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Mouse PSP94 Expression Is Prostate Tissue-Specific as Demonstrated by a Comparison of Multiple Antibodies Against Recombinant Proteins

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Abstract Prostate tissue-specific gene expression is crucial for driving potentially therapeutic genes to target specifically to the prostate. Prostate secretory protein of 94 amino acids (PSP94), also known as β-MSP (microseminoprotein), is one of the three most abundant secretory proteins of the prostate gland, and is generally considered to be prostate tissue-specific. We have previously demonstrated that the expression of the rat PSP94 gene is strictly prostate tissue-specific by an antibody against a recombinant rat PSP94. In order to study prostate targeting utilizing the PSP94 gene in a mouse pre-clinical experimental model, we need to establish antibodies against mouse PSP94 to confirm if it is prostate tissue-specific as well. In this study, firstly we raised a polyclonal antibody against a recombinant glutathione-*S*-transferase- (GST-) mouse mature form of PSP94. However, it showed very poor immunoreactivity against prostate tissue PSP94 as tested in Western blotting experiments. Neither antibodies against rat PSP94 nor mouse PSP94 showed significant cross-reactivity. Thus a second antibody was established against a recombinant mouse mature PSP94 containing N-terminal polyhistidines, and stronger immunoreactivity against mouse prostate tissue PSP94 was identified in Western blotting experiments. Both of these antibodies showed immunohistochemical reactivity, while the latter showed stronger reactivity in IHC when tested with different fixatives. By studying tissue distribution, we demonstrated that, as with rat PSP94, mouse PSP94 is strictly prostate tissue-specific in experiments of both Western blotting and immunohistochemistry (IHC). This conclusion was also derived from a comparison among antibodies against human, rat, and mouse PSP94, showing very different immunoreactivities in Western blotting and IHC. Finally, a competitive assay between different species was performed. We demonstrated that antibodies against PSP94 from different species (human, primate, rodents) have poor cross-reactivities. These observations also indicate that the PSP94 gene is a rapidly evolving gene in all species. Results from this study have led to the possibility of utilizing PSP94 as a targeting agent specifically to the prostate in a mouse experimental model. J. Cell. Biochem. 88: 999–1011, 2003.

Key words: PSP94; Prostate tissue specific expression; mouse; antibodies; recombinant proteins

One of the biggest challenges facing current gene therapy is to identify a tissue-specific vector gene to deliver therapeutic agents specifically targeted to the disease site. Prostate cancer (CaP) is the most frequently diagnosed malignancy among adult males in

Abbreviations used: PSP94, prostate secretory protein of 94 amino acids; GST, glutathione *S*-transferase; DLP, dorsolateral prostate; VP, ventral prostate; CG, coagulation gland; SV, seminal vesicles.

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North America [Steiner et al., 2000, review]. Currently, treatment options are very limited once the cancer had progressed to the point of being hormone-resistant. Gene therapy has been touted as one of the salvage options in hormone resistant CaP.

Only a few prostate specific genes have been tested for targeting of heterologous genes in the prostate [Shirakawa et al., 2000; Steiner et al., 2000; Matusik et al., 2001, review]. These include the rat prostate steroid-binding protein (PSBP or C3(1)) [Shibata et al., 1999], the human prostatic specific antigen (PSA) [Rodriguez et al., 1997; Wei et al., 1997], the rat probasin gene [Greenberg et al., 1995; Gingrich et al., 1996, 1997], and an osteocalcin-based cotargeting vector [Matsubara et al., 2001]. Because of the limited prostate tissue-specific genes available for gene therapy studies, current clinical trials utilize a combination of promoter/enhancer regions of human PSA and rat probasin sequences [Rodriguez et al., 1997; Yu et al., 2001].

The prostatic secretory protein of 94 amino acids (PSP94), also known as β-microseminoprotein [Abrahamsson et al., 1988; Hara et al., 1989; Hyakutake et al., 1993], is one of the three most abundant secretory proteins from the prostate gland (the others being PAP, prostatic acid phosphatase and PSA, prostate specific antigen) [Dube et al., 1987; Abrahamsson et al., 1988] and is generally considered to be a prostate tissue-specific protein [Hara et al., 1989, review]. Although, PSP94 is so abundantly (g/L) secreted in the semen, its real biological function is still unknown, and a variety of functions have been hypothesized [Lazure et al., 2001, mini review]. Studies in human, primate, pig, and rodent have showed that PSP94, in contrast to rPB and PSA, is a conserved, but also a rapidly evolving protein [Fernlund et al., 1996; Xuan et al., 1999; Lazure et al., 2001, mini review]. PSP94 or PSP94-like molecules have been identified in birds (ratite), bovids, batracians, and fishes [Lazure et al., 2001]. However, the only structural connection among these species is with the conserved cysteine-rich sites, and no definitive biological functions have been identified.

Rat PSP94 has been reported to exist almost exclusively in the dorsolateral prostate [Fernlund et al., 1996; Xuan et al., 1999; Imasato et al., 2001]. By developing and characterizing a polyclonal antibody using the glutathione *S*-transferase (GST) gene fusion technique, we previously reported that rat PSP94 has strong differential expression in different lobes of the rat prostate [Kwong et al., 1999; Kwong et al., 2000a; Imasato et al., 2001]. However, since this antibody does not cross react with mouse PSP94, and since all animal models (transgenic, knockout), and pre-clinical studies of gene therapy currently utilize the mouse species, we intend to test if the mouse PSP94 gene is also prostate tissue-specific by raising and comparing immunoreactivities of two antibodies against mouse PSP94 established in this study.

MATERIALS AND METHODS

Animal Handling and Dissection, Tissue Lysate, and Fixative Samples Preparation

Male with 6–8-week old mice (strain CD1) were anesthetized, and the prostate gland complexes were dissected. The prostate along with the male accessory glands, i.e., the ventral and dorsolateral prostate lobes (VP, DLP) respectively, along with seminal vesicles (SV) and coagulation gland (CG), were dissected out separately as per the description and definition reported [Sugimura et al., 1986; Lee, 1987]. Freshly dissected or frozen samples collected after dissection were homogenized at a concentration of 0.2 ml/50 mg (wet weight) tissue in 1% of sodium dodecyl sulphate (SDS), 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.01 M phosphate buffered saline (PBS). The supernatants were removed and quantified by their optical density (OD) at 280 nm. This clear cell extract was designated as the "lysate." Human and rat prostate samples were collected as previously reported [Xuan et al., 1995; Imasato et al., 2001]. For histological studies, fresh prostatic tissues were fixed in the following fixative solutions: (1) 10% Formalin (Fisher); (2) Carnoy's fixative (60% methanol, 30% chloroform, 10% glacial acetic acid) [Kwong et al., 1999, 2000b]; (3) 4% paraformaldehyde in PBS, pH 7.0 [Imasato et al., 2001]; and (4) acetic alcohol (96% ethanol, 1% glacial acetic acid) [Mentor-Marcel et al., 2001]. After fixation, samples were washed with 70% ethanol and embedded in paraffin. Slides, 4-μm thick, were then cut, deparaffinized, and rehydrated as

we previously reported [Kwong et al., 1999, 2000a].

Construction of GST and Polyhistidine Containing-PSP94 Fusion Plasmids in *Escherichia coli* Expression Vectors pGEX2T and pTrcHis A Vectors

A mouse PSP94 cDNA clone was isolated and sequenced as reported previously [Xuan et al., 1999]. A cDNA fragment coding for the mature form of mouse PSP94 was amplified by PCR with a forward (N-terminal) primer (5′ GGG TTG GAT CCG TAT GTT CTA TTG AAA ATC GTG AG 3′) and a reverse (C-terminal) primer (5′ GGG AAG AAT TCT TAC ATA GTC CAG CTG TCA ACT GG 3′). The N-terminal primer was synthesized at the excision site of the PSP94 precursor protein and contained a cloning site of the restriction enzyme *Bam*HI (Fig. 1A) to maintain the same reading frame as the carrier protein GST. The stop codon and the cloning site of restriction enzyme *Eco*RI were also included in the reverse (3′-end, C-terminus) primer. PCR reaction was performed as previously reported [Xuan et al., 1995] using 10 ng of mouse PSP94 cDNA template [Xuan et al., 1999]. PCR product was purified by a spin cartridge for ultrafiltration (Millipore, Bedford, MA) to eliminate primers and to desalt, then digested with *Bam*HI and *Eco*RI enzymes. This fragment was cloned into the *E. coli* expression vectors pGEX-2T (Amersham-Pharmacia, Montreal, Que) and pTrcHis A (Invitrogen, Carlsbad, CA) at the same two restriction sites (Fig. 1A). The whole insert and the linker sequences with vector GST and polyhistidines containing carrier protein were confirmed by complete DNA sequencing.

Expression and Purification of Recombinant GST-Mouse PSP94

The recombinant GST-mouse PSP94 was expressed in the *E. coli* strain BL21 (Novagen, Madison, WI.), which lacks the *lon* protease and the *omp*T out-membrane protease. *E. coli* bacterial culture was induced for the recombinant protein by 0.5 mM IPTG (isopropylthio-μ-D-galactoside) and the recombinant GST-PSP94 fusion protein was purified by an affinity column (glutathione-Sepharose 4B, Amersham-Pharmacia) as reported previously [Kwong et al., 1999]. Both recombinant GST-mouse PSP94 cytosol and inclusion body fractions were tested and most of the GST-mPSP94 was found only in the inclusion body portion of the *E. coli* cells. A denaturing solution (8 M urea, 0.1 M glycine, pH 9.0) was employed to dissolve inclusion bodies. The dissolved solution was then immediately desalted by passing it through a Sephadex G50 column in 0.1 M glycine buffer (pH 9.0) and renaturing of the desalted protein fractions was performed in the same buffer at 4°C overnight. Affinity column (glutathione Sepharose 4B) purification of the dissolved *E. coli* proteins from inclusion bodies was conducted according to the manufacturer's instructions. Thrombin digestion (10 U/mg protein) was performed by overnight incubation at room temperature [Kwong et al., 1999]. Protein concentration was determined by measurements of absorbency at OD = 280 nm and checked by 15% sodium dodecyl sulphate–polyacrylamide gels electrophoresis (SDS–PAGE) followed by staining with Coomassie Blue.

Expression and Purification of Polyhistidine Containing Recombinant Mouse PSP94

The recombinant polyhistidine containing mouse PSP94 (TrcHis-mPSP94) was first tested for the best yield in different *E. coli* strains: Top10, Bl21, and DH5α. Top10 was used to purify large amount of recombinant proteins. The preparation of bacterial culture and the induction of recombinant protein by 100-mM IPTG were prepared accordingly [Kwong et al., 1999], when the cell culture reached an $OD_{600\,nm}$ of 0.6 and continued incubation for another 2 h at 37°C. Recombinant TrcHis-mPSP94 fusion protein was purified by a $NiSO_4$ charged-Sepharose based affinity column (His.Bind, Novagen) following manufacturer's recommendations. Because the yield is poor (<500 μg/L), a large-scale (2L NZCYM medium, Invitrogen) preparation of the fusion protein was conducted. The recombinant TrcHis-mouse PSP94 was purified by a denaturing method, i.e., a 6 M guanidine lysis buffer pH 7.8 was employed to dissolve both cytosol and the precipitated proteins inside *E. coli* cells. The total lysate proteins were loaded onto the Ni^{++}-affinity column, and washed sequentially with a denaturing binding buffer (5 mM imidazole, 50 mM NaCl, 20 mM Tris-HCl pH 7.9) and a wash buffer (40 mM imidazole, 50 mM NaCl, 20 mM

Tris-HCl pH 7.9), and the protein was then eluted with a buffer of 1 M imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9. The concentration of protein is measured at OD = 280 nm and confirmed with a molecular weight standard (Invitrogen) in 15% SDS–PAGE followed by staining with Coomassie Blue.

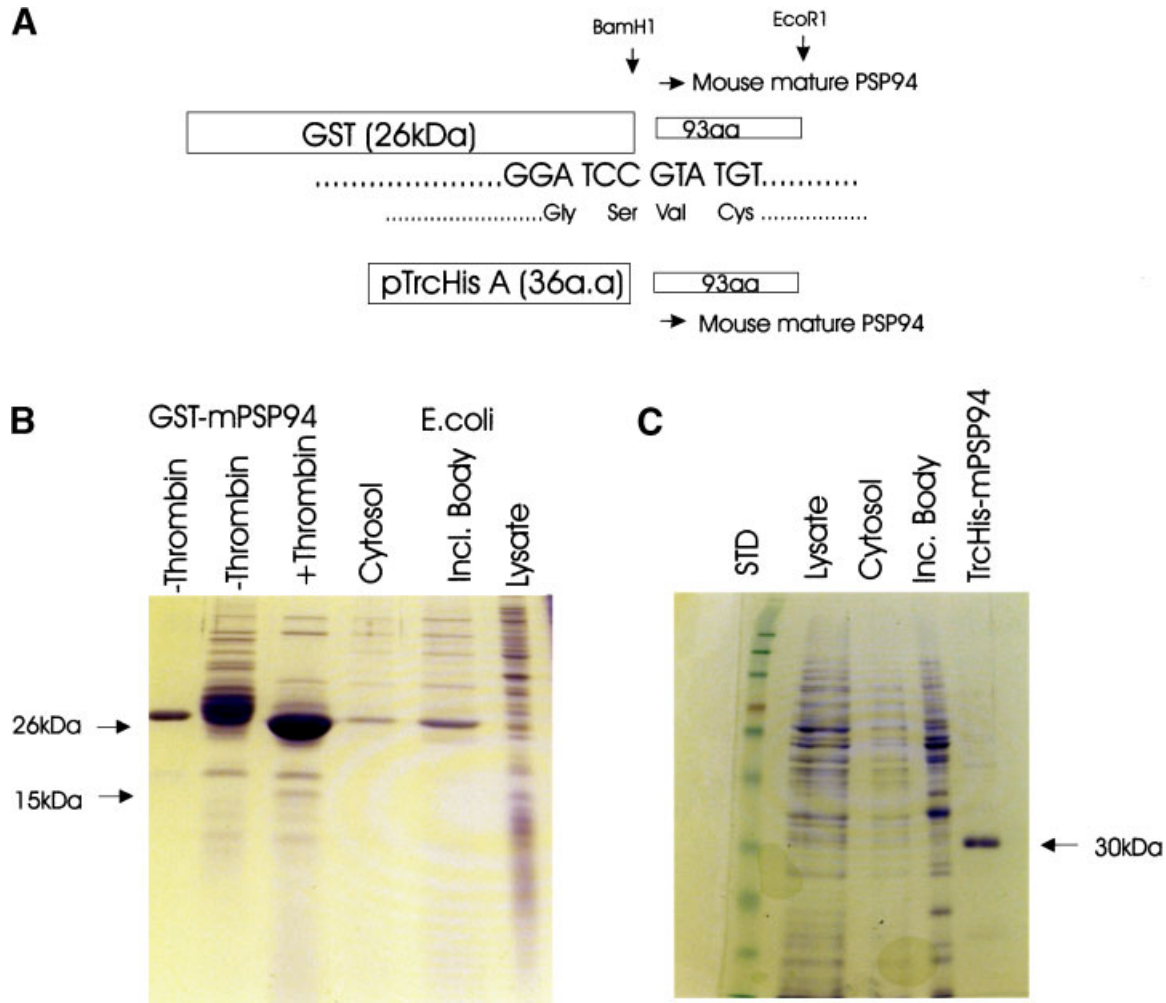

Fig. 1. Expression of recombinant glutathione *S*-transferase-(GST-) mouse PSP94 and pTrcHis-mouse PSP94 fusion proteins in *Escherichia coli*. **A**: Strategy for PCR cloning of the mature PSP94 cDNA sequence in *E. coli* expression vectors of pGEX2T and pTrcHis A. *Bam*HI recognition sequence (in capital) introduced by a N-terminal primer and an *Eco*R1 site (in C-terminal primer) in both vectors are indicated by arrows. The extra glycine and serine residues in recombinant mouse PSP94 are shown in small letters. The sequences also show that the *Bam*HI site in the vector and the N-terminal sequence of PSP94 are in the same reading frame. **B** and **C**: Fifteen percent sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the expression of GST-mouse PSP94 (B) and pTrcHis-mouse PSP94 recombinant fusion proteins (C). Purified recombinant mouse PSP94 protein was released from GST by thrombin (TBN) digestion. Recombinant mouse PSP94 is shown at apparent molecular weight of ~15 kDa. Prestained standard protein ladder (Invitrogen) used are (from top to bottom): 176, 113, 80, 63.8, 49.5, 37.4, 26, 19.6, 14.9, 8.4 kDa. **D**: Less exposure time of mouse PSP94 bands showing multiple isoforms of mouse PSP94 banding in 15% SDS–PAGE and with out dimer band. **E**, **F**, and **G**: Three identical Western blots of 15% SDS–PAGE loaded (from right to left) with purified recombinant mouse TrcHis-mouse PSP94 protein 10, 25, 50 ng, mouse ventral prostate lysate (1, 3, 5 µl) and rat dorsolateral prostate lysates (1, 3, 5 µl) sequentially. Results show Western blotting experiments reacting with rabbit antiserum to TrcHis-mouse PSP94 (D,E), to GST-mouse PSP94 (F), and to GST-rat PSP94 (G) separately (B). A control protein of 30 kDa band standing for recombinant TrcHis-mouse PSP94 was tested for moderate dilutions (Table I) of all polyclonal antibodies. The secondary antibody (horse radish peroxidase-conjugated goat anti-rabbit IgG) was diluted 1,000 times. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

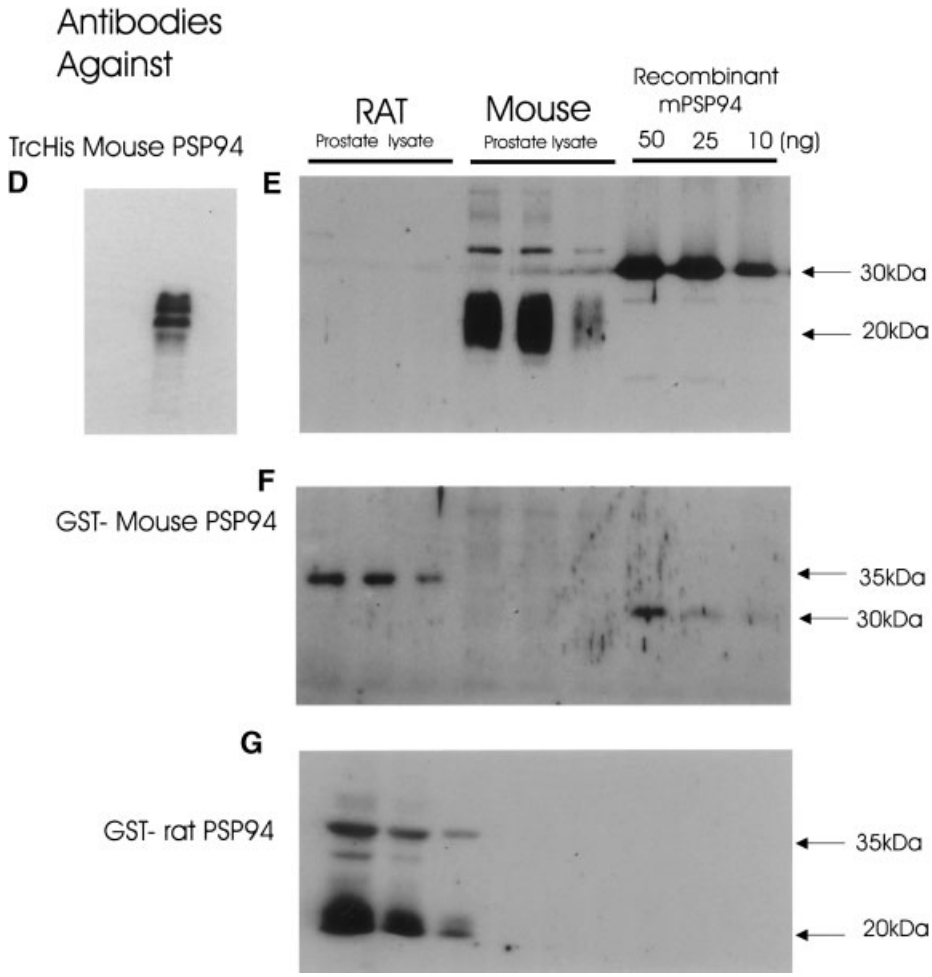

Fig. 1. (*Continued*)

Generation of Rabbit Antiserum Against Recombinant Fusion Proteins of GST and TrcHis-Mouse PSP94

Rabbit polyclonal antibodies against recombinant mouse PSP94 proteins were obtained using a standard procedure (SOP #370-01, University of Western Ontario Animal Care Committee). In brief, 0.6–1 mg of recombinant mouse PSP94 (0.5 ml) was emulsified in 0.5 ml of Freund's complete adjuvant (Sigma, St. Louise, MI) and injected intramuscularly into rabbits with the body of approximately 1 kg. A second booster injection was performed 2 weeks later. Adequate antibody activity was obtained within 1 month of the second injection.

SDS–PAGE, Western Blotting, and Competitive Western Blotting Tests

Tissue lysate samples (10 μg/lane for Coomassie Blue staining and 1 μg/lane for Western blotting) were boiled with standard loading dye and loaded for a 15% SDS–PAGE analysis. Western blots were prepared by electro-transference to a nitrocellulose membrane (Hybond-ECL membrane, Amersham-Pharmacia). Chemiluminescence technique using an ECL Western Blotting kit (Amersham-Pharmacia) was conducted according to the protocol provided by manufacturer. Horse radish peroxidase (HRP) conjugated swine anti-rabbit IgG (Dimension Laboratories, Mississauga, ON) was used as a secondary antibody.

For competitive Western blotting tests, rabbit antiserum to TrcHis-mouse PSP94 was used at 1:500 dilution as a first antibody. Competitors were tissue lysates (0.2 ml/50 mg wet tissue) and were added to the first antibody reaction buffer at different amounts including controls with no competitor. The other procedures were same as the Western blotting experiments.

Immunohistochemistry (IHC)

The hydrated sections were treated in 0.6% H_2O_2 in absolute methanol for 20 min to remove endogenous peroxidase activity. Sections were blocked in 10% goat serum for 30 min. After draining off the blocking solution, the sections were incubated with the recombinant GST-m PSP94 antiserum or TrcHis-mPSP94 antiserum at room temperature for 1 h in a humid chamber. After washing with PBS, the sections were incubated with a biotinylated goat anti-rabbit IgG secondary antibody (Oncogene, Boston, MA, diluted 1:200 in blocking solution) for 30 min at room temperature. The antibody binding sites were then revealed by the avidin-biotin peroxidase complex (ABC) procedure for 30 min at room temperature. After incubation with the ABC solution, the sections were rinsed in PBS three times. Peroxidase activity was then visualized with a glucose oxidase-diaminobenzidine (DAB)-nickel intensifying procedure [Kwong et al., 1999]. The sections were lightly counterstained with hematoxylin. Control sections were performed using PBS without a primary antibody.

RESULTS

Characterization and Comparison of Polyclonal Antibodies Against Recombinant Mouse PSP94 by Western Blotting Experiments

Although, PSP94 is a very abundant protein in semen and in prostatic secretions, the availability of fresh mouse semen and prostate fluid is very limited. We first expressed a mature form of mouse PSP94 as a GST fusion protein (Fig. 1A,B) and a rabbit antiserum was raised. This immunogen has only two extra amino acids, compared to the mature form of mouse PSP94 (93 amino acids (aa) residues) and was demonstrated in a Coomassie Blue staining of 15% SDS–PAGE gel by the appearance of additional bands (~15 kDa, Fig. 1B) after thrombin digestion of GST-mouse PSP94, and several truncated form of GST-mouse PSP94 were also observed (lane 2, Fig. 1B). The immunoreactivity of this antiserum was first tested by Western blotting experiments, in which a gradient recombinant mouse PSP94 protein and both rat and mouse prostate lysates were loaded as a natural PSP94 control (Fig. 1E). As shown in Figure 1F, the antiserum against GST-mouse PSP94 was poorly immunoreactive against all PSP94 antigens in Western blots, even up to 25 ng of recombinant PSP94. This antiserum could not detect PSP94 in mouse prostate lysate (Fig. 1F), which we considered as part of the epitopes of the antigen (~10 kDa) that had been embedded in the GST carrier (~26 kDa). Therefore, another recombinant mouse PSP94 immunogen with a polyhistidines (six times repeats) containing N-terminal leading sequences (36 aa, Fig. 1A) was tested. The apparent molecular weight of this immunogen is ~30 kDa as shown in 15% SDS–PAGE (Fig. 1C). The same Western blotting experiments determining the immunoreactivity of this second construct were performed, which revealed that this antibody is relatively strongly positive to recombinant TrcHis-mouse PSP94 protein (sensitive to ~3 ng at 500 dilution of the antiserum) and mouse prostate tissue (ventral prostate lobes) lysate (Fig. 1D). Since the immunoreactive bands of mouse PSP94 presented in the prostate tissue lysate (as shown in Fig. 1D) were wide when exposed for a longer period (5 s, Fig. 1E), a shorter exposure period (1 s) was tested, and as shown in the left of Figure 1D, there were several bands with different intensities of mouse PSP94 in the mouse prostate tissue. We may attribute these bands to different isoforms as previously reported in the pig [Fernlund et al., 1994], and rat [Fernlund et al., 1996; Imasato et al., 2001]. Only one additional weaker band for the mouse PSP94 bands (~20 kDa) was detected by the antibody of TrcHis-mPSP94, when Western blot was overexposed (E) and it disappeared when properly exposed (shown in Fig. 1D), indicating that it may be a dimer of PSP94 as we previously reported [Kwong et al., 1999]. In comparing these two antisera (Table I), the antiserum against GST-rat PSP94 showed strong reactivity to the rat prostate lysate and poor cross-activity with any mouse PSP94 antigens. Both antibodies of GST-ratPSP94 and TrcHis mPSP94 recognized an additional 35 kDa bands of rat prostate lysate (Fig. 1F,G), which was identified as non-specific binding as shown by competitive tests (rat lysate vs. mouse antibody, shown in Fig. 4E). In order to compare the activity of GST-rat PSP94 with these two mouse PSP94 antibodies (Table I), the same Western blotting experiments were conducted and no significant cross-reactivity was found between antibodies against mouse and rat PSP94 (Fig. 1F,G).

TABLE I. Comparison of Immunoreactivity of Antibodies Against Prostate Secretory Protein of 94 Amino Acids (PSP94) Used in This Study

Antibodies used for this study	Antigen expression		Immunoreactivity				
			In immunhistochemistry		In Western blotting		
	Location	Abundance	Reactivity	Dilution x	Reactivity	Dilution x	References
GST-Rat PSP94	Inclusion body	Poor (<1 mg/L)	++	500	+++	1,000	Kwong et al. [1999]
GST-mouse PSP94	Inclusion body	Poor (<1 mg/L)	++	200	+	200	This study
pTrcHis-mouse PSP94	Both cytosol and inclusion body	Poor (<1 mg/L)	++	500	++	500	This study
Human PSP94	Seminal plasma and prostate fluid	mg/ml	+++	1,000	+++	1,000–5,000	Xuan et al. [1997]

+++, very strong; ++, strong; +, weak.

Characterization and Comparison of Polyclonal Antibodies Against Recombinant Mouse PSP94 by IHC

Next, both of these two polyclonal antibodies against recombinant GST and TrcHis-mouse PSP94 were tested for their immunohistochemical reactivitiy. As shown in Figure 2A,B, antibodies against GST-mouse PSP94 and TrcHis-mouse PSP94 showed positive staining signals in IHC (Table I, Fig. 2A,B). IHC signals were specific to cytoplasm of epithelial cells, including part of the lumen of the secretory gland. No signal, or no significant signals were found in the stroma and other connective tissues in the prostate. Since these IHC signals were uniform and in some slides could not differentiate the intensities or extents of staining by adjusting dilutions (data not shown), we tested four different fixatives, under the moderate conditions of antigen retrieval in IHC. As shown in Table II, the antiserum of GST-mPSP94 showed good staining with formalin and paraformaldehyde fixatives, detectable only at higher concentration (1:200). The antiserum against TrcHis-mPSP94 showed increased IHC activity at a higher concentrations (1:200) with all four fixatives, and clearly showed different intensities and extents of staining in different glands (Fig. 2C,D). In lower concentrations (1:800), this later antiserum also produced strong IHC signals using Carnoy's fixatives (Fig. 2C) and acetic alcohol (Fig. 2D). Table II also showed that Carnoy's and acetic alcohol fixatives were more favorable to rabbit antiserum against TrcHis-mPSP94 than to rabbit antiserum against GST-mPSP94, whereas formalin and paraformaldehyde were more favorable to the antiserum against GST-mPSP94.

Prostate Tissue-Specific Expression of Mouse PSP94 as Demonstrated by IHC and Western Blotting Experiments

PSP94 expression was tested in 19 different tissues from freshly dissected adult mice ($n > 10$, Fig. 3). Fifteen percent SDS–PAGE staining with Coomassie Blue (Fig. 3A) of these 19 tissue lysates served as a control to show comparable amounts of tissue protein contents were tested. As shown in Figure 3B of a Western blot test of Figure 3A, mouse prostate (VP) lysate was the only tissue which showed a positive band at ~20 kDa. Next, in order to confirm results from the Western blotting test, IHC staining of these 19 tissue fixed blocks was performed and only prostate samples (from VP) were found to be strongly positive. No consistent strong signals were seen with other tissues, including kidney, bladder, lung, liver stomach, heart, and intestine tissues. Figure 2E,F showed examples of negative staining in the mouse lung (bronchial tissues as well) and coagulation gland by antibody to TrcHis-mouse PSP94.

Demonstration of the Prostate Tissue-Specific Expression of Mouse PSP94 by Testing in Different Species and by Competitive Western Blotting Experiments

Since there was some cross-reactivity between mouse and rat PSP94 antibodies, a Western blot was constructed by loading

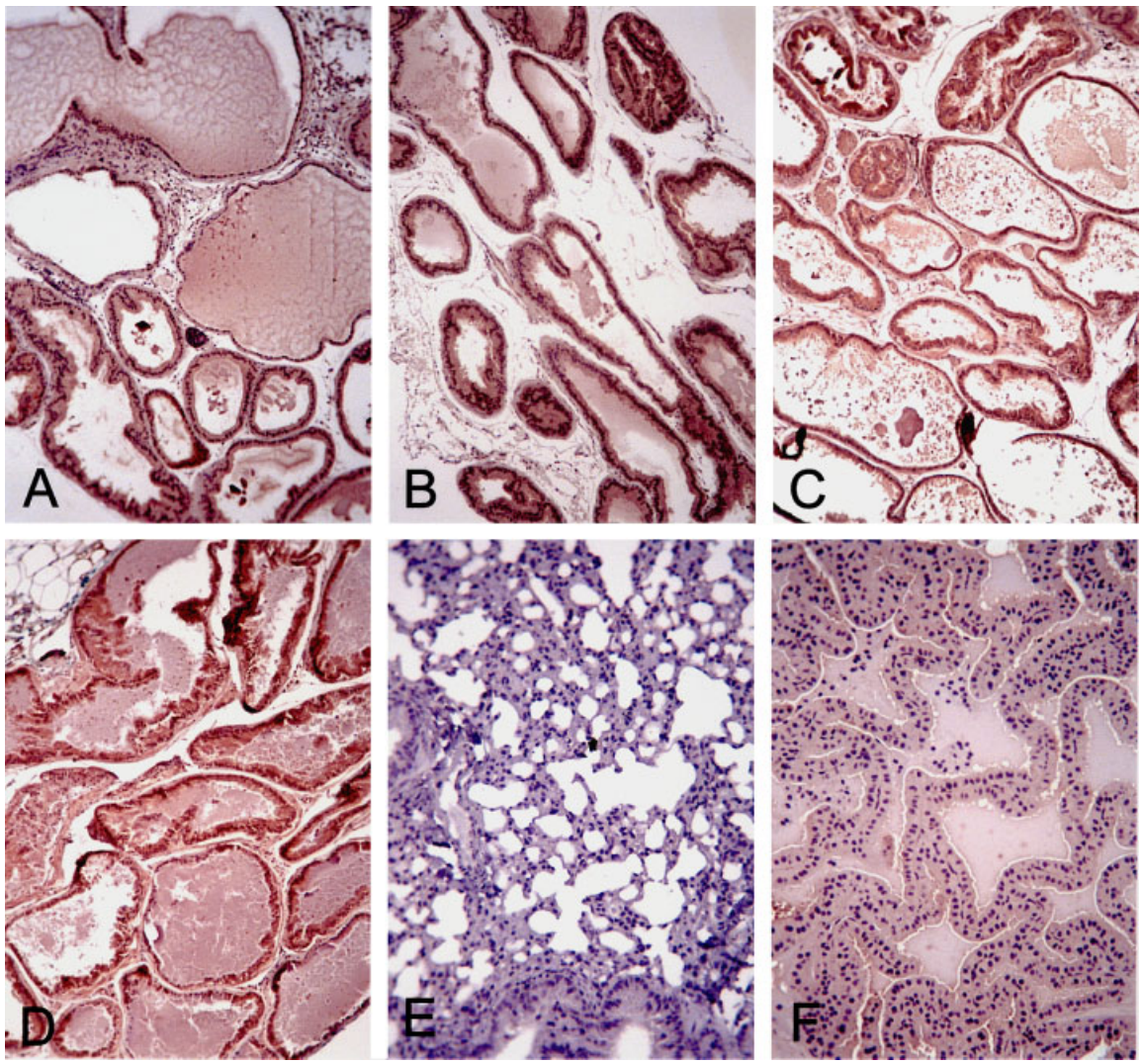

Fig. 2. Immunohistochemistry (IHC) of mouse PSP94. In prostate tissues, two kinds of polyclonal antibodies were compared in slides treated with four fixatives. **A**: Formalin fixed prostate (VP) tissues stained with rabbit antiserum against glutathione *S*-transferase- (GST-) mPSP94 (1:200), 10 times. **B**: Formalin fixed prostate (VP) tissues stained with antiserum against TrcHis-mPSP94 (1:200), 10 times. **C**: Carnoy's fixation of prostate (VP) tissues stained with antiserum against TrcHis-mPSP94 (1:800), 10 times. **D**: Acetic alcohol fixation of prostate (VP) tissues stained with antiserum against TrcHis-mPSP94 (1:800), 10 times. **E**: IHC of mouse lung tissue with antiserum against TrcHis-mPSP94 (1:200) showing negative staining, 20 times. **F**: IHC of mouse tissue of coagulation gland with antiserum against TrcHis-mPSP94 (1:200) showing negative staining. Faint yellow colour background was due to non-specific binding, 20 times. All IHC slides were counterstained briefly with hematoxylin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

comparable amounts (by Coomassie staining of a 15% SDS–PAGE, Fig. 4A) of tissue lysate samples prepared from human prostate, human semen, baboon prostate, rat, and mouse prostate (Fig. 4B). The Western blot reacting with rabbit antiserum against TrcHis-mouse PSP94 showed mostly mouse prostate tissue-specific signals (Fig. 4B). Some cross-reactivity of this antibody with human seminal plasma samples was observed in this experiment (Fig. 4B), which may be explained by the extraordinary high content (in grams per liter) of human PSP94 in semen. Therefore, a competitive binding test was performed to assess the specificity of the antiserum against recombinant mouse PSP94, by adding the first antibody reaction buffer in Western blotting experiments with prostate tissue lysates from different species as competitors. Western blots of mouse lysate were tested with rabbit antiserum against

TABLE II. Effects of Different Fixatives on Immunoreactivities of Antibodies Against Mouse Prostate Secretory Protein of 94 Amino Acids (PSP94)

Antiserum		Carnoy's	Acetic alcohol	Formalin	Paraformaldehyde
GST-mPSP94	1:200	+	+	++	++
TrcHis-mPSP94	1:200	+++	+++	++/+++	++
	1:800	++	++	+	+

+++, very strong; ++, strong; +, weak.

the TrcHis-mouse PSP94 antibody for competition with different amount (0, 50, and 100 μl) of tissue lysates of mouse ventral prostate (Fig. 4C), human prostate (from benign tissues, Fig. 4D), and rat dorsolateral prostate (Fig. 4E). When competing with increased mouse ventral prostate lysates, the mouse antibodies showed significantly decreased signals in Western blotting. In contrast, competitors of rat and human prostate tissue lysates showed negligible effect as compared with control (without competitors added). Using rat lysate as a competitor against mouse PSP94 antibody, the intensity of a 35 kDa band decreased with additional amounts of lysate (50–100 μl of rat lysate), as compared with no competitor (0) control (Figs. 1F,G and 4E). Therefore, the 35-kDa band in the rat prostate lysate binding is non-specific by both antibodies against GST-rat and mouse PSP94.

DISCUSSION

Current available systematic therapy of advanced prostate cancer are not sufficiently tissue-specific to precisely target prostate cancer tissue. Ideally, gene therapy aims to selectively target prostate tissue. A vector gene, which specifically directs prostate targeting is a crucial element of any effective gene therapy. Although, at least 15 different vector genes have

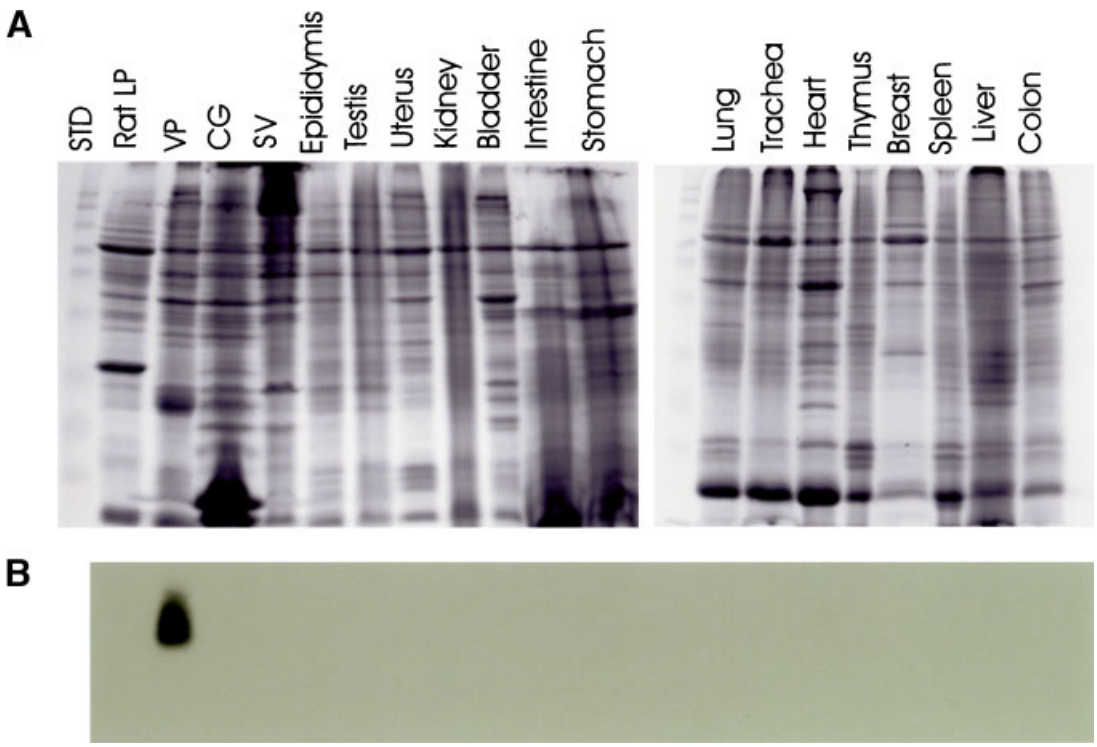

Fig. 3. Tissue distribution of mouse PSP94 shown by Western blotting experiments. **A**: Fifteen percent sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) staining of 19 tissue lysates showing comparable amount of tissue proteins were loaded for Western blotting analysis (**B**). Protein samples (10 μg/lane) were separated in 15% SDS–PAGE and stained in Coomassie Blue. Prestained standard protein ladder (Invitrogen) used are (from top to bottom): 176, 113, 80, 63.8, 49.5, 37.4, 26, 19.6, 14.9, 8.4 kDa. B: Western blotting analysis of (a) showing prostate tissue-specific expression of mouse PSP94. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

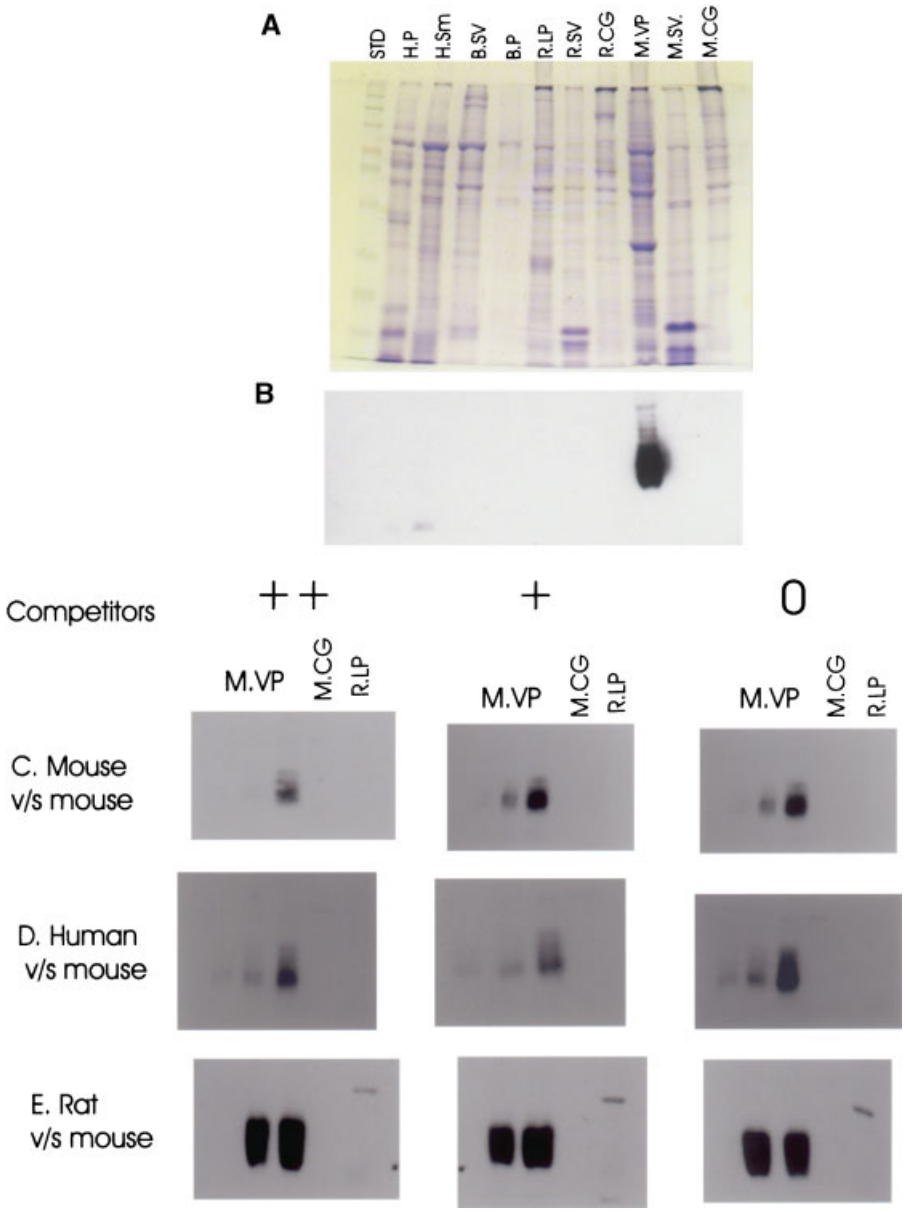

Fig. 4. Prostate tissue-specific expression of PSP94 in different species. **A**: Fifteen percent sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) staining of prostate tissue lysates of human prostate (H.P.) and seminal plasma (H.Sm.); baboon prostate (B.P.) and seminal vesicles (B.SV.); mouse ventral prostate (M.VP), seminal vesicles (M.SV.), and coagulation gland (M.CG.); and rat lateral prostate (R.LP.) and seminal vesicles (R.SV.), coagulation gland (R.CG.). Coomassie staining of (A) showed comparable amounts of tissue proteins were loaded for Western blotting analysis (B). Only mouse ventral prostate (VP) showed strong reactivity with rabbit antiserum to TrcHis-mouse PSP94. Prestained standard protein ladder (Invitrogen) used are (from top to bottom): 176, 113, 80, 63.8, 49.5, 37.4, 26, 19.6, 14.9, 8.4 kDa. **C**, **D**, and **E**: Competitive Western blotting tests of prostate tissue-specific expression of PSP94 in different species. For each competitive test, three identical blots were prepared loaded with a gradient of mouse prostate lysates (M.VP., 1, 2, 5 µl), 5 µl of mouse coagulation gland (M.CG.), and rat lateral prostate lobe (R. LP) lysate sequentially. C: First antibody (rabbit antiserum against TrcHis-mouse PSP94) was diluted at 1:500 and competing with no competitor (0), with 50 µl (+), and with 100 µl (++) of mouse VP lysate competitors. Similar competitive tests were shown with competitors of rat lateral prostate (D) and human prostate (E) lysate. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

been tested for prostate targeting, only a few vector genes are available for this purpose (review see 1998 NIH Workshop [Green et al., 1998; Shirakawa et al., 2000; Matusik et al., 2001; Gabril et al., 2002]). There is a need to identify more prostate tissue-specific genes as vector genes for prostate targeting. The prostate gland produces hundreds of gene pro-

ducts, although only a few of these have been characterized. Some of these gene products are relatively unique to the prostate and may be potentially exploited for vector targeting or gene vaccine immunization. Moreover, prostate-specific promoters and other enhancers that direct transcription of these prostate tissue-specific products may also be incorporated into vectors to direct prostate specific expression of therapeutic genes [Steiner et al., 2000].

PSP94 is the most abundantly secreted protein from the prostate. Studies on tissue distribution in human clinical samples have shown that PSP94 exists in most mucus secretory tissues, and in respiratory tract secretions (at ~25% of the level seen in prostate) [Ulvsback et al., 1989; Weiber et al., 1990]. However, these reports were largely based on human pathological samples and have not been confirmed in porcine [Fernlund et al., 1994; Tanaka et al., 1996] and rodent studies [Fernlund et al., 1996; Imasato et al., 2001]. We have previously demonstrated that rat PSP94 expression is strictly prostate tissue-specific [Kwong et al., 1999, 2000b]. This conclusion has been confirmed at both transcriptional level and protein expression level [Kwong et al., 1999, 2000b], and also by a comparative studies with the rat probasin gene [Imasato et al., 2001] and other genes [Kwong et al., 2000a]. We have reported, at least at a protein expression level, there is uncoupling of transcription and translation [Imasato et al., 2001]. For instance, rPB protein expression is prostate tissue (dorsolateral lobe) specific and but gene transcript may have less stringent control in male reproductive organs [Imasato et al., 2001]. By assuming that mouse PSP94 behaves in a similar manner as rat PSP94, being a prostate specific gene, transgenic mouse experiments were performed. We have demonstrated that the mouse PSP94 promoter/enhancer region can direct heterogenous gene expression specifically in the prostate (both ventral and dorsolateral lobes) [Gabril et al., 2002].

Our transgenic mouse prostate cancer model is a cancer model induced by the SV40 Tag oncogene expression directed by a 3.84 kb mouse PSP94 promoter/enhancer region, and we have found both ventral and dorsolateral prostate were targeted with prostate cancer development [Gabril et al., 2002]. In order to assess the specificity of prostate targeting, we need to study PSP94 expression in mouse tissue as well. The current study has established and characterized two antisera against two kinds of recombinant mouse PSP94 as immunogens. Our preliminary observation is that mouse PSP94, as with rat PSP94, is strictly prostate tissue-specific, and the only difference between these two PSP94s is that they exist in DLP (rat) and VP (mouse) separately. We are performing systematic studies on this interesting finding, by elucidating the mechanism of the completely different expression patterns in different prostate lobes of PSP94 between these two closely related rodent species.

As shown in Table I, human seminal PSP94 is a good immunogen for a polyclonal antibody as tested in Western blots [Xuan et al., 1997], enzyme linked immunosorbent assay (ELISA) [Abrahamsson et al., 1988; Hyakutake et al., 1993; Xuan et al., 1997], and IHC [Dube et al., 1987; Hyakutake et al., 1993; Imasato et al., 2000]. However, this is almost a unique situation, as most other antibodies, either monoclonal or polyclonal, have limited utility and must be used under favorable reaction conditions (concentrations, antigen retrieval, etc.) with antigens immobilized either in the Western blot or in tissue fixatives. The difference of antibody reactivity may depend on the methods and the extent of antigen retrieval (cf. Table II). Because of the complexity of the epitope structure, each antibody will show different activity to different antigens retrieved and also under different experimental conditions. In this study, we assessed for the optimal conditions for two antibodies established for mouse PSP94 separately (Tables I and II). We demonstrated that both antibodies could be used for IHC studies. In general, TrcHis mouse PSP94 as an immunogen produced better immunoreactivity compared to GST-mouse PSP94. We demonstrated that for a small peptide, such as the 93 aa long mouse PSP94 [Xuan et al., 1999], the 36 additional amino acids in recombinant fusion protein TrcHis-mPSP94 (Fig. 1A) is enough to induce immuno-response, as compared with the GST (26 kDa) carrier protein. As compared with GST-rat PSP94, GST-mouse PSP94 is a weaker immunogen, since it showed weaker immunoreactivities in both Western and IHC experiments (Table I). We assume that the epitope of mouse PSP94 may be embedded in the carrier GST protein. Another factor, which may affect

the immunogenicity of GST-mouse PSP94, is that it produces many truncated proteins from the C-terminus of the fusion protein in *E. coli* (Fig. 1B) as we observed previously [Xuan et al., 1997; Kwong et al., 1999], which may reduce the immunogenic response of mouse PSP94 (located in the C-terminus) in the rabbit.

The antibodies established in this study are important in characterizing our mouse prostate cancer model and also in determining prostate targeting by the PSP94 vector gene. With these antibodies, we can perform correlation studies of PSP94 expression in prostate cancer tissues, which is a prerequisite for utilizing our transgenic mouse prostate cancer model in preclinical drug testing. The establishment and characterization of antibodies against mouse PSP94 contribute to our understanding of the potential for PSP94 as a new vector gene for prostate cancer gene therapy.

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REFERENCES

Abrahamsson P-A, Lilja H, Falkmer S, Wadstrom LB. 1988. Immunohistochemical distribution of the three predominant secretory proteins in the parenchyma of hyperplastic and neoplastic prostate glands. Prostate 12:39–46.

Dube JY, Frenette G, Paquin R, Chapdelaine P, Tremblay J, Tremblay RR, Lazure C, Seidah N, Chretien M. 1987. Isolation from human seminal plasma of an abundant 16-kDa protein originating from the prostate, its identification with a 94-residue peptide originally described as β-inhibin. J Androl 8:182–189.

Fernlund P, Granberg L, Roepstorf P. 1994. Amino acid sequence of β-microseminoprotein from porcine seminal plasma. Arch Biochem Biophy 309:70–76.

Fernlund P, Granberg L, Larson I. 1996. Cloning of β-microseminoprotein of the rat: A rapidly evolving mucosal surface protein. Arch Biochem Biophy 334:73–82.

Gabril MY, Onita T, Ji PG, Sakai H, Chan FL, Koropatnick J, Chin JL, Moussa M, Xuan JW. 2002. Prostate targeting: PSP94 gene promoter/enhancer region directed prostate tissue expression in a transgenic mouse prostate cancer model. Gene Ther 9:1589–1599.

Gingrich JR, Barrios RJ, Morton RA, Boyce BF, DeMayo FJ, Finegold MJ, Angelopoulou R, Rosen JM, Greenberg NM. 1996. Metastatic prostate cancer in a transgenic mouse. Cancer Res 56:4096–4102.

Gingrich JR, Barrios RJ, Kattan MW, Nahm HS, Finegold MJ, Greenberg NM. 1997. Androgen-independent prostate cancer progression in the TRAMP model. Cancer Res 57:4687–4691.

Green JE, Greenberg NM, Ashendel CL, Barrett JC, Boone C, Getzenberg RH, Henkin J, Matusik R, Janus TJ, Scher HI. 1998. Workgroup 3: Transgenic and reconstitution models of prostate cancer. Prostate 36:59–63.

Greenberg NM, Demayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ, Rosen JM. 1995. Prostate cancer in a transgenic mouse. Proc Natl Acad Sci USA 92:3439–3443.

Hara M, Kimura H. 1989. Two prostate-specific antigens, gamma-seminoprotein and β-microseminoprotein. J Lab Clin Med 113:541–548.

Hyakutake H, Sakai H, Yogi Y, Tsuda R, Minami Y, Yushita Y, Kanetake H, Nakazono I, Saito Y. 1993. β-microseminoprotein immunoreactivity as a new prognostic indicator of prostatic carcinoma. Prostate 22:347–355.

Imasato Y, Xuan JW, Sakai H, Izawa JI, Saito Y, Chin JL, Moussa M. 2000. PSP94 expression after androgen deprivation therapy: A comparative study with prostate specific antigen in benign prostate and prostate cancer. J Urol 164:1819–1824.

Imasato Y, Onita T, Moussa M, Sakai H, Chan FL, Koropatnick J, Chin J, Xuan JW. 2001. Rodent PSP94 gene expression is more specific to the dorsolateral prostate and less sensitive to androgen ablation than probasin. Endocrinol 142:2138–2146.

Kwong J, Chan FL, Jiang S, Guo YZ, Imasato Y, Sakai H, Koropatnik J, Chin JL, Xuan JW. 1999. Differential expression of PSP94 in rat prostate lobes as demonstrated by an antibody against recombinant GST-PSP94. J Cell Biochem 74:406–417.

Kwong J, Xuan JW, Chan PSF, Ho SM, Chan FL. 2000a. A comparative study of hormonal regulation of three secretory proteins (PSP94, Probasin, SVSII) in rat lateral prostate. Endocrinol 141:4543–4551.

Kwong J, Xuan JW, Choi HL, Chan PSF, Chan FL. 2000b. PSP94 (or β-microseminoprotein) is a secretory protein specifically expressed and synthesized in the lateral lobe of the rat prostate. The Prostate 42:219–229.

Lazure C, Villemure M, Gauthier D, Naude RJ, Mbikay M. 2001. Characterization of ostrich (*Struthio camelus*) beta-microseminoprotein (MSP): Identification of homologous sequences in EST databases and analysis of their evolution during speciation. Protein Sci 10:2207–2218.

Lee C. 1987. Gross dissection of three lobes of the rat prostate. In: Coffey DS, Bruchovsky N, Gardner WA, Jr., Resnick M, Karr JP, editors. Current concepts and approaches to the study of prostate cancer. New York: Alan R. Liss, Inc. p 577–582.

Matsubara S, Wada Y, Gardner TA, Egawa M, Park MS, Hsieh CL, Zhau HE, Kao C, Kamidono S, Gillenwater JY, Chung LW. 2001. A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate

cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. Cancer Res 61:6012–6019.

Matusik RJ, Masumori N, Thomas T, Case T, Paul M, Kasper S, Shappell SB. 2001. Transgenic mouse models of prostate cancer. In: Matuk M, Brown CW, Kumar TR, editors. Comtemporary endocrinology: Transgenics in endocrinology. Totowa, NJ: Human Press, Inc. p 401–425.

Mentor-Marcel R, Lamartiniere CA, Eltoum IE, Greenberg NM, Elgavish A. 2001. Genistein in the diet reduces the incidence of poorly differentiated prostatic adenocarcinoma in transgenic mice (TRAMP). Cancer Res 61:6777–6782.

Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, Henderson DR. 1997. Prostate attenuated replication competent adenovirus (ARCA) CN706: A selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Res 57:2559–2563.

Shibata MA, Liu ML, Knudson MC, Shibata E, Yoshidome K, Bandey T, Korsmeyer SJ, Green JE. 1999. Haploid loss of bax leads to accelerated mammary tumor development in C3(1)/SV40-TAg transgenic mice: Reduction in protective apoptotic response at the preneoplastic stage. EMBO J 18:2692–2701.

Shirakawa T, Gotoh A, Wada Y, Kamidono S, Ko SC, Kao C, Gardner TA, Chung LW. 2000. Tissue-specific promoters in gene therapy for the treatment of prostate cancer. Mol Urol 4:73–82.

Steiner MS, Gingrich JR. 2000. Gene therapy for prostate cancer: Where are we now? J Urol 164:1121–1136.

Sugimura Y, Cunha GR, Donjacour AA. 1986. Morphogenesis of ductal networks in the mouse prostate. Biol Reprod 34:961–971.

Tanaka T, Itahana K, Andoh N, Takeya T, Sato E. 1996. Expression of prostate secretory protein (PSP)-like protein in porcine corpus Luteum: Isolation and characterization of a new gene encoding PSP94-like protein. Mol Reprod Dev 42:149–156.

Ulvsback M, Lindstrom C, Weiber H, Abrahamsson PA, Lilja H, Lundwall A. 1989. Molecular cloning of a small prostate protein, known as β-microseminoprotein, PSP94 or β-inhibin, and demonstration of transcripts in non-genital tissues. Biochem Biophys Res Commun 164: 1310–1315.

Wei C, Willis RA, Tilton BR, Looney RJ, Lord EM, Barth RK, Frelinger JG. 1997. Tissue-specific expression of the human prostate-specific antigen gene in transgenic mice: Implications for tolerance and immunotherapy. Proc Natl Acad Sci USA 94:6369–6374.

Weiber H, Anderson C, Murne A, Rannevik G, Lindstrom C, Lilja H, Fernlund P. 1990. β-microseminoprotein is not a prostate-specific protein. Am J Pathol 137:593–603.

Xuan JW, Chin JL, Guo Y, Chambers AF, Finkelman MA, Clarke MW. 1995. Alternative splicing of PSP94 (prostate secretory protein of 94 amino acids) mRNA in prostate tissue. Oncogene 11:1041–1047.

Xuan JW, Wu D, Guo Y, Garde SV, Baijal-Gupta M, Chin JL. 1997. Analysis of epitope structure of PSP94 (prostate secretory protein of 94 amino acids): (I) Immunodominant and immuno-recessive area. J Cell Biochem 65:172–185.

Xuan JW, Kwong J, Chan FL, Ricci M, Imasato Y, Sakai H, Fong GH, Panchal C, Chin JL. 1999. cDNA, genomic cloning and gene expression analysis of mouse PSP94 (prostate secretory protein of 94 amino acids). DNA Cell Biol 18:11–26.

Yu DC, Chen Y, Dilley J, Li YH, Embry M, Zhang H, Nguyen N, Amin P, Oh J, Henderson DR. 2001. Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel. Cancer Res 61:517–525.