Published in final edited form as:

Biochim Biophys Acta. 2011 July ; 1809(7): 295–305. doi:10.1016/j.bbagr.2011.06.004.

Identification and characterization of the major alternative promoter regulating *Bcrp1/Abcg2* expression in the mouse intestine

Karthika Natarajan^a, Yi Xie^{b,c}, Takeo Nakanishi^{c,d,1}, William T. Beck^e, Kenneth S. Bauer^f, and Douglas D. Ross^{b,c,d,g,h}

^a Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore MD 21201, USA

^b Department of Medicine, University of Maryland School of Medicine, Baltimore MD 21201, USA

^c Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore MD 21201, USA

^d Department of Pathology and Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore MD 21201, USA

^e Department of Biopharmaceutical Sciences (MC865), 833 South Wood Street, University of Illinois at Chicago, Chicago, IL 60612, USA

^f Department of Pharmacy Practice and Science, University of Maryland School of Pharmacy, 20 North Pine Street, PHN 415, Baltimore, MD 21201, USA

^g Baltimore VA Medical Center, Baltimore MD 21201, USA

1. Introduction

Expression of breast cancer resistance protein (BCRP/ABCG2) limits the transport of xenobiotics across cellular membranes; hence BCRP expression on the apical surface of the human intestinal epithelium might limit transport across the gut lumen, thereby limiting oral bioavailability of pharmaceuticals [1–5]. The effect of BCRP on the absorption of xenobiotics may be antagonized by inhibiting BCRP function or by BCRP gene knockdown [6–9]. To date, several classes of functional BCRP inhibitors are available [10–13] but modulating intestinal *BCRP* expression to improve drug absorption remains largely unexplored.

Intestinal *BCRP* expression is regulated transcriptionally by alternative promoter usage. Previous work from our lab at the University of Maryland identified tissue-specific expression of *BCRP* mRNA isoforms (E1U, E1A, E1B and E1C) in humans. The *BCRP*

^hCorresponding author. Greenebaum Cancer Center, University of Maryland School of Medicine, 655 West Baltimore Street, Room 9-031 Bressler Research Building, Baltimore, MD 21201, USA. dross@som.umaryland.edu; Phone: 410-328-8095; Fax: 410-328-6559.

¹Current Address: Department of Membrane Transport and Biopharmaceutics, Kanazawa University School of Pharmaceutical Sciences, Kanazawa, Japan, 920-1192

English lowercase letters are used throughout the manuscript to describe murine *Bcrp1* mRNA isoforms. The human *BCRP* mRNA isoforms are indicated by capital letters.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

mRNA isoforms identified shared the same coding region hence, they translated the same protein. However, their first exons were transcribed from distinct genomic regions upstream of *BCRP* Exon 2, indicating regulation of *BCRP* expression by alternative promoters. In the same study, analyzing the human small intestine for *BCRP* mRNA isoform expression identified only the E1B and E1C isoforms. The first exons of the E1B and E1C isoforms have unique 3' splice acceptor sites but their sequences overlap towards the 5' end [14]. This suggested transcription of intestinal *BCRP* mRNA from a single alternative promoter, which we designated as E1B/C. The E1B/C promoter is a functional promoter with confirmed *cis* regulatory elements [14, 15]. Interference with E1B/C promoter to enhance *in vivo* oral drug absorption requires the development of a suitable animal model.

Mouse *Bcrp1* protein and human *BCRP* protein are structurally and functionally similar. They share 87% sequence homology and efflux identical substrates [10]. Murine *in vivo* models harboring *Bcrp1* gene knockouts in FVB or C57BL/6J mouse strains are used to predict *BCRP* regulation of human small intestinal drug absorption [16, 17]. Mouse *Bcrp1* mRNA and protein are expressed throughout the mouse intestine with highest expression in the mouse ileum [18, 19]. Humans too may have region-specific variation in expression of *BCRP* but the data available in the literature are controversial [20–22]. Similarities in *BCRP* and *Bcrp1* regulation emerged when *Zong et al* identified three alternative *Bcrp1* mRNA isoforms (E1a, E1b and E1c) with transcription initiation from alternative promoters. Using an unspecified region of the mouse intestine, they attributed mouse small intestinal *Bcrp1* mRNA expression to the *Bcrp1* E1b mRNA isoform [23].

Since intestinal region-specific differences exist for mouse *Bcrp1* mRNA and protein expression [18, 19], we hypothesize that similar intestinal region-specific differences may also exist for *Bcrp1* alternative promoter usage. In addition, we recently identified a novel *Bcrp1* mRNA isoform designated E1u because of its location 5' upstream of the other alternative first exons on mouse chromosome 6 (to be published separately). E1u expression in the mouse intestine is yet to be characterized. Hence, in this study, we first quantified the four *Bcrp1* mRNA isoforms in the mouse duodenum, jejunum and ileum. We established the predominance of the E1b isoform in all regions of the mouse small intestine. Using reporter assays we established functional activity for the promoter upstream of E1b for the first time, narrowing the core promoter region to –231/–42 bps in relation to the transcription start site of E1b. Finally, using functional studies and ChIP assays we established phospho-CREB (p-CREB) as a positive regulatory factor of the mouse E1b *Bcrp1* promoter region.

2. Materials and Methods

2.0 Materials

The cAMP analog, 8Br-cAMP and phosphatidylinositol-3-kinase (PI3K), inhibitor LY294002 were purchased from Sigma-Aldrich, Inc (St. Louis, MO). The protein kinase A (PKA) inhibitor, H89 and the extracellular signal-regulated kinase (ERK) inhibitor PD98059 were obtained from LKT Laboratories, Inc (St. Paul, MN) and Cell Signaling Technology (Danvers, MA) respectively. The rat anti-mouse *Bcrp1* antibody (BXP-9) was purchased from Abcam (Cambridge, MA), the rabbit anti-mouse GAPDH and the horse radish peroxidase (HRP) labeled secondary antibodies were purchased from Cell Signaling Technology.

2.1. Collection of mouse and human intestinal tissues

The animal use protocol was approved by University of Maryland School of Medicine Institutional Animal Care and Use Committee. Male FVB/NCr mice (6 weeks old) purchased from the National Cancer Institute were allowed to acclimatize for a minimum of

two weeks with food and water ad libitum. Mice were euthanized by cervical dislocation, the entire intestine removed and the intestinal contents flushed three times with 1 ml of ice cold saline. The duodenum (1 cm region distal to the stomach), jejunum (16-19 cm from duodenum) and ileum (3 cm from the tip of the cecum) were collected in individual cryovials and flash frozen in liquid nitrogen [24]. Human intestinal samples were collected under an IRB-approved protocol as described previously [14].

2.2. Cell Culture

The mtsA58 transgenic mice, harboring a thermo-labile mutant of SV40 Large T antigen (tsA58) inserted downstream of an interferon- γ inducible H-2K promoter element [25]. The MSIE cell line is grown at a permissive temperature of 33°C (T-antigen stable) in RPMI media (Biosource, Invitrogen, Carlsbad, CA) supplemented with 5% FBS (Biosource), 10% Penicillin-Streptomycin and 5 units of mouse interferon- γ /ml (Peprotech Inc, Rocky Hill, NJ). Interferon- γ was added every two to three days. Growing the cells at the permissive temperature (33°C) preserves the activity of the temperature sensitive T-antigen (T-Ag) mutant tsA58 and facilitates MSIE cell proliferation while the interferon- γ supplementation drives the tsA58 mutant T-Ag expression. MSIE cells grown in interferon-free conditions for at least a week were used for the experiments. MSIE cells remain viable for at least 10–15 days in culture after interferon- γ withdrawal. MSIE cells grown to 90% confluence in 100 mm dishes for at least a week under interferon-free conditions were used to prepare mRNA and protein. The NIH3T3 cells (a kind gift from Dr. Anne Hamburger, University of Maryland, Greenebaum Cancer Center) were grown in DMEM media (Biosource) supplemented with 10% FBS (Biosource) and 10% Penicillin-Streptomycin.

2.3. RNA and cDNA preparation

Mouse intestinal sections were individually homogenized with a mortar and pestle and the resulting homogenate was used to prepare both RNA and protein. RNA was extracted from each intestinal section and also from the MSIE cell line using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed with M-MULV reverse transcriptase (Roche Diagnostics, Basel, Switzerland). Human small intestinal RNA was prepared as described previously [14].

2.4. Quantitative RT-PCR

The alternative *Bcrp1* mRNA isoforms from the different regions of the mouse intestine were absolutely quantified with standard curves generated by serial dilution of the respective full-length isoforms inserted in pcDNA3 vector. 1 μ g of the reverse-transcribed RNA was then PCR-amplified in real-time with IQ SYBR green mix (Bio-Rad, Hercules, CA) using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). Each reaction was performed in duplicate. Total *Bcrp1* mRNA was amplified with primers spanning Exon 6 and Exon 7 while the individual isoforms were amplified using isoform specific forward primers and a common reverse primer in Exon 2. The standard curves for the individual isoforms were prepared from the mouse *Bcrp1* cDNA inserted into pBluescript I KS (–) vector (kind gift from Dr. Naoko Takebe MD, PhD, CTEP, NCI). To prepare the full-length *Bcrp1* cDNA isoforms with alternative 5' UTRs, the *Bcrp1* cDNA from pBluescript I KS (–) vector was first restriction enzyme digested and inserted into the pcDNA3 expression vector. Next, the E1u, E1a, E1b and E1c 5'UTR sequences were PCR-amplified using 5' UTR specific forward primers spanning the 5' end of the individual 5' UTR sequences and a common *Bcrp1* specific reverse primer designated as *Bcrp1* OP R in Exon 2 (Table 1) and cloned into pCR2.1 vector using the TOPO TA cloning kit (Invitrogen). Each of the four TOPO cloned *Bcrp1* 5' UTRs were then restriction enzyme digested and inserted upstream of the translational start site in Exon 2 of the *Bcrp1* cDNA in the pcDNA3 vector. The sequence of each promoter construct was confirmed by sequence analysis (Biopolymer/

Genomics shared services). The data from three different mice were collected and analyzed for significant differences in mRNA isoform expression along the mouse small intestine. The data for the MSIE cell line is representative of at least three different passages. The sequences for 5' UTR cloning and qRT-PCR primers are given in Table 1.

2.5. 5' Rapid amplification of cDNA ends (RACE) PCR

RNA obtained from mouse jejunum epithelial cells and human small intestine was subjected to 5' RACE PCR according to First Choice RLM-RACE kit instructions (Ambion Inc, Austin, TX) using forward primers supplied in the kit and *Bcrp1* specific reverse primers designated as *Bcrp1* OP R and *Bcrp1* IP R in Table 1. The *Bcrp1* 5' RACE products were inserted into pCR2.1-TOPO vector (Invitrogen), cloned and sequenced at the Biopolymer/Genomics shared services at University of Maryland Greenebaum Cancer Center (UMGCC). The mouse *Bcrp1* 5' RACE sequences were compared with the 80kb genomic region upstream of mouse *Bcrp1* Exon 2 and human *BCRP* 5' RACE sequences were compared with 100kb genomic region upstream of human *BCRP* Exon 2 using the BLAST program at NCBI and mapped in relation to the translational start site in Exon 2. The *Bcrp1* 5' RACE products sequences are available in the NIH genetic sequence database, GenBank (Accession No: 1316841). See supplemental table S1.

2.6. Western Blotting

Intestinal protein supernatants were prepared by sonicating each intestinal homogenate in RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-42; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM sodium orthovanadate; 1 µg aprotinin/ml and 2 mM Pefabloc SC, Roche Diagnostics) and clearing the sample of cell debris by centrifugation at $10,000 \times g$ for 10 min. MSIE cells were gently scraped off the culture surface and the supernatant from the cells was prepared as mentioned above for intestinal homogenate. Protein concentrations were then measured with Bio-Rad Protein assay (Bio-Rad). 20 µg protein from each intestinal sample and 80 µg protein from cell culture samples were separated on 10% SDS-PAGE gel. The electrophoretically separated proteins were transferred to PVDF membranes and probed with rat anti-mouse *Bcrp1* (1:50) and anti-GAPDH (1:3000) primary antibodies followed by HRP labeled goat anti-rat and goat anti-rabbit secondary antibodies (1:3000 each). The immune complexes were visualized with a chemiluminescent substrate. The expression of *Bcrp1* protein and GAPDH protein in each intestinal section was quantified by densitometric analysis (Visionworks LS image acquisition and analysis software, UVP, Upland, CA).

2.7. Deletion constructs

The regions flanking the transcription start site of *Bcrp1* alternative 5' UTRs (E1u -1906/+64, E1a -1875/+10, E1b -1847/+60 and E1c -1904/+83) were PCR-amplified from BAC clones RP23-285A12 and RP24-314E24 (BACPAC Resources Center, Children's Hospital Oakland Research Institute, Oakland, CA) using restriction digestion site-inserted forward and reverse primers (Table 1). The PCR-amplified fragments were restriction digested and inserted into the pGL3-Basic vector (Promega, Madison, WI). Nucleotide sequences of the selected clones were confirmed by sequencing. The 5' end shortened deletion constructs for E1b -1847/+60 were prepared by shortening the 5' end to -1027, -305, -231 and -75 bps while maintaining the 3' end at +60 bps by using a common reverse primer and sequence specific forward primers (Table 1). On sequencing the clones from E1b -231/+60 deletion construct, some clones were found to have a deletion from bp -58 to bp -42, likely due to a PCR artifact. Clones with such deletions were hence designated as the Δ -58/-42 *Bcrp1* E1b -231/+60 deletion construct. Two point mutations T-33A and G-32C were introduced on the CRE in the E1b -1847/+60 deletion construct using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies Inc,

Santa Clara, CA) as per manufacturer instructions. The sense (E1b PM1S) and antisense (E1b PM1AS) primers for generating the two point mutant, designated as E1bPM -1847/+60, are given in Table 1. The CRE on the primers is given in bold and the point mutations are underlined.

2.8. Reporter assay

MSIE cells were seeded and grown in complete growth media in the absence of interferon- γ for at least a week before the experiments. On the day of the experiment, MSIE cells were plated at a density of 50,000 cells/well in 48 well plates and allowed to attach for 6 hrs. Each of the above-mentioned *Bcrp1* deletion constructs (100 ng/mL) and the pGL3-Basic vector (100 ng/mL, negative control) were then transiently co-transfected with the internal control, pRL-TK vector (20 ng/mL, Promega) using FuGENE 6 transfection reagent (Roche Diagnostics). Following transfection, the cells were cultured for an additional 48 hours at 33°C, after which the dual luciferase assay (Promega) was performed using the 20/20ⁿ Single Tube Luminometer (Turner Biosystems, Sunnyvale, CA) in accordance with kit instructions. Each experiment was performed at least thrice with triplicate samples per experiment. The luminescence of each deletion construct and of the pGL3-Basic vector relative to that of pRL-TK was calculated. To compare the promoter activity between the four alternative promoters, the relative luminescence of each alternative promoter construct was normalized over pGL3-Basic vector. For deletion analysis of the E1b promoter region, the relative luminescence of each shorter E1b deletion construct was normalized over luminescence of the -1847/+60 construct. For determining the promoter activity of the E1bPM -1847/+60, NIH3T3 fibroblasts cultured in 24-well plates at a density of 500,000 cells/well were transfected with either *Bcrp1* E1b -1847/+60 deletion construct, the E1bPM -1847/+60 deletion construct or pGL3-Basic vector. After 24 hrs of transfection, cells were serum starved overnight and then stimulated with 2 mM 8Br-cAMP (final concentration) in SFM for 6 hours. Following stimulation, cells were lysed in passive lysing buffer and dual luciferase assays were performed as detailed above. pRL-TK vector was used as internal control for transfection. For promoter assays in presence of analogs and inhibitors of cAMP, NIH3T3 fibroblasts were plated in 24-well plate with density of 500,000 cells/well. After 6 hr plating, cells were transfected with 1 μ g of pGL3-basic vector or *Bcrp1* E1b/PGL3 reporter plasmid and co-transfected with 4 ng of the internal control, pRL-TK in 20 μ l SFM plus 1.5 μ l of FuGENE 6 (Roche diagnostics). After 24 hr of transfection, cells were starved in SFM overnight, then incubated with a inhibitor mix (20 μ M of H89, 50 μ M of LY294002 and 50 μ M of PD98059, final concentration in SFM for 20 min. After washing, followed by stimulation with 2 mM 8Br-cAMP for 6 hrs, cells were lysed with passive lysing buffer and luciferase activity was determined with Dual-Luciferase reporter assay kit (Promega). The results are expressed as the ratio of the luciferase activity over the internal control.

2.9. Identification of cis and trans-elements on *Bcrp1* E1b and *BCRP* E1B/C promoters

A 500 bp region upstream of E1b 5' untranslated region (5' UTR) was scanned for transcription factor binding sites using the PROSCAN [26] promoter scan program as well as the PAGEN [27] program and also searched against the Transcription Regulatory Regions Database [28] using BLAST. All three programs were used to identify additional transcription factor binding sites on the 500 bp region upstream of human *BCRP* E1C 5' UTR.

2.10. ChiP assay

NIH3T3 fibroblasts, cultured in 100 mm dishes after serum starvation overnight, were stimulated with 2 mM 8Br-cAMP in SFM for 30 min and subjected to ChiP assay as per manufacturer instructions (Millipore, Temecula, CA). Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature. Cells were then harvested in sodium

dodecyl sulfate lysis buffer and the DNA sheared to 200–1000 bps by sonication. The sonicated cell lysates were precleared with protein A agarose beads pre-blocked in Bovine Serum Albumin (Millipore). Immunoprecipitation of protein-DNA complexes was then performed on the pre-cleared lysates with rabbit anti-mouse p-CREB antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Simultaneously, pre-cleared lysates were also incubated with anti-rabbit IgG (Cell Signaling Technology) to control for potential non-specific co-immunoprecipitations. DNA was quantified by real-time qPCR using promoter-specific primers for p-CREB site on E1b. The primers for qRT-PCR are given in Table 1.

2.11. Statistical analysis

Linear regression analysis was used to quantify the alternative *Bcrp1* mRNA isoforms as well as the total *Bcrp1* mRNA isoform expression from each region of the mouse intestine using MYIQ™ real-time PCR detection system software version 1.0. All other statistical analyses were performed with GraphPad Prism software version 4.03. Statistical significance for real-time qRT-PCR and the promoter activity of the alternative 5' untranslated regions was determined using the Kruskal-Wallis non-parametric test with post hoc Dunn's test. The statistical significance of the promoter activity for the shorter E1b deletion constructs was determined ($p < 0.005$) using non-parametric t-test with Welch's correction. The statistical significance of the promoter activity for the E1b –1847/+60 deletion construct and its point mutant, both in the presence and absence of analog/inhibitor mix of cAMP as well as the statistical significance of the ChIP assays was measured with non-parametric Student's t-test.

3. Results

3.1. E1b is the major mouse intestinal *Bcrp1* mRNA isoform

Alternative 5' UTR usage is indicative of alternative promoter usage. Hence to determine small intestinal region-specific alternative *Bcrp1* promoter usage, *Bcrp1* mRNA isoform expression in the mouse duodenum, jejunum and ileum was characterized using real-time qRT-PCR. The *Bcrp1* E1b mRNA isoform accounted for more than 50% of the total *Bcrp1* mRNA expression in each intestinal section studied (Figure 1) except for the high expression of E1c observed from the duodenum of one mouse. Since the sum of the individual isoforms failed to add up to the total *Bcrp1* expressed in this particular mouse, it was classified as an outlier. *Bcrp1* E1c and E1a mRNA isoforms were also detected in the mouse small intestine, although the median expression levels were much lower than that of the E1b isoform (Figure 1). The E1u mRNA isoform was not detected in any part of the mouse small gut (Figure 1). Taken together, these data suggest that the alternative promoter upstream of the *Bcrp1* E1b 5' UTR predominantly regulates *Bcrp1* expression in the mouse small intestine.

3.2. Utilization of variable transcription start sites by *Bcrp1* E1b promoter

In order to characterize the transcription start site of the mouse intestinal *Bcrp1* E1b mRNA isoform, 5' RACE PCR was performed on a representative mouse intestinal tissue, the jejunum epithelium. Gel electrophoresis of the mouse intestinal 5' RACE products yielded an intense 200 bp band (Figure 2A). This band was cloned and five clones were randomly selected and sequenced. These sequences have been deposited in GenBank (supplemental Table S1). On subsequent sequence comparison with the genomic region upstream of *Bcrp1* Exon 2, the five clones aligned exclusively with the *Bcrp1* E1b 5' UTR supporting the real-time qRT-PCR data for predominance of the *Bcrp1* E1b mRNA isoform expression in the mouse intestine. While all E1b clones shared a common 3' end, the intestinal *Bcrp1* E1b 5' UTRs from five clones were mostly of variable lengths (82 bp or 61 bp or 45 bp or 35 bp),

demonstrating variable transcription start site utilization in the mouse intestinal E1b promoter (Figure 2B).

3.3. *Bcrp1* mRNA and protein expression along the mouse intestine

Real-time PCR quantification identified high expression for *Bcrp1* mRNA in the mouse duodenum and jejunum and higher *Bcrp1* mRNA expression in the mouse ileum (Figure 3A). Immunoblotting followed by densitometric quantification established maximum *Bcrp1* protein expression in the mouse ileum as well (Figure 3B).

3.4. Reporter activity for alternative *Bcrp1* promoters in the MSIE cell line

To test the ability of the 5' region upstream of the alternative *Bcrp1* 5' UTRs to promote gene transcription in mouse intestine using reporter assays we chose the conditionally immortalized MSIE cells as a model of mouse intestinal epithelium. First, the MSIE cell line was characterized for *Bcrp1* alternative mRNA isoform expression using qRT-PCR. The MSIE cell line, like the small intestine, predominantly expressed the *Bcrp1* E1b mRNA isoform (Figure 4A). Comparatively lower levels of both *Bcrp1* E1a and E1c isoform were also expressed in the MSIE cell line, but not the E1u isoform (Figure 4A). *Bcrp1* protein expression in the MSIE cell line was confirmed by the appearance of a 72 kDa immunoreactive band on Western blotting (Figure 4B). To prepare luciferase reporter constructs, the 5' regions upstream of each *Bcrp1* alternative first exon was cloned into pGL3-Basic vector. A schematic showing the location of E1u -1906/+64, E1a -1875/+10, E1b -1847/+60 and E1c -1904/+83 promoter constructs in relation to the TSS of their respective longest 5' UTRs identified, as well as in relation to *Bcrp1* Exon 2, is given in Figure 5A. As expected from the expression of *Bcrp1* E1a, E1b and E1c mRNA isoforms in the MSIE cell line (Figure 4A), significant (Kruskal-Wallis non-parametric test, $p < 0.005$) promoter activity over the empty vector (pGL3-Basic) was observed only for deletion constructs upstream of the E1a, E1b and E1c *Bcrp1* 5' UTRs (Figure 5B) but not for E1u, the 5' UTR not expressed in the MSIE cell line. The highest promoter activity was observed for the promoter upstream of E1b 5' UTR (Fig 5B) which was the major *Bcrp1* 5' UTR expressed in the MSIE cell line (Figure 4A).

3.5. Identification of the core promoter region upstream of *Bcrp1* E1b 5' UTR

The core promoter region upstream of the major *Bcrp1* 5' UTR expressed in the MSIE cell line (E1b) was then characterized using deletion analysis (Figure 6). We made five progressively 5' end shortened *Bcrp1* E1b deletion constructs, -1847/+60, -1027/+60, -305/+60, -231/+60, and -75/+60 (Figure 6A) which all exhibited significant promoter activity ($p < 0.001$) compared to the empty vector control, pGL3-Basic (Figure 6B). The promoter activity remained comparable for the -1847/+60, -1027/+60, -305/+60 deletion constructs but increased significantly on additional 5' end shortening to the -231/+60 deletion construct (Figure 6B). The comparatively high reporter activity observed for the -231/+60 construct was lost on the successive 5' end shortened -75/+60 construct. Of note, the reporter activity observed for the -231/+60 construct was also lost when ~16 bps -58/-42 were deleted in the -231/+60 construct (-231/+60 Δ -58/-42 construct) (Figure 6B). Hence we conclude the regions between -231 and -75 bp and between -58 and -42 bp contain areas for binding of positive *trans* acting regulatory elements. We deduce the core promoter region occupies the region between -231 and -42 bps.

3.6. *In silico* characterization of E1b promoter region

The cis/trans factor prediction programs identified several transcription factor binding sites within the E1b core promoter region spanning -231 to +60 bps (Figure 7A). Most of the transcription factor binding sites predicted *in silico* lie within the -58 to -42 bps and -231

to -75 bps, regions identified by reporter assays to contain important *cis*-regulatory elements (Figure 7A). Several Sp1 sites with varying weights (increasing weight indicates preferential binding of the transcription factor to promoter regions compared to non-promoter regions) were predicted in the core promoter region by the PROSCAN promoter prediction program (Table 2). Interestingly, the Sp1 binding site predicted with weight = 34.423 (Table 2), occupied the region between -58 and -42 bp, the area deleted in the Δ -58/-42 variant of the -231/+60 construct. T-Ag, hypoxia-inducible factor (HIF) and GC-binding factor (GCF) binding sites were also identified in this region. The region between -231 and -75 bp contained two additional Sp1 binding sites predicted by PROSCAN (weight = 3 and 8) and also a T-Ag, CREB, NF-kB and AP2 binding sites. IgNF-A, ATF and E4F1 were additional sites predicted in the -231/+60 E1b promoter region. The mouse E1b promoter region and the human E1B/C promoter share only the Sp1, HIF, AP2, GCF and T-Ag transcription factor binding sites (Figure 7A, B). The CCAAT box and the estrogen response element [29] are unique to the human E1B/C promoter while NF-kB, CREB, IGNF-A, c-MYB, ATF and TIN-1 binding sites are unique to the mouse E1b promoter. The position of the *cis* elements, software program utilized and their weights are given in Table 2.

3.7 Characterization of the cAMP response element (CRE) on the E1b -1847/60 promoter in NIH3T3 cells

We used NIH3T3 murine fibroblasts for these studies because they obviated the potential interference of T-Ag/interferon- γ observed in the MSIE cell line. Also, NIH3T3 cells express significant amount of *Bcrp1* E1b mRNA isoform and exhibit E1b promoter reporter activity (Supplemental figure S1).

To understand intestine-specific regulation of mouse *Bcrp1* expression further, we introduced two point mutations in the CRE (indicated by * in Figure 7A), a CIS site predicted exclusively on the mouse E1b promoter, the major intestinal promoter. We then compared the reporter activity of the mutated E1bPM -1847/+60 construct with its non-mutated parent construct. The reporter activity of the -1847/+60 E1b construct was completely lost in the mutated E1bPM -1847/+60 construct and was comparable with the reporter activity of the negative control pGL3-Basic (Figure 8A). This confirmed the presence of a critical, positive *cis* regulatory element in the mutated region. Next we treated the mutated and non-mutated constructs with 8Br-cAMP, an analog of cAMP and compared their reporter activities with their respective non-treated controls. We observed that the reporter activity of the non-mutated construct but not the point mutant increased on treatment with 8Br-cAMP suggesting the *cis* regulatory element is responsive to cAMP (Figure 8B). We then tested the response of the non-mutated -1847/+60 deletion construct to inhibitors of cAMP (Figure 8C). The reporter activity of the -1847/+60 E1b deletion construct was significantly inhibited when treated with the cAMP inhibitor mix. The cAMP inhibitor mix contained a combination of inhibitors of the PI3K, PKA as well as the ERK pathways, the signal transduction pathways downstream of cAMP. This inhibition of reporter activity observed was reversed on inhibitor washout and subsequent treatment with 8Br-cAMP, substantiating the presence of a cAMP response element in the E1b promoter region at the site of the two-point mutation. Finally we performed ChIP assays for p-CREB, the trans-regulatory element of CRE, in the presence and absence of 8Br-cAMP. Phospho-CREB interaction with the mouse *Bcrp1* CRE was significantly increased on treatment with 8Br-cAMP when compared with untreated or with IgG isotype control immunoprecipitations (Figure 8D).

3.8. Alternative *BCRP* 5' UTR expression in human intestine

Using RT-PCR our lab at the University of Maryland has shown preliminarily that the promoter associated with the E1B and E1C alternative *BCRP* 5' UTRs may control *BCRP* expression in the human small intestine [14]. To investigate this further, we performed 5' RACE for *BCRP* mRNA using human intestinal RNA, and gel electrophoresed the final RACE products. A prominent 500 bp band (*) and disperse higher bands (#, ~550 to 850 bp) were observed (Figure 9A). Eighteen clones were selected from these areas, and sequenced (Figure 9B), and then the sequences were aligned with the genomic region upstream of *BCRP* Exon 2. Among the 18 clones sequenced, 17 clones aligned with E1C 5' UTR while only one clone aligned with E1B 5' UTR (Figure 9B) therefore clarifying that the E1C 5' UTR is the major human intestinal *BCRP* 5' UTR (Figure 9A and 9B). These sequences have been deposited in GenBank (supplemental Table S1).

4.0 Discussion

In the current study, we propose that *Bcrp1* mRNA expression in all regions of the mouse small intestine is regulated from a single functional alternative promoter (E1b). We further clarified transcription of *BCRP* mRNA isoforms in the human small intestine from a single alternative promoter (E1C). Based on the predominant expression of a single alternative promoter for *Bcrp1* and *BCRP* in mouse and human small intestine respectively, we hypothesized that similar pre-transcriptional mechanisms regulate *Bcrp1*/*BCRP* expression in mouse/human small intestine. However, based on *in silico* analysis, we report the existence of significant differences between the mouse intestinal alternative promoter E1b and the human intestinal promoter E1C. The qRT-PCR and 5' RACE data from the current study established a predominant expression pattern for the *Bcrp1* E1b isoform in the mouse small intestine. The predominance of a single 5' UTR in the intestine of both mice and humans suggests conservation of intestine-specific alternative promoter usage for *BCRP* across two species.

Alternative transcription initiation can occur from variable transcription start sites within a single promoter. Drug-resistant and drug-sensitive phenotypes were shown to initiate transcription from alternative transcription start sites from the same promoter for *BCRP* [14]. Tissue-specific variable transcription start site utilization has also been reported in the E1b promoter for *Bcrp1* in mouse fetal liver and bone marrow cells [23]. Likewise, we found variation in transcriptional start sites for the E1b promoter in mouse intestine. From these findings it is reasonable to conclude that the 82 bp E1b 5' UTR is the longest E1b 5' UTR and both the 82 bp and 49 bp E1b 5' UTRs have ubiquitous expression. BLAST analysis of dbEST identified a *Bcrp1* E1b expressed sequence tag with 35 bp from the mouse kidney but with a splice donor site from an 82 bp E1c 5' UTR (NCBI Accession: AW611052).

High *Bcrp1* mRNA as well as protein expression have been reported in the mouse ileum [18, 19]. However, *Bcrp1* mRNA and protein expression levels have not been studied simultaneously in different mouse intestinal regions. Hence a relationship between intestinal *Bcrp1* mRNA expression and protein expression, measured simultaneously in the different intestinal regions has never been reported. We measured both *Bcrp1* protein and *Bcrp1* mRNA levels from the same intestinal homogenates. A similar pattern of expression along the mouse small intestine was observed for both total *Bcrp1* mRNA and *Bcrp1* protein (Figure 3A and 3B). This suggests that intestinal *Bcrp1* mRNA expression for the most part might be regulated transcriptionally. Expression of a specific 5' UTR is accompanied by functional activity of the promoter upstream of the same 5' UTR. The reporter activity of the promoter constructs containing the 5' upstream region of the alternative first exons generally correlated with the expression of the *Bcrp1* mRNA isoforms in MSIE cells (Figure 4 and 5).

We found a similar correlation between *Bcrp1* promoter construct reporter activity and mRNA isoforms in NIH3T3 cells, where the E1a and E1b alternative first exons are most abundantly expressed (Supplementary data Figure S1). Reporter activity was not observed for the promoter upstream of E1u, the *Bcrp1* mRNA isoform not expressed in either cell line.

In silico secondary structure analysis of the deleted -58/-42 bp region (Figure 7A) using DINAmelt server [30, 31] indicated a stem loop structure. DNA polymerase mediated slippage across this stem-loop structure might explain why the spontaneous deletion of -58 to -42 bp was observed [32]. The -58/-42 bp region encompasses several cis factor binding sites suggesting the secondary stem-loop structure formed by this region might facilitate binding of these factors. The fortuitously prepared Δ -58/-42 *Bcrp1* E1b -231/+60 deletion construct therefore helped us identify this as a significant region within the core promoter spanning -243/-42bp.

Several of the *in silico* predicted sites on the E1b promoter were also predicted in the other alternative promoters of mouse *Bcrp1*. Hence to identify an intestine-specific regulator of mouse *Bcrp1* expression, the CRE, one of the cis-elements unique to the mouse E1b promoter was selected for further analysis. Introduction of two point mutations on the predicted CRE site resulted in complete loss of reporter activity for the -1847/+60 E1b deletion construct suggesting the CRE site is essential for transcriptional regulation of the E1b promoter (Figure 8A). The CRE or the cAMP response element in *Bcrp1* -1847/+60 E1b deletion construct was activated when treated with either 8Br-cAMP, an analog of cAMP or inhibited by cAMP inhibitor mix (Figure 8B,C). The downstream effect of cAMP treatment is phosphorylation of CREB, its subsequent binding with and activation from the CRE's in the promoter region of its effector genes. Subsequent ChIP assays revealed enhanced binding of p-CREB with mouse *Bcrp1* CRE in NIH3T3 cells treated with 8Br-cAMP but not in untreated cells (Figure 8D). Taken together these data indicate that *Bcrp1* expression in the mouse intestine is at least in part regulated by cAMP associated pathways.

Identification of Sp1 binding sites in the -231 to -42 bp promoter region indicated that the alternative promoter regulating *Bcrp1* expression in the mouse intestine is a TATA-less promoter similar to the human intestinal promoter [15]. Several identical and unique cis-regulatory elements were predicted in the mouse and human intestinal TATA-less promoters. Further, comparison of the human E1C 5' UTR and promoter region with genomic sequences across-species using BLASTN revealed 90% homology with chimpanzee and monkey. The mouse *Bcrp1* E1b 5' UTR and its genomic upstream region was conserved only in rat [33]. This suggests that although a single alternative promoter regulates intestinal BCRP expression in mouse and humans, differences might exist in regulation of BCRP expression from these promoters. The identification of dissimilarities in BCRP regulation in both species might help us understand any difference that may arise when data from pre-clinical studies are translated to clinical studies in the future.

We have been able to establish for the first time that a distinct functional alternative promoter (E1b) regulates *Bcrp1* expression in the mouse intestine. We have also been able to show regulation of *Bcrp1* E1b mRNA expression from a CRE by its direct interaction and activation by p-CREB. We were also able to confirm the utilization of a distinct alternative promoter in the human intestine. To date, transcription factors shown to regulate human and mouse BCRP/*Bcrp1* intestinal promoters remain divergent. However, human-mouse species cross-comparison studies of the transcription factors regulating BCRP/*Bcrp1* intestinal promoter activity is currently underway in our laboratory and will provide the data necessary to develop the mouse as an *in vivo* model to study the effects of diet or xenobiotics on the transcriptional regulation of intestinal *Bcrp1* expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by a VA Merit Review grant awarded to Dr. Douglas D. Ross and by the National Cancer Institute grant CA40570 awarded to Dr. William T. Beck.

5.0 References

1. Doyle LA, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A*. 1998; 95(26):15665–70. [PubMed: 9861027]
2. Miyake K, et al. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res*. 1999; 59(1):8–13. [PubMed: 9892175]
3. Allikmets R, et al. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res*. 1998; 58(23):5337–9. [PubMed: 9850061]
4. Kruijtzter CM, et al. Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. *J Clin Oncol*. 2002; 20(13):2943–50. [PubMed: 12089223]
5. Maliepaard M, et al. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res*. 2001; 61(8):3458–64. [PubMed: 11309308]
6. van Loevezijn A, et al. Inhibition of BCRP-mediated drug efflux by fumitremorgin-type indolyl diketopiperazines. *Bioorg Med Chem Lett*. 2001; 11(1):29–32. [PubMed: 11140726]
7. Ee PL, et al. Modulation of breast cancer resistance protein (BCRP/ABCG2) gene expression using RNA interference. *Mol Cancer Ther*. 2004; 3(12):1577–83. [PubMed: 15634651]
8. Kowalski P, et al. Selection and characterization of a high-activity ribozyme directed against the antineoplastic drug resistance-associated ABC transporter BCRP/MXR/ABCG2. *Cancer Gene Ther*. 2001; 8(3):185–92. [PubMed: 11332989]
9. Priebisch A, et al. Complete reversal of ABCG2-depending atypical multidrug resistance by RNA interference in human carcinoma cells. *Oligonucleotides*. 2006; 16(3):263–74. [PubMed: 16978089]
10. Allen JD, et al. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther*. 2002; 1(6):417–25. [PubMed: 12477054]
11. Cooray HC, et al. Interaction of the breast cancer resistance protein with plant polyphenols. *Biochem Biophys Res Commun*. 2004; 317(1):269–75. [PubMed: 15047179]
12. Zhou XF, et al. Effects of dihydropyridines and pyridines on multidrug resistance mediated by breast cancer resistance protein: in vitro and in vivo studies. *Drug Metab Dispos*. 2005; 33(8):1220–8. [PubMed: 15908473]
13. Henrich CJ, et al. Botryllamides: natural product inhibitors of ABCG2. *ACS Chem Biol*. 2009; 4(8):637–47. [PubMed: 19555120]
14. Nakanishi T, et al. Novel 5' untranslated region variants of BCRP mRNA are differentially expressed in drug-selected cancer cells and in normal human tissues: implications for drug resistance, tissue-specific expression, and alternative promoter usage. *Cancer Res*. 2006; 66(10):5007–11. [PubMed: 16707421]
15. Bailey-Dell KJ, et al. Promoter characterization and genomic organization of the human breast cancer resistance protein (ATP-binding cassette transporter G2) gene. *Biochim Biophys Acta*. 2001; 1520(3):234–41. [PubMed: 11566359]
16. Jonker JW, et al. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc Natl Acad Sci U S A*. 2002; 99(24):15649–54. [PubMed: 12429862]

17. Zhou S, et al. Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. *Proc Natl Acad Sci U S A*. 2002; 99(19):12339–44. [PubMed: 12218177]
18. Han Y, Sugiyama Y. Expression and regulation of breast cancer resistance protein and multidrug resistance associated protein 2 in BALB/c mice. *Biol Pharm Bull*. 2006; 29(5):1032–5. [PubMed: 16651740]
19. Enokizono J, Kusuhara H, Sugiyama Y. Regional expression and activity of breast cancer resistance protein (Bcrp/Abcg2) in mouse intestine: overlapping distribution with sulfotransferases. *Drug Metab Dispos*. 2007; 35(6):922–8. [PubMed: 17353350]
20. Gutmann H, et al. Distribution of breast cancer resistance protein (BCRP/ABCG2) mRNA expression along the human GI tract. *Biochem Pharmacol*. 2005; 70(5):695–9. [PubMed: 15998509]
21. Englund G, et al. Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells. *Eur J Pharm Sci*. 2006; 29(3-4):269–77. [PubMed: 16822659]
22. Urquhart B, et al. Breast cancer resistance protein (ABCG2) and drug disposition: intestinal expression, polymorphisms and sulfasalazine as an in vivo probe. *Pharmacogenet Genomics*. 2008; 18(5):439–48. [PubMed: 18408567]
23. Zong Y, et al. Expression of mouse Abcg2 mRNA during hematopoiesis is regulated by alternative use of multiple leader exons and promoters. *J Biol Chem*. 2006; 281(40):29625–32. [PubMed: 16885162]
24. Radanovic T, et al. Regulation of intestinal phosphate transport. I. Segmental expression and adaptation to low-P(i) diet of the type IIb Na(+)-P(i) cotransporter in mouse small intestine. *Am J Physiol Gastrointest Liver Physiol*. 2005; 288(3):G496–500. [PubMed: 15701623]
25. Whitehead RH, et al. Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2Kb-tsA58 transgenic mice. *Proc Natl Acad Sci U S A*. 1993; 90(2):587–91. [PubMed: 7678459]
26. Prestridge DS. Predicting Pol II promoter sequences using transcription factor binding sites. *J Mol Biol*. 1995; 249(5):923–32. [PubMed: 7791218]
27. Kamalakaran S, Radhakrishnan SK, Beck WT. Identification of estrogen-responsive genes using a genome-wide analysis of promoter elements for transcription factor binding sites. *J Biol Chem*. 2005; 280(22):21491–7. [PubMed: 15790569]
28. Kolchanov NA, et al. Transcription Regulatory Regions Database (TRRD): its status in 2002. *Nucleic Acids Res*. 2002; 30(1):312–7. [PubMed: 11752324]
29. Huls M, et al. The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane. *Kidney Int*. 2008; 73(2):220–5. [PubMed: 17978814]
30. Markham N, Zuker M. DINAMelt web server for nucleic acid melting prediction. *Nucleic Acids Res*. 2005; 33(Web Server issue):W577–81. [PubMed: 15980540]
31. Markham N, Zuker M. UNAFold: software for nucleic acid folding and hybridization. *Methods Mol Biol*. 2008; 453:3–31. [PubMed: 18712296]
32. Trinh T, Sinden R. The influence of primary and secondary DNA structure in deletion and duplication between direct repeats in *Escherichia coli*. *Genetics*. 1993; 134(2):409–22. [PubMed: 8325478]
33. Altschul SF, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 1997; 25(17):3389–402. [PubMed: 9254694]

Research Highlights

- The goals were to identify and characterize the major alternative promoter(s) regulating mouse intestinal Bcrp1 expression and to compare it with the alternative promoter(s) controlling human intestinal BCRP expression;
- A single alternative promoter (E1b) regulates mouse intestinal Bcrp1 expression; in humans, the E1C promoter controls intestinal BCRP expression;
- The promoter elements predicted *in silico* to control mouse and human intestinal Bcrp1/BCRP expression appear to be somewhat different.
- The murine intestinal specific promoter for Abcg2, E1b, contains a cAMP response element (CRE). Functional and ChIP assays reveal that this CRE binds phospho-CREB; hence cAMP plays a role in the transcriptional regulation of Bcrp1/Abcg2 in the mouse intestine.

Figure 1. Real-time quantification of *Bcrp1* 5' UTR expression in different regions of the mouse intestine

E1b was the major *Bcrp1* 5' UTR expressed in the mouse duodenum, jejunum and ileum accounting for at least 50% of the total *Bcrp1* mRNA expression. Each data point in the figure is the average of duplicate PCR reactions from individual mice. Median values are represented by horizontal bar. E1u, E1a, E1b and E1c represent *Bcrp1* alternative first exons 72kb, 58kb, 10kb and 5kb respectively upstream from the translation start site of *Bcrp1* in Exon 2.

Figure 2. RACE-PCR analysis of mouse jejunum RNA

A) Agarose gel electrophoresis of the final RACE product yielded a 200 bp band. B) Schematic representation of the mouse jejunum 5' RACE products in relation to Exon 2 of *Bcrp1* mRNA. E1b 5' UTR with multiple transcription start sites was identified as the major 5' UTR in the mouse jejunum

Figure 3. Mouse intestine region-specific Bcrp1 mRNA and protein expression

A) Real-time RT-PCR quantification of total *Bcrp1* mRNA expression in the different regions of the mouse intestine. Highest *Bcrp1* mRNA expression was observed in the ileum.

B) A representative immunoblot of Bcrp1 and GAPDH (housekeeping gene) from the various intestinal regions and the densitometric analysis of the relative Bcrp1 expression (n=3) in the different regions of the intestine using GAPDH as control is shown below the immunoblot. Highest Bcrp1 expression was observed in the ileum

Figure 4. *Bcrp1* 5' UTR and protein expression in MSIE cell line

A) *Bcrp1* mRNA was expressed in the MSIE cell line and E1b 5' UTR expression was higher than either E1a or E1c 5' UTR expression. B) Immunoblotting identified the 72 kDa *Bcrp1* protein monomer to be expressed in the MSIE cell line. The faint band of ~52 kDa is non-specific.

Figure 5. Promoter activity for the alternative *Bcrp1* 5' UTRs in the MSIE cell line

A) Schematic representation of the four *Bcrp1* 5' UTRs in relation to Exon 2 and the promoter deletion constructs for each 5' UTR in relation to the TSS (indicated by arrows) of the respective 5' UTRs. The distance between each 5' UTR is also shown. B) Promoter activity was higher than the empty vector control (pGL3-Basic) for E1a, E1b and E1c promoter constructs while E1u promoter activity was lower than pGL3-Basic promoter activity in the MSIE cell line. The experiment was repeated at least three times with triplicate samples per experiment.

Figure 6. Promoter activity of Bcrp1 E1b deletion constructs

A) Schematic representation of E1b deletion constructs in relation to the transcription start site of the longest E1b 5' UTR sequenced. The position of the deleted 17 bp in the -231/+60 bp Δ -58/-42 is indicated by ($\blacktriangle$). B) Reporter activity of the mouse *Bcrp1* E1b deletion constructs in the MSIE cells. Cells were grown in the absence of interferon- γ for at least a week before the experiments. The mean $\pm$ S.E.M. is shown for each deletion constructs. The experiments were repeated at least three times with duplicate samples per experiment.

A.	Promoter upstream of E1b (major Mouse intestinal isoform)	B.	Promoter upstream of E1B/C (major Human intestinal isoforms)
-501	TTCTGTACAGCCACCTAAGTGAAGTCCCAACTTCTAGCATAGGACCTT	-501	TTCTTCTCTGGGTAGATTAGTGCCTGATTTTTTAGGCCCTGGGGG
-451	CCATTGGGAGCTGCTGGTAAAGTCCCAACAAGTAGGAATCAGA EBNA-1	-451	CAAGCATCCATTTCTCAGAATCCATTCAACGAAACACCATTTAAC
-401	TCATTTTAGGAGCAGCTCTCTCTCTCATCAAAAGGCCATCCACCCAT	-401	TTGCTCTGGGTGCGAGCAGCGCTTTGTGACTGGGCACTGTGCTCAGCG XBBF AP1
-351	CTCGTAATCCCGGAGAGTCCACACAAGCCAGCCTGCCACAGTTAT	-351	TCCCGGTGCTTCGGCGCTCCGGCAGTGACGGGACCAACCCAGCTAG
-301	AATAGTAATTTGCCTAAATGGATCTTGGGTATCTTGGGTCTCCAGG IgNF-A	-301	GTCAGACGAGTACTGATCAGCCCAATGAGCGCTGTGATTCTGTAGT CAAT Box
-251	TTACAGGTGAAGTACTCTGAGTATATTTTCTTCCGAGGCTCTTAT -231	-251	TAATCACTCTGGTTCATTCCGTTCTGATCCCGAGCGGGAGTGTGGCT p300/SP1
-201	CGCAGGTACGCGCTTGCTCCGCATGACCGGGGCTCTCTGCTCCG T-Ag/NFkB	-201	TGTCCCTGCGTGTACGCGAGGTGACCTAGCCCGAGGGAGGGCGTG SP1/AP2/ERE SP1/SDR_RS/AP2
-151	CTCTCGGCGCTCCGTCAGGAGCCTGTGACGACAGGAAGCCCGCGCC Early-Seq1/SP1/AP2 -75 -58 SP1	-151	GTACAGTCTCTGCTGGCGGCTCAGCGCGCAGGACAGTGTGCGCTTCA SP1/GCF/HIF-1
-101	ACATGTGTCCAGCTGCTCTGGGAGCGGCTTCTCTGCGCGGCTGCGG -42 TGAACCA* TIN-1/HIF-1/SP1/	-101	GCGGGTCGCGAGGGCGCTTATCGCGGCCCGCAGTCTGGGGCCACGCTCA T-Ag
-51	GGGCGGCGCGGCTGAGCTTCAAGGCGCTGTCGGGGCGGGGCG T-Ag/GCF CREB/ATF/E4F1 SP1	-51	CCCCCGCCCGGAACCCGACCTGGGGAACCCGGGGCGCTGGGGAGGGG AP2/SP1/Early-Seq1
+1	AGACGCTCTTGGCGGGCGAGCGTGTGACGTCTGAGTGTGTGCGAGTTCT SP1	+1	CCACTGCGTTTACGCTCTGGCGGTCCACAGCCCGAAGCGCGGCTTAGGAAG
+51	GGCGCGGCTGCTGCTCCGCCAGGACCGGAGGTGAGCGAGTGCGGGA	-51	TTCTGTTCAGCGCTGCTGAGCTGCTCCCTGAGTGTGCGGTCTCCCA

Figure 7. Comparison of predicted and identified cis-elements on *Bcrp1* E1b and BCRP E1B/C promoter regions

Several SP1, Hif-1, T-Ag, AP2 sites were common to both the mouse and human promoter regions. A) The start site of the -231/+60 and -75/+60 deletion constructs as well as the -58/-42 spontaneous deletion region are shown in bold in the figure. The CREB binding site, selected for mutational analysis, is indicated by *. The position of the point mutations and the mutant nucleotides are given in bold face above the original nucleotide sequence. B) The previously predicted transcription factor binding sites on the human promoter region are in **BOLD** type face while sites predicted using PROSCAN, PAGEN and TRRD are in normal font color.

Figure 8. Analysis of interaction of p-CREB with the CRE on the mouse *Bcrp1* E1b core promoter region in NIH3T3 cells
p-CREB directly interacts with the CRE activating the E1b *Bcrp1* core promoter. A) Two adjacent point mutations were introduced in the CRE site on the E1b *Bcrp1* promoter. The promoter activity of the CRE site mutated (E1bPM -1847/+60) construct was then compared with the promoter activity of the non-mutated (E1b -1847/+60) construct. Shown is the representation of three independent experiments (n=3). **, p< 0.01; ***, p<0.001 compared to pGL3Basic control group. B) The promoter activity of the mutated (E1bPM -1847/+60) construct was compared with the non-mutated construct (E1b -1847/+60) in the presence and absence of treatment with 8Br-cAMP (2mM), an analog of cAMP. Shown is the representation of three independent experiments (n=3). *, p<0.05; **, p<0.01; ***, p< 0.001 compared to non-treated control group. C) The promoter activity of the non-mutated E1b -1847/+60 deletion construct was measured after treating NIH3T3 cells either individually with the cAMP analog, 8Br-cAMP or cAMP inhibitor mix or sequentially with cAMP inhibitor mix followed by inhibitor washout and subsequent treatment with 8Br-cAMP. Shown is the representation of three independent experiments (n=3). *, p< 0.05; **, p<0.01 compared to non-treated control group. D) ChIP analysis of p-CREB interaction with CRE was performed in the presence of 8Br-cAMP under serum staved conditions. ChIP assays with p-CREB (in the absence of 8Br-cAMP) and with IgG isotype control were performed to exclude non-specific interactions. The results are expressed as percentage of immunoprecipitated (IP) p-CREB-DNA to total DNA input (input). Shown is the representation of three independent experiments (n=3, *, p< 0.05) compared to non-treated control group.

Figure 9. Human intestinal 5' UTR analysis by RACE-PCR

A) * denotes the 500 bp band observed on agarose gel electrophoresis of the nested RACE PCR product from human small intestinal RNA while # indicates disperse higher bands observed B) Diagrammatic alignment of the human small intestinal BCRP 5' RACE sequences with the human E1B and E1C 5' UTRs and Exon 2 of Human BCRP gene. E1C 5' UTR was the highest 5' UTR identified by 5' RACE in the human small intestine. # and * indicate the 5' RACE clones obtained from the disperse higher bands and the prominent 500bp band respectively.

Table 1

Primers

Primer Name	Sequence	Function	Digestion site
Bcrp1 OP R	cgcctgtgggtccagaata	5' RACE, 5' UTR cloning	
Bcrp1 IP R	gcgacattgggtactaacacg	5' RACE	
E1u 5' Bcrp1 F	aggaatcacacgaatggaag	5' UTR cloning	
E1a 5' Bcrp1 F	ctccttgccagataagag	5' UTR cloning	
E1b 5' Bcrp1 F	agacgctcctggcggcgag	5' UTR cloning	
E1c 5' Bcrp1 F	aggttgaatatggagac	5' UTR cloning	
Bcrp1 F	cgaatgctgtcctttgct	Real-time qRT-PCR	
Bcrp1 R	ataccgaggctgatgaatgg	Real-time qRT-PCR	
E1u Bcrp1 F	agacaccctgatgttacc	Real-time qRT-PCR	
E1a Bcrp1 F	gacacctcattacacatagc	Real-time qRT-PCR	
E1b Bcrp1 F	gcggctgctgctcc	Real-time qRT-PCR	
E1c Bcrp1 F	gaagaaccagaccaataaggga	Real-time qRT-PCR	
E2 Bcrp1 R	ctccttctgcgagcgtcct	Real-time qRT-PCR	
E1a -1875 F	cacctgggagctctgctacagga	Deletion construct	SacI
E1a +220 R	gcctcaagagacagcgcaaatcaag	Deletion construct	
E1u -1906 F	tgcagtggaaagagctccctta	Deletion construct	SacI
E1u +64 R	gcagagaagctagcatcagggtgctc	Deletion construct	NheI
E1b -1847 F	caggtagcaggctgtgcttgccactgt	Deletion construct	KpnI
E1b +60 R	cggctagccagccgcgccagaactcg	Deletion construct	NheI
E1c -1904 F	gtctctggggaatgagctcaag	Deletion construct	SacI
E1c +83 R	tccctattggacgcttcttca	Deletion construct	MluI
E1b -1027 F	tttcaagccatggtaccgc	Deletion construct	KpnI
E1b -305 F	cccagcctggtaccgttat	Deletion construct	KpnI
E1b -231 F	cacagggtgaaggtaccctga	Deletion construct	KpnI
E1b -75 F	cctgtaccggcttct	Deletion construct	KpnI
E1b PM1S	cggcgcgagctgac ca cacggcggtc	Site directed mutagenesis	
E1b PM1AS	gaccgccgtg gtg tcagctcgccg	Site directed mutagenesis	
E1b CRE F	tctcatcaaaagccatcca	ChiP real-time qPCR	
E1b CRE R	aacctggaggaccacaaga	ChiP real-time qPCR	

The restriction enzyme digestion sites are underlined in the primers used for preparing the deletion constructs. For the primers used for site-directed mutagenesis, the CRE site is underlined; the sites of the point mutations are indicated in BOLD.

Table 2

Predicted cis/trans element location on E1b *Bcrp1* promoter

All PROSCAN identified signals on the positive strand within 500 bps upstream of the TSS of E1b 5' UTR is shown below.

Cis binding factor	Trans regulatory element	Position		Promoter Prediction Program	Weight
		Start	End		
EBNA-1	CATTTTGGGCAGCTG	-449	-435	TRRDsite	3.8*
IgNF-A	ATTGCAT	-282	-276	PROSCAN	1.434
NF-kB	GGGRNTYYC	-167	-159	PROSCAN	1.08
T-Ag	GGGGC	-167	-164	PROSCAN	1.086
EARLY-SEQ1	YYCCGCC	-147	-140	PROSCAN	6.322
AP-2	YCSCCMNSSS	-145	-136	PROSCAN	1.355
Sp1	CCGCC	-145	-140	PROSCAN	3.292
AP-2	CCCMNSSS	-109	-102	PROSCAN	1.108
(Sp1)	CCCCGCC	-107	-100	PROSCAN	8.117
Sp1	CCGCC	-106	-101	PROSCAN	2.755
TIN-1	TGCTCTGGGACCGC	-87	-72	TRRDsite	3.8*
c-MYB	TGCTCTGGGACCGC	-87	-72	TRRDsite	3.8*
HIF-1	GCGAGCTCGGGCGG	-60	-45	TRRDsite	3.8*
GCF	SCGSSC	-52	-46	PROSCAN	2.361
Sp1	CGGGCGGCG	-51	-42	PROSCAN	34.423
T-Ag	GGGC	-50	-46	PROSCAN	1.086
Sp1	GGGGCGGCG	-50	-41	PROSCAN	3.442
Sp1	GGGCGG	-49	-44	PROSCAN	3.013
beta-pol_CS	NTGACGTCAN	-37	-28	PROSCAN	8.603
CREB	TGACGTC	-36	-31	PROSCAN	3.442
CREB	TGACGTCA	-36	-29	PROSCAN	8.603
CREB	TGACGTYW	-36	-29	PROSCAN	3.886
ATF	TGACGYMR	-36	-29	PROSCAN	3.721
ATF	TGACGT	-36	-31	PROSCAN	1.157
CREB	KWCGTCA	-35	-29	PROSCAN	1.912

Cis binding factor	Trans regulatory element	Position		Promoter Prediction Program	Weight
		Start	End		
E4F1	ACGTCAC	-34	-28	PROSCAN	3.824
E4F1	ACGTMAC	-34	-28	PROSCAN	3.764
ATF/CREB	ACGTCA	-34	-29	PROSCAN	1.564
CREB	CTGACGTCACGG	-38	-27	PAGEN	94%**

Transcription factor binding sites with an E value less than 10 for TRRDsites indicated by* and a score higher than 90% for PAGEN indicated by ** are given below.