Regulation of the gene encoding the intestinal bile acid transporter ASBT by the caudal-type homeobox proteins CDX1 and CDX2

Li Ma,1 Moritz Jüttner,1 Gerd A. Kullak-Ublick,1,2 and Jyrki J. Eloranta1,2
1Department of Clinical Pharmacology and Toxicology, University Hospital Zurich; and 2Zurich University Research Priority Programme “Integrative Human Physiology” (ZIHP), Zurich, Switzerland

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Ma L, Jüttner M, Kullak-Ublick GA, Eloranta JJ. Regulation of the gene encoding the intestinal bile acid transporter ASBT by the caudal-type homeobox proteins CDX1 and CDX2. Am J Physiol Gastrointest Liver Physiol 302: G123–G133, 2012. First published October 20, 2011; doi:10.1152/ajpgi.00102.2011.—The apical sodium-dependent bile acid transporter (ASBT) is expressed abundantly in the ileum and mediates bile acid absorption across the apical membranes. Caudal-type homeobox proteins CDX1 and CDX2 are transcription factors that regulate genes involved in intestinal epithelial differentiation and proliferation. Aberrant expression of both ASBT and CDXs in Barrett’s esophagus (BE) prompted us to study, whether the expression of the ASBT gene is regulated by CDXs. Short interfering RNA-mediated knockdown of CDXs resulted in reduced ASBT mRNA expression in intestinal cells. CDXs strongly induced the activity of the ASBT promoter in reporter assays in esophageal and intestinal cells. Nine CDX binding sites were predicted in silico within the ASBT promoter, and binding of CDXs to six of them was verified in vitro and within living cells by electrophoretic mobility shift assays and chromatin immunoprecipitation assays, respectively. RNAs were extracted from esophageal biopsies from 20 BE patients and analyzed by real-time PCR. Correlation with ASBT expression was found for CDX1, CDX2, and HNF-1α in BE biopsies. In conclusion, the human ASBT promoter is activated transcriptionally by CDX1 and CDX2. Our finding provides a possible explanation for the reported observation that ASBT is aberrantly expressed in esophageal metaplasia that also expresses CDX transcription factors.

transcriptional regulation; bile acid transport; Barrett’s esophagus

Bile acids are synthesized as a result of cholesterol catabolism in the liver, and they function as physiological detergents to facilitate the intestinal absorption of dietary fats and fat-soluble vitamins. The apical sodium-dependent bile acid transporter (ASBT; gene symbol SLC10A2) is a 48-kDa transmembrane protein expressed in the terminal ileum, biliary tract, and renal proximal tubule cells. At the apical membrane of ileal enterocytes, ASBT is the chief mediator of active sodium-dependent bile acid absorption (11). Bile acids are then effluxed into the portal blood via the heterodimeric organic solute transporter-α/β (OSTα/β) at the basolateral membrane. Together with the basolateral bile acid uptake system Na+/taurocholate cotransporting polypeptide (NTCP) at the sinusoidal membrane of hepatocytes and bile salt efflux pump (BSEP) at the liver canalicular membrane, ASBT and OSTα/β maintain efficient enterohepatic bile acid circulation (1, 17).

Prior studies have shown that ASBT gene expression is controlled at the transcriptional level in a complex manner: known transcriptional regulators of the human ASBT promoter include the glucocorticoid receptor (GR) (22), the hepatocyte nuclear factor-1α (HNF-1α) (23, 40), and the peroxisome proliferator-activated receptor-α (PPARα) (23). Both serum (12) and certain bile acids (13) appear to elevate ASBT expression via a response element for the activator protein-1 (AP-1). In a knockout mouse model, the transcription factor Gata4 regulates the jejunal-ileal gradient of ASBT expression (4). Alterations in ASBT function or ASBT gene expression have been associated with intestinal diseases in humans: mutations in the ASBT gene can lead to bile acid malabsorption (33), whereas reduced ileal ASBT protein (22) or mRNA (45) levels have been found in patients with inflammatory bowel disease (IBD). It was recently shown that the expression levels of three bile acid transporters [ASBT, the multidrug resistance protein-3 (MRP3), and the ileal bile-acid-binding protein (I-BABP)] are elevated in esophageal epithelial cells from patients who are diagnosed with Barrett’s esophagus (BE) and are again decreased or lost in esophageal adenocarcinoma at both mRNA and protein levels (16). However, the precise mechanisms, which mediate the aberrant expression of the ASBT transporter gene in BE, are not known. BE is a premalignant lesion to esophageal adenocarcinoma, where squamous epithelium at the distal end of the esophagus is replaced by an intestine-like epithelium. The molecular origins of BE are not yet fully understood. One theory suggests that bile acids in the gastroesophageal reflux contents cause tight junctions to break in squamous cells, allowing them to leak into the basal layer, thus inducing cell transdifferentiation (37). Another recent study by Wang et al. (48) suggests that the transcription factor p63 plays a role in the etiology of BE (44).

Caudal-type homeobox-1 (CDX1) and -2 (CDX2) are transcription factors of the homeodomain family, and homologs of the Drosophila melanogaster protein Caudal that is essential for the early anterior-posterior development and body patternig (19). Despite the high level of amino acid identity (95%) within their DNA-binding domains, they play at least partially distinct roles in the intestine. Cdtx1−/− mice exhibit a shift in the body frame (39), and Cdtx2+/− heterozygous mutant mice develop colonic tumors (10). These two mouse models also show differential responses upon inflammatory challenge (7). Complete Cdtx2-null mice are embryonic lethals, as evidently are Cdtx1/Cdtx2 double-null mice. In adult mammals, Cdtxs/Cdtxs are expressed exclusively in the small and large intestine, playing important roles in proliferation and differentiation of intestinal epithelial cells. However, their levels have been reported to be aberrantly elevated in esophageal metaplasia, such as BE, compared with the adjacent normal tissue (36, 41). Similarly to the bile acid transporters, CDX2 expression has been reported to be decreased upon progression of esophageal metaplasia into high-grade adenocarcinoma (21, 42). A number

Address for reprint requests and other correspondence: J. J. Eloranta, Dept. of Clinical Pharmacology and Toxicology, Univ. Hospital Zurich, Rämistrasse 100, CH-8091 Zurich, Switzerland (e-mail: jyrki.eloranta@usz.ch).
of CDX1 and CDX2 transcriptional target genes have been identified so far. These include marker genes for intestinal differentiation, such as sucrase isomaltase (5), mucin 2 (32), cytokeratin 20 (9), and desmocollin 2 (20), as well as genes encoding intestinal transporters, such as peptide transporter 1 (PEPT1; gene symbol SLC15A1) (35) and sodium-coupled monocarboxylate transporter 1 (SCMT1; gene symbol SLC5A8) (24). Furthermore, it has been suggested that CDX1 and CDX2 can regulate their own expression in esophageal cell lines via both auto- and crossregulation (26).

Aberrant esophageal expression of both ASBT and CDXs in BE prompted us to study whether the expression of the ASBT gene is regulated by the CDXs.

MATERIALS AND METHODS

Chemicals. Deoxyadenosine 5'-[α-32P]triphosphate (6,000 Ci/mmol) was purchased from Perkin Elmer (Schwerzenbach, Switzerland). All chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland), unlabeled oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). Other chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland), unless stated otherwise.

Cell culture. All cell lines were obtained from LGC Promochem (Molsheim, France). Human esophagus-derived cell line Het-1A was cultured in bronchial epithelial cell growth medium (BEGM) supplemented with 5% FBS (Brunschwig, Basel, Switzerland), and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). The epithelial cell line IEC-4.1 (30) (a kind gift from Dr. Anthony Jevnikar), derived from small intestines of BALB/c mice, was cultured on collagen-coated plates in DMEM/F-12 (1:1) media supplemented with 5% FBS, 1 mM sodium pyruvate, 10 ng/ml epidermal growth factor, and antibiotics as above. All cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C.

Transfection of cells with siRNAs. Cells were seeded at 1 × 106 cells/well onto 6-well plates and transfected with hCDX1 SiGenome SMARTpool (Dharmacon, Lafayette, CO), hCDX2 SiGenome SMARTpool, or siCONTROL nontargeting siRNA #2 (Dharmacon) at a final concentration of 50 nM using the TransIT-TKO reagent (Mirus Bio, LabForce). The transfections were repeated after 24 h, and the cells were harvested in 1 ml of TRIzol (Invitrogen) 24 h after the second transfection.

Reverse transcription and real-time PCR. Four micrograms of RNA were reverse transcribed with high-capacity cDNA reverse transcription kit (Applied Biosystems, Rotkreuz, Switzerland) before the real-time PCR analysis using the TaqMan assays Hs00156451_m1 (CDX1), Hs00230919_m1 (CDX2), Hs00166561_m1 (ASBT), Hs00167041_m1 (HNF-1α) and Hs00167042_m1 (villin), or human ACTB (beta-actin) endogenous control (Applied Biosystems) on a 7900HT fast real-time PCR system (Applied Biosystems). The relative mRNA levels were calculated by the comparative threshold cycle method. Within an experiment, all tests were performed in triplicate.

Reporter gene constructs and expression vectors. The human CDX1 and CDX2 expression constructs (kind gifts from Dr. Juan Lucio Iovanna) in the pcDNA3.1 vector (Invitrogen) were created as described in Ref. 31. Mutations in the constructs were corrected to wild-type by site-directed mutagenesis (QuickChange, Stratagene, La Jolla, CA). Cloning of the human ASBT (−1,688/+525) (23) and ASBT (−830/+525) (22) promoter constructs has been previously described. The human ASBT promoter fragment (−99/+525) was amplified using oligonucleotides shown in Table 1 and the promoter was inserted into pCMV-SPORT6 (Invitrogen) and pGHSPORT (Invitrogen). The promoters were introduced into mammalian cells in the presence of the reporter gene luciferase (Promega). Cells were transfected with ASBT promoter constructs and the pCMV-luciferase plasmid (1:10 ratio) using the TransIT-LT2 transfection reagent (Mirus Bio, LabForce).

Table 1. Sequences of oligonucleotides used for cloning, EMSAs, and ChIP assays

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Where applicable, restriction sites introduced are underlined and the corresponding enzymes used are given in parentheses. Only the top strands are shown for oligonucleotides used in electrophoretic mobility shift assays (EMSAs). CDXRE consensus motifs are indicated in bold, and introduced mutations are in italics. ChIP, chromatin immunoprecipitation assay.
construct ASBT (~1.68/1.825) as the template. The PCR fragment was cloned into the pGEM-T vector (Promega, Dübendorf, Switzerland), and further subcloned into the pG3basic vector (Promega). The mouse ASBT promoter fragment (~1.81/0+3) and the rat ASBT promoter fragment (~2.975/1+174) were cloned from mouse or rat, respectively, genomic DNA (Clontech) using PCR oligonucleotides shown in Table 1. Both PCR products were cloned into pGEM-T vector and further subcloned into the pG3basic vector. To create the pTNT-CDX constructs for in vitro translation, the CDX1 and CDX2 cDNA fragments were cut from the pcDNA3.1 constructs with EcoRI and subcloned into the EcoRI restriction site of the expression vector pTNT (Promega). The identities of all PCR-cloned fragments were verified by DNA sequencing (Microsynth).

**Transient plasmid transfections and luciferase reporter assays.** To study the effect of CDX overexpression on endogenous ASBT mRNA expression, OE19 cells were seeded on 12-well plates at the density of 250,000 cells/well the day before transfections. Cells were transfected with 2.5 μg of either pcDNA3.1 vector control or 2.5 μg of either CDX expression construct, using 3 μl of the FuGene HD transfection reagent (Roche Diagnostics) per microliter of plasmid DNA. Twenty-four hours later, cells were harvested in 0.5 ml of TRIzol/well, and RNAs were processed as described above. For reporter assays, cells were seeded on 48-well plates at a density of 1 × 10⁵ cells/well for Caco-2, LS174T, and T84 cells, or 50,000 cells/well for Het-1A, OE19, and IEC-4.1 cells 24 h before transfections. Cells were cotransfected with 400 ng of the luciferase reporter construct and 200 ng of the expression plasmids at a ratio of 3 μl FuGENE HD per 1 μg DNA. To normalize the amount of DNA transfected, the pcDNA3.1 (+) vector was added where appropriate. To control for transfection efficiency, 100 ng of the renilla luciferase (pRL-CMV) reporter plasmid (Promega) were cotransfected. Cells were harvested 36 h after transfections, and luciferase activities were determined using the dual Luciferase assay system (Promega) and a Luminoskan Ascent microplate luminometer (Thermo Fisher Scientific, Wohlen, Switzerland). Reporter activities obtained for the empty pG3basic corresponding to each test condition, as well as for the test construct containing the test promoter in the control conditions, were set to one, and fold activities are shown relative to this. All experimental conditions were performed in triplicate, and the experiments were repeated at least three times.

**Electrophoretic mobility shift assays.** Oligonucleotides used in electrophoretic mobility shift assays (EMSAs) (Table 1) were designed to have a 5'-AGCT overhang in the top strand and a 5'-GATC overhang in the bottom strand when annealed, allowing radioactive labeling by fill-in reactions. Fifty nanograms of annealed oligonucleotides were labeled in 20-μl reactions containing 1 μl of MultiScribe reverse transcriptase (50 U/μl) (Applied Biosystems), 1 μl of 1 × RT buffer, 250 nM dGTP/dCTP/dTTP, and 1 μl of [α-32P]dATP. Unincorporated nucleotides were removed using Microspin G-25 columns (GE Healthcare). Six micrograms of LS174T nuclear extracts prepared using the NE-PER extraction kit (Pierce, Lausanne, Switzerland) or 1.5 μl of in vitro translated CDX1/CDX2 proteins each, generated with Tnt T7 coupled reticulocyte lysate system (Promega) using the plasmids pTNT-CDX1 and pTNT-CDX2 as templates, were used per DNA-binding reaction. Protein-DNA complexes were formed in the binding buffer [20 mM Tris-HCl, pH 8.0; 60 mM KCl; 2 mM MgCl2 12% (vol/vol) glycerol; 0.3 mM DTT; 87.5 ng/μl preboiled poly(dI-dC)-poly(dI-dC)] in a total volume of 20 μl for 10 min at +30°C. After this, 50,000 counts/million (0.5–1.5 ng) of the radioactive probes were added to reactions. For competition EMSAs, 10-, 50-, or 100-fold molar excess of unlabeled ASBT promoter CDX-binding site-containing oligonucleotides was added immediately before the radioactive probe containing a consensus CDX response element (CDXRE). In supershift experiments, 1 μl of the monoclonal CDX1 antibody (9) (a kind gift from Dr. Walter Bodmer and Dr. Carol Chan), polyclonal CDX2 antibody (C6747, Sigma), polyclonal RXRα antibody (sc-774, Santa Cruz, CA), or polyclonal PPARα antibody (sc-1985) were added to the extracts 1 h before the probe and incubated at +4°C. Immediately after the binding reactions, the samples were loaded onto preelectrophoresed 5% (acrylamide/bis 30:1) native acrylamide gels and run at 200 V in ×0.5 TBE for 3 h. The gels were fixed in 10% (vol/vol) acetic acid for 10 min, dried onto Whatman DE81 paper under vacuum, and exposed to Kodak BioMax MR-1 films at ~80°C.

**Chromatin immunoprecipitation assays.** Chromatin immunoprecipitation (ChIP) assays were carried out essentially as described previously (18). Briefly, T84 cells were grown on 10-cm plates to 80% confluence, and the cells were harvested by cross-linking with 1% methanol-free formaldehyde and processed through chromatin immunoprecipitations using the ChiP-IT Express Kit (Active Motif, Rixensart, Belgium). Shearing of the chromatin was achieved by five 20-s pulses of sonication with 30-s pauses on ice between each pulse, using a Branson Digital Sonifer (Branson Ultrasonics, Danbury, CT) at power setting of 25%. For the immunoprecipitations, the samples were incubated with 2 μg of negative control antiserum mouse IgG1 (Dako Denmark, Glostrup, Denmark) or 2 μg of one of the following antibodies: anti-CDX1 (CDX1 Ab1), anti-CDX2 (Ab24000, Abcam, Cambridge, UK) (CDX1 Ab2), anti-CDX2 (71–83, Sigma) (CDX2 Ab1), anti-CDX2 (MU392A-100, Biogenex, Fremont, CA) (CDX2 Ab2). Two amplicons were assayed for immunoprecipitated samples, using the oligonucleotide primers listed in Table 1, containing either the ASBT promoter region (~736–154) or a region from the first intron of the ASBT gene. After the initial denaturation stage at 94°C for 3 min, the PCR cycling conditions were 94°C for 20 s, 60°C for 30 s, and 72°C for 1 min. For the ASBT promoter region, 5 μl of each PCR product were taken after 38 cycles, and for the ASBT intron fragment, 5 μl of each PCR product were taken after 30 cycles. The ASBT promoter and intronic PCR fragments were resolved on 1.5% agarose gels, containing GelRed gel stain (Chemie Brunschwig, Basel, Switzerland).

**Human tissue collection and processing.** Signed, informed consent forms were collected for all human subjects. The study protocol and consent forms were approved by the Ethical Committees of the sample collections sites. A total of 35 patients were included in the study. BE tissue was collected at the Department of Gastroenterology, Qingdao Municipal Hospital, China; Department of Gastroenterology, Seventh People’s Hospital, Tongji University School of Medicine, Shanghai, China; Division of Gastroenterology, Chinese PLA General Hospital, Beijing, China, and Department of Gastroenterology, General Hospital of Chinese People’s Armed Police Forces, Beijing, China in 2011. BE were diagnosed endoscopically and histologically on the basis of typical features such as the presence of specialized columnar epithelium and goblet cell. Histology was performed by an experienced local pathologist. Of the 35 patients, 22 (63%) were men and 31 (65%) women. The mean age ± SD of the cohort was 52.60 ± 10.97 yr, and the median age was 52.00 yr. Among patients, 29/35 (83%) had detectable CDX1 mRNA expression, 35/35 (100%) had detectable CDX2 expression, and 20/35 (57%) had detectable ASBT expression. The BE samples were homogenized by ultrason. RNAs were isolated using the TRIzol reagent (Invitrogen) and were quantified with a NanoDrop ND-1000 spectrophotometer (Witec, Littau, Switzerland). Ileal biopsies were obtained from 48 healthy patients during routine surveillance colonoscopies in the endoscopy unit of the Division of Gastroenterology and Hepatology at the University Hospital Zurich between 2004 and 2008. Of the 48 patients, 17 (35%) were men and 31 (65%) women. The mean age ± SD of the cohort was 54.3 ± 13.7 yr, and the median age was 58.5 yr. Ileal samples were collected in RNA Later (Ambion, Austin, TX) and syringed into TRIzol reagent before RNA isolation. RNAs were quantified with a NanoDrop ND-1000 spectrophotometer.

**Statistical analysis.** All cell-based experiments were independently repeated at least three times, and representative experiments are shown. All quantitative data are reported as means ± SD. Differences between experimental groups were analyzed by one-way ANOVA.
with Tukey’s post hoc test. P values of <0.05 were considered significant. For the correlation of mRNA expression levels in human tissue material, logarithmic values of mRNA levels were analyzed using linear regression. All statistical analysis was carried out using GraphPad Prism (GraphPad Software, San Diego, CA).

RESULTS

Modulation of endogenous ASBT mRNA expression levels by CDX knockdowns or overexpression. To investigate whether endogenous human ASBT mRNA levels are regulated by CDX1 and CDX2, we performed knockdown experiments with pools of siRNAs specifically targeting CDXs. For this purpose, we chose the human colon cancer cell line T84, which endogenously expresses both CDX1, CDX2, and ASBT, at all detectable levels. By targeting the endogenous CDX1 or CDX2 with specific siRNAs, ASBT mRNA was significantly decreased compared with its expression in control cells (Fig. 1A). Upon CDX1 siRNA treatment, there was an 80% loss of endogenous CDX1 mRNA, and no decrease of the endogenous CDX2 mRNA level (Fig. 1B). Conversely, upon CDX2 siRNA treatment, there was a significant loss of endogenous CDX2 mRNA, but endogenous CDX1 mRNA expression level remained unchanged (Fig. 1C). The siRNA knockdowns thus worked in a gene-specific manner. In a converse approach, we transiently overexpressed CDX1 and CDX2 in human esophagus-derived OE19 cells. As shown in Fig. 1D, endogenous mRNA levels of ASBT were significantly increased upon exogenous expression of CDX1 and CDX2 in the esophageal cell line.

CDX1 and CDX2 transactivate the ASBT promoter. To further study the possible direct role for CDX1 and CDX2 in the regulation of the ASBT promoter, we transiently transfected two human esophagus-derived cell lines and three human colon-derived cell lines with expression plasmids for human CDX1 and CDX2 together with the ASBT (−1,688/+525) promoter-luciferase construct. Both CDXs increased the ASBT promoter activity in the two esophageal cell lines, OE19 (Fig. 2A) and Het-1A (Fig. 2B). Potent CDX-mediated activation of the ASBT promoter was also observed in intestinal T84 (Fig. 2C), Caco-2 (Fig. 2D), and LS174T cells (Fig. 2E). A comparative study with the human, mouse, and rat ASBT/Asbt promoter-luciferase constructs transfected into Caco-2 and murine IEC-4.1 cells revealed an increased activity of the mouse Asbt promoter by both CDX1 and CDX2 in both cell lines, whereas the rat Asbt promoter was significantly activated by both CDX1 and CDX2 in IEC-4.1 cells but only by CDX2 in Caco-2 cells (Fig. 1, F and G).

In silico analysis of the ASBT promoter region. Next, we performed an in silico analysis on the proximal 850 base pairs of the human ASBT promoter (NCBI reference sequence: NT_009952.14) to identify putative CDX response elements (CDXREs). The consensus core motif for CDX DNA-binding is 5′-TTTA/GC-3′ or 5′-G/CATAAA-3′ (19). We identified nine putative CDXREs within the proximal ASBT (−850/+1) promoter region by MatInspector software (8) and visual inspection (Fig. 3).

The region containing the predicted CDX binding sites mediates ASBT promoter activation. To study whether the proximal promoter region containing the predicted CDXREs can mediate the CDX-dependent activation, we used two ASBT promoter deletion constructs in transient transfections in

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**Fig. 1.** Modulation of endogenous apical sodium-dependent bile acid transporter (ASBT) mRNA expression levels by CDX1 and CDX2. A–C: ASBT mRNA expression levels are reduced upon knockdown of endogenous CDX1 and CDX2 expression in T84 cells. Cells were transfected with control nontargeting siRNA, siCDX1, or siCDX2 at a final concentration of 50 nM twice at a 24-h interval and harvested after 48 h. The ASBT, CDX1, and CDX2 mRNA expression levels are normalized to those obtained for beta-actin. A: knockdown of either CDX1 or CDX2 leads to a significant decrease in ASBT mRNA expression. B: CDX1 siRNAs significantly reduce CDX1 but not CDX2 expression. C: CDX2 siRNAs significantly reduce CDX2 but not CDX1 expression. D: exogenous expression of CDX1 and CDX2 significantly increases endogenous ASBT mRNA levels in human esophagus-derived OE19 cells. Significant difference: **P < 0.01; ***P < 0.001.
Caco-2 cells. The construct \textit{ASBT} (−830/+525), which contained all the predicted \textit{CDXRE}s, was potently activated by both CDX1 and CDX2, whereas the construct \textit{ASBT} (−99/+525), lacking all predicted \textit{CDXRE}s, exhibited significantly reduced activation (Fig. 4). The activation by CDXs was not entirely abolished, suggesting that there may be further \textit{CDXRE}s located in the 5′-UTR of the \textit{ASBT} gene. We further performed individual site-directed mutagenesis on all predicted \textit{CDXRE}s, but disruption of any single element was not sufficient to efficiently reduce the CDX-mediated transactivation of the \textit{ASBT} promoter in reporter assays, implying that several of the predicted \textit{CDXRE}s are involved in this regulation (data not shown).

\textit{CDX1} and \textit{CDX2} bind to six of the nine predicted \textit{CDX response elements} in vitro. To study the potential in vitro interaction between \textit{CDX1} and \textit{CDX2} with their predicted binding motifs within the \textit{ASBT} promoter, we performed EMSAs using nuclear extracts derived from LS174T cells, in which both \textit{CDX1} and \textit{CDX2} are endogenously expressed, using both wild-type and mutant predicted \textit{CDX response element} as radiolabeled probes. Two DNA-protein complexes were formed on the \textit{CDXRE}s 2, 3, 4, 7, 8, and 9, which were abolished by addition of either \textit{CDX1} (the faster complex) or \textit{CDX2} antibodies (the slower complex) (Fig. 5A). Addition of unrelated antibodies raised against nuclear receptor transcription factors PPARα or RXRα did not affect CDX-DNA com-

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Fig. 2. CDX1 and CDX2 transactivate the human \textit{ASBT} (A–E), mouse (\textit{mAsbt}; \textit{F} and \textit{G}), and rat (\textit{rAsbt}; \textit{F} and \textit{G}) \textit{Asbt} promoters. Two hundred nanograms of \textit{CDX1} or \textit{CDX2} expression plasmids were transfected into OE19, Het-1A, T84, Caco-2, and LS174T cells, together with 400 ng \textit{ASBT} (−1,688/+525) promoter luciferase construct. Reporter activities were measured 36 h later. A: OE19 cells. B: Het-1A cells. C: T84 cells. D: Caco-2 cells. E: LS174T cells. F: Caco-2 cells. G: IEC-4.1 cells. Significant difference: ** \(P < 0.01\); *** \(P < 0.001\); ns, not significant.
ABST PROMOTER IS REGULATED BY CDXS

lanes 6
binding sites (supershift. The interaction between the radiolabeled probes and CDXs was abolished or strongly reduced upon introducing mutations to critical bases within CDX

CDX2-probe complexes supershifted with antibodies. The anti-CDX1 antibody leads to the abolishment of the CDX1-probe complexes rather than a defined

image panels are derived from the same experiment and from film exposure times of equal length.

containing the consensus CDX response element derived from the human desmocollin 2 promoter was used as the radioactively labeled probe. The two EMSA

: competition EMSAs were carried out using in vitro translated CDX1 and CDX2 proteins, both simultaneously added to the binding reactions. Unlabeled

Further confirm the specificity of CDX1 and CDX2 binding to

specific complexes with CDXs in EMSAs (data not shown). To

CDX binding were introduced into the oligonucleotides and

plex formation. When mutations designed to interfere with

CDX binding were introduced into the oligonucleotides and

used as the radioactively labeled probes, the formation of the two CDX-DNA complexes was abolished or strongly reduced. The other putative CDXREs within the proximal ABST promoter (CDXREs 1, 5, and 6) predicted in silico failed to form specific complexes with CDXs in EMSAs (data not shown). To further confirm the specificity of CDX1 and CDX2 binding to the CDXREs within the ASBT promoter, we performed EMSA competition experiments (Fig. 5B), using the CDX consensus binding site from the human desmocollin 2 gene as a radiolabeled probe and increasing molar excesses of unlabeled competitor oligonucleotides harboring the ASBT promoter region. As an additional specificity control, we amplified a 221-bp region from the first intron of the ASBT gene (Fig. 6, bottom). None of the CDX antibodies was able to precipitate this intronic region of the GAS gene using the same ChIP samples as templates. None of the CDX antibodies was able to precipitate this intronic region of the ASBT gene (Fig. 6, bottom).

Correlation analysis of CDX, ABST, and HNF-1α expression levels in human Barrett’s esophagus tissue and ileal tissue. To study whether CDXs or the known transactivator of the ASBT promoter, HNF-1α (34), may be chief factors in maintaining the expression of ASBT mRNA in human Barrett’s esophagus tissue, we measured their expression in RNAs isolated from esophageal biopsies derived from BE patients. In total, tissue was obtained from 35 patients, out of which 20, who expressed mRNAs of all genes of interest, were included in the analysis. ASBT mRNA levels significantly correlated with those of CDX1 (Fig. 7A), CDX2 (Fig. 7B), and HNF-1α

Fig. 3. Sequence of the proximal ASBT promoter region (−850/+525). The transcription start site is indicated by an arrow. The consensus motifs found in the nine predicted CDXREs are shown in bold. The location of the oligonucleotide primers used in ChIP assays (Fig. 6) are indicated with underlining arrows. The positions of the predicted CDXREs are as follows: CDXRE1 (−722/−717); CDXRE2 (−672/−667); CDXRE3 (−621/−616); CDXRE4 (−583/−578); CDXRE5 (−541/−536); CDXRE6 (−480/−475); CDXRE7 (−424/−419); CDXRE8 (−337/−332); CDXRE9 (−161/−156).

Fig. 4. The region containing the predicted CDX binding sites mediates ASBT promoter activation. Two hundred nanograms of the CDX1 or CDX2 expression plasmids were transfected into cells, together with 400 ng of the ASBT (−830/+525) or ASBT (−99/+525) promoter luciferase construct. Reporter activities were measured 36 h later. Significant difference: **P < 0.5; ***P < 0.001.

Fig. 5. A: CDX1 and CDX2 bind to six predicted CDXREs in vitro. EMSAs were carried out using nuclear extracts from LS174T cells. Oligonucleotides containing the six predicted wild-type or mutant CDXREs within the ASBT promoter were radioactively labeled to be used as probes. The CDX1 and CDX2 complexes are indicated by black arrowheads (lanes 1, 7, 13, 20, 26, 32). The specificity of the complexes was confirmed by incubation with an anti-CDX1 (lanes 2, 8, 14, 20, 26, 32) or anti-CDX2 (lanes 3, 9, 15, 21, 27, 33) antibody. To further confirm the specificity of the anti-CDX antibodies used in supershifts, anti-PPARα (lanes 4, 10, 16, 22, 28, 34) and anti-RXRα (lanes 5, 11, 17, 23, 29, 35) antibodies were tested in parallel. The asterisks indicate the location of CDX2-probe complexes supershifted with antibodies. The anti-CDX1 antibody leads to the abolishment of the CDX1-probe complexes rather than a defined supershift. The interaction between the radiolabeled probes and CDXs was abolished or strongly reduced upon introducing mutations to critical bases within CDX binding sites (lanes 6, 12, 18, 24, 30, 36). The two EMSA image panels are derived from the same experiment and from film exposure times of equal length. B: competition EMSAs were carried out in vitro translated CDX1 and CDX2 proteins, both simultaneously added to the binding reactions. Unlabeled oligonucleotides containing the CDXREs from the human ASBT promoter were added in 10-, 50-, or 100-fold molar excess, as indicated. The oligonucleotide containing the consensus CDX response element derived from the human desmocollin 2 promoter was used as the radioactively labeled probe. The two EMSA image panels are derived from the same experiment and from film exposure times of equal length.
ASBT PROMOTER IS REGULATED BY CDXS

A

Antibody

CDX2 →
CDX1 →

WT MUT WT MUT WT MUT WT MUT

CDX2 CDX2 PPARα RXRγ CDX2 CDX2 PPARα RXRγ CDX2 CDX2 PPARα RXRγ

CDXRE2 CDXRE3 CDXRE4

CDXRE7 CDXRE8 CDXRE9

B

Cold competitors

CDX2 →
CDX1 →

Free probes

WT MUT WT MUT WT MUT WT MUT

CDX2 CDX2 PPARα RXRγ CDX2 CDX2 PPARα RXRγ CDX2 CDX2 PPARα RXRγ

CDXRE2 CDXRE3 CDXRE4

CDXRE7 CDXRE8 CDXRE9
CDX1 and CDX2 play important roles in early gastrointestinal development, and also in gastrointestinal diseases in adult humans. Their ectopic overexpression in esophageal and stomach metaplasia suggests that they may play a role in the pathogenesis of these diseases (38). Alternatively, CDX expression could be the consequence rather than the cause of metaplasia. Given the elevated and ectopic expression of both CDXs and ASBT in BE, we studied whether CDXs may act as regulators of the ASBT promoter. Here, we have demonstrated in human esophageal and intestine-derived cell lines that the human ASBT promoter is a direct target for transcriptional activation by the transcription factors CDX1 and CDX2. Our finding adds the intestinal bile acid transporter, ASBT, to the list of genes regulated by CDXs. Other intestinal transporters regulated by CDXs include PEPT1 (35) and SMCT1 (24). Furthermore, our study adds another component to the already known complexity of transcriptional regulation of ASBT gene expression. It is currently not known whether the CDXs interact with the previously identified regulators of the ASBT promoter, such as GR (22), HNF-1α (23, 40), and PPARα (23).

In this context, it is interesting to note that it has been previously proposed that HNF-1α and CDX2 have been proposed to cooperate with each other in the regulation of the intestinal genes sucrase isomaltase (5) and calbindin-D9k (43).

The correlation between the mRNA expression levels of CDXs and the bile acid transporter ASBT was confirmed in BE biopsy material. The role of bile acids in the molecular pathogenesis of esophageal metaplasia still remains somewhat unclear, although bile acids have been shown to directly augment CDX1 (26) and CDX2 (6, 27) expression. It may thus be that, secondary to gastrointestinal reflux, the bile acids that enter the esophagus may lead to elevated ASBT expression via induction of its transactivators CDXs. Excess level of bile acids are toxic and have shown to be carcinogenic to the gastrointestinal tract (14) and liver (28, 29, 46), and bile acids are the major component of the duodenal juice, which can cause severe esophageal mucosal damage (25). Dvorak et al. demonstrated ASBT, the ileal bile acid-binding protein (IBABP), and the multidrug-resistant protein 3 (MRP3) are elevated in BE and decreased in esophageal adenocarcinoma at both the mRNA and protein levels (15). It has been proposed that bile acid transporter expression in gastrointestinal metaplasia of the esophagus serves as a protective mechanism by the host to remove excess bile acids secondary to chronic biliary reflux. In contrast, in healthy human ileal tissue, CDX expression did not correlate with ASBT expression, indicating that this regulatory mechanism may be more relevant in BE than in healthy ileum.

We note that correlation has been previously reported between ASBT and CDX2 in ileal tissue of patients suffering from chronic diarrhea (2).

In both BE and healthy ileal tissue, the mRNA levels of HNF-1α significantly correlated with ASBT mRNA expression levels in both BE and healthy ileal biopsies. This finding is also consistent with previous reports that Hnf-1α-null mice have severely reduced Asbt expression (34) and that ileal HNF-1α protein levels correlate with ASBT mRNA levels in humans (3). Furthermore, we have demonstrated that CDX1 and CDX2 mRNA expression levels significantly correlate with each other in both human BE and ileal tissue, supporting the prior suggestion that they can mutually regulate each other’s expression (26).

It is interesting to note that the CDX-mediated activation was conserved in the mouse and rat Asbt promoters. Consistent with this, our preliminary in silico analysis revealed several putative CDXREs in both rodent Asbt promoters (data not shown), even though their overall homology with the human ASBT promoter is low. The level of activation of the rodent Asbt promoters appeared lower than for the human ASBT promoter. However, we note the limitation of the experimental setup in that human CDX expression constructs were used in these experiments, and these may have somewhat different coactivator requirements from the rodent counterparts. It will be interesting to study whether the intestinal ASBT expression and consequent bile acid absorption is reduced in Cdx mutant mice, although it should be noted that complete Cdx1/Cdx2-null mice cannot be analyzed for this purpose since they are not viable until birth.

Based on our present study, we conclude that CDX-mediated promoter activation may lead to aberrant esophageal expression of the bile acid uptake system ASBT and consequently to an increase in epithelial bile acid uptake activity by the mucosa in Barrett’s esophagus. Furthermore, our findings may provide an explanation for the correlation between the decrease in both ASBT and CDX expression in high-grade esophageal dysplasia.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

manuscript; L.M., M.J., G.A.K.-U., and J.J.E. edited and revised the manuscript; L.M., M.J., G.A.K.-U., and J.J.E. approved the final version of the manuscript.

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