The human secretin receptor gene: genomic organization and promoter characterization

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Abstract **Secretin is the most potent regulator of pancreatic bicarbonate, electrolyte and volume secretion. In this report, the organization of the human secretin receptor (hSR) gene was characterized by overlapping genomic phage clones. The hSR gene consists of 13 exons and 12 introns with all the splice donor and acceptor sites conforming to the canonical GT/AG rule. By transient reporter gene assays, the wild-type promoter, containing 3.0 kb of the hSR gene 5′ flanking region, was able to drive 5.8 ± 0.6 and 6.6 ± 0.2-fold ($P < 0.01$) increases in luciferase activities in pancreatic ductule-derived PANC-1 and BPD-1 cells, respectively. By subsequent 5′ and 3′ deletion analysis, a promoter element was identified within −408 to −158, relative to the ATG codon. This promoter element was found to be cell-specific since it could drive reporter gene expression in PANC-1 and BPD-1 cells but not in Hs 262.St, Hs 746T and αT3-1 cells. The study of the transcriptional control of human secretin and its receptor should shed light on the pathological developments of pancreatic cancer and autism in the future.**

Key words: Secretin; Secretin receptor; Promoter study; Gene structure; Autism

1. Introduction

Secretin was first discovered in 1902 by Bayliss and Starling [1]. The primary action of secretin is to stimulate bicarbonate, electrolyte and volume secretion from pancreatic ductule epithelial cells. Other actions of secretin were reported including inhibition of gastric emptying and acid output [2,3], stimulation of hepatic bile flow [4], stimulation of mucus, as well as bicarbonate and epidermal growth factor secretion from duodenal Brunner's glands [5]. The functions of secretin are mediated via its specific interactions with cell surface receptors. Recently, cDNAs corresponding to rat [6] and human [7–9] secretin receptors (SR) have been characterized. Similar to other members in the same G protein-coupled receptor family, the hSR contains a relatively large extracellular hydrophilic domain. The importance of this N-terminal ectodomain in ligand interaction was indicated by domain swapping experiments [10] and by a novel approach using the recombinant ectodomain to functionally antagonize the wild-type receptor [11].

All members of the secretin-glucagon receptor family are able to couple to Gs protein leading to the production of intracellular cAMP. By using a protein kinase A (PKA) inhibitor, H-89, PKA was indicated to be largely responsible for carrying out cellular responses upon secretin activation [11,12]. Recently, termination of hSR signaling mechanism was studied and it appeared that G protein-coupled receptor kinase and phosphorylation-independent receptor internalization are both important in receptor desensitization to protect receptor-bearing cells from over-stimulation [12–15]. However, this acute desensitization event is reversible and is followed by a rapid re-sensitization process [12].

The study of human secretin and its receptor has attracted much more attention recently because of the possible treatment of autistic patients with repeated intravenous infusion of secretin [16]. This new potential therapeutic application of secretin gives a totally new insight into the understanding of this traditional hormone and its receptor. It is highly possible that secretin is also a neuropeptide, and autism may be a genetic defect involving mutation of the structure and/or promoter of human secretin and/or secretin receptor genes. We have previously mapped the gene to 2q14.1 by fluorescent in situ hybridization [17], and now, we report here the genomic organization and the functional characterization of 3 kb 5′ flanking region of the hSR gene.

2. Materials and methods

2.1. Genomic library screening

Two human genomic libraries, HL1067j (Clontech Laboratories, Palo Alto, CA, USA) and LL02NS01 (ATCC, Rockville, MD, USA), were screened with either probe A (1166 bp, 249–1414 bp of the cDNA), probe B (427 bp, 108–534 bp of the cDNA), probe C (301 bp, 1–301 bp of the cDNA), probe D (137 bp, 1–137 bp of the cDNA) or probe E (446 bp, 1254–1699 bp of the cDNA). Probes B, C and E were obtained by polymerase chain reaction (PCR) amplification using primer pairs hSR5′ (CGGGATCCAT GCGTCCCCAC CTGTCGCCGC) and hSR3′ (CGGATTTCCA GCTTCAGCAG GTAGGAGTG), hSG5F (ACGAGGCCGG CCGGAGCCCG GGACCCTGCG) and hSG5R (CTGGCACTGG CTGCTCCGTG CCCAGGTCTC), or hSRG1 (GTGGTGGCCG TCCTCTACTG CTTCCTCA) and hSRG2 (ACCCCTGAAC TTCTCTTCCC GAAGAG), respectively. The PCR typically contained 50 pmol primers, 200 μM dNTPs and 5 units *Taq* polymerase (Life Technologies, Inc.) in the buffer provided by the manufacturer. The reaction times were 1 min at 94°C, 58°C and 72°C, respectively, for 30 cycles. The PCR fragments were subcloned into pBluescript SK^+ (Stratagene, La Jolla, CA, USA) for DNA sequence analysis using the T7 sequencing kit (Pharmacia, Uppsala, Sweden). The probes were labeled using a Megaprime DNA labeling kit and [α-^{32}P]dATP (Amersham). Library screening was performed essentially following the protocol described earlier [7]. Phage DNA was purified by a Qiagen lambda phage kit (Qiagen Inc., Santa Clarita, CA, USA) and subjected to restriction mapping and DNA sequence analysis. DNA sequences of the positive clones were analyzed by DNASIS v 3.6 (Hitachi, San Bruno, CA, USA).

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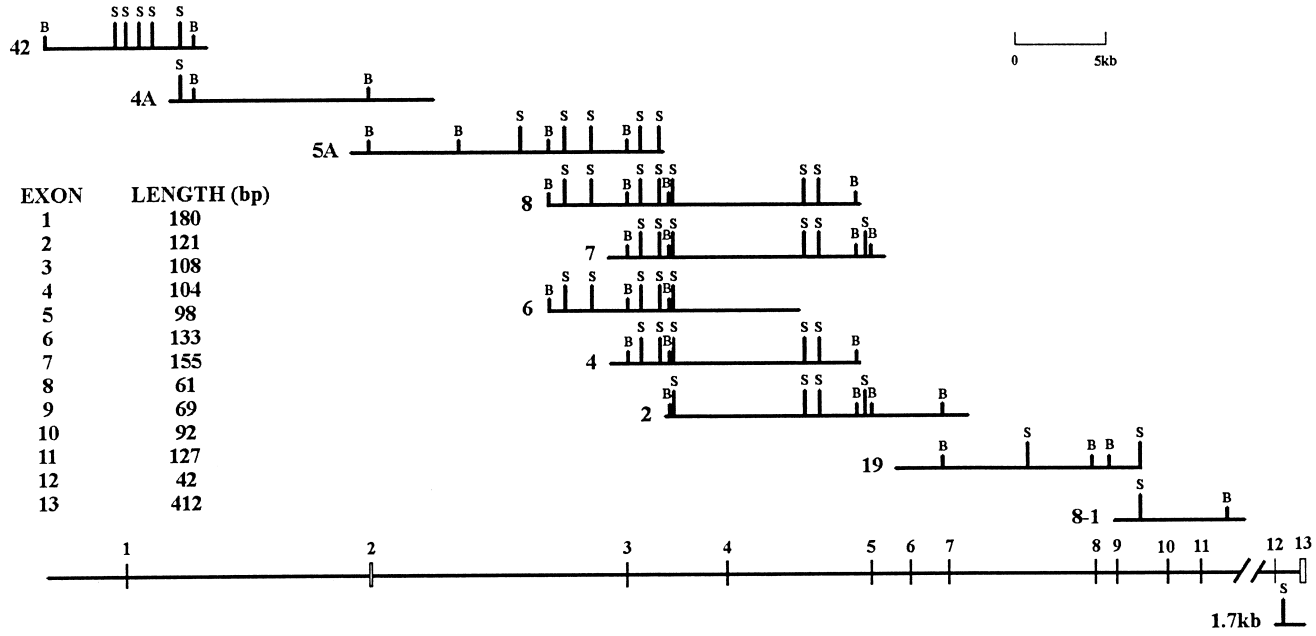

Fig. 1. Structure of the hSR gene. Positive clones obtained from library screenings were analyzed by DNA sequencing and restriction mapping. *Bam*HI (B) and *Sst*I (S) restriction sites are indicated. Exons are represented as bars and the size (bp) of each exon is shown on the left. Clones 42, 4A, 5A, 8, 7, 6, 4, 2, 19A, 8-1 and G1 are arranged and overlapping to reveal the genomic organization of the hSR gene.

2.2. Plasmid construction

A 3 kb restriction fragment (*Pst*I) corresponding to the 5′ upstream region of hSR gene was subcloned into pBluescript KS$^+$ (Stratagene) to produce p42Ab. The expression plasmids p3039luc and p3039Rluc were constructed by cloning the *Kpn*I/*Sst*I DNA fragment (−3039 to −69 relative to ATG) into the luciferase reporter vector pGL2-Basic (Promega, Madison, WI, USA) in forward and reverse orientations, respectively. Other 5′ and 3′ deletion clones were obtained by releasing restriction fragments from p3039luc. A nested family of 5′ deletion clones was generated from p681luc by exonuclease III/S1 nuclease digestion (Pharmacia).

2.3. Cell culture and transient transfection

All cells including PANC-1 (human pancreatic ductule carcinoma, ATCC) and BPD-1 (bovine pancreatic duct epithelium) were grown at 37°C with 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). For transient transfection assays, cells were seeded onto 6-well plates (Costar 3506 San Diego, CA, USA) at a density of 1.5×10^5/well. Transfection was performed 40 h later using a cocktail containing 1 μg promoter-reporter construct, 0.5 μg pSV-β-gal (Promega) and 5 μl LipofectAMINE reagent (Life Technologies, Inc.). Cells were incubated for 7 h before the addition of 1 ml DMEM/20%

Table 1
Exon-intron splice junctions of the hSR gene

Exon no.	Exon size (bp)	Sequence at exon-intron junction 5' splice donor 3' splice acceptor	Intron size (kb)	Amino Acid interrupted
1	180	CAC TCG gt.....ggacttgcag ACT GGA	12.9	Ser-24
2	121	CCA G gtatgaggct.....ttcttggcag GT TGT	13.6	Gly-65
3	108	AAT G gtaaccactc.....gcacccacag GT TCC	5.3	Gly-101
4	104	AAG CGG gtaagcctgt.....ctgcag CAC TCC	7.7	Arg-135
5	98	TTC CG gtgagaacac.....ctccctgcag G AGG	1.5	Arg-168
6	133	CAC AGG gtaatatgtc.....ctctctccag GCG GGC	2	Arg-212
7	154	TGG G gtatgtttct.....taatcctcag GT TCT	7.8	Gly-264
8	61	GTT GG gtaagctaac.....cctccctag G TGC	1.1	Gly-284
9	70	ATC CTG gtgagtggcc.....tgttttgcag ATT AAT	2.6	Leu-307
10	92	TAT AA gtaagtgaag.....gtgtccccag G CGC	1.9	Lys-338
11	127	TTC CAG gtaagtttcc.....cttctggcag GGA CTG	>1	Gln-380
12	42	GGG GAG gtgagtagtc.....tcccgggcag GTG CAG	1.3	Glu-394
13	412			

The positions at which introns interrupt the mRNA and protein sequences are indicated. Exon sequences are in capital letters. Introns were partially sequenced, and the sizes were estimated by PCR and genomic and phage Southern blot analysis. The sizes of introns 8, 9, 10 and 12 were confirmed by DNA sequence analysis.

FBS. Cell lysate was prepared 48 h later by washing the cells twice with PBS followed by the addition of reporter lysis buffer (Promega). Luciferase and galactosidase assays were performed essentially as described earlier [18]. For each transfection study, luciferase activity was determined and normalized based on the galactosidase activity. Plasmid DNA for transfection was prepared using the Qiagen Midi kit (Qiagen).

3. Results and discussion

3.1. Genomic organization of the human secretin receptor

Ten overlapping and one non-overlapping phage clones were obtained by screening the human genomic libraries (probe A: clone 8-1; probe B: clones 2, 4, 6, 7, 8 and 19; probe C: clones 4A and 5A; probe D: clone 42 and probe E: G1) (Fig. 1). The overlapping region was 69.6 kb in length which includes exons 1–11 and 10.4 kb 5′ flanking region of the gene. The 3′ region of the gene was characterized by the analysis of the non-overlapping clone G1 which spans exons 12 and 13. The minimal size of the gene was estimated to be 61 kb. The size of the intron between exons 11 and 12 is probably above 2 kb since we were unable to produce a PCR product using sequence-specific primers for exons 11 and 12 in a genomic PCR amplification. In summary, the hSR gene consists of 13 exons and 12 introns and spans over 61 kb of genomic DNA. The sizes of the introns were determined by PCR amplification coupled to agarose gel electrophoresis and/or by restriction mapping of the overlapping phage clones. The size of exons ranges from 42 bp (exon 12) to 412 bp (exon 13), and that of introns ranges from below 1 kb to 13.6 kb (Table 1). A comparison of the hSR genomic organization with that of other receptor genes in the same family shows that the hSR is, as yet, the largest gene identified [19–22]. The unusually large architecture of the hSR gene is attributable to the sizes of the first (12.9 kb) and second (13.6 kb) introns. The exon-intron splice junctions of the hSR gene agree with the canonical GT/AG rule [23] and all three types of splice phasing were observed.

The genomic structures of human GIP, human VIP1, human and rat glucagon receptors [19–22] have been previously described. With the exception that the rat glucagon receptor gene has only 12 exons, all other receptor genes have 13 exons. The 11th exon/intron splice junction, which interrupts the glutamine residue in human receptors, is absent in the rat glucagon receptor gene. In addition, the exon-intron organizations of all these genes in the secretin/glucagon family are remarkably similar.

3.2. Analysis of the 5′ flanking region of the hSR gene

The nucleotide sequence of 1.6 kb of the 5′ flanking region was determined (Fig. 2). No potential elements that are required for the accurate initiation of transcription, including the TATA and CCAAT boxes, are evident within this region. In fact, the absence of TATA and CCAAT motifs is not unique to the hSR; other G protein-coupled receptor genes such as the human α_{1b}-adrenergic receptor and the growth hormone-releasing hormone receptor [24,25] also lack these sequence motifs in their promoters. A search of the 5′ flanking region sequence against the TFMATRIX revealed the presence of several transcription factor binding sites. Two Sp1 binding GC-rich motifs are found at −205 and −403. Sp1 sites have been frequently linked to transcriptional control of genes that lack a functional TATA box. In addition, two

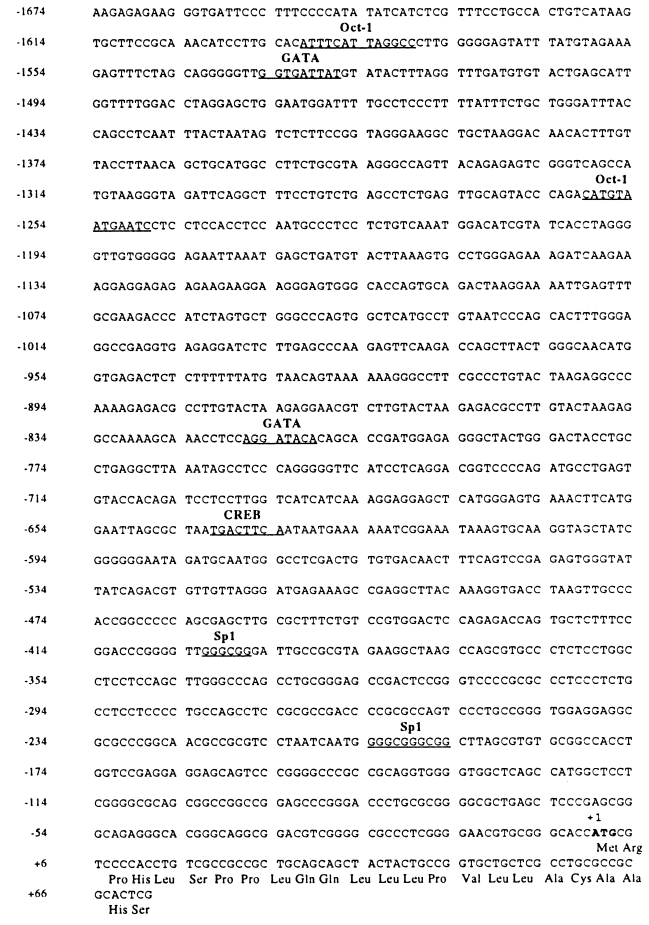

Fig. 2. Nucleotide sequence of the 5′ flanking region of the hSR gene. Bases relative to the ATG translation initiation codon (in boldface, designated +1) are numbered on the left. The amino acid sequence is shown below the nucleotide sequence. Consensus Oct-1, GATA, CREB and Sp1 sequences are indicated and underlined.

putative binding sites for the zinc finger transcription factor GATA are found at −818 and −1536. Recent findings have shown that GATA is expressed at high levels in the pancreas and gut-derived cells [26,27]. In addition, there are two octamer sites for the homeobox domain factor Oct-1 at −1261 and −1598 and a binding site for CREB (cAMP-response element binding protein) at −642. All these motifs may contribute cooperatively to the hormonal and developmental regulation as well as the basal expression of the hSR gene in various tissues.

3.3. Initial mapping of the hSR gene promoter element to a region from −681 to −69

To localize the region essential for hSR gene expression, several 5′ and 3′ deletion plasmids were constructed by linking restriction fragments to the luciferase reporter gene. The transcriptional activities of these promoter-luciferase constructs were tested by transfecting the plasmid individually into PANC-1 (human pancreatic ductule carcinoma) and BPD-1 (bovine pancreatic duct epithelium) cells (Fig. 3). It was found that 3 kb of the hSR gene 5′ flanking region (Fig. 3: p3039luc) was capable of directing significant ($P < 0.01$, ANOVA) increases in luciferase activities in both PANC-1 (5.8-fold) and BPD-1 cells (6.6-fold), when compared with the promoterless control, pGL2-basic. Subsequent

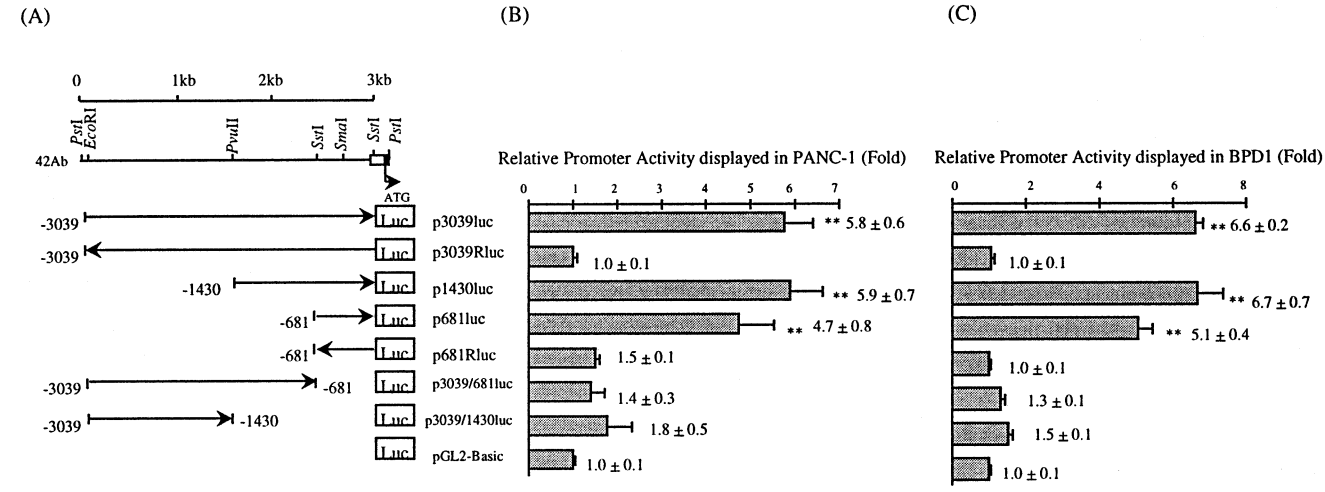

Fig. 3. Initial mapping of the hSR gene promoter element within −681 to −69 in PANC-1 and BPD-1 cells. A: Restriction map of the hSR gene 5′ flanking region and schematic diagrams of various promoter-luciferase constructs. In transient expression assays, these constructs were cotransfected with pSV-β-gal into (B) PANC-1 and (C) BPD-1 cells. Luciferase values are normalized by β-galactosidase expression and are shown as the fold changes in relative promoter activities compared with that in the promoterless control pGL2-Basic. Values reported in the figure represent the mean ± S.E.M. of six independent transfection experiments. Bars bearing '*' ($P < 0.05$) and '**' ($P < 0.01$) are statistically different.

5′ deletion constructs, p1430luc and p681luc, also supported transcription of the reporter gene to approximately the same extent in both cell lines suggesting that the 5′ boundary of the promoter resides within 681 bp from the translation start site. On the other hand, low levels of luciferase activities were observed in the 3′ deletion clones p3039/681luc and p3039/1430luc (Fig. 3), again indicating the importance of the sequences within the region from −681 to −69. Two other constructs, p3039Rluc and p681Rluc, with the putative promoter regions in reverse orientation showed similar luciferase activities when compared with the promoterless control, pGL2-basic (Fig. 3). These results confirmed the nature of this DNA fragment (−681/−69) as a promoter element since it functions in an orientation-dependent manner. In summary, we have identified a functional promoter element residing in the region from −681 to −69 within the 5′ flanking region of the hSR gene.

3.4. Fine Mapping of the hSR promoter element to a region within −355 to −158

In order to precisely map the location of the promoter element within the region −681 to −69, a number of progressive 5′ deletion clones were generated using the exonuclease III/S1 nuclease nested deletion method [28]. The abilities of these deletion clones to drive luciferase expression were again tested by transient transfection into PANC-1 and BPD-1 cells (Fig. 4). In PANC-1 cells, similar luciferase levels ($P < 0.05$, ANOVA) were observed in p681luc (4.7-fold), p505luc (5.0-fold), p470luc (5.7-fold), p408luc (4.8-fold) and p355luc (4.3-fold). However, a further deletion of 61 bp (p294luc, 2.9-fold)

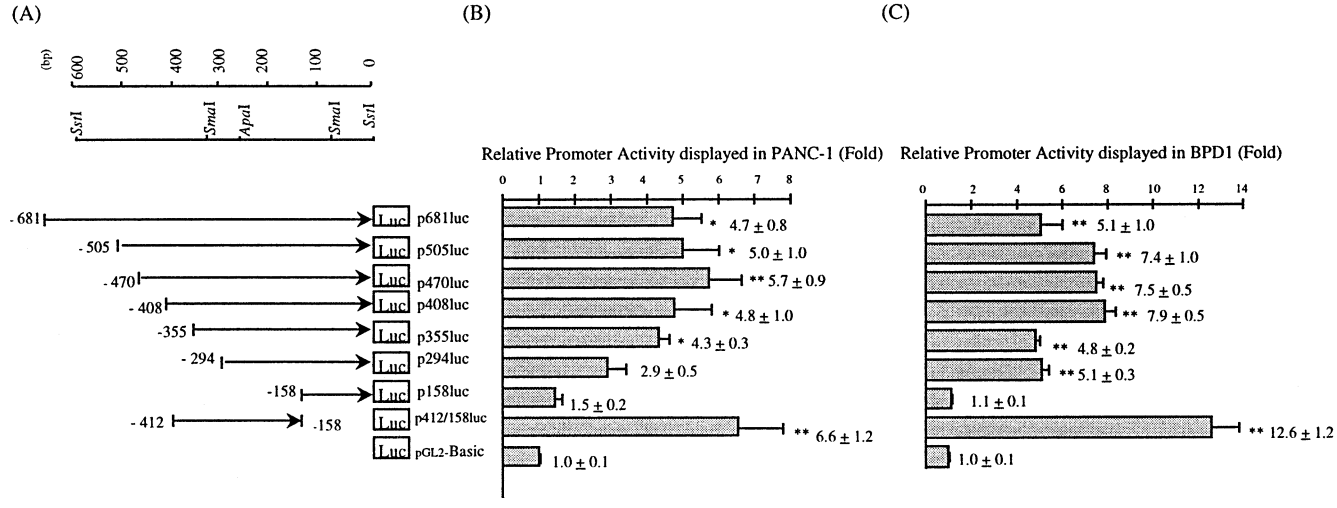

Fig. 4. Fine mapping of the hSR gene promoter element in PANC-1 and BPD-1 cells. A: A nested family of 5′ deletion clones was generated by ExoIII/S1 nuclease digestion. In transient expression assays, these constructs were cotransfected with pSV-β-gal into (B) PANC-1 and (C) BPD-1 cells. Luciferase values are normalized by β-galactosidase expression and are shown as the fold changes in relative promoter activities compared with that in the promoterless control pGL2-Basic. Values reported in the figure represent the mean ± S.E.M. of six independent transfection experiments. Bars bearing '*' ($P < 0.05$) and '**' ($P < 0.01$) are statistically different.

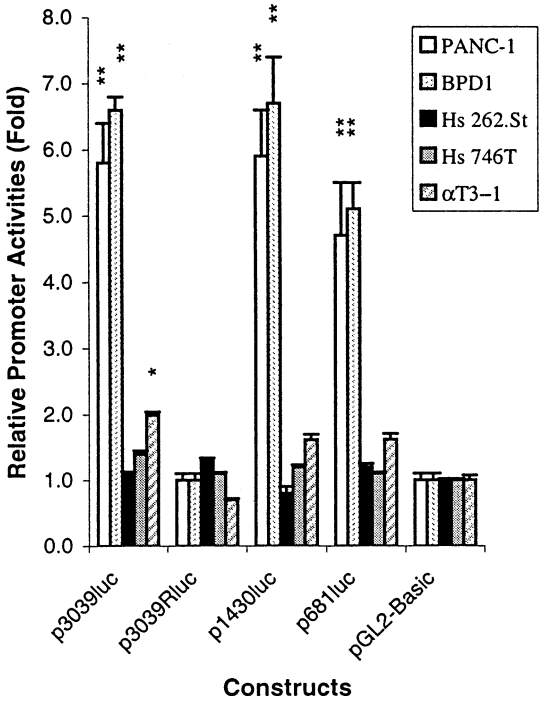

Fig. 5. Cell specificity of the hSR promoter. Promoter-reporter construct together with pSV-β-gal were transiently co-transfected into PANC-1, BPD-1, Hs 262, Hs 746T and αT3-1 cells. Luciferase activities are normalized by β-galactosidase expression and are expressed as the fold increase in relative promoter activities compared with the control pGL2-Basic. Values represent the mean ± S.E.M. of six independent transfection experiments. Bars bearing '*' ($P < 0.05$) and '**' ($P < 0.01$) are statistically different.

and 197 bp (p158luc, 1.5-fold) from the 5′ end of p355luc caused drastic reductions (33% and 65%, respectively) in promoter activities. Our data indicated that, in PANC-1 cells, the 5′ boundary of the promoter element is located within −355 to −294. In addition, the 3′ deletion clone (p411/158luc, −411 to −158) showed a high level of luciferase activity (6.6-fold), suggesting that the proximal 154 bp upstream from the start codon does not contain the 3′ boundary of the promoter. In summary, our data indicated that the region from −355 to −158 (197 bp) is sufficient as a promoter element to drive expression in PANC-1 cells. By searching the sequences against a database containing all the known *cis*-acting motifs (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html), only a consensus Sp1 site at −205 is identified within this region. It is highly possible that other novel transcription factors are needed for the transcriptional regulation of the hSR gene.

Although the overall expression profiles of the deletion clones are similar in PANC-1 and BPD-1 cells (Figs. 3 and 4), there are some minor but interesting differences. The sequences between −355 to −294 were important only in PANC-1 cells since deletion of this region led to a 33% reduction in promoter function in PANC-1 cells but had no effect in BPD-1 cells (Fig. 4: PANC-1: 4.3-fold and 2.9-fold; BPD1: 4.8-fold and 5.1-fold). On the contrary, the region between −408 to −355 was only functional in BPD-1 cells. In this cell line, there was a 40% drop (Fig. 4C, 7.9-fold to 4.8-fold) in promoter activity when this region of the promoter was deleted. In summary, our data suggest the presence of at least two distinct regions within the promoter. The proximal region from −294 to −158 is probably responsible for basal promoter functions and it appears that there was a differential use of upstream activator regions, −355/−294 and −408/−355, for transcription initiation in PANC-1 and BPD-1 cells, respectively.

3.5. Cell specificity of the hSR promoter element

Five mammalian cell lines, PANC-1, BPD-1, Hs 262.St (human stomach fibroblast-like), Hs 746T (human stomach carcinoma) and αT3-1 (mouse gonadotropes) were used to study the cell specificity of the hSR promoter element. All constructs with promoter sequences in the forward orientation were capable of driving the luciferase expression in PANC-1 and BPD-1 cells at levels significantly above that of other cells ($P < 0.001$, ANOVA) (Fig. 5). The same DNA sequences drove negligible luciferase expression in Hs 262.St and Hs 746T cells, but interestingly, as indicated by constructs p3039luc and p355luc, the hSR promoter is a weak promoter in αT3-1 cells ($P < 0.05$, ANOVA). The significance of this finding remains to be investigated.

Recently, the public have shown much interest in the possible treatment of autistic patients with secretin. It was reported that autism is linked to biological and neurological differences in the brain. For instance, the disorder is related to abnormalities in the structure of the brain such as differences in the size and the number of Purkinje cells within the cerebellum. In addition, it has also been suggested that autism is a genetic disorder [29]. The study of the regulation of human secretin and secretin receptor may provide a new window to investigate the genetic aspects of autism and to provide a better understanding of the pathophysiology of this receptor.

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