A Novel Human Prostate-specific Gene-1 (HPG-I): Molecular Cloning, Sequencing, and Its Potential Involvement in Prostate Carcinogenesis1

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ABSTRACT

Prostate-specific genes that have a role in normal and abnormal prostate growth are needed for early and specific diagnosis and treatment of prostate cancer. In the present study, the differential display-PCR technique was used to obtain a prostate-specific ~339-bp cDNA fragment. On screening the human-prostate Agt10 library with this fragment, a full-length 1468-bp human prostate-specific gene (HPG-I) with an open reading frame of 127 amino acids (aa) was retrieved. Extensive database search revealed that the HPG-I has novel nucleotide/aa sequences. It was localized on Homo sapiens 3q26 chromosomal locus, a region that has been shown to be involved in prostate carcinoma. The computer-generated translated protein has a calculated molecular mass of 14.8 kDa with several potential glycosylation and phosphorylation sites including two N-linked glycosylation, one tyrosine phosphorylation, and one N-mysristoylation sites. The in vitro transcription and translation procedures using HPG-I cDNA yielded a protein of similar molecular mass of ~15 kDa. Hydrophilicity analysis of the deduced aa sequence indicated that HPG-I is a membrane-anchored/attached protein. Analysis for tissue specificity by using the Northern blot and reverse transcription-PCR-Southern blot procedures using 19 different human tissues revealed that HPG-I is expressed specifically only in prostate tissue. To examine its involvement in prostate carcinogenesis, three prostate cancer epithelial cell lines, one androgen-responsive (LNCaP) and two androgen-nonresponsive (DU-145 and PC-3), were examined for the expression of HPG-I. Using the Northern blot and quantitative reverse transcription-PCR procedures it was found that LNCaP and DU-145 cells and not the PC-3 cells have HPG-I expression, with LNCaP cells having approximately 2.3-fold higher levels of HPG-I mRNA transcripts compared with DU-145 cells. In vitro culture of LNCaP cell with antisense and not the sense oligonucleotide decreased the HPG-I mRNA levels and inhibited the cell growth in a concentration-dependent manner; at 72 h there was an 86% inhibition of cell growth. HPG-I mRNA expression in LNCaP cells was found to be responsive to androgen. Thus, the novel androgen-responsive HPG-I, which has prostate-specific expression and seems to be involved in carcinogenesis, may have applications in the specific diagnosis and treatment of prostate cancer.

INTRODUCTION

Prostate adenocarcinoma is the most frequently diagnosed cancer and is second only to lung adenocarcinoma as having the highest cancer mortality (1, 2). Numerous factors are attributed to the increase in prostate cancer incidence. Two predominant factors include: (a) the explosion in the aging population demographic; age is the single most significant risk factor for development of prostate cancer (3); and (b) the availability of recent technologies (digital rectal examination and the prostate-specific antigen test) for the early detection of prostate cancer (4). These clinical diagnostic tests lack sensitivity and specificity in appropriately distinguishing between prostate cancer and benign abnormalities such as BPH3 and prostatitis (5, 6). Additional prostate-specific molecules need to be identified to design more sensitive/specific tests for early disease diagnosis and to develop more effective therapies.

Numerous laboratories, including ours, are actively searching for genes that are prostate-specific and have a role in carcinogenesis (7). Several genes have been identified using various approaches, such as, RNA DD analysis (8–10), expressed sequence tag database analysis (11–15), exon trapping (16), representational difference analysis (17), subtractive cDNA libraries (18, 19), suppression subtractive hybridization (20), microarray technology (21, 22), serial analysis of gene expression (23), and yeast two hybrid system analysis (24). The genes identified using these techniques include the cell surface antigens, namely, prostate carcinoma tumor inducing gene-1, prostate stem cell antigen, prostate-specific G-coupled receptor, six-transmembrane epithelial antigen of the prostate, and prostein (8, 13, 17, 20, 22, 24). Other genes reported are noncoding riboregulators, DD3 and PCGEM1 (9, 10), serine protease, Tmprss2 (16), small nuclear proteins, T-cell receptor γ-chain alternate reading frame protein, and prostate/rectum/colon gene (14, 15), tumor suppressor genes, Nkx3.1 and prostate androgen-regulated transcript-1 (11, 21), coregulator of the androgen receptor, PDEF (12), androgen-regulated gene, Pmeapa-1 (23), and the calcium channel protein, transient receptor potential-8 (19). Most of the genes have not been extensively examined for their prostate-specificity and/or for their role in carcinogenesis. Thus, their application in early diagnosis or immunotherapy of prostate cancer and BPH remains unclear. Only three, namely, prostate-specific antigen, prostatic acid phosphatase, and prostate-specific membrane antigen have been investigated for prostate-specificity and for their utility in diagnosis of human prostate disease (25–27). Additional molecules need to be identified that are prostate-specific and have a role in prostate carcinogenesis, and can thus be used for early diagnosis and for developing specific immunotherapeutic modalities for treatment of prostate abnormalities (cancer and BPH).

The objective of the present study was to identify genes expressed specifically in normal human prostate. To identify genes that are specifically expressed in human prostate, we used the DD-PCR technology. Herein, we describe the identification, molecular cloning, and sequencing of a novel HPG-I that is expressed only in the prostate. The expression of HPG-I is up-regulated in androgen-responsive LNCaP cells compared with androgen-nonresponsive DU-145 and PC-3 cells, and the expression in LNCaP cells is enhanced with androgens. Treatment of LNCaP cells with antisense oligonucleotide decreases cell growth in a concentration-dependent manner by decreasing the level of specific mRNA transcripts, indicating its role in prostate carcinogenesis.

MATERIALS AND METHODS

Isolation of Prostate-specific cDNA Fragment(s) by DD-PCR Procedure. The Delta RNA DD method (Clontech Laboratories, Inc., Palo Alto, CA) was used to obtain prostate-specific cDNAs, and the procedure was...
MOLcular Cloning of HPG-1

Carried out according to the manufacturer’s instructions (28). Briefly, first-
strand cDNA was synthesized from total RNAs from normal human prostate,
liver, and spleen (Clontech), and then reversed transcribed 42°C for 1 h. Each
DD primer-pair fingerprint consisted of two dilutions (1:10 and 1:40) of a
cDNA template of 20 μl of starting RNA, and the appropriate buffer and water
controls were subjected to PCR amplification in the presence of 50 pmol of a
selected P and T primer pair. 10× reaction buffer, 5 mM dNTP, 10 μCi [α-32P]dATP, and Taq polymerase; 12 different combinations of primers were
used in various experiments. The bands were resolved on a 6% urea denaturing
gel, the gel was dried, and exposed to X-ray film. On autoradiography, mRNAs
that appeared to be differentially expressed in prostate tissue were selected, and the
cDNA fragments were recovered, cloned into ph Bluescript II SK+ at the
SmaI site, and sequenced using the Sequenase 2.0 (USB Corporation, Cleve-
land, OH). The nucleotide sequence was subjected to a sequence homology
search in the GenBank database, and only those cDNA fragments that showed
no homology with any existing gene in the database were selected for addi-
tional studies. The DD-PCR procedure furnished a novel 339-bp cDNA frag-
ment, designated as P17, which was expressed only in the prostate and not in
liver or spleen, the tissues tested.

Library Screening. Isolation of Full-length cDNA, and Sequence Anal-
ysis. The human prostate 5’-STRETCH Agt10 cDNA library (Clontech) was
screened with the P17 cDNA fragment to obtain the full-length cDNA. The partial
cDNA fragment was cut from ph Bluescript II SK+ by digestion with PstI and BamHI and labeled with [α-32P]dCTP. The library was plated at a density of
≈10 × 10^4 plaque-forming units/100-mm Petri dish with Esche-
richia coli C600 Hfr as the host bacterium. After growth at 37°C for 5.5 h, the
plaques were plated onto nitrocellulose membranes, denatured, neutralized,
and UV cross-linked. The membranes were blocked with ExpressHyb solution
(Clontech) for 15 min, and then probed with the 32P-labeled P17 probe for 2 h.
The filters were washed with 0.1% SDS in 2× SSC solution (room tempera-
ture, 5 min), 0.1% SDS in 1× SSC (room temperature, 5 min), and then with
0.1% SDS in 1× SSC (55°C, 10 min). The putative positive clones were
subjected to secondary and tertiary screening, and the cDNA insert of the
selected clone was subcloned into ph Bluescript II SK+ at the EcoRI site, and
sequenced. The nt and aa sequence were performed using BLASTN and
BLASTX algorithms (29) using the National Center for Biotechnology infor-
mation web server.4 The sequence was analyzed for putative ORF and plotted
for hydrophobicity according to the Kyte and Doolittle (30), and Engelman et
al. (31) scales using the Vector Nti Suite software program (InforMax Inc.,
North Bethesda, MD). Potential phosphorylation sites, protein motifs, and
glycosylation sites were analyzed using the GCG program (University of
Wisconsin, Madison, WI). The full-length cDNA was designated as HPG-1.

Northern Blot Procedure. The human multiple tissue Northern blots were
obtained from Clontech, and the Northern blots of normal prostate tissue and
cultured prostate cancer cells were prepared in the laboratory (28). Total RNA
from cultured prostate cancer cells (LNCaP, DU-145, and PC-3) that had
reached 75–80% confluency was extracted using TRIzol reagent (Invitrogen,
Carlsbad, CA) according to manufacturer’s instructions. After electrophoresis
on a denaturing gel, all of the RNAs were found to have distinct 28S and 18S
ribosomal bands with intensity ratio of at least 1:5.1. The gel containing 2 μg of
poly(A)+ RNA per lane from normal human prostate (Clontech) or cultured
prostate cancer cells was subjected to electrophoresis, blotted onto nitrocel-
lose membrane, and UV cross-linked. Blots (multiple tissue Northern sites, pro-
tate, and prostate cancer cells) were prehybridized (60°C, 10 min) in ExpressHyb solution and then hybridized (65°C, 2 h) with [α-32P]dCTP-labeled
cDNA probe incorporating the HPG-1 ORF. The cDNA probe (ORF) was
prepared for PCR amplification of the HPG-1 cDNA, using forward
(5’-CGGGATCCATCTGATAAAAAAATCTT-3’) and reverse primer (5’-CGGA-
ATCTGGATGAACAAAGTC-3’) incorporating the EcoRI site. These primers correspond with the start and
termination codons, respectively, of ORF of HPG-1 cDNA. PCR amplification
cycles involved initial denaturation at 94°C for 10 min, and 30 cycles: 94°C for
1 min, 55°C for 2 min, and 72°C for 2 min, followed by a final extension at
72°C for 15 min. The final volume of the PCR reaction mixture was 50 μl
consisting of 20 ng cDNA, 50 pmol of the respective forward and reverse
primers, 1× PCR buffer, 1.5 mM MgCl2, and Taq polymerase. The PCR-

sequence, and the 28 nt reverse primer corresponds to its complimentary strand. The CT 36-nt primer was a hybrid with 28 nt in the 5'-end identical to the reverse primer and 28 nt in the 3'-end corresponding the complimentary strand of HPG-1 cDNA located in a region approximately 100–120 bp upstream of the original reverse primer. The 28 bp at the 3'-end of the CT primer allows it to bind to its complimentary sequence on the target gene, but it cannot be extended with Taq polymerase. The primer sequences, nt position, and the PCR product length for both the NT and CT of HPG-1 and β-actin are described in Table 1.

Synthesis and quantification of CT standards were performed as described by Willey et al. (35). The forward NT primers and CT primers of HPG-1 and β-actin, were used to amplify CT standards from cDNAs of LNCaP, DU-145, and PC-3 cells. CT standards were synthesized in a 50-μl reaction mixture that contained 5 μl of 10× PCR buffer, 50 pmol CT primer or primers, 50 pmol forward NT primer, 20 ng of cDNA, 1.5 μl of 50 mm MgCl₂, 0.5 μl of 10 mm dNTPs, and 0.2 μl of Taq polymerase (5 units/μl). PCR amplification cycles involved initial denaturation at 94°C for 10 min and then 30 cycles: 94°C for 1 min, 58°C for 2 min, and 72°C for 2 min followed by a final extension at 72°C for 15 min. HPG-1 and β-actin CTs were checked by electrophoresis for predicted sizes, and then cut from the gel, eluted, and purified. The concentrations of gel-purified CTs were determined by electrophoresis for a 2% agarose gel containing 0.5 μg/ml EtBr using a known amount of HindIII-digested λ DNA markers. The bands were visualized using a Foto/Eclipse Investigator image capture system (Fotodyne, Hartford, WI) and quantified using the GelPro densitometric scan software program (Fotodyne). CT molarity was calculated from the pixel quantity and compared with that of a known fragment of the HindIII-digested λ DNA marker. CTs were diluted in 10 nm Tris and 1 μM ethylenediaminetetraacetic acid buffer (pH 8.0), so that the forward and reverse primers yielded CT and NT PCR products of approximately equal intensity in EtBr-stained electrophoretic gels (31). CT mixtures and dilutions containing known amounts of both HPG-1 and β-actin CTs were prepared from the same original stock solutions (31). CT mixtures were then diluted to contain β-actin (A) and HPG-1 (P) in the following molar (M) concentration: A 15P–11, A 13P–14, A 13P–13, A 11P–16, A 13P–17, A 14P–15, A 14P–16, A 14P–17, and A 14P–18. Each quantitative competitive RT-PCR reaction (50 μl of total volume) required 5 μl of the CT mixture, and thus, the final CT molarity was 10-fold lower than the above concentrations. The appropriate CT mixture was selected based on the relative cDNA concentration in prostate cancer cells and on the relative expression of HPG-1 to β-actin. In our study, 1 μl of cDNA was found to compete equally with 10⁵–10⁶ molecules of β-actin CT and 10⁴–10⁵ molecules of HPG-1 CT in PCR. Primer pair for each gene was expected to amplify a single band from the PCR mixture when no native cDNA was present. The quantitative competitive RT-PCR was carried out as described elsewhere (35). Briefly, a 50-μl reaction mixture containing 50 pmol of β-actin forward primer, 50 pmol of β-actin reverse primer, 50 pmol of HPG-1 forward primer, 50 pmol of HPG-1 reverse primer, 5 μl of CT mixture, 2 μl of cDNA, 5 μl of 10× PCR buffer, 1.5 μl of 50 mm MgCl₂, 0.5 μl of 10 mm dNTPs, and 0.2 μl Taq polymerase, was subjected to PCR, and the products were quantified and analyzed after the conditions described above. The intensity of the EtBr-stained band of NT and CT products was mathematically corrected for size by the after equation as described by Allen et al. (36). IN = NT × C Ts INs / CTD × (iCT) where IN = the number of native molecules; NT = native band density; CTD = CT band density; C Ts = CT size in bp; INs = native size in bp; and iCT is the initial number of CT molecules:

The number of initial native molecules in a sample was calculated based on the amount of CT standard added, and the ratio of native gene product and CT gene products. Results were expressed as the number of molecules of HPG-1 mRNA per 100 molecules of β-actin.

### Effect of Antisense Oligonucleotide on mRNA Transcript Levels in LNCaP Cells

The human prostate cancer LNCaP cells were grown in RPMI 1640, supplemented with 2 mm l-glutamine, 10% FCS, 50 μg/ml lentamycin, and maintained in an atmosphere of 5% CO₂/95% air at 37°C. Exponentially growing LNCaP cells were seeded into 2 ml of RPMI 1640 with 10% charcoal/dextran-stripped, delipidated, heat-inactivated, FCS (Sigma, St. Louis, MO) in culture dishes at a concentration of 2 × 10⁶ cells/ml. After 24 h, the cells were washed with HBSS (Life Technologies, Inc., Grand Island, NY), and resuspended in fresh RPMI 1640 charcoal-stripped calf serum medium and 2.5–20 μM (final concentration) of sense/antisense phosphorothioate-conjugated oligonucleotides. The sense (5'-ATGTATTTTTTTATCAT-3') and antisense (5'-ATTITTTTTCATCAT-3') oligonucleotides were based on the translation initiation region (nt sequence 318–332) of the HPG-1 cDNA sequence, had 6.7% G+C content, and were synthesized at the MWG-Biotech (High Point, NC). LNCaP cells were incubated with the oligonucleotides for 24, 48, and 72 h, washed, and total RNA was extracted and examined for integrity as described above. Approximately 2 μg of poly(A)+ RNA per lane from LNCaP cells cultured with various concentrations of sense or antisense phosphorothioate oligonucleotides were subjected to gel electrophoresis, blotted onto nitrocellulose membrane, and UV cross-linked for Northern blot analysis. Blots were prehybridized (60°C, 10 min) in ExpressHyb solution, hybridized (65°C, 2 h) with [α-³²P]dCTP-labeled cDNA probe incorporating HPG-1 ORF, washed, and exposed to X-ray film. The bands were visualized and quantified as described above. HPG-1 transcript intensity in each lane was normalized to the β-actin band intensity.

### Effect of Androgens on Growth of LNCaP Cells

Exponentially growing LNCaP cells were seeded into 2 ml of RPMI 1640 with 10% FCS in tissue culture dishes at the concentration of 2 × 10⁶ cells/ml. After 24 h, cells were washed with HBSS and resuspended in 2 ml of fresh RPMI 1640 charcoal-stripped calf serum medium containing 2.5–20 μM of sense or antisense phosphorothioate oligonucleotide. LNCaP cells were incubated with the various concentrations of phosphorothioate oligonucleotides for 24, 48, and 72 h, trypsinized, and total cell counts were determined by hemocytometer. Cell viability was assayed using the trypan blue dye exclusion assay (Sigma). Five independent experiments were performed on different days using different passages of LNCaP cells. Significance of differences between sense- and antisense-treated and the control groups was analyzed by the one-way ANOVA test. Post-hoc analysis was performed by using the Bonferroni test. A P of <0.05 was considered statistically significant.

### Effect of Androgens on HPG-1 Expression in LNCaP Cells

LNCaP cells were cultured in phenol red-free RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with charcoal-stripped calf serum, and the experiments were performed on cells cultured between passages 12 and 16. Cells were incubated with (5α-DHT, Sigma) at 10⁻⁷ M, 10⁻⁸ M, 10⁻⁹ M, 10⁻¹⁰ M, and 10⁻¹¹ M concentrations for 6 h, washed with PBS, and the RNA was extracted and examined for integrity as described above. Approximately 2 μg of poly (A)+ RNA per lane from the LNCaP cells treated with the various concentrations of 5α-DHT were subjected to electrophoresis, blotted onto nitrocellulose membrane, and UV cross-linked for Northern blot analysis. Blots were prehybridized (60°C, 10 min) in ExpressHyb solution, hybridized (65°C, 2 h) with [α-³²P]dCTP-labeled cDNA probe incorporating HPG-1 ORF, washed, and exposed to X-ray film. HPG-1 mRNA levels were visualized and quantified as described above. LNCaP cells treated identically without 5α-DHT served as a control.

### Table 1: RT-PCR primers for HPG-1 and β-actin native and competitive templates

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<td>NT Forward</td>
<td>5’ TGG AAC AAG CCA AGA AGA ATCA CCA CCT GTC A 3’</td>
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<tr>
<td></td>
<td>NT Reverse</td>
<td>5’ GTT GTT ATG CCA ATT CCA TGC TGG TTG T 3’</td>
<td>1100–1127</td>
<td>718</td>
<td></td>
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<tr>
<td></td>
<td>CT</td>
<td>5’ R* + GAT TTT AGG ATT GTT TGG TCT ATT TCT GT 3*</td>
<td>979–1004</td>
<td>598</td>
<td>Genbank X00351</td>
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<tr>
<td>β-Actin</td>
<td>NT Forward</td>
<td>5’ GAT TCC TAT GTG GGC GAC G 3’</td>
<td>192–212</td>
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<td></td>
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<tr>
<td></td>
<td>NT Reverse</td>
<td>5’ CCA TCT CTT GCT AGT AGT CC 3’</td>
<td>704–723</td>
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<tr>
<td></td>
<td>CT</td>
<td>5’ R* + GCC AGC AGC GAT CAC AGC AC 3’</td>
<td>568–587</td>
<td>416</td>
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* R* refers to the NT reverse primer sequence that was added to the 5'-end of the CT primer.

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RESULTS

**HPG-1 Full-Length Clone.** To identify novel genes that are expressed exclusively in the normal prostate, we compared mRNA expression patterns of normal human prostate, liver, and spleen by the DD-PCR technique. A total of 5 cDNA fragments were found that were differentially expressed in the prostate. These fragments were subcloned into pBluescript II SK+, sequenced, and analyzed for nt and aa homology in the GenBank database, and for prostate-specific expression in the Northern blot analysis. Of these, a 339-bp cDNA fragment, designated as P17, did not show a significant homology with any nt/aa sequence in the database and demonstrated prostate-specific expression, and thus was selected for additional studies. Using P17 as a probe, the full-length 1,468-bp cDNA, designated as novel HPG-1, was obtained after screening 873,000 clones from the normal human prostate Agt10 cDNA library.

**HPG-1 Sequence Analysis.** HPG-1 cDNA was found to be a novel sequence without any homology with any known nt/aa sequence in the database. HPG-1 cDNA was found to be located on human 3q26 chromosome locus. Analysis of HPG-1 cDNA using the DNA analysis program revealed that it has an ORF starting with an ATG initiation codon at nt 318–320 with a termination codon at nt 306–308 (Fig. 1A). It has a 5'-UTR of 317 bp and a 3'-UTR of 767 bp. The canonical 3' polyadenylation signal, AATAAA, is located at nt positions 720–725.

Analysis of the predicted peptide using Vector NTI DNA analysis software program revealed that the predicted peptide of HPG-1 has 127 amino acid residues with a calculated molecular mass of 14,810 Da and a isoelectric point of 9.42. The motif search of the predicted peptide showed two potential N-linked glycosylation sites on the hydrophilic portions of the peptide at 35–38 and 97–100 aa, respectively, one Asn-myristoylation site at 10–15 aa, two casein kinase II phosphorylation sites at 42–45 and 75–78 aa, respectively, one Ser-threonine phosphorylation site at 8–19 (L...), one Tyr-phosphorylation site at aa 102–113, and two Ser/threonine phosphorylation sites at aa positions 8–19, two threonine phosphorylation sites at aa positions 59–67 and 71–79, and six serine phosphorylation sites at aa positions 25–33, 33–41, 39–47, 58–66, 95–103, and 109–117, respectively. The encoded protein contains ~15% serine/threonine residues, which are potential sites for O-linked glycosylation. The aa sequence has 52.8% hydrophilic and 47.2% hydrophobic amino acid residues, respectively. It seems that the HPG-1 has a slightly more hydrophilic characteristic. Analysis of the hydrophilicity profile of the deduced amino acid sequence of HPG-1 (ORF; Fig. 1B) indicated that this peptide has a hydrophobic N-terminal region, a central core with several hydrophilic and hydrophobic domains, and a tendency toward hydrophility at the COOH terminus. Both the Kyte and Doolittle (30) and Engelman et al. (31) computations indicated similar patterns.
Tissue-specific Expression of HPG-1 cDNA. The tissue-specific expression of HPG-1 cDNA was examined by the Northern blot and RT-PCR-Southern blot analyses. Human multiple northern blots containing ~2 µg of poly (A)+ RNA per lane from 16 human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, adrenal medulla, thyroid, adrenal cortex, testis, thymus, small intestine, stomach, and prostate) were probed with HPG-1 cDNA (ORF). The estimated transcript size of HPG-1 on human Northern blots corresponded to the size of the insert cDNA. The HPG-1 (ORF) cDNA probe did not hybridize with any other tissue even when the membrane was exposed for 2 weeks. Fig. 2A, bottom row, shows hybridization of all of the tissues to the control human β-actin cDNA probe revealing a positive signal of equal intensity of the ~2.0-kb transcript (Fig. 2B).

The tissue-specific expression of HPG-1 cDNA (ORF) was additionally confirmed by RT-PCR-Southern blot analysis. The total RNA from 10 various human tissues was examined using specific primers based on HPG-1 or β-actin cDNA. The HPG-1-specific primers amplified the expected 692-bp fragment only in the prostate RNA (Fig. 3A). The Southern blot hybridization additionally confirmed the specificity of the amplified 692-bp fragment (Fig. 3B). The β-actin-specific primers amplified the expected 256-bp fragment in all of the tissues by RT-PCR (Fig. 3C) that was specifically hybridized with the β-actin cDNA probe in the Southern blot procedure (Fig. 3D).

In Vitro Transcription and Translation Products. In vitro transcription and translation procedures were performed to examine the apparent molecular mass of the translated polypeptide. The subcloned HPG-1 cDNA in pBluescript II SK+ vector was analyzed for orientation of mRNA-like strand (sense strand) sequence. To obtain the flanking insert sequence with either T3 or T7 RNA polymerase, the XhoI- or NotI-restricted recombinant plasmid was used for the in vitro transcription. T7 and T3 RNA promoter polymerase-generated run-off transcripts (Fig. 4A) were subjected to the in vitro translation procedure by using the rabbit reticulocyte lysate system. The translated products were resolved in the SDS-PAGE and subjected to autoradiography. Autoradiography of the gel showed a single band of ~15 kDa translated product when the NotI-restricted recombinant plasmid with T7 RNA polymerase was used. The run-off transcript generated by T7 RNA polymerase using NotI-restricted recombinant plasmid indicated that it was a sense strand (Fig. 4B). The run-off transcript generated with T3 RNA polymerase using XhoI-restricted plasmid did not yield any translated product, indicating it to be an antisense strand (Fig. 4B).

HPG-1 Expression in Prostate Cancer Cells. To investigate the role of HPG-1 in carcinogenesis, its expression was examined in the androgen-responsive (LNCaP) and androgen-nonresponsive (DU-145 and PC-3) cells by the Northern blot analysis. HPG-1 mRNA transcript of ~1.5-kb was detected in the LNCaP and DU-145 cells and not in PC-3 cells; the LNCaP transcript band was stronger (~2-fold) than that of DU-145 cells (Fig. 5A). The control β-actin transcript of ~2.0-kb was present in equal amounts in all of the three lanes indicating equal loading and RNA integrity.

The quantitative measurements of HPG-1 transcript levels in the cultured prostate cancer cell lines were determined by the quantitative RT-PCR (Fig. 5B). LNCaP cells showed significantly higher levels of HPG-1 transcripts per 100 β-actin molecules (~2.3 HPG-1 transcripts/100 β-actin molecules) as compared with DU-145 cells (~1 HPG-1 transcript/100 β-actin molecules). There was no expression observed in the androgen-nonresponsive PC-3 cells (Fig. 5B).
Effect of Antisense Oligonucleotide on HPG-1 mRNA Transcript Levels in LNCaP Cells. The overexpression of HPG-1 in LNCaP cells implied that it may play a critical role in the cellular processes in the androgen-responsive cells. We proceeded to examine whether treatment of LNCaP cells with 2.5, 5, 10, or 20 μM of antisense oligonucleotide would reduce HPG-1 transcript levels. Sense and antisense oligonucleotides based on the initiation codon and the following four codons were selected for the study. These oligonucleotides were chemically modified with a phosphorothioate backbone that enabled it to be resistant to cellular nucleases, while maintaining the ability for the oligonucleotide to bind to the target mRNA and recruit RNaseH. Because of the cellular toxicity observed at higher concentration (20 μM) and no effect observed at lower concentration (2 μM) of antisense oligonucleotide in the pilot experiments, the 5 μM and 10 μM concentrations were selected for these studies. Treatment of LNCaP cells with 10 μM of antisense oligonucleotide resulted in a 54% decrease (P = 0.023) at 24 h and a 59% decrease (P = 0.028) in HPG-1 mRNA levels at 48 h as compared with control (Fig. 6A). The sense oligonucleotide did not significantly (P > 0.05) affect HPG-1 mRNA transcript level at these time points. There was no significant effect of sense or antisense oligonucleotide on HPG-1 mRNA levels at 72 h.

Effect of Antisense Oligonucleotide on Growth of LNCaP Cells. The effect of antisense oligonucleotide on LNCaP cells was also determined. The incubation of LNCaP cells with 10 μM of antisense oligonucleotide significantly (P < 0.05) decreased cell growth at all of the time points tested (days 1, 2, and 3; Fig. 7). The incubation of cells with 5 μM of antisense oligonucleotide significantly decreased cell growth at days 2 and 3. At day 3, there was a 63% reduction (P = 0.026) with 5 μM and an 86% reduction (P = 0.013) with 10 μM concentration of antisense oligonucleotide compared with control. The sense oligonucleotide did not significantly (P > 0.05) affect LNCaP cell growth.

Effect of Androgens on HPG-1 Expression in LNCaP Cells. LNCaP cells express high-affinity androgen receptors with the ability to respond to androgens. To examine the effect of androgen on HPG-1 mRNA levels, various concentrations of 5α-DHT were added to LNCaP cells cultured in charcoal-stripped serum and phenol red-free medium. HPG-1 mRNA levels were determined by using the Northern blot analysis, and their levels were quantified and normalized to β-actin mRNA levels. HPG-1 mRNA levels increased with increasing concentrations of 5α-DHT (Fig. 8). Significant increases in HPG-1 mRNA level occurred when LNCaP cells were treated with 10^{-10} M (P = 0.044), 10^{-9} M (P = 0.031), 10^{-8} M (P = 0.012), and 10^{-7} M (P = 0.003) concentrations of 5α-DHT as compared with controls.

**DISCUSSION**

The aim of the present study was to obtain a cDNA that is prostate-specific and is involved in carcinogenesis. The DD-PCR technique...
was used for this purpose. Using this technique, five cDNA fragments expressed only in the prostate and not in the other tissues tested were obtained. All were subcloned, sequenced, searched for homology with known genes in the database, and analyzed for prostate-specific expression in the Northern blot analysis involving RNAs from a limited number of tissues. Of these five, the PI7 cDNA fragment did not reveal homology with any known gene in the database and also indicated prostate specificity in the Northern blot analysis. Screening the human prostate 5'-stretch Agt-10 cDNA library with this prostate-specific 339-bp cDNA fragment yielded a full-length HPG-1 cDNA comprised of 1468 nt having an ORF encoding for a 127 aa peptide. Both nt and aa sequences did not show homology with any gene reported previously in the database, indicating it to be a novel cDNA. The presence of a termination codon at the 5' end before the ATG start codon, and a polyadenylation signal at the 3' end, indicate it to be a full-length cDNA. It has a 5'-UTR of 317-bp and a 3'-UTR of 767-bp. The UTRs may be involved in stabilizing mRNA for translation (37). Most eukaryotic mRNAs possess short 5' UTRs of 20–100 nucleotides that enable efficient cap-dependent ribosome scanning (38). Long 5' UTRs are found in proto-oncogenes, growth factors, growth factor receptors, and homeodomain proteins, and mutations in this region have been implicated in cancer progression (39).

The deduced aa of the HPG-1 ORF translated to a polypeptide of 14.8 kDa. The in vitro transcription and translation procedures confirmed the molecular mass of the HPG-1 protein to be ~15 kDa. The deduced aa of HPG-1 indicated two potential N-linked and several O-linked glycosylation sites; one tyrosine, two threonine, and six serine phosphorylation sites; six kinase C and two casein kinase II phosphorylation sites; and one N-myristoylation site. Hydrophilicity analysis of the deduced protein indicated a hydrophobic NH2-terminal region, a central core of several hydrophilic and hydrophobic domains, and a tendency toward hydrophilicity at the COOH-terminal region. This hydrophilicity profile indicates HPG-1 to be a membrane-anchored protein. The presence of potential N-myristoylation site close to the NH2-terminus also indicates that HPG-1 protein may have a propensity to bind to the plasma membrane of the cell (40). However, there is no signal peptide sequence at the NH2-terminus, and several O-linked and two N-linked glycosylation sites. It is possible that HPG-1 protein is present in both a membrane-attached/anchored and secreted forms. The presence of multiple potential phosphorylation sites renders the molecule with signal transducing ability that may have a role in cellular growth, differentiation, and proliferation (41, 42).

The HPG-1 has a prostate-specific expression as indicated by the Northern blot and RT-PCR/Southern blot procedures using 19 different tissues. The prostate-specific expression suggests a unique role of this molecule in normal and possibly abnormal prostate function and growth. The HPG-1 transcripts were present in LNCaP and DU-145 cells, and absent in PC-3 cells as indicated by the Northern blot and quantitative RT-PCR procedures. LNCaP is an androgen-responsive cancer cell line, and the DU-145 and PC-3 are androgen-nonresponsive cancer cell lines. There was a 2–3-fold higher HPG-1 mRNA expression in the androgen-responsive (LNCaP) cells compared with androgen-nonresponsive (DU-145 and PC-3) cells as observed in both procedures. These findings indicate that HPG-1 may play a role in regulating androgen-dependent responses in prostate adenocarcinomas. Indeed, the in vitro incubation with antisense and not the sense oligonucleotide caused a significant, concentration- and time-dependent inhibition in LNCaP cells; at 72 h there was an 86% inhibition of cellular growth. These findings indicate a role of HPG-1 in prostate carcinogenesis. The growth-inhibitory effects of antisense oligonucleotides were specific because they specifically reduced the levels of HPG-1 mRNA transcripts (and not the β-actin mRNA levels) that correlated with the inhibition of LNCaP cellular growth. The incubation of LNCaP cells with the androgen, 5α-DHT, that cannot be metabolized to estrogen, caused an up-regulation of HPG-1 mRNA levels in a concentration-dependent manner confirming that HPG-1 is an androgen-responsive gene.

The molecular mechanisms involved in the growth inhibition of LNCaP cells require additional study. It is possible that the HPG-1 is involved in a signal transduction cascade through phosphorylation of serine/threonine/tirosine residues (41–44). Many peptide growth factors, such as epidermal growth factor, fibroblast growth factor, insulin-like growth factor, and transforming growth factor-β, and phosphorylation events have been shown to participate in normal and abnormal prostate growth (45).

The computer-based genomic analysis mapped HPG-1 cDNA to Homo sapiens chromosome 3q26 locus. Studies by Sattler et al. (46) showed that 3q25–q27 chromosomal locus, including interleukin 12A, myelodysplasia syndrome 1, solute carrier family 2 member 2, and sex determining region-Y box 2 genes, is amplified in human prostate carcinoma. Because HPG-1 is localized in this chromosomal locus, it may be one of the genes amplified in this cluster.

The utility of an antigen in the specific diagnosis and treatment of prostate cancer is contingent on its prostate specificity and involvement in carcinogenesis. The novel androgen-responsive HPG-1 seems to fulfill both these criteria, and thus may provide an interesting molecular marker for the specific diagnosis of prostate cancer. The involvement of HPG-1 in prostate carcinogenesis is indicated by its prostate-specific expression, inhibition of cancer cell growth by antisense oligonucleotides, and its localization at the 3q26 chromosome locus that has been shown to be involved in prostate cancer. It appears to be a membrane-anchored/attached protein; however, it needs to be determined whether or not it is also secreted in blood and/or semen.
The immunotherapeutic strategies involving passive immunization with HPG-1 antibodies and a vaccine could be developed against this molecule for the treatment of prostate abnormal growth (47). The HPG-1 expression and its role in different tumor size and grade, in indolent and aggressive cancers, in androgen-independent cancer growth, and in BPH need additional investigation.

REFERENCES


A Novel Human Prostate-specific Gene-1 (HPG-1): Molecular Cloning, Sequencing, and Its Potential Involvement in Prostate Carcinogenesis

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