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SP1 Regulates the Transcription of *BMPR1A*

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Background. **BMPR1A is a cell surface receptor in the bone morphogenetic protein (BMP) pathway. Mutations in *BMPR1A* predispose to juvenile polyposis (JP). Sp1 and related proteins are widely expressed regulators of gene transcription, including members of the BMP pathway. We set out to identify important transcription factor binding sites (TFBS) in the recently identified *BMPR1A* promoter and to assess for the role of Sp1 and associated proteins in its regulation.**

Materials and Methods. **The *BMPR1A* promoter was cloned into a luciferase reporter vector. Deletion fragments of this promoter insert were then constructed, of varying lengths and opposing directions, and were used to transfect HEK-293 and CRL-1459 cells. *In silico* analysis was performed to screen for relevant TFBS. Site-directed mutagenesis (SDM) was then employed to individually disrupt these TFBS in the wild-type (WT) vector. SDM constructs were then assessed for activity.**

Results. **Light activity from the deletion constructs ranged between 3% and 129% of the WT promoter. ModelInspector identified eight potential binding sites for Sp1- and Sp1-associated proteins that mapped to areas of marked loss or gain of activity from the deletion constructs. SDM of these TFBS led to a drop in activity in five mutants, which included 3 Sp1 sites, an ETSF site, and NFκB site.**

Conclusions. **By combining *in silico* analysis and experimental data, Sp1 was found to be a candidate factor that likely plays a role in the transcriptional regulation of *BMPR1A*. This study potentially provides further insight toward the molecular basis of JP, and suggests that Sp1 plays a role in BMP signaling.**

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Key Words: **BMPR1A; promoter; bone morphogenetic protein; deletion mutagenesis; luciferase; modelInspector; Sp1.**

INTRODUCTION

BMPR1A is transmembrane cell surface receptor with serine-threonine kinase activity. When activated through phosphorylation by the type II receptor after binding bone morphogenetic protein (BMP) ligands [1, 2], BMPR1A phosphorylates SMADs 1, 5, and/or 8, which then bind to SMAD4. That complex then migrates to the nucleus, recruits DNA-binding proteins, and binds to promoters to regulate transcription of various genes [3]. The BMP pathway has been implicated in a number of cellular processes such as lineage commitment, differentiation, proliferation, cellular maintenance, and apoptosis [4–7]. The role of BMP signaling and related proteins in cancer and tumorigenesis is of increasing interest to researchers [8]. Germline mutations in coding regions of *BMPR1A* are known to cause the disease juvenile polyposis (JP) [9]. We have recently characterized the promoter for *BMPR1A* and found germline genetic alterations in JP probands that likely predispose to JP [10]. Therefore, understanding the transcriptional control of *BMPR1A* is important, as it will help us to better understand pathological processes affected by that pathway.

Sp1 is a transcription factor that either enhances or represses the activity of promoters of a multitude of target genes [11]. Sp1 belongs to the specificity protein/Krüppel-like Factor (SP/KLF) family of transcription factors. This family of transcription factors targets genes that play important roles in the process of tumorigenesis [12, 13]. Furthermore, Sp1 has been shown to be necessary in the regulation of genes that respond to TGF-β stimulation [14].

Understanding the transcriptional regulation of *BMPR1A* is important due to its central role in BMP

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signaling and related disorders. Furthermore, JP carries a >50% lifetime risk of colorectal cancer development [15], suggesting that defective BMPR1A production is central to the oncogenic steps in this process. The objectives of this study were 2-fold: to identify essential transcription factor binding sites (TFBS) involved in the regulation of *BMPR1A* transcription and to combine a modified approach to traditional promoter deletion mutagenesis and computational analyses to identify important TFBS.

METHODS

Construction of Luciferase Reporter Vectors

The previously described promoter region for *BMPR1A* [10], a 748-bp region (Chr 10: 88505860–88506607, NCBI36/hg18), was amplified from genomic DNA by PCR. Sense primers included the *MluI* restriction site at the 5′ end and antisense primers had the *BglII* site at the 3′ end. PCR products were purified using the Qiagen Gel Extraction kit (Qiagen, Valencia, CA), digested with *BglII* and *Mlu1,* then subcloned into the *BglII-Mlu1* site of the pGL3-basic vector (Promega, Madison, WI). *E. coli* cells were transformed, colonies were selected then cultured overnight. Plasmid DNA was extracted using the PureLink kit (Invitrogen, Carlsbad, CA), and inserts were verified by direct sequencing.

BMPR1A-Promoter pGL3 Constructs

To create different deletion constructs, PfuUltra (Stratagene, La Jolla, CA) was used to generate blunt-ended linear segments of DNA from the 748 bp BMPR1A pGL3 full promoter construct, leaving out 125 bp at a time. DNA from the linear constructs was purified using the Qiagen PCR purification kit (Qiagen). Phosphate groups were added to DNA ends using PolyNucleotide Kinase (New England Biolabs, Ipswich, MA), and then ends were ligated using T4 Ligase. Competent cells were transformed and bacterial colonies were grown under antibiotic selection. Plasmid DNA was extracted from individual colonies, insert sizes checked by gel electrophoreses, and sequences were verified by capillary sequencing (Applied Biosystems, Carlsbad, CA).

Cell Lines and Transfection

A normal human colon fibroblast cell line (CRL-1459) and human embryonic kidney cell line (HEK-293) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Cells were cultured to achieve 90%–95% confluence in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. pGL3 constructs were transfected using Lipofectamine 2000 (Invitrogen). Two μg of pGL3 construct and 500 ng of Renilla control vector (pRL-CMV; Promega) in each well. Cells were lysed 48 h later and light units were quantified with a TD 20/20 luminometer (Turner BioSystems, Sunnyvale, CA) using the dual luciferase reporter assay (Promega, Madison, WI). All experiments were done in triplicate, and measurements were normalized to the internal control.

In Silico Analysis

Computational analysis was performed using the Genomatix Software Suite (www.genomatix.de). MatInspector was used to locate TFBS [16], which utilizes a large library of matrix descriptions of TFBS to locate matches in DNA sequences. Similar or functionally related TFBS are grouped into matrix families and individual sites are assigned quality ratings based on available consensus sequence data. The *BMPR1A* promoter sequence was searched for possible TFBS, and then ModelInspector was used to search for predefined promoter models. This program scans DNA sequences for matches to previously described models. Models are groups of various individual elements (TFBS in this study) that are defined by strand orientation, sequential order, and distance ranges [17, 18]. Areas with significant loss or gain of promoter activity from our deletion constructs were then mapped and all TFBS determined.

Site Directed Mutagenesis

To assess the importance of the sites in our luciferase models, SequenceShaper (Genomatix) was utilized to delete individual bases in TFBS. Alterations were made such that a minimal number of other TFBS were created or lost. Complementary primers of varying lengths were made with the intended base changes in the center. Pfu-Ultra (Stratagene, La Jolla, CA) was then used to amplify the wild-type *BMPR1A* promoter pGL3 insert under the following conditions: 95°C for 30 s, 65°C for 1 min, and then 7 min at 68°C for 18 cycles. PCR products containing the desired mutations were then used to transform bacterial cells. Colonies were selected, DNA extracted, and sequenced for verification. These vectors were then transfected into HEK-293 cells in triplicate to evaluate changes in promoter activity and validate the functionality of binding sites.

RESULTS

Functional Areas

To define areas in the promoter that contain important regulatory sites, we designed a bidirectional promoter deletion mutagenesis experiment and tested it in two different cell lines. Ten *BMPR1A* pGL3 deletion constructs were generated; five were made sequentially shorter by 125 bases starting at the 5′ end and five were made sequentially shorter from the 3′ end (Fig. 1A). All constructs were transfected into HEK-293 and CRL-1459 cells. After adjusting for transfection efficiency, the same vectors generated more luciferase in HEK-293 compared with CRL-1459. The full construct generated 149.5 light units in HEK-293 compared with 4.8 units in CRL-1459, and these values were set as 100% to compare with other constructs in their respective cell lines. The trends were similar in both cell lines relative to gain or loss of promoter activity for each construct. The first four forward deletion constructs (F1-F4, Fig. 1B) demonstrated minimal activity of all segments extending out to 500 bases in length. The addition of the most 5′ 248 bases led to a notable increase in activity (84.3% of wild-type in HEK-293 and 82.2% in CRL-1459), suggesting important TFBS in that region. When the reverse segments were examined, there were more constructs with luciferase activity. The loss of the 125 bp between positions 126 and 250 (R2) led to an increase in activity in both cell lines (2.3-fold in HEK-293 and 2.8-fold in CRL-1459, relative to the 125 bp longer construct). Furthermore, the loss of the 125-base segment between R2 and R3 almost abolished luciferase activity in HEK-293. These results suggested that the regions between bases 126 to 375, and 501 to 748 had

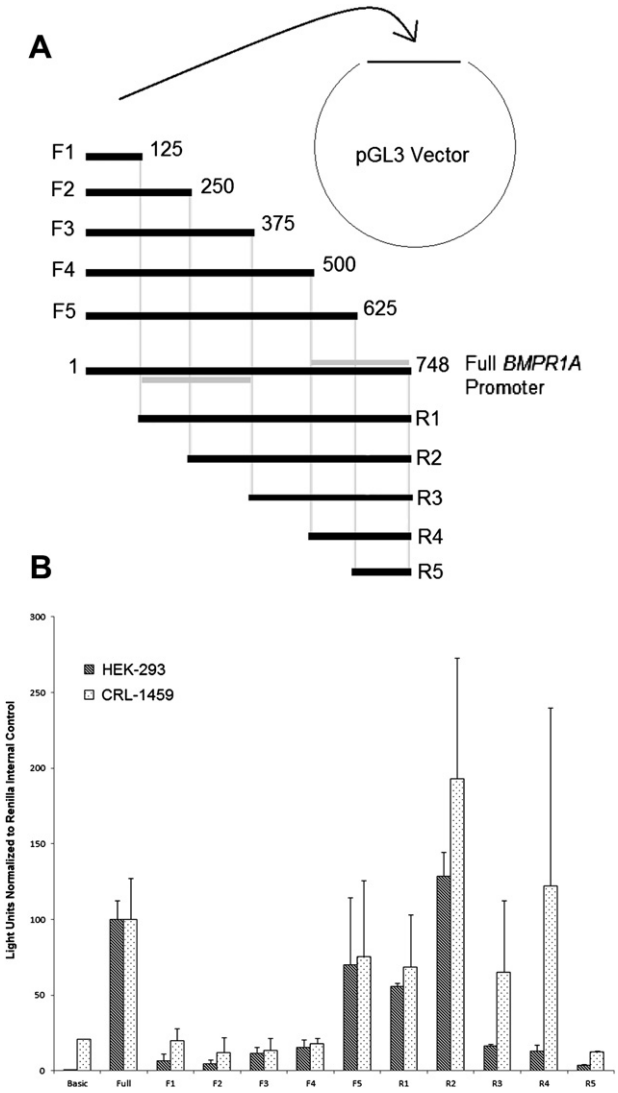

FIG. 1. (A) Deletion mutagenesis was used to make 10 constructs of the wild-type 748 bp *BMPR1A* promoter. F1 through F5 refer to forward deletion constructs consecutively shorter from the 3′ end, whereas R1 through R5 are reverse deletion constructs shorter from the 5′ end. (B) Transfection of deletion constructs into HEK-293 (diagonal stripes) and CRL-1459 (dotted) led to a similar trend in gain or loss of promoter activity. Y-axis on the right is in reference to HEK-293; on the left is in reference to CRL-1459. The "basic" vector is pGL3 with no promoter insert. WT (full) promoter is set to have 100% activity.

the most functional significance, which refined our search for relevant TFBS to these regions.

In Silico Analysis

The 748-bp sequence for the *BMPR1A* promoter was obtained from the UCSC genome browser (www.genome.ucsc.edu) assembly v36, hg18 from March of 2006, and was uploaded to MatInspector (Genomatix) to define predicted TFBS. This search yielded 249 different TFBS based on consensus sequences. To refine the search, this output was then analyzed with ModelInspector (Genomatix) to look for previously described models. Models are thought to be the functional units of promoters and allow filtering of those TFBS generated by MatInspector by eliminating factors that do not align with a reference model. After restricting to vertebrates, ModelInspector identified 24 candidate models that carried a high likelihood of being functional. These were not clustered in any particular area, and instead were found across the whole promoter region. Our general strategy was to next disrupt all models that were within the regions predicted to be important from our deletion constructs; i.e., between 126 to 375 bp; and 501 to 748 bp. There were five models that mapped to these areas, each composed of 2 TFBS (Table 1).

Site-Directed Mutagenesis of TFBS

To assess the significance of individual models, we set out to disrupt TFBS by creating mutations within their respective consensus sequences. Using SequenceShaper (Genomatix), mutations were designed such that a minimal number of TFBS were either created or lost. To disrupt all models predicted, we made eight mutants, as several sites were redundant (appearing in more than one model; Table 1). Out of the eight sites tested, four belonged to the Sp1/KLF family of transcription factors, two were for ETSF (human and murine ETS factors family), 1 was nuclear factor κ-light-chain-enhancer of activated B (NFκB) and 1 was for myogenic family of transcription factors (MYOD). The decrease in luciferase activity after SDM ranged between 2.7% and 25.5% of the wild-type construct, and mutations in five of the eight sites led to a statistically significant reduction in luciferase activity. These sites were the three Sp1 sites starting at positions 160, 214, and 599; the ETSF site at position 603, and NFκB at position 188 (Fig. 2 A and B, and Table 1). These sites belonged to four distinct models, and all models contained the consensus binding site for Sp1.

DISCUSSION

There are several lines of evidence to support the clinical significance of the *BMPR1A* promoter evaluated here. We have recently reported a germline deletion and point mutations in JP probands that involve this promoter region [10]. Moreover, when these changes were recreated in luciferase reporter assays, a significant decrease in luciferase activity was observed. This suggested that alterations in the promoter region likely underlie disease in these subjects, which could be explained by the change in affinity for key transcription factors, of which Sp1 is a plausible candidate.

Sp1 is one of the first transcription factors to be described in mammalian cells [19]. It utilizes zinc fingers

TABLE 1

TFBS and their corresponding sequences within the *BMPR1A* promoter

Model name	TFBS (base position)	Strand	TFBS sequence	Bases to next TFBS	Decrease in activity
SP1F, SP1F	Sp1 (160)	+	CCCGGGCGGCAGCTG	27	21.8%*
	Sp1 (187)	+	GTGGGGAGGGGCCGG	–	8.9%
NFKB, SP1F	NFKB (188)	+	TGGGGAGGGGCCG	27	11.9%*
	Sp1 (214)	–	TCGGGGCTGCTCCTC	–	25.5%*
SP1F, MYOD	Sp1 (187)	+	GTGGGGAGGGGCCGG	23	8.9%
	MYOD (163)	+	GGGCGGCAGCTGTGGGG	–	6.9%
ETSF, SP1F	ETSF (577)	+	AGGAGCGAGGAGGGAGGAGGG	19	2.7%
	Sp1 (599)	+	CAAGGGCGGGCAGGA	–	20.7%*
ETSF, SP1F	ETSF (603)	+	GGCGGGCAGGAAGGCTTAGGC	7	15.6%*
	Sp1 (599)	+	CAAGGGCGGGCAGGA	–	20.7%*

*Asterisks indicate drops that are statistically significant.

to bind GC-rich sites, and shares over 90% of its consensus binding sequence with its homologue Sp3 [11]. Essentially, Sp1 and Sp3 recognize the same sequence and work together to orchestrate the transcription of many genes. For promoters that contain functional Sp1/Sp3 sites, the question remains to be answered as to whether the effects of Sp1 and Sp3 are exerted preferentially or cooperatively. However, Sp1 is unique among its family of transcription factors in that it retains the ability to form multimers that can activate transcription in promoters that do not have a TATA-box for core promoter binding (commonly referred to as TATA-less promoters). Interestingly, the promoter analyzed in this study is TATA-less, which reinforces the potential important role of Sp1 as a core element in the regulation of *BMPR1A*.

Recently, members of the SP/KLF family of transcription factors have been implicated in the pathogenesis of a number of cancers and loss or reduction in their expression has been documented in breast cancer [20], colorectal cancer [21–23], and adenomas taken from patients with familial adenomatous polyposis [24]. This pattern of overexpression is a potential target for therapeutic interventions that inhibit Sp1. One example is the utilization of mithramycin A (MTA), a drug that binds Sp1 sites on chromatin and alters the transcription of genes regulated by such promoters [25]. In fact, since perturbed activation of Sp1 and related factors has been implicated in the overexpression of angiogenic molecules in gastric and pancreatic cancers, experiments with MTA in animal models have demonstrated suppressed cancer growth in both tumor types [26, 27].

A promoter is a sequence that initiates and regulates the transcription of a gene. TFBS are important elements within a promoter sequence that help regulate the transcriptional machinery. The crude scanning of a sequence for TFBS merely highlights the potential for a protein to bind to that sequence. It is now becoming more apparent that it is the context of a particular TFBS that renders a site as transcriptionally active [28]. Available TFBS prediction programs rely on sequence alone to generate an output, and this usually results in a list of a large number of functionally irrelevant sites. MatInspector found 249 TFBS based on sequence alone in this *BMPR1A* promoter, which was markedly reduced by running an algorithm incorporating available literature to five 2-element models.

Systematic deletion mutagenesis of promoter sequences (commonly referred to as "promoter bashing") is a technique that has been frequently utilized in molecular biology to localize sequences with important regulatory elements [29]. Traditionally, promoter sequences that are sequentially shorter from their 3′ ends are cloned into a reporter vector. Promoter activity is then assayed and data on loss or gain of activity is used to outline important regions. Valuable information on active promoter region boundaries is provided through this technique; however specific regulatory proteins and detailed descriptions of their interactions are hardly ever outlined with certainty. In this study, a modification of this method is introduced by analyzing deletion constructs that are shorter from the 5′ end. The rationale behind this approach is based on the notion that promoter activity is often reliant on the interaction of different proteins with DNA. These interactions could take place across the whole promoter region and unidirectional deletions could potentially miss factors at the either end that cooperatively are important in transcription. The reverse segments (R1-R5; Fig. 1) represent the traditional direction of deletion mutagenesis. By examining those areas alone, the region between 501 and 748 bases appears to have relatively negligible effects. However, the forward constructs revealed that this is not true, and mutations of two sites within this area [Sp1 (599) and ETSF (603)] led to a statistically significant drop in luciferase activity.

The regulation of genes is precisely controlled by the different combinations of transcription factor-bound sites under different conditions [30]. These so

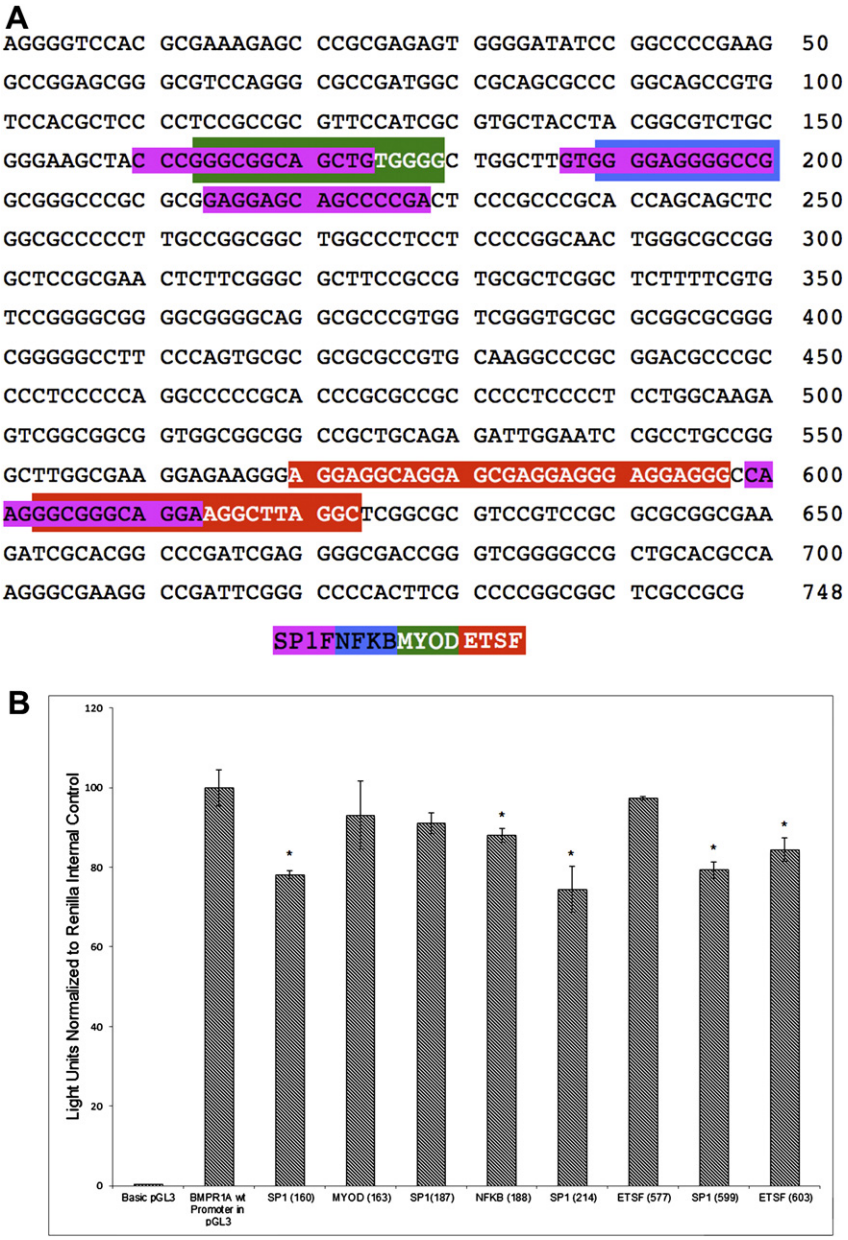

FIG. 2. (A) The 748-bp *BMPR1A* promoter sequence is shown. Eight TFBS sequences from five distinct models that map between bases 126 and 375; and 501 and 748 are highlighted. (B) These sequences were individually disrupted by site directed mutagenesis and then transfected into HEK-293 to test for their significance. WT (full) promoter is set to have 100% activity.

called modules are the functional units that allow for synergistic or antagonistic actions for the activation or repression of a gene. We utilized an experimental approach that combines a modified version of traditional promoter deletion mutagenesis and computational analyses to define TFBS with a higher likelihood to be functional, and found that Sp1 is a common binding site, and when mutated, leads to a significant decrease in promoter activity. These results define Sp1 as a potential transcriptional regulator of the BMP pathway, with important implications for further studies of regulation of BMP signaling.

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