Prostate-specific Antigen Promoter/Enhancer Driven Gene Therapy for Prostate Cancer: Construction and Testing of a Tissue-specific Adenovirus Vector

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ABSTRACT

A range of luciferase reporter vectors was constructed, incorporating 5′-flanking sequences from the prostate-specific antigen (PSA), human glandular kallikrein 2 (hKLK2), and cytomegalovirus (CMV) promoters for expression control. Tissue specificity was evaluated in the PSA-positive cell line LNCaP and PSA-negative cells from different tissues of origin (CoLo320, DG75, EJ, A2780, and Jurkat).

The minimal 628-bp PSA and hKLK2 promoters showed only low-level expression in either PSA-positive or PSA-negative cells and showed no increase with the addition of androgen. Tandem duplication of the PSA promoter slightly increased expression in PSA-positive LNCaP cells. The addition of CMV enhancer sequences upstream of a single PSA or hKLK2 promoter substantially but nonspecifically increased luciferase expression in all cell lines tested. However, placing a 1455-bp PSA enhancer sequence upstream of either the PSA or hKLK2 promoters increased expression 20-fold in the PSA-positive cell line LNCaP but not in the PSA-negative lines. Tandem duplication of the PSA enhancer increased expression to ~50-fold higher than either promoter alone while retaining tissue-specific control. The level of expression was reduced by the addition of a third copy of the PSA enhancer. Expression from all enhancer constructs was increased 100-fold above basal levels when induced with the androgen dihydrotestosterone, with the PSA-based constructs consistently exhibiting roughly twice the level of expression of the hKLK2-based constructs at all androgen concentrations. Adenovirus vectors were produced in which either enhanced green fluorescent protein or nitroreductase could be expressed from the optimized PSA double enhancer-promoter construct and evaluated in LNCaP cells and the bladder-derived line EJ. Control vectors with the CMV promoter gave good levels of expression in both cell lines, whereas the PSA constructs only produced detectable levels of protein in the LNCaP cells as assessed by fluorescence of enhanced green fluorescent protein or by Western blotting of nitroreductase. LNCaP but not EJ cells were selectively sensitized to the prodrug CB1954 following infection with Ad-PSA-EGFP-NR. The PSA-based nitroreductase virus produced comparable amounts of nitroreductase and sensitization to CB1954 approaching that of the CMV-driven virus.

Plasmid and adenovirus constructs combining PSA enhancer and promoter sequences demonstrate selective expression of linked genes in PSA-positive cells. The expression is induced by androgen and gives therapeutically relevant levels of effector proteins.

INTRODUCTION

Prostate cancer is the third most common cause of cancer death in men after lung and colorectal cancers. The increased use of PSA testing is resulting in a rapid rise in the numbers of cases diagnosed throughout the developed world and is producing a marked change in case-mix toward early disease. Treatment options for early disease include “watchful waiting” with deferred treatment, radical surgery, radical radiotherapy, hormone therapy, and combinations of these options. All of these options have substantial drawbacks, and alternative treatment strategies are urgently needed.

Gene therapy approaches to prostate cancer are attractive because they hold the possibility of selectively targeting therapy to affected tissue, thereby avoiding toxicities associated with treatments such as cytotoxic chemotherapy, which has thus far proved of limited benefit in prostate cancer. Because the prostate is a nonessential organ, treatment can be targeted with tissue-specific rather than tumor-specific promoters. A number of proteins are known to be expressed in a tissue-specific manner, including prostate-specific membrane antigen and the kallikreins PSA and hKLK2, and the regulatory DNA sequences controlling these genes are therefore suitable candidates for driving tissue-specific expression of therapeutic genes.

PSA is a protein expressed exclusively by benign, hyperplastic, and malignant prostatic epithelium (1–3). Rising levels in serum are indicative of prostate disease (Ref. 4; benign hyperplasia or malignant carcinoma), and this has allowed PSA to be used as a diagnostic marker. Expression of the related kallikrein hKLK2 gene encoding human kallikrein-2 protein (hK2), which shows considerable sequence homology with PSA (5, 6), is also restricted to prostate tissue (3). Expression of both PSA and hKLK2 is up-regulated by androgen (3, 7, 8). Detection of hK2 has also been shown to be related to disease both on immunohistology and in serum (9, 10). The restriction of PSA expression exclusively to prostate tissue has led to investigations of the PSA gene regulation (11) for the purposes of targeting prostate cancer therapy. Initial reports suggested that the immediate 5′ promoter region was sufficient to target expression to prostate tissue (11), and this was further supported by subsequent studies aimed at testing targeted expression constructs for gene therapy vectors (12). Subsequent research suggested that an upstream enhancer was required for tissue-specific expression (13). The present study aimed to further examine the control of PSA expression, to resolve apparent discrepancies between these papers, and to optimize possible promoter constructs for use in gene therapy vectors. In addition, we set out to investigate the possible use of the hKLK2 promoter for prostate gene therapy. A range of therapeutic options is available with tissue-specific control of gene therapy vector expression. These include expression of suicide genes, prodrug activating enzymes (virus- or gene-directed enzyme prodrug therapy), immunomodulatory proteins, and gene replacement therapy (for reviews, see Refs. 14 or 15). The present study reports the construction and evaluation of a prostate tissue-specific promoter and its incorporation into plasmid reporter constructs. The optimal control sequence was subsequently validated in an adenovirus vector in which it showed appropriate regulation of either EGFP or the prodrug activating enzyme nitroreductase. Expression of nitroreductase selectively sensitized the PSA-positive cell line LNCaP to the prodrug CB1954.

MATERIALS AND METHODS

Cell Lines. The prostate cancer cells LNCaP and the nonprostate lines CoLo320, DG75, EJ, A2780, and Jurkat were routinely grown in RPMI 1640 medium supplemented with 10% FCS, 100 units/ml penicillin, and 100 mg/ml
streptomycin. In DME with 10% FCS, 293 cells for adenovirus propagation were maintained; virus infections were carried out in DME with 2% FCS. Androgen sensitivity experiments were carried out in phenol red-free RPMI supplemented with 10% charcoal-stripped FCS and antibiotics as above. Di-hydrotestosterone was dissolved in ethanol and then diluted to the required concentration in growth medium.

Preparation of PSA Reporter Vectors for Transfection Studies. All vector maps are shown in Fig. 1.

PSA-Luciferase Construct. Promoter sequences from the PSA gene were PCR amplified using primers and conditions previously described (16). Template genomic DNA was extracted from an LCL prepared from a normal donor. The PCR product was cloned into the Smal site of the pUC19 plasmid to generate pUC-PSA. Two independent clones were sequenced and found to differ at three positions from the published sequence (-155 G to A; -203 A to G; -249 A to G; Ref. 16). One change (-155 G to A) lies within the androgen response element. The same deviations from the published sequence were also found in the PSA promoter similarly PCR amplified and cloned from LNCaP cells; an additional change (-555 A to G) unique to the promoter isolated from LNCaP cells was chosen for further study.

The PSA promoter was subcloned as an Asp718-HinDIII fragment into the pGL3-basic luciferase expression vector (Promega) to give plasmid pPSA-GL3. A second copy of the PSA promoter introducing an Asp718 site just upstream of the TATA box was obtained by PCR with primers primerGL2 (Promega) and 5'-'CCGGTACCTTCCATCCACCAGGACTC-3', which introduced an Asp718 site, and 3' primer 5'-CCAGTCCACGCGCCAGGTGGA-3', which introduced a HindIII site.

CMV-based Constructs. The CMV immediate early gene control region derived from plasmid pLNCX (kindly donated by Dusty Miller; Ref. 17) was inserted between the DIII site of plasmid pGL3-basic (Promega) and the minimal 5' promoter of PSA derived either from a laboratory donor (pGL3-PSA LCL) or the LNCaP cell line (pGL3-PSA LNCaP), or bearing the minimal 5' KLK2 promoter derived from the LNCaP cell line (pGL3-hKLK2 LNCaP). Cells were cotransfected with 10 μg of the pCMV-βgal control, and parallel control transfections were carried out with pCMV-GL3 and pCMV-βgal. Cells were harvested in PBS, lysed, and processed for assay of β-galactosidase and luciferase activity as described in “Materials and Methods.” For each cell line and construct, the ratio of luciferase activity:CMV control was calculated, and the results were expressed as the percentage ratio of test construct activity:CMV control.

Fig. 2. Comparison of luciferase expression in prostate and nonprostate cell lines following transfection with PSA or hKLK2 promoter constructs. The PSA-positive line LNCaP and control cells (CoLo320, DG75, EJ, A2780, and Jurkat) were transfected by electroporation with 10 μg of the unmodified pGL3-basic plasmid or constructs bearing the minimal 5' promoter of PSA derived either from a laboratory donor (pGL3-PSA LCL) or the LNCaP cell line (pGL3-PSA LNCaP), or bearing the minimal 5' KLK2 promoter derived from the LNCaP cell line (pGL3-hKLK2 LNCaP). Cells were cotransfected with 1 μg of the pCMV-βgal control, and parallel control transfections were carried out with pCMV-GL3 and pCMV-βgal. Cells were harvested, and results were analyzed as in Fig. 2.

Fig. 3. Comparison of luciferase expression following transfection of prostate and nonprostate cell lines with CMV enhancer-kallikrein promoter constructs. The PSA-positive line LNCaP and control cells (CoLo320, DG75, EJ, A2780, and Jurkat) were transfected in Fig. 2 by electroporation with the unmodified pGL3-basic plasmid or constructs bearing the minimal 5' promoter of PSA derived either from a laboratory donor (pGL3-PSA LCL) or constructs containing the CMV immediate early promoter upstream of the PSA (pGL3-CMV-PSA LCL) and hKLK2 (pGL3-CMV-hKLK2) 5' promoters. Cells were cotransfected with the pCMV-βgal control, and parallel control transfections were carried out with pCMV-GL3 and pCMV-βgal. Cells were harvested, and results were analyzed as in Fig. 2.

Fig. 1. Diagram of promoter and enhancer constructs used to express the luciferase reporter gene. Solid arrows, PSA promoter. Open arrows, hKLK2 promoter. Horizontally hatched arrow, CMV enhancer. Cross-hatched arrows, PSA enhancer. Open box, luciferase reporter gene. Scale bar, 1000 bp.
and inserted into the Asp718 site upstream of the PSA promoter in pPSA-GL3 in the forward orientation to generate pCMV$_b$-PSA-GL3.

**PSA and hKLK2 Enhancer-Promoter Constructs.** A DNA fragment containing sequences from -5323 to -3869 of the PSA gene flank ed by Asp718 sites was prepared from LNCaP genomic DNA by PCR with primers 5’-CCCGTACCTCTAGAATTCTTGGATGAATG-3’ and 5’-CCCGTACCAACGGTGAAGACTGTCCTGCAGAC-3’. This was cloned into the Asp718 site upstream of the PSA promoter in pPSA-GL3; to generate pPSA$_b$-GL3, three independent clones were evaluated in preliminary transfection assays with similar results, and one clone was selected for further studies. Second and third copies of the PSA enhancer were inserted as further Asp718 fragments to generate pPSA$_{2b}$-GL3 and pPSA$_{3b}$-GL3. Equivalent single and double enhancer constructs were made using the hKLK2 promoter and PSA enhancer in a similar fashion to give constructs pKLK2$_b$ and pKLK2$_{2b}$.

**Evaluation of Expression of Luciferase Reporter Constructs in Prostate and Nonprostate Cell Lines.** Cells were washed once in PBS, trypsinized, and then washed. Then, 5 × 10$^5$ cells were resuspended in 500 µl of RPMI and placed in a 1 ml electroporation cuvette with an electrode spacing of 4 mm with 10 µg of the relevant luciferase reporter plasmid and 1 µg pCMV-β-gal DNA as an internal control for transfection efficiency. The cells were pulsed at 125 µF and 0.45 kV using a Bio-Rad Gene Pulser, transferred to 10 ml of medium in a 25-cm$^2$ tissue culture flask, and incubated for 24 h. Cells were washed in PBS and then lysed in 500 µl of lysis buffer [100 mM HEPES (pH 8), 2 mM magnesium chloride, 5 mM DTT, 2% Triton X-100]. Luciferase activity was determined by the addition of 100 µl of luciferase assay reagent [20 mM glycylglycine (pH 7.9), 5 mM magnesium chloride, 0.1 mM EDTA, 33 µM DTT, 270 µM CoA. 470 µM beetle luciferin, and 530 µM ATP] to 100 µl of lysate and by the measurement of light emission for 10 s in a Berthold LB9501 luminometer. The luciferase activity in each sample was then normalized for variations in transfection efficiency by measuring the level of β-galactosidase expression from the cotransfected pCMV-β-gal plasmid. After incubating the residual cell lysate at 50°C for 1 h to inactivate endogenous β-galactosidase, a 50-µl aliquot was mixed with 200 µl of substrate (K2048–1, Clontech) and incubated for a further 1 h at room temperature. β-galactosidase activity was determined by measuring light emission for 5 s in the luminometer.

**Results were controlled for transfection efficiency in LNCaP cells by dividing the luciferase expression from the test construct by the β-galactosidase expression from the cotransfected pCMV-β-gal plasmid. Parallel transfections were also carried out using pCMV-GL3 and pCMV-β-gal, thus allowing activity of the promoter constructs to be expressed as a percentage of the CMV promoter activity. This double control was necessary because preliminary experiments had shown that expression from the CMV promoter was itself influenced by androgen, and the additional control removed any differences arising between androgen-sensitive and -insensitive cell lines from this effect. All plasmid transfection results are the mean of six experimental measurements.**

**Construction of Adenovirus Vectors.** The self-complementary oligonucleotide 5’-GTACGGATCTTCTGAAAGGATCC-3’ containing BamHI and BsrBI sites was inserted into the Asp718 site upstream of the two PSA enhancers after partial digestion of pPSA$_{EEP}$-GL3. The PSA sequences were then excised with BsrBI and HindIII and inserted between these sites upstream of the nitroreductase gene in a derivative of pPS1017A5/pxLNC-NR (17), replacing the CMV promoter. A PSA$_{EEP}$-EGFP cassette was assembled in a similar manner by insertion into a similar plasmid with the EGFP gene (Clontech) in place of NR. The PSA$_{EEP}$-NR and PSA$_{EEP}$-EGFP cassettes were then excised with BamHI and inserted individually into the BamHI site of the adenovirus transfer vector pSW115A5, a derivative of pSP971C5 (18) in which the EcoRI site at the left end of the adenovirus sequence has been converted to a SwaI site. The resulting plasmids with the PSA$_{EEP}$-NR and PSA$_{EEP}$-EGFP cassettes in the rightward orientation relative to adenovirus sequences were linearized with SwaI and separately cotransfected into 293 cells with pSP1092B1. The latter is a plasmid that contains an adenovirus type 5 sequence from nucleotide 3328 to the right end of the genome, with a deletion between the XbaI sites in the E3 region replaced with a oligonucleotide containing BsrI and PacI sites; it was linearized before transfection at a SwaI site inserted at the right end of the viral genome. The Ad-CMV-ntr virus is described elsewhere.4

For the rescue of adenovirus constructs, plasmid DNA containing the target sequences was transfected into 293 80% confluent cells using the calcium phosphate method, and the cells were harvested when a complete viral cytopathic effect was apparent (10–14 days). The cells were subjected to three cycles of freezing and thawing, and the virus was separated from cell debris by centrifugation at 900 × g for 10 min. The virus stock was amplified by serial passage in 293 cells and used at the fourth passage. Virus was banded in a cesium chloride/glycerol gradient (19), and the titre was determined by plaque assay on 293 cells.

**Assessment of Adenovirus Vectors.** Cells were plated out in 6-well plates at 10$^5$/well and infected with adenovirus at MOIs 1, 10, and 30/cell 24 h later. Twelve to twenty-four hours after infection, cells were trypsinized and plated onto poly-l-lysine-treated slides and incubated for a further 24 h prior to fixation in acetone-methanol and visualization under phase-contrast microscopy. Experiments were carried out in triplicate, and results shown are the mean of three experiments.

**Prodrug Activation Assays.** Cells were grown until 70–80% confluent and then infected with virus at MOIs 1, 10, or 30. After 24 h of incubation, cells were harvested by trypsinization, and an aliquot was saved for subsequent Western blot analysis using standard methodology using a sheep polyclonal antinitroreductase antibody (Cobra Therapeutics; Ref. 20). Remaining cells were plated at 5 × 10$^5$/well on 96-well plates and incubated for a further 24 h prior to the addition of the prodrug CB1954. Cell survival was quantified 72 h after the addition of the prodrug using alamarBlue (Serotec) by fluorimetry.

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4 Weedon et al. submitted for publication.
Experiments were carried out in triplicate, and results shown are the mean of three experiments.

RESULTS

Promoter Constructs. The PSA and hKLK2 promoters coupled to luciferase reporter constructs were compared for activity following electroporation into the PSA-positive cell line LNCaP and a panel of nonprostate cell lines (Fig. 2). The PSA promoter constructs derived from the LNCaP cell line and the normal donor LCL were examined. The level of expression was expressed as a percentage of that from a control pCMV-GL3 plasmid normalized against cotransfected p-CMV-βgal as described above. Expression from both PSA and the hKLK2 promoters was detectable but very low, 0.1–0.3% of the level of expression from CMV, and there were no significant differences between the expression from the PSA and hKLK2 promoters in LNCaP cells and the other cell types. A construct with a tandem repeat of the PSA promoter sequence upstream of the TATA box showed at best a marginal increase in the expression in LNCaP cells (data not shown). The PSA promoter derived from the LCL clone was chosen for further study.

Enhancer Constructs. In an attempt to increase the level of expression from the PSA and hKLK2 promoters, CMV enhancer sequences were inserted upstream. As shown in Fig. 3, the level of expression was increased 100-1000-fold over that obtained with the promoters alone; however, there was no apparent tissue-specificity because similar high levels of expression were observed in all tested cell lines.

Shuur and coworkers (13) identified enhancer sequences between 3.7 and 5.8 kb upstream of the PSA gene by analysis of 5′ and 3′ deletions. We cloned this region by PCR and inserted 1–3 copies upstream of the PSA and 1–2 copies upstream of the hKLK2 promoters in the luciferase expression vectors; expression was examined as for the promoter constructs alone (Fig. 4). A single enhancer copy caused a substantial increase in expression in LNCaP cells but not in other nonprostate cell types. The double and triple enhancers further increased tissue-specific expression with the double enhancer giving higher levels than the triple enhancer, ~50-fold higher than that seen with the promoter alone.

Androgen Responsiveness of Enhancer-Promoter Constructs. The androgen responsiveness of the PSA enhancer-containing constructs was evaluated together with the 5′ minimal promoters (Fig. 5). Both promoter constructs showed no detectable response to androgen. The enhancer constructs all gave increased expression with androgen, the PSA enhancer-PSA promoter constructs having approximately double the level of expression at each androgen concentration compared to the PSA enhancer-hKLK2 promoter constructs. This shows that the low level of activity of the minimal PSA and hKLK2 promoters, the PSA promoter contributed additional activity to the enhancer constructs compared to the closely homologous hKLK2 promoter. On the basis of these experiments, the double enhancer-PSA promoter construct was incorporated into a series of adenovirus constructs for further study.

Evaluation of Adenovirus Constructs. Two replication-defective adenovirus constructs were constructed in which the double enhancer-PSA promoter construct controlled the expression of either the EGFP reporter or the prodrug-activating enzyme nitroreductase. These constructs were compared with CMV-driven control adenoviruses in the PSA-positive prostate cancer line LNCaP and the bladder line EJ. Tissue-specific regulation was examined by fluorescence to detect EGFP expression (Fig. 6) and by Western blotting to detect nitroreductase following infection with the respective viruses (Fig. 7). The Ad-CMV-EGFP virus gave high levels of expression in both the LNCaP and EJ cell lines. In contrast, the Ad-PSAEEP-EGFP vector only expressed detectable levels of EGFP in the LNCaP line with no detectable expression in the bladder-cancer-derived EJ line. A lower level of expression was seen with Ad-PSAEEP-EGFP than with Ad-CMV-EGFP, which is consistent with the difference in expression seen with transient transfection of the plasmid constructs where the pPSAEEP-luc construct only possesses around 4–5% of the activity of the pCMV-based control (Fig. 3).

Fig. 7 shows levels of nitroreductase assessed by Western blotting following viral infection. As with the EGFP viruses, tissue-specific expression is retained with Ad-PSAEEP-NR. The control Ad-CMV-ntr viruses in the luciferase expression vectors; expression was examined as described in "Materials and Methods." As in Fig. 2, for each cell line and construct, the ratio of luciferase activity between the expression from the PSA and the hKLK2 enhancer-kallikrein promoter constructs. The LNCaP cells were transfected by electroporation with constructs bearing the minimal 5′ promoter PSA or constructs containing 1–3 copies of the PSA enhancer upstream of the PSA (pPSAEEP) and hKLK2 (phKLK2EEP) 5′ promoters. Cells were cotransfected with the pCMV-βgal construct, and control cells were transfected with pCMV-GL3. Cells were harvested in PBS, lysed, and processed for assay of β-galactosidase and luciferase activity as described in “Materials and Methods.” As in Fig. 2, for each cell line and construct, the ratio of luciferase activity/β-galactosidase activity was calculated, and results were expressed as the percentage ratio of test construct activity:CMV control. a, PSA promoter-based constructs; b, hKLK2 promoter-PSA enhancer constructs.
virus gave expression of nitroreductase in both cell types. Ad-PSA EEP -NR gave a comparable level of nitroreductase protein expression to the Ad-CMV-ntr construct in LNCaP cells but little or no expression in the EJ bladder cancer cell line. To test the selectivity and efficacy of target cell sensitization, the nitroreductase viruses were also evaluated in prodrug sensitization assays (Fig. 8). The LNCaP but not the EJ cells were found to be unusually sensitive to prodrug alone. Nonetheless, the experiment showed that additional CB1954-dependent cytotoxicity was observed in the LNCaP cells upon infection with either Ad-CMV-ntr or Ad-PSA EEP -NR (Fig. 8, a and b). As anticipated from the Western blots, the potency of the two viruses was comparable in LNCaP cells. In

Fig. 6. Expression of EGFP in LNCaP and EJ cells following infection of EJ and LNCaP cells with either adenovirus vectors driven by the double PSA enhancer-PSA promoter construct (Ad-PSA EEP -EGFP) or a control CMV-driven construct (Ad-CMV-EGFP). Cells were plated out at 10^5 cells/well and infected with virus at MOIs of 1, 10, and 30 pfu/cell. Untreated cells were prepared in parallel. Twelve to 24 h after infection, cells were trypsinized, plated onto slides, fixed, and visualized under phase contrast or UV light microscopy.
therefore set out to optimize tissue-specific control of expression. We anticipate that specific expression would be an acceptable therapeutic strategy. We recognize that coexistent morbidity from benign prostatic enlargement, tissue-specific control is acceptable (and may even be desirable to relieve the inadvertent destruction of normal prostate tissue by any therapeutic strategy that is not vector mediated).

The combination of both approaches together would potentially minimize toxicity to nontarget structures such as the urethral lining, but it requires either tumor- or tissue-specific expression vectors. Because the inadvertent destruction of normal prostate tissue by any therapeutic strategy is acceptable (and may even be desirable to relieve coexistent morbidity from benign prostatic enlargement), tissue-specific expression would be an acceptable therapeutic strategy. We therefore set out to optimize tissue-specific control of expression using the PSA promoter as a model.

Earlier reports with the minimal 5' PSA promoter suggested that the region from −539 to +12 from the gene was sufficient to give tissue-specific regulation of gene expression (11). We cloned the 5' promoters of PSA and the closely homologous kallikrein hKLK2 for our initial investigations aimed at developing promoter constructs for our gene therapy program. The PSA promoter cloned from two distinct sources differed from the published sequence: the sequence variations seen in the LCL-derived PSA clones were detected in the LNCaP clone and also in a third clone obtained from another research group (data not shown); an additional base change was seen in the LNCaP-derived clone. Because the LCL cells were derived from a nonprostate tissue in a normal donor, it was believed that the differences were likely to be normal variants rather than of any functional significance. However, in our hands, as shown in Fig. 2, neither the minimal 5' promoter nor the promoter of its close homologue hKLK2 gave tissue-specific regulation of expression. In addition, absolute levels of expression compared to the CMV promoter-driven control vectors were very low and were insufficient to produce immunologically detectable levels of the coimmunostimulatory molecule CD80 (B7.1; data not shown). The use of a tandem repeat of the minimal promoter resulted in only a minimal, nonsignificant increase in expression (data not shown). These results contrast with those of Pang and coworkers (12) who reported results with a similar 620-bp PSA promoter termed PCPSA and showed tissue-specific regulation and androgen inducibility with this promoter in LNCaP but not in PSA-negative cell lines both prostatic and nonprostatic in origin.

In an attempt to increase the level of expression, a CMV enhancer was introduced upstream of the PSA and hKLK2 promoters. This did give substantial increases in expression, but neither construct showed tissue specificity in our panel of cell lines (Fig. 3), which again contrasted to the results reported by Pang et al. (12) for their CMV-PSA construct. We are unclear as to how this discrepancy arose because we think it is unlikely that the sequence changes listed above would totally abrogate tissue-specific regulation from our promoter construct (see also Fig. 5 and discussion below). However, the PCPSA construct included the CMV TATA sequence upstream of the PSA promoter, which might have resulted in some transcription initiation upstream of the PSA promoter. In our attempt to avoid the possibility of upstream initiation, we excluded the CMV TATA sequence from our construct.

While the early stages of this work were in progress, Schuur et al. (13) described the location of enhancer sequences in the DNA upstream of the PSA gene. We therefore cloned this sequence and introduced one, two, or three copies of the 5' to the PSA promoter and one or two copies of the 5' to the hKLK2 promoter. The single enhancer, as predicted, gave increased expression in a tissue-specific manner (Fig. 4). The addition of further tandem repeats of this enhancer gave additional tissue-specific increases in expression, with two enhancers being the optimal configuration (Fig. 4). Comparison of the PSA and hKLK2 promoters with the PSA enhancers showed that, despite the low activity of the minimal PSA promoter (Fig. 2), it did contribute additional activity to the enhancers compared to the closely homologous hKLK2 promoter (Fig. 5). On the basis of these data, the tandem PSA enhancer-PSA promoter configuration PSA_EEP was chosen for further study. All promoter-enhancer constructs showed androgen inducibility (Fig. 5). Interestingly, the PSA-based constructs had approximately twice the activity of the hKLK2-based homologues, a ratio reflected in the relative concentrations of mRNA for the two genes in vivo (3).

A variety of gene therapy strategies are possible, including the introduction of genes encoding “suicide enzymes,” which activate a prodrug, and immune modulatory proteins, such as GM-CSF or CD80 and replacement tumor suppressor gene function, e.g., p53 (14, 15).
Possible delivery systems for gene therapy include viruses, in particular, adenoviruses, adeno-associated viruses, and retroviruses, and nonviral systems, such as liposomes, as well as direct injection of naked DNA. We have chosen an adenovirus delivery system because of the high yields of virus obtainable compared to retroviruses and the high infection efficiencies possible with adenoviruses. In addition, adenoviruses do not integrate into the host genome, lessening concerns about insertional mutagenesis.

We have evaluated the PSA EEP transcription regulatory construct in two adenoviruses, one encoding the reporter protein EGFP and the other encoding the prodrug activating enzyme nitroreductase. The test virus Ad-PSAEEP-NR contained only 7 bp of bacterial DNA 3′ to the nitroreductase open reading frame, compared to 81 bp in the Ad-CMV-ntr virus. We had recently observed that, in a retroviral vector, nitroreductase was expressed more efficiently from a construct with the shorter 3′ sequence (21). Seeking to maximize expression from the prostate-specific enhancer/promoter construct, we therefore used this more efficient version of the nitroreductase gene (which we abbreviated to NR to distinguish it from the original, unmodified version, ntr) in the construction of Ad-PSAEEP-NR. The differential expression seen with plasmid transfection is retained in the Ad-EGFP constructs in which the Ad-CMV-EGFP virus produced detectable green cells at MOI = 1 compared to MOI = 30 for the Ad-PSAEEP-EGFP virus (Fig. 6). In contrast, infection with the modified Ad-PSAEEP-NR resulted in similar expression to Ad-CMV-ntr as assessed by Western blotting (Fig. 7) and sensitization to prodrug (Fig. 8). This effect is consistent with an ~20-fold improvement in the expression of the nitroreductase protein with the shorter 3′ bacterial untranslated sequence while retaining tissue specificity. The nitroreductase/CB1954 system has been shown to produce bystander lysis in a variety of cell systems (21, 22). We anticipate that this would further amplify the effect of nitroreductase expression in PSA-positive cells in vivo.

The virus constructs were assessed using the bladder line EJ as the specificity control. This line was chosen because it is derived from transitional cell epithelium that comprises one of the cell types in the urethra and bladder and which may be dose-limiting in clinical studies. High infection efficiency was noted with the Ad-CMV virus in both cell lines (Fig. 6, a and b). In contrast, the LNCaP line is difficult to transfect using standard laboratory techniques, and transfection efficiencies of 0.1–10% were obtained with a variety of nonviral
transfection techniques (data not shown). In addition, virus infection appeared to be directly toxic to the LNCaP line but not to the EJ line (Fig. 8), which may prove to be therapeutically useful for a more generalized feature of prostate tissue. Direct toxicity resulting in objective tumor responses (including a complete pathological response) has been reported with the injection of replication-deficient adenovirus encoding a marker protein (β-galactosidase) in one clinical study (23), although it is possible that this effect may be immune-mediated rather than due to direct viral cytotoxicity (24). Additional cell killing was seen with the addition of produg to EJ cells infected with Ad-CMV-ntr and to LNCaP infected with either virus (Fig. 8), which is consistent with the expression of nitroreductase seen on Western blotting (Fig. 7). Studies are under way to investigate both these effects in other prostate cell lines and in cultured primary prostate tissue.

The lack of PSA-positive cell lines other than LNCaP has limited the evaluation of the construct; as a result, the expression seen may be restricted to this cell type rather than to prostate tissue in general. However, given the consistent expression of PSA on both benign and malignant prostate tissue and the androgen inducibility of the promoter construct, it seems likely that the promoter will behave in a similar fashion in other PSA-positive cells.

We are planning to evaluate the Ad-PSAEQP-NR virus in clinical trials. The simplest strategy uses direct virus injection into the prostate gland in patients with locally recurrent disease. A trial using HSV-tk plus ganciclovir by local intratumoral injection has demonstrated cell killing associated with the produg (Fig. 8), which is consistent with the expression of nitroreductase seen on Western blotting (Fig. 7). Studies are under way to investigate both these effects in other prostate cell lines and in cultured primary prostate tissue.

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