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Abstract:	Gene expression in different tissues is often controlled by alternative promoters that result in the synthesis of mRNA with unique - usually untranslated - first exons. Bcrp1 (Abcg2), the murine orthologue of the ABC transporter Breast Cancer Resistance Protein (BCRP, ABCG2), has at least four alternative promoters that are designated by the corresponding four alternative first exons produced: E1U, E1A, E1B, and E1C. Herein, in-silico protocols are presented to predict alternative promoter usage for Bcrp1. Furthermore, reporter assay methods are described to produce reporter constructs for alternative promoters and to determine the functionality of putative promoters upstream of the alternative first exons that are identified.
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TITLE:

Methods to discover alternative promoter usage and transcriptional regulation of murine Bcrp1

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SHORT ABSTRACT
With the murine ABC transporter Bcrp1 (Abcg2) as an example, *in-silico* protocols are presented to detect alternative promoter usage in genes expressed in mouse tissues, and to evaluate the functionality of the alternative promoters identified using reporter assays.

LONG ABSTRACT
Gene expression in different tissues is often controlled by alternative promoters that result in the synthesis of mRNA with unique – usually untranslated – first exons. Bcrp1 (Abcg2), the murine orthologue of the ABC transporter Breast Cancer Resistance Protein (BCRP, ABCG2), has at least four alternative promoters that are designated by the corresponding four alternative first exons produced: E1U, E1A, E1B, and E1C. Herein, *in-silico* protocols are presented to predict alternative promoter usage for Bcrp1. Furthermore, reporter assay methods are described to produce reporter constructs for alternative promoters and to determine the functionality of putative promoters upstream of the alternative first exons that are identified.

INTRODUCTION
More than half of human genes are regulated by alternative promoters[1]. Each alternative promoter can contain regulatory elements that may be different from those in other alternative promoters. The promoter(s) utilized in one tissue may differ from those used in another tissue. For example, it is possible that activation of a given signaling pathway may trigger the alternative promoter for a gene utilized in one tissue, yet have no effect on or repress a separate alternative promoter for the same gene that is utilized in another tissue.

Expression of the Bcrp1 gene is governed by alternative promoters. Bcrp1 is the murine orthologue of the human Breast Cancer Resistance Protein (BCRP) gene. BCRP is an ATP-binding cassette (ABC) transporter, formally designated ABCG2[2,3]. As an apical plasma membrane protein, BCRP/Bcrp1 functions to efflux a wide variety of natural and xenobiotic substrates[3,4]. In humans and mice, BCRP/Bcrp1 is highly expressed in pharmacologically relevant organs such as liver (bile canaliculi), intestine, and kidney, as well as organs with blood-tissue barriers such as

placenta, brain and testis[2,5-12]. Expression of BCRP/Bcrp1 in hematopoietic and other stem cells, including cancer stem cells, may confer resistance of these cells to xenobiotics and cancer chemotherapeutic drugs[3].

In early work to understand the regulation of BCRP expression in normal and neoplastic cells, 5' rapid amplification of cDNA ends (5'-RACE) analysis of BCRP mRNA was performed to determine its exact transcriptional start site[13]. Not only were multiple transcriptional start sites found; also encountered were three alternative forms of the first exon, which in BCRP is untranslated. These alternative first exons – designated E1a, E1b, E1c – were expressed differently in a variety of human tissues. Two additional first exon variants were discovered in a BLAST search of the human EST database using the second exon of BCRP[13]. Four matches revealed a first exon >70 Kb upstream from exon 2 which were designated E1u; four other matches revealed BCRP mRNA that lacked a first exon entirely, which were designated E1-.[13] The presence of alternative leader exons is considered to be a manifestation of alternative promoter usage[14].

In mice, four alternative first exons of Bcrp1 are described that may correspond to alternative promoters that govern *Bcrp1* transcription in different mouse tissues; these exons/promoters are designated E1U, E1A, E1B and E1C, and are located approximately 70, 58, 15, and 5 kb upstream from Bcrp1 exon 2[5,15]. The E1A mRNA isoform was found to be predominant in murine hematopoietic stem cells, heart, lung, spleen, and brain, whereas the E1B isoform was expressed in mouse intestine, fetal liver cells, and erythroid precursor cells in bone marrow[5,15]. The promoter upstream from E1B was shown to be the major alternative promoter governing Bcrp1 transcription in mouse intestine, regulated at least in part, by phospho-cyclic-AMP response element binding protein (p-CREB) and a CREB response element unique to that alternative Bcrp1 promoter[16]. The E1C mRNA isoform is predominantly expressed in adult murine liver and kidney[5]. The E1U isoform is undetectable in most tissues tested except for murine testis, where it is the predominant isoform expressed[5]. Bcrp1 expressed in rat testes is found in both somatic (endothelial tight junctions, peritubular myoid cells, and Sertoli cells) and germ cells (in the seminiferous endothelium, where it may protect late-stage spermatids[4]). The region upstream from E1U contains a functional response element for steroidogenic factor-1 (SF-1)[5]. Bcrp1 mRNA and protein are markedly reduced in the testes of Sertoli cell-specific SF-1 knockout mice, suggesting that Bcrp1 expression in murine Sertoli cells is controlled by SF-1[5].

The protocols presented detail methods to detect alternative first exons of Bcrp1 *in-silico*, and to establish luciferase-based reporter assays for putative promoters upstream from the alternative first exons identified.

PROTOCOLS:

1. *In silico* prediction of alternative first exons of Bcrp1 using BLAST analysis of mouse EST database

Note: This protocol describes how to search the mouse expressed sequence tag (EST) database for ESTs with sequence similarity to exon 2 of Bcrp1 (which contains the translational start site) and then how to align the matching EST sequences to genomic sequences to ascertain their

location in the mouse Bcrp1 gene relative to the 5' end of Bcrp1 exon 2.

1.1 Obtain the sequence for mouse Bcrp1 exon 2 by inputting the mRNA reference sequence ID (NM_011920.3) into the search window of the Ensembl Genome Browser[17], click on "GO." In the next screen, select a full-length sequence (contains 16 exons):

1.1.1 In the next screen ("Transcript-based Display"), select "16 Exons." This will display a screen containing the sequence of all exons of Bcrp1. Exon 2 of Bcrp1 will read "5'-AAAGGC…TATCAA-3'." The results obtained will be similar to those shown in reference [18].

1.1.2 Click on the "Download sequence" option. In the next screen, choose the FASTA format, and then click on "Preview." In the next screen, copy the Preview sequence of exon 2 into the clipboard.

1.2 Navigate to the BLAST homepage[19] on the National Center for Biotechnology Information (NCBI) website[20].

1.2.1 Select "Mouse" genome. Paste the exon 2 sequence from the clipboard into the query box.

1.2.2 Select "ESTs" from the database dropdown, optimize for "highly similar sequences," and then choose "BLAST." Run time will take a few minutes. When the BLAST run is complete, the "Results" page will appear.

1.2.3 Under the "Descriptions" subheading of the "Results" page, select "ALL," then "Download," then "FASTA (complete sequence)" in the dropdown, and then choose "Continue." A .txt file will appear; open and copy the entire file into the clipboard. The .txt file contains the sequences of all ESTs with high sequence similarity with the mouse Bcrp1 exon2 but not their position in relation to exon 2 in the Bcrp1 gene.

Note: An analysis performed on April 15, 2015 identified 14 murine ESTs that aligned with Bcrp1 exon 2. These are listed in Table 1.

1.3 Identify the location of the EST sequence that is 5' to exon 2 in the Bcrp1 gene. The mouse Bcrp1 gene is located in the chromosome 6 contig NC_000072.6 (GI:372099104).

1.3.1 On the BLAST homepage under the "Specialized Blast" subheading, select "Align two (or more) sequences using BLAST (bl2seq)."

1.3.2 Paste the text file from the clipboard into the query box and enter 372099104 in the subject sequence box. Optimize for "highly similar sequences" under program selection, and run BLAST.

1.3.3 Once the results window appears, view the alignments graphically by clicking on

"Graphics" in the "Alignments" window. Use the right and left arrows and zoom to focus on Bcrp1/Abcg2 and the sequence alignments.

1.3.4 Save the sequence alignments: select "ALL" in the "Descriptions" box, then under the "download" dropdown select "Hit table (CSV)," and then click on "continue." This file contains the sequence alignment of Bcrp1 exon 2 and the alignment of all the EST sequences with sequence similarity to Bcrp1 Exon2 relative to the numbering of the nucleotides in the mouse chromosome 6 contig. Each complete EST sequence might generate multiple alignments spanning regions 5' and 3' to exon 2 including the sequences overlapping with Bcrp1 exon 2.

1.3.5 As the position of the 5' end of exon 2 will correspond to nucleotide 58,655,638 in the chromosome 6 contig, designate this as +1, and then calculate the position of the partial sequences of each EST 5' to 58,655,638. The results for the 14 ESTs are given in Table 1.

Note: Be careful to analyze EST sequences that are 5' to +1 (i.e., have a negative nucleotide value) as potential first exons. For example, in two of the ESTs that aligned with Bcrp1 exon 2 shown in Table 1 (AI647825 and AI664571) the remaining sequence was 3' to exon 2.

2. Evaluation of Bcrp1 alternative promoter function

2.1) Design of reporter constructs for Bcrp1 E1U, E1A, E1B and E1C promoters

2.1.1 Using the sequence of E1U obtained from searching the EST database, designate the first 5' nucleotide of E1U as +1.

2.1.2 Obtain a bacterial artificial chromosome (BAC) clone of mouse chromosome 6 that contains the Bcrp1/Abcg2 gene sequence and the sequence at least 100 kb upstream of the Bcrp1/Abcg2 gene (see Table of Materials and Equipment).

2.1.2.1 Identify a suitable reporter vector such as the luciferase reporter plasmid pGL3-Basic.

2.1.2.2 Determine the multiple cloning sites in the reporter vector. KpnI, SacI, Mlu1, NheI, SmaI, XhoI, BglII and HindIII are the restriction endonuclease sites in the multiple cloning site of the pGL3-Basic vector, in the 5' to 3' orientation.

Note: The sequence of the digestion sites is available from product catalogs of companies that produce the restriction enzymes.

2.1.2.3 Identify all digestion sites in the E1U exon and the 2 kb region 5' of E1U using software programs such as NEB cutter. Identify the digestion sites that occur only once and are in common with those in the multiple cloning site of the pGL3-Basic vector; exclude all sites that occur more than once in the selected DNA region.

2.1.3 Prepare forward and reverse primers for PCR that contain restriction endonuclease sites using commercial gene/primer synthesis services or primer selection software (see Table of Materials and Equipment). Select a forward primer located ~2 kb upstream from the E1U

sequence and a reverse primer within the E1U sequence. The primers used in the authors' previous work amplified the genomic region from approximately −1906 to +64 with the first 5' nucleotide of E1U specified as +1 (see step 2.1.1 above), and are provided in reference [16].

2.1.3.1 PCR amplify the E1U genomic region using these primers, 0.01 to 1 µg of the BAC, and PCR master mix containing high-fidelity *Taq* polymerases (see Table of Materials and Equipment) with denaturing at 95˚C for 30 seconds, then 35 to 40 cycles of PCR, with extension at 72˚C (2 min), and annealing and melting temperatures based on the melting characteristics of the primers used.

Note: The use of high-fidelity *Taq* polymerases is essential for sequencing promoter regions that have high GC content. When using large genomic DNA fragments such as a BAC as the template, the secondary structure of the genomic DNA may make it difficult for PCR primers to bind. If this problem arises, better PCR results may be obtained if the long genomic DNA template is sheared gently by sonication before the PCR reaction.

2.1.3.2 Verify the length of the amplified PCR product by comparing against a 1 kb DNA ladder using 0.8% TAE agarose gel electrophoresis.

2.1.4 Digest the PCR product obtained and the pGL3-Basic vector, using the restriction endonucleases specific for the restriction digestion sites introduced into the forward and reverse primers as per instructions supplied with the restriction digestion enzymes.

2.1.4.1 Purify the digestion reactions using commercial SV Gel and PCR Clean-Up Systems kit (see Table of Materials and Equipment), following the kit protocol[21].

2.1.4.1.1 Verify digestion and linearization of the vector by comparing it against the uncut vector using agarose gel electrophoresis, then cut and purify the digested vector DNA band. Measure the concentration of the purified PCR product and the purified vector using UV spectrometry (see Table of Materials and Equipment).

2.1.5 Prepare the E1U reporter construct by ligating the purified, restriction enzyme digested PCR product and pGL3-Basic firefly luciferase reporter vector (contained in the reporter assay kit – see Table of Materials and Equipment) at insert : vector ratios of 1:1, 2:1 and 3:1, using the "T4 DNA ligase quick ligation kit" as per kit instructions[22], thereby producing the E1U reporter construct, named pGL3-E1U.

2.1.5.1 Use the pGL3-E1U plasmid to transform bacteria to clonally expand pGL3-E1U following instructions in the TA cloning kit (see Table of Materials and Equipment).

2.1.6 Prepare reporter constructs for E1A, E1B and E1C using similar methodology as for E1U. Confirm the fidelity of the inserts by sequencing as in section 2.1.5.2.

Note: The promoter inserts for E1A, E1B and E1C should be approximately −1875/+10,

−1847/+60, and −1904/+83 relative to the first 5' nucleotide (designated as +1) of E1A, E1B, or E1C, respectively.

2.2) Reporter assay methods

Note: General strategy of reporter assays: the putative promoter (5' upstream region) of a gene is inserted into the multiple cloning site of an empty "reporter" vector that contains a "reporter gene" (e.g., firefly luciferase) downstream from the multiple cloning site. The vector is then transfected into eukaryotic cells that express the gene of interest. The trans-acting factors that promote expression of the gene of interest in these cells should activate the promoter in the transfected reporter vector, causing expression of firefly luciferase, which can be easily quantified with a luminescence assay.

2.2.1 Select the appropriate cell line to use for the reporter assay.

Note: To evaluate the activity of the E1U alternative promoter reporter construct, it is necessary to transfect that construct into cells that express Bcrp1 under control of the E1U promoter. The TM4 murine Sertoli cell line (see Table of Materials and Equipment) expresses Bcrp1 protein and Bcrp1 mRNA containing E1U, as well as E1A, E1B, and E1C[5]. As a negative control, consider using a cell line that does not express Bcrp1 protein or mRNA.

2.2.2 Culture the TM4 cells in 24-well plates at an initial density of 200,000 cells/well in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium with 1.2 g/L sodium bicarbonate, 15 mM HEPES supplemented with 5% horse serum, 2.5% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 µg/mL), at 37˚C, 5% CO_2, as described previously[5].

2.2.3 Six hours after placing the cells in culture, transfect the cells with 0.2 µg of empty pGL3-basic vector or with 0.2 µg of pGL3 vector containing the −1906/+64 E1U or −1875/+10 E1A or −1847/+60 E1B or −1904/+83 E1C *Bcrp1* deletion construct along with 4 ng of pRL-TK (a Renilla luciferase-expressing vector) as internal control, using a commercial DNA transfection kit and the manufacturer's protocol[23] (see Table of Materials and Equipment).

2.2.4 30 hours following transfection, remove the growth media from the cultured transfected cells. Wash the cells once with 200 µL of PBS, then remove the wash solution.

2.2.4.1 Measure firefly and *Renilla* luciferase activity using a commercial kit according to manufacturer's protocols[24].

2.2.5 Express the activity for each tube as the ratio of the firefly luciferase activity divided by the internal (*Renilla* luciferase) control; overall results are usually expressed as the activity of each reporter construct relative to that of cells transfected with the empty pGL3 basic vector, which is given a value of 1.

REPRESENTATIVE RESULTS:

Identification of Bcrp1 alternative promoter utilization in mouse testis by analysis of leader exons

When the EST database at NCBI was probed (April 2015) using the steps outlined in Protocol 1, the ESTs found that were contiguous with the 5'end of exon 2 in *Bcrp1* mRNA are shown in Table 1, along with their position in genomic DNA relative to the start of exon 2. One EST derived from C57BL/6J mouse testis that is contiguous to exon 2 in *Bcrp1* mRNA has sequences in genomic DNA 70 kb upstream from exon 2, corresponding to E1U (Table 1). Similarly, ESTs corresponding to E1A, E1B, and E1C were detected. Of note is that the two ESTs corresponding to E1C also contained E1B spliced to the 5' end of E1C. The location of these predicted first exons in relation to Bcrp1 exon 2 on mouse chromosome 6 is shown in Figure 1.

Evaluation of Bcrp1 alternative promoter function

Typical luciferase assay results corresponding to E1U, E1A, E1B and E1C promoter-luciferase reporter constructs transfected into TM4 murine Sertoli cells (see section 2.2) are shown in Figure 2A.

In previous work, an SF-1 response element was predicted to be in the E1U promoter[5]. If this putative SF-1 response element is involved in the regulation of Bcrp1 transcription in mouse testis, then mutation (as described in a previous paper[5]) of that response element in the E1U promoter reporter construct will reduce the luciferase activity produced by that construct when transfected into TM4 cells. Results of a typical experiment are shown in Figure 2B. The mutated construct shows lower luciferase activity than the un-mutated construct, with activity comparable to that of the empty pGL3-basic vector, even in cells with enforced expression of SF-1 (described in a previous paper[5]). Enforced expression of SF-1 increases the activity of the un-mutated construct, but not that of the mutated one.

Figure 1. Diagram of the genomic alignment of ESTs with sequence identity to Bcrp1 exon 2 with murine chromosome 6. These alignments were identified in a dbEST BLAST search performed in April, 2015. Four distinct alignments were found, corresponding to Bcrp1 alternative first exons E1U, E1A, E1B, and E1C. This figure is reproduced from a previous publication[5].

Figure 2. A. Reporter assay for Bcrp1 promoters E1U, E1A, E1B, and E1C in BALB/c Sertoli cell-derived TM4 cells. Data are expressed as the luminescence of firefly luciferase normalized to that of Renilla luciferase, using methods described in section 2. Data shown are the mean and standard deviation of three different experiments, done on different days. The asterisk indicates a significant difference from the empty pGL3 vector control using the *t*-test ($P<0.01$). This figure is reproduced from a previous publication[5]. **B.** Effects of mutation of the SF-1 response element in the Bcrp1 E1U reporter construct on luciferase activity. Bcrp1 reporter constructs with the putative SF-1 binding region (mutated or un-mutated) were transfected into TM4 cells with (SF-1 transfected) and without (vector transfected) the enforced expression of SF-1. The data shown are the mean of 3 different experiments, done on different days; the error bars represent standard deviations. For each experiment, individual assays were done in duplicate. In the figure, **a** denotes a significant difference from the empty vector pGL3 control

(P<0.05) using the *t*-test; **b** signifies a statistically significant difference compared to the E1U promoter construct using the *t*-test (P<0.05). This figure is reproduced from a previous publication[5].

Table 1[25-28]**.** Blast search of the mouse EST database against the sequence of the second exon of Bcrp1.

DISCUSSION:

The majority of the methods and representative results presented are described in previous work entitled "*B*crp1 transcription in mouse testis is controlled by a promoter upstream of a novel first exon (E1U) regulated by steroidogenic factor-1," that was published in 2013[5]. In addition to the representative results depicted here, the previous paper provided estimates of alternative first exon utilization using 5'-RACE PCR and RT-PCR methodology. Furthermore*, in-silico* identification of a putative SF-1 response element in the promoter upstream from E1U was accomplished, and chromatin immunoprecipitation (ChIP) assays demonstrated that SF-1 bound to the putative SF-1 response element. Functional studies revealed that in murine Sertoli cells, *Bcrp1* transcription is controlled by SF-1 via the SF-1 response element in the E1U promoter. These functional studies included upregulation of SF-1 in TM4 cells by transfection or by the use of a histone deacetylase inhibitor (vorinostat), which resulted in enhanced expression of *Bcrp1* E1U mRNA, and an increase in *Bcrp1* protein as well as activity of the *Bcrp1* E1U promoter in a reporter assay. Finally, these studies provided evidence that in testes from adult Sertoli-cell specific SF-1 knockout mice, the expressions of *Bcrp1* E1U mRNA, total *Bcrp1* mRNA, and Bcrp1 protein were markedly diminished[5]. The same work also reported the expression patterns of Bcrp1 mRNA isoforms in a variety of murine tissues, including kidney, liver, testis, brain, heart, lung, muscle, and spleen[5].

The protocols described here – using tissue-specific regulation of *Bcrp1* as a model – provide a facile experimental framework to unravel the transcriptional complexity of any gene to determine its differential expression in various tissues or cellular subtypes. It must be emphasized that the results obtained from the protocol steps described for *in-silico* evaluations may be time-dependent since the various websites that house the software and databases used may be subject to change. The *in-silico* methodology presented here utilizes searching the EST database (dbEST) as reported previously[29], which estimated that approximately 18% of all human genes in the EST dataset employ alternative promoters. Searching the dbEST may underestimate alternative first exons, because other investigators – using an "oligo-capping" method to produce 1.8 million 5'-end sequences of cDNAs from many human tissues – were able to identify putative transcriptional start sites for human genes and estimate that 52% of the human RefSeq genes studied were possibly regulated by alternative promoters[1]. Interestingly, the latter study found that the tissues that utilized putative alternative promoters the most were testis and the brain; furthermore, they found that genes encoding proteins related to signal transduction pathways were more likely to employ tissue-specific alternative promoters[1]. In spite of the drawbacks mentioned, dbEST analyses provide a rough overview of 5' UTR usage for a particular gene in multiple tissues with minimal effort.

Although dbEST analysis provides a facile glimpse at alternative first exon usage, we highly recommend performing 5' rapid amplification of cDNA ends (5'-RACE) analysis to verify first exon usage in a tissue or cell line under investigation. Commercial kits are available for 5'-RACE, with detailed and straightforward procedures provided by the manufacturer (see Table of Materials and Equipment). Although somewhat time consuming, 5'-RACE studies provide actual estimates of the presence as well as the sequence of alternative first exons in the mRNA of interest; furthermore, the presence of multiple transcriptional start sites can also be detected[13]. To assure sufficient representation, sequencing of at least 15 to 25 clones is required. Before sequencing, it is essential to remove contaminating vector sequences from the 5' RACE products. If this is not done, BLAST analysis may fail completely to detect a sequence alignment with the mouse genome. Once first exon usage is established, the 5'-RACE findings can be used to validate subsequent quantitative RT-PCR assays for alternative first exons. In general, the authors' previous work found that there is good correlation between the percentage of first exon mRNA clones recovered by 5'RACE and the percentage of PCR product recovered for a given alternative first exon from the same tissue or cell line[5]. Additionally, good correlation was found between the activities of luciferase promoter constructs for alternative Bcrp1 promoters and the expression of the corresponding alternative first exon in a particular cell line[5].

In the search for tissue-specific alternative promoter usage, if whole organs are used, one must be aware that the alternative promoters/first exons found will reflect the tissue heterogeneity of the organ such as glandular elements, blood vessels, stroma, etc. For example, the testis is a mix of somatic (Leydig, Sertoli, myoid, and endothelial) cells and germinal (haploid) cells, as well as vasculature. To probe for tissue-specific first exon expression, it will be necessary to isolate specific tissues from the organs.

Other methods have been reported for identifying alternative promoter usage and regulation; however, these require next-generation sequencing and bioinformatics computing capable of analyzing the large amount of data produced. These methods include whole transcriptosome sequencing (RNA seq), and ChIP-seq of histones such as H3K4me3, which binds preferentially to promoters[30]. Although these methods can be expensive to perform *de-novo*, when the focus is on expression of a few specific genes and tissues, mining of existing data may be a more practical approach.

The assays for estimating promoter activity presented here employ the pGL3-basic vector, which contains a multiple cloning region upstream from the firefly luciferase gene. Promoter activity is measured by the production of firefly luciferase, which is easily quantified, after addition of cofactors and a luminescent substrate, by measurement of luminescence in a commercial luminometer (see Table of Materials and Equipment). The related pGL4 vector can be made to contain selectable markers (e.g., neomycin, hygromycin, puromycin), enabling production of stably transfected cells. The protocols given in this paper utilize transient transfection. Typically, reporter assays for promoter activity are done using co-transfection with a vector that constitutively expresses *Renilla* (Sea Pansy) luciferase (pRL-TK), to control for variations in cell number and the efficiency of transfection. A critical step in establishing a

promoter assay for a given gene is to be sure that the gene of interest is expressed in the cell line that is transfected with the reporter construct. Secondly, when alternative promoter usage is present, it is important to use the promoter construct that corresponds to the alternative first exon expressed in the cell line transfected. For example, do not expect to see luminescence for the E1U promoter construct that is transfected into cells that express Bcrp1 solely under the control of the E1B promoter. Alternatively, as a negative control, transfection of the reporter construct into a cell line that does not express the gene or mRNA isoform of interest should result in no production of firefly luciferase.

The consequences of alternative promoter usage include production of the same protein, production of proteins with different N-termini, or production of different proteins[29]. In the case of Bcrp1/BCRP, multiple (tissue- or cell type-specific) promoters control the production of the same protein. A similar pattern exists for the CYP19 (aromatase) gene[29,31]. The protocols presented here provide the fundamental steps one can use to decipher in a tissue or cell line the complex mechanisms of transcriptional control of a protein of interest.

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DISCLOSURES:
Douglas D. Ross and the University of Maryland, Baltimore hold patent rights to human BCRP.

REFERENCES:
1 Kimura, K. *et al.* Diversification of transcriptional modulation: large-scale identification and characterization of putative alternative promoters of human genes. *Genome Res.* **16** (1), 55-65, doi:10.1101/gr.4039406 (2006).
2 Doyle, L. A. *et al.* A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A.* **95** (26), 15665-15670 (1998).
3 Natarajan, K., Xie, Y., Baer, M. R. & Ross, D. D. Role of breast cancer resistance protein (BCRP/ABCG2) in cancer drug resistance. *Biochem Pharmacol.* **83** (8), 1084-1103, doi:10.1016/j.bcp.2012.01.002 (2012).
4 Qian, X., Cheng, Y. H., Mruk, D. D. & Cheng, C. Y. Breast cancer resistance protein (Bcrp) and the testis--an unexpected turn of events. *Asian J Androl.* **15** (4), 455-460, doi:10.1038/aja.2013.24 (2013).
5 Xie, Y. *et al.* Bcrp1 transcription in mouse testis is controlled by a promoter upstream of a novel first exon (E1U) regulated by steroidogenic factor-1. *Biochim Biophys Acta.* **1829** (12), 1288-1299, doi:10.1016/j.bbagrm.2013.10.008 (2013).
6 Maliepaard, M. *et al.* Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res.* **61** (8), 3458-3464 (2001).
7 Fetsch, P. A. *et al.* Localization of the ABCG2 mitoxantrone resistance-associated protein in normal tissues. *Cancer Lett.* **235** (1), 84-92, doi:10.1016/j.canlet.2005.04.024 (2006).
8 Cooray, H. C., Blackmore, C. G., Maskell, L. & Barrand, M. A. Localisation of breast cancer resistance protein in microvessel endothelium of human brain. *Neuroreport.* **13** (16), 2059-2063

(2002).
9 Kolwankar, D., Glover, D. D., Ware, J. A. & Tracy, T. S. Expression and function of ABCB1 and ABCG2 in human placental tissue. *Drug Metab Dispos.* **33** (4), 524-529, doi:10.1124/dmd.104.002261 (2005).
10 Xiong, H. *et al.* ABCG2 is upregulated in Alzheimer's brain with cerebral amyloid angiopathy and may act as a gatekeeper at the blood-brain barrier for Abeta(1-40) peptides. *J Neurosci.* **29** (17), 5463-5475, doi:10.1523/JNEUROSCI.5103-08.2009 (2009).
11 Woodward, O. M. *et al.* Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout. *Proc Natl Acad Sci U S A.* **106** (25), 10338-10342, doi:10.1073/pnas.0901249106 (2009).
12 Huls, M. *et al.* The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane. *Kidney Int.* **73** (2), 220-225, doi:10.1038/sj.ki.5002645 (2008).
13 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.* **66** (10), 5007-5011, doi:10.1158/0008-5472.CAN-05-4572 (2006).
14 Ayoubi, T. A. & Van De Ven, W. J. Regulation of gene expression by alternative promoters. *FASEB J.* **10** (4), 453-460 (1996).
15 Zong, Y., Zhou, S., Fatima, S. & Sorrentino, B. P. Expression of mouse Abcg2 mRNA during hematopoiesis is regulated by alternative use of multiple leader exons and promoters. *J Biol Chem.* **281** (40), 29625-29632, doi:10.1074/jbc.M606314200 (2006).
16 Natarajan, K. *et al.* Identification and characterization of the major alternative promoter regulating Bcrp1/Abcg2 expression in the mouse intestine. *Biochim Biophys Acta.* **1809** (7), 295-305, doi:10.1016/j.bbagrm.2011.06.004 (2011).
17 *Ensembl Genome Browser*, <http://Ensembl.org> (2015).
18 *Transcript-based Display from Ensembl*, <http://www.ensembl.org/Mus_musculus/Transcript/Exons?db=core;g=ENSMUSG00000029802;r=6:58584523-58692869;t=ENSMUST00000031822> (2015).
19 *Basic Logical Alignment Search Tool (BLAST) homepage*, <http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome> (2015).
20 *National Center for Biotechnology Information (NCBI)*, <http://www.ncbi.nlm.nih.gov/> (2015).
21 Promega. *Wizard® SV Gel and PCR Clean-Up System - INSTRUCTIONS FOR USE OF PRODUCT* (2009).
22 New_England_Biolabs. *Quick Ligation Kit Protocol - M2200S* (2014).
23 Roche. *XtremeGENE HP DNA Transfection Reagent Protocol - Manual version 1.0* (2010).
24 Promega. *Quick Protocol for the use of the Dual-Luciferase Reporter Assay* (2009).
25 Carninci, P. *et al.* The transcriptional landscape of the mammalian genome. *Science.* **309** (5740), 1559-1563, doi:10.1126/science.1112014 (2005).
26 VanBuren, V. *et al.* Assembly, verification, and initial annotation of the NIA mouse 7.4K cDNA clone set. *Genome Res.* **12** (12), 1999-2003, doi:10.1101/gr.633802 (2002).
27 Bonaldo, M. F., Lennon, G. & Soares, M. B. Normalization and subtraction: two approaches to facilitate gene discovery. *Genome Res.* **6** (9), 791-806 (1996).

28 Okazaki, Y. *et al.* Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. *Nature.* **420** (6915), 563-573, doi:10.1038/nature01266 (2002).
29 Landry, J. R., Mager, D. L. & Wilhelm, B. T. Complex controls: the role of alternative promoters in mammalian genomes. *Trends Genet.* **19** (11), 640-648, doi:10.1016/j.tig.2003.09.014 (2003).
30 Park, P. J. ChIP-seq: advantages and challenges of a maturing technology. *Nat Rev Genet.* **10** (10), 669-680, doi:10.1038/nrg2641 (2009).
31 Bulun, S. E. *et al.* Regulation of aromatase expression in breast cancer tissue. *Ann N Y Acad Sci.* **1155** 121-131, doi:10.1111/j.1749-6632.2009.03705.x (2009).

Figure 1.

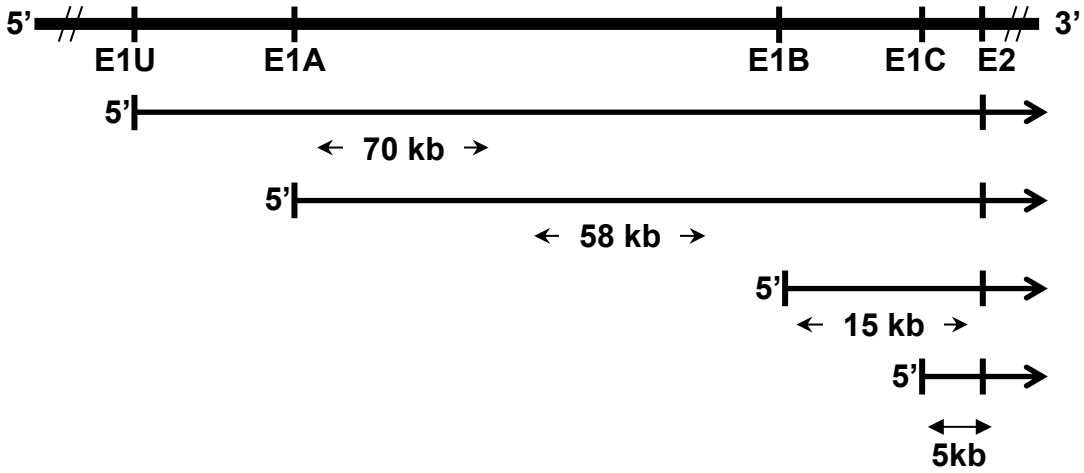

Figure 2A.

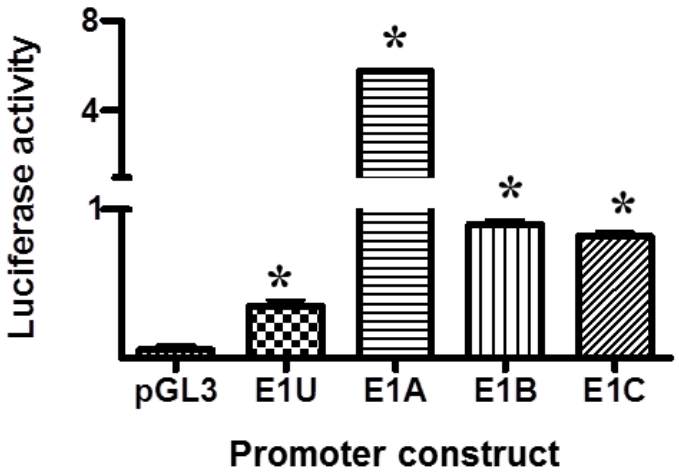

Figure 2B.

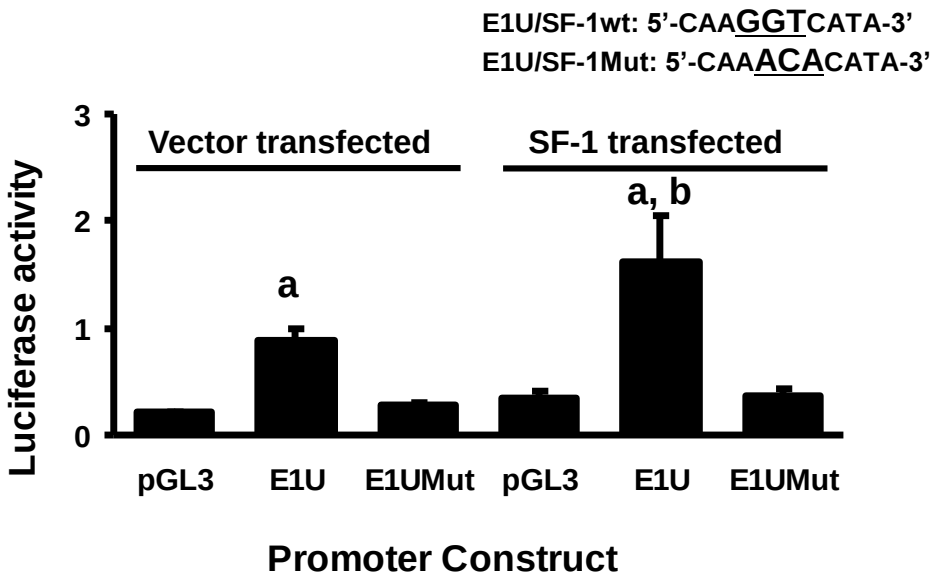

Table 1. Blast search of the mouse EST database against the sequence of the second exon of Bcrp1. Adapted from [5].

Accession #	bp in EST	Reference	Tissue source	Description	TSS*	Splice donor*	Exon name
CJ042273	465	Hyashizaki Y[25]	Testis	CJ042273 RIKEN full-length enriched mouse cDNA library, C57BL/6J testis male adult Mus musculus cDNA clone 1700028L09 5-, mRNA sequence	-70664	-70578	E1U
BQ561807	515	Yong Quian[26]	Mixed	H4072E04-5 NIA Mouse 7.4K cDNA clone Set Mus musculus cDNA clone H4072E07 5-, mRNA sequence	-58967	-58530	E1A
CA570499	604	Dudekula DB-u	Hematopoietic Stem cell (Lin-/c-Kit+/Sca-1+)	K0507B12-5N NIA Mouse Hematopoietic Stem Cell (Lin-/c-Kit+/Sca-1+) cDNA Library (Long) Mus musculus cDNA clone NIA:K0507B12 IMAGE:30061535 5-, mRNA sequence	-58967	-58530	E1A
CO039662	697	Strausberg R-u	Hypothalamus	UI-M-BH2.3-aoc-c-04-0-UI.s1 NIH_BMAP_M_S3.3 Mus musculus	-58607	-58530	E1A
BE648996**	364	Chin H[27]	Brain	UI-M-BH2.3-aoc-c-04-0-UI.r1 NIH_BMAP_M_S3.3 Mus musculus cDNA clone UI-M-BH2.3-aoc-c-04-0-UI 5-, mRNA sequence	-59663 -58665	-59621 -58528	E1A/ E1A1
CB952822	722	Strausberg R-u	Kidney	AGENCOURT_13687590 NIH_MGC_176 Mus musculus cDNA clone IMAGE:30304782 5-, mRNA sequence	-15059	-15011	E1B
AI466665	263	Marra M-u	Whole embryo	mg85b06.y1 Soares mouse embryo NbME13.5 14.5 Mus musculus cDNA clone IMAGE:439763 5-, mRNA sequence	-15073	-15011	E1B
AI882010	525	Marra M-u	Kidney	Ul30b11.y1 Sugano mouse kidney mkia Mus musculus cDNA clone IMAGE:2099805 5- similar to TR:Q02785 Q02785 LPE14P.;, mRNA sequence	-15078	-15011	E1B
BY730713	675	Hayashizaki Y[28]	Kidney	BY730713 RIKEN full-length enriched, 0 day neonate kidney Mus musculus cDNA clone D630015I16 5-, mRNA sequence	-15092	-15011	E1B
AA008579	926	Marra M-u	Embryo	mg85b06.r1 Soares mouse embryo NbME 13.5 14.5 Mus musculus cDNA clone IMAGE:439763 5- similar to PIR:S48442 hypothetical protein – yeast;, mRNA sequence	-15074	-15011	E1B
AW611052***	652	Marra M-u	Kidney	un76b05.y1 Sugano mouse kidney mkia Mus musculus cDNA clone IMAGE:2536785 5- similar to TR:O95374 BREAST CANCER RESISTANCE PROTEIN. ;, mRNA sequence	-15043 -5179	-15007 -5095	E1B/ E1C
AI226912****	562	Marra M-u	Kidney	uj11b07.y1 Sugano mouse kidney mkia Mus musculus cDNA clone IMAGE:1907701 5- similar to TR:Q02785 Q02785 LPE14P. ;, mRNA sequence	-15054 -5179	-15007 -5095	E1B/ E1C
AI647825#	529	Marra M-u	Kidney	uk44f03.x1 Sugano mouse kidney mkia Mus musculus cDNA clone IMAGE:1971869 3' similar to TR:P78577 P78577 ATP-BINDING CASSETTE MULTIDRUG TRANSPORTER. ;, mRNA sequence			#
AI664571#	458	Marra M-u	Embryo	uk25h09.y1 Sugano mouse embryo mewa Mus musculus cDNA clone IMAGE:1970081 5' similar to SW:YOH5_YEAST Q08234 PROBABLE ATP-DEPENDENT TRANSPORTER YOL075C. ;, mRNA sequence			#

Footnotes:

* TSS (Transcription Start Site) and splice donor site are in relation to 5' end of Exon 2 (+1)

** A novel alternative Bcrp1 Exon 1 spliced upstream of E1A in the EST BE648996, and hence designated as E1A1.

*** E1C spliced to E1B on its 5' end and E2 on its 3' end (EST accession # AW611052.2); 5' UTR comprised a combination of two first exons

**** E1C spliced to E1B on its 5' end and E2 on its 3' end (EST accession # AI226912.3); 5' UTR comprised a combination of two first exons

EST does not include 5' end of Bcrp1 exon 2, so no inferences to the 5' UTR can be made

u Unpublished

MATERIALS AND EQUIPMENT

Methods to discover alternative promoter usage and mechanisms of *Bcrp1* transcriptional regulation in mouse testes.

Name of the Material/Equipment	Company	Catalog Number	Comments/ Description (optional)
COMMERCIAL BIOLOGIC MATERIALS AND KITS:			
First Choice RLM-RACE kit	Ambion Inc., Austin, TX, currently available through Life Technologies	AM1700	5'-RACE PCR kit
TOPO TA cloning kit	Life Technologies	K450001	This kit contains the TOP10 chemically competent *E. coli* bacteria.
T4 DNA ligase quick ligation kit	New England Biolabs	M2200	
Faststart high-fidelity Taq- DNA polymerase	Sigma-Aldrich/Roche	3553400001/RMB-4738284001	Contains a high-fidelity Taq polymerase enzyme blend
XtremeGENE HP DNA transfection reagent	Roche	6366236001	
Dual-luciferase reporter assay kit	Promega	E1910	Includes the pGL3-basic empty reporter vector (firefly luciferase), pRLTK renilla luciferase expressing vector, and other control vectors.
QIAprep	Qiagen	27104	For plasmid miniprep - isolation and purification of plasmid DNA from bacterial colonies
pCR2.1 vector	part of the TOPO TA cloning kit		
pGL3-basic luciferase containing vector	Promega	E1751	
pRL-TK Renilla luciferase expressing vector	Promega	E2241	
Bacterial artificial chromosome	BACPAC Resources Center, Children's Hospital Oakland Research Institute, Oakland, CA	RP23-285A12 and RP24-314E24	http://bacpac.chori.org/clones.htm
REAGENTS/CHEMICALS/MEDIA/SUPPLIES			
TRIzol	Life Technologies	15596026	
Wizard® SV Gel and PCR Clean-Up System	Promega	A9281	Useful for purifying PCR products following digestion with restriction endonucleases
CRYOVIALS	Denville Scientific	V9012	
SOFTWARE:			
Ensembl software	Ensembl project	http://Ensembl.org	The Ensembl project produces genome databases for vertebrates and other eukaryotic species, and makes this information freely available online.
Blast software	NCBI	http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome	
Primer 3 software	Simgene	http://simgene.com/Primer3	Useful for designing primers for 5'-RACE PCR
NCBI Nucleotide database	NCBI	http://www.ncbi.nlm.nih.gov/nucleotide/	
CELL LINES:			
TM4 murine Sertoli cells	ATCC, Manassas, Virginia	CRL-1715™	
INSTRUMENTS:			
Luminometer	Turner Biosystems	TD-20/20	This is a relatively inexpensive, manually operated luminometer. Automated systems are also available from the same manufacturer that utilize 96 well plates.
NanoDrop spectrophotometers	Thermo Scientific, Inc.	NanoDrop 2000	Spectrophotometer can use very small quantities of sample
DU 800 UV/VIS spectrophotometer and Nucleic Acid Analysis II software	BeckmanCoulter Inc, Fullerton, CA	DU 800	

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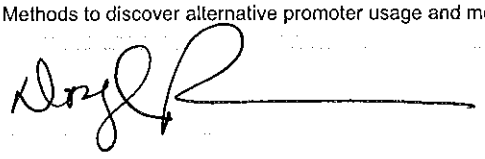
Signature:	 Date: June 10, 2015

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October 23, 2015

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Dear Editors:

On behalf of the authors, I am resubmitting a manuscript entitled "Methods to discover alternative promoter usage and transcriptional regulation of murine Bcrp1" by Karthika Natarajan, Yi Xie, Takeo Nakanishi, Rebecca S. Moreci, Pancharatnam Jeyasuria, Arif Hussain, and Douglas D. Ross that has been revised in response to your editorial review. The revised manuscript has been read and approved by all of the authors.

The following are your editorial comments; our responses to them are given in ***bold italics***.

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2. Please revise the highlighting of the protocol text for the video. There is a 2.75 page limit to ensure that the videography can occur in a single day and you have 3.5 pages currently highlighted. ***We eliminated protocol steps in section 2.1.5.1 and 2.2.5-9, which contain details of procedures that are clearly outlined in kit manufacturers' instructions. In all, we have eliminated 22 lines of highlighted text. When the highlighted text is copied and placed in a separate document, it comes to a few lines over 2 pages (with no spacing between paragraphs or steps).***

On behalf of the authors, I thank you for your thoughtful consideration and evaluation of our manuscript.

Should you have any questions, please do not hesitate to contact me at 410-328-8095 or by email at dross@som.umaryland.edu.

Sincerely yours,

Douglas D. Ross, MD, PhD, FACP
Professor of Medicine and Pathology

SCREENSHOTS