Identification of a Positive Regulatory Element Responsible for Tissue-specific Expression of Prostate-specific Antigen

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ABSTRACT

The prostate-specific antigen (PSA) promoter (PSA-P) has been identified, characterized, and determined to be tissue specific. Compared with high expression of the genomic PSA gene in prostate cells, expression of the transgene driven by the putative PSA promoter is low. This suggests that the identified promoter may be incomplete or may function optimally with additional regulatory elements. To identify the presence of additional regulatory elements, we screened sequences upstream of the PSA promoter and identified a DNA fragment of 822 bp, which enhances PSA gene expression. Combining the newly identified PSA gene regulatory sequence (PSAR) with our previously identified PSA promoter (PCPSA-P) exhibited enhanced expression activity in the PSA-producing LNCaP cell line. With the addition of 10 to 100 nM dihydrotestosterone, a more than 1000-fold increase in expression was observed as compared to androgen-negative controls. Furthermore, although the combined regulatory element (PSAR)-PSA promoter (PCPSA-P) sequence resulted in high transgene expression in LNCaP cell lines, the combined regulatory element-promoter sequence resulted in minimal expression in the non-PSA-producing prostate cell line PC-3, renal tumor cell line R11, and cervical adenocarcinoma cell line HeLa. The newly identified 822 bp alone could also function as a promoter. Compared with the combined promoter, however, the 822-bp fragment alone demonstrated lower activity and lower responsiveness to androgen stimulation. Our results suggest that coupling the PSA promoter with an upstream regulatory element results in a marked increase in PSA expression, suggesting that the complete PSA promoter contains two functional domains: a proximal promoter and a distal promoter, which can also function as an enhancer. The enhanced gene expression of the new construct, combined with its tissue specificity and androgen responsiveness, in turn provides a foundation for the development of tissue-specific vectors for prostate cancer gene therapy.

INTRODUCTION

PSA is a well-characterized prostate-specific protein (1-4). The 5'-640-bp flanking sequence of the PSA gene has been studied and identified as a promoter sequence for the PSA gene (5). Previously, we cloned a PSA promoter sequence (PCPSA) from a patient with advanced prostate cancer and determined that the PSA promoter (PCPSA) was both tissue specific and androgen responsive (6). Its tissue specificity renders this PSA promoter an ideal regulatory element for conferring prostate-specific transgene expression. However, compared to genomic PSA expression, the expression of transgenes linked to this PCPSA promoter sequence is significantly lower. This decreased expression may point to the presence of genomic positive co-regulatory elements that may be essential for enhanced PSA expression (7).

In this study, we attempted to determine whether such additional positive regulatory elements exist. PCR was used to clone the upstream sequence of the PSA promoter. DNA isolated from a patient with an extremely high serum PSA level (approximately 6000 ng/ml) served as the PCR template. A 2.4-kb DNA fragment was obtained and cloned into a plasmid containing our tissue-specific PCPSA promoter and luciferase reporter gene (6). DNA transfections were performed to test the activity of the cloned fragment. Subsequently, deletions were made in the cloned sequence to identify the essential regions responsible for gene regulation. A sequence of 822 bp was identified and sequenced. Our results demonstrate that the 822-bp fragment can dramatically increase gene expression when combined with our previously identified PCPSA promoter while preserving tissue specificity and androgen responsiveness.

MATERIALS AND METHODS

PCR Cloning and Construction of Plasmids. Our PSA promoter sequence was used as the probe. We searched GenBank and found a 5.8-kb DNA sequence of locus HSU376721 upstream of the PSA gene (8). Because most sequences in the 3'-half region containing that PSA promoter have already been characterized (5), we focused on the 5'-region. PCR primers were designed to amplify sequences from nucleotides 503 to 2950 of the GenBank sequence. DNA isolated from a patient with advanced prostate cancer was used as the template (6). The amplified PCR fragment, approximately 2.4 kb in length, was inserted into the pUCBM20-PCPSA-Luc plasmid upstream of the PCPSA promoter (Fig. 1).

The plasmid containing the 2.4-kb DNA fragment was further digested using restriction endonucleases to remove an approximately 1-kb DNA fragment from the 3' end to obtain a 1.4-kb DNA fragment. The 1.4-kb fragment was further digested by restriction enzymes to remove the 5' 570-bp DNA sequence to generate a fragment 820 bp. Both 1.4-kb and 820-bp DNA fragments were also cloned into the pUCBM20-PCPSA-Luc plasmid (Fig. 1). The positive control plasmid contains the immediate early gene promoter of CMV (the CMV promoter), whereas the negative control plasmid contains no promoter.

Tumor Cell Line Culture and Maintenance. The culture of cell lines LNCaP, PC-3, and R11 has been described previously (6). HeLa cells were cultured according to the conditions suggested by American Type Culture Collection. Cells subjected to androgen stimulation tests were maintained in media with 10% FBS 2 days before DNA transfection. The procedure to prepare CBS was as follows. Charcoal (0.625 g; Mallinckrodt) and 12.5 mg of dextran sulfate were washed with 500 ml of PBS once before being mixed (by shaking or vortex for 30 min) with 500 ml of PBS. The charcoal was removed from the serum by centrifugation (1500 × g), followed by 0.2-μm filtration.

DNA Transfections and Luciferase Assay. Electroporation was used for DNA transfection. Cells were transplanted into 175 mm flasks and washed twice using electroporation medium (1.2× RPMI with 10% fetal bovine serum). The cells were resuspended in electroporation medium at 2 × 10^6 cells/ml. Cell suspensions of 0.25 ml were mixed with 10 μg of DNA on ice for 10 min before electroporation. The cells were pulsed at 230 V with 960 μF using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA). The treated cells were kept on ice for another 10 min before replating. At 16 h, the plates were washed with serum-free medium once before adding the new culture medium. Cells were collected approximately 48 h after transfection with 1× tissue lysis buffer (luciferase kit; Promega Corp., Chicago, IL). Firefly luciferase activity of the cell lysates was measured by luminescence. For each assay, 10 μl of cell lysate was used. The protein amount of 10 μl of cell lysate was measured using a...
**RESULTS**

**Tissue Specificity and Androgen Response of the Cloned PSA Regulatory Sequences.** PSA gene expression was characterized by its tissue specificity and androgen responsiveness (1–5, 9, 10). To test the tissue specificity and androgen responsiveness, the electroporation method was used. The PSA-producing prostate cancer cell line LNCaP, the non-PSA-producing prostate cell line PC-3, and the renal tumor cell line R11 were transfected with the newly constructed plasmids. After transfection, varying concentrations of DHT were added to the media. Two days after transfection, cells were collected for luciferase assays to characterize the function of the cloned PSA regulatory fragments.

The negative control plasmid showed extremely low activity and did not respond to DHT stimulation in any of the tested cell lines. The CMV promoter showed high activity (Fig. 2) in all tested cell lines. In LNCaP cells, the CMV promoter activity increased by 3–4-fold with the addition of DHT (Fig. 2a). In cell lines PC-3 and R11, the CMV promoter did not show any increase with the addition of androgen (Fig. 2, b and c). In the same transfection conditions, plasmids containing 2.4-kb, 1.4-kb, and 822-bp DNA fragments showed the following expression profile in LNCaP cells. The promoter activity was extremely low when no androgen was added. With the addition of DHT, the expression activity rapidly increased more than 1000-fold. In other cell lines, the activity of PSAR 2.4-kb, 1.4-kb, and 822-bp DNA fragments did not show any difference compared with the negative control, and the addition of androgen showed no effect.

This lack of response to added DHT may have been due to the lack of androgen receptors in the PC-3 and R11 cells. To determine whether the increase in transgene expression in the LNCaP cells and the lack of expression in the PC-3 and R11 cells was due primarily to the presence of functional androgen receptors, we also transfected HeLa cells, which in our previous experiments were determined to possess functional androgen receptors (data not shown) with the 2.4-kb, 1.4-kb, and 822-bp DNA fragments. DHT was similarly added to the transfected HeLa cells. With the addition of DHT, the CMV promoter showed a 3–4-fold increased activity, similar to the LNCaP cell line. However, the 2.4-kb, 1.4-kb, and 822-bp regulatory sequences did not show any function in HeLa cells, suggesting that tissue-specific factors other than androgen receptors are required for activating the regulatory sequences (Fig. 2d).

In non-PSA-producing cell lines R11 and PC-3, the CMV promoter did not show any response to androgen stimulation (Fig. 2). This suggests that the 3–4-fold stimulation by androgen may result from the presence of androgen receptors in both LNCaP and HeLa cell lines. In cell lines R11 and PC-3, the luciferase gene driven by our PSA regulatory fragments and the PSA promoter does not exhibit significant reporter-gene expression compared with the negative control plasmid. The PSAR2.4-kb fragment showed a slight androgen response in PC-3 cells; however, this may be due to limitations of the luciferase assay. The sensitivity of luciferase assays is around 300 light units. Any results around 300 light units or lower are not accurate. Because the PSAR1.4-kb and the PSAR822-bp fragments did not show any response to androgen stimulation, it is more likely that the expression increase of the PSAR2.4-kb fragment stimulated with androgen was because of the limited sensitivity of luciferase assays.

**Localization of the Essential Sequence.** The 2.4-kb DNA fragment was digested using restriction endonucleases. The 3′-1-kb fragment was cleaved, resulting in a 1.4-kb DNA fragment. The 1.4-kb fragment was further digested. Its 5′-570 bp were cleaved, resulting in an 822-bp fragment. Comparison of the 2.4-, 1.4-, and 822-bp fragments reveals that the 822-bp fragment confers a similar or higher activity of gene expression as compared to the 2.4- and

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**Diagram:**

- **CMV-Lux**
- **PSAR2.4k-PCPSA-P-Lux**
- **PSAR1.4k-PCPSA-P-Lux**
- **PSAR822**
- **PSAR**
- **PCPSA-P-Lux**
- **Lux (negative)**
Fig. 2. Tissue specificity and androgen responsiveness of PSA gene positive regulatory sequences. Cells were transfected with plasmids containing different lengths of the PSA regulatory sequence and grown in varying concentrations of DHT. Cells were transferred from regular medium to medium containing 10% FBS for 2 days prior to electroporation. The cells were trypsinized and resuspended in electroporation medium (6) at 2 × 10^6 cells/ml. Cell suspensions of 0.25 ml were mixed with 10 μg of DNA on ice for 10 min before electroporation. The cells were pulsed at 230 V with 960 μF using a Bio-Rad Gene Pulser (Bio-Rad). The treated cells were kept on ice for another 10 min before replating into six-well plates. RPMI 1640 containing 10% FBS and varying concentrations of DHT (0—100 nM) was added to each well. At 48 h, cells were lysed and assayed for luciferase activity. a, LNCaP; b, PC-3; c, R11; d, HeLa. Luciferase activity was measured as light units (LU) per microgram of cellular protein isolated from cells transfected by plasmids containing the CMV promoter ( ), plasmids containing no promoter ( ), plasmids containing the PSAR2.4-kb-PCPSA-P sequence ( ), plasmids containing the PSAR1.4-kb-PCPSA-P sequence ( ), and plasmid containing the 822-bp PSAR-PCPSA-P sequence ( ). For each assay, 10 μl of cell lysate was used. The protein amount of 10 μl of cell lysate was measured using the Bio-Rad kit. The luciferase activity was finally calculated by the concentration of protein in cell lysates.

1.4-kb fragments. These findings suggest that the 822-bp fragment possesses the essential co-regulatory sequence.

Promoter Function of the Cloned PSAR 822-bp Fragment. To assess whether this cloned sequence is an enhancer or potentially a distal promoter as well, we reversed the orientation of our 1.4-kb fragment and noted that the transcriptional activity of the reversed orientation was almost identical to the activity of the plasmids containing the 1.4-kb sequence in the original orientation (data not shown). The orientation-independent property of the 1.4-kb fragment suggested that the 5' upstream sequence is an enhancer.

Sequence analysis revealed that there is a potential TATA box in our 822-bp fragment (Fig. 4). This finding suggests that the 822-bp fragment may also serve as a distal promoter. To detect promoter activity of our 822-bp fragment, we deleted the PCPSA promoter from our plasmid PSAR 822-PCPSA-P-Luc, as shown in Fig. 1. The PSA promoter-deleted plasmid, the parental plasmid PSAR822-PCPSA-P-Luc, and the negative control plasmid were transfected into the LNCaP cell line and control HeLa cell line. Our results demonstrate that the sequence alone can significantly activate the expression of the transgene. Compared with the negative control, the expression level in is approximately 100-fold higher in the LNCaP cell line (Fig. 3). In the HeLa control cell line, the expression of the luciferase gene driven by the 822-bp fragment did not show a significant difference compared with the negative control plasmid. Although the androgen receptor is present in HeLa cells, the pro-

Fig. 3. Promoter activity and androgen responsiveness of the PSAR 822-bp fragment. LNCaP cells and HeLa cells were prepared and transfected using the same method as described in Fig. 2. Compared with the negative control, the expression of the luciferase gene transfected by the plasmid containing the PSAR sequence alone was 100-fold higher in LNCaP cells. In HeLa cells, the 822-bp PSAR fragment showed neither promoter activity nor androgen responsiveness. However, compared with the parental PSAR822-PCPSA-P-Lux plasmids, both promoter activity and androgen responsiveness were decreased in LNCaP prostate cells (a).
TATA box for RNA transcription starts at position 672 and the putative androgen-responsive element sequence at position 611 to 625 are underlined. The sequence at the top lines TACT, position 440—454; Ref. 6). In the GenBank sequence, the PSA promoter may be important to maintain both high expressional activity and high androgen responsiveness for the expression of the PSA gene. Our clone derived from a prostate cancer patient. and the sequence at the bottom line is as published by Shuur et al. (8, 16). Nine nucleotide differences were detected compared with the sequence present in GenBank. These nucleotides are underlined and in italic typeface. The sequence that may serve as a putative androgen-responsive element found in the PCPSA promoter (AGAACAgcaAGTACT, position 611 to 625 of HSU37672, nine nucleotide changes were noted (Fig. 4). A sequence GGAACAtatTGTATF was found at position 611 to 625 of the sequence PSAR (PSA gene positive regulatory sequence). In our study, we found that this DNA fragment performs not only enhancer stimulation; however, compared with the PSAR-PCPSA-P combined promoter, both the promoter activity and the androgen responsiveness of PSAR are much lower.

Sequencing the 822-bp PSAR Regulatory Element. The PSAR 822 bp was sequenced using the USB sequencing kit (purchased from United States Biochemical Corp.). Compared with the GenBank sequence HSU37672, nine nucleotide changes were noted (Fig. 4). A sequence GGACAtataTGATTT was found at position 611 to 625 of the 822-bp fragment. This sequence is very similar to the androgen-responsive element found in the PCPSA promoter (AGAACAgcaAGTACT, position 440—454; Ref. 6). In the GenBank sequence, the element is GGACAtataTGATTC.

DISCUSSION

The use of a tissue-specific promoter to drive therapeutic gene expression in target cells is a novel approach for target-specific gene therapy (11—15). Our newly identified PSA regulatory DNA fragment possesses tissue specificity and androgen sensitivity, thus rendering this particular regulatory fragment promising for use in gene therapy of prostate cancer.

The DNA sequence upstream of the PSA gene promoter was recently characterized as an enhancer by Schuur et al. (16). In our study, we found that this DNA fragment performs not only enhancer functions but also promoter functions. We, therefore, named our sequence PSAR (PSA gene positive regulatory sequence). In our study, the PSAR alone demonstrated a 100-fold increase in transgenic expression. The PSAR sequence alone also responded to androgen stimulation; however, compared with the PSAR-PCPSA-P combined promoter, both the promoter activity and the androgen responsiveness of PSAR are much lower.

Fundamental differences were observed between our results and those published recently by Schuur et al. (16). Their results documented that the 5' region from XbaI to Psrl sites is essential to maintain the positive regulatory activity. In contrast, our data demonstrate that the deletion of this region does not decrease the expres-
sion of the transgene (Fig. 2). We also noticed a different responsiveness to androgen stimulation in LNCaP cells. With the addition of androgen, an activity increase of more than 1000-fold was observed. This increase in activity is significantly higher than that observed by Schuur's group. These differences in results may be attributed to several points: (a) our promoter sequence was derived from fresh prostatic tumor of a patient with advanced prostate cancer that expressed high PSA levels; Schuur et al. (16), on the other hand, derived positive regulatory activity, and the 5' 570 bp is no longer needed. A normally required to maintain the positive regulatory function. In our higher. The roles of other sequence changes are under investigation.

We hypothesize that the 5' 570-bp sequence that is essential in Schuur's clone contains a functional domain. This functional domain is normally required to maintain the positive regulatory function. In our isolated fragment, the mutations in the 822-bp region greatly increase the positive regulatory activity, and the 5' 570 bp is no longer needed. A similar finding was also demonstrated for the PSA promoter. Schuur's clone contains a functional domain. This functional domain is higher binding affinity for the androgen receptor because the similarity to the identified androgen-responsive element of our element is higher. The roles of other sequence changes are under investigation.

Our combined PSAR-PCPSA-P promoter resulted in a significantly higher expression. The high potency of the PSAR-PCPSA-P promoter-enhancer sequence suggests that generating an androgen-responsive, tissue-specific vector system with high efficacy is feasible. Furthermore, the combined promoter with a ~1.4-kb size can be easily cloned into adenoviral, retroviral, or adeno-associated viral vectors without associated packaging difficulties. Further studies are needed to explore its potential use for prostate cancer specific gene delivery.

REFERENCES

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