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Cloning and Analysis of the Promoter Activity of the Human Prostatic Acid Phosphatase Gene

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Human prostatic acid phosphatase (PAP) has been proposed to be a prostate-epithelium differentiation antigen and its expression can be regulated by androgen. Nevertheless, the regulatory mechanism at the molecular level is not completely understood. In this communication, we demonstrated the tissue-specific expression of PAP in the normal prostate epithelium. Furthermore, results of nuclear run-on experiments indicated that androgen could regulate the transcriptional rate of the PAP gene. This mode of regulation was modulated by cell density. To investigate the transcriptional regulation, we cloned and characterized a 1.4- kilobase (kb) fragment of DNA that flanks the 5′ region of the PAP gene from LNCaP human prostate carcinoma cells. The results demonstrated that this 1.4-kb DNA fragment can drive a chloramphenicol acetyltransferase (CAT) gene expression in LNCaP cells. Also, the promoter activity was inversely correlated with the growth of those cells. © 1998 Academic Press

Prostate cancer is the most commonly diagnosed solid tumor in men. Reports from the American Cancer Society have predicted that there could be 209,000 new cases and 41,800 deaths from prostate cancer for 1997. Between 1976 and 1994, prostate cancer rates have doubled and mortality has increased by 20%. The reasons for this increase in cancer incidences are not known, but increasing life expectancy, growing disease prevalence resulting from environmental carcinogens, and increasing use of novel diagnostic modalities have been suggested as some causes (1).

In the past few years, several new approaches including gene therapy for treating advanced cancer have been proposed. Since the differential expression of a desired product in the target tissue is the central to the concept of gene therapy, several strategies for targeting specific gene expression have been developed. One such approach is to use a tissue-specific promoter to drive therapeutic genes (2-4). Because a tissue-specific promoter can only be activated in the targeted tissue, the genes driven by the promoter will be expressed differentially in these cells, minimizing systemic toxicity. Promoters of prostate-specific genes including prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) are possible candidates serving for that approach. However, the expression of PSA gene is not specific only to the prostate. Its expression was observed in several breast tumors and endometrium (5).

PAP has a long history of serving as a tumor marker of prostate cancer and has been proposed to have a tissue-specific manner of expression (6,7). Nevertheless, controversial results exist (8). Additionally, since the expression of PAP can be regulated by androgen, this gene is suitable for studying the mechanism of androgen action in prostate. Although DNA sequences of PAP promoter have been reported (9-11), no information is yet available on regulation of the promoter activity of PAP gene in human prostatic cancer cells.

In this study, we examined the tissue-specific expression of PAP in normal human tissues. We also investigated the mechanism by which androgen regulates the level of PAP mRNA by nuclear run-on experiments. Furthermore, we cloned and characterized a 1.4 kilobase (kb) DNA fragment from the promoter region of the PAP gene (−1356/+87). In this communication we report that this 1.4 kb DNA fragment exhibits a promoter activity in LNCaP cells, a PAP producing human prostate cancer cell line.

MATERIALS AND METHODS

Materials. Cell culture medium, fetal bovine serum (FBS), gentamicin and Lipofectin reagent were obtained from Life Technologies, Inc. The MasterAmp PCR Optimization kit was from Epicentre Tech-

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nologies Corp. Zero Blunt PCR cloning kit, and pCR-Blunt vector were obtained from Invitrogen Corp. pCATBasic, pCATEnchancer, pCATPromoter, pSV-β-galactosidase vectors and CAT assay kit were purchased from Promega Corp. DNA manipulations of plasmids were performed by conventional molecular biology techniques (12).

Cells culture. LNCaP cells were routinely maintained in RPMI-1640 medium supplemented with 5% FBS, 1% glutamine, and 0.5% gentamicin. To examine androgen effect on PAP expression, cells were maintained in a steroid-reduced medium, i.e., phenol red-free RPMI-1640 medium containing 5% heat-inactivated steroid-reduced FBS (SR-FBS) (13, 14).

β-Galactosidase histochemistry. Cultured LNCaP cells were transfected with a pSV-β-galactosidase vector, containing β-galactosidase gene driven by a SV 40 promoter. After 24h cells were rinsed twice with phosphate-buffered saline (PBS), pH 7.3, and fixed for 5 min in 2% formaldehyde plus 0.2% glutaraldehyde in PBS. The cells were washed with PBS, overlaid with 1 ml per well of histochemical reaction mixture, containing 1 mg/ml 4-Cl-5-Br-3-indolyl-β-galoctosidase (X-gal), and incubated at 37°C for 18 hours to obtain visible staining (15).

Northern blot. Multiple tissue membrane was purchased from Clontech Laboratories, Inc. Northern blot hybridization was performed as described previously (13, 14).

Nuclear run-on experiment. Nuclear extracts and run-on assay was carried out essentially as described (16,17). Briefly, nuclear extracts were prepared from LNCaP cells grown in the presence or absence of 5α-dihydrotestosterone (DHT) in a steroid-reduced medium as described (13, 14). Transcription was continued in the presence of ^{32}P-labeled UTP (Amersham Life Science Inc.). The radioactive RNA was hybridized with a slot blot membrane containing the full length of PAP cDNA.

Cloning of the PAP promoter. The promoter fragment of PAP gene was obtained by a polymerase chain reaction (PCR) amplification using genomic DNA isolated from LNCaP cells as the template. PCR reaction was conducted in a volume of 100 μl in the presence of Pfu DNA polymerase (Stratagene), and Buffer F from the MasterAmp PCR Optimization kit utilizing a Perkin-Elmer GeneAmp PCR System 2400 (Perkin-Elmer). Two oligonucleotide primers were utilized: 5′ TTG TAG GTT TGG GCT TTT TGC 3′ and 5′ ATT CTT AAT CTG TTG GGA GTC 3′. PCR mixture was first denatured by heating at 95°C for 5′. The amplification was performed for 30 cycles using following conditions: 30‟ at 94°C, 1′ at 64.7°C, 1′30′ at 72°C. A DNA fragment of 1.4 kb was obtained and cloned into the pCR-Blunt vector. The obtained DNA insert was sequenced and compared with reported sequences to ensure the accuracy of PCR product (9-11).

Plasmid constructs. To assess the promoter activity, a HindIII/XbaI fragment of PAP promoter from pCR-Blunt vector was cloned into pCATBasic and pCATEnchancer plasmids. Resulting plasmids, pCATPAP and pCATEPAP, contained a 1.4 kb promoter fragment of the PAP gene covering the region −1356 to +87 in the sense orientation. A plasmid pCATasPAP containing the same 1.4 kb fragment in the antisense orientation was constructed as a control.

Transfection and reporter assays. For transfection, LNCaP cells were routinely plated 2.5 × 10^5 cells per well in a 6-well plate in RPMI 1640 medium containing 5% FBS. To examine steroid effect on the promoter activity, cell were plated in a steroid-reduced medium. 5 μg plasmid DNA were introduced into LNCaP cells by complexing with the Lipofectin reagent as described previously (18). After 6 hours incubation, an equal amount of medium containing 10% FBS was added and incubated for 16 hours. For CAT assays, cells were washed once with PBS, scraping, and lysed in 1× reporter lysis buffer (Promega). The protein concentration of cell extracts was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories) with bovine serum albumin as a standard.

Quantitative CAT assays were performed with the same amount of total cell lysates in a reaction volume of 125 μl in the presence of ^{14}C-chloramphenicol (Amersham Life Science Inc.) as described in the Promega CAT-assay manual accompanying with the assay kit. Samples were incubated overnight followed by a single extraction with 300 μl xylene. The 250 μl organic phase was transferred to scintillation vials containing 2 ml EdoLume scintillation fluid (ICN Corp.) and counted by Beckman LS 1801 scintillation counter. All experiments were repeated four times in triplicates.

RESULTS

Tissue specificity and transcriptional regulation of PAP by androgen. To analyze the tissue specificity of PAP expression, Northern hybridization was performed utilizing a specific PAP cDNA probe with a membrane containing mRNAs from eight different normal human tissues. As shown in Fig. 1A, only normal prostate cells expressed a detectable level of PAP mRNA. To investigate whether PAP expression is regulated by androgen at the transcriptional level in LNCaP cells, nuclear run-on experiments were conducted. As shown in Fig. 1B, in low density cells, DHT stimulated the transcription of PAP mRNA; while, in high density cells, the transcription of PAP mRNA was suppressed by DHT. Thus, PAP exhibits a tissue-spe-

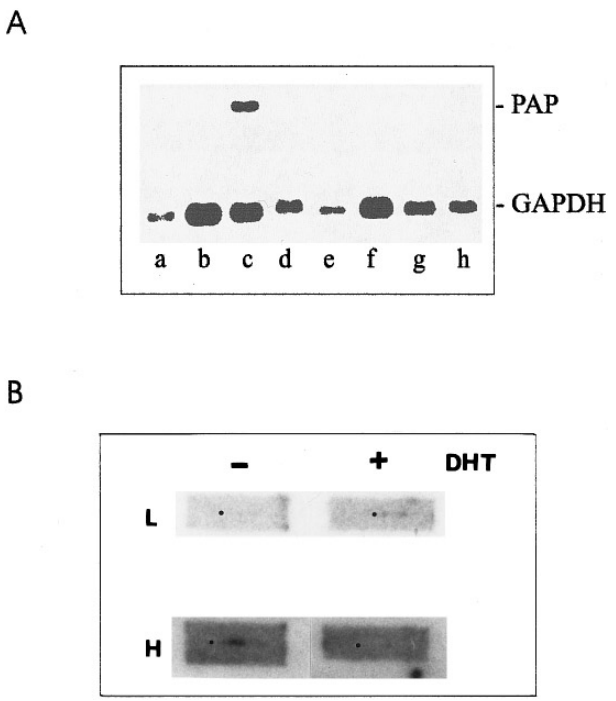

FIG. 1. (A) Expression of PAP mRNA in normal human tissue. The multiple tissue membrane contains 2 μg poly(A^+) mRNA per line; a-spleen; b-thymus; c-prostate; d-testis; e-ovary; f-small intestine; g-colon; h-peripheral blood leukocyte. The membrane was first hybridized with a PAP cDNA probe. After stripping, the membrane was rehybridized with a GAPDH cDNA probe. (B) Slot blot of nuclear run-on assay. Nuclei were isolated from LNCaP cells that were treated with 10 nM DHT (+) or solvent alone (−); L- low, and H-high density of LNCaP cells.

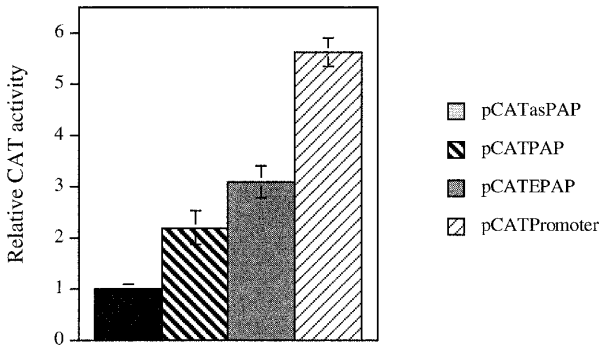

FIG. 2. Promoter activity of the 1.4 kb fragment of PAP 5′-flanking region. LNCaP cells were transiently transfected with pCATPAP, pCATasPAP, pCATEPAP, and pCATPromoter vectors, as a reporter, respectively. The relative CAT activity were calculated from results of triplicate samples after normalizing to pCATasPAP. Similar results were obtained from four independent experiments. Bar represents standard deviation.

cific expression which can be regulated by androgen at the transcriptional level.

Transfection efficiency. For monitoring the transfection efficiency, LNCaP cells were transfected with a pSV-β-galactosidase vector utilizing a Lipofectin transfection method and subsequently cells were stained *in situ* for β-galactosidase activity. Only after 18 hours of incubation the cells could be visibly stained. This prolonged incubation indicated a low activity of the SV40 promoter in LNCaP cells. Furthermore, our data demonstrated a low efficiency of transfection since only approximately 20% cells were transfected (data not showed).

Analysis of PAP promoter activity. To investigate whether a fragment of PAP gene upstream of the starting codon (−1356/+87) has a functional activity, the CAT assay was performed. As shown in Fig. 2, in LNCaP cells, the −1356/+87 fragment of the 5′- flanking region could drive the expression of CAT activity which was approximately 2.5-fold higher compared to the vector containing the same DNA fragment in the antisense orientation. Approximately 3-fold induction of activity was observed when LNCaP cells were transfected with a vector containing the −1356/+87 PAP gene fragment and a SV40 enhancer. Transfection with a pCATPromoter vector containing the CAT gene driven by a SV40 promoter resulted in an approximately 5.5-fold activity of the control vector. These data indicated that this 1.4 kb of 5′ flanking region contains the basic promoter activity although the activity is low.

To examine whether this DNA fragment contains steroid response elements, after transfection, cells were grown in medium containing 5% FBS, 5% or 1% SR-FBS. Cells were then harvested and used for CAT activity assays. We observed a decrease of cell grown in the steroid-reduced medium (5% SR-FBS) with a simultaneous increase of CAT activity, in comparison with cells growth in 5% FBS (Fig. 3). Interestingly, when SR-FBS concentration was decreased from 5% to 1%, the cell growth further diminished and the CAT activity was even higher. Thus, the PAP promoter activity of this 1.4 kb fragment inversely correlated with the cell growth.

DISCUSSION

PAP has been reported to be a useful marker of differentiated prostate epithelium cells and may play an important role in regulating the growth of those cells (13,18,19). However, its tissue-specific expression in normal prostate cells has not been investigated at molecular level. Here we have shown for the first time a tissue-specific expression of PAP in normal prostate epithelium by Northern blot analyses at the mRNA level. The reported non-prostate expression of PAP could be due to the cross-reactivity of anti-PAP antibody or the non-specificity of inhibitors in the enzyme activity assay (8). Thus, our report of the tissue-specific expression of PAP in normal prostate cells supports the hypothesis that the promoter of the PAP gene could potentially be used for the cell-specific targeting in gene therapy.

It is a long-standing interesting question of androgen regulation of PAP expression. Although it has been shown that androgen could regulate PAP expression at the mRNA level, it is not known whether androgen regulates the transcriptional rate of PAP gene or the stability of its mRNA (14,20). Our nuclear run-on experiments clearly demonstrated that androgen could regulate the expression of PAP gene at the transcriptional level. Furthermore, this transcriptional regulation by androgen could be modulated by cell density

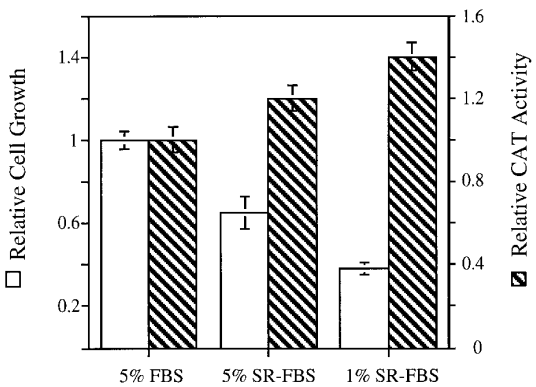

FIG. 3. Serum effect on the PAP promoter activity in LNCaP cells. LNCaP cells were transiently transfected with the pCATPAP vector. After 16 hours of incubation in the steroid-reduced medium, cells were grown in different serum conditions for two days. The relative CAT activity were calculated as the mean of triplicates from three independent experiments. Cell growth was calculated based on total cellular protein. Bar represents standard deviation.

and/or cell growth, consistent with results of Northern analyses (14, 20, 21).

While the genomic DNA sequence of PAP gene has been reported (9-11), the regulation of the PAP promoter activity is still poorly understood. It has been proposed (22, 23) that androgen regulation of PAP expression could be mediated via steroid response elements (SRE) present in the regulatory region of gene since the SREs of PAP gene could bind to androgen receptor *in vitro* (9). However, results in a previous study indicated that androgen could not directly regulate the expression of reporter genes driven by a PAP promoter in LNCaP cells (5).

In this communication, our data clearly demonstrated that a promoter construct of the PAP gene covering the region of −1356 to +87 is functional in LNCaP prostate cancer cells, although the activity of this cloned promoter region is relatively low. It is possible that the low transcriptional activity of PAP promoter is in part due to a low transfection efficiency of LNCaP cells. When a promoter fragment (−1600/+12 bp) of PSA gene was used to drive a CAT gene in LNCaP cells, no significant CAT activity was detected even when it was cotransfected with an androgen receptor (AR) cDNA expression vector. It was proposed that the low activity is due to a low transfection efficiency of LNCaP cells (24). However, no direct experiment was performed. Our results utilizing a SV40 promoter clearly demonstrated that LNCaP cells exhibit a low transfection efficiency mediated by liposome complexes (data not shown). LNCaP cells also exhibit a low transcriptional activity which is indicated by a very slow growth rate (18). This hypothesis is supported by the observations that a SV40 promoter displays a low level of β-galactosidase (data not shown) and CAT activity (Fig. 2). The low transcriptional activity of the PAP promoter is also demonstrated by the weak hybridization bands in nuclear run-on experiments. The low promoter activity could be due to a low amount of basic transcription factors essential for the promoter activity in those cells. Additionally, there is no significant difference in the CAT activity between the plasmid containing the PAP promoter region with a SV40 enhancer and the PAP promoter alone (Fig. 2). Thus, it is possible that the cloned promoter part of PAP gene lacks a specific enhancer element. The data taken together provide us with an explanation regarding the low PAP promoter activity in LNCaP cells, consistent with a recent report (5). Further experiments are needed to clarify the molecular mechanisms.

Interestingly, the promoter activity is increased even under non-permissive conditions of cell growth (Fig. 3), indicating that the transcriptional factors for PAP expression are actively functioning despite suppression of the growth machinery. The results are consistent with our previous observations that cellular PAP activity as well as its protein level is inversely correlated with the growth rate of LNCaP cells (13, 14, 18, 19). Additionally, a reduced growth rate with an increased promoter activity in steroid-reduced FBS, in comparison with cells grown in presence of normal FBS indicates that the cloned promoter region contains some steroid response elements. Thus, the negative results in a previous report could be due to different cell growth conditions (5).

The expression of PAP gene in prostate epithelium is regulated by a complicated process. The cloning and characterization of the promoter region of the PAP gene should enable us to investigate the regulatory mechanisms of PAP expression at the molecular level.

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