat ileal mucosa contained proteins that supershifted with both c-Fos and c-Jun antibodies. A number of potential explanations exist to explain the differential expression of c-Fos. Caco-2 cells derived from a human epithelial tumor cell line, while rat ileal mucosal scrapings are derived from a number of different cell types. The functional significance of the difference in expression in c-Fos will need to be assessed. The enhancement of ASBT promoter activity, in both Caco-2 and MDCK cells, by co-expression of c-Jun strongly supports the hypothesis that AP-1 plays an important role in the transcriptional regulation of ASBT.

AP-1-binding proteins are composed of a dimer containing Jun, Fos, or other activating transcription factor proteins depending on the individual targeting gene (38). Given the diversity of potential elements that may bind to the AP-1 site, a wide variety of regulatory phenomenon may be mediated by AP-1. There is a growing body of evidence that suggests that AP-1 may play an important role in intestinal gene expression. In the rat intestinal cell line, IEC-6, dexamethasone lead to an increase in c-*jun* mRNA and increased AP-1 DNA binding (50). Enterocyte-like differentiation of the human colon cancer cell line, Caco-2, has been associated with an increase in c-Jun, JunD, c-Fos, and Fra-2 gene products (51). Intestinal cell growth as mediated by either L-glutamine or polyamines appears to be mediated at least in part through AP-1 (52, 53). After surgically induced intestinal ischemia and subsequent reperfusion, AP-1 activity is markedly up-regulated via c-*fos* (54). Finally, the intestinal specific expression of the neurotensin/neuromedin N gene may be dependent upon at AP-1/CRE-like motif in the proximal promoter of this gene (55).

One potential regulatory role for AP-1 may be the control of tissue and region-specific expression. Analysis of the expression of AP-1 *trans*-acting factors in the developing mouse and chicken support this hypothesis (56–58). For example, JunB expression appears to be restricted to the developing mouse skin and intestine (56). Our studies demonstrate both region- and developmental stage-specific expression of c-Jun and c-Fos in the rat small intestine. The correlation of the expression of these AP-1 elements with ASBT DNA-binding protein patterns on gel shift analysis and the expression of ASBT strongly suggests that AP-1 is involved in determining region and developmental stage specificity. This is a major advance, since transgenic mouse studies have indicated that *cis*-acting factors may impart specific patterns of expression in the intestine, although the specific *cis*- and *trans*-acting elements have not been determined (46, 59–61).

ASBT 5′-directed gene expression is cell line specific, although the *in vitro* transcriptional activity cannot be completely reconciled with the presence of ASBT transcripts in specific cell lines. Clearly, Caco-2 cells contain all of the necessary transcriptional machinery for ASBT expression. In contrast, HeLa cells do not generate ASBT transcripts and do not express ABP2. On the other hand MDCK cells contain the necessary machinery to generate ASBT transcripts, but do not support promoter activity. The absence of ABP2 in MDCK cells may indicate that a different set of binding proteins mediate ASBT expression in kidney and/or in dogs. It is especially important that c-Jun expression in MDCK cells endows this cell line with the capacity to support ASBT promoter activity. Finally, the ABP2 detected in HepG2 cells appears to bind to DNA but does not trans-activate ASBT expression. This indicates that HepG2 cells contain an AP-1 complex with a different composition. Liver cells have a distinct but related sodium-dependent bile acid transporter system whose transcriptional regulation is not mediated through AP-1 (62). Further experiments are needed to characterize the biochemical properties of the two ABP2s found in Caco-2 and HepG2 cell lines, respectively.

Recent knock-out studies indicate that HNF-1α also plays a key role in the transcriptional regulation of ASBT (24). The knock-out mice have increased fecal and urinary excretion of bile salts, which is coupled with reduced ileal expression of ASBT protein and mRNA. Analysis of the human ASBT promoter revealed a functional hepatocyte nuclear factor-1α-binding site. This site is highly conserved and is immediately proximal to the conserved AP-1-binding site (see Fig. 10). These findings strongly suggest that the complex patterns of expression of ASBT may be controlled by combinatorial regulation utilizing both hepatocyte nuclear factor-1α and AP-1 (63, 64). This is supported by the fact that the expression of the HNF-1 protein during intestinal development and along the longitudinal axis of the intestine is similar to the pattern seen for ASBT.

In summary, the rat ASBT gene has been cloned and its promoter analyzed by luciferase reporter gene assays and studies on DNA/nuclear protein interactions. A 208-bp region of the proximal promoter appears to be sufficient to impart basic promoter activity and cell line specificity. These effects appear

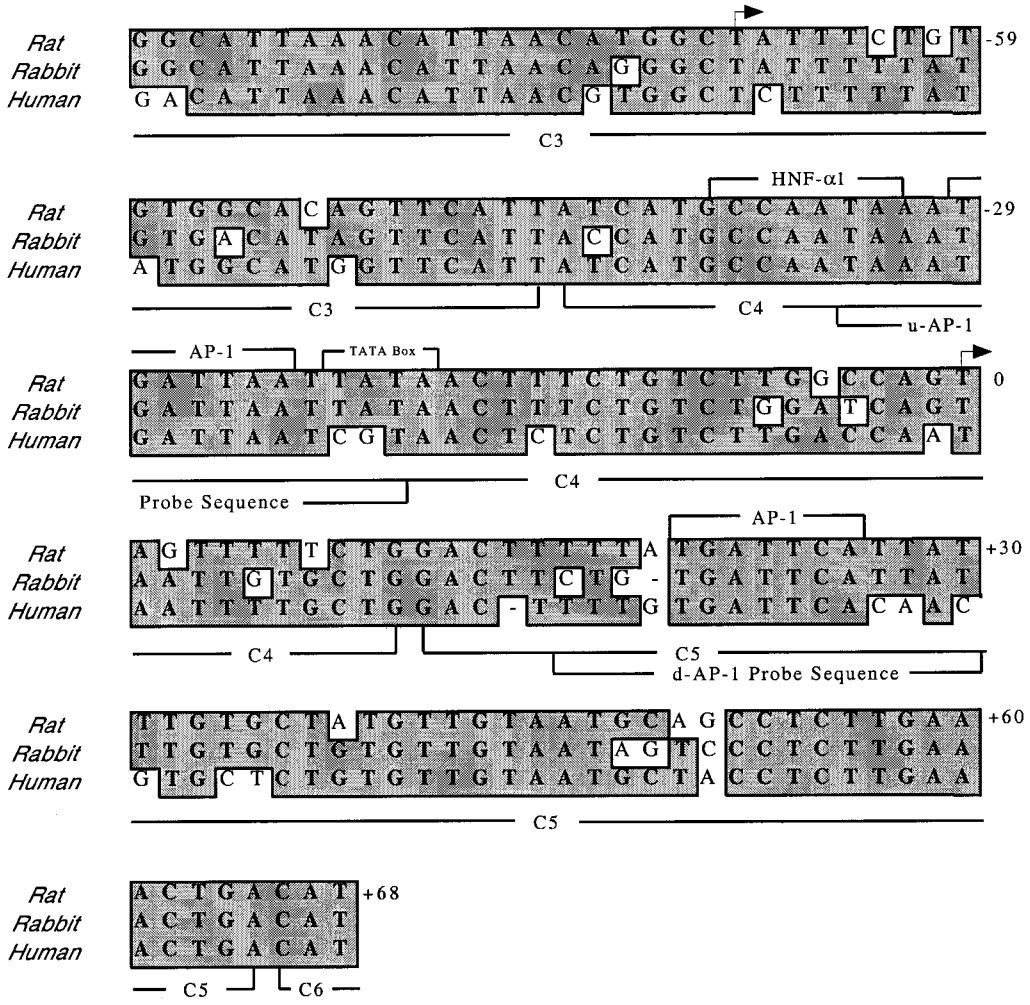

FIG. 10. **Homology of ASBT 5′ sequences of rat, rabbit, and human genes.** Computer-aided nucleotide sequence comparisons reveal that there is an average of 88% homology between rat and rabbit and 88% homology between rat and human ASBT 5′ sequences (rat genomic ASBT nucleotides −89 to +68). The two AP-1 *cis*-elements and the HNF-1α sites involved in rat ASBT regulation are absolutely conserved in the 5′ regions of both rabbit and human species. The location of the oligos, C3, C4, C5, u-AP-1, and d-AP-1 are indicated by *bars*. The site of the point mutations in the AP-1 elements are indicated by the *arrowheads*.

to be mediated in large part through AP-1. Mutations of the AP-1 *cis*-elements lead to suppression of ASBT directed gene expression. The trans-acting factors involved in ASBT expression include at least four nuclear proteins ABP1–4, among which ABP2 has been identified as an AP-1 element binding both c-Jun and c-Fos. Formation of the complex ABP2 appears to be correlated with cell line specificity, and region- and developmental stage-specific expression in the rat small intestine. Further analysis of this system will provide important insights into intestinal gene regulation and many aspects of the enterohepatic circulation of bile acids.

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