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Regulation of Human Prostate-Specific G-Protein Coupled Receptor, PSGR, by Two Distinct Promoters and Growth Factors

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Abstract PSGR is a newly identified human prostate tissue-specific gene belonging to the G-protein coupled receptor (GPCR) family. Overexpression of PSGR is associated with human prostate intraepithelial neoplasia (PIN) and prostate tumors, suggesting PSGR may play an important role in early prostate cancer development and progression. To understand the regulation of tissue-specific expression of human PSGR and its upregulation mechanism in prostate cancers, we characterized the promoter region of *PSGR* and analyzed the control mechanism for PSGR expression in human prostate tissues/cells. In this report, we demonstrate that two distinct promoters control the transcriptional regulation of *PSGR* in human prostate cells. The first promoter region includes exon 1 and a TATA box at −31 site. The minimal DNA sequence with promoter activity is about 123 bp upstream of exon 1. Exon 1 contains tissue specific regulatory activity for the first promoter of *PSGR* gene. The second promoter is located in the upstream region of exon 2, which is a TATA-less and non-GC-rich promoter. Primer extension and RNA protection assays (RPA) revealed that the transcription driven by the second promoter is initiated at the junction of intron and exon 2 within a cluster of nucleotides located about 250 bp upstream from the junction. Both promoters show prostate cell-specific characteristics in our luciferase assays in transfected cells. Furthermore, we investigated the regulation of the promoter activities of the *PSGR* gene by different growth factors and cytokines, and demonstrated that interleukin-6 (IL-6) activates the promoter activities of *PSGR* in human prostate cancer cells. These data suggest that two functional promoters regulate the transcriptional expression of *PSGR* in human prostate tissues and *PSGR* is a new target for IL-6 transcriptional regulation. J. Cell. Biochem. 96: 1034–1048, 2005.

Key words: prostate-specific G-protein coupled receptor; PSGR; OR51E2; transcriptional regulation; prostate-specific promoters; prostate cancers; IL-6

Receptors coupled to heterotrimeric GTP-binding proteins (G-proteins) are integral membrane proteins involved in the transmission of signals from the extracellular environment to the cytoplasm. A variety of external stimuli, including neurotransmitters, hormones, phospholipids, and peptides can activate the G-protein coupled receptors (GPCRs). Therefore, the GPCRs and signal transduction pathways represent important specific targets for a variety of therapeutic approaches, ranging from the control of blood pressure, allergic response, kidney function, hormonal disorders, neurological diseases, and to tumorigenesis [Edwards et al., 2000; Neubig, 2002; Stafford et al., 2002]. Approximately 50% of the pharmaceutical products sold in the United States target the GPCRs and their signal transduction pathways.

Several studies have demonstrated that GPCRs have oncogenic potential and can induce tumor formation. For example, the *mas* oncogene, a GPCR, can induce the formation of tumors in mice [Young et al., 1986]. LPA and its GPCRs (Edg subfamily) were first implicated in human carcinogenesis by the observation that

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LPA is present at elevated levels in ascites of ovarian cancer patients [Xu et al., 1995]. Other GPCRs, such as serotonin 1C and muscarinic acetylcholine receptors m1, m3, and m5 transform contact-inhibited cultures of rodent fibroblasts when activated, suggesting that GPCRs can behave as oncogenes [Gutkind, 1998a]. Constitutively active mutants of GPCRs have been implicated in a number of human neoplasias, including thyroid adenomas, small cell lung carcinoma, colon adenomas and carcinomas, and gastric hyperplasia and cancers [Gutkind, 1998a,b]. Furthermore, a number of DNA viruses, such as the Kaposi's sarcoma associated herpesvirus (KSHV) and Herpesvirus saimiri, encoding GPCRs and acting as cellular proliferation oncogenes, further support an important role for GPCRs in normal and aberrant growth control [Allen et al., 1991; Arvanitakis et al., 1997; Gutkind, 1998a].

In our effort to identify prostate-specific biomarkers and to understand signaling pathways in human prostate cancers, we and others have identified and characterized the prostate-specific GPCRs, PSGR [Xu et al., 2000; Yuan et al., 2001; Xia et al., 2001b]. PSGR shares sequence homology with other GPCRs in the seven putative transmembrane domains. Northern blot analysis indicates that the expression of PSGR is restricted to human prostate gland and is not expressed in other human tissues. PCR and Matched Normal/Tumor Tissue Array study shows significant overexpression of PSGR mRNA in prostate tumor tissues [Xia et al., 2001b]. In situ RNA hybridization and quantitative real-time PCR analysis of more than 140 human prostate tissues (normal and tumors) demonstrate that PSGR was overexpressed approximately 10-fold in human prostate intraepithelial neoplasia (PIN) and prostate cancers compared to normal human prostate tissues and benign prostatic hyperplasia (BPH) tissues, suggesting PSGR as a sensitive and specific biomarker in human prostate cancers [Weng et al., 2005]. However, the mechanisms that regulate the specific expression of PSGR in prostate tissues and in tumor progression are still not clear. Information about the molecular regulation of this prostate-specific receptor would help us understand how it could be modified by disease states and by pharmacologic manipulations. To study the transcriptional control of the human *PSGR* gene, we cloned and characterized its promoter region and analyzed the regulation of *PSGR* expression. We found that the transcriptional expression of *PSGR* was regulated by two distinct promoters. The first promoter proximity region contains exon 1 and its upstream region with a TATA box at −31 site. The minimal promoter activity can be achieved with 123 bp DNA upstream of exon 1 and shows tissue specific regulation activity. The second sequence with promoter activity lies in a region upstream of exon 2, and contains no TATA box or no GC-rich sequences. Primer extension and RNA protection assays (RPA) assay revealed that transcription from the second promoter is initiated at a cluster of nucleotides about 250 bp upstream from the junction of intron and exon 2. The SV40 enhancer showed strong activating activity for the second promoter while it inhibited the first promoter, suggesting that these two promoters are under different regulatory mechanisms. The identification of prostate-specific functional promoters in the prostate-specific *GPCRs* gene will provide us with tools for the study of transcriptional regulation of *PSGR* and its potential role in human normal prostate function and in prostate cancer progression.

MATERIALS AND METHODS

Cell Culture

The human prostate cell lines, LNCaP, PC-3, DU-145, PNT1A, and non-prostate cell lines, SW480, Hela, MDA231, and HEK293 were maintained in our laboratory. All prostate and SW480 cells were cultured in RPMI1640 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (CELLect GOLD, ICN Flow, Irvine, CA) at 37°C in a humidified atmosphere containing 5% CO_2. Hela, MDA231, HCT116, N2A, HNSC, HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Mediatech) supplemented with 10% fetal bovine serum (CELLect GOLD, ICN Flow) at the same condition.

RNA Extraction and RT-PCR

Cells were seeded in six-well plates and cultured for 36 h in media. Total RNA was extracted with 1 ml Trizol reagent (Invitrogene). Three micrograms RNA was reverse-transcripted to cDNA in 30 μl volume and 1 μl cDNA was used in subsequently PCR reaction with a pair of *PSGR* specific primers. Primer 1:

5′-GAAGATCTATGAGTTCCTGCAACTTC-3′, primer 2: 5′-CCCA AGC TT TCACTTGCCTCC-CACAG-3′. A pair of β-actin primers was used as a control: primer 1: 5′-CA TGATG GAGTT-GAAGGTAGTTTCG-3′; Primer 2: 5′-CAGAC-TATGCTGT CCCT GTACGC-3′. PCR was carried out under the following conditions: 94°C for 2 min, 94°C for 30 s, 56°C for 30 s, 72°C for 1 min 20 s, total 35 cycles, 72°C for 10 min. β-actin was run for 25 cycles. Equal amount PCR product were then loaded and detected in the gel electrophoresis.

Computer Analysis of Human and Mouse PSGR Gene Proximal Promoter Region

The gene structure of human PSGR and the 5′-promoter proximal region of human *PSGR* gene were obtained from Human Genome resources on NCBI and by PCR from LNCaP genomic DNA. Promoter Prediction software from Berkeley Drosophila Genome Project (http://www.fruitfly.org/) and TESS (http://www.cbil.upenn.edu/tess) were used to examine the potential promoter and transcription factor binding sites.

PSGR Luciferase Constructs and Luciferase Assays

The human PSGR proximal promoter region and first intron were obtained by PCR using LNCaP genome and the following primers. For the first exon region: primer P3R: 5′-GGAGA-TCTCATCTAGAGTC AAGCATTTTC-3′ (reverse) and primer F483KpnI, 5′-GGGGTACCTCTG-TAAGAG AAA GACTGCTGC-3′ (forward), primer F951KpnI 5′-GGGGTACCAACTGACAGAA GG TGACATCATTG G-3′ (forward), primer F1.5kbKpnI 5′-GGGGTACCAG GTGACA TATTC ACAGGTTC C-3′ (forward). For the minimal promoter region, another primer R904700BgIII 5′-GGAAGATCTATTC CCA-GAAGACTTTTGCC-3′ (reverse) was designed. For the proximal promoter region before exon2: primer F677KpnI, 5′-GGGGTACCGCTG TGC TTCTTATTCCTCTGG-3′ (forward) and primer R889569BgIII, 5′-GAAGATCTAGCT GGAA CTGAGGAG GGG TGAC-3′ (reverse). The Luc-123 and Luc-360 were obtained by cutting in HindIII site in Luc-483 vector. The primers for the two proximal promoter regions contained a KpnI and a BgIII site, respectively. Reverse promoter constructs were made via another vector pcDNA3.1. PCR products encompassing these two regions were cloned into the promoter-less pGL3 Basic Vector (Promega). All PCR products were verified by sequencing on an ABI automatic DNA sequencer (PerkinElmer Life Sciences).

For luciferase transfection experiments, cells were plated at a density of 1×10^5 cells/well of a 24-well plate the day before transfection. Growth in the appropriate complete medium for 20–24 h generally resulted in 50%–70% cellular confluence in each well at the time of transfection as previously described [Xia et al., 2003]. Transient transfection of luciferase constructs and their mutants into the various cultured cell lines using lipofectamine effectene transfection reagent (Invitrogen). After incubation of the transfected cells at 37°C for 48 h, luminescence assays of cellular lysates were performed for a semi-quantitative measure of luciferase production driven by each cloned segment of the PSGR 5′-flanking region. Within a given assay, plate wells were set up in triplicate for each transfected construct or control vector. The strength of the promoting activity for each construct was assessed by comparison to luciferase expression from the PGL3-Control vector transfected into triplicate samples of the same cell type within the same assays. PGL3-Control contains the SV40 promoter and the SV40 enhancer and shows activity constantly in all cells tested. To allow for normalization of firefly luciferase values based on transfection efficiency, a co-reporter vector expressing beta-galactosidase (pcDNA3-LacZ) was included at a ratio of 1:20 of co-reporter plasmid to experimental promoter construct (or control vector) in the transfection mixture. Where indicated, statistical analysis of luciferase reporter data was performed using the Mann–Whitney *U*-test.

Primer Extension

Total RNA was extracted from LNCaP cell using Trizol (Life Technologies, Inc., Carlsbad, CA), and primer extension was carried out using the Primer Extension System (Promega). Briefly, an oligodeoxynucleotide complementary to nucleotides −1 to −24 in exon 2 (5′-TCTG GTGGTGACAGGAGATTCTCCCCTTCT-3′) (Fig. 2C) was 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Thirty micrograms of total RNA from LNCaP cell and 0.1 pmol of labeled primer were mixed in the presence of 2× primer extension buffer and annealed at 65°C for 10 min followed by cooling to room temperature for 10 min. The annealed

primer was extended with avian myeloblastoma virus reverse transcriptase at 42°C for 90 min and ethanol-precipitated. The sample was resuspended in 5 μl of gel loading dye, separated in a denaturing 8 M urea and 6% polyacrylamide gel.

Ribonuclease Protection Assay (RPA) and Northern Blot Analysis

RPA was performed with the RPA III ribonuclease protection assay kit (Ambion, Inc., Austin, TX). Constructs Luc-677 was sub-cloned into pCMV-Tag-1 in reverse orientation and was linearized at the 3′-end of the insert with BgI II and transcribed in vitro from the upstream T3 promoter to generate [α-32P]UTP-labeled antisense RNA probes. Approximately 25 μg of total RNA from LNCaP cells were precipitated with about 1×10^5 cpm of the appropriate probe and resuspended in 10 μl of hybridization buffer. Samples were denatured at 90°C for 3 min and incubated at 45°C for 20 h. Following hybridization, 150 μl of RNA digestion buffer containing RNase A and RNase T1 were added, and the samples were incubated at 37°C for 30 min. RNA was subsequently precipitated, resuspended in 10 μl of gel-loading buffer, and separated in a denaturing 8 M urea, 4% polyacrylamide gel. The sizes of the protected bands were compared with concurrently electrophoresed labeled X174 *Hin*fI DNA marker (Promega).

For Northern blot analysis, total prostate cellular RNA was isolated with Trizol (Life Technologies, Inc.). The RNA samples were separated by electrophoresis on 1% agarose-formaldehyde gel and transferred to a Nytron membrane (Schleicher & Schuell). cDNA of PSGR were labeled with [^{32}P]dCTP by random oligo-nucleotide priming. The hybridization reaction was carried out using a standard process as previously described [Xia et al., 2001a].

Growth Factors and IL-6 Stimulation Assays

LNCaP cells were seeded in 24-well plates overnight before transfection with Luc-318 and Luc-677. After transfection, cells were grown for 6 h in 10% FBS medium and then serum starvation overnight. The media were then replaced with RPMI1640 containing 2 ng/ml TGF-B1, 10 ng/ml EGF, 5 μg/ml insulin, 5 ng/ml HGF, 10 ng/ml TNF-α, 10 ng/ml VEGF, 5 ng/ml FGF-basic, 5 ng/ml IL-6, or 10 nM R1881 in 0% FBS. Cells were then grown for 24 h before luciferase activity were measured.

RESULTS

Specific Expression of PSGR in Human Prostate Cell Lines

In order to study the expression patterns of PSGR and its regulatory mechanisms, we examined the specific expression of PSGR in human prostate cell lines and other non-prostate cell lines. RNA was collected from human prostate and non-prostate cell lines, including LNCaP, PC-3, DU145, PNT1A, Hela, SW480, HCT-116, MDA231, HNSC, N2A11, and HEK293. RT-PCR was performed to detect the expression levels of *PSGR* gene. As shown in Figure 1, *PSGR* is expressed exclusively in human prostate cell lines. The highest expression level was found in LNCaP cells while weak expression of PSGR was detected in PC-3, DU145, and PNT1A prostate-specific cell lines (Fig. 1). Similar results were obtained using real-time quantitative PCR (data not shown). No *PSGR* expression was detected in non-prostate cell lines. Together, the expression of

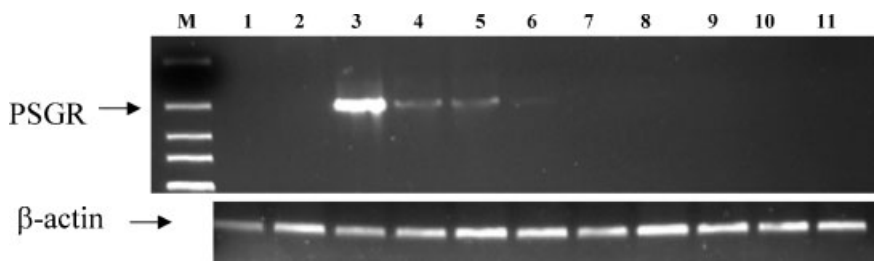

Fig. 1. Specific expression of PSGR in human prostate cell lines: 1, Hela; 2, SW480; 3, LNCaP; 4, PC-3; 5, DU145; 6, PNT1A; 7, HCT116; 8, MDA-MB-231; 9, HNSC; 10, N2A; 11, HEK293. A 1-kb band of PSGR was detected only in human prostate cell lines after gel electrophoresis. The expression of *PSGR* gene was found the highest in LNCaP cells, followed by PC-3, DU145 cells, and PNT1A cells. No *PSGR* expression was seen in non-prostate cell lines. β-actin was used as a cDNA control.

PSGR in different human cell lines are in agreement with our previous studies in which PSGR was specifically expressed in human prostate tissue, not in other human tissues [Xu et al., 2000; Xia et al., 2001b].

Genomic Structure and Potential Regulatory Sites of Human *PSGR* Gene

The human *PSGR* gene has one short non-coding exon (exon 1) and one long coding exon (exon 2) separated by a 14.9 kb intron. The exon 2 of *PSGR* encodes the full protein sequence (all the amino acids) of PSGR protein (Fig. 2B). The DNA sequence upstream of exon 1 contains two TATA boxes at sites of −31 and −171, a region with potential promoter activity. In exon 1, there exist DNA *cis*-elements for STAT-3, NF-kB, CACCC binding factor, and AP1 binding site (Fig. 2A). The upstream region of exon 1 contained multiple activator and repressor binding sites. To analyze the potential promoter region for PSGR, a Eukaryotic Promoter Prediction Program (Berkeley Drosophila Genome Project) was used to analyze the DNA region upstream of both exons of *PSGR*. For the region upstream of exon 1, a 40-bp sequence 5′-GGAACAGATATAAAAGGACTGTATGAG-GCAAAAGAAGTCT-3′ was predicted to be the promoter of *PSGR*, which was described as the transcription start site of *PSGR* previously [Yuan et al., 2001]. For the sequence about 250-bp upstream of exon 2, a 40-bp sequence 5′-GGGCCAACAAA ATATCCA G GC AACGAAG GTATGG-3′ was also predicted to be the promoter site of *PSGR* with the highest score (1.00). The sequence contains no TATA box elements and is a non-GC rich region, but did contain putative *cis*-acting elements that have been implicated in regulating mammalian gene expression (Fig. 2C). Two GATA factor binding-sites are also identified in the promoter region (Fig. 2C). GATA factors are highly expressed in prostate tissues and have been shown to be very important prostate gene enhancer [Perez-Stable et al., 2000]. Overlapping the first GATA site, a transcription element Lom2 was identified (Fig. 2C). The LIM-only protein Lom2 is a bridging molecule assembling an erythroid DNA-binding complex, including the TAL1, E47, GATA-1, and Ldb1/NLI proteins [Wadman et al., 1997]. Furthermore, a recognition sequence for the Forkhead family of transcription factors (Freac-6) is identified. Freac-6 factor is a potent transcriptional activator that shares sequence homology in the winged helix DNA binding domain. The existence of a Foxo transcription factor-binding site suggests potential regulation of *PSGR* by the signals coupled to PI_3K-Akt-Foxo signaling pathways. Other sites in this region include two GKLF sites and the binding sites for the Pit-1 protein. GKLF sites have been shown to be involved in the regulation of endogenous gene expression [Shields and Yang, 1998], and Pit-1 protein is known to regulate differentiation in several tissues [Andersen and Rosenfeld, 1994].

Functional Analysis of the Human PSGR Promoters

To characterize the promoter activity of *PSGR* gene, a series of sequences containing 5′-flanking regions of both exon 1 and exon 2 were inserted into a mammalian expression vector, pGL3-Basic, which contains luciferase as the reporter gene. The resulting plasmids were transiently transfected into various cultured cell lines, including prostate cell lines (LNCaP, PC-3, DU145, PNT1A) and non-prostate-cell lines (Hela, SW480, MDA231). The luciferase activities corresponding to different regions of the potential promoter were determined. As shown in the following sections (Figs. 3 and 4), constructs containing either promoter region directed high levels of luciferase expression in prostate cells, especially in LNCaP cells. These data are in consensus with the *PSGR* mRNA expression profile in which the highest expression level of PSGR was detected in LNCaP cells, and no PSGR expression was detected in other human cell lines and tissues (Fig. 1) [Xu et al., 2000; Xia et al., 2001b].

The first promoter contained exon 1 and the 5′-flanking region of exon 1. To characterize the first promoter and its regulatory region, various lengths of 5′-flanking regions were created by PCR in the pGL3 basic vector (Fig. 3A) and the luciferase activities were investigated (Fig. 3B). Constructs containing only 5′-flanking region of exon 1 show low promoter activity compared to the constructs containg exon 1, which suggested exon 1 contains positive transcription factors binding sites for the transcription activity of *PSGR* gene expression. The dramatic increase of transcription activity of constructs containing exon 1 in LNCaP cells as compared to other cells suggests that the exon 1 region contain binding sequence for cell specific factors. Further extending the upstream sequences of

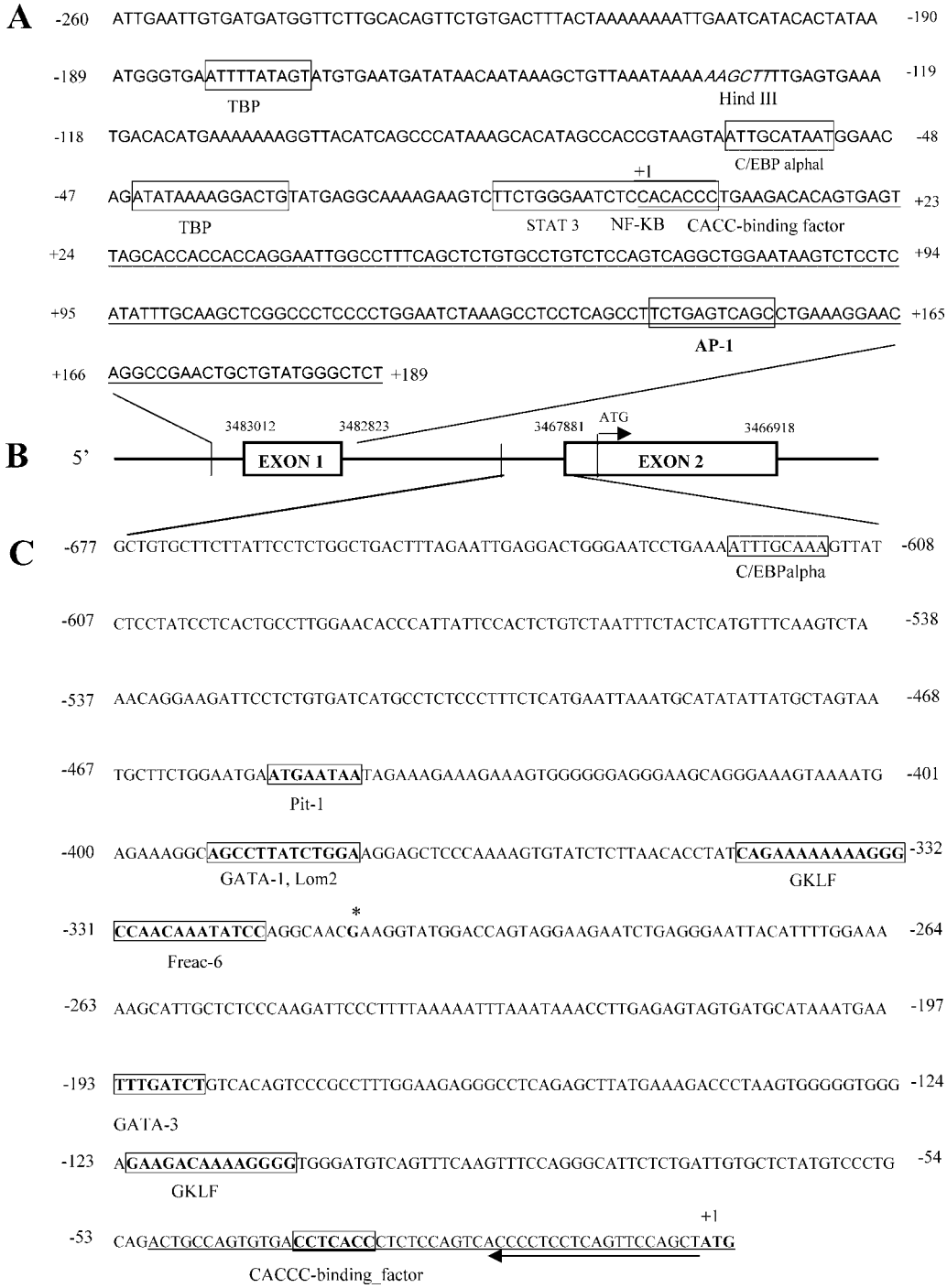

Fig. 2. Gene structure of human *PSGR*. **A**: Putative transcription factor binding sites in the 5′-flanking region of exon 1 and exon 1 of human *PSGR* gene was analyzed with TESS web software. Sequences representing *cis*-acting DNA regulatory elements are boxed. Exon 1 sequence is underlined. The numbering of sequence is based on the principle in which exon 1 start-site is indicated as +1 site. **B**: A schematic representation of human *PSGR* genes (obtained from the NCBI human). The human *PSGR* gene is located on chromosome 11p15 and composed a short non-coding exon (exon 1) and a coding exon 2, which encodes all the amino acids of PSGR. **C**: Putative transcription factor binding sites in the 5′-flanking region of exon 2 of human *PSGR* gene. Sequences represented *cis*-acting DNA regulatory elements are boxed. A potential transcription initiation site predicted by Promoter Prediction Software (Berkeley Drosophila Genome Project) is indicated by a star sign. The partial sequence of exon 2 is underlined. A primer designed for primer extension assay is shown with an arrow. The numbering of sequence is based on the principle which translation start site (ATG) is indicated as +1 site.

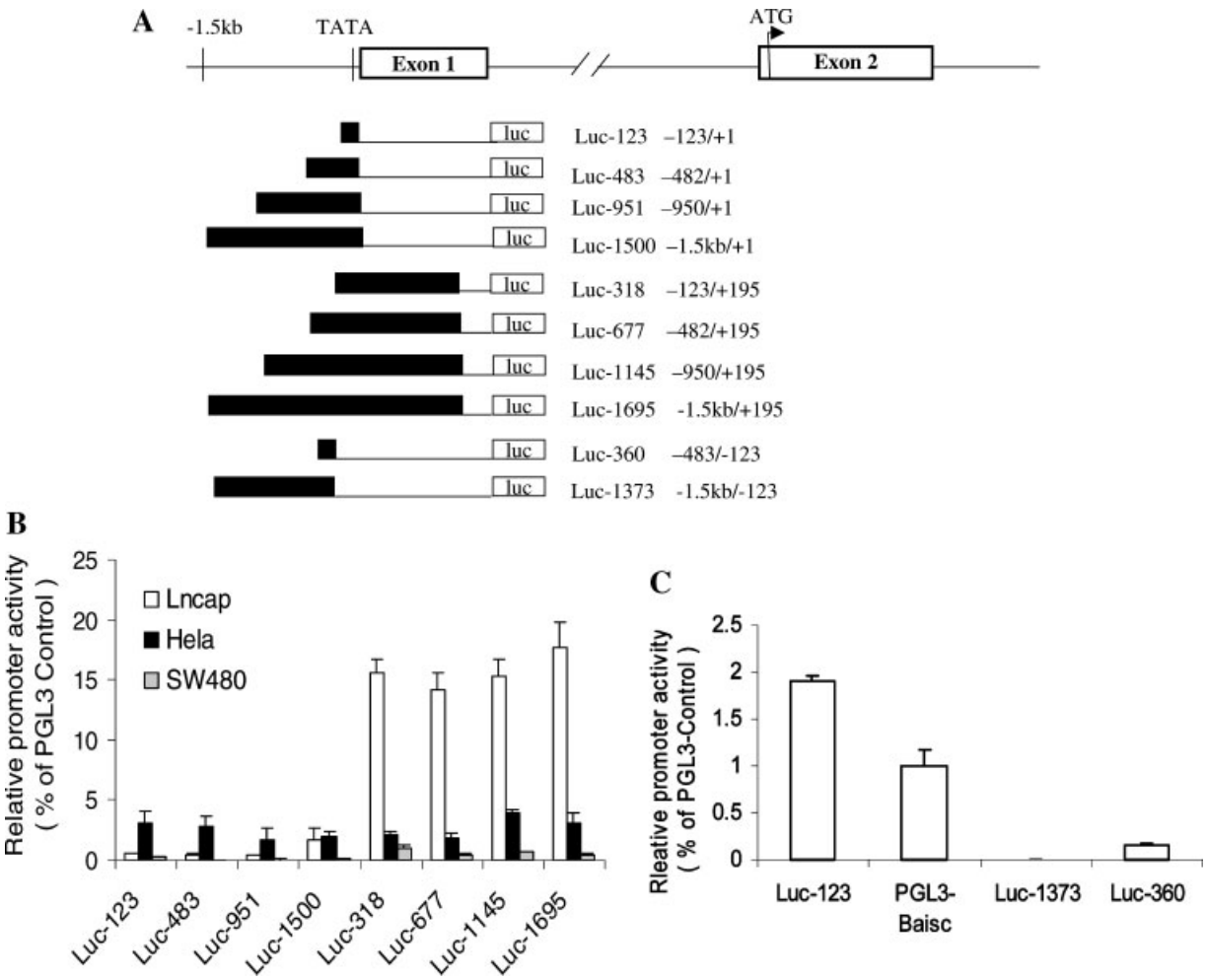

Fig. 3. Functional analysis of human PSGR promoters. **A**: Series of heterologous luciferase reporter constructs were generated by PCR amplification and contained progressive deletions of the 5′-flanking regions of *PSGR* as illustrated. The relative positions of the constructs are shown as in the picture. For the constructs, the start site of exon 1 is regarded as +1. **B**: These constructs were transfected into LNCaP, Hela, and SW480 cells with lipofectamine. Luciferase activities were calculated as percentage activity of PGL3 control vector activity. The Luc-123, Luc-483, Luc-951, Luc-1.5 kb showed no difference between LNCaP, Hela, and SW480 cells. Constructs that contain the exon 1 region of *PSGR* showed much higher activity in LNCaP cells but not in Hela and SW480 cells ($P < 0.05$). Extending the upstream region of 5′UTR of exon 1 showed little difference ($P > 0.05$) in luciferase activity. **C**: The core promoter activity was detected in the Luc-123 construct, containing the sequence upstream of exon 1 and the TATA box at −31 site. The two constructs, Luc-360, and Luc-1373, contain no TATA box at −31 site and show no promoter activity compared the control PGL3-basic activity.

the luciferase constructs (Luc-1145 and Luc-1695) did not result in any significant change of promoter activity ($P > 0.05$) (Fig. 3B), suggesting the upstream sequence contains little promoter activity for *PSGR* expression.

To find the minimal promoter of *PSGR*, we serially deleted the 5′-flanking region and generated the minimal −123 promoter construct (Luc-123) upstream of the exon 1. The promoter activity of Luc-123 was about two-fold compared with the promoter-less pGL3-basic (Fig. 3C). The 5′ upstream of exon 1 contains two TATA boxes (one at −31 and the other at −171). Luc-123 contains only one TATA box at −31 site. To investigate whether the second TATA box has functional promoter activity, we have made two other constructs that contain the TATA box at site −171. As showed in Figure 3C, the construct contains the first TATA box (−31) showed a minimal promoter activity (Luc-123) while the TATA box at −171 has no promoter activity for *PSGR* gene expression (Luc-360, Fig. 3C). Deletion of the TATA box at −31 site completely deleted the promoter activity of the

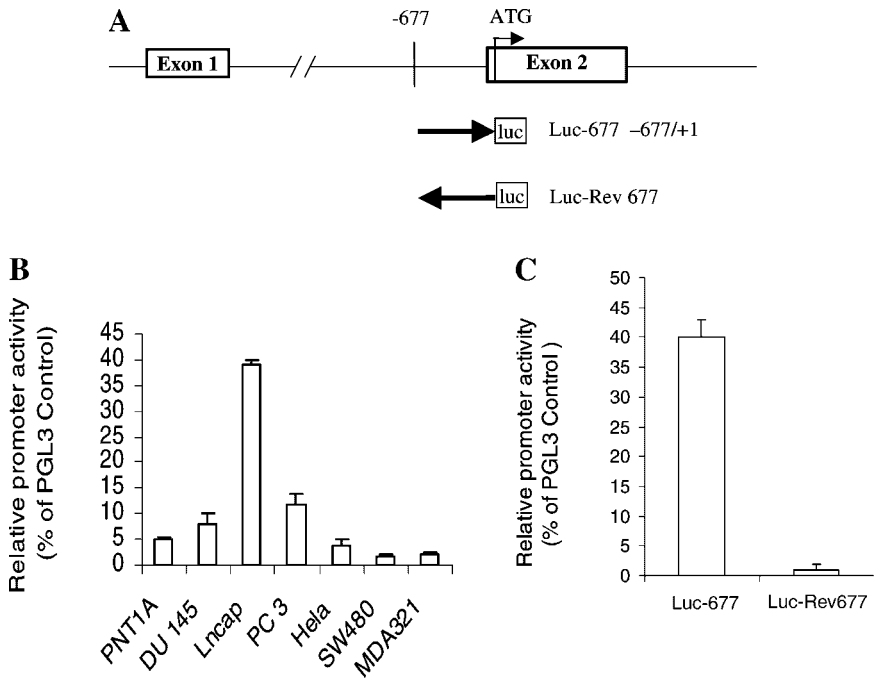

Fig. 4. Identification of the second promoter region of *PSGR* gene. **A**: Diagrams of the genomic structure and the constructs for analysis of potential second promoter region for *PSGR* gene. DNA sequence of the promoter region upstream exon 2 of *PSGR* was amplified by PCR and inserted into promoter less PGL-3 basic plasmid in two directions. **B**: The forward 677 construct (Luc-677) was transfected into different cells and assayed for luciferase activity. The Luc-677 construct shows the strongest activity in LNCaP cells, followed by other prostate cell lines (PC-3, DU145, PNT1A). Little activity was detected in other cell types, such as Hela, SW480, and MDA231 cells. **C**: Determination of promoter or enhancer. The forwarding construct (Luc-677) shows strong promoter activity while the reverse orientation construct (Luc-Rev 677) has little activity, suggesting orientation-dependent promoter activity for this region of *PSGR* (Luc-677) instead of enhancer activity.

constructs (Luc-1373) (Fig. 3C), indicating that the TATA box at −31 site contains the core basic promoter activity for *PSGR* gene expression.

The second promoter is located in the upstream region of exon 2, which is a TATA-less and non-GC-rich promoter (Figs. 2 and 4A). In human prostate cancer cell lines (LNCaP and PC-3), the second promoter shows much stronger promoter activity than in non-prostate cancer cell lines (Hela, SW480, and MDA-MB-231 cells) (Fig. 4B). To test whether the transcription activity of the second promoter region is orientation dependent, we inversed the orientation of this region and inserted into PGL3-basic plasmid. As show in Figure 4C, reverse orientation of the 677-promoter region almost completely abolished the luciferase activity of this construct, indicating this region contains promoter activity rather than enhancer activity. To further characterize the second promoter in human prostate cells, series of heterologous luciferase reporter constructs were generated by PCR amplification and contained progressive deletions of the 5′-flanking regions of exon 2 of *PSGR* as illustrated (Fig. 5). These constructs were transfected into LNCaP, PC-3, and PNT1A cells. Deletion of 5′-flanking region of 677 did not significantly change the luciferase activity of the promoter region in LNCaP cell until −377 site. The highest promoter activity is achieved at −331/+1 construct, suggesting potential negative regulators exist between −677 and −331 sequences of the second promoter. On the other hand, serial deletion of 677 results in decreased promoter activity in PC-3 and PNT1A cells, suggesting the 5′ region of 677 contain functional *cis*-binding motif that are bound by different transcription factors in different human cell lines.

Identification of the Transcription Initiation Sites of the *PSGR* Gene by Primer Extention Analysis and RNA Protection Assays (RPA)

The transcriptional initiation sites driven by the intron promoter 5′ of exon 2 were determined by primer extension analysis and by RPA. For primer extension, an oligonucleotide primer located in exon 2 was 5′-end-labeled,

hybridized with total RNA from LNCaP cells, and extended by avian myeloblastoma virus reverse transcriptase. A prominent band at the position −311 bp upstream from ATG site in the exon 2 was identified (Fig. 6A). Two other small clusters of transcription start sites located at about −175 and −118 bp upstream from ATG site in exon 2 were also observed on the autoradiogram (Fig. 6A). Multiple initiation sites are commonly observed with promoters lacking a TATA box. To verify the data obtained from primer extension, RPA was performed using ^{32}P-Labeled antisense RNA probe. The antisense RNA probe was hybridized with total RNA from LNCaP and digested with RNase A and T1. A fragment protected by the mRNA probe was detected between the 311 and 249 DNA markers (Fig. 6B). The position of this band was slightly different from those obtained with primer extension because mRNAs move faster than the DNA of the same size in denatured condition. The transcripts from the two minor initiation sites were not detected by RPA, probably due to the less abundance of the transcripts from these sites. Protection was not observed in liver, COS-7, and Hela cells (data not shown). These results demonstrate the prostate-specific expression of *PSGR* gene, which is in agreement with our results in prostate cell lines (Fig. 1) and our previous Northern blot analysis [Xia et al., 2001b; Weng et al., 2005].

Expression of Two Different Sizes of PSGR Transcripts in Human Prostate Tissues

The presence of two distinct promoters and a cluster of transcription factors binding motifs upstream of position −311 suggest that the expression of PSGR may have two mRNA transcripts. To test this hypothesis, we investigated the expression pattern of the *PSGR* gene by Northern blot analysis. A Northern blot membrane containing prostate tissue mRNA was hybridized with a labeled *PSGR* cDNA probe (Fig. 6C). Two major *PSGR* transcripts were detected (Fig. 6C). The upper band corresponds to the transcript from first promoter while the lower band corresponds to the

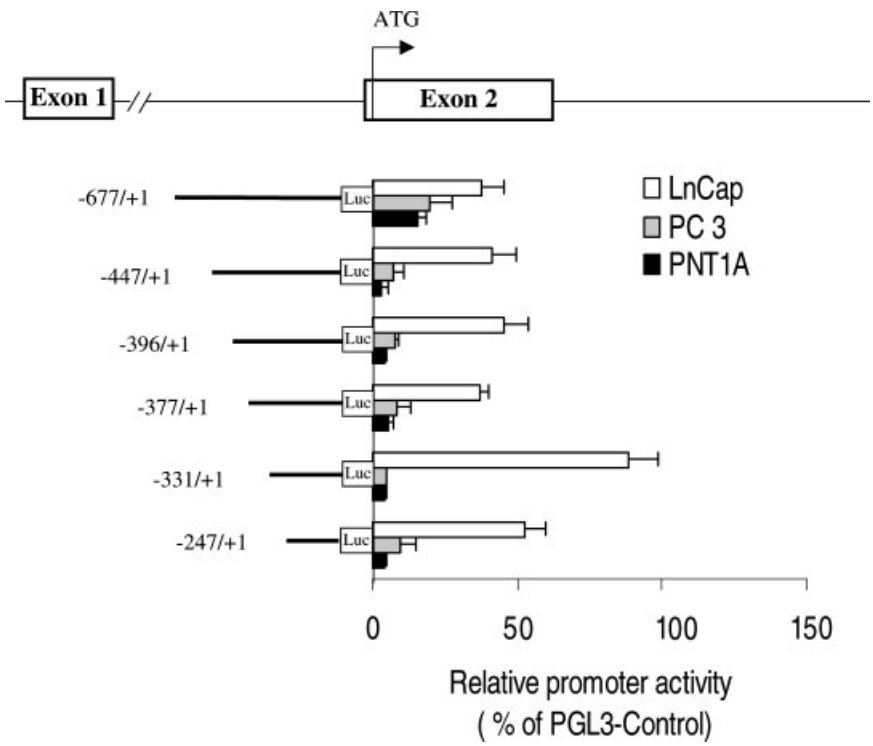

Fig. 5. Characterization of the second promoter activity of *PSGR* gene. A series of heterologous luciferase reporter constructs were generated by PCR amplification. These constructs contain progressive deletions of the 5′-flanking regions of exon 2 of *PSGR* gene and were transfected into LNCaP, PC-3, and PNT1A cells. Serial deletion of 5′-franking region of 677 has no significant effect on the luciferase activity in LNCaP cell until −331 site. The highest activity is achieved at −331/+1 construct. On the other hand, serial deletion of 677-Luc results in decreased promoter activity in PC-3 and PNT1A cells.

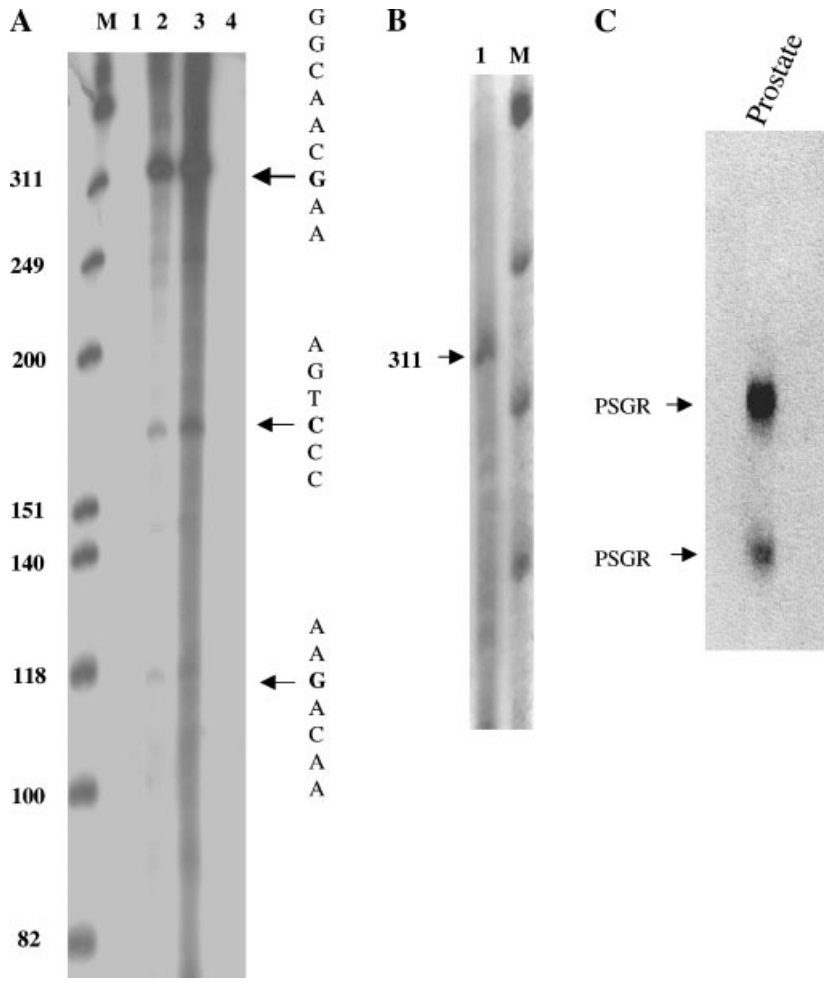

Fig. 6. Identification of transcription initiation sites of the second promoter of PSGR. **A**: Analysis of transcriptional starting sites of 5′-upstream region of exon 2 by primer extension, showing multiple transcription initiation sites at −311, −175, and −110 site. The primer (bases from −20 to 1 in exon 2) (see Fig. 2C) was labeled at the 5′-end, hybridized with RNA from LNCaP cells, and extended by avian myeloblastoma virus reverse transcriptase. All transcription start sites were indicated by an arrow and corresponding DNA sequence. M, marker; 1, no RNA control; 2, primer extension 1.5 h; 3, primer extension overnight; 4, yeast RNA control. **B**: Transcription initiation site identified by RPA (RNA protection assay). A labeled antisense RNA probe transcribed from the pCMV-tag-1-s/b vector that includes the intron of *PSGR* was hybridized with RNA from LNCaP. The sizes of the protected bands were compared with concurrently electrophoresed labeled X174 HinfI DNA marker (Promega). One 311 bp-band was protected by RNA transcribed by the intron promoter and are indicated by the arrow. 1, LNCaP RNA; M, marker. **C**, Detection of PSGR mRNA from human prostate tissues by Northern blot analysis. Specific *PSGR* cDNA probe was synthesized and hybridized to the prostate tissue mRNA membrane. Two different sizes of mRNAs specific for PSGR can be detected in prostate tissues.

transcript from the second promoter region. The relative abundance of the larger transcript suggests that the longer message was expressed at a higher level in prostate tissues and is regulated differentially.

Differential Regulation of PSGR Promoters by SV40 Enhancer

To further analyze the regulatory mechanism of the two *PSGR* promoters, we examined how an enhancer changes the promoter activities by inserting a SV40 enhancer (SVE) downstream of the two promoters, respectively (Fig. 7A). The first promoter Luc-318 was not activated by SV40 enhancer but actually inhibited by the presence of SV40 enhancer (Fig. 7B). On the other hand, the second promoter, Luc-677, was significantly enhanced in the presence of SV40 enhancer element (Fig. 7C $P < 0.05$). These data demonstrate that the same SV40 enhancer have totally different regulatory activities for the two individual promoters of *PSGR* gene, suggesting

that the two promoters of *PSGR* are modulated by different transcription factors and regulatory mechanisms.

Regulation of PSGR Promoter Activities by IL-6 and Other Growth Factors

To examine whether different growth factors and cytokines are involved in the regulation of *PSGR* in human prostate cancer cells, we tested the two promoter activities of *PSGR* in LNCaP cells under different stimulation of growth factors and cytokines. Transfected LNCaP cells were stimulated with TGF-β1, EGF, Insulin, TNF-α, VEGF, FGF-basic, R1881, and IL-6, the promoter activities of Luc-318 and Luc-677 were assayed. As shown in Figure 8, IL-6 increased the luciferase activity of both promoters by 2-3 fold, respectively ($P < 0.05$), suggesting possible regulation of *PSGR* by IL-6. Other growth factors have little effect in regulating the two promoter activities of *PSGR* gene expression (Fig. 8). Clinical data demonstrated that serum Il-6 levels are elevated in men with hormone-refractory prostate cancer and high levels of serum IL-6 are accompanied by high levels of serum prostate specific antigen (PSA) [Drachenberg et al., 1999]. The biological effects of IL-6 on prostate cancer cell growth and proliferation are still not clear and IL-6 may exert divergent effect in human prostate cancers, including the transcriptional regulation of human prostate-specific GPCRs, PSGR, in human prostate cancers.

DISCUSSION

PSGR is originally identified as a prostate specific GPCRs in human prostate tissues [Xu et al., 2000; Xia et al., 2001b]. Both the gene and the encoded protein are highly conserved across species from human, mouse, and rat [Yuan et al., 2001; Vanti et al., 2003]. However, the expression pattern of *PSGR* gene is quite different across each species. In human, PSGR is specifically expressed in the prostate tissue, with very low expression in the olfactory bulb and no

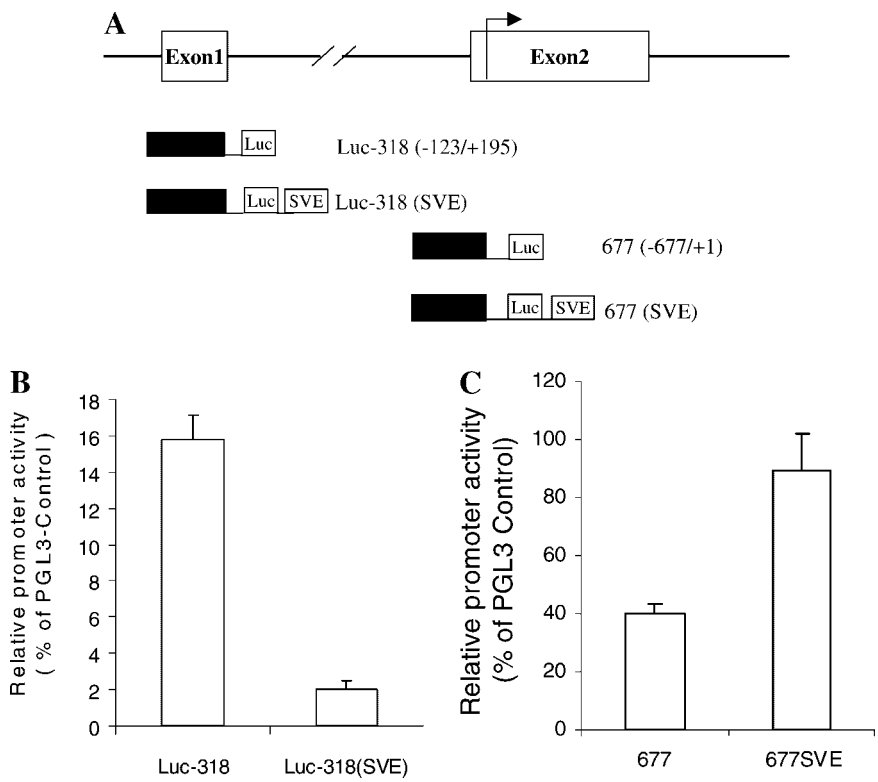

Fig. 7. Differential regulation of *PSGR* promoters by SV40 enhancer. **A**: Promoter constructs with and without SV40 enhancer. The first and second promoter region was cloned into a vector with the luciferase reporter and a SV40 enhancer element (SVE). **B–C**: Effects of SV40 enhancer on the promoter activities of *PSGR* gene. The above constructs were transfected into PNT1A cells and assayed for luciferase activities as described. The presence of SV40 enhancer greatly enhanced the Luc-677 promoter activity but decreased the Luc-318 promoter activity.

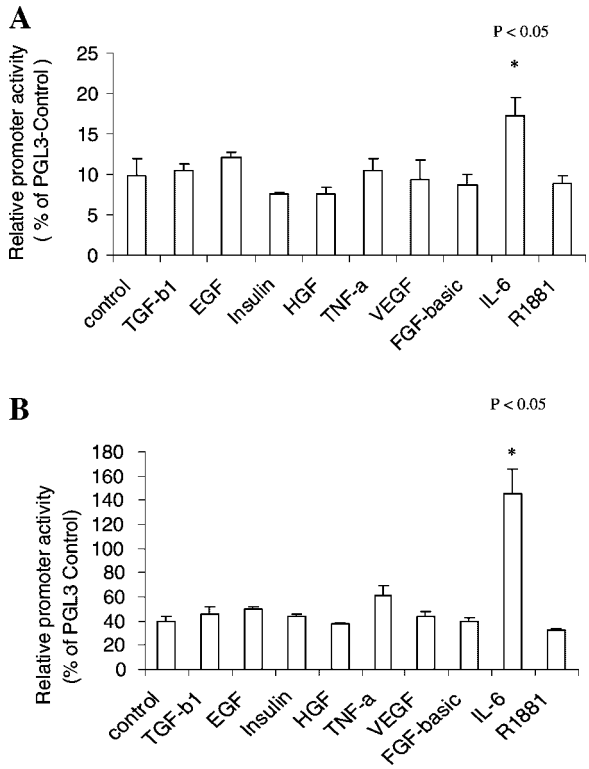

Fig. 8. Regulation of PSGR promoters by growth factors and cytokines. Regulation of the first (**A**) and the second (**B**) promoters of *PSGR* by different growth factors and cytokines in LNCaP cells. The cells were grown in RPMI 1640 medium and transfected with the two promoter constructs, Luc-318 (A) and Luc-677 (B), respectively. The transfected cells were stimulated with different growth factors and cytokines, and luciferase activity were measured. IL-6 significantly increased the activities of the two PSGR promoters ($P < 0.05$) while EGF, TGF-β1, Insulin, HGF, FGF-basic, VEGF, and R1881 showed little effects for both promoters of *PSGR*.

detectable expression of *PSGR* in other tissues. In mouse and rat, PSGR is expressed in brain, liver, and colon [Yuan et al., 2001]. In our most recent studies, we have demonstrated that *PSGR* is overexpressed in human PIN and prostate cancers [Xia et al., 2001b; Weng et al., 2005]. The specific expression patterns of PSGR in human prostate tissues and its overexpression in human prostate cancers suggest a potential role of this specific GPCR as a biomarker and drug target in our fight against human prostate cancers. In an effort to characterize the regulatory mechanisms of human *PSGR* that specifically expressed in prostate tissues and correlated well with human prostate cancers, we have identified and characterized the promoter regions of human *PSGR* gene and their potential regulation by growth factors and cytokines. Our results indicate that human *PSGR* gene is transcriptionally regulated by two distinct promoters and that the activities of the two promoter proximity regions are significantly activated by interleukin-6 (IL-6).

The *PSGR* receptor gene consists of two exons, an upstream non-coding exon 1 and a long exon 2 where the entire receptor protein is encoded (Fig. 1B). These two exons are separated by a large intron of ~15 kb in human *PSGR*. The distinct localization of human *PSGR* two exons prompts us to suspect that multiple promoters control the gene expression of human *PSGR*. Using Neural Network Promoter Prediction and TESS program, a strong promoter (score = 1.0) and multiple transcription factor binding sites, such as Freak-6, GATA-1, GATA-3, C/EBP alpha1, Pit-1a, and Lom2 were found for the second promoter proximity region, suggesting possible function of this region as an independent promoter in transcriptional regulation of human *PSGR* gene.

The first promoter region in human *PSGR* gene contains TATA box and consensus binding sequences for AP1, NF-*k*B, and CACCC binding factor in the first exon. Deletion of exon 1 resulted in much lower activity, indicating that exon 1 contains strong promoter activity for human *PSGR* gene transcription. Extension of the upstream region of exon 1, however, shows no increased activity, suggesting the 5′-flanking region of exon 1 contain less binding motifs for regulatory factors. The second promoter region contains no TATA box, nor CAAT box, and is not GC-rich. Furthermore, we found no consensus sequences for Sp1 binding sites that are seen as features in many housekeeping and regulated genes. We further confirmed the existence of two different sizes of mRNA in human prostate tissues by Northern blot analysis using specific *PSGR* probe. Together, our data showed that the 5'-flanking region of exon 2 acted as a functional second promoter rather than as an enhancer region since reversion of the orientation of the promoter regions dramatically reduced the promoter activity. To date, a number of genes in various species have been discovered to have more than one promoter, including the genes for dystrophin, brain-derived neurotrophic factor, growth hormone, growth hormone-releasing hormone, protein tyrosine kinase (blk), the A1 adenosine receptor, human D1A dopamine receptor gene [Chelly et al., 1990; Boyce et al., 1991; Gonzalez-Crespo and Boronat, 1991; Courtois

et al., 1992; Timmusk et al., 1993; Lin et al., 1995; Ren and Stiles, 1995; Lee et al., 1996]. The function of the dual promoters in human *PSGR* gene and their regulation in normal and malignant prostate tissues is an interesting question, and is under active studies.

The two promoters of the *PSGR* gene appear to be subject to regulation by different transacting factors, accounting for differences in their transcript abundance. For instance, the activator sequence, SV40 enhancer, activates the second promoter region but tends to repress the first promoter (Fig. 8). Since exon 1 in this gene is non-coding, the resultant receptor protein is not different whether translated from the short or long transcripts. The conserved structural organization of the *PSGR* gene among rats, mouse, and humans [Yuan et al., 2001] might suggest evolutionary significance of the intron and the resultant two different transcripts. The presence of two promoters and many unique potential transcription factor binding sites may be required to ensure transcription of this important gene in the prostate tissues. For example, forkhead family of transcription factors have been shown to control core promoter activity in mammalian tissues and regulate diverse cellular functions, such as differentiation, metabolism, proliferation, survival, and tumorigenesis [Overdier et al., 1997; Kitamura et al., 2002; Nakae et al., 2003; Tran et al., 2003; Accili and Arden, 2004; Brunet et al., 2004; Hu et al., 2004]. The existence of Foxo transcription factor binding sites in the second promoter regions of *PSGR* gene suggests potential regulation of *PSGR* by the PI3K-Akt-Foxo pathways.

The cytokine, IL-6, has many physiological functions, and has been implicated in a number of pathophysiological processes, including human prostate cancers [Chen et al., 2000; Deeble et al., 2001; Giri et al., 2001; Culig et al., 2002]. A variety of tumors, including melanoma, renal cell carcinoma, ovarian carcinoma, lymphoma and leukemia, multiple myeloma, Kaposi's sarcoma, and prostate carcinoma are stimulated by IL-6 [Keller et al., 1996; Qiu et al., 1998; Smith et al., 2001; Culig et al., 2002; Steiner et al., 2003]. The finding that IL-6 significantly activated the two promoters of *PSGR* and induced the expression of *PSGR* in human prostate cancer cells suggests that PSGR is a potential target of IL-6 activation. It has been shown that serum IL-6 level is elevated in men with hormone-refractory prostate cancer, and that IL-6 increase is accompanied by high levels of serum PSA [Drachenberg et al., 1999]. The exact effects of IL-6 on prostate cancers are still controversial. Some studies demonstrated that IL-6 could promote prostate cancer cell growth, while others suggest that IL-6 can suppress prostate cancer cell growth [Hobisch et al., 1998; Chung et al., 1999]. Binding of IL-6 to its receptor could activate three distinct downstream signaling pathways, including the PI3-Kinase pathway [Lin et al., 1999; Yang et al., 2003], the Stat3 pathway, and the MAPK pathway [Ueda et al., 2002; Yang et al., 2003], that could potential regulate the transcriptional regulation of a large number of genes, such as *PSGR*. The exact signaling mechanism and pathways that IL-6 regulates the transcriptional activation of *PSGR* in human prostate cancer cells are under active investigation in our laboratory.

In summary, we have demonstrated that two distinct promoters regulate the specific expression of human *PSGR* gene and that these two promoters are subject to regulation by different transacting factors coupled to different growth factors and cytokine, such as IL-6. As GPCRs mediate important cellular functions, two distinct PSGR promoters may be required to allow efficient transcription regulation of human *PSGR* gene throughout normal prostate development and prostate cancer progression. Investigations are underway to identify these factors and to further elucidate the regulatory mechanisms and signaling pathways that control *PSGR* expression in human prostate and prostate cancers.

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