Detection of Prostate-Specific Antigen mRNA by Reverse Transcription Polymerase Chain Reaction and Time-Resolved Fluorometry

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We have developed a time-resolved fluorometric hybridization assay for detecting prostate-specific antigen (PSA) mRNA amplified by reverse transcription polymerase chain reaction. During PCR, digoxigenin-11-dUTP is incorporated into the amplified product. An oligonucleotide internal to the primers is used as a specific probe, being biotinylated and captured on streptavidin-coated microtiter wells. Denatured PCR product hybridizes with the probe, and the hybrids are detected with an alkaline phosphatase-labeled anti-digoxigenin antibody. We used the phosphate ester of fluorosalicylic acid as the substrate. The fluorosalicylate produced forms a highly fluorescent ternary complex with Tb^{3+}-EDTA, which we can measure by time-resolved fluorometry. A signal-to-background ratio of 10 was obtained when 160 PSA cDNA molecules were present in the preamplification sample. Also, mRNA corresponding to one LNCaP cell in the presence of 10^6 PSA-negative cells can be detected (signal-to-background ratio of 3.1). Samples containing 100, 1000, and 50 000 LNCaP cells gave CVs of 12.4%, 4.9%, and 6.8%, respectively (n = 10).

Indexing Terms: *mRNA hybridization/biotin–streptavidin interaction/immunoenzymometric assay/prostate cancer/digoxigenin label*

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths (behind lung cancer) in the US male population (*1*). Prostate-specific antigen (PSA) is a glycoprotein with serine protease activity produced by the prostate epithelial cells, the cells involved in prostatic oncogenesis.[4] Serum PSA has been a valuable marker for diagnosis, screening, and clinical management of patients with prostate cancer (*2*, *3*).

The therapeutic approaches for prostate cancer depend on the stage of the malignancy. Patients with stage A or B cancer are treated with radiation or radical prostatectomy. Stage C disease is usually treated with radiation. In metastatic (stage D) disease, surgery is not suggested and the patients are offered systemic treatment aimed at slowing down the progression of the malignancy. After the initial diagnosis, therefore, it is important to assess whether the cancer is organ-confined or has already spread beyond the prostate. However, the sensitivity of current staging techniques is inadequate, and as many as 40% of patients who undergo prostatectomy are found at the time of surgery to have metastasis (*4*, *5*).

Polymerase chain reaction (PCR) (*6*, *7*) is a powerful technique with the ability to detect a few tumor cells in the presence of a vast excess of normal cells. This is accomplished by amplifying nucleic acid sequences that are specific and characteristic of tumor cells and subsequently analyzing the amplified fragments. During the last 2–3 years, reverse transcription polymerase chain reaction (RT-PCR) assays for PSA mRNA have been developed and applied to the detection of metastatic prostate cancer cells in lymph nodes and in peripheral blood (*8–12*), offering greater sensitivity than standard histological and immunohistochemical methods for detecting lymph node metastasis. Furthermore, Katz et al. (*11*), using RT-PCR in whole blood, found that 78% of metastatic prostate cancer patients had circulating cancer cells and that 38% of patients with clinically localized disease were positive by RT-PCR performed on peripheral blood specimens before surgery. A significant observation in all these studies is that circulating PSA-expressing cells were not detected in any of the patients who did not have prostate cancer. Thus, the detection of PSA mRNA is emerging as a new test that may be useful for the correct staging and the clinical management of patients diagnosed with prostate cancer. These issues have recently been reviewed (*13*).

Up to now all the assays for PSA mRNA have been based on the analysis of PCR products by agarose gel electrophoresis and ethidium bromide staining or by Southern transfer. Here, we report the first microtiter-well-based hybridization assay for detecting PSA mRNA amplified by RT-PCR. The probe is immobilized in microtiter wells and the amplified DNA (target) is labeled during PCR with the hapten digoxigenin. The hybrids are detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase. The enzymatic activity is monitored by using fluorosalicyl phosphate as substrate. The fluorosalicylate produced forms highly fluorescent complexes with Tb^{3+}-EDTA, which are measured by time-resolved fluorometry (*14*, *15*).

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[4] Nonstandard abbreviations: PSA, prostate-specific antigen; RT-PCR, reverse transcription polymerase chain reaction; MMLV-RT, Moloney murine leukemia virus reverse transcriptase; dig-dUTP, digoxigenin-11-dUTP; FSAP, phosphate ester of 5′-fluorosalicylate; and PBS, phosphate-buffered saline.
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Materials and Methods

Materials

The human prostate adenocarcinoma cell line, LNCaP, which expresses PSA mRNA, was obtained from the ATCC (Rockville, MD; CRL 1740) and cultured in 25-cm^2 polystyrene culture flasks (Corning, Oneonta, NY), in media consisting of 900 mL of RPMI 1640, 100 mL of fetal bovine serum, 100 kU of penicillin, 100 mg of streptomycin, and 0.25 mg of fungizone per liter. The human promyelocytic leukemia cell line, HL-60, which does not express PSA mRNA, was also obtained from ATCC (CCL 240) and cultured as above. All cell-culture reagents, Trizol® reagent, Moloney murine leukemia virus reverse transcriptase (MMLV-RT), oligo $(dT)_{12-18}$ primer, and biotin-14-dATP were purchased from Life Technologies (Gaithersburg, MD). dNTPs and Sephadex G-25 columns (Nap-5) were purchased from Pharmacia (Montreal, PQ, Canada). Digoxigenin-11-dUTP (dig-dUTP), terminal deoxynucleotidyl transferase, and the alkaline phosphatase-labeled polyclonal anti-digoxigenin antibody from sheep (Fab fragments) were obtained from Boehringer Mannheim Biochemica (Laval, PQ, Canada). Streptavidin, diethylpyrocarbonate, mineral oil, and Tween 20 were from Sigma (St. Louis, MO). The Bio-ladder DNA markers, used in agarose gel electrophoresis of PCR products, were from Biosynthesis (Lewisville, TX). Opaque, polystyrene Microlite 2 microtiter wells were purchased from Dynatech Labs. (Chantilly, VA). The phosphate ester of 5′-fluorosalicylic acid (FSAP) was from CyberFluor Div., Nordion International (Toronto, ON, Canada); a 0.01 mol/L stock solution of FSAP was prepared by dissolving 11.8 mg of the compound in 5 mL of 0.1 mol/L NaOH and kept at 4 °C. Terbium chloride hexahydrate was obtained from Aldrich Chemical Co. (Milwaukee, WI). The recombinant plasmid pA75 was a gift from J. Trapman, M.D. Anderson Cancer Center, Houston, TX. This plasmid contains a 1.4-kb PSA cDNA insert (*16*). Tris, NaCl, and all other general laboratory chemicals used in preparation of buffers were from BDH (Toronto, ON, Canada).

Three oligonucleotides (shown below) were designed for this work. Two of them, each 20 bases long, were used as PCR primers. The upstream (U) primer and the downstream (D) primer are complementary to sequences in exons 2 and 3, respectively, of the PSA gene. PCR amplification of PSA cDNA with these primers yields a single 289-bp fragment. The third oligonucleotide (a 24-mer) was used as a probe and binds to a specific sequence in exon 3 of the PSA gene, a sequence within the PCR product. All oligonucleotides were synthesized by DNAgency (Aston, PA).

PSA U-primer: 5′-ATT GTG GGA GGC TGG GAG TG-3′
PSA D-primer: 5′-GGT CGT GGC TGG AGT CAT CA-3′
PSA probe: 5′-TCA GGA TGA AAC AGG CTG TGC CGA-3′

All solutions used in the isolation of total RNA and reverse transcription were prepared with diethylpyrocarbonate-treated water. Pipettes, pipette tips, solutions, and the mineral oil used for RNA isolation, reverse transcription, and PCR were irradiated with ultraviolet light for 2 h to avoid contamination (*17*).

RNA Isolation

Cells were washed three times with phosphate-buffered saline (PBS; 0.14 mol/L NaCl, 10 mmol/L sodium phosphate, and 1.7 mmol/L potassium phosphate, pH 7.4) and pelleted by centrifugation at 12 000*g* for 1 min. After removing the supernate, we isolated the total RNA by the guanidinium thiocyanate/phenol/chloroform extraction method described by Chomczynski (*18*), using the Trizol reagent. The procedure for total RNA isolation was carried out according to the manufacturer's instructions. The RNA pellet obtained after precipitation with isopropanol and washing with 750 mL/L ethanol was allowed to air-dry and then was redissolved in 20 μL of water. The RNA concentration was determined spectrophotometrically at 260 nm.

Reverse Transcription

An aliquot containing 5 μg of total RNA was added to 0.5 μg of oligo$(dT)_{12-18}$ primer and brought to a final volume of 12 μL with water. The solutions were then heated to 70 °C for 10 min and quickly chilled on ice. Then, 8 μL of reverse transcription reaction buffer (125 mmol/L Tris-HCl, pH 8.3, 188 mmol/L KCl, 7.5 mmol/L $MgCl_2$, 25 mmol/L dithiothreitol, 2.5 mmol/L of each dNTP, and 200 U of MMLV reverse transcriptase) was added. After incubation for 1 h at 37 °C, the reverse transcriptase was inactivated by heating the solutions to 95 °C for 4 min. The synthesized cDNA was stored at −20 °C.

PCR

PCR was carried out in a total reaction volume of 100 μL containing 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 9.0), 1 mL/L Triton X-100, 2.5 mmol/L $MgCl_2$, 0.1 mmol/L of each dNTP, 5 μmol/L of dig-dUTP, 2.5 U of Taq DNA polymerase, and 10 μL of the sample cDNA. The PCR mixture was layered with 80 μL of mineral oil to prevent evaporation. The "hot-start" protocol was applied (*19*). The mixture was first heated to 95 °C for 5 min, during which time 50 pmol of each of the PSA-U and PSA-D primers was added to each tube. Thirty cycles of PCR were carried out; each cycle consisting of a denaturation step at 95 °C for 30 s, primer annealing at 60 °C for 30 s, and primer extension at 72 °C for 1 min. The Perkin-Elmer Cetus (Norwalk, CT) DNA Thermal Cycler was used. At the end of the cycles the reaction mixture was kept at 72 °C for 10 min and then cooled to 4 °C. A PCR-negative sample, which contained all the reagents but no template, was included in each PCR series to check for contamination.

Tailing the Probe with Biotin-14-dATP

The oligonucleotide that was used as a PSA-specific probe was enzymatically tailed with biotin-14-dATP by using terminal deoxynucleotidyl transferase. The tailing reaction was carried out in a final volume of 20 μL containing 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl (pH 6.6), 0.25 g/L bovine serum albumin, 5 mmol/L $CoCl_2$, 125 μmol/L of each dNTP, 20 μmol/L biotin-14-dATP, 50 U of terminal transferase, and 5 μmol/L of the probe. The reaction mixture was incubated for 30 min at 37 °C, after which the tailed probe was purified from the excess of biotin-14-dATP by gel filtration with the Nap-5 columns equilibrated with 10 mmol/L sodium phosphate buffer, pH 6.8.

Microtiter Well Hybridization Assay

Opaque, polystyrene microtiter wells were coated overnight (at room temperature) with 100 μL of 1.4 mg/L solution of streptavidin in PBS. Before use, the wells were washed three times with wash solution (50 mmol/L Tris, 0.15 mol/L NaCl, and 1 mL/L Tween 20, pH 7.5). Then 100 μL of 33 nmol/L biotin-tailed PSA probe diluted in the wash solution was pipetted into each well. After a 45-min incubation (with shaking) at room temperature, the wells were washed three times and 80 μL of PBS containing 1 mL/L Tween 20 (preheated to 42 °C) was pipetted into each well. PCR products were denatured by heating at 95 °C for 10 min, and immediately cooled on ice. A 20-μL aliquot of the PCR product was then added into each well, which already contained the 80 μL of the PBS Tween solution. All assays were carried out in duplicate. Hybridization was carried out for 45 min at 42 °C with shaking. The wells were then washed three times, and 100 μL of a 750 U/L alkaline phosphatase-labeled anti-digoxigenin antibody (diluted in wash solution) was added. After a 30-min incubation at room temperature, the wells were washed three times and 100 μL of the substrate (1 mmol/L FSAP, 0.1 mol/L Tris, pH 9.1, 0.1 mol/L NaCl, and 1 mmol/L $MgCl_2$) was added. The enzymatic reaction was allowed to proceed for 30 min at room temperature; then 100 μL of developing solution (0.4 mol/L NaOH, 2 mmol/L Tb^{3+}, 3 mmol/L EDTA, and 1 mol/L Tris) was pipetted into each well and mixed for 1 min. The fluorescence produced was measured with the CyberFluor 615 Immunoanalyzer (CyberFluor, Toronto, Canada), a time-resolved fluorometer in which the excitation and emission wavelengths were set at 337 nm and 615 nm, respectively.

Results and Discussion

The effect of the biotin-tailed probe concentration was studied by preparing various dilutions of the probe (concentration range, 1.5–50 nmol/L) and using them to measure amplified DNA from 10 000 PSA cDNA molecules. The fluorescence increases steadily with the probe concentration up to 25 nmol/L and then reaches a plateau, as the streptavidin binding sites become saturated. Thus, the maximum amount of probe that can be immobilized is 2.5 pmol/well.

The effect of the volume of PCR mixture used in the hybridization assay was also studied with amplified DNA corresponding to 10 000 and 1000 LNCaP cells. The fluorescence increases as the volume of product applied per well increases. We chose to use a 20-μL sample volume because this provides adequate sensitivity (see below) and allows for analysis of replicates.

The time required for completion of the hybridization reaction was studied in the range of 15 to 90 min; the results are presented in Fig. 1. As the incubation time is extended, a continuous increase of the fluorescence is observed. However, the signal-to-background ratio reaches a plateau after 45 min of incubation. Longer incubations have a concomitant increase in background, because of nonspecific binding of the excess (unincorporated) dig-dUTP present in the PCR mixture. The short incubation period required for hybridization in microtiter wells is a substantial advantage over the Southern transfer technique, in which hybridization requires several hours and is followed by long, tedious washing steps.

To assess the sensitivity of the proposed assay, we prepared serial dilutions of a plasmid containing PSA cDNA. We then subjected to PCR aliquots that contained 0, 16, 160, 1600, 16 000, and 160 000 molecules and assayed the products by hybridization. The fluorescence and the signal-to-background ratio (determined in duplicate) were plotted against the number of molecules (Fig. 2). A signal-to-background ratio of 10 was obtained with 160 cDNA molecules. We also directly compared the sensitivity of the proposed assay with that of agarose gel electrophoresis and ethidium

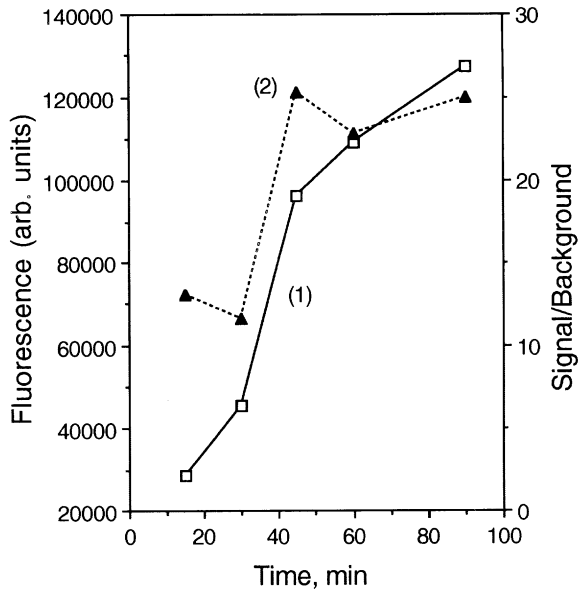

Fig. 1. Optimization of the time allowed for hybridization of the immobilized probe with amplified DNA produced by PCR of mRNA corresponding to 1000 LNCaP cells.

The fluorescence (*1*) and the signal-to-background ratio (*2*) are plotted against the incubation periods. The background is the fluorescence obtained when no PSA cDNA is present in the PCR mixture.

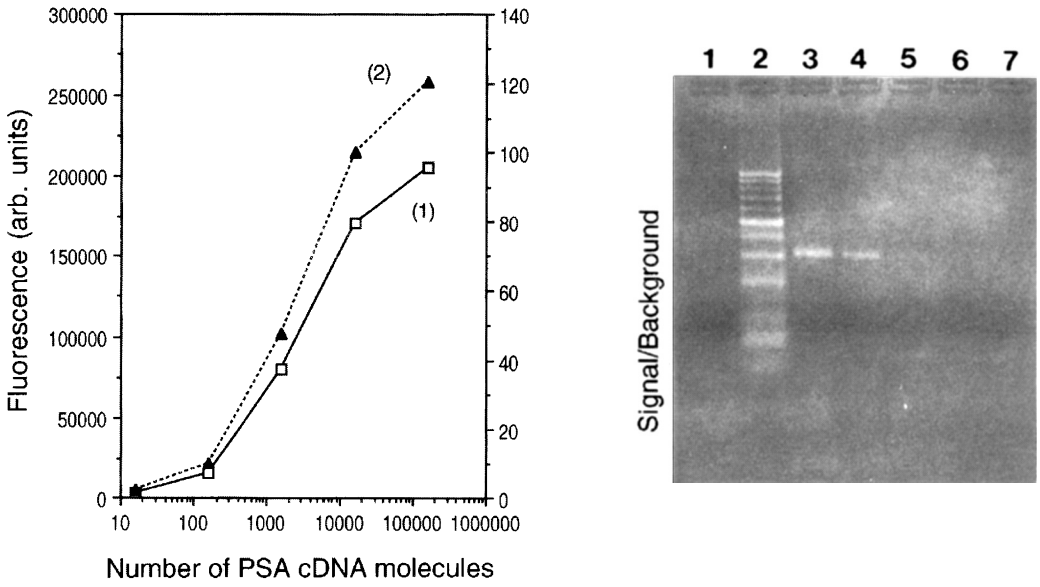

Fig. 2. (*Left*) Sensitivity of the assay studied by preparing serial dilutions of a plasmid containing the PSA cDNA sequence; (*right*) agarose gel electrophoresis and ethidium bromide staining of amplified PSA cDNA.

(*Left*) Plots of (*1*) the fluorescence and (*2*) the signal-to-background ratio vs the number of PSA cDNA molecules originally present in the PCR mixture. The assay detects 160 PSA cDNA copies with a signal-to-background ratio of 10. (*Right*) A 2% agarose gel was run with 20-μL aliquots of PCR products. *Lane 1*: PCR negative (all reagents present except for the template); *lane 2*: molecular mass markers (the first band represents 1000 bp and each band that follows decreases by 100 bp); *lanes 3–7* contain PCR product representative of mRNA corresponding to 160 000, 16 000, 1600, 160, and 16 PSA cDNA copies, respectively.

bromide staining: 20-μL aliquots from the same PCR mixtures were loaded onto a 2% agarose gel, electrophoresed, and stained with ethidium bromide. A photograph of this gel (Fig. 2) shows that only the samples corresponding to 16 000 and 160 000 PSA cDNA molecules appear as visible bands. Thus, the detection limit for electrophoresis is >100 times that for the time-resolved fluorometric hybridization assay.

The ability of the proposed system to detect a few PSA-expressing cells in the presence of a large excess of cells that do not express PSA was tested by preparing and analyzing samples containing PSA-mRNA corresponding to 0, 1, 10, 100, 1000, and 10 000 LNCaP cells in the presence of 10^6 HL-60 cells. Amplification always produced a 289-bp fragment. If genomic DNA had been coamplified, it would have given a 1918-bp fragment (based on the positions of the primers). However, no such fragment was detected, either by electrophoresis or by time-resolved fluorometry. In Fig. 3, the fluorescence and the signal-to-background ratio are plotted vs the number of LNCaP cells. The assay can detect amplified mRNA from a single LNCaP cell with a signal-to-background ratio of 3.

Within-run precision studies for the hybridization assay were performed by using amplified DNA generated from mRNA representative of 100, 1000, and 50 000 LNCaP cells in the presence of 300 000 HL-60 cells. The reproducibility of the fluorescence was 12.4%, 4.9%, and 6.8% for 100, 1000, and 50 000 LNCaP cells, respectively. To assess the overall reproducibility, including the PCR step, we amplified in seven side-by-side reactions a sample containing mRNA from 1000 LNCaP cells in the presence of 200 000 HL-60 cells and determined the amplified products by the proposed hybridization assay. The CV obtained for the signal was 8.8%.

Deguchi et al. (*9*) proposed a PCR-based assay for PSA mRNA involving agarose gel electrophoresis, Southern transfer, membrane hybridization, and immunodetection of the hybrids. The assay was able to detect a single LNCaP cell in the presence of 10^6 PSA-negative cells. Katz et al. (*11*) also applied a single

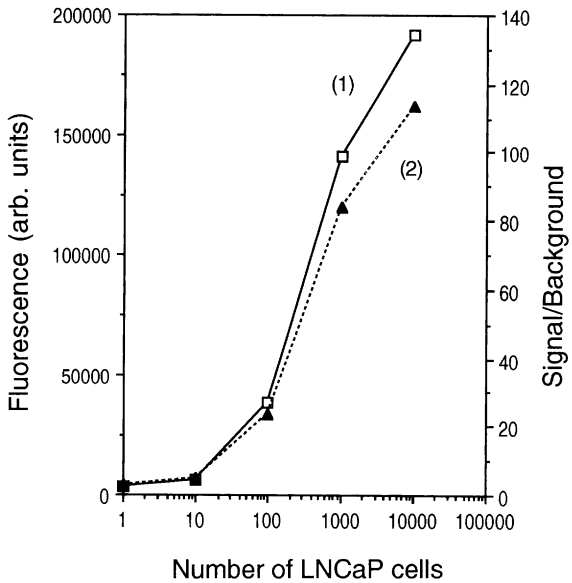

Fig. 3. Study of the variation of (*1*) the fluorescence and (*2*) the signal-to-background ratio with the number of LNCaP cells.

Mixtures containing mRNA from 1 to 100 000 LNCaP cells each in the presence of 10^6 HL-60 cells were subjected to RT-PCR, and the amplified fragments were analyzed by hybridization. The background is defined as the fluorescence obtained in the presence of 10^6 HL-60 cells with no LNCaP cells.

PCR, followed by electrophoresis and ethidium bromide staining of the amplified products. The reported sensitivity (detection limit) was 10 LNCaP/10^6 PSA-negative cells. The sensitivity was further enhanced by Southern transfer and immunodetection of PCR products. Israeli et al. (*10*) devised a nested PCR (two rounds, 25 cycles each) and analyzed the products by electrophoresis and ethidium bromide staining to achieve a sensitivity of 1 LNCaP/10^6 negative cells. They also performed Southern transfer and hybridization with ^{32}P-labeled probes but found no improvement in sensitivity. Jaakkola et al. (*12*), performing nested PCR (two rounds, 30 cycles each) with electrophoretic analysis of the amplified DNA, achieved a sensitivity of ~2 LNCaP/10^6 negative cells. Compared with the above approaches, the proposed assay achieves equivalent sensitivity and specificity (1 LNCaP/10^6 PSA-negative cells) without the need for nested PCR. This obviates the possibility of contamination associated with a second round of PCR. Furthermore, hybridization in microtiter wells allows for confirmation of amplified product identity without Southern transfer and membrane hybridization. Thus, the proposed assay is automatable and adaptable to the routine clinical laboratory and may be useful in the detection of metastatic cancer cells in lymph nodes and in peripheral blood of patients diagnosed with prostate cancer.

In new findings (*20–22*) recently reviewed (*23*), PSA is also present in 30–40% of female breast tumors as well as in other tumors. Because RT-PCR is capable of being more sensitive than immunoassay technology, we are currently investigating with the proposed RT-PCR procedure the role of PSA in these tumors and its clinical significance.

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