A 6-kb Promoter Fragment Mimics in Transgenic Mice the Prostate-Specific and Androgen-Regulated Expression of the Endogenous Prostate-Specific Antigen Gene in Humans

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Prostate-specific antigen (PSA) is a kallikrein-like serine protease, which is almost exclusively synthesized in the luminal epithelial cells of the human prostate. PSA expression is androgen regulated. Previously, we characterized *in vitro* the proximal promoter, and a strong enhancer region, approximately 4 kb upstream of the PSA gene. Both regions are needed for high, androgen-regulated activity of the PSA promoter in LNCaP cells. The goal of the present study is the *in vivo* characterization of the PSA promoter. Three transgenic mouse lines carrying the *Escherichia coli* LacZ gene, driven by the 632-bp proximal PSA promoter, and three lines with LacZ, driven by the 6-kb PSA promoter, were generated. Expression of the LacZ reporter gene was analyzed in a large series of tissues. Transgene expression could not be demonstrated in any of the transgenic animals carrying the proximal PSA promoter. All three lines carrying the 6-kb PSA promoter showed lateral prostate-specific β-galactosidase activity. Transgene expression was undetectable until 8 weeks after birth. Upon castration, β-galactosidase activity rapidly declined. It could be restored by subsequent androgen administration. A search for mouse PSA-related kallikrein genes expressed in the prostate led to the identification of mGK22, which was previously demonstrated to be expressed in the submandibular salivary gland. Therefore, the 6-kb PSA-LacZ transgene followed the expression pattern of the PSA gene in humans, which is almost completely prostate-specific, rather than that of mGK22 in mice. In conclusion, the 6-kb promoter fragment appears to contain most, if not all, information for androgen regulation and prostate specificity of the PSA gene. (Molecular Endocrinology 11: 1256–1265, 1997)

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INTRODUCTION

Prostate specific antigen (PSA) is a 30- to 33-kDa glycoprotein, which is almost exclusively produced by the luminal epithelial cells of the human prostate. It is one of the predominant proteins secreted into the prostatic fluid. Serum PSA is a well known marker for diagnosis and monitoring of prostate cancer (1, 2). The PSA gene (or KLK3) is a member of the human kallikrein gene family. Other members of the kallikrein gene family are the hGK-1(KLK2) gene, which is also expressed in the prostate, and the tissue kallikrein gene (KLK1), which is mainly expressed in the pancreas and kidney (3–6). The three genes are clustered within the 60-kb kallikrein locus on chromosome 19 (7, 8). PSA expression can be regulated by androgens (9–11). Previously, we and others characterized *in vitro* the 632-bp proximal promoter (11, 12) and a strong 440 bp-enhancer region, approximately 4 kb upstream of the transcription start site of the PSA gene (13, 14).

Both regions are required for high, androgen-regulated activity of the PSA promoter in LNCaP cells. Two functionally active androgen receptor-binding sites (androgen response elements, or AREs) were identified in the proximal PSA promoter, at positions −170 (ARE-I) and −394 (ARE-II), respectively (11, 12). The upstream enhancer showed synergistic cooperation with the proximal PSA promoter and was found to be composed of at least three separate, but cooperating, regulatory regions. At −4.2 kb, the presence of a functionally active, high-affinity androgen receptor-binding site (ARE-III) was established (14). Transient transfection of a 6-kb PSA promoter fragment, containing both the proximal promoter and the upstream enhancer linked to the luciferase reporter gene, to prostate and nonprostate cell lines showed largely LNCaP prostate cell-specific activity (13, 14). The strong tissue specificity of the endogenous PSA gene *in vivo* and the 6-kb PSA promoter fragment in transient transfection experiments makes the PSA promoter a candidate to deliver therapeutic genes to prostate cancer cells. To explore this view, the goal of the present study is the *in vivo* characterization of the PSA promoter in transgenic mice.

In mice, the kallikrein gene family is composed of 24 members, half of which are probably pseudogenes (15). Although structurally related to the PSA gene, none of the mouse kallikreins can be considered as the mouse homolog of human PSA, because of the different tissue distribution (16). All functional mouse kallikrein genes are expressed in the submandibular gland. Individual genes show additional expression in pancreas, kidney, spleen, and/or testis. Mouse kallikrein expression in the prostate has not yet been demonstrated. Two members of the closely related rat kallikrein gene family have been found to be expressed in both prostate and submandibular gland (17). To compare PSA promoter specificity in transgenic mice with the promoter specificity of endogenous mouse kallikreins, we determined which, if any, of the mouse kallikrein genes was expressed in prostate.

RESULTS

Activity of the PSA Promoter LacZ Fusion Constructs in LNCaP Cells

Previously, in transfection experiments, we characterized the proximal promoter and a strong 440-bp core enhancer region, approximately 4 kb upstream of the transcription start site of the PSA gene (11, 12, 14). Two functionally active AREs were identified in the proximal PSA promoter, at positions −170 (ARE-I) and −394 (ARE-II), respectively (11, 12). In the center of the 440-bp upstream enhancer region, a third functionally active ARE, ARE-III (−4200), could be demonstrated (14). Although both the proximal promoter and the upstream region contributed to maximal androgen-regulated and cell-specific activity of the PSA promoter, the upstream enhancer was found to be essential for high activity (12, 14). To investigate the regulatory regions of the PSA promoter in transgenic mice, two LacZ reporter gene constructs were designed (Fig. 1A). In these constructs, the LacZ gene is driven by the 632-bp proximal PSA promoter (PSA-4-LACH) or by the 6-kb PSA promoter fragment (PSA-61-LACH). The hormone-induced activity of the constructs was tested in transiently transfected LNCaP cells. The PSA-4-LACH construct, cotransfected with the human androgen receptor expression plasmid pSVARo, was 7-fold more active in the presence of 1 nM R1881 than in its absence (Fig. 1A). In the absence of pSVARo, PSA-4-LACH showed a limited androgen inducibility (1.8-fold). Under these conditions, PSA-61-LACH activity was induced 600-fold by R1881. These results are essentially identical to those obtained with comparable luciferase reporter gene constructs (12, 14).

Identification of Transgenic Mice

Both PSA-4-LACH and PSA-61-LACH were used to generate transgenic mice. Three PSA-4-LACH and five PSA-61-LACH founder animals were identified by PCR of tail DNA with primers PSA-s and LacZ-as (data

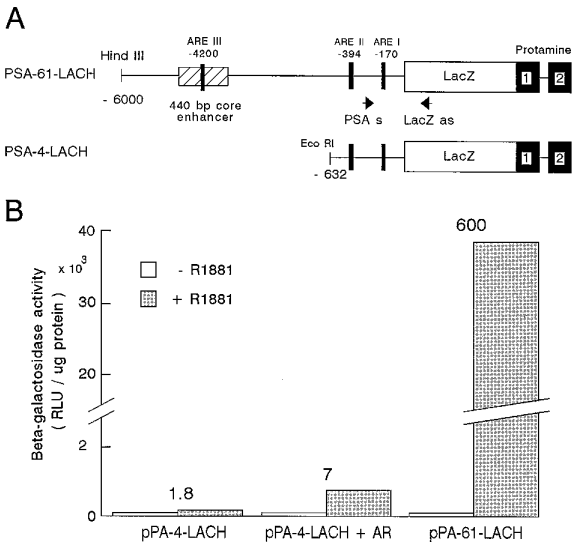

Fig. 1. Structure and Activity of the PSA-LACH Constructs Introduced in Transfected LNCaP Cells

A, Schematic representation of constructs PSA-61-LACH and PSA-4-LACH. The 440-bp core enhancer region (−4380 to −3940) is represented by a *hatched box*; ARE sequences are indicated by *black bars*. The *open box* represents the LacZ open reading frame; *numbered black boxes* indicate exons 1 and 2 of the mouse protamine gene. Positions of primers used to identify transgenic animals are indicated *below* PSA-61-LACH. B, LNCaP cells were transiently transfected with the PSA-4-LACH and PSA-61-LACH constructs or with PSA-4-LACH plus the androgen receptor expression plasmid as described in *Materials and Methods* and Ref. 14. Incubation with the plasmid precipitate was for 4 h. In indicated cases, cells were incubated with 1 nM R1881 for 24 h. Induction values are given at the *top* of the *bars*.

not shown). Transmission of the transgene to their offspring was demonstrated for three PSA-61-LACH and all three PSA-4-LACH transgenic lines. One PSA-61-LACH male founder did not transmit the transgene; another PSA-61-LACH male founder was infertile. Comparison of the hybridization signals of the transgene and the endogenous mouse protamine-1 gene on Southern blots of *Kpn*I-*Sac*I digested genomic DNA revealed the presence of 4, 2, and 38 copies of the transgene in lines PSA-61 TG2, TG28, and TG31, respectively (Fig. 2A, lanes 4–6). PSA-4 TG1, PSA-4 TG2, and PSA-4 TG6 carried approximately 150, 100, and 126 copies of the transgene (Fig. 2A, lanes 1–3). Note that the endogenous mouse protamine-1 gene showed a restriction fragment length polymorphism, resulting in 6- and/or 8-kb hybridizing fragments (Fig. 2A, lanes 7, 8).

The 6-kb, but Not the 632-bp, PSA Promoter Directs Lateral Prostate-Specific Transgene Expression

To determine the expression pattern of the transgene, male mice were killed at 8 to 16 weeks of age, and β-galactosidase activity was measured in 26 different tissue lysates (see *Materials and Methods*). Thorough

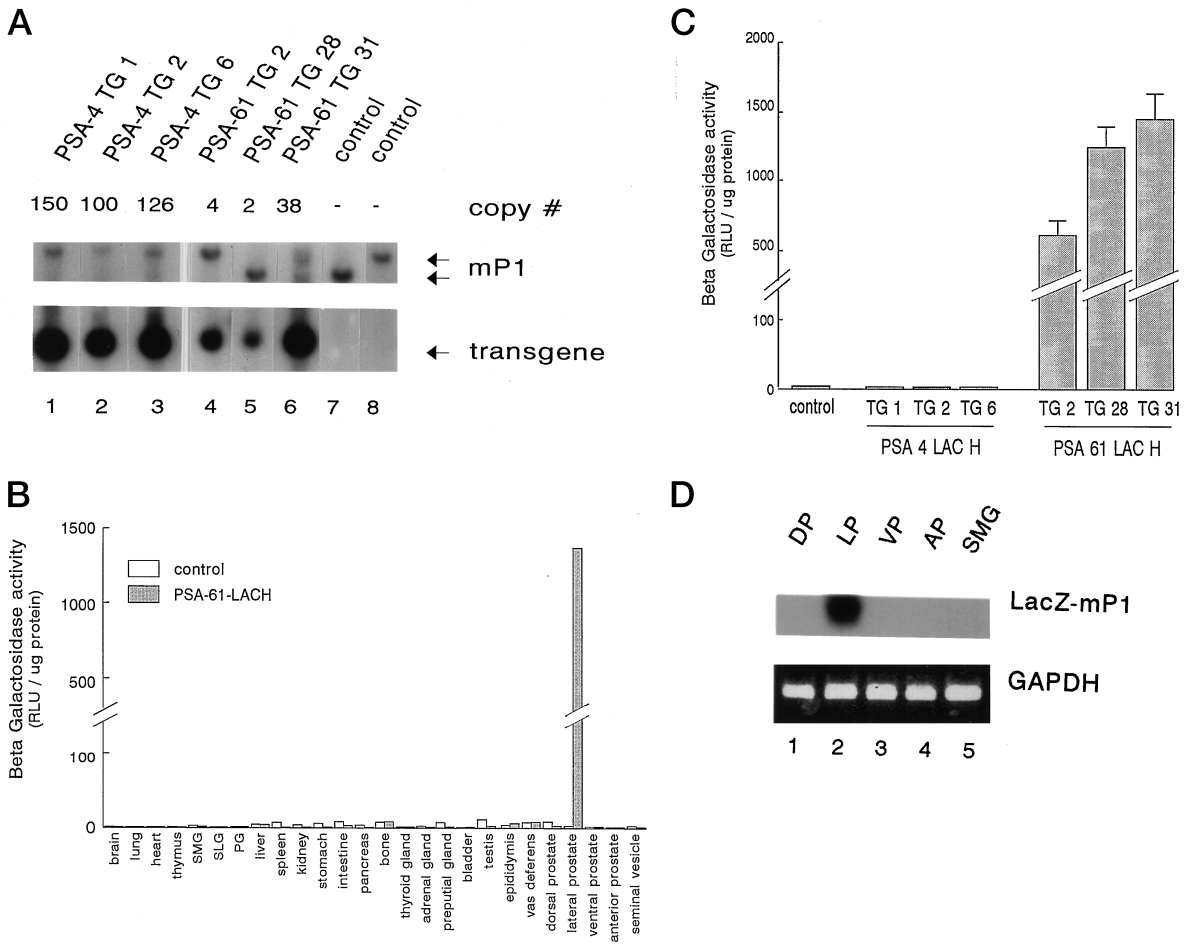

Fig. 2. Characterization of Transgenic Mouse Lines

A, Southern blot analysis of the *Kpn*I-*Sac*I-digested genomic DNA of PSA-4-LACH (lanes 1–3) and PSA-61-LACH (lanes 4–6) transgenic (TG) lines. Lane 7 contains DNA of a control mouse. DNA (10 μg/lane) was hybridized with a 175-bp mouse protamine cDNA probe (see *Materials and Methods*). By comparison of the intensity of the endogenous (see *arrowheads*) and transgene bands, the number of transgene copies present in the individual transgenic lines was determined (*numbers on top* of each lane). For PSA-4-LACH transgenic animals, two different exposure times of the same Southern blot are shown (a 4-h exposure of the transgene-hybridizing fragment and a 40-h exposure of the endogenous mouse protamine gene). Note that the endogenous protamine fragment is polymorphic, leading to a hybridizing fragment of 6 or 8 kb. B, Liquid β-galactosidase assay of tissue extracts of 10-week old PSA-61-LACH TG28 male mice. SMG, Submandibular gland; SLG, sublingual gland; PG, parotid gland. C, β-Galactosidase activity in lateral prostate lysates of PSA-4-LACH TG 1, 2, and 6 and PSA-61-LACH TG 2, 28, and 31 animals as compared with activity in control mice. D, RT-PCR analysis of LacZ/protamine transgene mRNA in RNA obtained from dorsal (DP), lateral (LP), ventral (VP), and anterior prostate (AP) and SMG of PSA-61-LACH TG 28 male mice. Experimental details are described in *Materials and Methods*. The *lower* panel shows the result of RT-PCR analysis of ubiquitously expressed glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA. PCR products were separated over a 2% agarose gel.

analysis of all three PSA-61-LACH transgenic mouse lines showed exclusive β-galactosidase activity in extracts from lateral prostate. In all other tissues, including the dorsal, ventral, and anterior prostate lobes, LacZ expression was undetectable (as shown in Fig. 2B for PSA-61 TG28). β-Galactosidase activity could also not be detected in extracts from tissues of virgin or lactating female transgenic mice (data not shown). β-Galactosidase expression could not be found in any of the tested tissues of PSA-4-LACH mice (data not shown, and Fig. 2C). For PSA-61-LACH, transgene activity was independent of the number of integrations because β-galactosidase activity in the lateral prostate was comparable, despite the difference in copy numbers (4, 2, and 38, respectively). The level and specificity of transgene expression appeared independent of the integration site.

To screen for the presence of low levels of transgene expression in the different prostate lobes and submandibular gland, a known expression site of mouse kallikreins, we performed RT-PCR with transgene cDNA-specific primers, and GAPDH as a control (see *Materials and Methods*). Again, transgene expression could only be detected in the lateral prostate (lane 2, Fig. 2D).

PSA-61-LACH Expression Is Restricted to the Luminal Epithelial Cells of the Lateral Prostate

Whole mount β-galactosidase staining, followed by sectioning of the paraffin-embedded tissue, was performed to investigate the cell type in the prostate expressing the LacZ gene. As demonstrated in Fig. 3B, β-galactosidase staining was restricted to the luminal epithelial cells. Staining was concentrated at the basal site of the cytoplasm. No staining was found in the lateral prostate from age-matched control mice (Fig. 3A). To further evaluate PSA-61-LACH expression, sections of the paraffin-embedded lateral prostate of PSA-61-LACH-positive and control mice were analyzed by *in situ* hybridization using sense and antisense digoxygenin (DIG)-labeled protamine riboprobes. Results obtained with the antisense protamine probe revealed that transgene mRNA was localized within the cytoplasm of the luminal epithelial cells of the lateral prostate (Fig. 4, C and D). No hybridization signal was detected in control mice or with a sense protamine riboprobe (Fig. 4, A and B). The restricted expression of the transgene to the luminal epithelial cells is consistent with endogenous PSA expression in the human prostate (18).

Developmental and Hormonal Regulation of PSA-61-LACH Expression

PSA gene expression has been shown to be developmentally regulated and to follow plasma testosterone levels (19). In *in vitro* studies, expression of PSA mRNA and protein PSA promoter activity are strongly androgen regulated (9–14). To determine the pattern of the PSA-61-LACH transgene expression during development, lysates of lateral prostate tissues were prepared from line 28 males between 2 and 52 weeks of age. As indicated in Fig. 5A, the dorsolateral prostate of 2-week-old and the lateral prostate of 4-week-old mice did not show significant β-galactosidase activity. In contrast, sexually mature males, ranging from 8 to 52 weeks of age, showed an almost constant, high level of β-galactosidase activity (~1500 relative light units (RLU)/μg protein).

To obtain information on androgen inducibility of the 6-kb PSA promoter in transgenic mice, sexually mature PSA-61-LACH males of line 28 were castrated, and β-galactosidase activity in the lateral prostate was

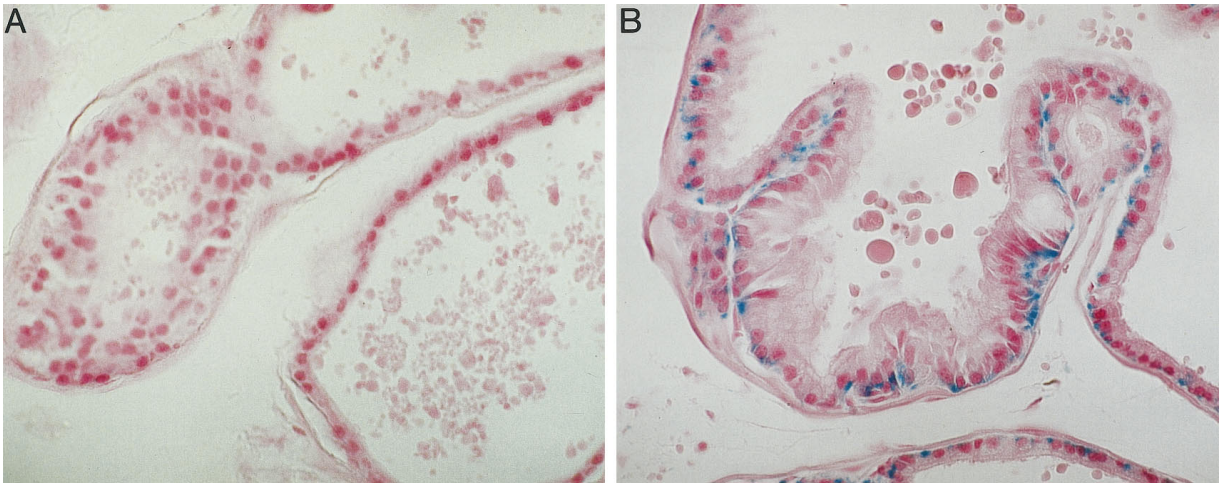

Fig. 3. Transgene Expression in the Lateral Prostate

A and B, Whole mount X-gal staining, followed by neutral red counterstaining, of 5-μm paraffin-embedded sections of lateral prostate of a 10-week PSA-61-LACH TG 28 male (B) and lateral prostate of a nontransgenic littermate (A) (magnification 400×). Blue X-gal staining is shown as *blue spots* in the cytoplasm of luminal epithelial cells.

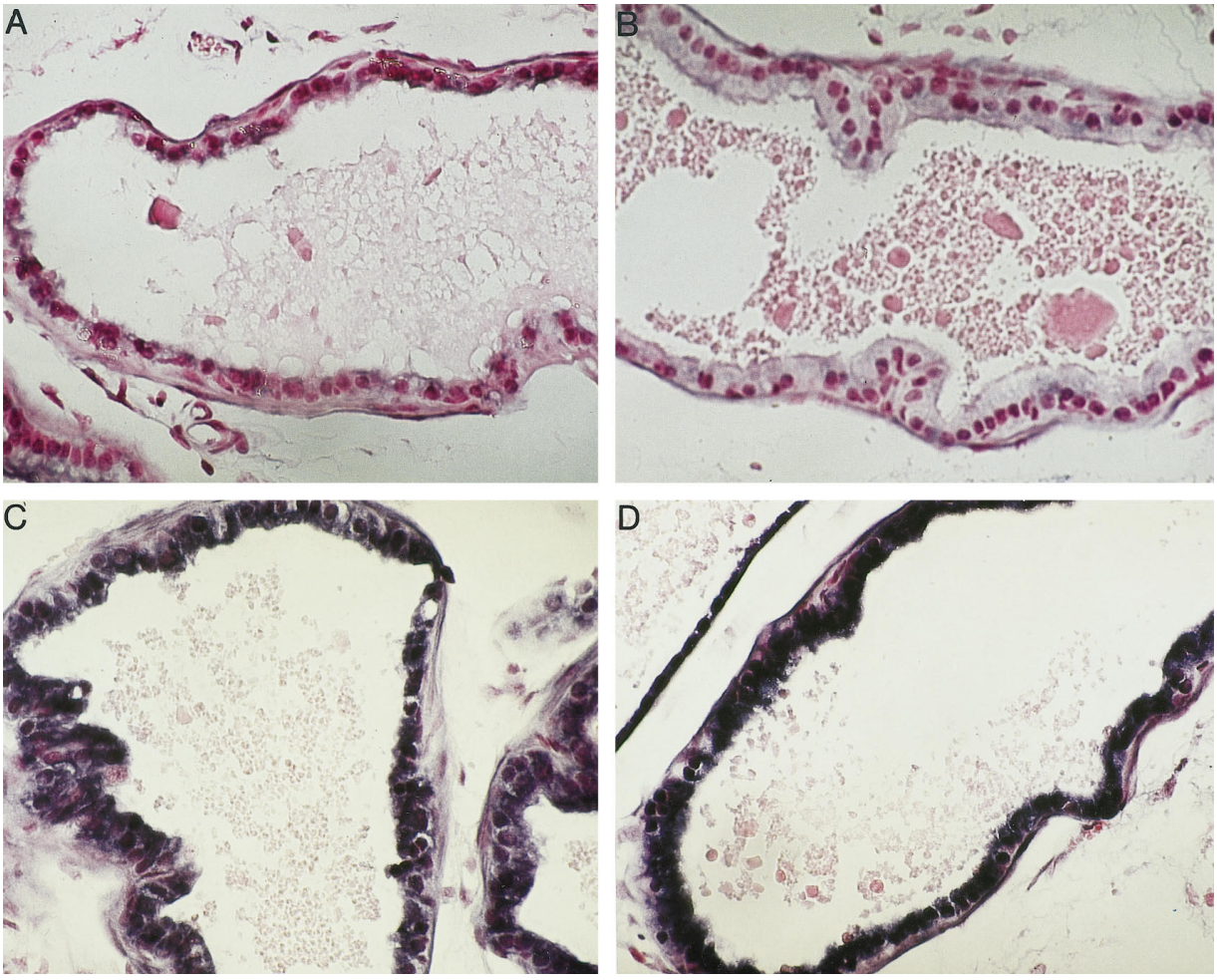

Fig. 4. RNA *in Situ* Hybridization Analysis of Lateral Prostate Tissue Sections of a 10-Week-Old PSA-61-LACH TG 28 Male Transgenic Mouse

Five micrometer sections of paraffin-embedded tissue were incubated with a DIG-labeled protamine RNA probe. Hybridization was visualized with alkaline-phosphatase-conjugated anti-DIG antibody (see *Materials and Methods*). C and D, Tissue sections of a 14-week-old PSA-61-LACH male transgenic mouse; B, nontransgenic littermate (antisense probe; 400 × magnification). A, Incubation of a transgenic mouse prostate with a sense protamine riboprobe.

determined at 4 days after castration and at 4 days after castration followed by 2 days of hormone replacement [(5 mg dihydrotestosterone (DHT)/kg body weight)]. As demonstrated in Fig. 5B, transgene activity decreased dramatically after castration and returned very rapidly to precastration levels after DHT administration. This finding strongly indicates androgen regulation of transgene expression.

Mouse Kallikrein Expression

To investigate mouse kallikrein gene expression in the prostate, RNA was isolated and RT-PCR was performed with primers overlapping highly conserved regions in exon 3 (KALK-3-s) and exon 4 (KALK-4-as) of all known mouse kallikrein genes (see GenBank data for mouse kallikrein sequences). Thirty-four cloned, 146-bp PCR fragments were sequenced. Thirty-two clones contained a mGK22 fragment (20), the two additional cDNA fragments were 94% identical, and both contained novel kallikrein sequences, with highest homology to mGK16 (91 and 92%, respectively) (21). Previously, mGK22 was found to be expressed in both male and female salivary glands, but absent in all other tissues tested (22). RT-PCR with mGK22-specific primers confirmed the presence of mGK22 mRNA in lateral prostate and submandibular gland. mGK22 was absent in dorsal, ventral, and anterior prostate (Fig. 6). The expression level in submandibular gland was much higher than in lateral prostate.

DISCUSSION

Previously, we investigated the properties of the 632-bp proximal promoter and a strong far upstream (−4 kb) 440 bp enhancer region of the PSA

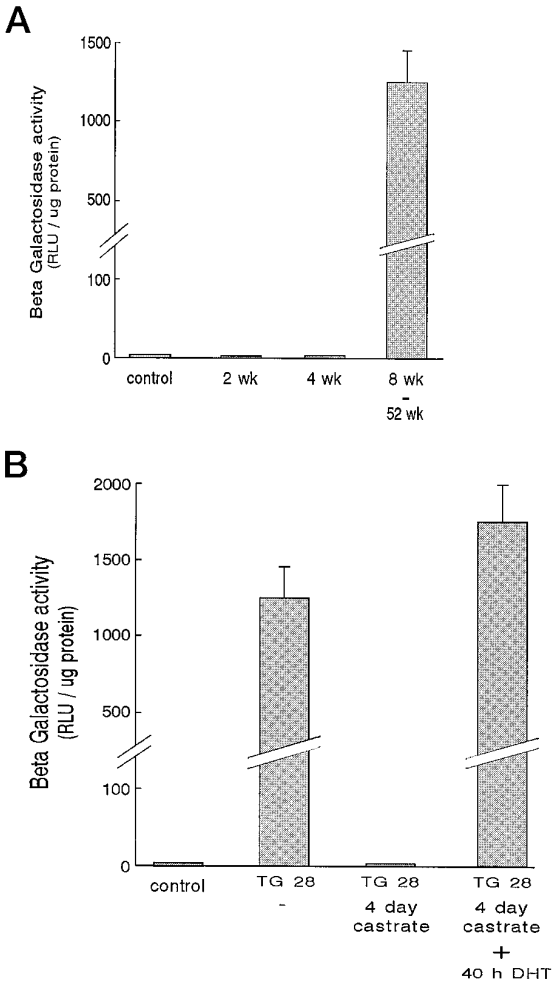

Fig. 5. Developmental (A) and Androgen (B) Regulation of PSA-61-LACH Expression

A, β-Galactosidase activity in extracts of dorsolateral prostate of 2-week, and lateral prostate of 4-week and 8- to 52-week old PSA-61-LACH TG 28 mice. B, Androgen regulation of β-galactosidase activity in lateral prostate of PSA-61-LACH TG 28 mice. Mice were castrated at 10 weeks of age. After 4 days, part of the mice were supplemented with DHT or vehicle. In indicated cases, mice were supplemented once a day with DHT (5 mg/kg body weight; in 100% ethanol mixed with 9 vol sesame oil, and injected subcutaneously). Lateral prostate of 10-week-old PSA-61-LACH mice and nontransgenic littermates served as control. Data shown are the average of three mice, except for the 8- to 52-week group in Fig. 5A, which is the average of ten animals (±SEM).

gene in transfected LNCaP cells (11, 12, 14). Although both regions contributed to androgen-regulated activity of the promoter, the presence of the 440-bp core enhancer was a prerequisite for high activity. A 6-kb PSA promoter fragment, which contains both the proximal promoter and the upstream enhancer region, was mainly active in LNCaP prostate cells. However, PSA promoter activity was also observed in T47D mammary tumor cells (14).

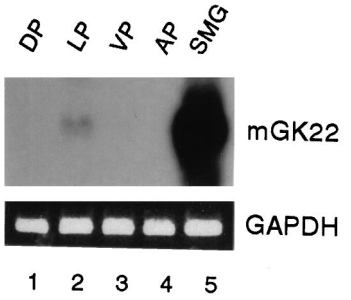

Fig. 6. RT-PCR Analysis of Mouse Glandular Kallikrein 22 Expression in the Various Lobes of the Mouse Prostate and Male Submandibular Gland

The RT-PCR products were blotted to Hybond N^+ membrane and hybridized with a random primed ^{32}P-labeled probe specific for the expected 634-bp cDNA fragment. RT-PCR of GAPDH mRNA in the RNA preparations of the different tissues is shown in the *lower* part. For abbreviations see legend to Fig. 2D.

In the present study we demonstrate the *in vivo* prostate specificity of the PSA promoter. We showed that the 6-kb, and not the 632-bp, PSA promoter is able to direct reporter gene activity in transgenic mice. In three independent transgenic lines, carrying a LacZ reporter gene under control of the 6-kb PSA promoter, hormonally and developmentally regulated expression of the transgene was exclusively targeted to the luminal epithelial cells of the lateral prostate, which mimics the expression pattern of the endogenous PSA gene in the human prostate. This strongly suggests that the 6-kb PSA promoter contains most, if not all, information for prostate-specific activity. The specific expression of the transgene in the mouse lateral prostate is in agreement with the structural homology between the human prostate and the mouse lateral prostate, and the mouse kallikrein expression in the lateral prostate. The variable level of PSA expression in human breast cancer (23) and the activity of the 6-kb PSA promoter in transiently transfected T47D human mammary tumor cells (14) could not be confirmed in normal breast tissue of female PSA-61-LACH transgenic mice (data not shown).

Transgene expression was assessed in a liquid β-galactosidase assay by RT-PCR and by RNA *in situ* hybridization. Additionally, X-gal staining of the different tissues was performed. X-Gal staining of adult mouse tissues is complicated due to high endogenous β-galactosidase activity present in many tissues, including testis, epididymis, vas deferens, liver, intestine, and prostate. This problem was overcome by modification of the standard protocols (24, 25). Incubation at elevated temperature before staining (1 h at 50 C), and a raised pH (8.6) during the various incubation steps (see *Materials and Methods*) suppressed endogenous β-galactosidase activity, without noticeable loss of activity of the *E. coli*-derived transgene. Only in epididymis, vas deferens, and anterior prostate could endogenous β-galactosidase activity be found

at a long (>16 h) staining period, which precludes detection of a low level of transgene expression in the X-gal assay (data not shown).

β-Galactosidase expression was undetectable in the PSA-4-LACH mice, despite the presence of 100 or more copies of the transgene in all three transgenic lines. Transient transfection of LNCaP cells with PSA-4-LACH (Fig. 1) and also PSA-4-LUC constructs (12, 14) showed low activity of this 632-bp promoter fragment, especially in the absence of a cotransfected androgen receptor expression plasmid. The observation by Schaffner *et al.* (26), that transgenic mice carrying a Ha-*ras*T24 oncogene, driven by the 632-bp proximal PSA promoter, developed salivary gland and gastrointestinal tract tumors seems to be in contrast to our findings for this promoter. However, mutant Ha-*ras* expression was only confirmed in salivary gland tumors, and not in gastrointestinal tumors. Furthermore, the late onset of tumorigenesis could indicate that Ha-ras expression was a secondary event. This might be related to PSA expression in a subset of salivary gland tumors in humans (27). An alternative hypothesis is that Ha-*ras* intron or exon sequences affect the selectivity and level of expression of the oncogenic transgene.

The three PSA-61-LACH transgenic lines show a comparable level of lateral prostate-specific, but copy number-independent, β-galactosidase expression. This could indicate that the PSA-61-LACH transgene cassette lacks elements, such as matrix attachment regions or locus control regions, that might determine boundaries in chromatin structure, leading to copy number-dependent and position-independent activity of transgenes (see Ref. 28 and references therein). The 6-kb PSA promoter fragment contains all DNaseI-hypersenstive sites (which indicate important regulatory regions) in the 31 Kb region upstream of the PSA gene (see Ref. 14). However, it might lack putative, so far unidentified regulatory sequences within the PSA gene, or in the flanking region downstream of the PSA gene, or even downstream of the hGK-1(KLK-2) gene, which is also prostate specifically expressed, and which is at a distance of 12 kb in the human genome (7, 8). Alternative explanations for copy number-independent activity are also possible. Although difficult to compare, the RT-PCR and X-gal staining experiments suggest that the expression level of the transgene in PSA-61-LACH mice is not as high as that of the endogenous PSA gene in the human prostate. Although this might be due to the integration site and the properties of the LacZ and protamine part of the transgene cassette, it is a real possibility that one or more *trans*-acting factors that direct high level PSA expression in the human prostate are absent, or present in a much lower concentration in the mouse prostate. If this is indeed the case, these factors could limit expression of the transgene, which corresponds to a comparable activity of the 6-kb PSA promoter in the three independent transgenic lines. This might also explain the low expression level of the mouse kallikrein mGK22 in the prostate. On the other hand, the latter might be caused by differences in promoter make up. Further analysis of mGK22 mouse kallikrein promoter activity in human prostate cell lines should provide additional information. In this regard, it is also interesting that the 6-kb PSA promoter-driven transgene expression pattern was different from that of mGK22, which is expressed at a high level in submandibular glands. The PSA-61-LACH transgene follows the expression pattern of the endogenous PSA gene in humans, and not that of mouse kallikreins.

The 6-kb PSA promoter is the first human promoter that directs prostate-specific expression in transgenic mice. Previously, three rat promoters, rKLK8, C3(1), and probasin, have been studied with respect to prostate specificity and applicability in the development of rodent prostate cancer models (29–35). Transgenic rats carrying a 2.5-kb rKLK8 rat kallikrein promoter fragment did not show tissue specificity. Expression of the transgene was demonstrated in almost all tissues tested, including prostate, but was absent at the major sites of endogenous gene expression, the submandibular and sublingual salivary glands (29). Transgenic mice carrying a 6-kb 5′-flanking region of the rat C3(1) gene linked to the β-galactosidase reporter gene (30) or a 9.5-kb fragment carrying the C3(1) gene with 4-kb upstream and 2-kb downstream flanking sequences (31) did not direct transgene activity strictly to the prostate. Depending on integration site, expression was also detected in testis, heart, lung, and skeletal muscle. Transgenic mice bearing a 5.7-kb C3(1) promoter linked to the SV40 large T antigen region developed at 7 months a prostate adenoma or adenocarcinoma (32). Female mice carrying this transgene acquired mammary adenocarcinomas. The mice also developed other phenotypic changes including several proliferative lesions and malignancies leading to premature death. Greenberg *et al.* (33) reported a 426-bp promoter fragment of the rat probasin gene directing chloramphenicol acetyltransferase (CAT) reporter gene expression to the prostate of transgenic mice. These transgenic mice showed CAT expression in dorsal, lateral, and ventral prostate. Low levels of transgene expression were observed in the anterior prostate and in the seminal vesicles. Although prostate specific, the expression level of the transgene was dependent on the integration site and did not strictly follow the expression pattern of the endogenous rat probasin gene, which is selectively expressed in the dorsolateral prostate. Cointegration of chicken lysozyme matrix attachment regions resulted in transgene expression in dorsolateral prostate of adult mice. Cointegration of matrix attachment sites was insufficient to facilitate high-level and copy number-dependent expression. Transgenic mice carrying the 426-bp probasin promoter-driven SV40 large T antigen oncoprotein developed progressive forms of prostatic cancer (34, 35).

Progress toward the understanding of the biology of prostate cancer benefits enormously from the avail-

ability of proper animal models displaying the whole range of clinical stages. The present study provides a baseline for the generation of such models, utilizing the 6-kb PSA promoter hooked to the appropriate oncogenes. Because of its tissue specificity and integration site-independent, constant activity it might even be preferred above the probasin and C3(1) promoter-driven prostate cancer models.

The observations presented in this study are not only relevant to the generation of mouse prostate cancer models, but also to gene therapy programs of human prostate cancer. The PSA gene is not only expressed in the luminal epithelial cells of the normal human prostate, but also in almost all prostate cancers. Therefore, the regulatory elements that determine PSA expression in prostate cancer are of potential interest for building a promoter to drive expression of therapeutic genes in prostate cancer cells. The strict prostate specificity of the 6-kb PSA promoter fragment strongly supports the applicability of this large promoter fragment, or derivatives, in gene therapy of human prostate cancer. Preliminary experiments, indicating prostate specificity of the 6-kb promoter-driven TK gene in an adenovirus construct, are in accordance with this view (A. Gotoh, A. S. C. Ko, C. Kao, L-J. Ho, K. B. J. M. Cleutjens, J. Trapman, F. L. Graham, and L. W. K. Chung, unpublished results).

MATERIALS AND METHODS

Cell Culture

LNCaP prostate cells were cultured as described (36). For examination of androgen-driven promoter activity, the synthetic androgen, R1881 (DuPont NEN, Boston, MA), was added to steroid-depleted medium to a final concentration of 1 nM.

Construction of Plasmids

All plasmid constructs were prepared according to standard procedures (37). The human androgen receptor expression plasmid pSVARo and the LacZ-containing reporter plasmid pLACH were described previously (38, 39). A mouse protamine gene fragment (mP1, +95 to +625, see Ref. 24) provides the LacZ cassette with an intron and the 3′-untranslated region, including the polyadenylation signal. PSA-61-LACH was generated by integration of the blunt ended *Hin*dIII/*Hin*dIII (−6 kb/+12) fragment of the PSA promoter into the *Sma*I site of the pLACH multiple cloning site. PSA-4-LACH was generated by integration of the *Eco*RI/*Hin*dIII (−632/+12 bp) PSA promoter fragment into pLACH.

Transient Transfections

Cells were transfected according to the calcium phosphate precipitation method, essentially as described (14).

Generation and Identification of Transgenic Mice

The 632-bp and 6-kb PSA promoter-driven LacZ genes were released from vector sequences by restriction digestion, purified by gel electrophoresis, and prepared for injection according to standard methods (40). The appropriate fragments were microinjected into the male pronuclei of fertilized eggs of C57BL6×DBA2C (F1) mice. The presence of the transgene was established by PCR amplification on DNA from tail biopsies (40), using oligonucleotide primers PSA-s: 5′-TTGTCCCCTAGATGAAGTCTCCATGA-3′ and LacZ-as: 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′ (indicated in Fig. 1).

Transgene copy numbers were quantitated by phosphoimage analyses of Southern blots of tail DNA. To this purpose, 10 μg DNA were digested with *Kpn*I and *Sac*I, electrophoresed on 0.8% agarose gel, and transferred to Hybond N^+ membrane (Amersham, Cardiff, UK). Filters were hybridized at high stringency with a random primed ^{32}P-labeled protamine probe (see *RNA in Situ Hybridization*). DNA transfer and filter hybridization were carried out according to the protocol of the manufacturer.

Liquid β-Galactosidase Assay

β-Galactosidase activity was measured in lysates of LNCaP cells and mouse tissues using the Galacto-Light Plus chemiluminescent reporter assay (Tropix Inc., Bedford, MA). Two to 5 mg of mouse tissue were incubated in 100 μl lysis solution, and transfected LNCaP cells were collected in 350 μl lysis solution. β-Galactosidase activity in 10 μl extract was corrected for variations in protein concentrations (protein microassay, Bio-Rad, München, Germany).

Whole Mount β-Galactosidase Staining

Immediately after death, mouse tissues were fixed by perfusion fixation in 2% paraformaldehyde in a 0.1 M piperazinebis (ethane sulfonic acid) buffer (pH 6.9), containing 2 mM $MgCl_2$ and 1.25 mM EGTA. Tissues were dissected and fixed for an additional 60–90 min at room temperature. To inactivate endogenous β-galactosidase activity, tissues were washed three times for 30 min in PBS (PBS, pH 8.6: 1.5 mM KH_2PO_4/6.5 mM Na_2HPO_4/2.7 mM KCl/135 mM NaCl). Subsequently, tissues were incubated in PBS for 60 min at 50 C. After cooling to room temperature, tissues were incubated in prestaining solution (containing 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 5 mM EGTA in PBS) for 60 min. After transfer to staining solution (prestaining solution supplemented with 0.5 mg/ml X-Gal), incubation was continued for 6–24 h at room temperature. The reaction was stopped by extensive washing in PBS, and tissues were postfixed in 4% paraformaldehyde in PBS before paraffin embedding. Five-micrometer sections were counterstained with neutral red.

RT-PCR

Isolation of total cellular RNA was carried out according to the guanidinium isothiocyanate method (41). RT-PCR amplification of LACZ-protamine (primers LACZ-s and PRO1/2-as), mouse kallikreins (primers KALK- 3-s and KALK-4-as), mGK22 (mGK22–1/2-s and mGK22–4/5-as), and GAPDH (GAPDH-s and GAPDH-as) were performed on 1 μg total RNA in the single tube Access RT-PCR* system (Promega, Madison, WI), according to the protocol of the manufacturer. Annealing steps were at 58 C, except for the kallikrein cDNAs expressed in mouse prostate (primers KALK-3-s and KALK-4-as), which was at 50 C.

RT-PCR Primers

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LACZ-s: 5′-AGCCATCGCCATCTG-3′
PRO1/2-as: 5′-GACGGCAGCATCTTCGCCTC-3′
KALK-3-s: 5′-TGCGGATCCTCAGGCTGGGGCAGCA-3′
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KALK-4-as: 5′-TGTCAGATCTCCTGCACACAA/GCAT-3′
mGK22–1/2-s: 5′-CTAGGAGGGATTGATGCTGC-3′
mKG22–4/5-as: 5′-CCTCCTGAGTCTCCCTTACA-3′
GAPDH-s: 5′-GGTCTACATGTTCCAGTATGACTCC-3′
GAPDH-as: 5′-GAGACAACCTGGTCCTCAGTGTAGC-3′

The resulting PCR products were separated over a 2% agarose gel and, in indicated cases, transferred to Hybond N^+ membrane. Filters were hybridized at high stringency with random primed ^{32}P-labeled probes specific for the expected cDNA fragment. The PCR product obtained with primers KALK-3-s and KALK-4-as was cloned in PCR-II (Invitrogen, Leek, The Netherlands), and resulting clones were sequenced.

RNA *in Situ* Hybridization

Sense and antisense DIG-labeled protamine RNA probes were generated on a 175-bp protamine cDNA fragment, obtained by RT-PCR on mouse testis RNA with primers PRO-s (5′ GAAGATGTCGCAGACGGAGG 3′) and PRO-as (5′ GATGTGGCGAGATGCTCTTG 3′). The PCR fragment was first cloned in pCR-II. After sequencing, the *Eco*RI-*Eco*RI cDNA fragment was recloned in pTZ19 (Pharmacia, Uppsala, Sweden). After linearization with *Hin*dIII, DIG-labeled RNA was transcribed from the T7 promoter. Hybridization of 5-μm paraffin-embedded sections and visualization with alkaline phosphatase-coupled anti-DIG antibodies and indoxil-nitroblue tetrazolium substrate were done essentially as described (42). Sections were counterstained with neutral red.

Experimental Animals

In accordance with the NIH Guidelines for Care and Use of Laboratory Animals, all experiments were conducted using the highest standard for humane care.

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